Proteomics-based approaches to the identification of bladder tumour markers

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

To Jess

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Abstract

Transitional cell bladder cancer (TCC) is characterised by a tendency to recur and, less commonly, to progress requiring extended cystoscopic surveillance which entails significant morbidity and expense. Tumour size, stage and grade are associated with risk of recurrence and progression but are not significantly accurate prognostic markers to plan the management of individual patients. Contemporary biomarkers of TCC derived from studies of cell biology and genetics tend to assay single molecules and do not add significant clinical information above that provided by grade and stage. Extensive investigations to develop urinary biomarkers to detect the presence of TCC recurrence have also failed to deliver reliable clinical tools.

Novel biomarkers for diagnosis, disease monitoring and prognosis may be discovered with a new approach to profiling TCC. "Proteomics" refers to the analysis of global protein expression. It is complementary to but has several theoretical advantages over genomic-based approaches to cancer biology. Proteins are the functional units of the cell and thus protein expression more closely reflects its current state. Traditional proteome separation and identification requires two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and mass spectrometry (MS). More recently, MS based profiling, often combined with liquid chromatography (LC) has improved the sensitivity and rapidity of protein profiling. The development of modified mass spectrometry, Surface-Enhanced Laser Desorption/Ionisation mass spectrometry (SELDI-MS) allows rapid profiling of the proteome of biological samples which was not previously possible. This thesis describes the application of a proteomic approach including 2D PAGE and SELDI-MS to identify novel biomarkers of TCC diagnosis and progression.

Methodological and quality control standards required for this new technology were determined and SELDI-MS proteome-profiling was applied to 350 urine samples collected from patients suffering from TCC and appropriate controls (including those with benign urothelial inflammatory processes). The resulting proteomic

profiles produced, each containing several hundred peaks corresponding to a protein or peptide, were processed and analysed with novel algorithms and machine learning tools. After repeated profiling experiments, associated with improved methodological and analytical SELDI-MS techniques, a Random Forest classifier was trained to identify TCC from controls using a training set (n=130). In an initial test set (n=54), bladder cancer was diagnosed with a sensitivity of 71.7% and specificity of 62.5%. A second validation test set (n=43) tested 6 months later maintained this test performance with a sensitivity of 78.3% and specificity of 65.0%.

Additionally, 6 low grade (G1-2) Ta and 6 high grade (G3) Ta TCC were analysed by 2D PAGE to identify differentially-expressed proteins as possible prognostic biomarkers. The study was prospective because of the need for fresh tissue, so that follow-up is as yet limited. Therefore grade was used as a surrogate marker of tumour aggressiveness. Seven differentially-expressed proteins were successfully identified by mass-spectrometry. Protein and RNA expression of these markers was validated with immunohistochemistry (IHC) and quantitative real-time RT-PCR in another TCC tissue panel of 41 TCC.

Most interestingly, formyltetrahydrofolate dehydrogenase (FDH) for the first time was shown to be down-regulated in high-grade Ta TCC. FDH downregulation has been recently described in several other cancer types. It has been proposed that in normal tissues, FDH suppresses purine metabolism by depletion of the intracellular folate pool and thus acts as an inhibitor of cell proliferation, while FDH down-regulation is accompanied by tumour proliferation.

Our novel proteomic approach to biomarker discovery has identified a promising non-invasive diagnostic test and new prognostic markers for TCC. SELDI-MS urine analysis appears to have genuine clinical utility and may alter the ongoing management of patients with TCC, although further development and assessment is needed.

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1 Introduction

1.1 Transitional cell carcinoma of the bladder

1.1.1 Epidemiology

There are in excess of 11,000 new cases and nearly 5,000 deaths from bladder cancer in the UK annually (http://www.cancerresearch.org). Transitional cell carcinomas (TCC) account for 90-95% of malignant bladder tumours. In 1998, a WHO consensus conference defined "urothelial cancers" as the preferred synonym of TCC. The term, urothelial cancer, does not include rarer forms of bladder cancer that arise from the urothelium: squamous cell carcinoma (SCC), adenocarcinoma and neuroendocrine tumours. While mesenchymal tumours are very rarely found in the bladder, adenocarcinoma from other pelvic organs (prostate, ovary, endometrium and rectum) may develop as metastatic secondary lesions. SCC accounts for ~5% of bladder cancers in industrialized countries, but in East Africa and the Middle East where bilharzia (Schistosoma *haematobium*) is endemic, it accounts for ~75% of bladder cancers. However, the scope of this thesis is limited to bladder TCC only.

Bladder TCC affects a population predominantly in their 6^{th} and 7^{th} decades with an incidence in men 3-4 times higher than in women. Familial associations account for a small number of cases while environmental factors are unusually well characterised; most notably smoking habit and occupation. Bladder cancer has been historically associated with aniline dye exposure in factory workers and modern exposure to industrial carcinogens including 2-napthylamine and benzidine in the dye, chemical, rubber and paint industries. In contemporary European society it was estimated that 1-19% of TCC are occupation-related (Vineis and Simonato, 1991). Recently, the urine from 117 Spanish Textile workers and controls was subjected to a cell based mutagenicity assay. The textile workers' urine was significantly more mutagenic than controls, despite the implementation of regulatory norms forbidding the use of most carcinogenic arylamines (Fanlo et al., 2004). The incidence of urinary bladder cancer across various occupations was also assessed in a cohort of 1.6 million Swedish men

from 1960 to the present (Ji, Granstrom, and Hemminki, 2005). The standardised incidence ratios (corrected for age, socioeconomic status and smoking) revealed a slightly higher rate of bladder cancer in administrators, managers and physicians, and a lower rate in mechanics, iron and metalware workers. The authors speculate that sedentary working may predispose to prolonged urinary contact of carcinogenic agents with the urothelium, while physicians may have easier access to diagnostic facilities.

Cigarette smoking increases the level of urinary amines and is associated with a strong increased risk of urinary tract cancer. A meta-analysis of 43 epidemiologic studies (8 cohort and 35 case-control) calculated summary odds ratios by meta-regression analyses for different smoking characteristics. Even though the component studies differed in methodology, the results were consistent. The age- and gender-adjusted odds ratios for current and former cigarette smokers compared with those for non-smokers were 3.33 (95% confidence interval 2.63-4.21) and 1.98 (CI, 1.72-2.29), respectively (Zeegers et al., 2000).

Other risk factors associated with bladder cancer include increased coffee and low vegetable consumption, a history of cystitis and exposure to drugs such as phenacetin and cyclophosphamide (D'Avanzo et al., 1995; DeVita, 1997). Dietary soy was associated with an increased risk of bladder cancer (relative risk 2.2 for highest vs. lowest quartile of intake) in a study of 18,000 Chinese men (Sun et al., 2004). Hepatic metabolism of aromatic amines via O-acetylation enzymes produces carcinogenic metabolites, while N-acetylation produces benign metabolites. Humans homozygous for the so-called “fast-acetylator” polymorphic form of the N-acetyltransferase-2 gene, have a reduced incidence of bladder TCC (Marcus, Vineis, and Rothman, 2000; Mommsen, Barfod, and Aagaard, 1985).

In England and Wales the incidence of bladder cancer has increased by 57% between 1971 and 1998 (Hayne et al., 2004). It is not clear what proportion of this rise is due to a real increase in the disease, and what proportion is due to increased diagnosis and reporting. In particular, the age standardized incidence has increased by 37% in women and only 16% in men. This reflects the rise in female smoking habits, yet overall, the trend in bladder cancer rates correlates poorly with smoking and other

smoking–related disease rates such as lung cancer. There is no compelling evidence for any proposed cause of this phenomenon.

1.1.2 Diagnosis and treatment of TCC

Bladder cancers are often discovered during investigations following the report of blood within the urine (haematuria). Haematuria may be classified into that which is visible to the naked eye (macroscopic) or not (microscopic). Microscopic haematuria is often detected on "dipstick urine assays" that are a readily available point of care assay used by many health workers and the public. In fact 80% patients with TCC report intermittent, painless haematuria (Cummings, Barone, and Ward, 1992). Additionally, about a third of TCC patients report lower urinary tract symptoms of an "irritative" nature such as the need to void frequently and urgently, often at night and often associated with pain. More rarely, patients may present with the symptoms of advanced disease, such as weight-loss, abdominal pain or an abdominal mass arising from the pelvis. In tumours that obstruct the ureters, additional symptoms of renal obstruction and /or infection may be present.

Diagnosis is usually made upon the basis of visualisation of the urethra and bladder with a cystoscope (usually a flexible fibre-optic endoscope). Cystoscopy can be regarded as the "gold standard" for the diagnosis of TCC, with high sensitivity and specificity, particularly following recent advances in fluorescence techniques (Joudi and Konety, 2004). Additionally, cystoscopy allows assessment of the tumour extent and location to aid surgical management. TCC lesions appear as frondular (papillary) or solid or velvety masses arising from the urothelium. Direct visualisation of the upper tracts (comprising the renal collecting systems and ureters) is more challenging and these areas are usually assessed with X-ray contrast studies or ultrasonography.

The mainstay of treatment is surgical removal (resection) of the tumour. Small tumours are usually resected via a rigid instrument passed through the urethra into the bladder, in a procedure known as a Trans-Urethral Resection of Bladder Tumour (TURBT). Histological examination of the tumour will determine the depth of invasion (pathological stage), given the limitations of tissue sampling. Additional

information about tumour extent can be obtained by cross-sectional computer tomography (CT) or magnetic resonance (MR) imaging and an examination of the pelvis under anaesthesia (clinical stage). The presence of TCC metastases may be revealed by radioactive isotopic bone scans, chest X-ray and liver function tests.

The TNM system is widely used for pathological staging of bladder TCC (Table 1.1 (Sobin and Wittekind, 2002)). The TNM staging of bladder cancer is based on the depth of invasion (T), involvement of regional lymph nodes (N) and presence or absence of metastases (M). Additional treatment of localised bladder cancer varies according to the grade and stage of the disease as well as patient age, co-morbidity and preference. There is a wide range of adjuvant medical treatments, principally chemotherapy (mitomycin, doxyrubicin and epirubicin) and immunotherapy (bacilli Calmette-Guerin: BCG) administered directly into the bladder. There is limited evidence that BCG reduces TCC recurrence and progression, whilst other agents only reduce recurrence (Lamm, 2000; Nilsson et al., 2001).

A meta-analysis undertaken by the European Organisation for Research and Treatment of Cancer (EORTC) in 2002 demonstrated that ongoing (maintenance) BCG therapy for superficial bladder cancer reduced the risk of progression from 14% to 10% compared with controls (Sylvester, van der, and Lamm, 2002). However, a review from the Cochrane Collaboration in 2003 comparing BCG with MMC concluded that BCG only reduced recurrence in high-risk cancers, and had no effect on progression or survival compared to MMC (Shelley et al., 2003). Additional immunological agents such as Interferon alpha 2B are under investigation to augment BCG or as salvage treatments in those who fail BCG therapy (Witjes, 2006).

Table 1.1. TNM (2002) staging of urinary bladder cancer

Adapted from TNM Classification of Malignant Tumours (Sobin and Wittekind, 2002)

T	**Primary Tumour**
Ta	Non-invasive papillary carcinoma
Tis	Carcinoma in situ:
T1	Tumour invades sub-epithelial connective tissue
T2	Tumour invades muscle
T2a	Tumour invades superficial muscle (inner half)
T2b	Tumour invades deep muscle (outer half)
T3	Tumour invades perivesical tissue
T3a	Microscopically
T3b	Macroscopically (extravesical mass)
T4	Tumour invades any of: prostate, uterus, vagina, pelvic wall, abdominal wall
T4a	Tumour invades prostate or uterus or vagina
T4b	Tumour invades pelvic wall or abdominal wall
N	**Regional Lymph Nodes**
N0	No regional lymph node metastases
N1	Metastasis in a single lymph node 2 cm or less in greatest dimension
N2	Metastasis in a single node more than 2 cm but less than 5 cm or multiple lymph nodes, none more than 5 cm in greatest dimension
N3	Metastasis in a lymph node more than 5 cm in greatest dimension
M	**Metastases**
M0	No distant metastases
M1	Distant metastases

Muscle invasive tumours may be treated with radical surgery or radiotherapy. The operative mortality of cystectomy is 2% and major complications occur in 20-30% of patients. Overall five-year survival is reported at 40–60% (Hassen and Droller, 2000; Stein et al., 2001). Survival and complications are broadly similar for those treated by radiotherapy (Hamdy et al., 2002). Indeed there is no clear-cut evidence for the overall superiority of either treatment modality; although surgery appears to offer better disease control it has more serious adverse effects (www.nice.org.uk). Recent studies have reported a survival advantage with administration of chemotherapy before radical surgery, although this is not practised widely in the UK at present (Grossman et al., 2003; Vale, 2003). The 5-year UK survival rate for all TCC in 1996-99 was 64.4% for men and 56.0% for women (Office for National Statistics, www.statistics.gov.uk) but varies according to pathological and clinical features.

1.1.3 Clinical phenotype of TCC

Approximately 75% of patients present with non-invasive tumours that are confined to the urothelium (Ta) or have invaded the subepithelial stroma only (T1) (www.baus.org.uk). These tumours have been termed “superficial” (i.e. non muscle invasive), and this remains the suggested terminology for clinical use in some contemporary guidelines (Oosterlinck et al., 2002). However T1 TCCs are not truly superficial, as they have invaded the sup-epithelial connective tissue. “Superficial” will not be used further in this thesis to avoid confusion (Bryan and Wallace, 2002).

The majority (80%) of Ta/T1 tumours are papillary and these lesions tend to be multifocal and recur in over 50% of patients. However, only 10-15% subsequently invade the muscle of the bladder wall (Heney et al., 1983; Lutzeyer, Rubben, and Dahm, 1982). A minority (10-15%) of superficial tumours grow as flat carcinoma *in situ* (cis) (Heney et al., 1983). Carcinoma-*in-situ* may present in isolation but is usually seen in association with high-grade papillary and invasive tumours. It is also non-invasive but behaves differently to the other Ta/T1 tumours with about 60% of patients developing invasive disease over 5 years if untreated. The probability of disease progression increases with the extent of cis (DeVita, 1997; Hamdy et al.,

2002). Muscle invasive disease (T2+) accounts for approximately 30% of new diagnoses of bladder cancer. These are almost entirely high-grade aggressive lesions and have the potential to spread locally through the structures within the pelvis, to loco-regional lymph nodes and to distant sites such as lungs, liver and brain (DeVita, 1997; Hamdy et al., 2002).

In summary, TCC is characterised by an unusual clinical phenotype; at presentation, 75% of tumours are either non-invasive or minimally invasive (stages Ta and T1) and although only 10-15% of these tumours develop into muscle invasive disease, 50-60% recur following initial resection. This disease is heterogeneous in behaviour, and prone to local recurrence.

1.1.4 Prognostic parameters of TCC clinical phenotype: histological stage, size and multifocality

The appropriate timing and nature of intervention to treat bladder cancer remains unclear, as clinicians try to identify those at risk of disease progression. For example, the management of poorly differentiated T1 tumours is highly controversial with some advocating early cystectomy and others suggesting alternative "bladder-sparing" strategies. It is clear that the ability to identify those tumours likely to progress would greatly improve patient management.

Histopathological tumour characteristics provide some clues to tumour behaviour. The grouping together of TNM stages Ta and T1 as "superficial TCC" is somewhat arbitrary as it combines truly superficial Ta tumours and T1 tumours that have invaded subepithelial connective tissue. The risk of cancer progression is significantly higher in T1 than Ta TCC. Of a cohort of 362 TCC patients monitored for 20 years in Denmark, 14% of patients with Ta tumours had died from cancer after 15 years compared with 63% of patients with T1 tumours (Zieger et al., 2000). Within each stage grouping, other features such as tumour size, location, recurrence and multi-focality also suggest increased risk of progression. In particular, Parmar's report in

1989 has influenced the practice of UK Urologists, stratifying TCC into risk groups for recurrence (Parmar et al., 1989).

1.1.5 Prognostic parameters of TCC clinical phenotype: histological grade

Histological grade is a critical risk factor. The most poorly differentiated (G3) TCC are particularly aggressive with a relative risk of progression and mortality compared with all Ta/T1 tumours of 19.9 and 14.0 respectively (Millan-Rodriguez et al., 2000). The risk of progression of G3 pT1 TCC is c.50% irrespective of management (Jenkins et al., 1989; Kaubisch et al., 1991). High and low grade Ta TCC seem to have the greatest divergence in clinical phenotype: high-grade tumours behave aggressively with approximately a 25% risk of progression (Heney et al., 1983), by contrast, only 3 of 178 (1.6%) patients with G1 Ta TCC progressed to muscle invasive disease after 1-10 years of prospective surveillance in a National Bladder Cancer Group study (Prout et al., 1992).

The grading of TCC remains controversial and presently several grading classifications are used. Revision of the 1973 World Health Organisation classification (Mostofi and Sobin, 1973) by the 1998 WHO/ISUP (World Health Organisation/International Society of Urologic Pathology) consensus classification has introduced the term “papilloma of low malignant potential (PNLMP)” and low and high-grade “papillary urothelial carcinomas (LGPUC, HGPUC)”(Epstein et al., 1998). The subsequent 1999 WHO classification (Mostofi, Davis, and Sesterhenn, 1999) stratifies HGPUC into 2 further classes (WHO II & III) and these systems are compared in Table 1.2 The latest WHO classification (2004) is identical to the 1998 WHO/ISUP classification (Lopez-Beltran and Montironi, 2004b). However, it is not yet clear if these latest classifications will improve correlation with histological features and tumour behaviour (Holmang et al., 2001; Lopez-Beltran and Montironi, 2004; Oosterlinck, 2001). Therefore, all TCC in this study were subjected to the 1973 WHO classification.

Table 1.2 Summary of WHO (1973, 1998, 1999) grading classification

A comparison between systems adapted from Holmang (Holmang et al., 2001) and (Lopez-Beltran and Montironi, 2004).

<table>
<tr><th>WHO 1973</th><th>WHO 1999</th><th>WHO/ISUP 1998 (WHO 2004)</th></tr>
<tr><td>Grade 1</td><td rowspan="2">PNLMP
Grade I</td><td>PNLMP</td></tr>
<tr><td rowspan="2">Grade 2</td><td>Low grade</td></tr>
<tr><td>Grade II</td><td rowspan="2">High grade</td></tr>
<tr><td>Grade 3</td><td>Grade III</td></tr>
</table>

Several authors have devised systems to stratify this risk and identify the characteristics of TCC of differing clinical phenotypes (Allard et al., 1998; Kurth et al., 1995; Millan-Rodriguez et al., 2000). Most recently, Sylvester *et al* have completed a combined analysis of over 2500 patients who had participated in EORTC phase III trials involving Ta/T1 TCC between 1979-89 (Sylvester et al., 2006). Six prognostic parameters were identified: tumour number, tumour size, prior recurrence rate, T stage, grade and cis, which were subjected to statistical modelling to generate a weighted scoring system that stratified the Ta/T1 TCC into 4 risk groups. The average risk for recurrence and progression was calculated for each group. The lowest-risk tumours were associated with a recurrence rate of 31%, and a progression rate of 0.8% at 5 years compared to 78% and 45% respectively for the highest-risk tumours.

These clinical parameters of TCC are used to stratify individuals into groups of differing risk for progression and recurrence, and form the basis for management. However the association between these parameters and behaviour of an individual TCC may be poor. It would be greatly beneficial to accurately identify those tumours

that will progress to allow targeting of additional anti-tumour therapy, whilst sparing the majority of patients the morbidity of unnecessary procedures.

1.1.6 Diagnosis of recurrent TCC

Although only 10-15% of Ta/T1 tumours progress to muscle invasive disease, 50-60% recur following initial resection, some after many years of apparent quiescence (Heney et al., 1983; Lutzeyer, Rubben, and Dahm, 1982). Most patients require a prolonged period of surveillance and this is usually performed with a flexible fibreoptic instrument under local anaesthesia in a day-case or office environment. Less frequently these "check-cystoscopies" utilise a rigid instrument, usually under a general anaesthetic or regional anaesthetic block. Traditionally, both instruments illuminate the bladder with white light.

Recently, the technique of photodynamic diagnosis (PDD) has been developed to enhance the performance of cystoscopy. The principle is based upon the interaction between a fluorochrome (eg. 5-aminolaevulinic acid), with a high selectivity for tumour cells, re-emitting light of appropriate wavelength at a longer wavelength, thus demarcating tumour and normal tissue (Zaak et al., 2005). White-light cystoscopy was believed to approach 100% sensitivity and specificity in the diagnosis of recurrent disease and is the standard against which other surveillance techniques are measured. However, clinical studies comparing white-light cystoscopy and PDD have consistently reported that while PDD was associated with reduced specificity, i.e. a high number of false positive diagnoses of TCC, it identified TCC within bladders considered normal in up to 20% of conventional cystoscopies. In particular, many flat high-grade lesions became apparent only with PDD (Zaak et al., 2005).

It is important to remember that to determine a test's sensitivity the true status of each subject tested must be known. Thus a careful histological examination of the entire bladder, following a major surgical resection would be required to determine the true frequency of TCC in such studies, which is clearly impractical. Moreover, in most studies assessing TCC-biomarkers (including this) the sensitivity of the putative marker is determined by the proportion of tumours diagnosed compared to

cystoscopically-diagnosed tumours. This is usually with white light cystoscopy, which has been shown to be imperfect. As the vast majority of studies determine the “true status” of TCC in this way, we can usefully compare different markers, but we must recall that the quoted sensitivity values are only relative, and will be (slightly) lower in reality.

Cystoscopy is expensive, invasive, uncomfortable and may provoke urinary infection (Burke, Shackley, and O'Reilly, 2002) and so the benefits of repeated endoscopic procedures in terms of identifying recurrent disease must be balanced by its morbidity and burden upon healthcare systems. There is no consensus as to the appropriate form and schedule of cystoscopic surveillance. In the USA, cystoscopic monitoring is recommended at least 3–6 monthly for the first 3 years after diagnosis and annually thereafter (Scher et al., 1998). The European Association of Urology guidelines (http://www.uroweb.org) suggest cystoscopy at 3 and 9 months then annually for low risk TCC. For high-risk TCC the recommended schedule is every 3 months for 2 years, every 4 months for the third year, every 6 months for the fourth and fifth years and annually thereafter. Unsurprisingly, a postal survey of UK Urologists suggests there is wide variation in current practice (Wazait et al., 2003).

To avoid the morbidity and expense of recurrent cystoscopy, a non-invasive alternative is highly desirable. Currently, urinary cytology is the only established non-invasive adjunct to endoscopy but is dependent on skilled personnel and is operator dependent. Furthermore, although cytology is sensitive (70-80%) and highly specific (90-95%) for diagnosing high-grade disease including carcinoma *in situ*, sensitivity is as low as 6-38% for well-differentiated lesions (Bastacky et al., 1999; Landman et al., 1998; Lokeshwar and Soloway, 2001).

Several non-invasive tests are also used to assess the presence of TCC. Recent developments have led to FDA approval (at the time of writing) for 5 assays detecting TCC biomarkers in urine (see Chapter 1.5.2). However there is no consensus as to their efficacy and role in TCC surveillance and their use is uncommon in the UK at present.

1.2 Biomarkers of TCC

Chapter 1.1, above, describes failings in current practice to accurately predict TCC prognosis from clinical parameters and diagnose recurrent disease without invasive cystoscopy. Advances in the understanding of tumour biology have provided new approaches to assess bladder cancers. Patterns, levels and interactions of a wide number of molecules and genes within tumour cells have been studied. Many of these have been correlated with TCC progression and recurrence in order to supplement clinical parameters. If such "biomarkers" reliably predicted the prognosis of an individual, they would improve the targeting of therapy. Similarly, an easily-measured biomarker associated with the presence of TCC would reduce the need for repeated cystoscopic surveillance providing it had adequate sensitivity and specificity.

Biomarkers have been defined as "cellular indicators of the physiological state and also of change during a disease process [and reflect] the molecular signature of a cell" (Srinivas, Kramer, and Srivastava, 2001). The utility of a biomarker lies in its ability to provide early indication of disease (or disease progression) and thus it is hoped that the timeliness of this knowledge will allow intervention that will positively affect the disease outcome. Some markers indicate increased risk, and are based mainly upon inherited or somatically acquired susceptibilities to certain tumours, such *BRCA1* and *BRCA2* gene mutations associated with an increased incidence of breast cancer, or indeed acetylator status of the N-acetyltransferase-2 gene associated with TCC incidence. More usually, biomarkers aim to identify established tumours before a clinical diagnosis of disease presence or behaviour can be made. A widely used example is prostate specific antigen (PSA) that is used to detect prostate cancer diagnosis and recurrence after treatment and in some cases predict tumour stage and treatment response. Although its use is widespread, PSA is not an ideal biomarker with only moderate sensitivity and specificity for prostate cancer diagnosis.

There are several challenges that must be overcome to develop a useful biomarker (Pritzker, 2002; Srinivas, Kramer, and Srivastava, 2001). Biological heterogeneity in a patient population suggests that relevance of a biomarker test result is unique for each individual. Moreover, the heterogeneous population of cells forming the tumour tissue

suggests it is unique for each tumour cell-group. The biomarker must possess high sensitivity and specificity (dependant on the clinical setting) and the assay must be relatively cheap and easy to perform, with minimal morbidity and inconvenience to the patient. These attributes have been previously described in relation to screening tests (Wilson and Jungner, 1968).

Diagnostic tests can be classified as having either positive or negative results. Even those tests that have a continuous distribution can be defined as having an arbitrary cut-off point distinguishing positive from negative. Subjects undergoing a test for a putative biomarker either have or do not have an underlying disease or condition, and the relationship between these debatables can be most elegantly expressed by a two-by-two contingency table. The sensitivity of a test is the proportion of people with the disease who have a positive test result, the higher the sensitivity, the greater the detection rate and the lower the false negative rate (Table 1.3).

The specificity of the test is the proportion of people without the disease who have a negative test. The higher the specificity, the lower will be the false positive rate and the lower the proportion of people who have the disease who will be unnecessarily worried or exposed to unnecessary treatment.

A perfect biomarker would identify the disease as described with 100% accuracy. That is, the biomarker test would identify 100% of the true cases of the disease, i.e. it has 100% sensitivity. Additionally a biomarker test should never wrongly predict disease where it is not present, i.e. it has 100 % specificity. It follows that a positive test result from such a test is 100% predictive of the presence of the disease, i.e. the test has a 100% positive predictive value (PPV), and a negative result is 100% predictive of the absence of the disease, i.e. it has a 100% negative predictive value (NPV). These parameters are routinely used to describe putative biomarker assays.

	Actual status	
Result of biomarker test	**Disease positive** (a+c)	**Disease negative** (b+d)
Test positive; (a+b)	True positive (a)	False positive (b)
Test negative (c+d)	False negative (c)	True negative (d)

Table 1.3 Two by two contingency table notation for expressing the results of validation study for diagnostic or screening test. Test performance can be calculated: Sensitivity = a/a+c; Specificity = d/b+d; Positive predictive value (PPV) = a/a+b; Negative predictive value (NPV) = d/c+d

1.3 Genetics-derived prognostic tissue-biomarkers of TCC

The divergent clinical phenotypes of early TCC and alteration in phenotype seen with tumour progression are underpinned by genetic events including the loss of tumour suppressor genes and the activation of proto-oncogenes. In addition to understanding the mechanism of disease process, the differential expression of these genes and their proteins may provide prognostic information to predict the progression of individual tumours. The identification and characterisation of these genes have been undertaken using techniques including mutation screening, loss of heterozygosity (LOH) analysis, comparative genomic hybridisation (CGH) and classical cytogenetics.

1.3.1 Cytogenetics of TCC

The cytogenetic alterations identified in TCC have been comprehensively reviewed and demonstrate the heterogeneity and complexity of TCC karyotypes (Gibas and Gibas, 1997; Knowles, 1999; Sandberg, 2002). Structural abnormalities of chromosomes 1, 3, 5, 8, 9, 11, 13 and 17 have been identified with G (giemsa stain)-banding studies. A recent study additionally demonstrated losses of parts or all of chromosomes 1p, 9p, 9q, 11p, 13p and 17p, loss of an entire copy of chromosomes 9, 14, 16, 18 and Y and gains of chromosome arms 1q and 13q and of chromosomes 7 and 20 (Fadl-Elmula et al., 2000).

CGH analysis allows a more precise demarcation of genetic alterations within the whole genome. In brief, the DNA sequence copy number along each chromosome for the entire genome is identified using differentially labelled test DNA and normal reference DNA hybridised simultaneously to normal metaphase spreads. Changes in the ratio of intensities of the two fluorochromes indicate gain or loss of material. Many authors from the Institute of Pathology and University of Basel, Switzerland (Richter et al., 1998; Richter et al., 1997; Richter et al., 1999; Zhao et al., 1999) and others (Hovey et al., 1998; Simon et al., 1998) have demonstrated similar findings, summarised in Table 1.4.

Table 1.4. Summary of CGH findings in TCC

From Knowles 2001

Tumour stage	Losses	Gains	Amplifications
Ta	9p, 9q, Y	1q, 17	11q
T1	2q, 4p, 4q, 5q, 6q, 8p, 9p, 9q, 10q, 11p, 11q, 13q, 17p, 18q, Y	1q, 3p, 3q, 5p, 6p, 8q, 10p, 17q, 20q	1q22-24, 3p24-25, 6p22, 8p12, 8q12, 10p12-14, 10q22-23, 11q13, 12q12-21, 17q21, 20q13
T2-4	As for T1 + 15q	As for T1 + 7p, Xq	As for T1

All studies have commented upon the variability of changes within individual TCC, except for the striking consistency of chromosome 9 losses. While large genetic alterations between stages are apparent (although not universally identified (Richter et al., 1998)), any differences within each stage, associated with grade or prognosis are subtle. The genetic changes within T1 TCC are much closer to those of T2 TCC in contrast to Ta TCC, supporting the view that T1 are “invasive TCC”. The number of total aberrations detected increases with loss of differentiation for Ta and T1 TCC (Richter et al., 1999; Simon et al., 1998; Zhao et al., 1999) however

overrepresentation of 6p22 was the only significant individual aberration for T1 tumours (Richter et al., 1999). Specific chromosomal imbalances including 2q-, 5p+, 5q-, 6q-, 8p-, 10q-, 18q- and 20q+ were seen significantly more frequently in G3 verses G2 Ta TCC (Zhao et al., 1999). These findings correlate with the diverse clinical phenotypes of Ta TCC. Improved mapping studies of the TCC genome will be possible with the recent development of high-resolution CGH arrays (Hurst et al., 2004).

1.3.2 LOH in TCC

Additional evidence for the loci of critical genes lost in the development of TCC is provided by LOH analyses (reviewed by Knowles, 2001; Orntoft and Wolf, 1998)). LOH studies commonly utilise microsatellite marker polymorphisms. These highly variable short stretches of DNA contain multiple repeats of a 2-5 nucleotide sequence of DNA such as the (CA)n dinucleotide sequence. The polymorphic marker sequence length is frequently different in homologous chromosomes. This generates heterozygosity. Allelotypes provide a screen of markers on all chromosome arms in a particular tumour type. An allelotype of 83 TCC identified the most frequent sites of allelic loss as 9p (51%), 9q (57%), 11p (32%), 13q (15%), 17p (32%), 4p (22%) and 8p (23%) (Knowles et al., 1994). LOH on chromosome 9 was seen in all stages and grades of tumour but LOH of the other chromosomes was associated with high stage and grade only. The losses identified by LOH and CGH broadly correlate.

1.3.3 Oncogenes in TCC

A proto-oncogene, a gene with important regulatory actions upon cellular function may become erroneously activated and function as an oncogene. Activation may occur by point mutation, gene amplification or translocation. Several specific oncogenes have been identified in the development of TCC.

1.3.3.1 Ras genes

The *RAS* gene family (initially described in RAt Sarcomas) has been implicated in the development of many tumours notably pancreatic and colon where *KRAS* is often point mutated within codons 12, 13 and 61. Its protein product, p21, participates in the mitogen activated protein kinase (MAPK) signalling pathway that modulates various cellular functions including apoptosis and the cell cycle (reviewed by Macaluso et al., 2002). *HRAS* mutations (11p15) have been identified in up to 10% of TCC (Fujita et al., 1984). However evidence correlating RAS expression and TCC phenotype is contradictory, particularly when mutations are seen in only a small fraction of TCC. (Fujita et al., 1984; Knowles and Williamson, 1993; Malone et al., 1985; Theodorescu et al., 1990). Recently a screen of 98 bladder tumours identified mutations in all three *RAS* genes including *KRAS* and *NRAS* (not previously associated with TCC) in 13% of tumours (Jebar et al., 2005).

1.3.3.2 FGFR3

Fibroblast Growth Factors (FGF) and their receptors have the ability to stimulate wound healing, embryonic development, angiogenesis, chemotaxis, cell differentiation and mitogenesis (Johnson and Williams, 1993). Several of the FGF receptors and their large family of ligands have been implicated in the pathogenesis of TCC (reviewed by Munro and Knowles, 2003). In particular, mutations in *FGFR3* (4p16) were identified in 30-40% of all TCC (Billerey et al., 2001; Cappellen et al., 1999). It is proposed that the mutation S249C accounting for 70% of *FGFR3* mutations results in ligand independent dimerisation and thus constitutive activation. Interestingly, *FGFR3* mutation is associated with low stage disease and a reduced recurrence rate. In one study of the recurrence rate in 57 patients with superficial disease followed prospectively by cystoscopy for 12 months, 14 of 23 patients in the wild-type *FGFR3* group developed recurrent bladder cancer compared with only 7 of 34 patients in the mutant group ($P = 0.004$) (van Rhijn et al., 2001).

1.3.3.3 ERBB2

The epidermal growth factor receptor (EGFR) tyrosine kinases include 4 members: B1-B4, which bind soluble ligands including epidermal growth factor (EGF) and transforming growth factor alpha (TGFα). EGFRs activate numerous intracellular pathways such as MAPK and phosphatidylinositol-3 kinase (PI-3K) pathways. This leads, in turn, to increased cell proliferation and cell survival and other aspects of the transformed phenotype (Holbro, Civenni, and Hynes, 2003). Amplification of *ERBB2* (also known as HER2/Neu) and increased expression of its protein, ERBB2 (B2-EGFR) has been identified in 6-26% of TCC series (Coombs et al., 1991; Sato et al., 1992; Sauter et al., 1993). In contrast to FGFR3, increased ERBB2 expression is associated with increased tumour grade and stage. Several studies have assessed the role of ERBB2 as a prognostic biomarker, some have found it predictive of disease progression and recurrence (Sato et al., 1992) however others have found it predictive for disease survival only (Underwood et al., 1995). A monoclonal antibody against ERBB2 (trastuzumab, Herceptin®) has been shown to improve the survival of women with metastatic breast cancer (Slamon et al., 2001). Evidence from these studies suggests only tumours with *ERBB2* amplification respond to trastuzumab treatment. A more recent IHC assessment of a panel of 75 muscle-invasive TCCs showed only 5% expressed this amplification, and so questioned the potential utility of trastuzumab treatment in TCC (Latif et al., 2004). As yet no clinical trials with this agent for TCC have been published.

Increased EGFR protein expression has been widely reported in bladder cancer and has been associated with adverse disease characteristics and behaviour. In a cohort of 101 subjects with bladder TCC, 48% were identified as positive for EGFR by IHC. All those staining positively for EGFR were associated with an increased risk of death ($p<0.0001$), and of Ta/T1 TCC, time to recurrence was reduced ($p<0.03$) and the risk of progression increased ($p<0.0001$) (Neal et al., 1990). In a further study of 212 TCC with a mean follow up of 26 months, EGFR status was an independent predictor of progression. In those with G3 T1 TCC, EGFR positivity correlated highly with progression to a muscle invasive tumour, equivalent to a biomarker with a sensitivity of 80% and specificity of 93% (Mellon et al., 1995).

1.3.3.4 Cyclin D1

Cyclin D1 (11q13), in combination with cyclin dependent kinases (cdks) is essential for G1/S phase transition within the cell cycle (Sherr, 1996). Cyclin D1 is over-expressed in several cancer types resulting from amplification and translocation. Amplification of 11q13 is only seen in 10-15% of TCC (Bringuier et al., 1996) yet about 50% of all TCC show increased expression (Shin et al., 1997; Tut et al., 2001). Cyclin D1 expression studies in TCC are conflicting. Several studies have demonstrated significantly higher cyclin D1 expression in low-stage, well differentiated tumours (Suwa et al., 1998; Tut et al., 2001), and tumours with low expression have a more aggressive clinical course (Bringuier et al., 1996). However, strong IHC staining for cyclin D1 has been associated with early recurrence of TCC (Shin et al., 1997) and a further large survey found no prognostic significance of increased cyclin D1 protein levels at all for pTa/T1 TCC (Wagner et al., 1999).

1.3.3.5 MDM2

The *MDM2* gene (12q14) is amplified in about 7% of all human cancers, and the associated protein can also be over-expressed by increased transcription or translation. It has a ubiquitin ligase function and it regulates p53 by promoting its degradation, blocking its transcriptional activational activity and exporting it to the cytoplasm (reviewed by Alarcon-Vargas and Ronai, 2002). *MDM2* gene amplification is found in only 1-4% of TCC (Habuchi et al., 1994; Lianes et al., 1994), however increased protein expression has been reported in 29-49% of TCC (Lianes et al., 1994; Schmitz-Drager et al., 1997). Prognostic information from studies assessing MDM2 protein levels is contradictory. It was shown to predict low stage, low grade TCC (Lianes et al., 1994) but not disease recurrence (Pfister et al., 1999).

1.3.4 Tumour suppressor genes in TCC

Tumour suppressor gene (TSG) products are needed for normal cell function. Their loss contributes to the development of cancer. They may act by directly controlling cellular proliferation and thus keeping tumour growth in check or by indirectly suppressing tumour development by involvement in DNA repair, replication and

recombination and by reducing genomic instability. These functions have been described as “gatekeeping” and “caretaking” respectively (Kinzler and Vogelstein, 1996). Loss of both alleles at a TSG locus is generally required for phenotypic effect, while retention of one normal allele will generally prevent tumour development. This “Two hit hypothesis” suggests an inactivating mutation (often point mutation) of one allele (inherited or somatic) and often a loss of the second copy of the gene by deletion, transcriptional silencing, chromosomal non-disjunction, mitotic recombination, gene conversion or translocation (Knudson, 2001). Several TSGs have been implicated in the development of TCC, identified by both CGH and LOH studies. These tend to fall into two categories; those concerned with the cell cycle such as *TP53*, *RB1* and *CDKN2A* (p16, p14) that have been identified in other cancers and well studied, or genes identified within critical regions of LOH in bladder cancer such as *TSC1* and *DBC1* which are not known to be involved in other sporadic cancers.

1.3.4.1 TP53

The *TP53* gene (17p13.1) codes for a 53 kDa phosphoprotein, p53 and its loss is thought to contribute to more than half of human cancers (Hollstein et al., 1991). Functions of *TP53* are complex but its protein increases in activity in response to a variety of genotoxic insults and results in cell cycle arrest or apoptosis. Detection of *TP53* mutation may be assessed by direct molecular methods (eg. single strand conformation polymorphism analysis [SSCP] and sequencing) or indirectly by IHC. A concordance of approximately 90% has been demonstrated between both approaches (Cordon-Cardo et al., 1994). Wild type p53 in normal cells cannot usually be detected by IHC, however many mutations increase the protein’s stability and lead to its accumulation and detection. This relationship is made more complex as wild type p53 may also be detected by IHC if abnormally expressed or bound to increased levels of other cell-cycle proteins, such as MDM2 (Hall and Lane, 1994).

The loss of normal p53 function in TCC was first reported in 1991 (Sidransky et al., 1991; Wright et al., 1991) and many investigators have subsequently sought to assess the prognostic value of p53 protein expression in bladder tumours. These studies have

been carefully assessed by several authors (Keegan, Lunec, and Neal, 1998; Schmitz-Drager et al., 1997; Smith et al., 2003). Most studies have shown that p53 staining is positively correlated with increasing grade and stage of TCC, although fewer identify it as an independent marker by multivariate analysis. Keegan and Schmitz-Drager independently suggest that the heterogeneity of the methods of IHC, outcome measures, patient selection and follow-up makes overall interpretation of most published trials difficult. Recently, a study was published that attempted to overcome many of these limitations. Tissue from a Medical Research Council trial that recruited 502 patients with a median follow-up of 10 years was subjected to p53 IHC (Masters et al., 2003). In agreement with many of the earlier studies, p53 immunostaining had prognostic significance in patients that had progressed to advanced TCC (pT2+, metastases or death) compared with matched controls. The adjusted hazard ratio for time to progression for p53 IHC positive versus negative group was 2.5, with 95% confidence intervals of 1.05-5.98 (p=0.039). However the authors concluded that neither the sensitivity nor the specificity of association of p53 staining with progression was sufficient to recommend cystectomy in individual patients.

1.3.4.2 RB1

The retinoblastoma gene *RB1* (13q14) was the first tumour suppressor gene to be identified (Friend et al., 1986). Its central role in regulating the cell cycle at the G_1-S transition is complex and continues to be elucidated (Classon and Harlow, 2002). Reduced expression of the Rb protein is seen in many tumours of high grade and stage (Cairns, Proctor, and Knowles, 1991; Ishikawa et al., 1991). Fluorescent multiplex PCR analysis of 236 TCC cold-cup biopsies showed LOH of loci in the region of *RB1* increased with both TCC grade and stage (Wada et al., 2000). Reduced Rb protein levels measured by IHC correlate with advanced disease and reduced survival (Wada et al., 2000; Xu et al., 1993).

Several studies have assessed Rb status in combination with several other cell cycle regulators. Cordon-Cardo et al assessed 59 Ta/T1 TCC Rb and p53 levels by IHC (Cordon-Cardo et al., 1997); the rate of progression and death was associated with abnormal levels of both proteins, but their correlation was much greater when

combined. More recently, 164 archival radical cystectomy samples were stratified on the basis of TP53, p21 and RB1 expression by IHC. In those with zero, one, two or all the proteins altered, the 5-year survival rates were 70%, 58%, 33% and 8% respectively (log rank $p< 0.001$) (Chatterjee et al., 2004). A similar study of 80 cystectomy specimens undergoing IHC for TP53, p21, RB1 and p16 showed similar results (Shariat et al., 2004).

1.3.4.3 PTEN

PTEN (10q23) was identified in 1997 as a tumour suppressor gene. It functions as a lipid phosphatase, in opposition to phosphatidylinositol 3-kinase (PI3K), regulating signal transduction pathways and is involved with embryonic development, cell migration and apoptosis (Yamada and Araki, 2001). LOH at 10q23, gene mutation, or reduced expression is seen with cancers of the breast, prostate and ovary and in glioblastoma and melanoma (Simpson and Parsons, 2001). Studies examining bladder cancers of differing grade and stage have identified LOH at the *PTEN* locus in 6.6-20% Ta/T1 and 29-58% in T2+ TCC (Aveyard et al., 1999; Cairns et al., 1998; Cappellen et al., 1997). However, identified mutation rates are much lower and it has been proposed that either the retained copy is transcriptionally silenced or that loss of a single copy of the allele may be enough to lose sufficient gene function to have a phenotypic effect (Aveyard et al., 1999). Recently, a panel of 29 (Ta/T1, n=11, T2+, n=18) TCC were subjected to Western blotting to measure *PTEN* protein expression. Four patients showed a significant reduction in PTEN protein, of which 3 were muscle invasive (Koksal et al., 2005).

1.3.4.4 CDKN2A (p16, p14)

The *CDKN2A* gene is located at 9p21 which is a region of early deletion in the development of TCC. It encodes two structurally unrelated proteins, CDKN2A/$p16^{INK4a}$ and ARF/$p14^{ARF}$ (henceforward referred to as p16 and p14 respectively) that function as cyclin dependent kinase inhibitors in the regulation of the cell cycle. These genes are inactivated in several human tumours including haematological, skin (especially melanoma), lung, oesophageal and bladder (Ruas and

Peters, 1998). In a panel of 110 TCC, the frequency of p16 genomic alterations was 18%, and the mechanism of loss included LOH, hemizygous and homozygous deletions, but no point mutations (Orlow et al., 1995). Loss of p16 protein was seen in 29% of another series of 139 TCC, and increased loss was associated with advanced stage but not grade (Korkolopoulou et al., 2001). In a third series of 79 TCC and normal bladder mucosa specimens the expression of p16 protein fell from 100% in normal tissue to 40.3% in TCC and was reduced in the invasive tumours (Yang et al., 2002). In contrast Droller *et al* examined a series of 18 Ta and 22 T1 TCC for genomic alterations and protein expression of p16 and correlated these data to clinical outcome. LOH was seen in 37% of TCC with no evidence of homozygous deletion or point mutation. IHC revealed reduced p16 expression (<5% positive nuclei) in 25% patients; 75% patients had a positive reaction (>=5% positive nuclei) and 25% patients a strong positive reaction (>=50% positive nuclei). There was no correlation between any p16 parameter and clinical stage, grade, invasion, recurrence or outcome (Friedrich et al., 2001). Recently, this laboratory has subjected 50 microdissected TCC to comprehensive analysis to detect all forms of inactivation of *CDKN2A.* Exon 2 was underrepresented in 43% and exon 1beta (p14) in 46% of cases associated with LOH of 9p in 37% (Chapman et al., 2005).

1.3.4.5 TSC1

Another critical region 9q34, lost in 50-60% of all TCC, encodes the tuberous sclerosis gene (*TSC1*). *TSC1* is associated with an autosomal dominant disease Tuberous Sclerosis Complex (TSC), characterised by hamartomatous growths in multiple organ systems. *TSC1* encodes the protein hamartin that interacts with the product of *TSC2*, tuberin. They act as integrators of the actin cytoskeletal network (Haddad et al., 2002) and play a role in the PI3K pathway (also affected by PTEN as above), that has been linked to the development of human malignancies (Jaeschke et al., 2002). TSC 1/2 function in a signal transduction pathway through the regulation of mTOR (Brugarolas and Kaelin, 2004). Loss of 9q34 is associated with development of ovarian, gallbladder and nasopharangeal tumours but to date mutations of TSC1 have been described only in bladder cancer (Knowles, Hornigold, and Pitt, 2003). A preliminary mutation screen of 36 TCC with 9q34 LOH identified 4

54% (R), p=0.035) (Edwards et al., 2002). Expression profiling of TSC1 protein in TCC has not been published to date. As data for this gene accumulates it will become easier to assess its utility as a prognostic biomarker of TCC.

1.3.4.6 DBC1

Another candidate TSG, *DBC1*, (previously designated *DBCCR1*) has recently been identified within 9q32-33, a region of critical deletion of chromosome 9 (Habuchi et al., 1998). To date, no mutations of *DBC1* have been identified in bladder tumours although the second allele may be inactivated by hypermethylation-based silencing (Habuchi et al., 2001). When DBC1 was over-expressed in NIH3T3 cells and bladder tumour cells, proliferation was suppressed by negative regulation of the cell cycle G_1/S transition (Nishiyama et al., 2001). This is good evidence for the TSG role of DBC1 in the absence of identified mutations. While DBC1 is expressed in the brain in high levels, and in normal urothelium, its function is unknown although it has been shown to bind the putative acid-spingomyelinase ASML3a. The function of this protein is also unclear but it has homology with sphingomyelinases that are closely involved in biological membrane function (Wright, Messing, and Reeder, 2002). It has been shown to mediate death of cultured TCC cells independent of classical apoptotic pathways (Wright, Messing, and Reeder, 2004). The relative novelty of this gene's discovery and the absence of a reliable antibody have prevented protein based expression analysis. It is hoped that future studies will elucidate any correlation with disease progression and allow assessment of its role as a putative biomarker for TCC.

1.3.5 Genetic pathways in TCC development

Genetic models of disease progression incorporating the evidence described above have been developed (Cordon-Cardo, 1998; Hoglund et al., 2001; Knowles, 1999; Richter et al., 1998; Sandberg, 1992; Spruck et al., 1994). Figure 1.1 identifies pathways by which early tumours may progress.

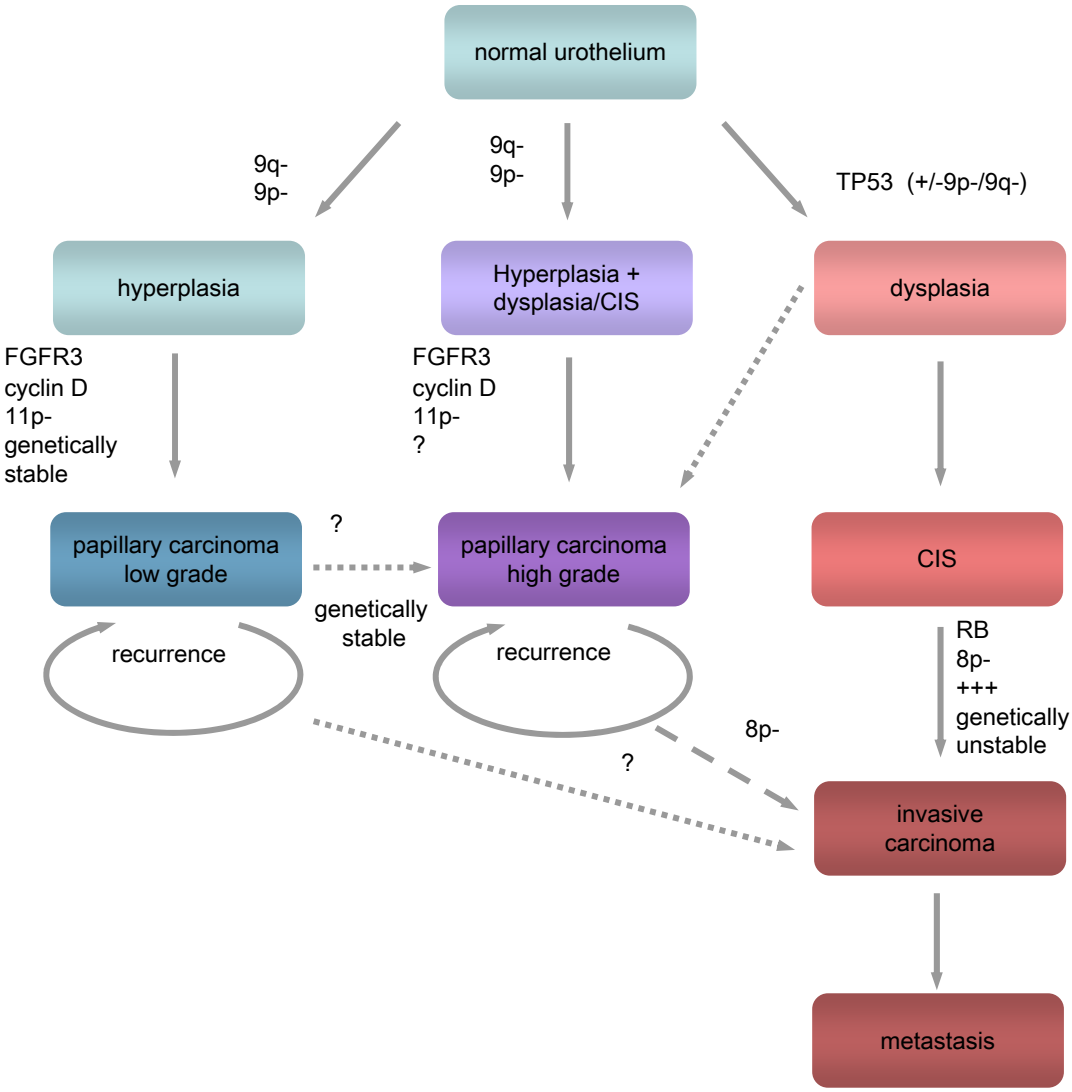


Figure 1.1 Potential pathways of TCC tumorigenesis.

From Knowles 2006.

1.4 Cell biology-derived prognostic tissue-biomarkers of TCC

In addition to putative biomarkers derived from observed mutations within TCC tumours, a multitude of molecules associated with TCC prognosis have been identified from the more general study of gene expresssion in these tumours that are summarised in Table 1.5. A recent article more comprehensively reviews prognostic markers of TCC (Duggan and Williamson, 2004).

1.4.1 Limitations of contemporary prognostic biomarkers of TCC

To date, these studies have not resulted in the development of clinical tools for routine practice. Most of the markers studied provide no additional information relative to grade and stage. Some do predict disease behaviour, such as survival or recurrence, but not as an independent factor in multi-variate analyses. The few molecules that have been demonstrated to predict disease behaviour in an independent manner (such as Ki-67 (Santos et al., 2003)) have a low predictive power. Even the most promising molecules such as FGFR3 and p53 appear to be incapable of informing upon the individual patient. Many of these studies are relatively small and differences in methodology make meta-analyses challenging. It is clear that applying classical genetic and cell biology techniques to profiling individual molecules or limited systems has not added significantly to clinical parameters in the assessment of TCC. This conclusion is similar to that reached by an international consensus panel that has recently comprehensively reviewed this issue (Habuchi et al., 2005).

Table 1.5 Summary of most reported prognostic biomarkers for TCC developed from cell biology studies.

Molecule	Putative Role(s)	Association with TCC prognosis	References
FGF1, FGF2	Growth and/or angiogenic factors	Associated with high stage	(Chopin et al., 1993; Gazzaniga et al., 1999; Nguyen et al., 1993)
VEGF		Variable association with stage	(Crew et al., 1999; Crew et al., 1997)
TGF-α		Associated with high stage	(Gazzaniga et al., 1998; Ravery et al., 1997; Thogersen et al., 2001)
Thrombospondin		Loss associated with progression and reduced survival	(Goddard et al., 2002; Grossfeld et al., 1997)
Ki-67	Proliferation antigens	Associated with progression and reduced survival	(Lianes et al., 1998; Mulder et al., 1992; Nakopoulou et al., 1998; Santos et al., 2003; Wu et al., 2000)
PCNA			(Blasco-Olaetxea, Belloso, and Garcia-Tamayo, 1996; Chen, Lin, and Li, 1997; Cohen et al., 1993)
Fas-ligand	Markers of apoptosis	Variable association with prognosis and survival	(Mizutani et al., 2001; Perabo et al., 2001)
Survivin			(Lehner et al., 2002; Nakanishi et al., 2002; Swana et al., 1999)
Bc12/bax			(Gazzaniga et al., 1996)
p21		Strong association with survival	(Lacoste-Collin et al., 2002; Primdahl et al., 2002)
Osteoprotegrin	Protects tumour cells from lymphocyte cytotoxicity	Raised levels correlated with stage, grade and early recurrence	(Mizutani et al., 2004)

Molecule	**Putative Role(s)**	**Impact upon TCC prognosis**	**References**
E-Cadherin	Cell adhesion molecules	Associated with grade, stage and poor survival.	(Popov et al., 2000; Shariat et al., 2001b; Shimazui et al., 1996)
CAMs, CD44			(Chow et al., 1998a; Miyake et al., 2002; Muller et al., 1997)
Laminin		Variable associations with all parameters	(Abou Farha et al., 1993b; Kirkali et al., 2001)
Cathepsin			(Dickinson et al., 1995; Ozer et al., 1999)
Urokinase plasminogen activator		Strong association with survival	(Hasui et al., 1996)
Motility-related protein 1 (CD9)		Associated with grade, stage and progression	(Mhawech et al., 2003)
Matrix metalloproteases	Proteolytic enzymes	Associated with high grade and stage	(Gohji et al., 1996; Ozdemir et al., 1999)
Cytokeratins 8,18,19	Structural proteins	Associated with high grade and stage and poor survival	(Silen et al., 2000)
Cytokeratin 20		Associated with recurrent, low-grade TCC	(Harnden et al., 1995; Harnden, Mahmood, and Southgate, 1999; Ramos et al., 2003)
Telomerase	Telomere maintainence	Associated with grade alone	(De Kok et al., 2000; Longchampt et al., 2003)
CEA	Oncofetal protein	Variable associations	(Feil et al., 1999; Jautzke and Altenaehr, 1982)

1.5 Biomarkers of *de novo* and recurrent TCC

Whereas it may be feasible to subject tissue from a tumour specimen to analysis to determine prognostic factors, there are potentially many more subjects that may undertake a biomarker test for diagnosis of *de novo* or recurrent TCC. Requiring tissue for each assay would be impractical, and it would be much better if TCC could be identified in a less invasive way from more easily accessible clinical material. The challenge of diagnostic TCC biomarker discovery is not necessarily to improve upon cystoscopic diagnostic accuracy, but to match its performance with a less invasive test. Many investigators target serum to measure putative biomarkers, although saliva, nipple aspirate and semen have also been used. The urothelium is bathed in urine that would appear to provide an ideal medium in which to search for novel TCC markers. Currently, urine cytology is the only established non-invasive adjunct to endoscopy. However, although cytology is sensitive (70-80%) and highly specific (90-95%) for diagnosing high-grade disease including carcinoma *in-situ*, sensitivity is as low as 6-38% for well differentiated tumours (Bastacky et al., 1999; Landman et al., 1998; Lokeshwar and Soloway, 2001).

The use of biomarkers to detect *de novo* cancers is a challenging task. The performance requirements for clinically useful diagnostic tests depend on the prevalence of the disease in the population sampled. The positive predictive power of a test of a given sensitivity increases with increasing incidence of the disease tested. Biomarkers that attempt to screen a large population for an uncommon disease require high sensitivity and an almost 100% specificity and are thus difficult to develop. Even prostate specific antigen (PSA), perhaps the most successful, widely-used biomarker ever developed, is associated with high false positive rates and its role in screening men for prostate cancer is unproven. The diagnosis of d*e novo* TCC would be similarly difficult, but as has been stated, one of the characteristics of bladder cancer is its tendency to recur. About 50% of patients undergoing endoscopic surveillance will suffer disease recurrence, resulting in a high prevalence of disease in this population. A putative biomarker to diagnose TCC with good but imperfect performance parameters would still have important clinical utility in diagnosing recurrent TCC (although would not be useful as a screening tool in the general

population). In comparison, PSA is successfully used to detect disease recurrence after radical therapy for prostate cancer, and in this context (unlike *de novo* diagnosis and screening) rising PSA is highly specific for recurrent cancer, as the prevalence of prostate cancer in this group is high.

1.5.1 Contemporary diagnostic urinary biomarkers of TCC

The last decade has seen an explosion of interest in the development of urinary markers of TCC, (reviewed by Burchardt et al., 2000; Duggan and Williamson, 2004; Fradet and Lacombe, 2000; Glas et al., 2003; Grossman and Dinney, 2000; Han and Schoenberg, 2000; Helpap et al., 2003; Kausch and Bohle, 2001; Konety and Getzenberg, 2001; Lokeshwar and Soloway, 2001; Lotan and Roehrborn, 2003; Orntoft and Wolf, 1998; Tiguert et al., 2002; van der Poel and Debruyne, 2001). In particular, van Rhijn provides a contemporary, clear systematic review of urinary biomarkers for bladder cancer surveillance (van Rhijn, van der Poel, and van der Kwast, 2005). Overall, they conclude that many markers have not demonstrated durable performance, others, however, are promising, but there is currently insufficient evidence to recommend their use outside of a clinical trial.

1.5.2 FDA approved urinary biomarkers of TCC

The use of these markers varies geographically and much of the development has been in the United States, where several assays are sold commercially with current FDA (www.fda.gov/cder) approval for TCC monitoring and diagnosis. Two tests, AuraTek and Accu-Dx tests for Fibrin-Fibrinogen degradation products are no longer produced, despite gaining FDA approval. From 1 April 2003, the Medicines and Healthcare products Regulatory Agency (MHRA) replaced the Medical Devices Agency (MDA) and the Medicines Control Agency (MCA) for the licensing of such diagnostic tests in the UK although there appears no reference to urine based tests for TCC in their published work (http://www.mhra.gov.uk). Additionally a large number of putative markers are under investigation in formal clinical trials or on a more *ad hoc* basis.

1.5.2.1 Nuclear Matrix Protein (NMP22) tests

The nuclear matrix anchors enzymatic machinery involved in DNA replication, transcription, RNA processing and gene expression (Berezney, 1991; Berezney and Coffey, 1974). NMP22 distributes chromatin among daughter cells during mitosis and it is suggested that it is released from the nuclei of tumour cells during apoptosis. Generally, higher levels of NMP22 are seen in the urine of those with bladder cancer compared to those without. The protein may be detected in urine by an ELISA kit – NMP22® or more recently a point of care NMP22 BladderChek™ immunoassay (Matritech, Newton, MA). Both have been approved for the diagnosis and monitoring of bladder cancer by the FDA. The prognostic value of the test is limited. Urinary NMP22 levels were unrelated to grade or stage in a series of 175 TCC samples (Carpinito et al., 1996) although recently such an association was demonstrated in a series of 92 TCC (Boman et al., 2002). A correlation with outcome data for tissue or serum samples has not been identified although there is a report of increasing urinary NMP22 as a predictor of recurrence independent of grade or stage (Poulakis et al., 2001).

Two recent systematic reviews and meta-analyses of NMP22 performance have been published (Glas et al., 2003; Lotan and Roehrborn, 2003). Glas et al report a comprehensive survey of all trials of suitable methodological quality between 1990 and 2001, including 14 trials (total population 2,290) assessing the NMP22 ELISA. Sensitivity was 67% (60-73: 95%CI) and specificity 78% (72-83: 95% CI). Similarly Lotan *et al*, using less stringent criteria, identified 15 trials from 1966-2001 with a median sensitivity of 73% (47-87: 95% CI) and specificity of 80% (58-91; 95% CI). Despite excellent attempts to overcome the inherent difficulties in assessing heterogeneous studies, often with different cut-off values for TCC diagnosis, the limits of these analyses must be remembered. Numerous (often small) studies have been published in the last 5 years which have reported sensitivities of 31-81% at specificities of 73-95% which give broadly similar results allowing for differing cut-off values (eg. Boman et al., 2002; Friedrich et al., 2002; Mahnert et al., 2003; Poulakis et al., 2001; Saad et al., 2002). The median sensitivities calculated by another systematic review of trials up to 2004 are 41, 53 and 80 % for G1, G2 and G3

TCC respectively, with a median specificity of 59% (van Rhijn, van der Poel, and van der Kwast, 2005).

NMP22 BladderChek™ has recently been shown to increase the diagnosis rate of recurrent TCC (Grossman et al., 2006). Over 700 consecutive patients with a history of bladder cancer underwent cystoscopy, the NMP22 assay and a biopsy where indicated. Of the 103 TCC identified, 94 were identified at cystoscopy. The NMP22 assay identified 8 of the 9 TCC not identified at cystoscopy, in comparison to 3 of 9 by urine cytology. This study is open to criticism in that tumours were classified as "missed" at cystoscopy if they were seen at cystoscopy up to 5 months subsequently; an imperfect method of determining the true disease state from which to assess test performance. However, using such a biomarker to improve cystoscopic performance warrants further consideration.

It is reported by many studies that inflammatory processes within the bladder, such as UTI or stone disease give rise to a high rate of false positive results (reviewed by Konety and Getzenberg, 2001). Control groups with varying levels of such conditions further hinder direct comparison of studies. Excluding patients with a known inflammatory process increased the test specificity to 95.6% (Sharma et al., 1999) and this is the current test protocol recommended by the assay manufacturers; Matritech.

1.5.2.2 Bladder Tumour Antigen (BTA) Tests.

The original bladder tumour antigen was a basement membrane protein antigen released into the urine upon stromal invasion by TCC. Its performance assessed by meta-analysis of 661 subjects demonstrated a disappointing sensitivity of 50% and specificity 79% and it is no longer used routinely (Glas et al., 2003). The BTA Stat®, a point of care test and BTA TRAK® an ELISA (Polymedco, previously Bard Diagnostics, Redmond, WA) detect a human complement factor H-related protein. This factor is also known as the bladder tumour associated antigen. It is produced by many TCC cell lines but not other epithelial cell lines and is believed to suppress the host anti-cancer immune response.

A meta-analysis by Glas *et al* (2003) examined the performance of BTA stat and reported a sensitivity of 70% (66-74: 95% CI) with a specificity of 75% (64-84: 95% CI) (n=1,160) and for BTA TRAK sensitivity of 66% (62-71%) with specificity of 65% (45-81: 95% CI) (n=829). The analysis by Lotan *et al* (2003) of a similar number of subjects calculated BTA stat sensitivity of 71% (57-82:95% CI), specificity of 73% (61-82: 95% CI) and BTA TRAK sensitivity of 69% (55-80: 95% CI) and specificity of 90% (38-98: 95% CI). The similar values for BTA stat reflect its qualitative nature, while the quantitative-ELISA allows adjustment of the cut-off value and hence gives more variable results. Many studies published in the last 5 years have assessed the performance of BTA stat (Babjuk et al., 2002; Friedrich et al., 2002; Halling et al., 2002; Quek, Chin, and Lim, 2002) and BTA TRAK (Babjuk et al., 2002; Gibanel et al., 2002; Mahnert et al., 2003). The results are again in line with those predicted from the earlier analyses. For instance, a study of 265 subjects comparing a telomerase assay, haemoglobin dipstick and BTA stat urinary test reported a sensitivity for BTA stat of 72% and a specificity of 74% (Halling et al., 2002). The median sensitivity calculated by another systematic review of trials up to 2004 is 55, 59 and 74 % for G1, G2 and G3 TCC respectively, with a median specificity of 59% (van Rhijn, van der Poel, and van der Kwast, 2005). The level of BTA recorded by the BTA TRAK test was associated with grade and stage (Ellis et al., 1997). Unfortunately, false positive results are often obtained with inflammatory genito-urinary conditions, instrumentation and immunotherapy (Konety and Getzenberg, 2001), and particularly when haematuria is present, as the complement protein is found in normal serum (Lokeshwar and Soloway, 2001).

1.5.2.3 Immunocyt

Recently Immunocyt (DiagnoCure Inc. Sainte-Foy QC, Canada) has gained FDA approval for the monitoring of bladder cancer. The assay comprises three monoclonal antibodies: 19A211, labelled with Texas Red immunoflouresent dye, binds to a high molecular weight form of carcinoembryonic antigen (CEA) and M344 and LDQ10, labelled with flourescein, bind to mucin glycoproteins. Exfoliated cells within the urine are stained for these epitopes in combination with cytological examination. The

assay was initially reported in 1997 (Fradet and Lockhard, 1997) and has been subjected to several trials since. Mian (Mian et al., 1999) evaluated 264 subjects with Immunocyt and subsequent cystoscopy and diagnosed TCC with 86% sensitivity and 79% specificity while Olsson (Olsson and Zackrisson, 2001) reported 100% sensitivity with 68% specificity from a survey of 121 subjects. These promising results have not been confirmed in other studies, which identified TCC in only 14-50% of Ta/T1 TCC at specificities of 73-84% (Feil et al., 2003; Vriesema et al., 2001). The median sensitivity calculated by another systematic review published in 2005 (albeit of 5 trials with 51 patients only) is 78, 90 and 100 % for G1, G2 and G3 TCC respectively with a median specificity of 59% (van Rhijn, van der Poel, and van der Kwast, 2005). Of those biomarker assays with FDA approval, this seems most promising, but it requires trained personnel and is subject to high inter-observer variation (Vriesema et al., 2001).

The association of sensitivity of these markers with grade and stage is shown in Table 1.6 (modified from Lotan 2003). Clearly overall results will be influenced by the grade and stage of tumours tested. Series often include a significant proportion of T2+ disease that is not encountered as often as Ta/T1 TCC.

Table 1.6 Median sensitivity (%) by grade and stage for 3 FDA approved biomarkers based on Bayesian meta-analyses (modified from Lotan 2003).

Marker	Studies (n)	G1	G2	G3	Ta	T1	T2+
NMP22	7	61	71	79	60	85	89
BTA stat	8	47	73	94	57	82	91
BTA TRAK	2	63	70	92	57	93	90

1.5.3 Other biomarkers of TCC

There are additional non-invasive techniques to identify TCC that rely on multiple changes to the appearance of shed cells. A karyometric analysis of bladder-washing microscopy images (Quanticyt) diagnoses TCC with a reported sensitivity of 60-70% and specificity of 70% (van der Poel et al., 1996; Wiener et al., 1998). Flow cytometry evaluation of urine is an automated measurement of cellular DNA to determine synthesis and ploidy associated with higher grade TCC and can identify 85% of recurrent TCC (Mora et al., 1996) and aid the prediction of survival (Schapers et al., 1993). Microsatellite markers may be used to diagnose TCC recurrence. TCC microsatellite analysis identifies frequent LOH in 4p, 8p, 9, 11p and 17p (Steiner et al., 1997; van Rhijn, van der Poel, and van der Kwast, 2005) and can be used to accurately detect recurrence. Most recently, a combined microsatellite and FGFR3 mutation analysis identified bladder cancer from a panel including 59 TCC with a sensitivity of 89% and specificity of 93% (van Rhijn et al., 2003). However, the authors concede that leucocyte contamination lowers test performance, and they suggest that the test requires automation and a wider assessment before entering general clinical practice.

Urinary and serum biomarkers to detect TCC have been derived from all aspects of cell biology. Published reports are numerous and are tabulated in Table 1.7 together with further details on prognostic biomarkers of TCC previously summarised in Table 1.5.

Table 1.7 Biomarkers of TCC. excluding oncogenes and TSG detailed in 1.2.3-4 and FDA approved markers detailed in 1.3.2. (IHC= immunohistochemistry, ELISA= enzyme-linked immunosorbant assay, BPH= benign prostatic hyperplasia, UTI=urinary tract infection)

Marker	Biological Function(s)	Diagnostic value	Prognostic Value	Comments	References
Acidic Fibroblast Growth Factor (FGF1)	Growth factor, Pro-angiogenic	A urinary ELISA on a panel of 236 TCC-urine samples and 343 controls demonstrated 72% sensitivity and 92% specificity for TCC[1].	IHC of a tissue panel (n=60) showed increased FGF1 expression was associated with high-stage TCC ($p<0.02$)[1]	TCC cell lines expressed FGF1[2]. FGF1-transfected NBT-II produced invasive, vascular TCC[3,4]	[1](Chopin et al., 1993). [2](Ravery et al., 1992). [3](Jouanneau et al., 1994). [4](Jouanneau et al., 1995).
Basic Fibroblast Growth Factor (FGF2)	Growth factor, Pro-angiogenic	FGF2 was initially detected in 6/11 TCC and 2/32 normals[1], subsequent ELISA studies (n= 39 and 83)[2,3] demonstrated sensitivity 62-81% and specificity 64-70%. Serum FGF2 levels were unhelpful[4].	FGF2 mRNA in TCC tissue correlated with stage and recurrence[5]. Increased urinary FGF2 levels were associated with higher stage ($p<0.1$)[2].	FGF2 was also elevated with BPH[3]. Bladder cell lines transfected with FGF2 increase MMP expression.[6]	[1](Chodak et al., 1988). [2](Nguyen et al., 1993). [3](O'Brien et al., 1995). [4](Edgren et al., 1999). [5](Gazzaniga et al., 1999). [6](Miyake et al., 1997).
Vascular Endothelial Growth Factor (VEGF)	Growth factor, Pro-angiogenic	A urinary ELISA of 98 TCC and 83 controls diagnosed recurrent TCC, with sensitivity 83% and specificity 48%[1].	High urinary[1] and tissue[2] VEGF were associated with recurrence, stage and grade. Other studies found no associations, with grade, stage or survival[3]. VEGF mRNA x10 in T1 and x4 in T2+ TCC c.f. normal bladder tissue[2]*.	*TCC internal tissue levels of VEGF were similar in TCC of all stages[2].	[1](Crew et al., 1999). [2](Crew et al., 1997). [3](Edgren et al., 1999).

Marker	Biological Function(s)	Diagnostic value	Prognostic Value	Comments	References
Epidermal Growth Factor (EGF)	Growth factor	A urinary radioimmunoassay reported reduced levels of EGF in TCC (n=54)[1*]. A urinary ELISA of 207 pts identified TCC with sensitivity 57% and specificity 66%[2]. EGF expression was identified in 45% of TCC tissue[3].	EGF expression had no association with grade, stage or tumour number [1,2]. Heparin-binding EGF-like growth factor measured with tissue IHC was associated with poor outcome[5].	[*]Reduced levels of urinary EGF were possibly due to increased EGFR binding. EGF induced MMP9 expression[4].	[1](Messing and Murphy-Brooks, 1994) [2](Saika et al., 2000) [3](Ravery et al., 1997) [4](Nutt et al., 2003) [5](Adam et al., 2003)
Transforming Growth factor alpha (TGF-α)	Growth factor EGFR ligand.	Urinary TGF-α was raised in TCC c.f. controls, although was poorly correlated with serum or tissue levels[1]. TGF-α was identified in 48-60 % of TCC by tissue IHC[2,3].	Increased tissue TGF-α strongly correlated with poor outcome[2,4] and tumour recurrence[3,5]. TGF-α expression was correlated with EGFR expression[6].	Increased protein levels were seen in the urine of patients with BPH c.f. normals[7].	[1](Chow et al., 1998b) [2](Ravery et al., 1997) [3](Turkeri et al., 1998) [4](Thogersen et al., 2001) [5](Gazzaniga et al., 1998) [6](Thogersen et al., 1999) [7](Monga, Gabal-Shehab, and Stein, 2001)
Transforming Growth factor beta (TGF-β)	Growth factor. Modulates the cell cycle via Rb.	Reports have identified a lack of correlation between TGF-β tissue, serum and urinary mRNA and protein expression[2].	One study has associated tissue overexpression of TGF-β with TCC grade and disease progression[3], yet, another found reduced TGF-β in high grade TCC[4]. Increased plasma protein was associated with nodal spread (n=59)[5].	Loss of TGF-β receptor I IHC expression was associated with poor outcome (n=59)[5,6].	[1](Eder et al., 1996) [2](Eder et al., 1997) [3](Kim et al., 2001) [4](Booth et al., 2002) [5](Shariat et al., 2001a) [6](Tokunaga et al., 1999)

Marker	Biological Function(s)	Diagnostic value	Prognostic Value	Comments	References
Thrombospondin-1(TSP)	Anti-angiogenic factor	No reports of serum or urinary levels of TSP to diagnose TCC were identified.	Panels of 163 invasive[1] and 220 pTa/T1[2] TCC reported that low TSP IHC expression was highly associated with disease recurrence progression and decreased survival.	In vitro data suggested TSP is the dominant anti-angiogenic factor in urothelium[3].	[1](Grossfeld et al., 1997) [2](Goddard et al., 2002) [3](Campbell et al., 1998)
Ki-67	Proliferation associated nuclear antigen identified by Ki-67 and MIB-1 MAB	No reports of serum or urinary levels of Ki-67 to diagnose TCC were identified.	A strong association has been seen between high Ki-67 IHC staining and high grade, stage and poor prognosis in Ta/T1[1,4] and advanced TCC[2,3] (although not nodal disease)[2]. Ki-67 is an independent factor in Ta/T1 TCC progression and recurrence (n=159)[5] , (n=114)[6].		[1](Wu et al., 2000) [2](Lianes et al., 1998) [3](Nakopoulou et al., 1998) [4](Mulder et al., 1992) [5](Santos et al., 2003) [6](Popov et al., 1997)
Proliferation Cell Nuclear Antigen (PCNA)	Proliferation associated antigen. DNA polymerase-δ cofactor	No reports of serum or urinary levels of PCNA to diagnose TCC were identified.	Several studies have associated high PCNA IHC staining with high grade and stage (n=50)[1], (n=60)[2], recurrence (n=48)[3], and reduced survival (n=48)[4,5].	Correlation with p53[6] and Ki-67[7] expression has been reported.	[1](Skopelitou et al., 1992) [2](Iizumi et al., 1997) [3](Blasco-Olaetxea, Belloso, and Garcia-Tamayo, 1996) [4](Chen, Lin, and Li, 1997) [5](Lavezzi et al., 2001) [6](Cohen et al., 1993)

Marker	Biological Function(s)	Diagnostic value	Prognostic Value	Comments	References
Mini-chromosomal maintenance proteins (Mcm)	Proliferation associated antigens: components of pre-replication complex	Mcm5 measured in the urine sediments of 353 subjects (n=71 TCC) identified TCC with 87% sensitivity and specificity[1].	Increased Mcm2 & 5 nuclear staining was associated with poor grade of T2+ TCC[2]. IHC of 54 T1 TCC correlated increased Mcm2 with early TCC recurrence and progresion[3].		[1](Stoeber et al., 2002) [2](Freeman et al., 1999) [3](Kruger et al., 2003)
p21	Cell cycle regulatory protein: Cyclin dependant kinase inhibitor (CDKI).	No difference in p21 was measured in urine from subjects with TCC (n=25) and controls (n=30)[1].	Increased p21 expression doubled the chance of recurrence in 114 Ta/T1[2] and was an independent prognostic indicator of recurrence and survival in 242 advanced TCC[3] and CIS[4], yet was found not to add any further prognostic info than p53 status in series of 363[5], 244[6] and 207[7] low-stage TCC.	It has been suggested that p21 maintenance abrogates the deleterious effect of p53 alterations on TCC progression[3,4].	[1](Scobbie, Anderson, and Horwich, 1994) [2](Fontana et al., 1996) [3](Stein et al., 1998) [4](Shariat et al., 2003a) [5](Holmang et al., 2001) [6](Pfister et al., 1999) [7](Liukkonen et al., 2000)
p27	Cell cycle regulatory protein: Cyclin dependant kinase inhibitor (CDKI)	No reports of serum or urinary levels of p27 to diagnose TCC were identified.	Several series have associated reduced p27 with high grade, advanced stage and reduced survival of TCC[1-5].		[1](Del Pizzo et al., 1999) [2](Korkolopoulou et al., 2000) [3](Kamai et al., 2001) [4](Primdahl et al., 2002) [5](Lacoste et al., 2002)

Marker	Biological Function(s)	Diagnostic value	Prognostic Value	Comments	References
Bcl2/bax	Marker of apoptosis (Bcl anti-apoptotic, bax pro-apoptotic)	No reports of serum or urinary levels of apoptotic markers to diagnose TCC were identified.	Many reports associated increased Bax [1,2] or increased Bax/Bcl2 ratio[3] of tissue protein with good outcome, yet others found no correlation with other disease parameters[4] or outcome[5].		[1](Hussain et al., 2003) [2](Giannopoulou et al., 2002) [3](Gazzaniga et al., 1996) [4](Cooke et al., 2000) [5](Kirsh, Baunoch, and Stadler, 1998)
Fas (Fas ligand)	Markers of apoptosis, members of TNF family	Soluble Fas was identified in higher levels in TCC c.f. controls, but not of a level to have been proposed as a diagnostic test for TCC[1].	Some reports correlated increased TCC recurrence with raised serum Fas or Fas ligand[1],yet others found no such correlation[2]. Tissue Fas was not associated with grade[3].		[1](Mizutani et al., 2001) [2](Perabo et al., 2001) [3](Lee et al., 1999)
Survivin	Inhibitor of apoptosis (IAP)	Detection of survivin protein in urine using a slot blot method was recently developed. From a panel of 46 TCC and 47 non-cancer controls TCC was predicted with a sensitivity of 100% and specificity of 95%[1].	Survivin was identified in 65% of G1, 90% G2 and 100% G3 TCC[2]. Survivin +ve T1 TCC recurred more quickly than –ve[2,3]. However, RNA expression was not correlated with recurrence (n=30)[4] or IHC staining with upper-tract recurrence[5]. Urinary survivin levels correlated with stage[1] and predicted recurrence after intravesical chemotherapy[6].	A commercial ELISA is now available (Assay Designs Inc. Ann Arbor, MI, USA) so larger trials will be reported. RNA expression of another IAP-Livin also correlated with recurrence[4].	[1](Smith et al., 2001) [2](Swana et al., 1999) [3](Lehner et al., 2002) [4](Gazzaniga et al., 2003) [5](Nakanishi et al., 2002) [6](Hausladen et al., 2002)

Marker	Biological Function(s)	Diagnostic value	Prognostic Value	Comments	References
E-Cadherin	Cell adhesion molecule	Soluble E-Cadherin was detected in urine of 42 subjects by immunoblot and levels were increased in high-grade, invasive TCC c.f. normals[1]. But in a subsequent ELISA based trial (n=35) no difference was seen between benign controls and Ta TCC[2*]. Serum E-Cadherin is higher in TCC c.f. normals[3].	Several studies have correlated tissue E-Cadherin expression with grade, stage and outcome[4-6], although not as an independent prognostic factor in multivariate analysis[7]. Prognostic value was given for nodal status[8], (but not upper-tract TCC[9]). Serum E-Cadherin protein levels correlated with TCC grade and recurrence, but not stage or with tissue levels[10].	*Protein/ creatinine index gave more diagnostic information than soluble E-cadherin levels[2]. E-Cadherin levels were associated with FGFR2 in high-grade TCC[11].	[1](Banks et al., 1995) [2](Protheroe et al., 1999) [3](Griffiths et al., 1996) [4](Bringuier et al., 1993) [5](Popov et al., 2000) [6](Shariat et al., 2001b) [7](Shimazui et al., 1996) [8](Byrne et al., 2001) [9](Nakanishi et al., 1997) [10](Durkan, Brotherick, and Mellon, 1999) [11](De Medina et al., 1999)
Integrin	Cell adhesion molecule	No reports of serum or urinary levels of apoptotic markers to diagnose TCC were identified.	A6β4 integrin was found in normal urothelium, but was overexpressed in TCC[1]. IHC correlated weak staining with improved survival (n=57)[2]. Derranged integrin expression positively correlated with high grade and advanced TCC[3].		[1](Liebert et al., 1994b) [2](Grossman et al., 2000) [3](Liebert et al., 1994a)
C-CAM I-CAM	Cell adhesion molecules (Ig Superfamily)	Preliminary studies have identified ICAM-1 levels in serum (n=120)[1] and urine (n=53)[2] were higher in TCC than controls.	ICAM-1 was overexpressed in high grade, invasive TCC tissue[3]. Urinary ICAM-1 was not related to other disease parameters[2].		[1](Ozer et al., 2003) [2](Chow et al., 1998a) [3](Tomita et al., 1993)

Marker	Biological Function(s)	Diagnostic value	Prognostic Value	Comments	References
CD44 (Hermes Ag, Pg-1, ECM-III)	Cell adhesion molecule (binds hyaluronic acid)	RNA levels of CD44 identified TCC with a sensitivity 0f 91% and specificity of 83% (n=100)[1]. A RNA expression ratio of CD44 v8-10/ v10 >1 identified TCC with a sensitivity of 75% and specificity of 100%[2]. A urinary ELISA (n=108) detected TCC with a sensitivity of 81% and specificity of 100%[3]. Serum measurements were not useful[4].	Increased tissue CD44v6 expression measured by IHC was seen in low stage/grade TCC[5] and was associated with good outcome of T2+ TCC[6]. Loss of v6 and v3 correlated with early recurrence of Ta TCC (n=241)[7]. CD44 v2 RNA but not protein was associated with TCC diagnosis[8] and high urinary CD44v8-10/CD44 ratio predicted increased Ta disease free survival[9].	CD44 expression was increased in Genito-urinary trauma, BPH and haematuria[3].	[1](Matsumura et al., 1994) [2](Okamoto et al., 1998) [3](Woodman et al., 2000) [4](Lein et al., 1997) [5](Ross et al., 1996) [6](Lipponen et al., 1998) [7](Toma et al., 1999) [8](Muller et al., 1997) [9](Miyake et al., 2002)
Hyaluronic acid (HA)	Cell adhesion molecule	An ELISA-like assay for urinary HA identified TCC with a sensitivity of 92%, specificity of 93% (n=144)[1] and sensitivity 81%, specificity 90% (n=513)[2] against controls.	IHC staining of a series of 83 TCC showed a trend towards increased HA expression and increasing stage and grade. There was 97% agreement of tissue and urine findings[3].	Bladder tumour cells were induced HA production by fibroblasts in co-culture[4].	[1](Lokeshwar et al., 1997) [2](Lokeshwar et al., 2000) [3](Hautmann et al., 2001) [4](Knudson et al., 1989)
Hyaluronidase (HAase)	HA cleaving enzyme	Hyaluronidase levels in urine were significantly higher in G2/3 (but not G1) TCC compared to controls[1].	IHC staining of a series of 83 TCC showed a trend towards increased HA expression and increasing stage and grade[2].	A combined HA-HAase urinary assay was 92% sensitive and 85% specific[3].	[1](Pham, Block, and Lokeshwar, 1997) [2](Hautmann et al., 2001) [3](Lokeshwar and Block, 2000)

Marker	Biological Function(s)	Diagnostic value	Prognostic Value	Comments	References
Laminin	Basement membrane protein	Studies diagnosed TCC by measuring serum laminin-P1 levels (n=72)[1], (n=113)[2]. Urinary laminin diagnosed TCC with a sensitivity of 58% and specificity of 96% (n=102)[3].	Laminin IHC of 66 TCC revealed that interruption of the basement membrane was correlated with tumour stage, recurrence and metastatic spread, although not grade[3]. Another study find no such relationship[4]. Raised urinary laminin levels were seen with advanced stage[1,2], high grade[2] and recurrence[1].	The invasive human TCC cell line EJ, but not the non-invasive RT4 cells, degraded basement membrane laminin[5].	[1](Mungan et al., 1996) [2](AbouFarha et al., 1992) [3](Abou Farha et al., 1993) [4](Kirkali et al., 2001) [5](Weiss et al., 1990)
Cathepsins	Basement memebrane-degrading proteases	A pilot study found significantly higher levels of urinary Cathepsin-L in TCC compared with controls (n=127)[1]. Cathepsin-B levels were raised in urine and serum from subjects with TCC compared to controls[2].	Several series (n=105)[3], (n=77)[4], (n=60)[5] (but not all[6]) identified a loss of Cathepsin-D IHC staining with increasing stage and high grade although not as independent prognostic factors in multivariate analyses[3,6].	Urothelial Cathepsin E expression was increased in the BNN-induced rat carcinogenesis model[7].	[1](Staack et al., 2002) [2](Eijan et al., 2000) [3](Dickinson et al., 1995) [4](Ioachim et al., 2002) [5](Iizumi et al., 1997) [6](Ozer et al., 1999) [7](Yamamoto et al., 1996)
Urokinase-type plasminogen activator (uPA)	Serine protease that activates plasmin an enzyme that degrades extracellular proteins	Urinary (n=229)[1] and serum (n=95)[2] uPA levels measured by ELISA, were elevated in those with TCC compared with controls (both $p<0.001$).	uPa in tissue from 52 Ta/T1 TCC was the most important risk factor for prognosis cf. stage, grade size and multiplicity[3] and associated with invasion and outcome in another study of 194[4].	uPA receptor, receptor and ligand were required for in-vitro cell invasion in TCC cell line models[5]	[1](Casella et al., 2002) [2](Shariat et al., 2003b) [3](Hasui et al., 1996) [4](Seddighzadeh et al., 2002) [5](Hudson and McReynolds, 1997)

Marker	Biological Function(s)	Diagnostic value	Prognostic Value	Comments	References
Matrix metalloproteases (MMP) 2,9 (gelatinises) and tissue inhibitor of MMPs (TIMP)	Proteolytic enzymes that degrades ECM TIMP inhibits MMPs.	Urinary MMP 2 and/or 9 levels detected by zymography[1,2] and from bladder washes[3] were increased in high-grade invasive TCC. Raised serum MMP2/TIMP ratios were also seen in TCC[4].	IHC analyses have shown an association with high tissueMMP2 and/or 9 and advanced disease and/or poor survivial[4-6]. An increased tissue MMP2/TIMP RNA ratio was found in TCC that recur[8].	MMP9 was induced by EGF in bladder cell lines[9].	[1](Sier et al., 2000) [2](Gerhards et al., 2001) [3](Bianco et al., 1998) [4](Gohji et al., 1996) [5](Davies et al., 1993) [6](Ozdemir et al., 1999) [7](Grignon et al., 1996) [8](Hara et al., 2001) [9](Nutt et al., 2003)
Matrix metalloproteases (MMP) 1	Proteolytic enzyme that degrades ECM.	MMP-1 was detected in urine samples from 21 of 131 (16%) patients with bladder cancer but was undetectable in samples from all other groups ($P < 0.0001$)[1].	MMP 1 protein and RNA levels correlated with invasive, high-grade TCC (n=59)[2].	MMP1 was induced by EGF in bladder cell lines[9].	[1](Durkan et al., 2001) [2](Nakopoulou et al., 2001) (Nutt et al., 1998)
Autocrine Motility Factor (AMF)	Recently identified as phosphoglucose isomerase[1]	Studies examining urinary AMF, (n=49)[2] and (n=117)[3] identified higher levels in TCC compared to controls.	Tissue AMF (inversely associated with E-Cadherin) was associated with poor TCC prognosis (n=83)[4].	anti-HER2 monoclonal antibody also inhibited AMF in cancer cell lines[5].	[1](Tsutsumi et al., 2003) [2](Guirguis et al., 1988) [3](Korman et al., 1996) [4](Otto et al., 1994) [5](Talukder et al., 2002)
Carcino--embryonic antigen (CEA)	Oncofetal protein, cell adhesion properties	CEA was assayed in serum and urine from 371 subjects and was raised in many TCC samples but not to a useful extent[1,2].	Early reports associated high CEA tissue levels with advanced stage[3]. However, IHC of 37 TCC with a specific Mab for CEA indicated it had little value as a prognostic marker of TCC[4].	Urinary CEA was increased in UTI[5].	[1](Guinan et al., 1975) [2](Coombers et al., 1975) [3](Jautzke and Altenaehr, 1982) [4](Feil et al., 1999) [5](Tanaka et al., 1994)

Marker	Biological Function(s)	Diagnostic value	Prognostic Value	Comments	References
Telomerase	Ribonuclearo--protein enzyme that maintains telomere length and chromosomal stability.	Urinary TRAP assays of telomerase reported sensitivity between 0[1]and 92% (n=232, specificity 89%)[2], typically, 85% (n=104, sensitivity 100%)[3]. RT-PCR detection of urinary hTERT is more practicable and identified TCC with sensitivity 55[4], 80[5], 94[6]%, and specificity of 100[4], 92[5], 96[6]%. Similar results were obtained from bladder washings[7,8].	Telomerase activity in TCC tissue did not appear to be related to grade or stage[8-11]. However, hTERT mRNA expression was significantly associated with TCC grade and stage[12,13]. A urinary TRAP analysis of 41 Ta/T1 TCC found telomerase activity was not predictive of tumour recurrence[14].	A meta-analysis of all methods of telomerase detection in the urine of 855 subjects recorded a sensitivity of 75%, specificity 86%[15].	[1](Linn et al., 1997) [2](Bhuiyan, et al, 2003) [3](Kavaler et al., 1998) [4](de Kok et al., 2000) [5](Ito et al., 1998) [6](Bialkowska et al., 2000) [7](Kinoshita et al., 1997) 8(Gelmini et al., 2000) [9](Rahat et al., 1999) [10](Yokota et al., 1998) [11](Yoshida et al., 1997) [12](De Kok et al., 2000) [13](Longchampt et al., 2003) [14](Wu et al., 2003) [15](Glas et al., 2003)
A, B, O(H) antigens	Blood group antigens	Blood group antigen status was identified from bladder cell washings of TCC and controls but not quantified[1-2].	Loss of ABO antigens correlated with a higher incidence of progression and metastasis (n=22)[3] but this has not been substantiated in other studies[4].	Deletion of ABO antigen cannot be determined in non-secretors (20% lack ABO expression)[4].	[1](Tsujihashi et al., 1987) [2](Sadoughi et al., 1980) [3](Decenzo, Howard, and Irish, 1975) [4](Sheinfeld et al., 1992)
Lewis Antigen	Blood group antigen	The detection of the Lewis antigen in bladder washings with a Mab identified TCC with a sensitivity of 80-85% and specificity of 70-86%[1-4]	There is a limited association with tissue Lewis Antigen levels and grade and stage, (and outcome pTa TCC only)[5].	Urine expression of Lewis antigen was not associated with other grade or stage[6].	[1](Sheinfeld et al., 1990) [2](Pode et al., 1998) [3](Planz et al., 1998) [4](Planz et al., 2001) [5](Juhl, Hartzen, and Hainau, 1986) [6](Golijanin et al., 1995)

Marker	Biological Function(s)	Diagnostic value	Prognostic Value	Comments	References
Fibrin-Fibrinogen degredation products (FDP)	Products of degredation by plasmin, in turn activated from plasminogen by tumour factors and lost from leaky tumour micro-vasculature.	Meta analysis of 4 trials reported a sensitivity of 77% (41-93: 95% CI) and specificity of 87% (77-94: 95% CI) in urinary FDP assays. Specificity was reduced in the presence of blood that also contained fibrinogen that cross-reacts with FDP assays[1,2].	Urine FDP levels were increased with higher grade and stage[2]. A single pilot study has also associated tissue FDP levels with grade and stage[3]. There appears to be no outcome data associated with FDP levels.	Commercially traded as Aura Tek FDP and Acc-Dx FDP[4] with FDA approval now out of production due to manufacturing issues[5]	[1](Lokeshwar and Soloway, 2001) [2](Lotan and Roehrborn, 2003) [3](Alsabti, 1979) [4](Burchardt et al., 2000)
Bladder Cancer NMP 4 (BLCA-4)	Nuclear matrix protein	BLCA-4 was isolated in the tissue of TCC and was not detected in normal urothelium[1]. Urinary BLCA-4 identified TCC in a sample of 52 TCC and 51 normal controls with sensitivity of 96.4% and specificity 81-100%.	No data regarding the prognostic value of BLCA-4 has yet been identified.	A multicentre prospective trial is underway to validate these findings[3].	[1](Konety et al., 2000a) [2](Konety et al., 2000b) [3](Konety and Getzenberg, 2001)
DD23	Monoclonal antibody to tumour associated antigen[1].	Used as a quantitative urinary marker in 3 trials, the sensitivity for bladder cancer detection was 85%[2], 81%[3] and 70%[4], with specificity 95%, 60% and 60% respectively	No data regarding the prognostic value of BLCA-4 has yet been identified.	Manufactured by UroCor Inc.[5]	[1](Grossman et al., 1992) [2](Bonner et al., 1996) [3](Sawczuk et al., 2002) [4](Gilbert et al., 2003) [5](Oklahoma City OK, www.urocor.com)

Marker	Biological Function(s)	Diagnostic value	Prognostic Value	Comments	References
T138	Monoclonal antibody to cell surface glycoprotein[1]	No reports of serum or urinary levels of T138 to diagnose TCC were identified.	Increased T138 expression in TCC, measured by IHC was associated with increased progression (n=57)[2] and recurrence (n=368)[3] of TCC.		[1](Fradet et al., 1984) [2](Ravery et al., 1995) [3](Allard et al., 1995)
Cytokeratin 20	An intermediate filament expressed in superficial cells of normal urothelium[1].	CK 20 was identified in serum by the PCR (n=66)[2] and (n=20)[3] with poor sensitivity (15%) and specificity. Initial reports of urinary TCC diagnosis (total n= 575) reported sensitivity 82-91%, with specificities of 67-100%[4-7], more recent studies report similar sensitivities of 78-81% but with poor specificities of 55%[8],36%[9].	Disruption of normal CK20 expression was highly correlated with recurrent Ta TCC[10] and low-grade papillary neoplasms[11]. Increased CK20 by IHC was associated with advanced grade and stage of 120 TCC[12].	Ta Bladder tumours with normal CK20 expression may be identified as benign papillomas[13]	[1](Harnden et al., 1996) [2](Gazzaniga et al., 2001) [3](Retz et al., 2001) [4](Golijanin, et al, 2000) [5](Rotem et al., 2000) [6](Klein et al., 1998) [7](Buchumensky et al., 1998) [8](Cassel et al., 2001) [9](Retz et al., 2003) [10](Harnden et al., 1995) [11](Ramos et al., 2003) [12](Desai et al., 2000) [13](Harnden, Mahmood, and Southgate, 1999)
Cytokeratin 19 CYFRA 21-1	An intermediate filament in TCC and normal urothelium and its soluble fragments (CYFRA 21-1)	Urinary CYFRA 21-1 ELISA studies identified TCC with sensitivities of 80-97% at specificities 67-95%[1-3]. Serum detection of CK 19 was poor at identifying TCC micrometasteses[4].	Tumour volume was strongly correlated to urinary CYFRA 21-1 in a study of 220 subjects[5].	CK19 is not organ specific and is elevated in other cancers and UTI[5].	[1](Nisman et al., 2002) [2](Pariente et al., 2000) [3](Sanchez-Carbayo et al., 1999) [4](Gazzaniga et al., 2001) [5](Senga et al., 1996)

Marker	Biological Function(s)	Diagnostic value	Prognostic Value	Comments	References
Cytokeratins 8 and 18 (Urinary bladder cancer, UBC antigen: CK8/18)	An intermediate filament protein expressed in normal urothelium.	Urinary CK 18 identified TCC in 402 subjects with a sensitivity of 66% and specificity of 88%[1]. The UBC antigen identified TCC with 66-76% sensitivity at 95% specificity[2,3] (total n=840) yet in another series only 20% of TCC were diagnosed at 85% specificity (n=101)[4]. Serum CK18 levels were not useful for Ta/T1 diagnosis[5].	Elevated urinary UBC were associated with advanced grade and stage and reduced survival[2].	The UBC ELISA and point of care UBC rapid™ test kit is manufactured by IDL biotech[6]. Elevated UBC levels were found in some benign disorders: anaemia, thyroid disorders, diabetes, hyperlipemia, and UTI[2].	[1](Sanchez-Carbayo et al., 2000) [2](Silen et al., 2000) [3](Heicappell et al., 2000) [4](Mungan et al., 2000) [5](Ramazan Sekeroglu et al., 2002) [6](IDL Biotech, Bromma Sweden, www.idl.se).

1.5.4 Limitations of contemporary TCC diagnostic biomarkers

Although several commercial kits are marketed to diagnose or monitor TCC, they have not gained widespread acceptance in the urological community because of the reduced sensitivity and specificity of these biomarkers in comparison to cystoscopy, particularly in the presence of benign inflammatory processes. These biomarkers have recently been subjected to a comprehensive review by an international consensus panel whose conclusions are in broad agreement with those here (Lokeshwar et al., 2005).

Many of the studies of newer molecules promise significantly higher performance but do not seem to have progressed into formal trials and it is doubtful that reliance on a single urinary biomarker will be sufficient (Knowles, 2006). The benefits of high-throughput techniques to assess gene-expression are yet to be seen, although these are moderately complex experiments that are difficult to translate into routine clinical tests.

Technological developments have allowed a high-throughput global assessment of the protein expression of tumours, but their application to TCC has been limited.

1.6 Analysis of the proteome

Proteomics, the global analysis of cellular proteins, is complementary to but has several theoretical advantages over genomic-based approaches to cancer biology. Proteins are the functional units of the cell and thus protein expression more closely reflects its current state. The genetic code cannot always indicate which proteins are expressed, in what quantity and in what form (Banks et al., 2000) as the level of mRNA often does not correlate with the amount of active cellular protein (Anderson and Seilhamer, 1997). In addition, post-translational modifications such as phosphorylation and glycosylation cannot be determined at the transcriptional level. For example, increased branching of N-linked glycans is a common feature of many

proteins in malignant cells (Orntoft and Vestergaard, 1999). The modifications possible from gene to protein are summerised in Figure 1.2.

Proteomic technologies have traditionally been divided into methods for displaying and/or seperating the component proteins such as one or two-dimensional polyacrylamide gel electrophoresis (PAGE) or liquid chromatography (LC), and subsequent identification of those proteins, using mass spectrometry (MS) or other techniques such as Edman degradation. At present the analytical sensitivity of proteomic techniques is limited. Unlike nucleic acid expression analysis, proteomics lacks an amplification methodology such as polymerase chain reaction (PCR). When profiling physiologically relevant sources, i.e. tumour tissue, sample abundance is a critical factor and may limit analysis. However, as proteomic technology improves (as detailed below), this problem is likely to be overcome.

Proteomics allows the simultaneous measurement of many thousand proteins as part of a systematic search for differentially expressed novel biomarkers in the same way that cDNA microarrays are being used to profile gene expression at the RNA level. There has been an increase in the frequency of reports of biomarker detection using proteomic techniques (Ludwig and Weinstein, 2005) and the Early Detection Research Network (EDRN) of the National Cancer Institute (NCI) within the USA uses proteomic approaches to complement genomic strategies to identify and characterise novel cancer biomarkers (Verma et al., 2001).

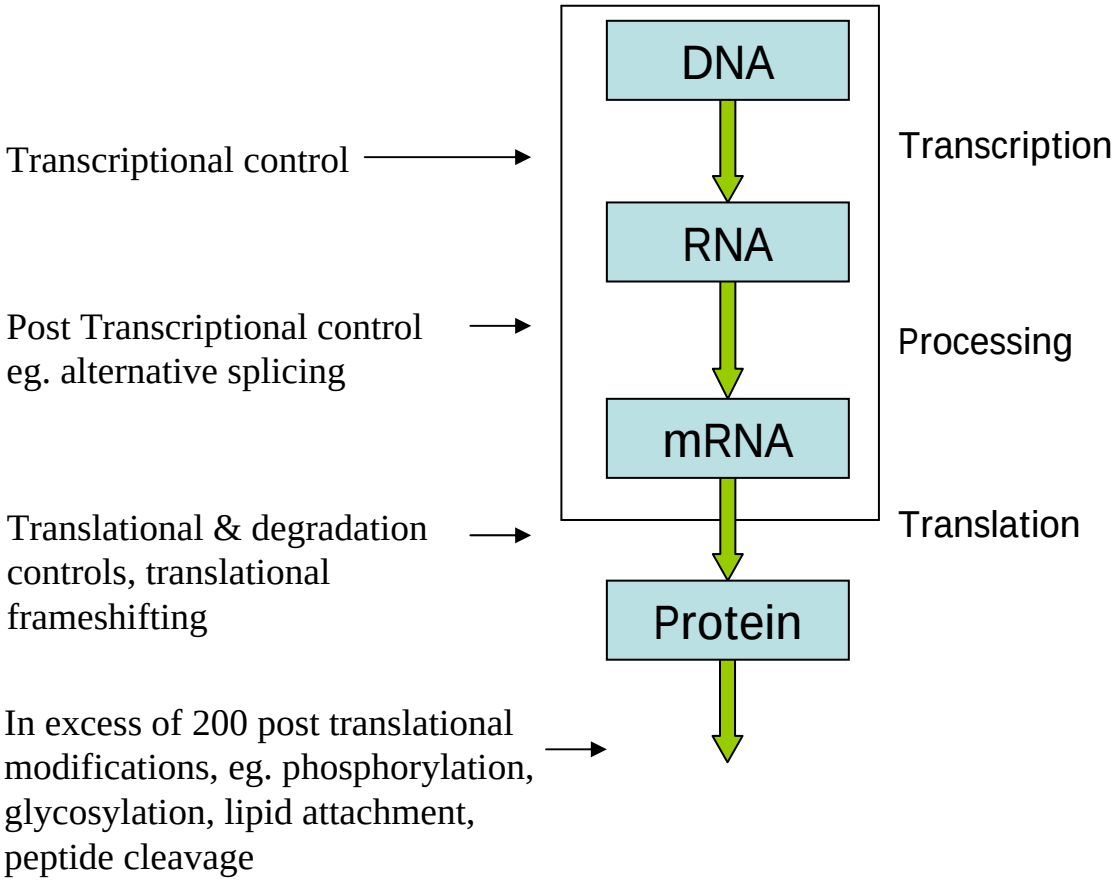


Figure 1.2 Diagram showing sites of modification in the process of protein synthesis from DNA. The human genome has been sequenced, revealing about 30,000 genes. The flow diagram shows the information encoded in genes first transcribed to RNA, processed into messenger RNA and translated into protein. The proteins produced represents the human proteome. At each stage during this process and particularly after translation the proteins may be modified, and so a potential huge range of proteins may be generated that cannot be predicted from the genomic sequence alone. An excess of 200 post translational modifications such as phosphorylation, glycosylation, lipid attachment and peptide cleavage have been described.

1.6.1 Two dimensional gel electrophoresis (2D PAGE)

2D PAGE was described initially by O'Farrell (O'Farrell, 1975). Solubilised proteins are separated from a complex mixture on the basis of their isoelectric point (pI) in the first dimension and subsequently by molecular weight in the second dimension (Molloy, 2000). The principals of 2D PAGE are shown in Figure 1.3. Silver staining, being more sensitive than Coomassie staining methods, has been widely used for high sensitivity protein visualisation on 2D PAGE. It has a limited dynamic range, and the most sensitive of silver staining methods are also incompatible with protein identification methods based on mass spectrometry. More recently, the Sypro post-electrophoretic fluorescent stains (Molecular Probes, Eugene, Oregon, USA) have emerged as alternatives, offering a better dynamic range, and ease of use. Sypro Ruby has been shown to be more sensitive than silver, and is compatible for subsequent peptide mass mapping. A careful analysis of the mass spectrometry compatibility of several Sypro dyes, colloidal coomassie and silver stains concluded that Sypro orange and red give the best results (Lilley, Razzaq, and Dupree, 2002).

The introduction of an immobilised pH gradient (IPG) within a polyacrylmide gel has greatly increased reproducibility of first dimension gels, protein loading capacity and is less technically involved than the carrier ampholyte system previously used (Gorg et al., 1999). Narrow-range IPG strips are commercially available and allow high-resolution (ultra zoom) protein separation by separating the protein mix by a reduced pH range, a typical reduction from 7 to 1 pH unit spread across a single gel (Hoving, Voshol, and van Oostrum, 2000). Differential fractionionation and/or solubilisation of the protein mix before first dimensional separation will similarly increase the relative concentration of less abundant protein species (Cordwell et al., 2000; Corthals et al., 1997). The combination of pre-fractionation and multiple, overlapping narrow-range pH gels is reported to significantly increase the detection of low abundance proteins in a complex proteome (Zuo and Speicher, 2002).

Digitised images of multiple gels are compared to search for differences between sample populations. These may be compared with comprehensive databases on the internet (Celis et al., 1999a). Experimental reproducibility is imperfect and requires complex matching of gel images to identify true differences between test samples.

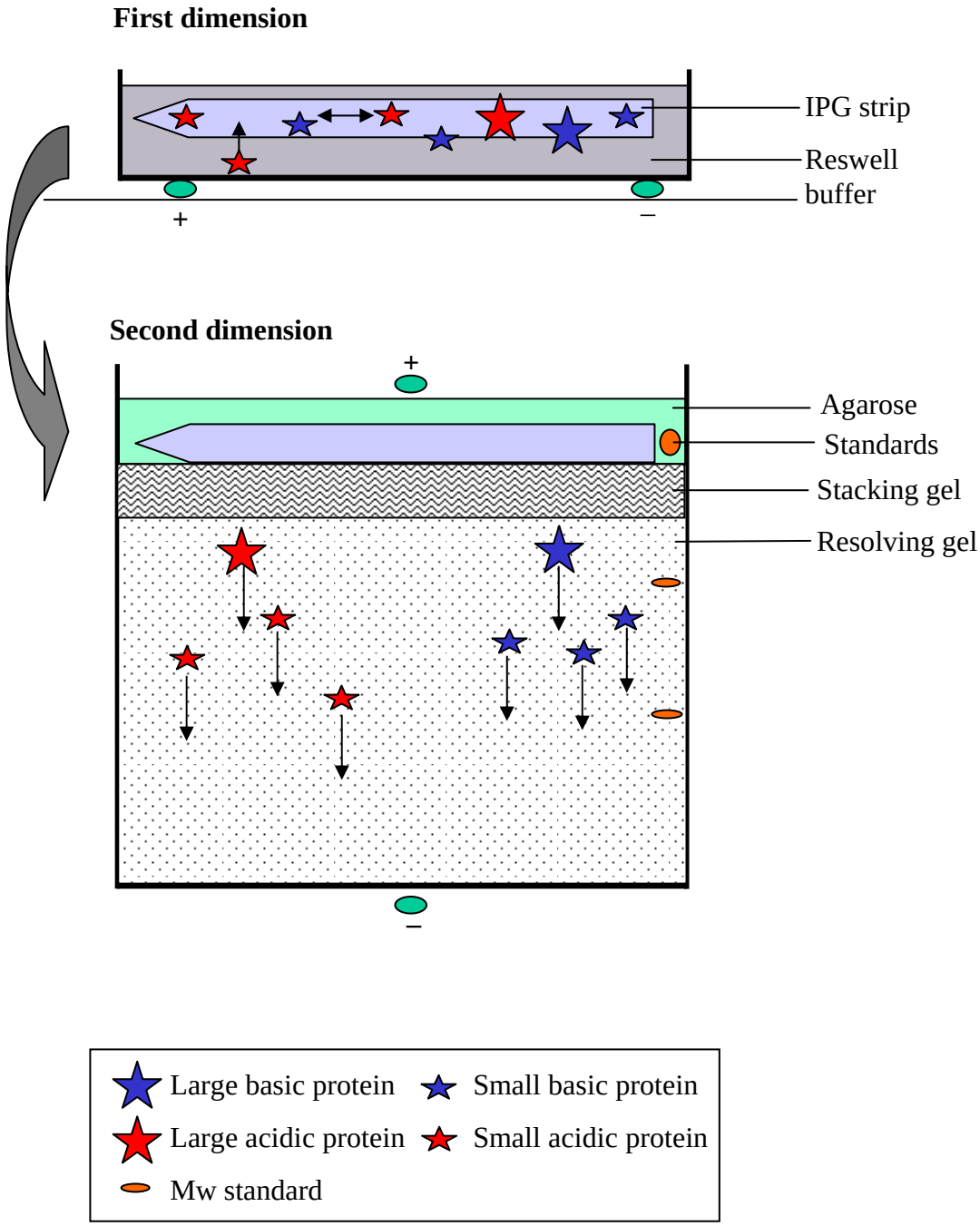


Figure 1.3 Schematic diagram of 2D PAGE In the first dimension, a protein-mix enters an IPG strip upon reswelling and resolves according to charge to become arrayed along the strip according to the isoelectric point of each constituent protein. The strip is transferred to one side of a gel for separation in the second dimension. The charged proteins are drawn through the gel matrix by a current at a speed proportional to their Mw.

Recently, 2-dimensional differential gel electrophoresis (2D-DIGE) has been developed that allows multiple samples to be loaded on a single gel, each labelled with a different flourophore (Tonge et al., 2001). This technology has been applied to identify biomarkers of oesophageal cancer (Zhou et al., 2002).

Several tumour types including breast, liver, prostate, ovarian, kidney, colorectal and leukaemia have been subjected to 2D PAGE biomarker study (reviewed by Simpson and Dorow, 2001). Despite the increase in reports of alternative proteomic technologies there are continuing reports of tumour profiling using 2D PAGE. For example, epithelial tissue from 5 invasive breast cancers and adjacent normal tissue was recently subjected to laser microdissection and 2D PAGE analysis. Thirty two differentially expressed proteins were identified by MS, including 13 that had not been previously associated with breast cancer (Hudelist et al., 2006).

1.6.2 Mass spectrometry

Mass spectrometry (MS) has arguably become the core technology in proteomics as it provides the key tools for the analysis of proteins. MS is routinely used to identify the proteins separated by 2D PAGE (Gevaert and Vandekerckhove, 2000) but increasingly to analyse the proteome independently. Mass spectrometers consist of three basic components: an ion source, a mass analyser, and an ion detector. MS measurements are carried out on ionised proteins in the gaseous phase, requiring a method to transfer molecules from solution or solid phase into this state. The two most commonly used techniques are matrix assisted laser desorption/ionisation (MALDI) and electrospray ionisation (ESI) (Guerrera and Kleiner, 2005). The importance in the development of these techniques in the late 1980s was recognised by the 2002 Nobel Prize in Chemistry (http://nobelprize.org). ESI is based on spraying an electrically generated fine mist of ions into the inlet of a mass spectrometer. This ionises molecules directly from solution. It is usually coupled to a mass analyser that can measure either a complete mass spectrum, or trap the ions for subsequent mass analysis. ESI MS was not used in this thesis and shall not be described in more detail.

In contrast, MALDI samples are co-crystallised with an organic matrix on a metal target. A pulsed laser is used to excite the matrix, which causes rapid heating of the molecules and eventually desorption of ions into the gas phase, producing packets of ions. The mass analyser separates ions by their mass-to-charge (m/z) ratios. Ions within the analyser can be manipulated by electric or magnetic fields to direct ions to a detector, which registers the number of ions at each m/z value. MALDI are usually coupled to time-of-flight (TOF) analysers which separate ions according to their flight time down a vacuum tube. The time of flight of a particular ion is directly related to its m/z value and thus a mass spectrum may be acquired. Other types of mass spectrometry such as the Fourier transform ion cyclotron are beyond the scope of this introduction.

Some mass analysers are able to subsequently fragment petide ions: tandem (MS/MS) mass spectrometry. Tandem MS allows the determination of a peptide's amino acid sequence in addition to its mass, and hence determination of its identity. Standard TOF analysers are unable to perform tandem MS. However, recently developed TOF/TOF instruments incorporate a collision cell between the two TOF sections. Ions of one m/z ratio are selected in the first TOF section, fragmented in the collision cell against inert gas molecules, and the fragments are separated in the second TOF section. Alternatively, hybrid quadrapole time-of-flight (Q/TOF) instruments incorporate a collision cell between a quadrapole mass filter and TOF analyser. The quadrapole filter selects only ions of a particular m/z for subsequent fragmentation and TOF analysis (Aebersold and Mann, 2003).

Within the field of proteomics, protein identification is almost exclusively achieved by MS. The most common strategies analyse peptides rather than full length proteins, which are digested with a sequence-specific enzyme such as trypsin. Using a process of peptide mass fingerprinting (also referred to as peptide mapping) these peptides are (usually) analysed with MALDI-TOF MS to give fragments of m/z 800-3000. These are compared against a database comprising peptide masses from a virtual digest of all proteins from a given organism with the same sequence specific protease. These

databases are readily available through internet-based resources, such as http://www.expasy.ch. This technique requires a purified target protein and so is often used in conjunction with 2D PAGE, as in this thesis. Because protein identification relys on matches with sequence databases it is largely restricted to those species for which comprehensive sequence databases are available.

Complex protein mixtures may be analysed with liquid chromatography-tandem MS (Hunt et al., 1992). However a number of issues have needed to be addressed in the development of this tool (reviewed by Aebersold and Mann, 2003). Firstly single-dimension chromatography does not provide sufficient peak capacity to separate peptide mixtures of complex biological samples. Various combinations of separation schemes have been explored including pre-fractionation with one dimensional PAGE, tryptic digestion and multiple chromatological surface separation. In most MS the relationship between the amount of analyte and measured signal intensity is complex and poorly understood. This makes quantitative MS analysis difficult. This may be addressed with metabolic isotopic labelling, in which isotopic labels are incorporated during the process of protein synthesis. For example, in 2002 a method for stable isotope labeling by amino acids in cell culture (SILAC) was developed (Ong et al., 2002). This describes in vivo incorporation of specific amino acids of differing mass (such as leucine and deuterated leucine) which may be separately identified by subsequent MS.

1.6.3 Surface Enhanced Laser Desorption/Ionisation (SELDI-MS)

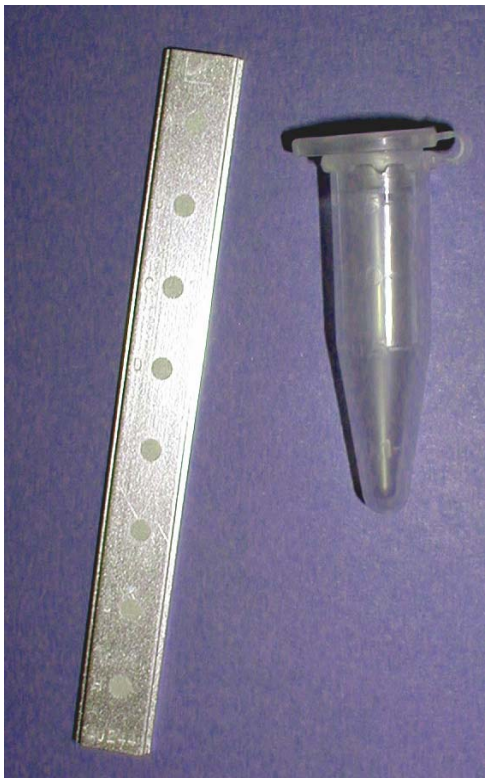
A modification of MALDI-TOF-MS; SELDI-MS, was developed through the 1980s and 1990s by Hutchens and Yip (Hutchens and Yip, 1993) and the system was manufactured from 1996 by the Ciphergen® company. SELDI-MS combines the analytical power of mass spectrometry with selective affinity capture of different protein chip surfaces derived from classical chromatological separation moieties. It allows rapid profiling of the proteins within a crude biological sample; by selectively binding and thus purifying proteins from samples it avoids the need for 2D PAGE or other protein separation techniques. The method detects protein fragments between 2-100 kDa (in fact optimal to 2-20 kDa) as larger ions are not generated. This is smaller

than are often identified with 2D PAGE. Additionally the sample size required for the generation of a profile is in the femptomolar range, several orders of magnitude smaller than with 2D PAGE. The ProteinChip® system comprises the "ProteinChip Reader", a MALDI-TOF MS, and a series of novel ProteinChip arrays ("chips"). The Reader is controlled via a PC using Ciphergen software to collect and analyse MS data (Figure 1.4).

The ProteinChip arrays are aluminium strips approximately 10 by 1 cm in size (Figure 1.3a), comprising a number of coatings applied to the basic chip in a series of 8, 16 or 24 spots. These coatings bind proteins in solution according to their characteristics. Several array types have been or are currently produced that are detailed below (Table 1.7). The proteins that bind to the chip surface are dissolved in an energy-absorbing molecule (EAM) in an organic solvent. This solution forms a crude crystal with the EAM upon drying. Once placed inside the vacuum of the MALDI mass spectrometer a 337nm wavelength nitrogen laser illuminates each spot in a series of pulses. The energy of the laser is transferred by the EAM to the crystallised proteins that are desorbed and ionised into the vacuum. The protein ions are accelerated across a voltage differential and impact upon an ion detector at the far end of a flight tube. The time of flight (TOF) is recorded, which is inversely proportional to the square root of a particle's molecular mass (assuming similarly charged ions accelerate with identical energy).

Various biological samples have been been analysed by SELDI-MS to diagnose human tumours. These include tissue homogenate, nipple aspirate, pancreatic secretions, ascites, serum and urine. SELDI-MS has been used to identify cancer-associated protein profiles from breast cancer (Aboagye-Mathiesen et al., 1999; Alexander et al., 2004; Li et al., 2002; Li et al., 2005; Ricolleau et al., 2006; Wulfkuhle et al., 2001), hepatic cancer (Ward et al., 2006), gastric cancer (Qian et al., 2005), colorectal cancer (Yu, Chen, and Zheng, 2004), head and neck cancer (Cho et al., 2004; Soltys et al., 2004; von Eggeling et al., 2000; Wadsworth et al., 2004), lung cancer (Yang et al., 2005), pancreatic cancer (Koopmann et al., 2004) and prostate cancer (Adam et al., 2002; Grizzle et al., 2004; Petricoin et al., 2002a; Qu et al., 2002; Wagner et al., 2004; Wellmann et al., 2002; Xiao et al., 2001).

a)



b)

Figure 1.4 Photographs of a) 8-spot SAX2 SELDI chip b) SELDI Protein Chip Reader™ The1.5ml ependorf vial is shown for scale with the chip. Note the black square lid on upper portion of mass spectrometer to insert chip. SELDI profiles viewed on monitor in real time.

Table 1.8 SELDI ProteinChip array types used in this thesis

Chip name(s)	Surface Chemistry	Functionality	Target amino acids
H4	Hydrophobic	Chains of 16 methylene groups that bind through reverse phase chemistry	Alanine, valine, leucine, isoleucine, phenylalanine, tryptophan and tyrosine.
NP1 **NP2**	Normal phase	Silicon oxide	Serine, threonine and lysine.
SAX2 **Q10**	Strong ion exchange	Quaternary ammonium groups that interact with negative charged amino acids	Aspartic acid and glutamic acid.
WCX2	Weak cation exchange	Anionic carboxylate groups that interact with positively charged amino acids	Lysine, arginine and histidine.
IMAC3	Immobilised metal affinity capture	Nitrilotriacetic (NTA) groups that chelate metal ions that bind protein	Histidine, cysteine, tryptophan and phosphorylated amino acids
PS1 **PS2**	Preactivated surface	PS1 carbonyl diimidazole, PS2 expoxy gropus for covalent binding.	Specific biomolecules (eg antibodies) for capture of target proteins.

Ovarian cancer has been diagnosed from serum with a sensitivity of 100% and a specificity of 95% (Petricoin et al., 2002b), although other reports are more modest (Zhang et al., 2006). Bladder cancer has been diagnosed from urine using SELDI-MS, and this will be discussed below (Vlahou et al., 2001). Preliminary SELDI studies have concentrated on the diagnostic function of cancer biomarkers, that is, simply to determine the presence or absence of a cancerous state. More recent reports have also addressed prognostic function such as stratifying disease behaviour, predicting clinical outcome or response to treatment. For example, using OAW42 and 2780 cell line models of ovarian cancer, induction of cisplatin resistance was consistently associated with alteration of 2 peaks (m/z 5041 and 7324) by SELDI-MS analysis (Britten et al., 2005).

One limitation of SELDI-MS is that it incorporates a standard MALDI-TOF MS, thus identification of the proteins and peptides within a sample cannot be performed directly. Tandem MS of SELDI protein chips would allow peptide sequencing but this is not presently in widespread use. Indirect methods such as immunodepletion or chip based purification techniques can aid protein identification but they require significantly greater amount of sample. Hence most studies using SELDI to discover cancer biomarkers rely on spectral features (peaks) without knowing the identity of the protein ion or indeed protein fragment ion that corresponds to that peak.

1.6.4 Proteomic studies of bladder cancer

Much pioneering work in the development of proteomic biomarkers has been undertaken in the field of bladder cancer, principally by Professor J. Celis and collegues who have analysed several hundred bladder tumour specimens by 2-D PAGE. A database of 2-D PAGE images from a variety of TCC, squamous cell carcinomas (SCC) and normal tissues has been established and is accessible via the internet (http://biobase.dk/cgi-bin/celis) (Celis et al., 1999c). Their stated aim was to develop a comprehensive set of biomarkers for squamous and transitional cell cancers (Celis et al., 2000).

2D PAGE-MS protein profiling of 6 SCC identified several differentially expressed proteins including keratins 5,6,10,13-17,19 & 20 (Ostergaard et al., 1997). Psoriasin has been identified in the urine of many of these patients and has been proposed as a marker of SCC (Ostergaard et al., 1997; Ostergaard et al., 1999). Subsequently, immunostaining of keratins and psoriasin was used to identify premalignant or metastatic SCC lesions from cystectomy specimens (Celis et al., 1999b). Adipocyte-type fatty acid binding protein (A-FABP) expression was found to be reduced in poorly differentiated TCC lesions (Celis et al., 1996). More recently, 153 TCC and control specimens have undergone 2D PAGE and IHC, including a tissue microarray comprised of 2317 samples from 1849 TCC (Ohlsson et al., 2005). A-FABP expression appeared to become increasingly reduced in tumours of increasing grade and stage and the authors propose it may have utility as a prognostic biomarker.

In 2005, urine samples collected from 45 patients with TCC and controls were subjected to 2D PAGE (Irmak et al., 2005). Two proteins, orosomucoid a 41 kDa acute phase protein and human zinc alpha-glycoprotein a 45 kDa protein (both associated with other cancer types such as breast) were increased in those with TCC, particularly of high grade and stage. Such a study on a relatively large number of subjects, including Western blotting and IHC validation represents a significant contribution to this field.

Sheng *et al* have just published a study of differences between 10 grade 2 and 10 grade 3 TCC using 2D PAGE and LC MS/MS (Sheng et al., 2006). In contrast to our

study, the high and low-grade tumours analysed were of various stages, unmatched, including only 2 Ta TCC. Seven proteins were down-regulated in grade 3 TCC, including A-FABP described by Celis previously; and 3 proteins, including annexin I and V were upregulated.

Before the beginning of our study in 2000, there were no published reports of SELDI-MS profiling of urine to diagnose TCC. In 2001, using SAX2 chips, a sensitivity of 87% (78% for low-grade lesions) and a specificity of 66% was reported using 5 peak-complexes identified by eye (3352/3432, 9495, 44647, 100120, 133190 Da) (Vlahou et al., 2001). Subsequently this group achieved similar predictive accuracy with the application of decision tree algorithm analysis (Biomarker Patterns Software®;Ciphergen) on a further 191 training and 36 blinded test set sample spectra using weak cation exchange chips (Vlahou et al., 2004). Using a similar analysis approach trained on a group of 118 samples and tested on a blinded set of 38 samples profiled using IMAC (metal binding) chips, higher sensitivity (93.3%) and specificity (87.0%) were achieved using 5 significant peaks of 3896, 4977, 9638, 15103 and 15509 Da (Zhang et al., 2004a). More recent studies with decision tree analysis reported figures for sensitivity and specificity in excess of 90% in the training set, but falling to 70-73% (Liu et al., 2005) or 50-62% (Mueller et al., 2005) sensitivity in the test set.

Recently, by combining SELDI analysis of 12 invasive TCC biopsy samples and associated normal urothelium, with LC ESI-MS/MS, Tolson *et al* identified calgranulin A to be highly expressed in tumour cells in contrast to normal urothelium (Tolson et al., 2006). The tumours selected were all muscle invasive and it is possible that calgranulin expression occurs late in the development of TCC and this may reduce its value as a prognostic or diagnostic marker of TCC. However, this appears to be the first published study to use MS-based proteomic techniques to examine bladder cancer.

1.7 Overall aims

The aim of this project was to investigate aspects of the proteome of TCC to identify biomarkers of TCC for disease diagnosis, recurrence and prognosis.

SELDI-MS techniques were developed to assess global protein expression within urine samples for the detection of TCC, to discriminate between patients harbouring TCC and a wide range of non-tumour bearing controls with benign urological conditions matched for age and sex. This assay was compared with other non-invasive TCC tests.

Low and high grade non-invasive TCC tumour tissues were compared to identify molecules potentially associated with recurrence or progression. The study was prospective because of the need for fresh tissue and therefore long-term follow-up is not available. Hence grade was used as a surrogate marker of tumour aggressiveness, as high grade tumours are associated with a significantly higher risk of recurrence and progression.

The proteome of bladder TCC has been investigated by Celis *et al* but only a few specific changes in protein expression in superficial TCC have been reported. Additionally, only preliminary reports have been published utilising SELDI-MS to profile urinary proteins as potential biomarkers of TCC. There is clearly considerable scope for the identification of novel and important proteins in the biology of TCC and these approaches offer new opportunities for the development of markers for diagnosis, prognosis and monitoring of patients with bladder cancer.

1.7.1 Specific aims

A. To identify novel proteomic urinary markers to detect (recurrent) TCC using SELDI-MS:

- To collect and store a cohort of urine samples from TCC and control patients
- To determine optimal tissue handling and storage methods
- To determine the optimal SELDI-MS parameters
- To identify candidate proteins from SELDI-MS profiles
- To develop a robust statistical model to analyse SELDI-MS profiles
- To train the neural network and test a cohort of TCC and control samples
- To validate the primary test set with a further prospective sample cohort

B. To develop and validate novel proteomic markers associated with high risk of TCC progression using 2D PAGE

- To collect and store a cohort of "fresh" Ta TCC tissue samples
- To separate the proteins within 6 TaG3 and 6TaG1/2 TCC with 2D PAGE
- To analyse composite proteomes of TaG3 and TaG1/2 TCC and identify differentially expressed candidate proteins
- To identify candidate proteins with preparative-2D PAGE and mass spectrometry
- To validate candidate protein expression in an independent panel of tumours by immunohistochemistry where possible
- To compare candidate protein expression with RNA levels measured by quantitative real time RT-PCR

2 Development of SELDI-MS urine profiling

2.1 Introduction

This chapter describes the use of SELDI-MS to develop novel proteomic markers to diagnose bladder cancer from urine. Initial reports describing the use of SELDI-MS were published after 2000, when this study was initiated, and so there was very little accumulated understanding to guide the development of this new proteomic tool. The methods used to profile urine with SELDI-MS developed as the study progressed. Therefore the materials and methods have been incorporated into each of the subsequent results chapters where relevant.

2.2 Methods: Sample acquisition and processing

2.2.1 Consent

Tumour tissue from TCC and voided urine from patients with TCC and controls were required for this study. Approval from the Leeds (East) Research Ethics Committee was sought and obtained. Subjects gave informed consent for their participation by completing a consent form with the aid of a doctor or nurse specialising in urological disease (Appendix II).

2.2.2 Urine collection and processing

Urine samples were collected from patients with presumed bladder TCC before surgery. Other urine samples were also collected from hospital patients and staff, some without medical problems and some with a range of benign urological conditions. Inclusion and exclusion criteria for study participants are shown in Table 2.1. In all cases the subject was provided with a 500 ml sterile jug in which to void. Up to 100ml of the urine sample was immediately transferred into a sterile screw capped container on ice containing one mini Complete^{TM} protease inhibitor tablet

(Roche) and mixed. These samples were transported on ice to the laboratory within 45 min for processing. The sample was tested for haematuria, proteinuria and leucocyturia using a Multistix™ dipstick (Bayer). Sample pH was measured with a hand-held meter and adjusted to pH 7 with 1M NaOH. Urines were centrifuged at 800g for 10 min at 4°C. The supernatant was stored at –80°C in 1ml aliquots; a 5ml aliquot was reserved for protein and creatinine concentration assays performed in the Clinical Chemistry Department of St James's Hospital.

Table 2.1 Inclusion and exclusion criteria for tissue collection

Sample Type	Inclusion Criteria	Exclusion Criteria
TCC-associated urine samples	Patient with subsequent histological diagnosis of TCC bladder	TCC from outside the bladder or other bladder tumour types
		Patient unable to give informed consent
		Any non-cutaneous malignancy
Control (normal) urine samples	Apparent good health upon questioning	Subject unable to give informed consent
		Any non-cutaneous malignancy or genitourinary condition
Control (non-normal benign) urine samples	Patients with benign genitourinary conditions, eg. urinary infection, renal calculi	Unable to give informed consent.

Patient and tissue details were anonymised and stored on secure computer databases. All histological samples were evaluated by a single pathologist (Dr P. Harnden). Obtaining patient consent, tissue retrieval and processing is part of an ongoing programme within the laboratory and as such was performed by several members of the scientific and clinical staff at St James's. However, the majority of work was undertaken by Ms. J. Robinson, Mr. J. Laye, Mr. S. Sak and the author.

2.3 Methods: SELDI urine analysis

Initially all 5 chip-surface types produced by Ciphergen (at the time of the study) were used although subsequently only 3 chip types (SAX2, IMAC3 and Q10) were used for the majority of experiments; Q10 replaced SAX2 as the anion-binding chip-type in 2004.

2.3.1 Manual preparation of SELDI protein chips

The exact protocol for each chip-type is shown in Table 2.2, but the procedures common to all chips are described here. Each 8-spot chip was placed within a humidity chamber to prevent drying of the applied solutions and each spot circled with a wax "mini-PAP" pen (Zymed) to keep the applied solutions in contact with the spots. Several chip-types required pre-sample treatments and buffer solution washes (Table 2.2).

Urine samples were thawed, microfuged at 10000g for 7 minutes and diluted with H_2O to a standard protein concentration of 0.05 mg/ml. All samples were stored on ice. Each urine sample was diluted 1:1 with 2x sample buffer and 50 µl applied to the prepared chip using a bioprocessor (Ciphergen). The bioprocessor was covered with parafilm (Pechiney) to avoid evaporation of the sample and incubated at 18-20 °C for 30-60 min on an orbital shaker at 200 rpm. After incubation, the urine samples were pipetted from the bioprocessor, the chip surface washed 3-5 times for a minute with 100µl/spot of the appropriate buffer, twice with 100µl/spot H_2O and allowed to air dry before the addition of matrix (except NP2 chip, see Table 2.2).

A total of 0.7 µl of matrix (an energy absorbing molecule solution) was applied to each spot on the chip surface in 2 applications of 0.35µl and allowed to dry before insertion into the ProteinChip Reader. The matrix comprised a saturated solution of sinipinic acid (Ciphergen) in 50% (v/v) acetonitrile (BDH), 0.5% (v/v) trifluroacetic acid (Pierce). This matrix solution was made fresh each day and stored in the dark, before use it was centrifuged at 10000g for 3 minutes.

Table 2.2 Summary protocol for preparing SELDI-MS Chips according to type.

Chip type	Surface Chemistry	Chipwash/ sample Buffer composition	Chip pre-sample treatment(s)?	Chip pre-sample buffer wash?	Urine sample diluted 1:1 with 2x sample buffer?	Sample/chip Incubation time (min)	Post incubation (1x sample buffer/ and H_2O) 1min washes
NP2	Normal Phase	N/A	No	No	No	30	0
H4	Reverse (hydrophobic) phase	10% acetonitrile/0.1 % TFA /0.2% NP40	30 sec 2µl 50% acetonitrile	No	No	30	3/2
SAX2	Strong anion exchange	20mM sodium acetate pH 7.9 /0.02% NP40	No	2 x 5 min 5µl SAX2 buffer	Yes	30	5/2
Q10	Strong anion exchange	20mM TRIS, pH 8.0	No	2 x 5 min 5µl SAX2 buffer	Yes	30	3/2
WCX2	Weak cation exchange	40 mM ammonium acetate /0.2% NP40 pH 6.5	5 min10mM HCl, 3 x 5µl H_2O washes, 5min 5µl/spot 100 mM ammonium acetate pH 6.6	2 x 5 min 5µl WCX2 buffer	Yes	30	5/2
IMAC3	Metal (nickel) binding	500mM sodium chloride/ PBS /0.02% NP40	4 x 5 min 5µl 50mM nickel sulphate	2 x 5 min 5µl IMAC3 buffer	Yes	60	5/2

2.3.2 SELDI ProteinChip Reader (MALDI-TOF)

Following matrix addition each chip was immediately inserted into the ProteinChip Reader and protein profiles generated. With automated chip preparation the processed Q10 chips were read after a delay of 30 minutes to reduce variability (see Chapter 3). Sample ionisation and flight was achieved upon illumination of each spot with a 337nm wavelength nitrogen laser. A standard protocol was used for all samples, which varied over the course of the project. Each MS setting will be described in Chapter 3.

Before each day's experimentation with manual or automated chip preperation, the ProteinChip Reader was externally calibrated. An H4 chip was loaded with 2μl of matrix containing 400 fmoles bovine ubiquitin (8564.8 Da), 400 fmoles of bovine superoxide dismutase (15591.4 Da) and 400 fmoles of bovine beta lactoglobulin A (18363.34 Da) callibrants (Ciphergen). Upon air drying the chip was inserted into the ProteinChip Reader and analysed as described above. The callibrants were displayed as singly and doubly charged peaks in the protein profile generated. Within the ProteinChip software the calibration dialog box was used to assign the actual mass values to these callibrant-peaks and so update the calibration equation used by the instrument.

2.4 Results: Determination of the optimal SELDI technique parameters

2.4.1 Selection of chip type

All available chip types: NP2 (normal phase), H4 (reverse phase), SAX2 (anion), WCX (cation) and IMAC3 (metal binding) were used to analyse a normal and TCC (G3pT1) urine sample to identify the chip type(s) that were likely to produce the most number of discriminating peaks. As the effect of protein load on profile was initially unknown, each urine sample was analysed at a range of abundances. The two urine samples selected had identical protein concentrations (0.1g/l) and each test urine was applied in volumes of 5, 50, 100 μl to a chip of each type. The

number and intensity of the peaks in the profiles binding to NP2 (normal) and H4 (reverse phase) chips (Figure 2.1a) were significantly less than with SAX2 (anion), IMAC3 (metal binding) chip types (Figure 2.1b) and WCX (cation) chips (Figure 2.1c). Initial profiling and developmental experiments used all chip types. The results of the early profiling experiments were used to determine the most promising chip type for increasing larger experimental runs, which through limited resources could not use all chip types. The details for each chip type used are given with each experiment. In summary SAX2 & IMAC were initially used, and finally SAX2 (later designated Q10) alone.

2.4.2 Determining protein load

The optimal protein load and sample volume for SELDI urine profiling was unknown. A TCC (G3pT2) urine sample of relatively high protein concentration (0.5g/l) was used to determine the effect of protein concentration upon profile quality. The urine sample was serially diluted with water to generate 25µl aliquots with protein concentrations of 0.5, 0.2, 0.1, 0.05 and 0.025 g/l. These were mixed with an equal volume of the appropriate buffer (Table 2.2) and applied to SAX2, IMAC3 and WCX chips. The quantity and magnitude of the peaks was greatest at 0.1 and 0.05g/l. Of the 20 TCC samples collected at that time, 3 had a protein concentration less than 0.1g/l and 1 less than 0.05g/l. Of the 22 normal samples collected, only 4 contained protein above 0.1g/l and only 12 samples contained protein above 0.5g/l. Thus a decision to set a standard protein concentration to maximise profile quality might exclude a significant proportion of potential subjects unless the urine was manipulated. We considered dialysing samples to increase their protein concentrations but felt that this process may affect the urinary proteome in an unknown manner and would be practically difficult to perform for a large number of samples. We concluded that 25µl of urine diluted with water to give a protein concentration of 0.05g/l (total protein load 1.25µg) would give good profile quality and allowed most urine samples to be used. In fact, the subsequent samples that were collected showed a higher mean protein concentration, and in excess of 95% of samples were measured to have protein concentrations of 0.05g/l and above, allowing their use in this study.

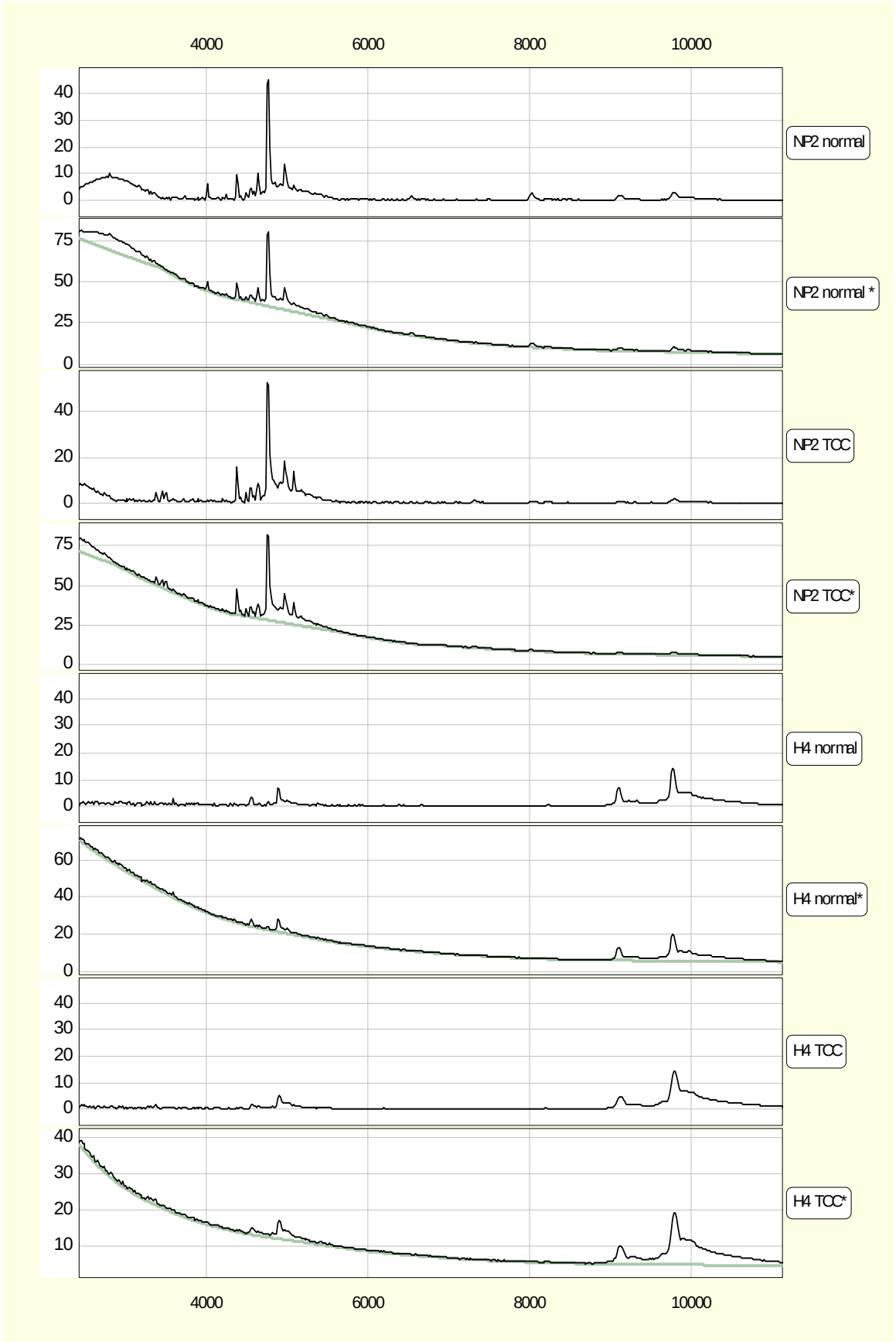


Figure 2.1a SELDI profiles of urine from a normal & TCC subject on NP2 and H4 Chips. Each profile is shown with the matrix signal subtracted and included (shown*) Horizontal scale is 2000-11000 Da. Vertical scale is relative intensity 0-100.

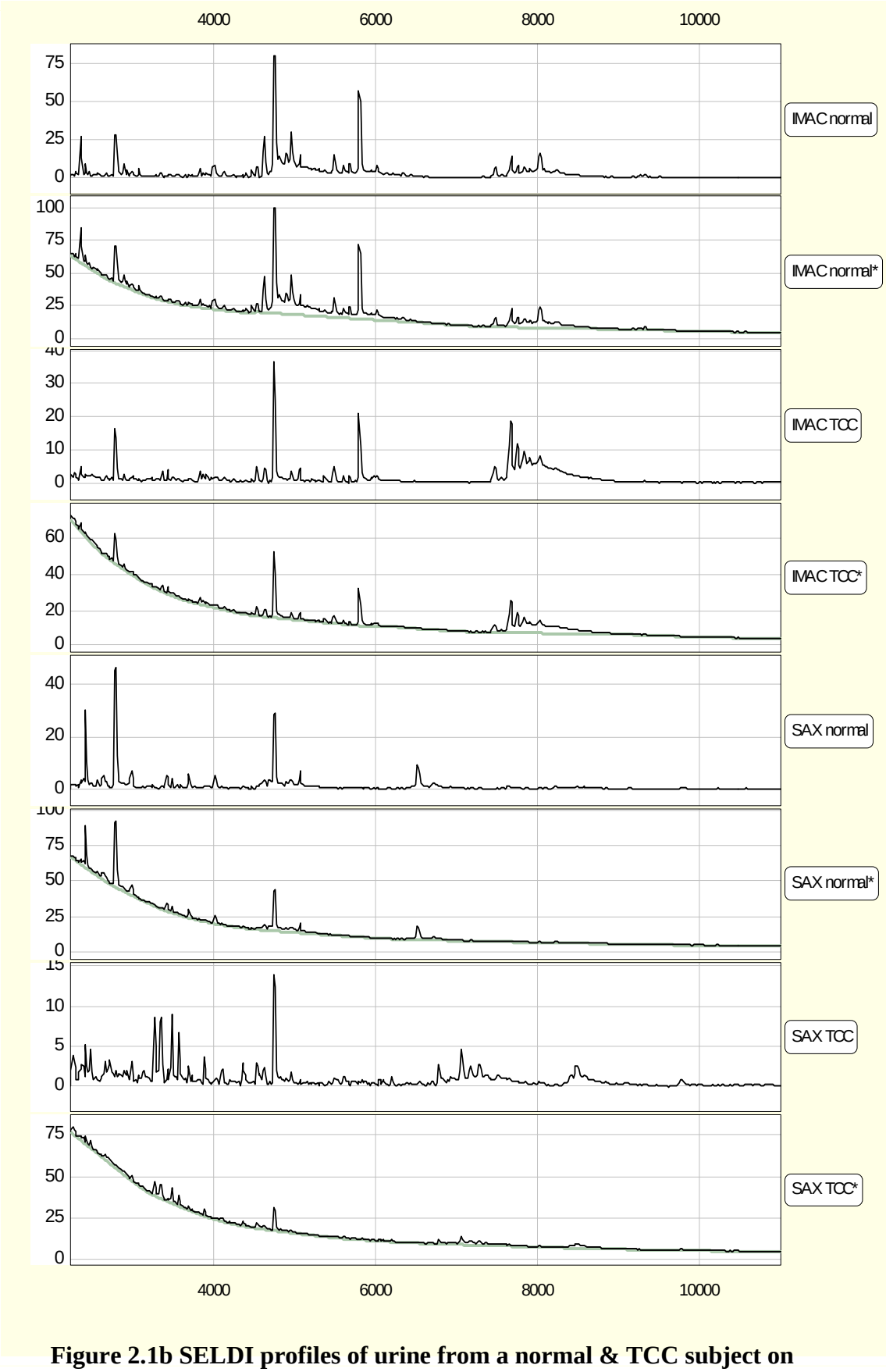


Figure 2.1b SELDI profiles of urine from a normal & TCC subject on IMAC and SAX Chips. Each profile is shown with the matrix signal subtracted and included (shown*). Horizontal scale is 2000-11000 Da. Vertical scale is relative intensity 0-100.

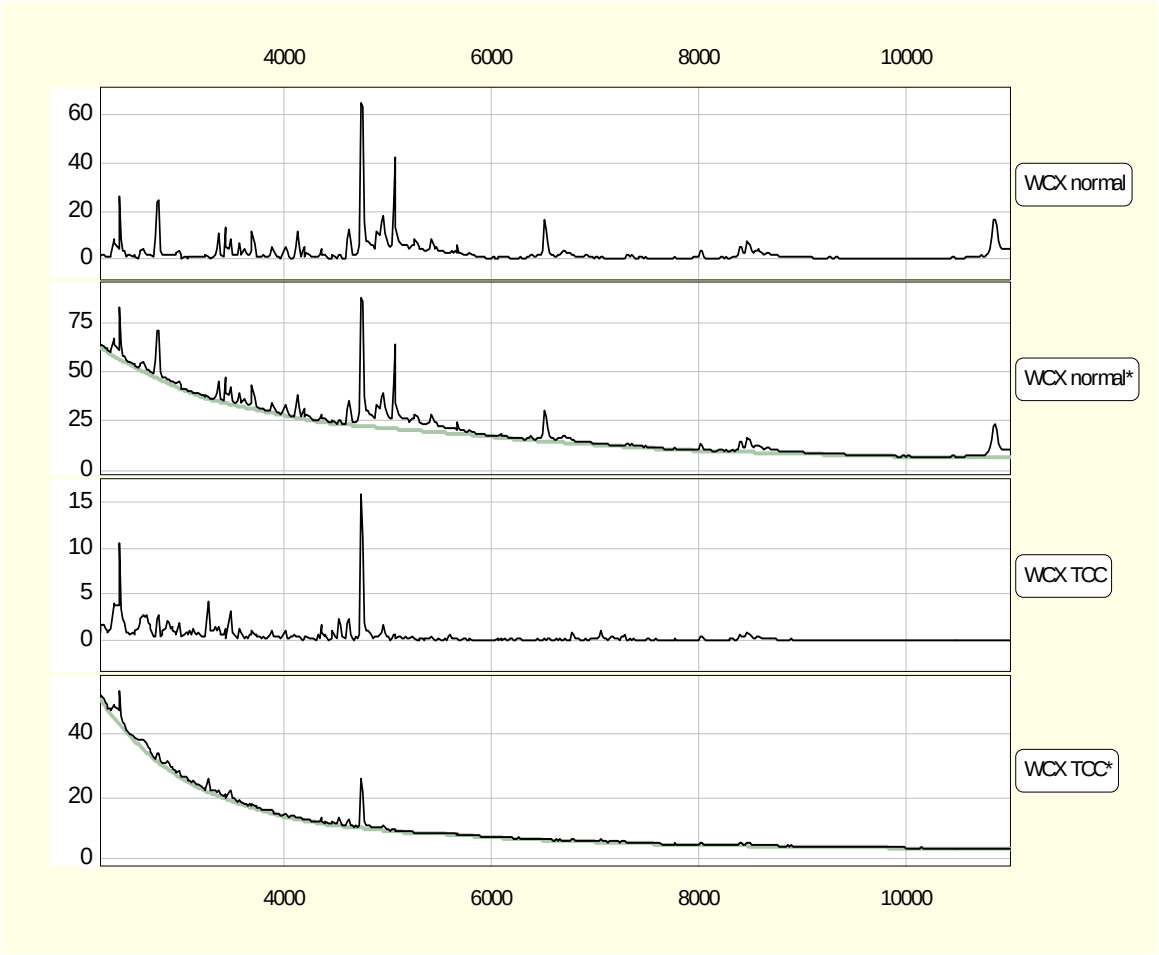


Figure 2.1c SELDI profiles of urine from a normal & TCC subject on WCX Chips. Each profile is shown with the matrix signal subtracted and included (shown*). Horizontal scale is 2000-11000 Da. Vertical scale is relative intensity 0-100.

2.4.3 Assessment of reproducibility of manual SELDI-MS profiling

Initial quality control (QC) assays were performed on all chip types (NP2, H4, SAX, IMAC, WCX) to assess reproducibility in mass values. Three prominent peaks were chosen and their masses recorded from the SELDI profiles on 4 spots on the same chip and 3 spots on different chips. Coefficients of variation (CV) were calculated (standard deviation/mean, expressed as a percentage). All CV were less than 0.1%, mean 0.04% (range 0.001-0.097%) and did not vary with chip type. However, it became clear that selecting only 3 large-magnitude peaks was insufficient to accurately assess QC when profile analysis was based on numerous peaks of smaller-magnitude.

Therefore the QC assessment was repeated; aliquots of a representative pooled urine sample from 3 TCC and 3 normal control urines were applied to spots on 3 SAX2 chips to produce protein profiles. Twenty identifiable peaks were selected at random within the mass range of interest (3000-20000 Da) and their m/z and intensity values recorded from 4 spots on different chips and 4 spots on the same chip. The 20 peaks used had a median intensity of 11.76 (range 2.82-29.62), the mean CV for mass values were 0.028% (range 0.008-0.052%) within a single chip and 0.036% (range 0.013-0.068%) between chips. The intensity variation was higher with mean CV of 32.9% (range 8.2-58.6%) within a single chip and 42.6% (range 17.5-107.0%) between chips.

One technique to reduce intensity variation between spots on different chips is to express the intensity values as a fraction of the total ionised current for that chip profile. This corrects for differences in global ionisation levels between chips and is discussed further below. When the spectra were normalised for total ion current, the intensity variation decreased to a mean CV of 20.2 % (range 3.5-42.9%) within a single chip and 30.3% (range 9.6-57.9%) between chips.

2.4.4 Assessment of protein degradation following warm and cold storage

Protein degradation in samples not processed immediately may adversely affect the quality of the profiles produced, so we undertook experiments to determine the stability of the urine samples. One normal urine sample (protein concentration 0.11g/l) was collected and processed with large, mini or no protease inhibitor (PI) tablets and applied to a SAX2 chip immediately or after 1 or 7 days cold storage at -80°C. Half of the samples received a further 10 µl solublised PI upon thawing and the urine was applied to the chip after 0, 1, 2 or 4 hours storage on ice. The profiles produced were examined for differences in peak size, number and signal/noise ratio.

No deterioration in the quality of the profiles was seen after 1 hour of storage on ice, a negligible deterioration was evident at 2 hours, and a marked deterioration was evident at 4 hours compared to samples run immediately. The degradation seen after 2 hours was abrogated by the addition of PI solution upon thawing and less apparent when a large PI tablet was mixed with the sample before freezing. Storage for 1 week made no difference to the quality of the spectra. These findings were incorporated into our method: standard samples were mixed with a mini PI tablet upon collection, and processed within 1 hour of thawing.

3 Urinary SELDI-MS profiling of TCC and controls

This chapter describes the application of SELDI-MS techniques developed in Chapter 2 to profile increasing numbers of urine samples for putative biomarker discovery. This chapter is structured around 6 major profiling experiments. After reviewing the data from each we altered the profiling techniques to improve test performance and reliability. These alterations in method and analytical technique are included in the text between these experimental runs. The second part of this chapter describes our work to identify some of the protein peaks using immunodepletion and the last part describes subjecting our urine samples to the latest commercial urinary biomarker (NMP22) by way of direct comparison with SELDI profiling.

3.1 Qualitative assessment of SELDI-MS profiles

3.1.1 Profiling experiment 1

A pilot study to diagnose TCC based upon SELDI-MS profiles was undertaken on urine samples from 30 TCC and 30 healthy normal subjects. The TCC subjects were of mean age 71.2 years (range 61-84) and comprised 21 Ta, 6 T1 and 3 T2 TCC, of whom 5 were grade 1, 16 grade 2 and 9 grade 3. The normal subjects were of mean age 54.6 years (range 23-78 years). The samples were randomised and run singly on 8 chips of each chip type (NP2, H4, SAX, IMAC, WCX). Each sample comprised 25µl of urine diluted with water to give a protein concentration of 0.05g/l (total protein load 1.25µg) as previously determined (Section 2.4.2). The high peak-intensity variation between identical samples demonstrated by the QC experiments (Section 2.4.3) led us to avoid quantitative analysis. Hence, the profiles generated were qualitatively examined by eye to identify the presence or absence of individual peaks within the profiles (Figure 3.1). However, we did identify apparent differences in the peaks from TCC and control urines which suggested that further study was warranted. The vast majority of apparently differential peaks were seen on SAX and IMAC chips, in line with the initial chip-type experiments, and further analysis using the other chip types was halted.

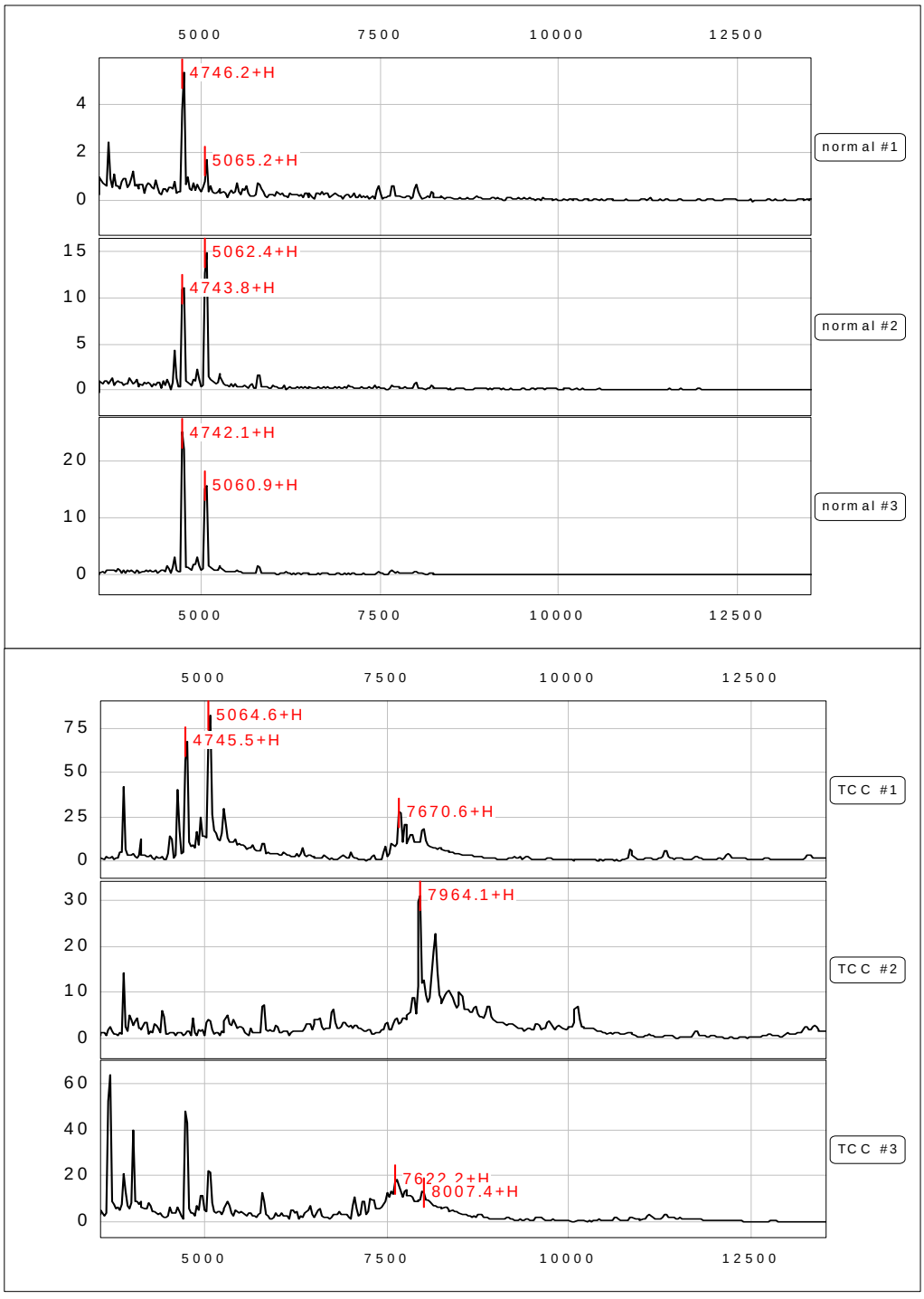


Figure 3.1 Representative SELDI profiles from 3 TCC and 3 normal urine samples using IMAC3 chips. Qualitative analysis suggests that the peaks seen 7600-8007 Da are more commonly identified in TCC vs. normal urine samples. Horizontal scale 4000-13000 Da, Vertical scale is relative intensity 0-100. (Note this varies for each sample). This Figure was presented as part of a poster in the British Electrophoresis Society Annual Meeting (York) 2001.

3.1.2 Profiling experiment 2

A further profiling experiment was undertaken to assess the performance of an assay based upon this approach. We decided to include additional controls representative of the normal population who develop TCC (UTI, BPH, urinary calculi etc.). This increased the sample size to 86, comprising 40 TCC and 46 controls (33 normal and 13 benign controls). The TCC subjects were of mean age 72.4 years (range 61-94) and comprised 27 Ta, 8 T1 and 5 T2 TCC, of whom 7 were grade 1, 21 grade 2 and 12 grade 3. The normal subjects were of mean age 62.3 years (range 23-83 years). The benign controls comprised 6 BPH subjects; patients attending hospital for prostatic surgery (without a catheter in-situ); 5 UTI subjects were identified from patients with clinical evidence of infection and an organism cultured from their urine and two urinary calculi subjects from patients with clear radiographic evidence of calculi.

All peak spectra were inspected by eye and the presence or absence of each peak recorded (IMAC3: 88 peaks, SAX2: 75 peaks). We used the peak-detection algorithm incorporated in the SELDI software (Ciphergen) to select features in the spectra as peaks based upon their signal/noise ratio exceeding 3. However this criterion requires the estimation of noise based upon further complex algorithms. We spent a considerable effort to optimise the automated peak detection by manipulating the variable parameters with only moderate results. Hence, the peak detection in each profile was checked by eye, which for 14,018 data points took many days. However we identified several peaks that appeared to be differentially expressed in tumour and normal samples and examples are shown in Figure 3.2 (SAX2 chips) and Figure 3.3 (IMAC3 chips). No single peak was tumour-specific. Several peaks were identified that were differentially expressed between TCC and control samples. A group of 9 peaks was selected that was differentially expressed between TCC and controls (Table 3.1). This method of analysis was time consuming, subjective and difficult to reproduce.

However, despite these difficulties, a selection of peaks clearly determined by eye from both chip types was identified that appeared to discriminate between TCC and normal subjects. All TCC samples expressed one or more of these peaks. If the presence of one or more of these peaks was considered as a diagnostic test, these results suggest 100% sensitivity. One control urine sample expressed one of these peaks (a false positive) equivalent to 97% specificity between these TCC and normal subjects. However, these peaks were present (albeit it smaller numbers) in the non-normal benign controls such as UTI and urinary calculi. When TCC urine sample profiles were compared with these non-normal controls, specificity fell to 31-37%. This disappointing result suggested that many of the peaks that were apparently associated with TCC were also seen in benign inflammatory states, often associated with infection. This finding reflects the experience of many proposed non-invasive TCC biomarkers whose performance is significantly impaired in comparison with similar controls (Duggan and Williamson, 2004).

Table 3.1 Qualitative SELDI-MS profiling of 25 TCC and 61 controls using SAX2 and IMAC3 chips. When the presence of any of the peaks identified on either chip is combined (or the absence of peak M/Z 6274*) the frequency in the sample groups is shown on the bottom row, in *italics*.

M/Z (Da)	Chip type	TCC (n=25)	Normal c. (n=29)	Benign c. (n=16)	Malignant c. (n=16)
6945	IMAC3	8	0	2	0
10265	IMAC3	7	0	2	0
10528	IMAC3	9	0	5	1
10840	IMAC3	13	0	4	1
13270	IMAC3	6	0	3	1
7557	SAX2	20	1	2	1
11310	SAX2	4	0	0	1
16950	SAX2	5	0	4	10
6274*	SAX2	12	0	5	7
combined	*both*	*25*	*1*	*11*	*10*

We also felt that identifying the presence or absence of peaks by eye was a crude method to understand the urinary proteome, which limited our collection of data with which to discriminate TCC from controls. Each SELDI-MS profile consisted of approximately 32,000 data points, each comprising a mass/charge (M/Z) ratio and an intensity value. Within the area of most peaks (2500-20000 Da) there are ~10,000 data points. Manipulating this amount of data for further study required robust automated tools.

Therefore, despite the limitations imposed by variable intensity of the peaks an attempt was made to examine these data quantitatively using software supplied with the SELDI-MS: "Biomarker Wizard" (Ciphergen). This package measured the intensity of peaks (selected using the Ciphergen peak-detection software) in each profile. Each profile was assigned to a class (ie. TCC or normal) and the average intensity values of each peak for each class was graphically displayed. A t-test was used to generate a statistical value of difference. Again this tool proved only moderately useful. Critically, the peak-detection algorithm seemed incapable of robustly identifying peaks, either failing to identify features of the spectra that appeared by eye to the investigator to represent peaks, or identifying peaks from what appeared to represent noise to the eye. Without being able to attribute a molecular identity or otherwise to these spectral features, it was impossible to know which features represented a peak, ie. a protein/peptide ion. Both the standard peak detection software and analysis software developed by Ciphergen were inadequate for our needs, and peak detection by eye remained to insensitive, subjective and laborious. Hence we attempted to develop better approaches to both peak detection and subsequent analysis to overcome this problem.

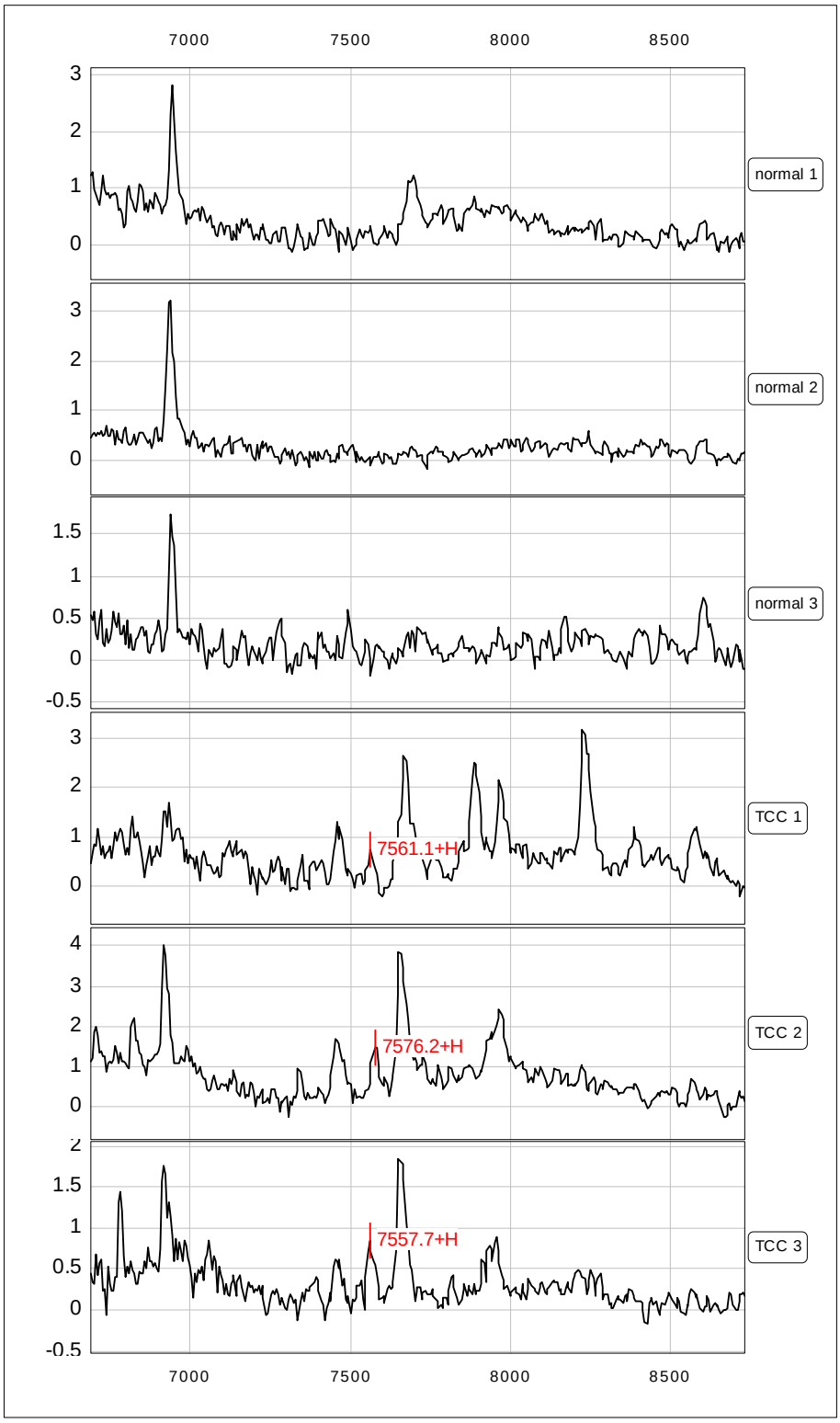


Figure 3.2 SAX2 spectra in range 6500-9000 Da from profiling experiment 2. Three representative normal and 3 TCC sample profiles are shown. Peak at 7557-7576 Da is only present in TCC. Vertical scale is relative intensity 0-100.

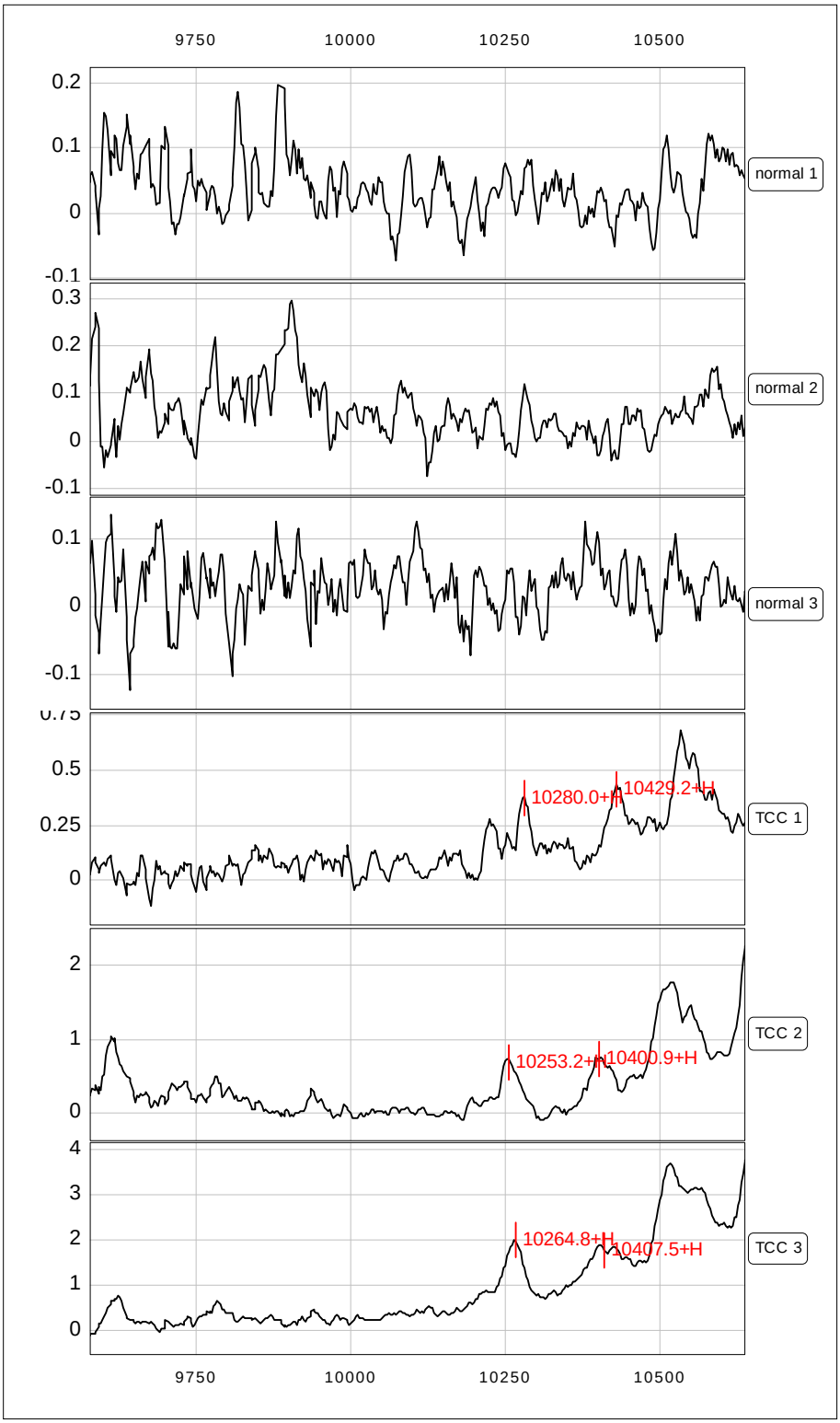


Figure 3.3. IMAC3 spectra in range 9500-11000 Da from profiling experiment 2. Three representative normal and 3 TCC spectra selected. Peaks at 10253-10280 and 10400-10429 Da are only present in TCC. Vertical scale is relative intensity 0-100.

3.2 Development of a peak detection algorithm and artificial neural network (ANN) to analyse SELDI profiles

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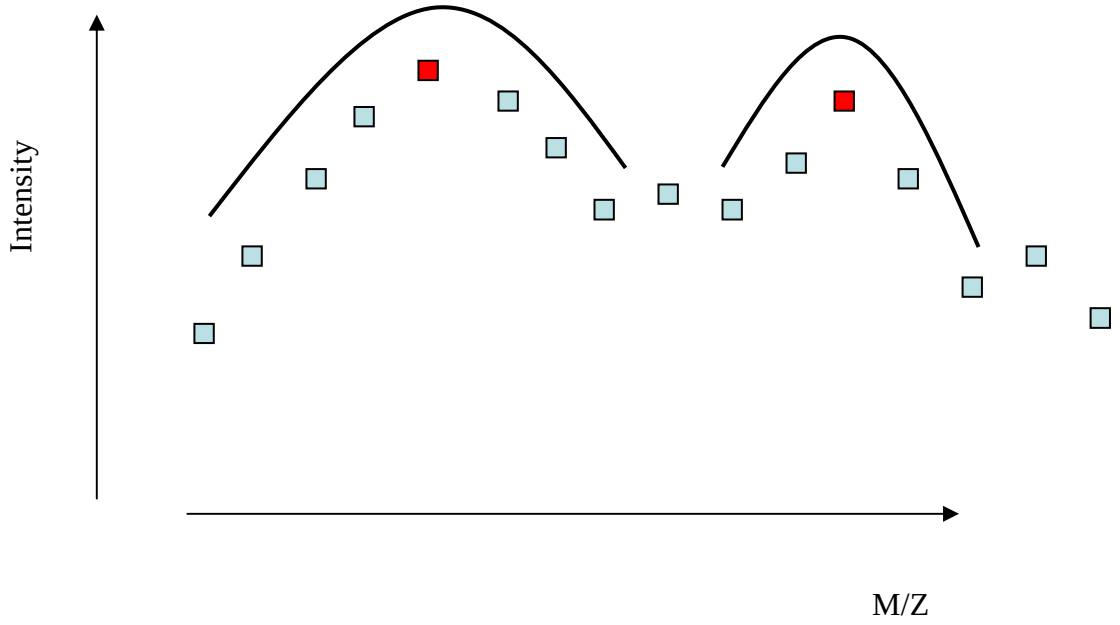
Our collaborators from the School of Computing, University of Leeds, Paul Clarke and Jason Noble, developed a novel algorithm to detect peaks within this data set. "Windows" were passed across each profile, identifying potential peaks as data points with a higher amplitude than the points on either side (Figure 3.4a). This simple approach appeared to correlate most closely with our visual definition of a peak, and was able to consistently and reproducibly identify peaks within SELDI profiles. Each peak was subjected to a chi-squared test to determine its ability to distinguish between cancer and control cases. Initially, the 50 peaks with the highest chi-squared values (i.e. independently the most discriminating) were selected for use as key variables in further analysis.

Paul Clarke also established a fully interconnected feed-forward artificial neural network (ANN). Detailed discussion of machine learning tools such as ANN is beyond the scope of thesis, but in summary the ANN comprised 50 binary inputs, 5 hidden layer neurons and one output neuron. The hidden neurons mapped the sum of their inputs to their outputs using a standard sigmoid function ($1/1+e^{-x}$), and a prediction of cancer corresponded to a high signal on the output neuron. All connection weights were randomly initialised in the range (-1, +1), the network was sequentially presented with useful peak data for each subject in the training set and trained using back propagation. A diagrammatic representation of such a network is shown in Figure 3.4b. For ANN classification of a data set it must be split into completely separate groups, one to train the ANN to separate TCC/controls and one to present for testing to the trained ANN. Clearly, testing profiles derived from subjects used in training the ANN would give a false improvement in test performance. Within a limited subject set there is a need to identify the best size for these groups. We were advised by our collaborators following their experimentation that the training set should be a minimum of approximately 100 profiles (data not shown).

a)



b)

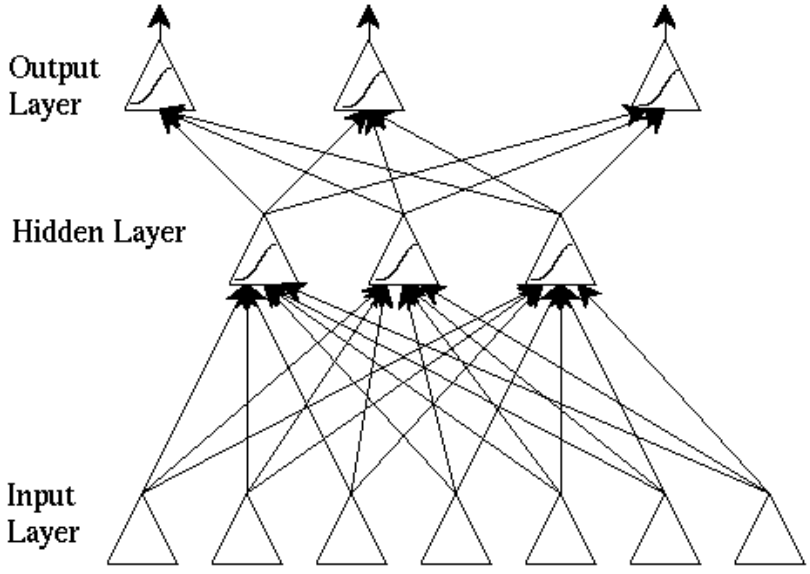


Figure 3.4 Diagrammatic representations of (a) the peak detection algorithm and (b) the architecture of a typical Feed-forward, back propagated ANN. In 3.4a the 2 data points in red are designated peaks as they are surrounded by adjacent data points of lower intensity. In 3.4b the ANN is shown to comprise 7 input layers, with 3 nodes in the hidden layer and in the output layer. The 3 output layers map their values to a single node, to generate a single value that describes the input data. The sigmoid curves drawn within the nodes represent the mathematical function applied to the values at each node.

3.3 Profiling TCC & control urine samples with ANN

3.3.1 Profiling experiment 3

We repeated the profiling with 149 urine samples. This group comprised additional aliquots of 66 of the 86 subjects used in experiment 2, (20 subjects' urine stocks were exhausted), and 83 newly collected samples. The clinical details of this group are shown in Table 3.2a. The samples for profiling experiment 4 were randomly divided into a training set of 112 samples (56 TCC, 40 normal controls and 16 benign controls) and a test set of 37 (18 TCC, 11 normal controls and 8 benign controls). The sample details are shown in Table 3.4a. The training set spectra were analysed and the 50 most discriminating peaks used as input values into the ANN as before.

Several modifications to the data analysis were undertaken in an attempt to improve the accuracy of the system and each was examined independently. In the simplest case, the presence or absence of the peak within a sample was recorded as [0,1]. For subsequent models the intensity value of any sample peak within the window of the discriminating-peak (+/- 0.1%) was used. The values were expressed as a fraction of the maximum peak intensity: 100 (assigned the value 1) to give all peaks a value between 0 and 1.

These intensity values were additionally corrected for variations between experimental runs. The total ionisation (total ion current) of each spectrum was compared to a standard to obtain a coefficient for each spectrum. All peak-intensity values within each spectrum were multiplied by this coefficient, in effect expressing each peak intensity value as a fraction of the total ion current for that spectrum rather than as an absolute value. We had previously shown that this did reduce peak intensity CV (Section 2.4.3) but its effect on ANN performance was untested.

a)

TCC n=74	Mean age (range)	75 (44-95)	
	Male/female	55/19	
	Tumour Stage	Tx*	3
		pTcis†	4
		pTa	46
		pT1	15
		pT2+	6
	Tumour Grade	Gx‡	1
		G1	9
		G2	32
		G3	32
Normal Controls n=51	Mean age (range)	53 (23-95)	
	Male/female	32/19	
Benign Controls n=24	Mean age (range)	62 (29-89)	
	Male/female		17/7
	Type	UTI	7
		BPH	12
		Stone	5

b)

TCC N=33	Mean age (range)	71 (37-90)	
	Male/female	22/11	
	Tumour Stage	Tx*	2
		pTcis†	0
		pTa	23
		pT1	6
		pT2+	2
	Tumour Grade	Gx‡	0
		G1	3
		G2	19
		G3	11
Normal Controls N=30	Mean age (range)	64 (37-92)	
	Male/female	22/8	
Benign Controls N=10	Mean age (range)	62 (34-86)	
	Male/female	8/2	
	Type	UTI	2
		BPH	3
		Stone	5

Table 3.2 Characteristics of sample groups in SELDI-MS profiling experiments 3 and 4. a) Combined Test/Training set in profiling experiment 4 (n=149). b) Late validation set in Profiling Experiment 5 (n=73). 1973 WHO Tumour Grade (Mostofi, 1973) and 1997 UICC TNM stage (Sobin, 1997). *Tx= staging not possible, †pTcis=carcinoma in situ, ‡Gx= ungradeable tissue

The hydration status of patients was considered by multiplying the peak intensity by a correction factor based on the urine creatinine concentration. Clinical chemistry performed upon urine routinely expresses analytes present as a ratio with urinary creatinine to correct for the patients hydration status. We were unable to simultaneously dilute the urine to correct for both protein (to generate the most informative SELDI spectra) and creatinine (to correct for hydration) as they vary independently within a sample. We therefore attempted to overcome this by applying a correction factor to the intensity values of each peak proportional to the protein/creatinine ratio within a sample before ANN processing.

The test spectra were then analysed and a prediction of TCC/normal produced with accompanying sensitivity, specificity, and positive and negative predictive values (Table 3.3). For the type of analyses that seemed most promising, a further 10 test sets (of an identical TCC/control mix to the original test group) were randomly created from the original group and tested with ANNs trained on the remaining samples. The median values are shown in Table 3.3 in parentheses.

Differentiation between TCC and controls was enhanced by using intensity rather than binary peak values. The highest predictive accuracy of the ANN was obtained using intensity values without correction for ionisation or hydration status (sensitivity 83%, specificity 74%) or values corrected for the level of total ionisation (sensitivity 78%, specificity 84%). Correcting for hydration did not improve predictive sensitivity or specificity.

Analysis type	Sensitivity	Specificity	PPV*	NPV†
Binary values, no corrections	55	100	100	70
Intensity values, no corrections	78(83)	84(74)	82(75)	80(82)
Intensity values, corrected for total ionisation	67(78)	95(84)	92(82)	75(80)
Intensity values, corrected for hydration status	67	95	92	75
Intensity values, corrected for total ionisation & hydration	61	89	85	71

Table 3.3 Summary of predictive accuracy (%) of assay analyses according to peak data analysis strategy. *PPV= positive predictive value, †NPV= negative predictive value. Classification threshold 0.5.Values () represent median values from a further 10 test sets.

3.3.2 Profiling experiment 4

The most predictive analysis determined from the analysis of experiment 3 using uncorrected intensity values, was applied to a second validation set of urines collected prospectively over the subsequent 6 months (Table 3.2b). Unfortunately, the predictive accuracy was completely lost with a sensitivity of 6% and specificity 92.5%, (PPV= 40%, NPV 54%). This poor predictive accuracy was not improved by applying additional correction factors described above.

To identify the cause of this change we performed experiments that showed the spectra produced from frozen aliquots of identical samples varied in peak mass and particularly peak intensity compared with the original spectra generated. When these samples were rerun on chips from the original batch, spectral differences were reduced but not abolished. Similar work in our laboratory to profile renal tumours from urine suffered identical change in the spectra produced. This is explained in detail elsewhere (Rogers et al., 2003). We concluded that the

errors inherent in this method of SELDI profiling required a new approach for this technique to increase its robustness, described below.

3.3.3 SELDI mechanical reliability and reproducibility

In 2002/3 the widespread issues of poor reproducibility and data quality associated with SELDI-MS profiles were reported in the scientific literature. The SELDI-MS in our unit became increasingly unreliable and several chips could not be processed to generate a profile due to mechanical failure. Problems were encountered with the unit's firmware, computer hardware, and vacuum pump. It became apparent that in particular the energy imparted by the laser was gradually declining. This accounted for many of the poor results from profiling experiment 5; samples were simply not achieving similar ionisation when subjected to MS with unchanged laser parameters. Such difficulties were compounded with chip manufacturing errors shown in Figure 3.5. It was also clear that our QC measures were insufficient to detect subtle changes in SELDI performance and would need improvement.

3.4 TCC profiling with improved SELDI-MS techniques

Each of these issues was addressed in turn. The SELDI-MS was replaced and upgraded and carefully calibrated to identify and correct performance characteristics. Preliminary experiments were undertaken as previously described (Rogers et al., 2003) to optimise processing and for chip selection with the SAX2 chip initially selected. This was subsequently superseded in the main part of the study by the replacement Q10 anion exchange chip. For the main study profiling, a Biomek 2000 robot (Beckmann Coulter, Fullerton, CA) was used for sample loading.

The SELDI profiles were normalised by total ion current in the region of analysis, excluding the matrix region, and baseline subtracted using the Ciphergen software. Data was then exported and peaks detected and aligned using the in-house algorithm previously described.

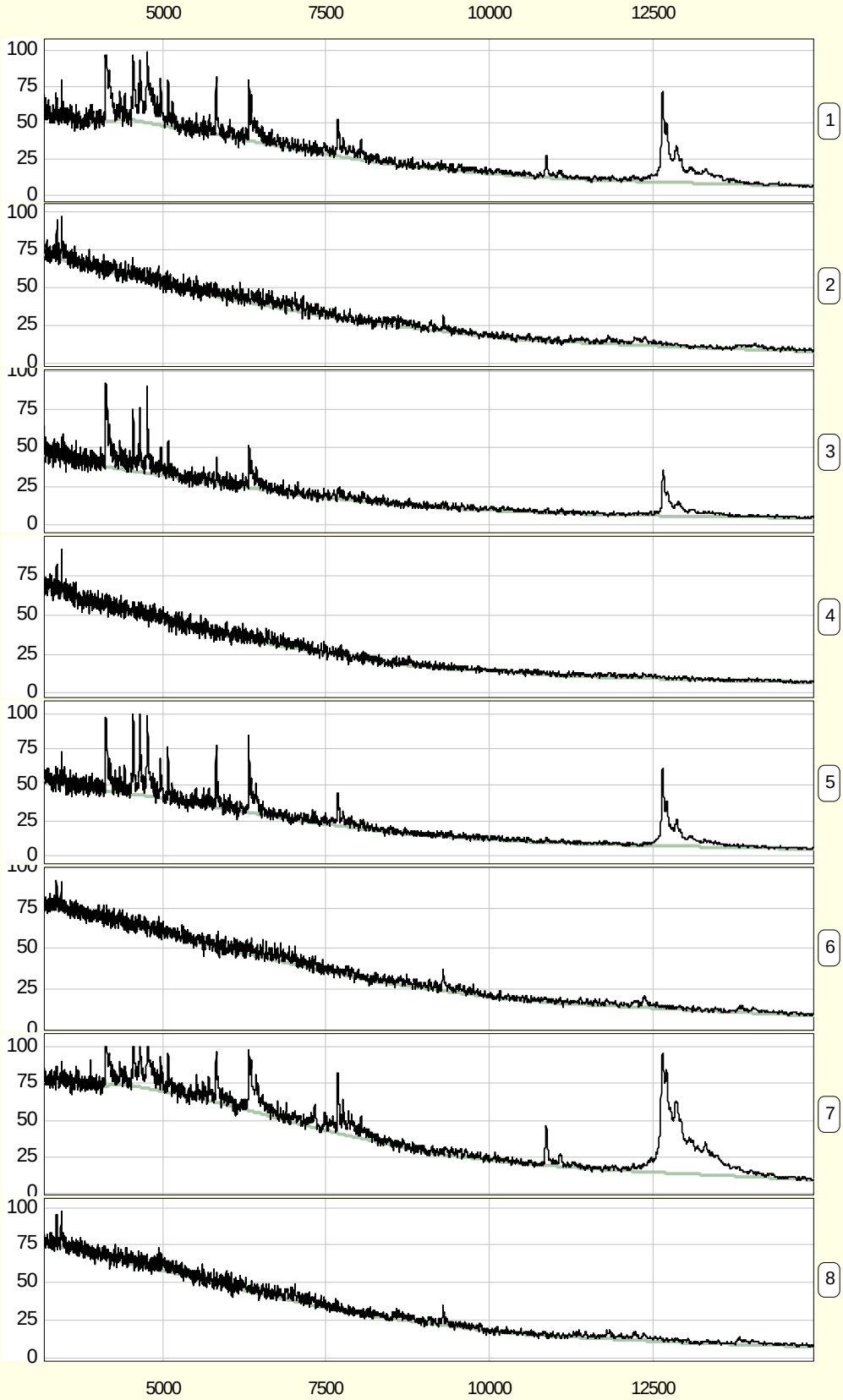


Figure 3.5 SELDI profiles showing IMAC chip flaws 2002. A single test (TCC) urine sample was applied to all spots (1-8) on an IMAC chip to test for concerns from poor spectra from IMAC profiling experiments. Urine proteins appear to only bind to alternate spots. A test sample of IMAC chips were subsequently recalled worldwide and the manufacturing process revised. No repeat episode has been encountered in our laboratory

The QC protocol was largely developed by others and analysis of the QC data is described in detail elsewhere (Rogers et al., manuscript in preparation) but in brief, the QC spectra from each chip were analysed in terms of total ion current (TIC), peak intensity, numbers of matched peaks and intensity deviation across the whole spectra and in five mass sub-ranges. Any chips with QC spectra outside the standard variation range were not examined further. SELDI performance was checked daily using a calibrant chip and a QC serum sample on a CM10 chip.

3.4.1 Methods: Automated Preparation of SELDI-MS ProteinChips

Urine profiling in experiment 5 and 6 was undertaken with the chip preparation performed by a Biomek 2000 robot (Beckmann Coulter). Up to 12 Q10 chips (anion binding chips similar to SAX2) were placed in the bioprocessor upon the robot and washed twice for 5 minutes with 200µl 1X Q10 buffer (20mM TRIS, pH 8.0). The urine samples were added to wells in a 96-well plate, diluted to 0.05mg/ml protein concentration with water and mixed 1:1 with 2X Q10 buffer. A total of 1.25µg urinary protein in 50µl was applied to each spot on the chips and incubated at 18-20°C for 30 min at 200 rpm. Subsequently the spots were washed 3 times with 1X Q10 buffer (200µl) and then twice with water (200µl). After removing the bioprocessor, the chips were allowed to air dry before two 0.6µl aliquots of matrix were applied by the robot.

3.4.2 Profiling experiments 5 and 6

After collecting an additional 72 samples and exhausting supplies of 5 of the 149 samples used for experiment 3, a new cohort of 216 urine samples were subjected to SELDI-MS profiling: experiment 5. The samples were analysed in duplicate, and each duplicate of each sample was randomised both in terms of the chip used and the day of experiment. For identification of systematic errors during the runs, an experimental QC sample (pooled urine prepared from 7 TCC, 5 normal and 2 benign samples) was included on each chip, assigned to spot A for the first chip, spot B for the second and so on to ensure representative coverage. Thirty two

duplicate profiles did not satisfy the QC parameters described above (section 3.4) and were rejected from further analysis. The remaining 184 duplicate profiles were randomly subdivided into a training set (n=130) and an initial test set (n=54). After a 6 month delay a second test (validation) set (n=43) was also profiled, which shall be discussed below (Section 3.4.5). The experimental design incorporating both experiments is shown in Figure 3.6 and the clinical details for all are shown in Table 3.4.

3.4.3 Statistical analysis using linear mixed effects modelling

Additional statistical approaches were used to investigate these SELDI profiles more thoroughly by our collaborators David Cairns and Jenny Barrett of Cancer Research UK, Leeds. From the 184 duplicate profiles, a total of 357 peaks were detected in the region 2,000-20,000 Da. These data were initially explored by hierarchical clustering using a Euclidean distance metric and Ward's linkage agglomeration method. To identify differentially expressed peaks a linear mixed effects model was used. The linear mixed effects model is a flexible extension of the linear model that can accommodate more than one source of random variation. In this case, a random effect to describe the variation between duplicate SELDI determinations for patients was used.

For identification of differentially expressed peaks between cases (or groups) and controls, significance levels of 0.5% ($p<0.005$) were used. This convention is observed as an *ad-hoc* measure to account for the large number of tests being performed to attempt to minimise the number of false-positive significant effects observed. Conversely, when identifying significant age, sex and run effects in the control group, a significance level of 5% ($p<0.05$) was used as we are less concerned about false positives than about including peaks in subsequent analysis that may have significant age, sex or run effects.

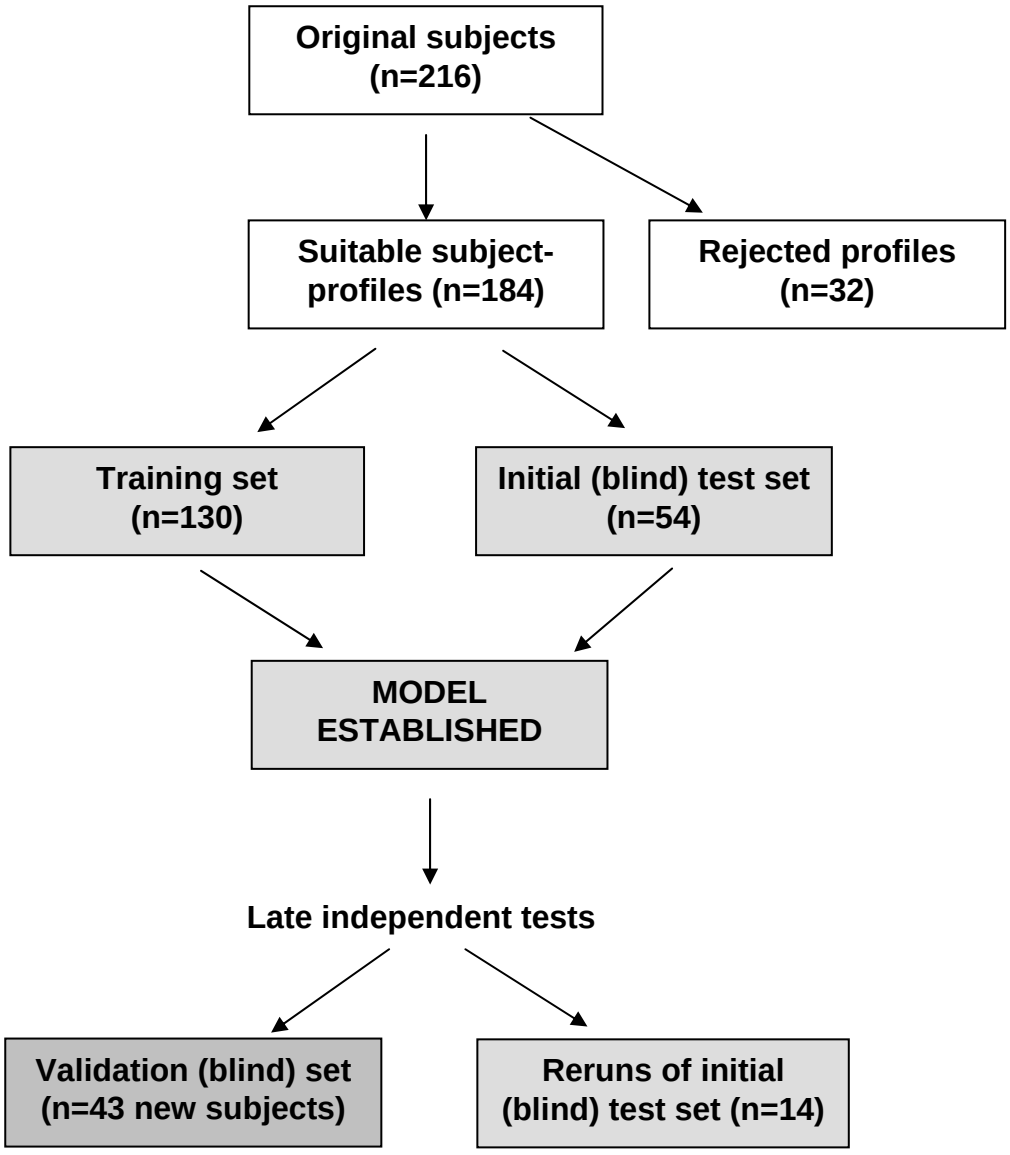


Figure 3.6 Diagram illustrating the design of the study in terms of the different sample sets used for the Random Forest analysis. The specific details of the patient and controls groups making up these sets are described in Table 3.4.

Sample type	Parameters		Training set (n=130)	Initial Test Set (n=54)	Validation Set (n=43)
TCC	**Median age (range)**		76 (31-100)	74 (57-92)	73(66-94)
	Male/female		45/20	24/6	16/7
	Tumour stage	*Tx**	1	1	2
		pTcis	1	2	0
		pTa	44	17	12
		pT1	8	6	4
		pT2+	11	4	5
	Tumour grade	*1*	13	1	3
		2	26	16	10
		3	25	11	10
		cis	1	2	0
Healthy controls	**Median age (range)**		56 (27-84)	50 (27-84)	58(24-81)
	Male/female		30/14	13/4	5/11
Benign Controls	**Median age (range)**		71 (34-89)	68 (33-87)	64(29-77)
	Male/female		17/4	5/2	1/3
	Type	*UTI*	10	5	1
		BPH	10	1	1
		Stone	1	1	2

Table 3.4. Characteristics of subjects in profiling experiments 5 and 6. These comprise Training Set (n = 130; 65 TCC, 44 healthy controls and 21 benign controls), the Initial Test Set (n = 54; 30 TCC, 17 healthy controls, 7 benign controls) and Late Blind Validation Set (n = 43; 23 TCC, 16 healthy controls, 4 benign controls). 1973 WHO Tumour Grade (Mostofi, 1973) and 1997 UICC TNM stage (Sobin, 1997

3.4.4 Identification of discriminatory peaks and potential confounding effects of age, sex and analytical run.

Significant differences in ages between the TCC and control groups were apparent with the normal control subjects on average 20 years younger than TCC subjects (Table 3.4). Several complementary statistical approaches were undertaken to address and correct for the effect of age and sex as possible confounding factors. To establish a point of reference to compare against corrected analyses, the data were analysed using the linear mixed effects model without considering differences in age. Twenty-two peaks were identified as significantly different between TCC and control groups ($p<10^{-7}$). Duplicate profiles of 83 healthy controls were then analysed (with the rationale that healthy controls should not be affected by as many unknown factors as the benign controls and TCC patients) with linear mixed effect models to identify peaks that differ according to run (i.e. initial or 6 months later), sex or age.

At the 5% level (p=0.05), 86 peaks (24% of the total) were identified as changing to some extent with age, sex or run, 3 of which were previously identified as discriminatory between TCC cases and controls (Table 3.5). At the 0.5% level, 16 peaks changed significantly, 5 of which were associated with age (2976.39, 3600.75, 6214.70, 6599.49, 15131.7 Da), 2 with sex (4911.26 and 10766.5 Da) and 9 with experimental run. An attempt was made to correct for these confounding variables by fitting these parameters as further explanatory variables in the linear effects model but this was unsuccessful and was abandoned.

To overcome the problem of age differences, matched subsets of TCC subjects and normal controls were generated from 5-year interval groups, resulting in 39 TCC and control matched pairs in duplicate. The linear effects model described above, correcting for run and sex but not age, was applied to these data and 4 highly significant peaks were identified that were differentially expressed between TCC and controls (Table 3.6), 2 of which were previously identified in the uncorrected and corrected analyses described above. These peaks did not differ between 54 matched pairs of benign controls and TCC (8182.67 most significant

at p=0.08). However a different selection of 9 peaks was identified that were differentially expressed between TCC and the benign controls irrespective of run or sex at the p=0.005 level (Table 3.6). Finally, when healthy and benign controls were compared with TCC patients within the age range 55 to 85 only (127 TCC cases, 20 benign controls and 39 normal controls in duplicate), 23 peaks significant at the 0.5% level were seen (Table 3.7). These include those of 6214.7, 6974.36 and 8182.67 Da previously identified as being significantly different in the age-matched subset between healthy controls and TCC.

2510.65	2567.98	2581.01	2624.08	2709.36	2880.07
2976.39	2993.43	3005.49	3037.77	3070.22	3118.19
3202.78	3484.98	3560.12	3600.75	3641.6	3950.18
4099.19	4156.96	4175.91	4197.28	4234.21	4547.88
4626.18	4753.1	4803.83	4881.73	4911.26	4980.95
5137.57	5154.68	5511.08	5642.64	5693.75	5868.15
5894.89	6100.94	6214.7	6237.87	6599.49	6929.99
6974.36	7176.55	7270.13	7310.87	7397.41	7568.85
7942.12	8020.81	8638.69	9948.71	10202.9	10292.1
10443.4	10766.5	11156.5	11459	11622.6	12166.9
12396.7	12484.7	12540.1	12608	12798.3	12873.1
12983.7	13379.8	14242.3	14608.1	14929.3	15131.7
15306	15657.6	15839.6	15964.5	16180.9	16342.3
17619	17797.3	17873.2	18045.4	18344.9	18591.7
18747.2	19085.4				

Table 3.5 List of m/z values for peaks (n=86) in urine from healthy controls which change to some extent based on age, sex or analysis run at the 5% significance level

Comparison	Peak m/z	Mean intensity controls	Mean effect in TCC	p-value
TCC and normal controls (n=78)	5069.40	1.485	-0.904	0.00009
	6214.70	1.733	-1.966	0.00004
	6974.36	0.435	-0.211	0.00045
	8182.67	0.782	-0.428	0.00034
TCC and benign controls (n=104)	3453.63	1.251	+1.480	0.00105
	3528.44	3.698	+3.377	0.00359
	4409.12	2.949	+1.534	0.00423
	9467.81	1.122	-0.497	0.00203
	17619.0	0.155	-0.099	0.00380
	17648.2	0.124	-0.095	0.00134
	18344.9	0.226	-0.106	0.00175
	18412.0	0.246	-0.107	0.00114
	18591.7	0.229	-0.111	0.00429
TCC and all controls (normal and benign) (n=186)	2808.63	0.643	-0.229	0.00447
	2907.71	0.487	-0.234	0.00349
	3181.01	0.689	-0.347	0.00233
	5303.29	1.253	-0.333	0.00053
	6214.70	0.675	-0.842	0.00253
	6732.82	0.809	-0.332	0.00002
	6974.36	0.494	-0.139	0.00316
	7828.06	1.698	-0.438	0.00212
	7904.55	1.632	-0.563	0.00056
	7978.13	1.917	-0.699	0.00380
	8058.65	1.982	-0.527	0.00259
	8142.88	2.143	-0.570	0.00056
	8182.67	0.871	-0.293	0.00032
	8222.56	1.940	-0.507	0.00065
	8886.03	0.642	-0.201	0.00185
	9467.81	1.092	-0.313	0.00098
	10936.70	0.295	-0.179	0.00015
	17944.4	0.129	-0.046	0.00245
	18270.4	0.137	-0.078	0.00156
	18591.7	0.217	-0.077	0.00083
	18747.2	0.253	-0.067	0.00349
	19141.2	0.299	-0.086	0.00388
	19972.6	0.193	-0.071	0.00207

Table 3.6 Urinary peaks on a Q10 SELDI chip significantly differentially-expressed between groups using age-matched subsets and correcting for sex and analysis run.

3.4.5 Discrimination of TCC samples from controls with Random Forest classification.

The ANN developed for profiling experiments 4 and 5 was superseded by another machine learning tool: A Random Forest classifier programme applied by David Cairns and Jenny Barrett of Cancer Research UK, Leeds. This is a classification method based on "growing" an ensemble of decision tree classifiers. In order to classify a new object from an input, in this case a SELDI profile, the profile is analysed using each of the classification trees in the forest. Each tree gives a classification, "voting" for that class. The forest chooses the classification having the most votes (over all the trees in the forest). A measure of the importance of classification variables is also calculated by considering the difference between the results from original and randomly permuted versions of the dataset.

The 86 peaks identified as significantly ($p<0.05$) associated with age, sex or analytical run were removed from further analyses. The remaining 271 peaks were used for Random Forest classification on the basis of the different groups. The study design is shown in Figure 3.6 and the group characteristics in Table 3.4. In the initial test set (n=54), a sensitivity of 71.7% and specificity of 62.5% was attained with corresponding figures for the late blind validation group (n=43) of 78.3% and 65.0%. In the initial test set, only 2 of 54 duplicate pairs (1 normal, 1 inflammatory) were differently classified between the replicates, one as normal and one as TCC. For the new-subject group of 43 within the late validation group, 8 pairs were differently classified within their replicates.

Of the 14 duplicate pairs that were rerun, 26/28 spectra (93%) were correctly classified (one normal duplicate classified as TCC in both replicates) on initial testing and after 6 further months of storage, 25/28 spectra were correctly classified. Peaks selected by the Random Forest classifier as important discriminators in classifying TCC and controls are shown in Table 3.7 and an example of duplicate SELDI spectra shown as Figure 3.7.

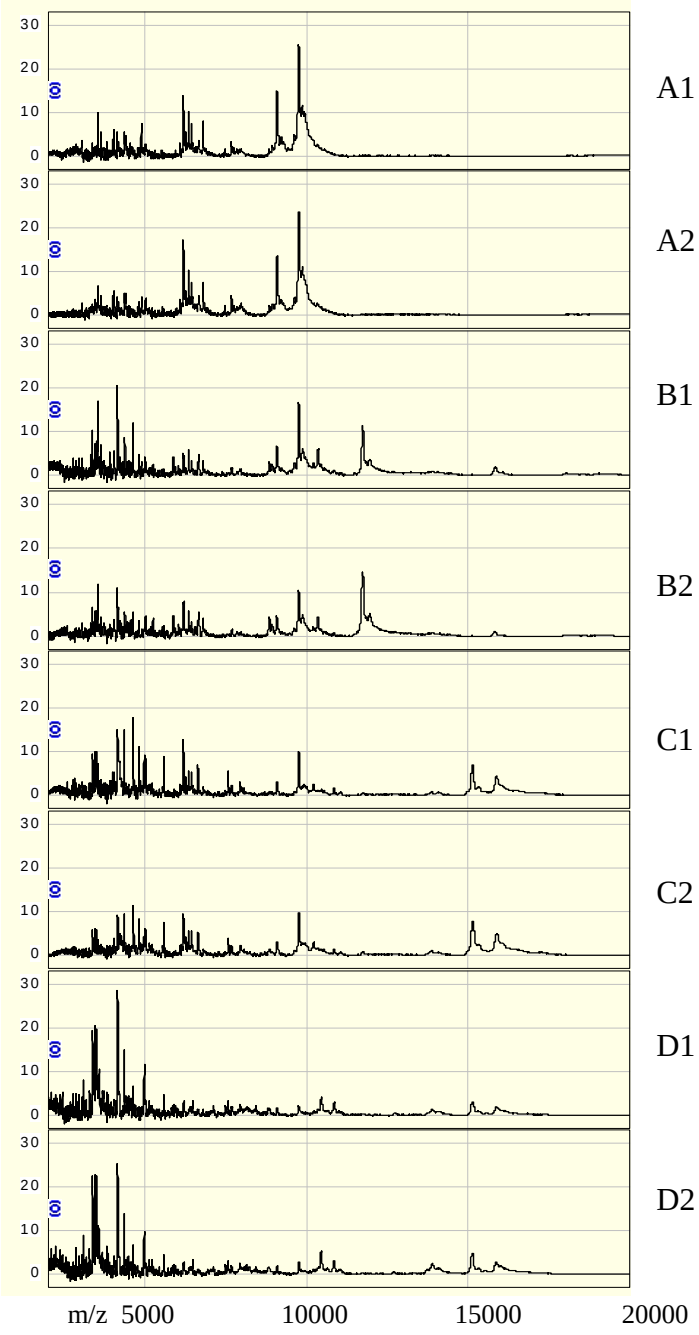


Figure 3.7 Examples of SELDI profiles of urine samples (1.25 µg protein/chip) from 4 patients (A-D; 3 TCC and 1 benign control) on a Q10 anion exchange chip illustrating the heterogeneity of profiles and the reproducibility between duplicates (1-2).

Important peaks to classify TCC	Important peaks to classify controls (benign and healthy)
*8142.88	*3329.67
*3842.53	3670.45
*3239.23	*8142.88
*7904.55	*3842.52
8182.67	*7828.06
*8058.65	*8058.65
*7828.06	*3368.96†
*8222.56	*8222.56
9215.96	*7904.55
9467.81	13887.6
*3329.67	*3239.23
8374.67	3612.87
*7978.13	*7978.13
10565.6	4386.00
9618.39	16031.8
6732.82	*7742.25
*7742.25	10147.4
9080.82	5303.29
8426.81	5914.63
3972.13	3287.45
*3368.96†	3780.21
19541.9	3579.86
8475.73	5069.40
9283.03	4777.16
19804.4	4356.88

Table 3.7 M/z values for the major peaks used by the Random Forest classifier (ranked by importance). Many peaks (marked *) are important to discriminate both TCC and controls. †α-defensin (HNP-2) as confirmed by immunoprecipitation

3.4.6 Comparisons of subgroups of TCC.

To identify peaks that were differentially expressed between high and low stage (T1/2+ vs. Ta) or grade (G3 vs. G1/2) again we used the linear mixed effects model on each peak in the SELDI profile. The age distribution of the TCC groups are quite similar when subdivided on the basis of high and low grade or high and low stage although there were no patients below 60 years of age with low grade and/or stage. To exclude any confounding effect of age the subjects were matched in 5-year age groups as before. This produced 78 patient profiles in duplicate in the high/low stage groups and 98 patient profiles in duplicate in the high/low grade groups.

Hierarchical clustering performed by David Cairns produced dendrograms, with some separation between high and low grade and stage TCC cases (Figure 3.8). By applying the linear mixed effects model and assigning grade and stage in turn as the unknown parameter, we identified 11 peaks that differed significantly between high and low grade and 21 peaks that differed between high and low stage (below p=0.005; Tables 3.8 and 3.9). Five peaks were common to both the stage and grade differential, namely a cluster between 19141.2 and 19884.5 Da.

3.5 SELDI-MS peak identification by immunodepletion.

To identify selected peaks within SELDI spectra, protein databases were searched for candidate proteins with masses similar to observed peak mass values. Where a suitable antibody could be obtained we attempted to deplete and subsequently elute this protein from the urine and show the loss and return of the associated peak in SELDI spectra without a similar effect from control antibodies. The SAX2 chip was used for immunodepletion experiments as the peaks of interest were found to bind to them.

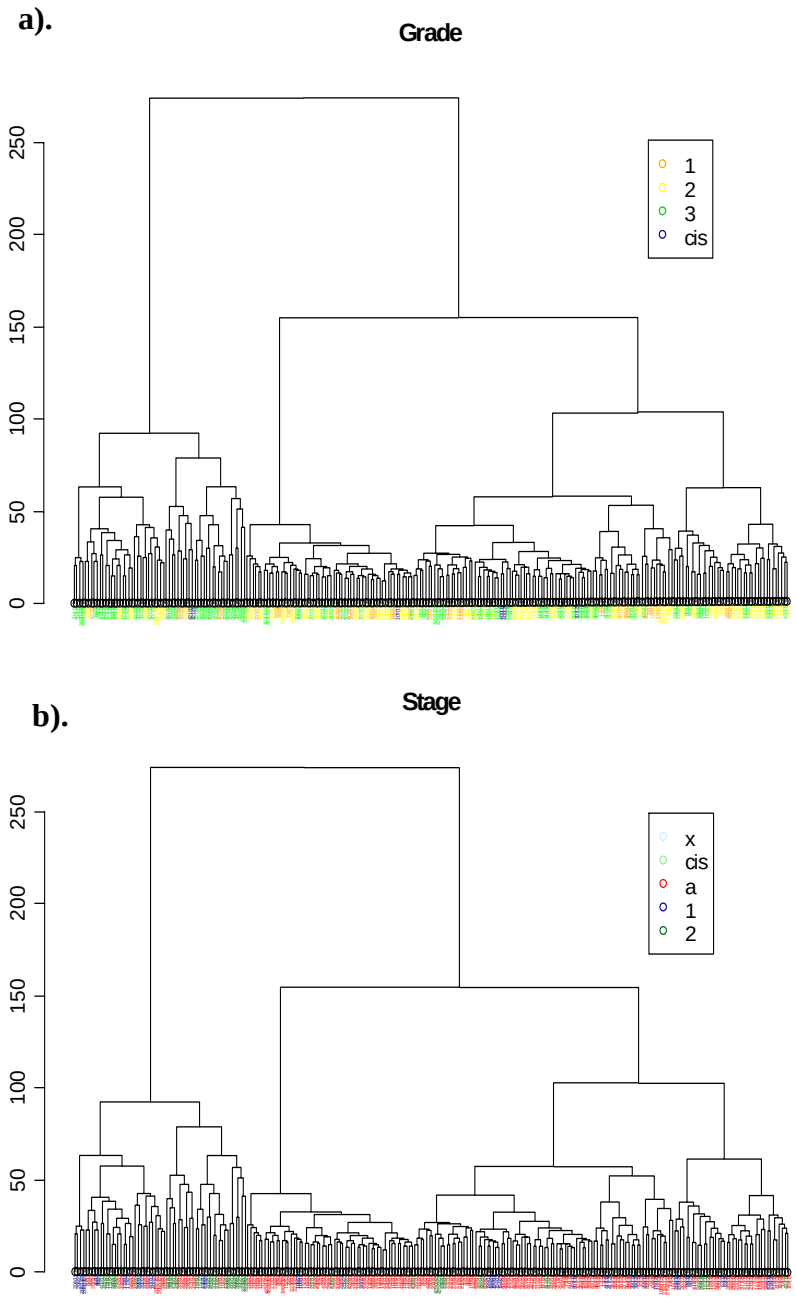


Figure 3.8 Dendrogram showing segregation of TCC by a) grade and b) stage. Hierarchical clustering using a Euclidean distance metric and Ward's linkage agglomeration method were used to generate this figure

Peak, M/Z	Value in Ta	Effect in T1/2+	p-value
3087.53	0.665	+0.561	0.00392
6339.73	6.310	-2.339	0.00116
6538.51	1.620	-0.487	0.00216
7540.14	0.297	-0.162	0.00323
7904.55	0.861	-0.434	0.00379
3978.13	1.403	-0.537	0.00276
9618.39	2.427	-1.056	0.00135
9753.83	15.186	-5.018	0.00399
9864.7	5.862	-2.571	0.00133
10696.2	0.491	-0.215	0.00379
10936.7	0.299	-0.146	0.00118
15306.0	0.677	+0.742	0.00380
17255.5	0.075	+0.148	0.00145
18114.5	0.184	-0.083	0.00334
18669.4	0.166	-0.086	0.00142
19141.2*	0.214	-0.129	0.00071
19242.9*	0.183	-0.106	0.00241
19321.9*	0.200	-0.139	0.00002
19541.9*	0.216	-0.111	0.00220
19652.3	0.211	-0.108	0.00416
19884.5*	0.135	-0.089	0.00161

Table 3.8 Significantly differentially-expressed peaks between age-matched subgroups of high (T1/2+) and low (Ta) stage TCC (n=78, duplicated). *indicates peaks significantly differentially-expressed in both increasing grade and stage

Peak, M/Z	Value in grade 1/2	Effect in grade 3	p-value
4219.9	2.075	+0.739	0.00310
6873.58	0.647	-0.647	0.00478
10715.20	0.529	-0.230	0.00275
16726.9	0.100	+0.070	0.00493
19141.2*	0.250	-0.106	0.00124
19242.9*	0.222	-0.094	0.00338
19321.9*	0.211	-0.119	0.00007
19541.9*	0.227	-0.107	0.00073
19804.4	0.204	-0.089	0.00311
19884.5*	0.152	-0.088	0.00059
19972.6	0.127	-0.076	0.00202

Table 3.9 Significantly differentially-expressed peaks between age-matched subgroups of high and low grade TCC (n=98, duplicated). *indicates peaks significantly differentially-expressed in both increasing grade and stage

3.5.1 Methods: Urine immunodepletion and SELDI-MS

Components from Seize™X Protein G Immunoprecipitation Kit (Pierce) were used and the manufacturer's protocol modified. In summary the protocol was scaled down in volume by 50%, the antibody/urine incubation time was increased by 800% and the antibody cross-linking stage was not used. Initially, 100µl of 50% protein G slurry was added to a spin X cup (2ml spin column and tube) and spun at 10,000g for 1 min in a microcentrifugue (Sanyo Gallenkamp). The remaining protein G was twice incubated with 200µl binding/wash buffer 1 for 1 min and spun at 10,000g for 1 min. The flow through was discarded. 20µg of each target antibody was made up with Binding/wash buffer 1 to 200µl and incubated in the prepared Spin X cup for 60 min at 4°C to bind protein G. The unbound fraction was removed with 3 x 500µl binding/wash buffer 1 washes interspersed with 10000g 1 min spins.

Freshly thawed 1ml urine samples were diluted to 0.05 g/l with water and diluted 1:1 with SAX buffer (#A). Preliminary experiments identified protein degradation during the prolonged timecourse of this technique. Subsequently 10 µl of solubilised PI tablet (1 Mini Complete™ tablet (Roche) /1ml water) was added to the urine upon thawing. This urine solution and antibodies were incubated together within the spin X cup prepared as above for 120 min at 4°C, spun at 10000g for 1 min and the flow-through collected (#B), 95 µl of elution buffer (pH 2.8) was added to the remaining antibody-protein complex, spun at 10000g for 1 min and the flow through collected. To neutralise the eluate, 5µl of 1M TRIS (pH 9.5) was added (#C). Undepleted urine (#A), depleted urine (#B), and eluate (#C) were run immediately on a SAX2 chip in a volume of 50 µl as described above.

3.5.2 Results: Identification of alpha defensins 1-3 and haemoglobin by immunodepletion

A characteristic triplet peak complex of 3380.3, 3452.0 and 3496.4 Da was readily apparent in many urine samples. The first peak of the complex at 3380.3 Da corresponded to one of the significant peaks used by the Random Forest classifier although the mean mass was slightly different when patterns were compared. A similar pattern was shown by Vlahou *et al* (Vlahou et al., 2001) to represent urinary alpha defensins 1-3 which have theoretical masses of 3377.01, 3448.09 and 3492.10 Da (HNP-2, 1 and 3 respectively).

We successfully depleted and subsequently eluted this peak complex using a monoclonal mouse antibody to alpha defensins (Biogenesis) in 2 separate urine samples, one from a patient with TCC (G3pT1) and one from a patient with a urinary tract infection. This effect was not seen with an Ig type and species-specific control antibody (Mouse IgG1 M9269, Dako) (Figure 3.9).

A peak complex at ~15,300 and 16,000 Da was also readily apparent in many TCC samples which we hypothesised on the basis of previous reports (Zurbriggen et al., 2005) and on the correlation with the presence of haematuria detected by dipstick to be haemoglobin alpha (HBA1) and beta (HBB) chains. The human haemoglobin alpha chain comprises 141 amino acids (15126 Da) and the beta chain 146 amino acids; (15867 Da). These proteins may undergo post translational modification glycosylation and acetylation that increase the m/z value in a variety of forms (Zurbriggen et al., 2005). We subjected a TCC urine sample (G3pT2) that contained this peak complex to immunodepletion (Figure 3.10). We demonstrated that this peak complex could be depleted and eluted using an antibody specific for haemoglobin, (Haem 1, Abcam). This effect was not seen with a specific control antibody (Mouse IgG1 M9269, Dako).

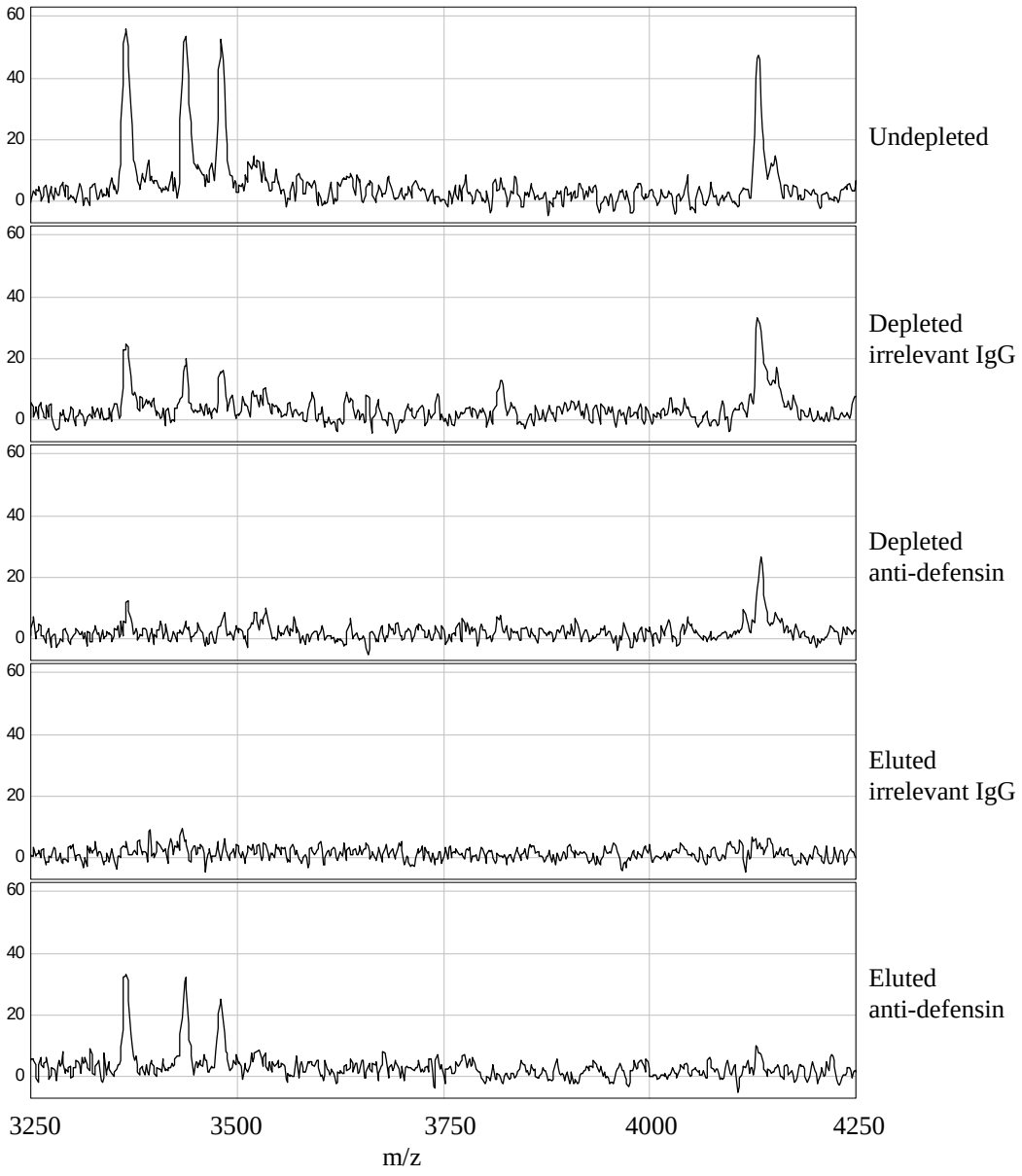


Figure 3.9. Identification of alpha-defensin in urine by immunoprecipitation and monitoring by SELDI. The triplet peak complex at 3380.3, 3452.0 and 3496.4 Da is removed upon incubation with an antibody to a-defensins and can be recovered in the eluate from the antibody. This is not seen with irrelevant control antibody and other dominant peaks, e.g. 4130.5 Da are unaffected.

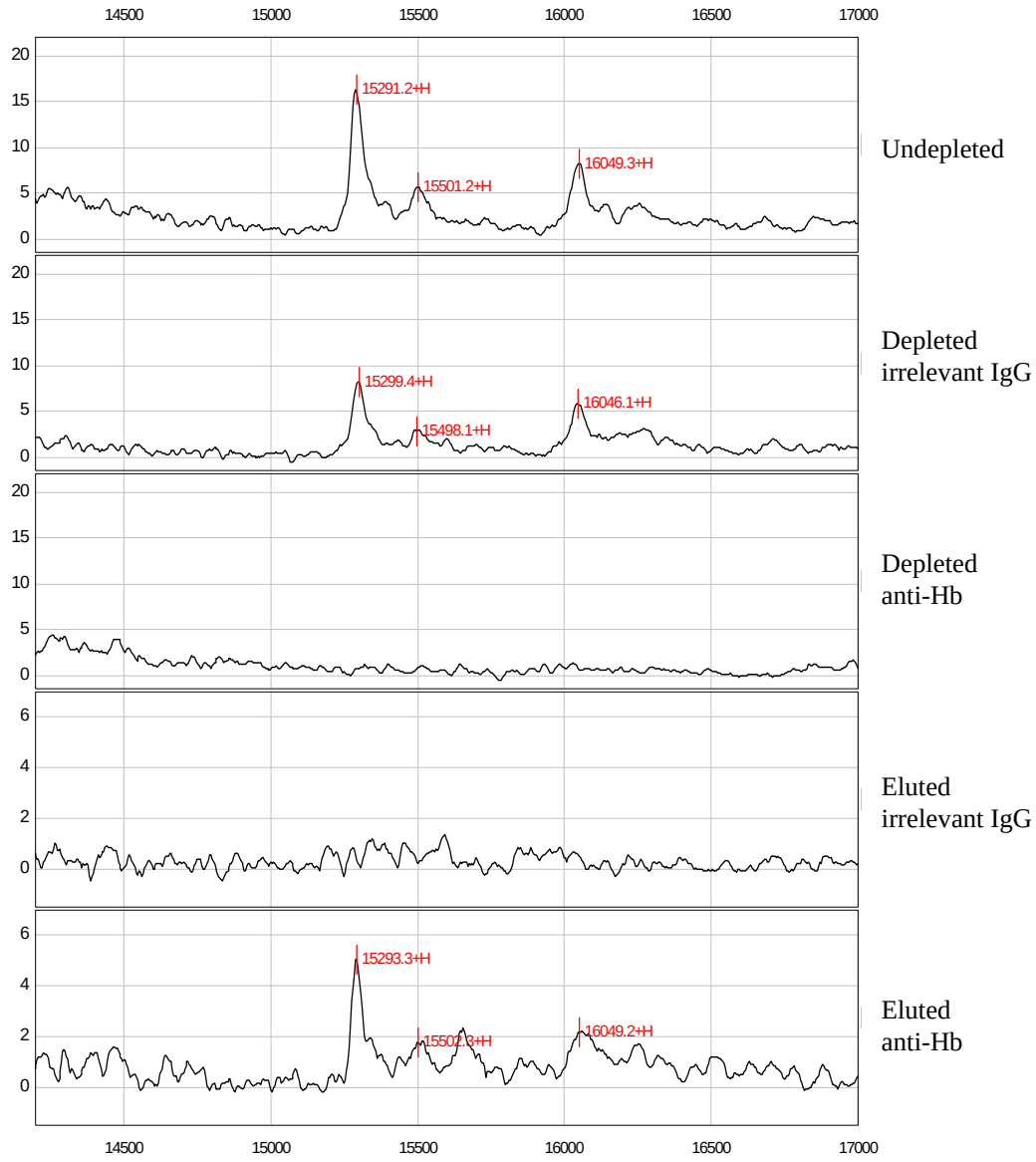


Figure 3.10 Identification of haemoglobin chains alpha and beta by immunodepletion. SELDI-MS profiles showing a complex of peaks, corresponding the alpha and beta haem chains that depleted and subsequently eluted by anti-Hb antibody (but not control antibody).

3.6 NMP22 urinary assay

To compare the performance of a contemporary commercially available assay to detect TCC we subjected a subset of samples to an NMP22 ELISA (Matritech). The manufacturers recommended urinary collection in their patent plastic pots containing a buffer of unknown composition. We prospectively collected 40 samples (23 TCC, 17 controls) in the supplied pots in addition to our standard collection technique that included the addition of a mini-Complete protease inhibitor tablet to the urine sample. We wished to assess the accuracy of this test using our samples and also the effect of the collection buffer used. If we demonstrated that our results were unaffected by buffer type it would allow further investigations using our previously collected sample bank. In the event no further NMP22 experiments were undertaken in this project.

3.6.1 Methods: Urinary NMP22 ELISA

The NMP22 (Matritech) ELISA kit requires urine collection vials containing undisclosed chemicals to stabilise NMP22 and prevent its degradation. The manufacturers stated that this assay was unreliable if the specific collection vials were not used (personal communication). However we wished to measure NMP22 levels in samples already subjected to SELDI-MS. These samples had been previously collected with mini Complete ^{TM} protease inhibitor tablet (Roche) and stored at –80ºC, albeit not in the specified Matritech buffer.

To assess the ability of this ELISA to identify TCC from urine, we prospectively collected a cohort of 40 test-samples (23 TCC and 17 controls). Each urine sample was divided so that aliquots were stored in the method we developed for SELDI-MS and according to the Matritech protocol. These instructions were followed exactly: 10 ml of urine was placed into the collection vial, mixed and stored on ice until processing within 1 hour. The sample was spun at 100g for 10 min at 15ºC and the supernatant frozen in 1ml aliquots at –80ºC.

In total, two 96-well plates were used, each comprising 5 standards, 3 control samples and 40 test samples in duplicate. Twenty urine test sample pairs, comprising the same urine sample were collected in both ways described above. The test samples were randomly allocated to wells on both plates to avoid potential bias. In outline, the ELISA performed was as follows: frozen urine samples were defrosted on ice, briefly centrifuged at 1000g and decanted to remove debris. Meanwhile, the kit wash-buffer, standards and test samples were reconstituted with water. The 96 well ELISA plate was washed 3 times with 300µl wash buffer in an automated aspiration system (Denville) and incubated with 200µl/well of standards, controls and test samples for 2 hours at 20ºC. After 3 further washes 200µl/well of Digoxigenin anti-NMP22 reagent was added and incubated for 1 hour, followed by 3 washes and the addition of 200µl/well of HRP-SAD reagent for 30 min. After 3 final washes 200µl/well of OPD solution was added and incubated for 30 min in darkness. The reaction was stopped upon the addition of 50µl/well of 2M sulphuric acid and the plate incubated for a further 10 min in darkness. The colour absorbance at 490nm was immediately measured with a FLUOstar Galaxy microplate reader (BMG Lab technologies) and a standard curve was constructed from 5 callibrator solutions to determine the NMP22 sample concentrations. The test performance was assessed with 3 control samples of known NMP22 concentration.

3.6.2 Results: NMP22 urinary assay

Two 96-well plates were filled with 5 calibration samples, 3 control samples and 40 urine samples in 2 buffer-types, all in duplicate. Calibration was satisfactory using standards from the kit. A linear regression line was generated, Y=mx + b where m=5.73/3.93E^{-03}, b=1.7008/-1.002$E^{-01/02}$ and r=0.9989/0.9996 respectively for plates 1 and 2 for concentrations 0-140E. The values for NMP22 concentration are shown in Table 3.10. These show good reproducibility with a small standard deviation between duplicates for each buffer-type. The NMP22 concentrations recorded were consistently higher with the Matritech buffer, although the overall distribution of values for tumour types was similar.

These data were imported into a statistical engine for Receiver Operating Characteristic (ROC) Curves (via www.anaesthetist.com/mnm/stats/roc). The ROC curves shown in Figure 3.11 identified the threshold values for each buffer-type that generated the highest sensitivity and specificity (Matritech buffer: 27.82 U/ml, PI tablet alone: 17.06 U/ml). The test accuracy using these thresholds is shown in Table 3.11. However this produces an unacceptably poor sensitivity of below 60%.

We decided to manipulate the threshold to increase sensitivity at the expense of specificity by lowering the threshold. This resulted in a marked reduction in specificity (Table 3.11). Between 4 and 7 TCC (17-30%) depending on buffer used, were measured with no NMP22 at all, and so would never be identified as TCC irrespective of the threshold chosen. Further analysis of the test using the Matritech buffer at an optimal threshold (where the ROC analysis determines the maximal sensitivity/specificity) showed that 11 TCC were misclassified as normal. Of these 8 were Ta (7 G1/2, 1 G3), 2 T1 and 1 T2 TCC. The test has an improved accuracy rate in diagnosing more advanced tumours, and if the test was designed to classify Ta G1/2 TCC as “non-significant TCC” it would correctly identify 11/15 more advanced TCC, resulting in a sensitivity of 73% for this group, with a specificity of 88%.

Type	Stage	Grade	Urine id	A1	A2	A mean	A S.D.	B1	B2	B mean	B S.D.
TCC	a	1	389	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TCC	a	1	365	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TCC	a	2	408	68.70	79.40	74.05	0.10	74.05	73.74	73.90	0.00
TCC	a	2	385	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TCC	a	2	376	2.57	1.55	2.06	0.35	1.01	2.67	1.84	0.64
TCC	a	2	371	4.78	7.54	6.16	0.32	0.00	0.00	0.00	0.00
TCC	a	2	377	6.27	7.89	7.08	0.16	7.48	8.19	7.84	0.06
TCC	a	2	378	14.57	16.34	15.46	0.08	0.00	0.00	0.00	0.00
TCC	a	2	363	25.06	30.78	27.92	0.14	21.38	25.58	23.48	0.13
TCC	a	2	366	29.27	32.37	30.82	0.07	18.36	19.39	18.87	0.04
TCC	a	3	362	23.11	17.93	20.52	0.18	21.96	16.39	19.17	0.21
TCC	1	3	407	878.93	955.53	917.23	0.06	780.85	760.16	770.51	0.02
TCC	1	3	405	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TCC	1	3	379	5.01	4.91	4.96	0.01	0.00	0.00	0.00	0.00
TCC	1	3	359	58.60	55.72	57.16	0.04	29.61	32.28	30.95	0.06
TCC	2	3	404	25.93	28.48	27.21	0.07	36.67	28.46	32.57	0.18
TCC	2	3	402	62.42	51.02	56.72	0.14	27.27	21.00	24.14	0.18
TCC	2	3	380	64.76	56.86	60.81	0.09	27.75	30.21	28.98	0.06
TCC	2	3	358	116.32	97.93	107.13	0.12	89.38	90.64	90.01	0.01
TCC	2	3	370	225.60	200.69	213.15	0.08	115.89	120.44	118.17	0.03
TCC	2	3	403	503.79	394.92	449.36	0.17	325.11	252.80	288.95	0.18
TCC	2	3	381	992.71	972.58	982.65	0.01	940.77	710.95	825.86	0.20
TCC	cis	cis	400	286.42	273.89	280.16	0.03	124.65	106.60	115.63	0.11
Stone	na	na	N21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Stone	na	na	N22	5.83	6.27	6.05	0.05	6.48	5.48	5.98	0.12
BPH	na	na	N28	31.31	27.82	29.57	0.08	15.71	12.23	13.97	0.18
Uti	na	na	N31	3.32	2.12	2.72	0.31	0.00	0.00	0.00	0.00
Uti	na	na	N27	3.17	3.11	3.14	0.01	0.00	0.00	0.00	0.00
Uti	na	na	N30	9.95	8.55	9.25	0.11	8.06	8.63	8.35	0.05
Uti	na	na	N25	18.11	13.54	15.83	0.20	10.54	12.76	11.65	0.13
Uti	na	na	N24	45.83	53.93	49.88	0.11	35.75	42.12	38.93	0.12
Normal	na	na	N26	2.21	2.64	2.43	0.13	2.90	0.94	1.92	0.72
Normal	na	na	N23	2.09	3.30	2.70	0.32	3.96	2.16	3.06	0.42
Normal	na	na	414	5.12	6.48	5.80	0.17	0.93	2.01	1.47	0.52
Normal	na	na	N29	6.34	7.44	6.89	0.11	5.66	4.83	5.25	0.11
Normal	na	na	N34	6.08	8.29	7.18	0.22	7.42	6.39	6.91	0.11
Normal	na	na	409	7.42	8.08	7.75	0.06	7.38	6.15	6.77	0.13
Normal	na	na	N35	7.30	8.27	7.79	0.09	1.16	1.69	1.43	0.26
Normal	na	na	N33	13.89	11.20	12.55	0.15	14.79	9.63	12.21	0.30
Normal	na	na	N32	22.78	17.06	19.92	0.20	18.70	16.51	17.61	0.09

Table 3.10. Concentration of NMP22 for 40 urine samples (U/ml) Matritech Buffer (A1 & A2) and St James's Urine collection buffer (B1& B2) (S.D.=standard deviation).

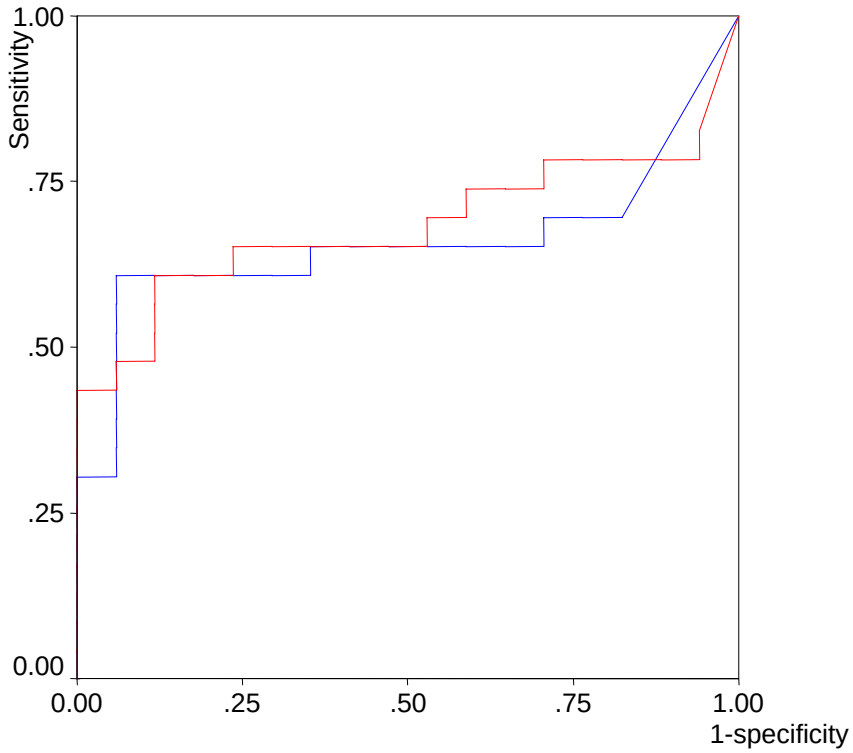


Figure 3.11a ROC curve analysis of NMP-22 Elisa Samples collected in Matritech collection buffer: red, with PI tablet only added: blue.

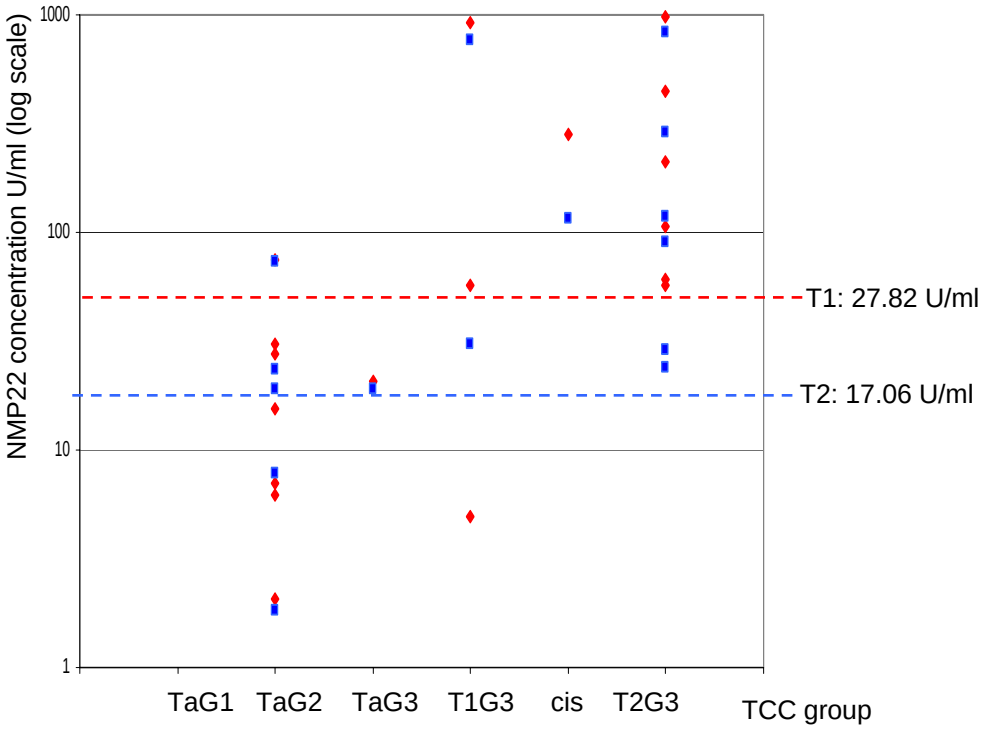


Figure 3.11b NMP-22 levels by TCC grade and stage. Samples collected in Matritech collection buffer: red, with PI tablet only added: blue.

Table 3.11 Results tables for NMP-22 ELISA for different buffers and threshold values

	TRUE TCC	TRUE Normal	
TEST TCC	12	2	14
TEST Normal	11	15	26
	23	17	

Matritech buffer, threshold 27.82 U/ml

Sensitivity: 52%

Specificity: 88%

Positive Predictive Value: 86%

Negative predictive Value: 58%

	TRUE TCC	TRUE Normal	
TEST TCC	13	2	15
TEST Normal	9	15	24
	23	17	

Standard buffer, threshold 17.06 U/ml

Sensitivity: 57%

Specificity: 88%

Positive Predictive Value: 87%

Negative predictive Value: 63%

	TRUE TCC	TRUE Normal	
TEST TCC	19	16	35
TEST Normal	4	1	5
	23	17	

Matritech buffer, threshold 1.00 U/ml

Sensitivity: 83%

Specificity: 6%

Positive Predictive Value: 54%

Negative predictive Value: 20%

	TRUE TCC	TRUE Normal	
TEST TCC	16	14	30
TEST Normal	10	3	10
	23	17	

Standard buffer, threshold 1.00 U/ml

Sensitivity: 70%

Specificity: 18%

Positive Predictive Value: 53%

Negative predictive Value: 30%

3.7 Discussion

The tendency of low stage TCC to recur and the consequent requirement for long-term cystoscopic surveillance places a large burden on both patients and health care providers. The development of a non-invasive test for bladder cancer would therefore be of significant benefit. Despite continuing development of urinary biomarkers of bladder cancer, none have gained widespread use (Quek et al., 2004). Many of these assays measure a single, or a small number of molecules in the urine.

In contrast, profiling approaches such as SELDI potentially allow combinations of many putative biomarkers to be assessed simultaneously, which may be more informative given the general heterogeneity of disease with multiple genetic and epigenetic influences. Such methods are also optimal in examining the mass range <20 kDa which is not readily visualised by other methods (Oh et al., 2004; Petricoin et al., 2002b; Qu et al., 2002) and includes many small proteins such as growth factors and cytokines, which potentially may be perturbed in neoplasia. However such approaches also have many challenges ranging from developing enrichment strategies to allow detection of lower abundance molecules to ensuring long-term robustness and addressing quantitation aspects.

The performance requirements for clinically useful diagnostic tests depend on the prevalence of the disease in the population sampled (the positive predictive power of a test of a given sensitivity increases with increasing incidence of the disease tested). Biomarkers that attempt to screen a large population for an uncommon disease require an almost perfect performance to ensure that unacceptably high numbers of false-positives do not arise, and are thus difficult to develop. The majority of TCC patients under surveillance will suffer recurrences and so prevalence of TCC in this group of patients is high. Failing to identify recurrent TCC (a false negative error) would be detrimental to a patient's outcome. This is more serious than a false positive error that would subject them to a cystoscopy as is current routine practice. Hence identifying recurrent TCC can be successfully addressed using an assay with high sensitivity even if specificity is only moderate.

In this study we have used samples collected from a UK teaching hospital. The grade and stage of TCC patients providing urine samples reflect the local patient population that requires monitoring. The majority of these TCC are of low stage. Similarly, our controls include a large proportion of benign urological diseases that are concurrently present in this population and hence our figures for specificity are more likely to reflect the true situation if used clinically than studies only comparing urine diagnosis of advanced TCC with young healthy volunteers. Thus for the late independent validation set, the figures of 77.6% for sensitivity and 67.9% specificity using the Random Forest classification approach are extremely promising although improvements in sensitivity are needed. It is encouraging that these results were maintained over a period of 6 months with no decrease in either sensitivity or specificity compared with the initial test results and a low rate of misclassification (7%) of samples rerun at the later time point. To further assess the reliability of this approach a multi-centre validation study should be undertaken, although none is planned at present.

Other studies examining urine samples from bladder cancer patients have also reported promising results. Using SAX2 chips, a sensitivity of 87% (78% for low-grade lesions) and a specificity of 66% using 5 peak-complexes identified by eye (3352/3432, 9495, 44647, 100120, 133190 Da) were reported (Vlahou et al., 2001). Subsequently this group achieved similar predictive accuracy with the application of a decision tree cluster algorithm analysis (Biomarker Patterns Software®;Ciphergen) on a further 191 training and 36 blinded test set sample spectra using weak cation exchange chips (Vlahou et al., 2004). Utilising a similar analytical approach trained on a group of 118 samples and tested on a blinded set of 38 samples profiled using IMAC (metal binding) chips, higher sensitivity (93.3%) and specificity (87.0%) were achieved using 5 significant peaks of 3896, 4977, 9638, 15103 and 15509 Da (Zhang et al., 2004a).

A more recent study from the same group but using WCX chips with decision tree analysis reported figures for sensitivity and specificity in excess of 90% in the training set, but falling to 70-73% sensitivity in the test set (Liu et al., 2005). Similar but more marked declines in sensitivity and specificity between the training and test sets were also found using either CM10 cation exchange or

SAX2 chips with sensitivities and specificities declining from 80% and 90-97% respectively to 50-62% (Mueller et al., 2005). Interestingly, unlike most other studies, this latter study examined the higher molecular weight regions of the spectra (up to 200 kDa) and found these to be more discriminatory.

The predictive accuracy of our study is generally lower than other studies despite using large training and test groups to maximise machine learning classification algorithms. However, we have shown reproducibility over time which has yet to be demonstrated in other studies. One possible explanation for the lower sensitivity and specificity obtained here is that our patient group contained a large proportion of low stage/grade TCC (75 Ta vs. 39 T1/T2+), whereas when TCC stage has been recorded in other studies, advanced disease predominates (19 Ta vs. 61 T1/T2+ (Liu et al., 2005); 10 Ta vs 14 T1/T2+ (Vlahou et al., 2001) and 45 Ta vs. 56 T1/T2+ (Vlahou et al., 2003). Our results are comparable with or better than approved tumour marker tests and it is possible that the combination of our method with such tests in the future may improve accuracy of prediction.

We identified numerous peaks that were significantly differentially-expressed between the TCC and control samples profiled. Most of the significant peaks in the TCC group were decreased compared with controls and many were of very low intensity. These decreases may not reflect loss of expression in TCC patients compared with controls as much of the urothelium is still normal in these patients and would therefore still shed these proteins. It may reflect increased production of proteases by the malignant tissue and hence degradation of some proteins. The profiles generated illustrate relative rather than absolute amounts of protein, and peak intensities are influenced by factors such as ionisation, relative affinities for the binding surface and potentially the effects of other proteins. Hence these decreases may be a consequence of other tumour-related factors such as haematuria which potentially could result in competition with tumour-derived proteins for binding to the chip surface.

Peak identification is critical to determine the biological significance of the results. Most peaks identified have not been described before, although in the earliest study which used the predecessor anion exchange chips (SAX2) with pH

9.0 buffers, the presence of some overlapping peaks can be seen (Vlahou et al., 2001). Comparison of peak profiles and our immunoprecipitation experiments, identified a peak at 3368.96 Da found to be significant in the Random Forest classification of TCC (Table 3.7) as a member of the alpha-defensin family (HNP-2). This had been identified in an earlier study as a doublet at 3300-3400 Da with a sensitivity of 47% and specificity of 86% if used as a single marker to predict TCC (Vlahou et al., 2001). However, in agreement with Zhang *et al* (Zhang et al., 2004a), we identified alpha-defensins not only in TCC, but also in normal and benign controls, suggesting reduced specificity if used as a solitary marker. The alpha-defensins are the major constituents of the azurophilic granules within neutrophils and are also found within intestinal Paneth cells, and the walls of coronary vessels. Their proposed roles include host anti-microbial defence and tissue inflammation and repair. Alpha defensins may be present in urine in response to infection or inflammation associated with benign conditions or TCC. However, increased protein or RNA expression has been reported in renal (Muller et al., 2002), lung (Bateman et al., 1992) and oral (Mizukawa et al., 2001) carcinomas. Thus it is possible that bladder tumours themselves secrete alpha-defensins. Increased expression of defensin with increasing stage of TCC has been demonstrated by immunohistochemistry (Holterman et al., 2003).

A protein cluster at 15,000-16,000 was previously identified in 63% of TCC and 23% of controls (Vlahou et al., 2001). MALDI analysis of haemoglobin chains identified peaks with masses of 15,127 and 15,868 for α and β chains respectively with other derived peaks arising from post-translational modifications (Zurbriggen et al., 2005). We identified a collection of peaks of similar intensity and morphology in this mass range, whose presence correlated with degree of haematuria recorded by dipstick, and we confirmed using antibodies that this was indeed haemoglobin. Haematuria is commonly found in TCC, and its significance within these protein profiles is not surprising. What is more surprising however is that these peaks were amongst those discounted due to the possible confounding effects of age, sex or analysis run. Similarly within the TCC comparisons, these haemoglobin peaks were found to be significantly different between high grade and stage but again significant effects of age and sex abrogated this grade- or

stage-related difference. Thus, some peaks found to be influenced by these factors may also have utility as potential markers, reinforcing the need for completely age-and sex-matched groups so that no markers are discounted but rather controlled for at the point of sample analysis.

This effect of factors such as age may have influenced the outcome of previous studies on bladder cancer which have recorded different median ages in control and patient groups (Liu et al., 2005; Vlahou et al., 2001; Zhang et al., 2004a). In clinical profiling studies, the importance of controlling for pre-analytical effects is increasingly clear. For example a marked effect of sample processing times on serum and plasma profiles has been shown (Banks et al., 2005). Similarly the effect of analytical run has been highlighted (Hu et al., 2005), although we found that relatively few peaks were affected by this, possibly due to the stringent QC routines used.

A previous study examining urine found matrix composition and instrument settings to be critical in determining urinary profiles with freeze-thawing or storage at 4^{o}C having little effect on profiles (Schaub et al., 2004). Blood in the urine, urine dilution and first-void compared with mid-stream samples in females were found to be significant factors. In urinalysis the issue is complicated by the highly variable and relatively dilute protein concentrations and the consequent need to standardise by protein load to avoid SELDI profiling simply being a reflection of total protein (Rogers et al., 2003). Clinical chemistry analyses of urine are usually normalised for creatinine concentration to correct for hydration status. However it is impossible to correct for this simultaneously with protein correction in SELDI profiles.

The novelty of SELDI-MS profiling to biomarker detection is naturally associated with changing perceptions as to its validity. In particular, controversy has been generated following reports relating to prostate and ovarian cancer detection. In 2002 a paper published in the Lancet claimed detection of eary-stage ovarian cancer from serum with a sensitivity specificity of nearly 100% (Petricoin et al., 2002b). In the subsequent years this paper has been fiercely attacked and defended in the scientific literature (Diamandis, 2003; Petricoin and Liotta, 2003). Plans to

introduce a commercial screening test were delayed by the FDA amid questions raised about the reliability and reproducibility of the SELDI analysis. The current consensus is that this research was flawed and this has tainted the introduction of similar approaches to other diseases (Ransohoff, 2005). More generally, concern has also been raised over the long-term robustness of SELDI profiling, various aspects of experimental design, the interpretation of statistical and machine learning tools and the inability to directly identify significant proteins (Diamandis, 2004; Rodland, 2004; White, Chan, and Zhang, 2004). We believe that our study illustrates that such approaches can be used reproducibly provided that adequate technical controls are used, and these issues are addressed more fully below.

Instrument variability, processing error and biological variability have been identified as sources of error within SELDI-MS analyses (White, Chan, and Zhang, 2004). Varying instrument settings, matrix composition and urine composition significantly altered the spectra produced in a systematic assessment of these issues (Schaub et al., 2004). We encountered such variablity in the initial experiments. Certainly the SELDI mass spectrometer used in these experiments and by most other groups is a relatively low-resolution instrument and cannot completely resolve 2 species differing by a M/Z below 20 Da (at 3000 Da) (Petricoin and Liotta, 2004). Incorporation of improved MS technology will potentially improve the resolution and reproducibility of profiles. The latest version of the SELDI ProteinChip reader (Series 4000) has been released and has replaced the machine used for this study in our laboratory. It has been recently demonstrated to improve the performance of diagnosis of ovarian cancer (Conrads et al., 2004). Similarly the use of robots in chip handling has been demonstrated here to dramatically improve both mass and intensity reproducibility.

We have always been aware of the errors generated by poor or unsystematic collection and handling of clinical samples. The sensitivity of the SELDI profiling may identify minimal differences between samples collected in different ways, at different times and by techniques of processing. Such artefacts may have influenced earlier studies (Baggerly, Morris, and Coombes, 2004). While such “noise” may be less critical in an established test assay, indeed is inevitable of the clinical setting, it must be eliminated in these discovery and validation sample

sets. All our tumour and control samples have been collected in the same institute, in the same way, by the same people. Similarly all samples were processed and analysed in a randomised blinded manner.

There has been much discussion of the statistical approaches to analyse SELDI-MS profiles but as yet no consensus exists over the most appropriate methods. Artificial neural networks are well-suited to the analysis of these multi-dimensional data sets but lack transparency and do not provide an explanation or mechanism to affirm findings to the sceptical. They also tend to require much larger sample sets. Classification trees are based on a smaller number of potentially identifiable proteins and an ensemble of trees within a Random Forest algorithm appears to offer simplicity, robustness to noise and reliability (Izmirlian, 2004). When analysis is based on individual data points, of which 30,000 are produced with each profile, it is possible to select a series of points that are predictive of cancer within the test cohort but are in fact erroneous; a statistical fluke based on data points from noise alone. We partly avoided such "overfitting" by excluding data masses within the region of maximum noise (below 2000 Da) and this is a practice undertaken by many but not all groups using SELDI-MS. Additionally, our novel peak detection algorithm identifies peaks representing an individual protein ion only and so avoids using raw data points altogether. This reduces the volume of data by approximately 100 times; from ~30,000 to ~300 for each protein profile.

With these evolving technologies there is a clear need for robust statistical analysis in experimental design and analysis and in interpretation of the data so bias is avoided (Ransohoff, 2004). Complex statistical manoeuvres to maximise performance with small sample sizes may also produce epiphenomena as we initially discovered. It also makes assessment of such studies difficult without specialist statistical knowledge. A conservative design has been applied in this study, namely to split the initial sample group randomly into a discovery (training) set and test set. A later validation test set based on samples collected prospectively in the months following the initial analysis was subjected to the classification tree based entirely on the initial training set. This approach requires more samples than

those that validate findings from new test sets derived from the initial sample group, but is less prone to error.

While SELDI-MS is able to quickly generate discriminatory protein profiles, the identity of the proteins that constitute these profiles cannot be ascertained directly. It is not crucial to the development of a diagnostic test to obtain these identities, but it is required to allow further work to understand mechanisms of disease or to develop fully quantitative alternative assays (e.g. ELISA) for specific proteins. Advanced, Tandem MS of SELDI protein chips allows peptide sequencing and this technique is in early development in our unit and elsewhere. Indirect methods such as immunodepletion (as described here) or chip-based purification techniques can aid protein identification (Zhang et al., 2004b) but they require a significantly greater amount of sample, are technically demanding and are unlikely to be able to identify all proteins required.

We decided to assess the performance of SELDI-MS compared with another non-invasive test to detect TCC, and identified NMP22 as the most established contemporary assay for this comparison. We felt it was important to subject our samples collected at St James's Hospital to an NMP22 ELISA, as comparing results from different laboratories, based upon different samples may lead to unreliable comparisons. In particular, many published series reporting NMP22 performance include a higher proportion of high stage and grade tumours than were collected for this study.

The NMP22 data were analysed with a ROC curve for each buffer type. With an assay that generates a continuous distribution of values, a threshold value must be determined to decide those samples that are to be identified as positive. Depending upon the threshold value used, the test performance will vary. If a very low value for NMP22 is chosen, the test will be very sensitive but not specific, and conversely a high threshold will generate good specificity at the cost of poor sensitivity. By plotting sensitivity against specificity for differing threshold values a ROC curve is generated. The threshold value with maximum sensitivity and specificity for that test thus lies at the point of the curve which is nearest to the

origin of the x-axis and closest to the maximum on the y-axis (ie. top left-hand corner). This assumes that sensitivity and specificity are to be given equal weighting, but in some circumstances, this is not the case. In bladder cancer surveillance, greater significance must be placed upon sensitivity over specificity as a false-positive diagnosis would simply lead to an unnecessary cystoscopy whereas a missed bladder cancer is potentially fatal.

We found that the NMP22 assay results were affected by using samples collected in our standard collection buffer, with the values obtained generally lower than with the buffer supplied by Matritech. However the test performance, using a threshold generated from the ROC curve, was similar for both buffers. In fact we did not undertake further NMP22 profiling as planned for this study and so this comparison was not essential. However it does offer the opportunity of NMP22 testing of the samples collected at St James's in future studies. We found that the NMP22 assay performed only moderately well. It was able to detect G3 pTa and pT1+ TCC with a sensitivity of 73% and specificity 88%, but overall, sensitivity fell to below 60% when the G1-2 pTa TCC were included. When the threshold was manipulated to maximise sensitivity for the reasons detailed above, although a sensitivity of 83% was obtained, specificity fell to below 10%. These findings are broadly in line with the results for published series when the proportion of low grade and stage TCC are matched.

Whether machine-based profiling and computer-assisted diagnosis or prognosis will translate into clinical practice is uncertain. Progress is being made in improving performance over time, as shown here, and in improving comparability between centres. However, much further information will be gained if the identities of the discriminatory peaks can be determined. In addition to providing biological information, this may also facilitate quantitative assay development which may be more amenable to translation into routine clinical use. The results from this study clearly indicate that identification of urinary peaks may have future value in bladder cancer and which may be particularly important in patient management following diagnosis.

4 Identification of differentially expressed proteins in high and low grade Ta bladder cancers

4.1 Introduction

This chapter describes the use of 2D PAGE to perform a comparative analysis of protein expression profiles between high and low grade *de novo* Ta TCC to identify differentially-expressed proteins. It is well established that low-grade tumours (G1-2) are less likely to progress than high-grade (G3) tumours. Therefore, in the absence of long-term follow-up we have used grade as a surrogate for tumour aggression. Proteins from tumour tissue samples from two groups of six patients were separated by 2D PAGE and their relative abundance calculated. Those differentially displayed protein species were subjected to mass spectrometry to determine their identity. We hoped to identify proteins that were differentially expressed between the two groups that might constitute putative biomarkers for TCC progression.

4.2 Materials and methods

4.2.1 Tissue collection and processing

Tissue collection was undertaken by the Urological Surgeons at St. James's Hospital. Under a general anaesthetic, presumed papillary bladder tumours from consenting study participants (Table 4.1) were subjected to (1-8) cold-cup biopsies before diathermy loop resection. All biopsies were promptly transported to the laboratory in 50ml ice-cold RPMI 1640 medium (Invitrogen) containing a mini-Complete^{TM} protease inhibitor tablet. The tissue was washed in ice-cold PBS (pH 7.2) and 8.6 % w/v sucrose solution, dabbed dry, embedded in OCT (BDH), snap frozen in liquid nitrogen and stored at -80°C.

Table 4.1 Inclusion and exclusion criteria for tissue collection

Sample Type	Inclusion Criteria	Exclusion Criteria
Bladder tumour biopsy	Subsequent *de novo* histological diagnosis of TCC bladder	TCC from outside the bladder or other bladder tumour types
		Patient unable to give informed consent
		Any other non-cutaneous malignancy
Control (normal) urothelial biopsy	Patient undergoing unrelated cystoscopic surgery	Patient unable to give informed consent
		Any non-cutaneous malignancy
TCC-associated urine samples	Patient with subsequent histological diagnosis of TCC bladder	TCC from outside the bladder or other bladder tumour types
		Patient unable to give informed consent
		Any non-cutaneous malignancy
Control (normal) urine samples	Apparent good health upon questioning	Subject unable to give informed consent
		Any non-cutaneous malignancy or genitourinary condition
Control (non-normal benign) urine samples	Patients with benign genitourinary conditions, eg. urinary infection, renal calculi	Unable to give informed consent.

Patient and tissue details were anonymised and stored on secure computer databases. All histological samples were evaluated by a single pathologist (Dr P. Harnden). Obtaining patient consent, tissue retrieval and processing is part of an ongoing programme within the laboratory and as such was performed by several members of the scientific and clinical staff at St James's.

4.2.2 Two Dimensional Polyacrylamide Gel Electrophoresis

4.2.2.1 General considerations

The visualisation of TCC proteins by 2D PAGE is best achieved with a lower protein load than is required for MS protein identification. Hence, 2 types of 2D PAGE experiment were performed, those to produce a good protein spot pattern (analytical gels) and those for spot excision for MS (preparative gels). Differences in method are detailed below where necessary. The avoidance of protein contamination is critical to the success of 2D PAGE and measures were undertaken to minimise contamination. All solutions were made up in MilliQ H_2O. At all times powder free gloves were worn, and when handling gels, rinsed in MilliQ H_2O. General glassware was autoclaved and rinsed in MilliQ H_2O and gel plates and trays were cleaned with neutracon detergent (Decon Labs), rinsed with MilliQ H_2O and then methanol.

4.2.2.2 Sample preparation

Frozen TCC biopsy tissue was transferred to a cryostat (Leica) and excess OCT trimmed off with a scalpel. 30-50 50μm sections of a single biopsy were added to 200μl 2D PAGE lysis buffer: 7M urea (Valeant), 2M thiourea, 4% (w/v) CHAPS, 64 mM DTT (Sigma) and 0.8% (v/v) Pharmalyte pH 3-10 (Amersham Biosciences). The sample was vortexed and incubated at room temperature for 30 min, sonicated 3 times for 30 seconds on ice, then centrifuged in an Optima T1 ultracentrifuge (Beckman) at 42,000 g for 60 min at 15°C. The supernatant was stored at –80°C in aliquots.

To generate protein solutions for preparative gels, multiple sample aliquots from single or multiple tissue samples were combined and concentrated. Upon defrosting, protease inhibitor liquid (Sigma) was added to form a final concentration of 50ul/ml lysis buffer. The samples were added to a YM-10 spin cup (Millipore) and centrifuged at 800g until the sample volume had reduced to 160μl.

4.2.2.3 Protein concentration assay

The protein content of prepared samples was determined with a protein assay kit (Biorad) based upon a modified Bradford assay. 10µl of serial concentrations of bovine serum albumin (BSA) standards in lysis buffer (0-5 mg/ml) and unknown samples in triplicate were mixed with 10µl 0.1M HCl, 80µl H_2O and 3.5 ml of Bradford Dye Reagent diluted 1 in 5. The absorbance at 595nm was read immediately on an Ultrospec III spectrophotometer (Amersham Biosciences), a standard curve constructed and the protein in the sample calculated by interpolation using Fcalc software (Perkin Elmer).

4.2.2.4 First dimension: IsoElectric focusing

Protein was subject to isoelectric focussing with 18cm pH 3-10 non-linear pre-cast IPG gel-strips (Amersham Biosciences) using an IPG Phor gel system (Amersham Biosciences). For analytical gels 33µg was used and for preparative gels 1.15-1.34 mg was used. The protein sample in lysis buffer was made up to 450µl with 2D PAGE re-swelling buffer: 7M urea, 2M thiourea, 4% (w/v) CHAPS, 30mM DTT, 0.2% (v/v) Pharmalyte 3-10 and a trace of Bromophenol Blue (Sigma). This mixture was pipetted into a ceramic strip holder, the IPG gel-strip placed in the solution and covered with a mineral oil: PlusOne Immobiline Drystrip Cover Fluid (Amersham Biosciences) to prevent evaporation. In-gel rehydration was performed at 30V for 12 hours. This was followed by isoelectric focusing at 200V (1hr), 500V (1hr), 1000V (1hr) and by gradient incline to 8000V (1hr), and 8000V for a total of 65000 Vh, (with a maximum current of 50mA/gel). On completion, the IPG strips were removed from the holder, excess oil drained and stored at -80°C.

4.2.2.5 Second dimension: SDS PAGE

Resolving Gels were cast with the ISO-DALT multicasting system (GE Healthcare) according to the manufacturer's instructions. Briefly, 22 gel cassettes were packed into a Perspex box, separated with plastic sheets. Small labels were placed in the bottom right corner of each cassette for identification and

orientation. Acrylamide solution was slowly poured into the box via a funnel then followed by a displacement buffer. Final gel compositions were 10%T acrylamide with 4%C bis/acrylamide (National Diagnostics), 0.36M Tris-HCl (pH 8.8), and 0.1% (w/v) SDS (BDH). Polymerisation was initiated with ammonium persulphate (Valeant) and TEMED (Sigma) and the gels overlaid with water, saturated isobutanol (5 ml) was applied to the upper edge of each gel. After polymerisation, the isobutanol was replaced with MilliQ H_2O and stored up to 30 days at 4°C.

The resolving gels were allowed to warm to room temperature and drained of covering water. Stacking gels [4% (v/v) acrylamide, 0.125M Tris-HCl (pH 6.8) and 1% (w/v) SDS, polymerised with ammonium persulphate and TEMED] were added to the upper edge of each gel, allowed to polymerise over 30 mins and washed with 2D PAGE running buffer.

The IPG strips were thawed and equilibrated in 10% (w/v) DTT (Sigma) in equilibration buffer for 15 min; [6M urea, 30% (v/v) glycerol, 2% (v/v) SDS in 0.05M Tris-HCl, pH 6.8], followed by 10 min in 40% (w/v) iodoacetemide (Sigma) in equilibration buffer. Strips were washed in 2D PAGE running buffer;[0.025M Tris, 0.2M glycine, 0.1% (w/v) SDS] and placed onto the stacking gel with the acidic end of the IPG strip lying above the gel label. Novex mark 12 molecular weight standards (Invitrogen) were mixed 1/10 with 1.2% (w/v) LMP ultra-pure agarose (Invitrogen) and 20µl solid droplets placed adjacent to each IPG strip and both sealed in place with 1% w/v agarose diluted in 2D PAGE running buffer. Up to 10 gels were run simultaneously using a Hoefer-Dalt tank filled with 2D PAGE running buffer at 12.5 °C, 18-22 mA/gel for c.18 hours until the dye front had run across the entire gel.

4.2.2.6 Silver staining PAGE gels

Proteins separated by 2D PAGE were visualised by staining using the Owl Silver staining Kit (Owl Separation Systems); in summary the gels were placed in

individual glass bowls and incubated with sequential solutions to fix, sensitise and stain with silver, develop the stain and stop the reaction. The standard kit protocol was previously modified in our laboratory. The initial protein fixation was performed by an overnight incubation in a solution of 50% methanol, 10% acetic acid, 40% water (500 ml/gel) in a clean glass dish, followed by a solution of 30% methanol, 10% acetic acid, 55% water and 5% Owl Reagent-1 (300 ml/gel) for 30 mins. After washing with a pre-treatment solution of 50% methanol, 45% water and 5% Plus-One-Reagent-2 (300 ml/gel) the gels were washed twice for 10 min with 500 mls water per gel. The staining solution of 90% water with 5% Owl Reagent-3 and 5% Owl-Reagent-4 was applied for 40 min. After this the gels were washed with water for 2 min, three times, before a developing solution of 95% water with 5% Owl-Reagent-5 was added for 1-5 min. When the gels developed, 15 ml/gel Owl Reagent-6 was added to stop the reaction. Preparative gels were stained with the Plus One™ kit (Amersham Biosciences) also according to a modified protocol (Westbrook et al., 2001) omitting gluteraldehyde and only adding 0.1mls formaldehyde per gel volume of developer.

4.2.2.7 Image analysis

Immediately after staining the gels were scanned as 12 bit images using a laser densitometer (Amersham Biosciences). Gel analysis was performed with Melanie III software (Gene Bio).

4.2.2.8 Protein excision from preparative 2D PAGE

Selected protein spots were excised from preparative gels. The gel was placed upon a glass plate atop a light box. The spots were excised from the gel with a disposable scalpel and placed into a 500µl eppendorf tube containing 15µl of water and frozen at -80°C.

4.2.2.9 Tryptic Digestion and mass spectrometry

The analysis of the excised spots was undertaken by Dr Nick Totty and Dr Sarah Hanrahan at the Cancer Research UK Protein Sequencing Laboratory at 44 Lincoln's Inn Fields, London using tryptic digestion and electrospray tandem- MS techniques.

4.3 Results: Analysis of high and low grade Ta TCC by 2D PAGE

4.3.1 Tissue sample collection

Cold cup biopsy samples from 12 patients in total were collected (Table 4.2). The comparative rarity of Ta G3 TCC required 14 months in total to collect enough samples. Frozen sections of all cold cup biopsies were examined by a single pathologist (Dr. P Harnden) to confirm the nature of each sample and ensure that all samples used contained in excess of 80% tumour cells. The cold cup biopsies were approximately spherical with a diameter of 2-4mm, giving a tumour sample volume of ~4-33 mm^3. Their small size required careful handling to maximise protein extraction.

Initial experiments to determine the optimal method found that crushing frozen tissue with a pestle and mortar under liquid nitrogen gave a low yield of protein. Sectioning the frozen sample on a cryotome was preferred although the excess OCT led to a very viscous solution that was difficult to handle. Slicing excess OCT away from the sample with a scalpel overcame this problem so that the tumour comprised on average 80% of the block rather than 10-40%. without trimming. The samples were sectioned in their entirety into 200µl of lysis buffer. The protein concentration ranged between 0.80-7.50 mg/ml (median 3.70mg/ml) to give a total protein extraction of 160-1500µg (median 740µg) per sample, enough for approximately 20 analytical gels.

Table 4.2 Details of patient high/low grade Ta TCC samples used for 2D PAGE

Sample number	Sample id number	Date sample collected	Patient age at collection	Sex	Grade
Low-1	100	Aug 2000	85	F	2
Low-2	101	Aug 2000	81	F	2
Low-3	103	Sep 2000	64	F	2
Low-4	118	Sep 2000	61	F	2
Low-5	239	Apr 2001	62	M	1
Low-6	248	Jun 2001	59	M	2
High-1	94	Aug 2000	85	M	3
High-2	110	Nov 2000	49	M	3
High-3	113	Nov 2000	74	M	3
High-4	124	Dec 2000	72	M	3
High-5	261	July 2001	81	M	3
High-6	322	Oct 2001	82	F	3

4.3.2 2D PAGE analysis

Scanned images of the 2D gels were analysed with Melanie III software. Each silver-stained spot within a gel was measured for stain intensity and area to give its effective volume (expressed as a fraction of the total staining volume: % volume). The images were edited by eye to exclude artefacts such as streaks or dust spots on the captured gel images.

The product of the size and intensity of a single spot is termed its “volume”. Within limits, this spot volume is proportional to the abundance of stained protein and so spots on different gels may be compared to assess their relative protein abundance. Such direct comparison is open to error, as each gel will be subjected to a different level of overall silver staining. To reduce this error, a spot’s volume can be expressed as a fraction of the volume of all spots on a single gel (usually expressed as % volume). This normalises the relative protein expression for each spot against total silver staining for each gel.

Before quantitative comparison, the 12 gels required manipulation to line-up spots corresponding to the same protein. Forty corresponding spots were identified by eye on each gel and were used as reference points to aid matching. Using mathematical algorithms based upon Fourier Transformations, the Melanie III software distorted the gel images so these spots were perfectly overlaid. The matching process using Melanie III software required the selection of a reference gels that the other gels were transformed to. However, spots that were not present in the reference gel would not have been identified by this method. Therefore the analysis was performed twice, once with a low-grade gel as reference to identify spots with increased expression in low grade TCC, and once with a high-grade reference gel to identify increased expression of spots associated with high-grade TCC. A representative gel for each class was chosen that displayed the highest spot numbers: #103 (TaG2) for the low-grade TCC and #113 (TaG3) for the high-grade TCC. The details of the number of spots in each gel successfully matched by the Melanie III software to these reference gels are shown in Table 4.3.

The raw % volume data were exported into Excel software. The spot volume for each member of the high and low grade classes was compared to identify putative differentially-expressed proteins. Initially the criterion applied for defining differential expression for an individual spot-group was a 2-fold difference in the mean % volume between the high and low-grade classes. However it was subsequently felt that this would conceal any strong differences in expression of individual TCC. Given the suggested heterogeneous nature of TCC, many

important molecular changes will not be seen in all tumours of the same grade. So an alternative criterion was introduced to identify proteins for further investigation: a spot-group was defined as significantly differentially-expressed if 3/6 or more samples displayed more than 2-fold increased expression compared to the mean of the comparison group.

Gel id	Grade	**Matching with #103(low)**		**Matching with #113(high)**	
		No. matches	% matches	No. matches	% matches
94	High	1060	68	914	63
100	Low	1095	69	1088	72
101	Low	1193	70	1051	65
103	Low	Na	Na	1061	68
110	High	1250	80	1050	71
113	High	1061	68	Na	Na
118	Low	1092	67	1005	65
124	High	991	67	934	67
239	Low	1051	68	1004	69
248	Low	1079	70	933	65
261	High	1085	69	992	67
322	High	1004	70	883	65

Table 4.3 Melanie III matches for each gel with low-grade (#103) and high-grade (#113) reference gels.

A shortlist of spot-groups (37 expressed more in low-grade, 46 expressed more in high-grade) was drawn-up that fulfilled the revised criterion and these were checked by eye to ensure that the differential spot volumes were not caused by artefact. Additionally, spot-groups with mean absolute volumes below 0.05% of the total spot volume were excluded as previous work in our laboratory had found it to be difficult to obtain MS identification on these very small spots. After this further selection a total 14 spots, 7 over-expressed in low-grade TCC (L1-7) and 7 over-expressed in high-grade TCC (H1-7), were selected for identification (Figure 4.1 and 4.2).

Analytical 2D PAGE gels contain a relatively low protein load to allow clear display of the proteome. However to aid protein identification, preparative-grade 2D PAGE gels were run, containing 1.15-1.34 mg protein per gel compared with 33µg for each analytical gel. Four preparative-grade gels were run, to allow excision of all chosen spots.

The analysis of the excised spots was undertaken by Dr Nick Totty and Dr Sarah Hanrahan at the Cancer Research UK Protein Sequencing laboratory at 44 Lincoln's Inn Fields, London. Tryptic digestion and electrospray tandem- MS techniques were used that are not within the scope of this thesis. The spot identities generated are detailed in Table 4.4; some spots were different isoforms of the same protein and some spots could not be identified.

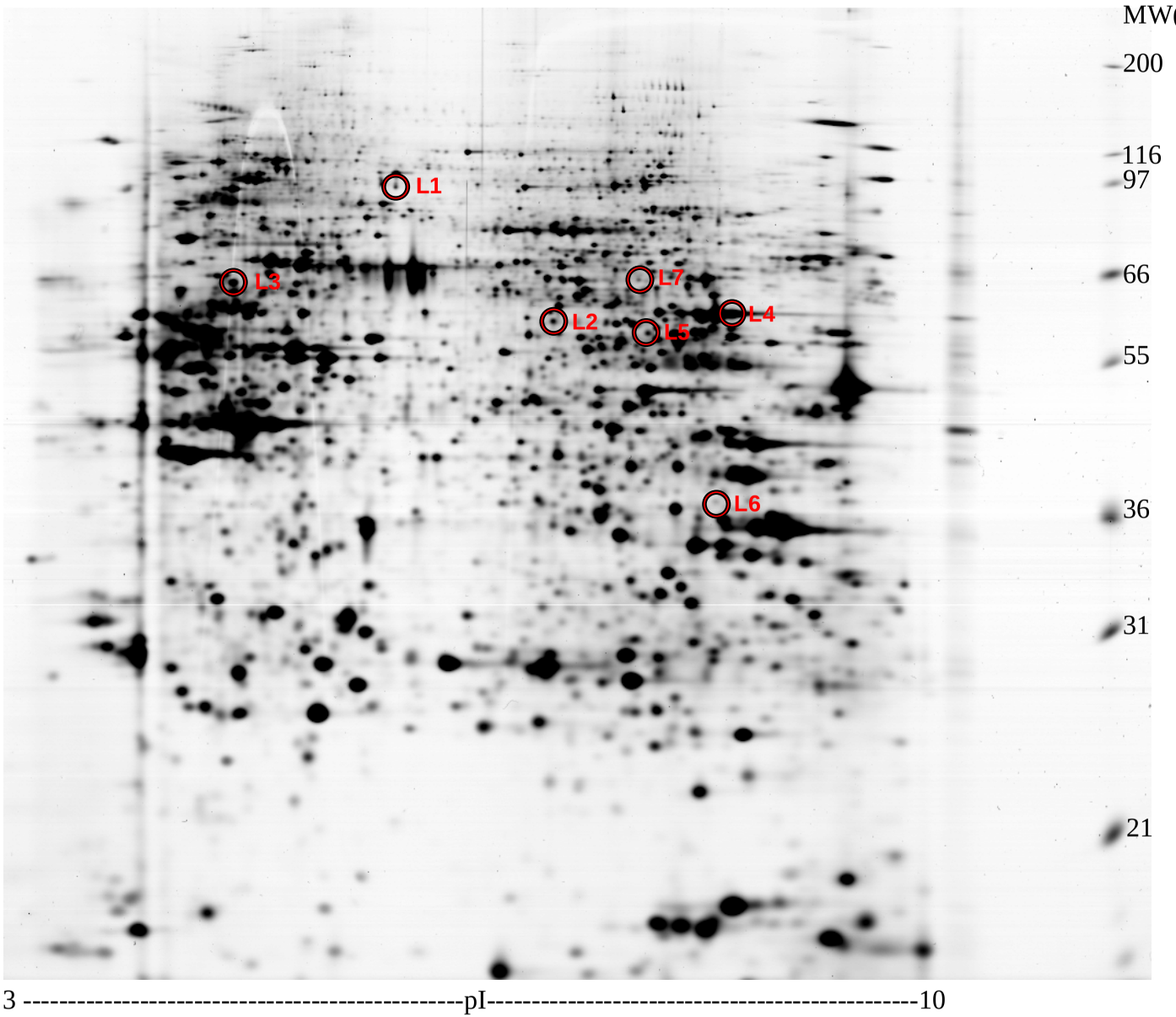


Figure 4.1 Silver Stained analytical gel (30µg) of human TCC tissue from patient #103 (low-grade reference gel) Locations of 7 (L1-7) up-regulated protein spots are marked in red

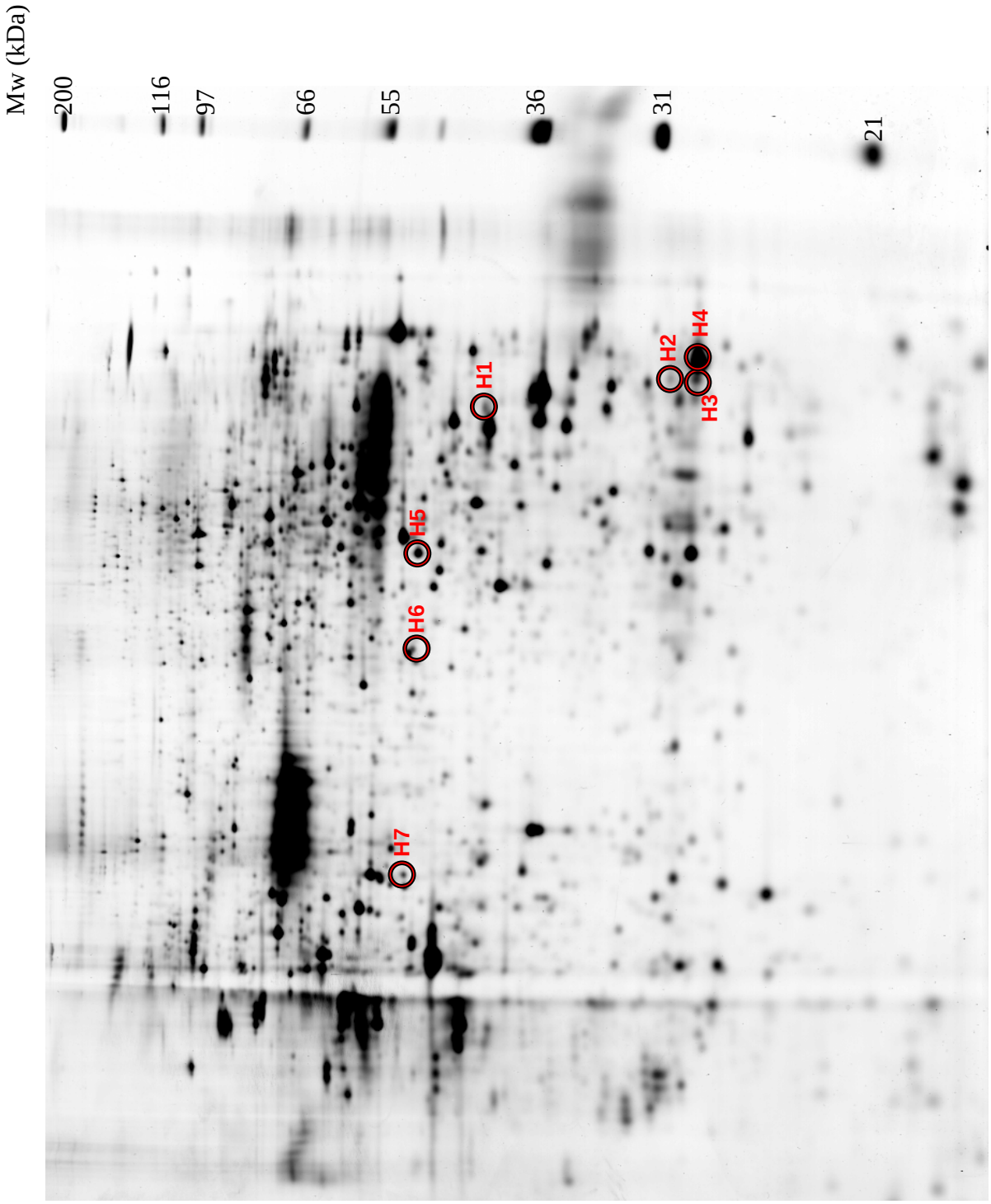
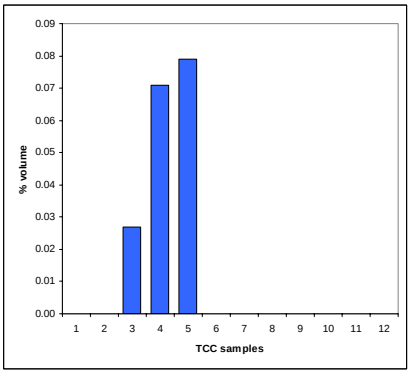


Figure 4.2 Silver Stained analytical gel (30µg) human TCC tissue from patient #113 (high-grade reference gel) Locations of 8 (H1-8) up-regulated protein spots are shown in red.

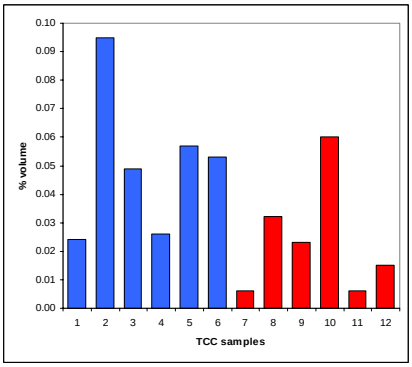
Table 4.4 Details of identified proteins differentially expressed between high and low grade Ta TCC

Spot No	Identity	Ratio of class means	samples >2-fold expression difference	Gene	Locus	Mw (Da)	pI	Gel MW (Da)	Gel pI	Accession No.
L1	10-Formyltetrahydrofolate dehydrogenase (FDH)	∞	3/6	FTHFD	3q21	98701	5.1	100000	5.5	6225390
L2	Coronin 1A	2.1	4/6	CORO1A	16p13	51027	6.3	61000	6.6	5902134
L3	Heteogenous nuclear ribonucleoprotein K (isoform1) (hnRNPK)	2.1	3/6	HNRNPK	9q21	51029	5.2	66000	4.8	14165437
L4	Pyruvate kinase	1.8	3/6	PKM2	15q22	57914	8.0	62000	7.8	4505839
L5-7	Not identified	-	-	-	-	-	-	-	-	-
H1	Aldolase A	∞	6/6	ALDOA	16p11	39420	8.3	40000	8.5	4557305
H2-4	Short-chain alcohol dehyderogenase family member-/HEP27	4.2-14.4	6/6	DHRS2	14q11	29927 27439	9.2 8.9	30000 27000	8.9 8.9, 9.2	5031737 3915733
H5	HMG Co A Synthase	1.7	2/6	HMGCS2	1p13	56636	8.4	55000	7.9	5031751
H6-7	Not Identified	-	-	-	-	-	-	-	-	-

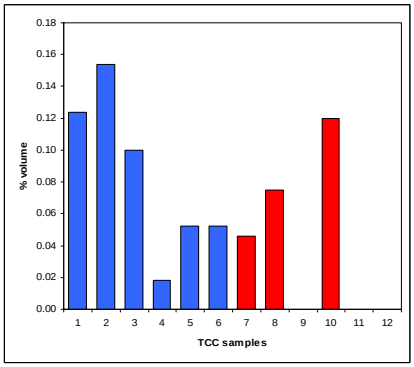
L1-FTHDH



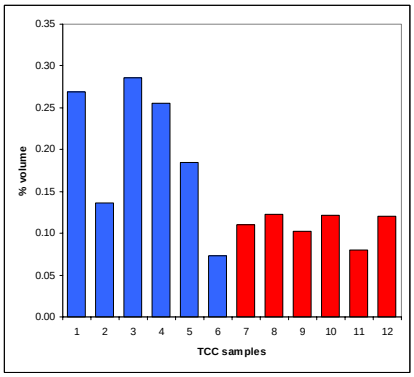
L2- coronin



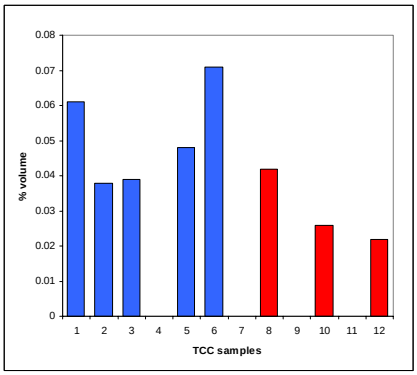
L3- HNRNPK



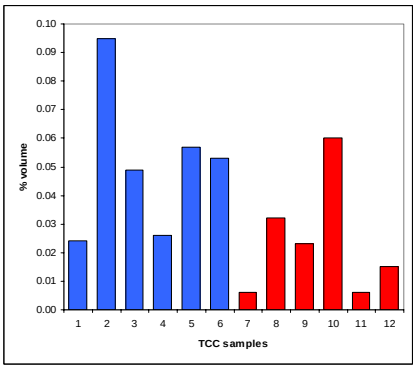
L4 -pyruvate kinase



L5-unknown



L6-unknown



L7-unknown

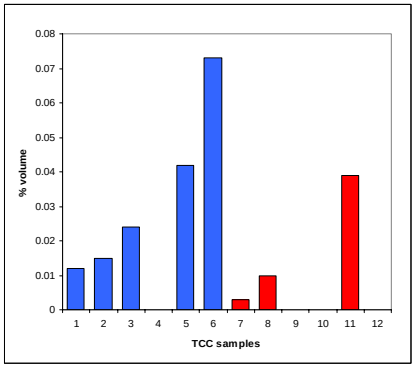
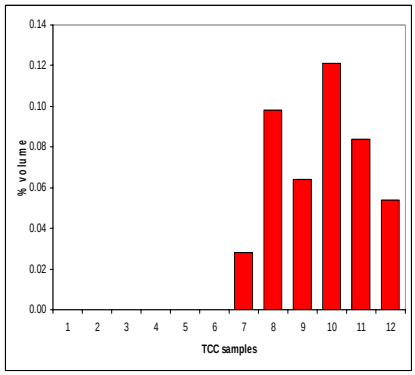
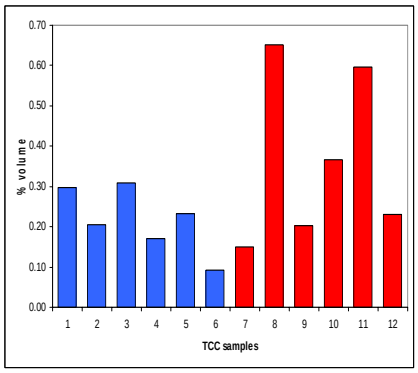


Figure 4.3 Expression levels on silver stained 2D gels of 7 proteins up-regulated in low-grade Ta TCC. Blue bars low-grade TCC samples (1:100, 2:101, 3: 103, 4:118, 5:239, 6:248) red bars high-grade TCC samples (7: 94, 8:110, 9:113, 10:124, 11:261, 12:322).

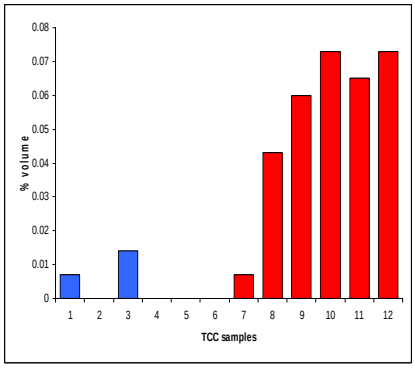
H1-Aldolase



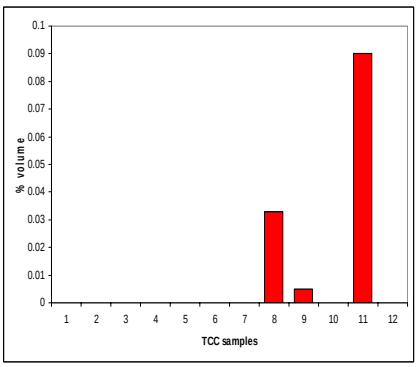
H5-HMG CoA Reductase



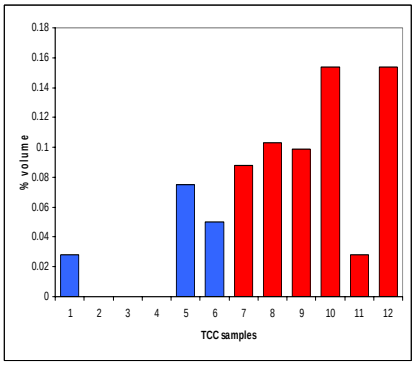
H2-SC alcohol DH



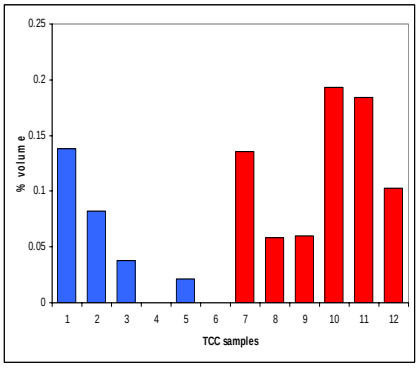
H6-unknown



H3-Hep 27



H7-unknown



H4 – Hep 27/ SC alcohol DH

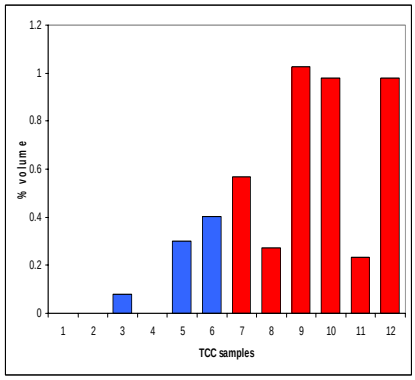


Figure 4.4 Expression levels on silver stained 2D gels of 7 proteins up-regulated in high-grade Ta TCC. Blue bars low-grade TCC samples (1:100, 2:101, 3: 103, 4:118, 5:239, 6:248) red bars high-grade TCC samples (7: 94, 8:110, 9:113, 10:124, 11:261, 12:322).

4.4 Discussion

This study has identified 4 proteins that are down-regulated, and 3 proteins that are up-regulated in high-grade, in comparison with low-grade, bladder tumours in a series of 12 Ta TCC. These seven proteins are tabulated in Table 4.5.

Up-regulated in G3 Ta TCC	**Down-regulated in G3 Ta TCC**
Hep 27	Formyltetrahydrofolate dehydrogenase (FDH)
HMG Co A synthesase	Coronin
Aldolase A	Pyruvate Kinase
	Heterogenous Nuclear Ribonuclear Protein K (hnRNP K)

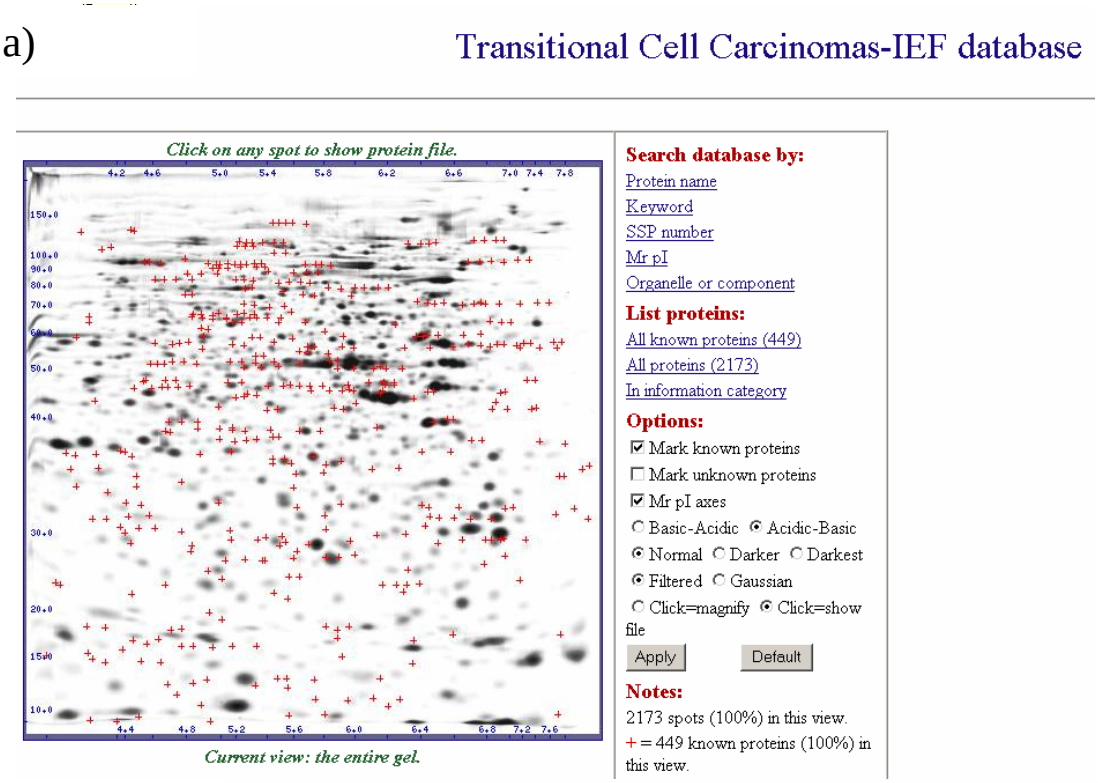
Table 4.5 Summary table of differentially-expressed proteins identified from profiling 12 Ta TCC by 2D PAGE.

Proteomic profiling of several tumour types has been performed including breast (Franzen et al., 1996; Wulfkuhle et al., 2001), colon (Lawrie et al., 2001; Stulik et al., 2001), ovary (Alaiya et al., 2002; Jones et al., 2002), prostate (Ahram et al., 2002; Meehan, Holland, and Dawkins, 2002), and kidney (Unwin et al., 2003a; Unwin et al., 2003b). Extensive 2D PAGE proteomic analysis of diseased tissues is a difficult and lengthy process. Only a few diseases have been subjected to detailed investigation including lung (Chen et al., 2002; Chen et al., 2003; Hanash, Brichory, and Beer, 2001) and bladder cancers. The Department of Medical Biochemistry and Danish Centre for Human Genome Research, The University of Aarhus, Denmark under the direction of Professor Julio E Celis has published many studies of 2D PAGE analyses of human fibroblasts, keratinocytes, squamous and transitional cell carcinomas and normal urothelium. In particular Celis *et al* have produced comprehensive proteomic databases for the study of

TCC bladder cancer (Celis et al., 1999c; Gromov et al., 2002). These can be accessed via http://proteomics.cancer.dk (previously http://biobase/dk/cgi-bin/celis). However, this group have not published any work similar to this study; the proteomic characteristics of Ta TCC have not previously been assessed to identify prognostic markers. Non-equilibrium pH gradient electrophoresis (NEPHGE) and isoelectric focus gel (IEF) techniques with ^{35}S methionine-labelled or silver-stained proteins have been used to analyse several hundred-bladder tumour specimens (a detailed method is available from http://proteomics.cancer.dk). To date, 2173 spots have been catalogued and 449 identified from IEF gels, 990 spots (144 identified) from NEPHGE gels and 459 (197 identified) from normal urine samples (Figure 4.5). The spot identities have been determined with mass spectrometric, immunoblotting and Edman Degradation techniques. The IEF database spans proteins of 10-150kDa with pI 4.0-7.6, and NEPHAGE 10-150 kDa with pI 6.6-11.4.

One of the proteins identified in this study; heteogenous nuclear ribonuclear protein K (hnRNP K) has been identified by Celis *et al* in the IEF database, and several variants of hnRNP K are also listed. It has a pI of 5.2 in the middle of the pI coverage of the IEF gels. Interestingly, although the predicted MW of the gene product of hnRNP K is 51029 Da, the mass of the proteins in this study and on the database are ~66000 and 64600 Da respectively. This high level of concordance between the 2 values gives extra support to their validity and suggests the addition of other molecular groups to the protein during post-translational modifications.

a)



b)

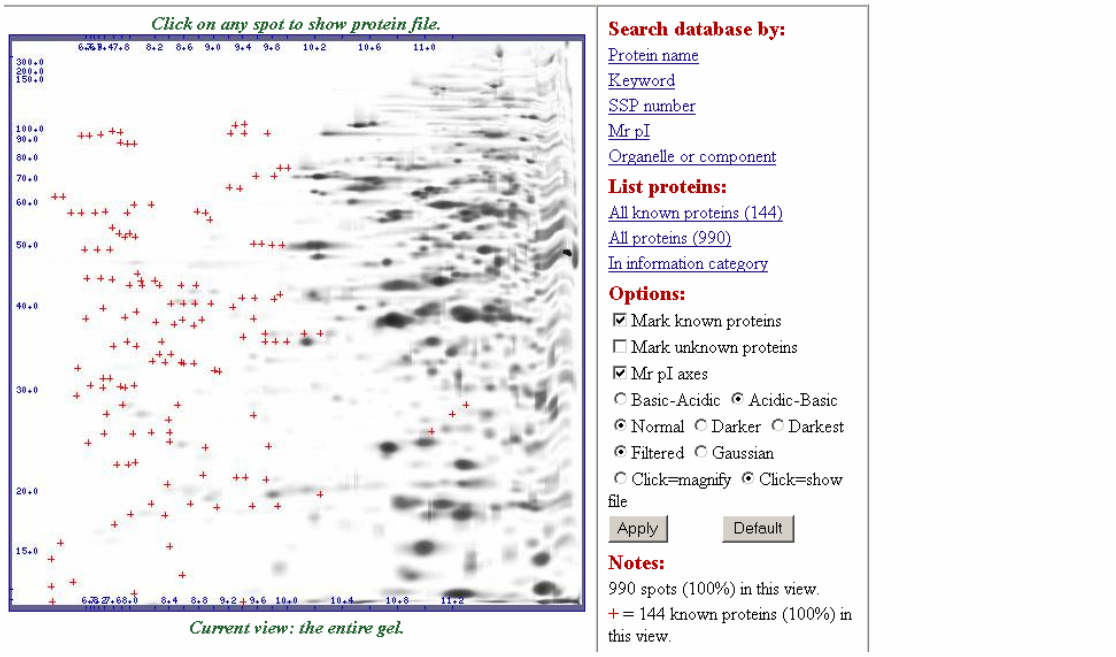


Figure 4.5 Screen images displayed from http://proteomics.cancer.dk

Composite synthetic images of proteins separated by a) IEF and b) NEPHGE 2D-PAGE displayed. Red crosses correspond to known proteins.

None of the other proteins identified are represented in either Celis-TCC database. This may be explained by incomplete protein identification in the databases. In fact of 3163 spots identified in both Celis databases, 2570 (81%) remain unidentified. Also, the ^{35}S methionine labelling performed by Celis *et al* will only detect synthesised proteins, whereas this study has compared all proteins that stain with silver, including for example those from serum within the tissue. The database also comprises many proteins expressed by TCC as cell lines such as T24 developed from a grade 3 invasive TCC, rather than Ta TCC used in this study. In addition, many proteins identified in this study are relatively basic, HMG Co A synthase, aldolase and Hep 27, up-regulated in high-grade TCC, have isoelectric points of pH 7.9, 8.5 and 9.2 respectively which are outside of the range of the IEF database that contributes the majority of the proteins identitified by Celis *et al*. However, this factor cannot help explain the absence of FDH (pI 5.1) and coronin (pI 6.3) from the databases. Hence, it is not surprising that the majority of proteins differentially expressed in this study have not been previously identified by 2D PAGE.

Several of the differentially-expressed spots could not be identified in this study (L5-7 and H6-7) and so the Celis-databases were examined to attribute possible identities to them. However, the differences between the 2D gel techniques and samples used in this study and the Celis-database gels make it difficult to use the database to identify the unknown spots with confidence. If differential expression is confirmed in future studies it will be important to identify these proteins.

Previous proteomic analysis by 2D PAGE has identified several molecules that are differentially expressed between normal urothelium and TCC of various grades and stages. A protein expression profile of normal urothelium and 63 TCC (5 G1Ta, 25 G2Ta, 2 G3Ta, 12 G3T1, 1 G2T2+, 18 G3+T2+) was generated by ^{35}S methionine-labelled IEF gels (Celis et al., 1996). Four proteins were expressed by normal urothelium and the majority of low stage low grade TCC but not by more advanced TCC. Adipocyte fatty acid binding protein (A-FABP) (Mw 14587 pI 7.2) was expressed in 24/31 (77%) low-grade TCC compared with 10/32 (32%)

of high-grade TCC (p=0.0006). Similarly it was expressed in 7/14 (50%) of grade 3 Ta/T1 TCC but only 3/13 (23%) of G3T2+ TCC (p=0.02). Data specifically comparing high/low grade Ta TCC were not shown. Cellular functions of the FABP family that have been described include intracellular lipid transport, metabolism and signalling (reviewed by Veerkamp, Peeters, and Maatman, 1991). In particular, some demonstrate growth inhibitory properties in various tumour cell culture models of breast cancer (Huynh et al., 1995) that would support a putative tumour suppressor role. Subsequent measurement of protein and mRNA levels linked the reduced protein expression with decreased gene transcription rather than translational regulation (Gromova et al., 1998). Since this discovery, additional studies have confirmed the differential protein expression between advanced and less-advanced TCC (Orntoft et al., 2002). Recently, Celis subjected a large panel of 46 benign and 107 TCC to IHC for A-FABP (FABP4), which also showed reduced expression, confirmed on a tissue microarray made from 2317 tumour samples (Ohlsson et al., 2005). Sheng *et al* have just published a study into differences between 10 grade 2 and 10 grade 3 TCC using 2D PAGE and LC MS/MS (Sheng et al., 2006). In agreement with previous studies A-FABP was consistently down-regulated in TCC on increasing grade and stage.

There was no differentially expressed spot identified in our study corresponding to MW 14587, pI 7.2. In fact a protein of MW 14587 is too small to be resolved in the gels run in our study, as any protein of this MW would have migrated through the entire gel. This is clearly a limitation of the present study as it would have been desirable to examine A-FABP expression in Ta TCC.

Celis *et al* also correlated a reduction of Glutathione S-transferase μ (GSTM) protein expression (Mw 25580, pI 6.7) with a reduction in tumour differentiation. GSTM was expressed in 18/31 (58%) of grade 1 and 2, but only 6/32 (19%) of grade 3 tumours. While we are unaware of further proteomic-based studies of GSTM and bladder cancer there has been an explosion of interest in this molecule as a marker of susceptibility to TCC. GSTM is an enzyme involved in the detoxification of polycyclic aromatic hydrocarbons found in tobacco smoke, a known risk factor for bladder cancer. In a meta-analysis of 17 studies (2149 cases

and 3646 controls) GSTM1 null status (accounting for 50% of persons of European descent) was associated with a modest increase in the risk of bladder cancer (odds ratio 1.44, 95%CI 1.23-1.68) (Engel *et al*., 2002). However, no significant difference was identified in GSTM1 status between 103 superficial and invasive TCC patients (Aktas *et al*., 2001). In contradiction, enzyme activity of GSTM in a panel of 83 TCC tissues and normal urothelial controls was found to be increased (mean 2.8 fold) in half of the TCC compared to normal urothelial samples (Berendsen et al., 1997), and so it appears that the role of GSTM in the development of TCC is unclear at present. In our study no protein with a similar Mw/pI profile to GSTM was identified, thus no additional evidence for its role has been provided.

Our study failed to identify prostaglandin dehydrogenase (PGDH) (Mw 28977, pI 5.7) as a differentially expressed protein although Celis *et al* found expression in 17/32 (46%) of high grade, compared to 27/31 (87%) of low grade, TCC. We are unaware of further reports of this molecule in relation to TCC. Similarly, keratin 13 (Mw 49643, pI 4.7) was not identified as a putative marker in our study but was identified by Celis *et al* as a marker of high-grade tumours. This is in agreement with previous immunohistochemical surveys of TCC panels that also found reduced CK13 expression with muscle invasive disease (Schaafsma et al., 1990). Celis *et al* noted that the overall protein expression profiles of grade 1-3 Ta/T1 TCC were very similar. This correlates well with our analysis that identified only 14 differentially expressed spots with certainty from approximately 1000 matched gel features. It is to be remembered that the different gel, staining and analysis techniques between these studies limit the value of their comparison.

Further reports from Celis *et a*l have identified additional protein markers associated with the invasive phenotype of TCC. Four TCC (2 invasive, 2 non-invasive) were subjected to comparative genomic hybridisation, high-density oligonucleotide array analysis of transcript level and 2D PAGE (Orntoft et al., 2002). The main aim of the study was to correlate protein and transcript alterations, which were broadly similar. However, additionally they identified

increased levels of annexin II and IV, CK 17 and 20, Fructose-1,6-bisphosphatase 1, plasma gelsolin, prohibin and prolyl-4-hydroxyl in invasive TCC. Invasive TCC expressed lower levels of PA-FABP and heterogenous ribonuclear protein B1 (hnRNP B1). Again, none of these proteins were identified in our study, but this is not surprising as both studies analysed protein differences between different types of TCC.

Most recently, Celis *et al* have examined protein expression differences between biopsies of normal urothelium and invasive TCC and within a group of G1Ta TCC (termed papillomas in the paper) (Celis et al., 2002). 2D PAGE of 116 normal urothelium biopsy samples were compared with 30 G2T2+ TCC. A significant difference between protein levels in each group was defined as a 2-fold variation in excess of 40% of samples (including CK20 that was differentially expressed in only 33% of invasive lesions). This criterion was adopted in our study as it was felt that a simple comparison of mean protein expression values could miss true differences if they were only seen in a proportion of tumours. Twenty identified-proteins were found to be deregulated in invasive TCC in comparison with normal urothelium (upregulated: CK 18 and 20, heat shock protein 28, elongation factor 1-γ, the proteosome ζ subunit, migration inhibitory factor-related protein 14 and proliferating cell nuclear antigen; downregulated: CK 5 and 13, annexin 5, high affinity bile-binding protein DD2, lactate dehydrogenase H chain, maspin, plasminogen activator inhibitor 2, phosphoglycerate kinase, purine nucleoside phosphorylase, a tropomyosin isoform, stratifin, A-FABP and PA-FABP). However, none were identified as differentially expressed in our comparison between high and low grade Ta TCC. This may indicate that these proteins show altered expression in all bladder tumours. It would be interesting to make specific measurements in a panel of low and high grade Ta in the future.

In the study of Celis (2002), several patterns of cytokeratin (CK) expression were described among the proteome of 30 G1Ta TCC, although the authors stated that it was not possible to comment on any correlation between these CK expression-

patterns and clinical phenotype as the study numbers were small and the follow-up short. In our study the use of a surrogate marker of poor outcome in Ta lesions (high-grade) may partly overcome the need for extended follow up although such studies provide the most direct evidence for the utility of prognostic biomarkers.

This study has identified a reduction of hnRNP K protein in 3 of 6 high-grade TCC compared to the mean of low-grade TCC with an overall 2.1 fold decrease in expression. Several members of the heterogenous ribonuclear protein family (hnRNP B, C, E, F, H, K and L) have been identified in the proteome of TCC (http://proteomics.cancer.dk). Indeed hnRNP B1 was down regulated in a comparison of invasive vs. non-invasive TCC (Orntoft et al., 2002). The hnRNP proteins influence pre-mRNA processing and encode much of the information specifying nuclear export, subcellular localisation, translation and stability required by mRNA, (reviewed by Dreyfuss, Kim, and Kataoka, 2002). In particular, hnRNP K is characterised by the K nuclear shuttling domain allowing its traffic between the nucleus and cytoplasm (Michael, Eder, and Dreyfuss, 1997). It interacts with many protein kinases such as Src, protein kinase C and the Y-box binding protein (Shnyreva et al., 2000) many of which have been associated with the development of tumours.

HnRNP K has been identified as a marker of keratinocyte proliferation by 2D immunoblotting (Dejgaard et al., 1994). In breast cancer, increased hnRNP K expression was seen in grade 3 vs. grade 2 tumours and in a breast cancer cell model, EGF induced hnRNP K protein expression that was blocked with the addition of anti-EGFR (Her1) antibody. Antibody administration to a mouse xenograft tumour model reduced tumour size and hnRNP K levels (Mandal et al., 2001). Interestingly, autoantibodies to hnRNP K appear highly specific to patients with systemic autoimmune lupus (SLE) (Hayer et al., 2000), and those women have an increased risk of developing breast cancer (Bernatsky et al., 2003). In lung cancer models, hnRNP K expression was altered in a complex manner, for example it became overexpressed in the nucleus of lung tumour cells, where its expression correlated with that of c-myc gene amplification status in a series of

human lung cancer cell lines (Pino et al., 2003). These findings seem at odds with our findings of the loss of this protein in more aggressive Ta TCC. It is clear however that the complex and incompletely understood functions of this protein are of great importance to cell growth and function and it is consistent that its deregulation may be associated with the development of cancer.

Coronin (coronin 1) was down regulated by a mean of 2.1 fold in high-grade Ta TCC compared to the mean of the low-grade Ta TCC in our study (by more than 2 fold in 4/6 samples). Coronin is a homodimeric 55kDa protein that was first isolated from the slime mould *Dictyostelium discoideum* in 1991 and is also found in humans. Its structure and functions have been recently reviwed (Rybakin and Clemen, 2005). Coronin is localised to the cell surface, particularly in actin-rich crown-like projections (hence *coronin*). It binds actin via WD interacting motifs (conserved 40-amino acid sequences ending with tryptophan-aspartic acid) that form into a so-called β-propeller structure (reviewed by de Hostos, 1999). Coronin null cells are reduced in locomotion and impaired in their ability to divide and phagocytose (de Hostos et al., 1993; Maniak et al., 1995). Coronin appears to promote the the assembly of actin and microtubular structures in yeast (Goode et al., 1999) and cell spreading and lamellipodia extensions in *Xenopus* cultured cells (Mishima and Nishida, 1999). Several mammalian coronin sub-types have been described (1-5), and we have identified coronin 1 whose transcripts are seen in cells of a haemopoietic lineage, lung and weakly in the brain of mammals (Okumura et al., 1998). In summary, coronin function is not clearly established in human cells and we are unaware of published studies examining its expression in human cancers.

Recently, cDNA microarray analysis of 9 TCC cell lines identified a group of molecules deregulated in TCC (Sanchez-Carbayo et al., 2002). Immunohistochemical validation experiments identified the loss of moesin protein expression was associated with advanced tumour grade and stage and also survival. Moesin is a member of the ERM (Ezrin-radixin-moesin) actin binding proteins and it may interact with coronin via cytosolic phagocytic oxidase (phox)

proteins (Wientjes et al., 2001). If moesin and coronin are functionally linked in support of the cellular microarchitecture of normal cells then it is possible to speculate that the loss of coronin may also be a marker of advanced TCC.

Aldolase A was expressed in all 6 high-grade Ta TCC but no low-grade TCC in our study. Aldolase catalyses the sixth step in the glycolysis pathway namely the conversion of D-fructose 1,6, bisphosphate to glycerone phosphate D-glyceraldehyde 3-phosphate. Three forms of this ubiquitous enzyme are found in humans that predominate in (A) muscle, (B) the liver and (C) the brain. Glycolysis metabolises glucose to pyruvate while liberating a small amount of energy, pyruvate enters the Kreb's cycle in the presence of oxygen to liberate significantly more energy. Cancer cells often maintain raised aerobic glycolytic rates and produce high levels of pyruvate, a phenomenon known as the Warberg effect (reviewed by Semenza, 2000). Consitutent members of the glycolysis pathway may be upregulated in tumours and most, including aldolase have been identified as potential tumour biomarkers. Aldolase A and C and other glycolytic enzymes were upregulated in 6/6 renal cell carcinoma tumour/normal tissue pairs analysed by 2D PAGE (Unwin et al., 2003a), and an increase in Aldolase A mRNA was seen in hepatocellular carcinomas compared to cirrhotic or normal liver tissue (Castaldo et al., 2000). Three studies published 1968-80 in German and Japanese investigated aldolase expression in TCC and English translations of these abstracts suggest that it was up-regulated. We must presume that the absence of aldolase in low-grade lesions in this study reflects its expression below the level of detection of our 2D PAGE. Aldolase may be increased in low-grade Ta TCC in comparison with normal urothelium, although this relationship was not examined in our study.

Pyruvate kinase (PK) is also an enzyme that catalyses a step in the glycolytic pathway, converting phosphoenolpyruvate to pyruvate. It exists as 4 isoforms in humans that are predominantly expressed in the liver (L), erythrocytes (R) and muscle (M1), while M2 is expressed in early fetal tissue. This study detected increased levels of PKM1 in low, compared with high-grade Ta TCC. The

difference between the mean expression levels of the 2 groups was only 1.8 fold although 3/6 low-grade tumours displayed more than twice the expression of the mean of the high-grade group. This finding seems counter-intuitive in light of the Warburg effect discussed above, although as cell biology is so complex we may be identifying a novel phenomenon. The true situation may be no difference between the 2 groups at all, and the small difference we have noted may not be maintained in a larger survey. Only a few reports assess tumour PKM1 expression, and most notably it was upregulated (with aldolase) in RCC by 2D PAGE analysis (Unwin et al., 2003a). In contrast, there has been much interest in (fetal) PKM2 that has been associated with cancers of the lung, kidney and breast (Brinck et al., 1994; Luftner et al., 2000; Schneider et al., 2002) although PKM2 is not increased in the plasma of patients with bladder cancer (Roigas et al., 2001).

One spot was identified as mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase (HMG CoA synthase) that was expressed with a mean 1.7 times in the high-grade, compared to the low grade Ta TCC, with 2/6 of high-grade TCC expressing more than twice the mean low-grade expression. These values was below the threshold we set for differential expression that was likely to be significant. HMG CoA synthase catalyses the condensation of acetyl-CoA and acetoacetyl-CoA to HMG-CoA, the first step in ketogenesis; ketone bodies transfer lipid-derived energy from the liver to other organs (Boukaftane and Mitchell, 1997; Mascaro et al., 1995). There appears to be no previous published studies of an association between HMG CoA synthase and cancer.

Three separate spots were significantly up regulated (4.2-14.4 fold) in high vs. low-grade Ta TCC with similar Mw (27-30kDa) and pI (8.9-9.2) in this study. Mass spectrometry revealed that all 3 spots comprised the human short chain alcohol dehydrogenase protein DHRS2 (synonymous with Protein D, Hep 27). Professor Franco Gabrielli at the University of Pisa initially described Hep 27 in 1991. This protein was synthesised in the nuclei of HepG2 (a human hepatoblastoma cell line) after growth inhibition with butyrate. Subsequently it was cloned and identified as a member of the short chain alcohol

dehydrogenase/reductases (SDR) family (Donadel et al., 1991; Gabrielli et al., 1995). SDR familiy members are functionally active to modulate a wide range of steroid hormones, retinoids or prostaglandins that are critical to the control of normal cellular functions, such as the cell cycle, and have been preliminarily associated with some human cancers (reviewed by Oppermann, Filling, and Jornvall, 2001).

The function of Hep 27 remains unclear although it has been recently characterised and its expression analysed in normal human tissues with Western blotting. Two bands at c. 27kDa and 30kDa are seen in tissue lysates from the liver, placenta, breast, ovary, testis, parotid and endometrium although not from lung, brain, renal, prostate colon or adrenal tissues (Pellegrini et al., 2002). These correspond with the different Mw of spots identified in this study, and can be explained by the presence of an alternative upstream promotor in the Hep 27 gene. Two main transcripts of 1.9 and 1.5 kb are produced in human liver that result in proteins of 30 and 27 kDa respectively. These variants were seen in tissues in varying ratios (Pellegrini et al., 2002). The expression and function of Hep 27 in TCC is unknown and requires further investigation.

Formyltetrahydrofolate dehydrogenase (FDH) was expressed in 3 of 6 low-grade TCC and none of the high-grade Ta TCC in our study. It is a multi-domain chimeric enzyme, formed by the fusion of two unrelated genes (Cook, Lloyd, and Wagner, 1991). It has been detected in abundance in many human tissues including lung, prostate, brain, muscle, heart, ovary, thymus, testis, kidney and pancreas (normal urothelium has not been examined) and comprises about 1% of the soluble cell protein of the liver (Krupenko and Oleinik, 2002). FDH structure and function are shown in Figure 4.6. FDH is believed to recycle excess 10-formyltetrahydrofolate (10-formylTHF) utilised for *de novo* purine synthesis to tetrahydrofolate (THF) by hydrolysis and thus replenish the folate pool for other one-carbon reactions (Krupenko, Vlasov, and Wagner, 2001; Krupenko and Wagner, 1999; Krupenko, Wagner, and Cook, 1997a; Krupenko, Wagner, and Cook, 1997b; Reuland, Vlasov, and Krupenko, 2003). One-carbon metabolism is

important in the biosynthesis of a number of essential molecules such as methionine and DNA (Schalinske and Steele, 1996). Additionally, the hydrolase reaction removes carbon units from the folate pool as carbon dioxide and this may serve to protect the cell from formic acid toxicity (Krebs, Hems, and Tyler, 1976; Tephly, 1991). The carboxyl-terminal domain shares about 48% sequence identity with members of class I and II aldehydedehydrogenases (ALDH), although it is unclear if FDH functions in this manner (Krupenko, Wagner, and Cook, 1997b). The ALDH family oxidize a variety of aldehydes into their corresponding acids, (reviewed by Yoshida et al., 1998). Altered ALDH expression has been reported in a variety of human cancers such as liver, colon and breast (Lindahl, 1992). Proteomic studies reported ALDH-II proteins were diminished to undetectable levels compared to normal controls in 10 cases of hepatocellular carcinoma (Park et al., 2002), and 12 cases of renal cell carcinoma (Balabanov et al., 2001). ALDH expression and activity profiles changed markedly (both increasing and decreasing) over time in a rat bladder-cancer model system (Campbell, Irving, and Lindahl, 1989).

The down-regulation of FDH expression has been recently reported in a number of human cancers (Krupenko and Oleinik, 2002). Protein levels were measured by IHC using an FDH-specific antiserum, and mRNA levels were measured by *in situ* hybridisation in a panel of 15 tissue-pairs representing human lung, breast and ovarian tumours and normal controls. FDH was expressed in all normal tissues but was not detected in any of the tumours examined. PCR amplification of cDNA from these samples produced much stronger bands in a gel from normal compared with tumour DNA (although this technique to assess RNA levels is severely limited, see Chapter 5). The human cell lines PPC-1 (prostate cancer), A549 (lung cancer) and Hep G2 (hepatocellular cancer), which did not expresses endogenous FDH protein, were subjected to transient transfection with FDH cDNA. In those cells expressing FDH, cell proliferation was inhibited, in contrast to those infected with an empty vector or mutant (inactive) FDH controls. These effects were not seen with a kidney embryonic (non-cancerous) cell line (293A) that was found to endogenously express FDH protein.

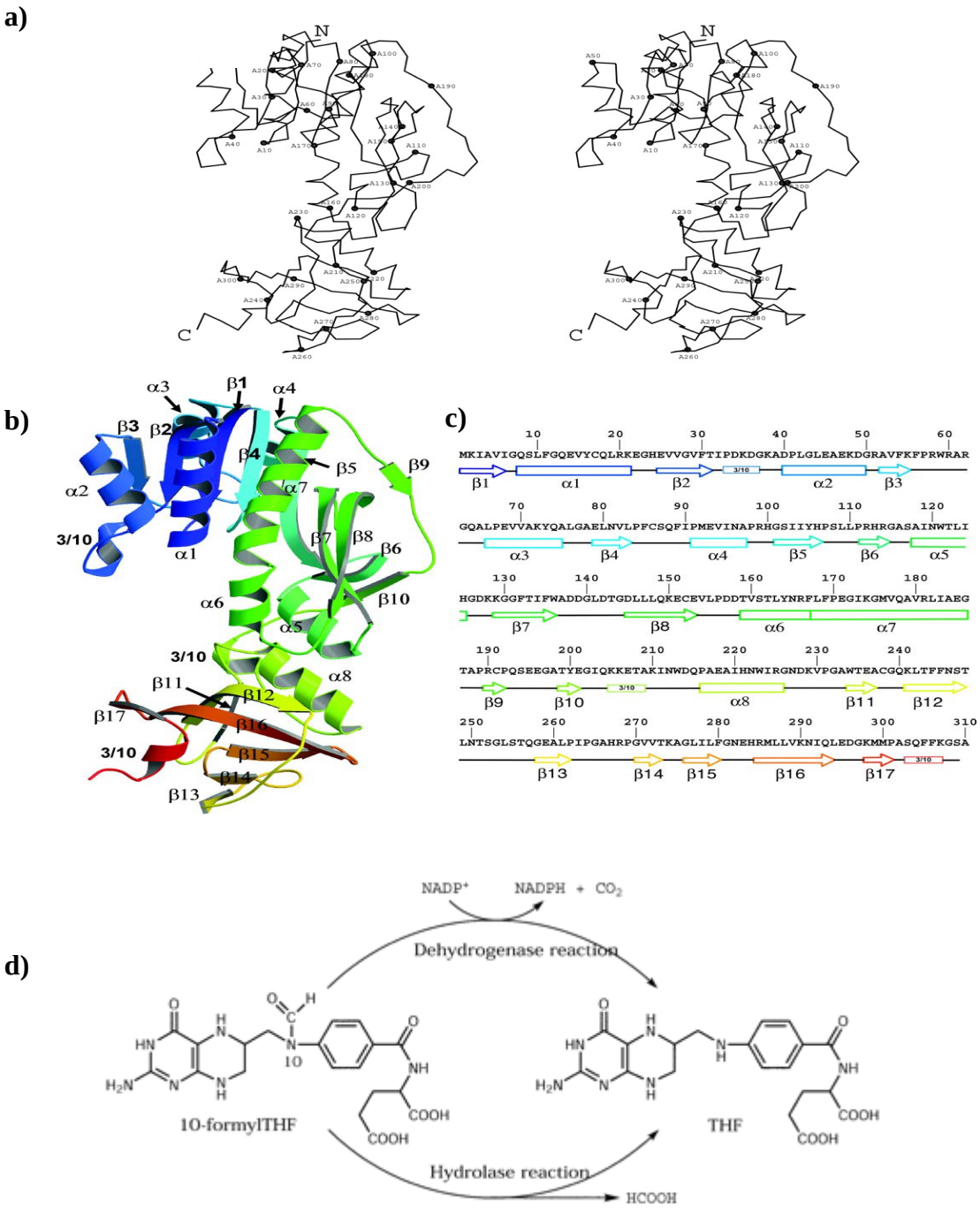


Figure 4.6 N terminal (Nt) -FDH structure and function. a) Stereoview showing the structure as a Cα trace in which every 10th residue is represented as a small sphere and labeled. **b)** A ribbon representation prepared with MOLESCRIPT and Radster 3D, in which the molecule is colour ramped blue to red in the N>C terminal direction. α helices and β strands are labelled individually. **c)** The sequence of N_t-FDH with secondary structure notation as (b) (Chumanevich et al, 2004). **d)** Full length FDH performs a dehydrogenase reaction while the amino-terminal domain alone may catalyse the hydrolysis reaction, from (Krupenko and Wagner, 1999).

Increased 10-formyl THF had been previously identified in tumour tissues (Barford and Blair, 1978) and Krupenko and Olenik hypothesised that this may be due to FDH loss. They proposed that in normal tissues, FDH suppresses purine metabolism by depletion of the intracellular folate pool and thus acts as an inhibitor of cell growth, while FDH down regulation is accompanied by tumour proliferation (Krupenko and Oleinik, 2002). In later studies, the A549 cell line was manipulated to allow inducible FDH expression. Increased FDH expression was associated with initiation of apoptosis and G_1 cell cycle arrest (Oleinik and Krupenko, 2003). When p53 function of the A549 cells was abrogated with RNAi and a dominant negative mutant the FDH-suppressor effect was lost. The mechanism of p53 interaction remains unclear although FDH elevation was shown to result in phosphorylation of the p53 transactivation domain (Oleinik et al., 2005).

This evidence supports the hypothesis that FDH loss is a critical primary or secondary event in the development of tumours. FDH appears to have properties of a p53 tumour suppressor that were not apparent until recently (Oleinik et al., 2005). No data have been previously reported regarding FDH and bladder cancer, although the results of our study are consistent with cell models examined by Krupenko *et al*. We speculate that the loss of FDH function may have an important role in the development of TCC, in addition to its role as a putative prognostic biomarker.

We have found considerable variability in the expression levels of proteins between individual tumour tissue biopsies. This reflects findings in a wide range of experiments based on human samples and may reflect the fundamental differences that exist in the development of cancer between individuals. However such variability may mask the identification of subtle changes in protein expression in a tumour panel. This issue may be overcome by increasing the size of the sample panel, although this may present practical problems (14 months was required to collect the small cohort used in this study from a large clinical unit). Cell lines are extensively used to model human biology and display more

homogeneous proteome expression than tumours. However protein expression may be artefactual, in response to stimuli upon *in vitro* culturing. This effect has been recorded in the culturing of 9 low-grade Ta TCC (Celis et al., 1999a).

A limitation of 2D PAGE is the large amount of sample required for analysis (in comparison to many genomics-based analyses). This may be heightened by the requirement to extract pure tumour tissue. Some tumour types are associated with a relatively large resected tissue specimen (eg kidney for RCC) but may require additional purification techniques (eg. cellular fractionation, laser capture micro-dissection). The Ta papillary lesions used in this study were composed almost exclusively of TCC cells and did not require such processing, but were much smaller.

It must also be remembered that proteins selected for MS identification from the gels in this study comprised in excess of 0.05% of the total % volume of the stained protein. In other words the technique we employed only compared the expression of the higher-abundance proteins, rather than the proteome in its entirety. Similarly it is well recognised that lipid soluble (often membrane-bound) proteins are not well visualised on unmodified 2D PAGE. These considerations may account for a failure to identify specific low-abundance proteins that have been identified by other techniques to be deregulated in TCC (discussed in Chapter 1). These problems may be overcome with narrow-range pH gradient gels or sub-cellular fractionation techniques but these were not incorporated into this study, although could be used in the future.

Despite these limitations, we have used 2D PAGE successfully to identify a number of proteins whose expression is altered between high and low-grade Ta TCC. We selected the most interesting proteins (aldolase, FDH, Hep27, hnRNP K and coronin) for further protein and mRNA expression profiling in a larger cohort of TCC samples. Pyruvate kinase and HMG Co A synthase were not investigated further. The additional analyses are described in Chapter 5.

5 Validation of differentially expressed proteins in TaG2/ Ta G3 TCC by RNA and protein expression profiling

5.1 Introduction

This chapter describes the use of immunohistochemistry (IHC) to validate the findings of the 2D PAGE experiments by determining the protein expression of putative biomarkers in a panel of Ta TCC. Real-time quantitative PCR (RT-QPCR) was also used to investigate the RNA expression of these proteins in this Ta TCC panel, and the protein and RNA expression was also investigated in a wider selection of more advanced TCC tumours (T1-T2+).

5.2 Immunohistochemical analysis of protein expression

5.2.1 Materials and methods

The patterns and level of expression of the proteins identified by 2D PAGE were investigated with immunohistochemistry (IHC). Sections 5µm thick were cut from paraffin embedded samples and mounted on Superfrost™ (BDH) glass slides. A 3-stage steptavidin-HRP immunolabelling protocol was used incorporating avidin/biotin and serum blocking and microwave antigen retrieval.

Sections were dewaxed and rehydrated by 3 x 5 min incubations in xylene, 3 x 1 min incubations in absolute alcohol, a minute in 90% v/v, then 70% v/v ethanol and a water wash. Antigen retrieval was achieved by placing weighted slides in a dish of citric acid (pH 6), covered with pierced cling film and microwaved on full power (750W) for 13 min. The dish was immediately cooled on a bed of ice and the slides washed in water. The slides were mounted in a sequenza system (Thermo) and subjected to blocking with an Avidin-Biotin blocking kit (Vector Labs) and then with normal serum according to the antibody used. The primary antibody (100µl) was incubated with the tissue for 1 hour, washed twice with TBS and the secondary antibody added for 30 min. After 2 further TBS washes 200µl

of Streptavidin ABC complex prepared according to the manufacturer's instructions (Dako) was added for 30 min, washed twice with TBS and removed from the sequenza system. A freshly made DAB-chromagen solution was added for 15 min and then washed from the slides. The details of the antibodies used are shown in Table 5.1.

All sections were counterstained with Mayer's haematoxylin for 15 sec, followed by a minute in the following solutions in order: water, Scott's Tap water, water, 70% v/v ethanol, 90% v/v ethanol, absolute ethanol and xylene. The slides were placed in a second xylene bath and mounted in DepeX mounting medium (BDH) under a coverslip and left to dry overnight.

Table 5.1 Details of the antibodies used for IHC experiments

Antibody	**Type**	**Optimal Primary concentration.**	**Secondary type and concentration**	**Blocking serum**
Anti-Hep 27	Mouse polyclonal	1:400	Rabbit α-mouse 1:200 (Dako)	Rabbit normal serum 1:10 (Dako)
Anti-FDH-1	Rabbit polyclonal	1:200	Goat α-rabbit 1:400 (Dako)	Goat normal serum (1:10) (Dako)
Anti-FDH-2	Rabbit polyclonal	1:200	Goat α-rabbit 1:400 (Dako)	Goat normal serum (1:10) (Dako)

5.2.2 Results: Immunohistochemistry

5.2.2.1 Samples and antibodies

A new panel of 41 *de novo* TCC was assembled (Table 5.2). This panel comprised frozen sections and paraffin sections from 19 Ta, 14 T1, 8 T2+ and 1 Tcis TCC. We wished to examine the protein expression by IHC of the 12 samples used for the 2D PAGE studies, although we were limited by the availability of paraffin-embedded tissue only.

Aldolase and pyruvate kinase protein and gene expression are well established markers of tissue metabolism as described in Chapter 4 and therefore were not investigated further with IHC. Unfortunately, antibodies to all the remaining putative biomarkers were not commercially available and we were unable to generate them within the timescale of this study. However an antibody to Hep 27 was kindly supplied by Professor Gabrielli (University of Pisa) and two antibodies to FDH were kindly supplied by Dr Krupenko (University of South Carolina). Thus, 2 candidate prognostic markers were examined by IHC: Hep 27 (upregulated in G3Ta TCC) and FDH (downregulated in G3Ta TCC).

5.2.2.2 FDH immunohistochemistry

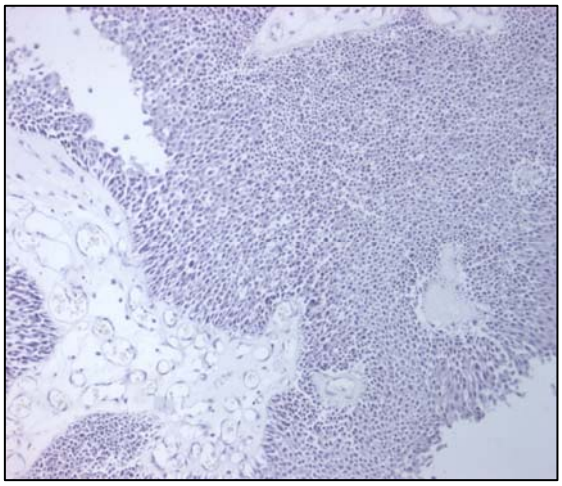
Optimisation of immunohistochemical staining for FDH proved unsuccessful, with the antibody appearing to be poorly specific for FDH. We selected normal ureter, lung and kidney tissue as positive controls and Hep G2 cells as a negative control based on published experiments using the antibody (Krupenko and Oleinik, 2002). The slides showed a generalised brown wash that was unsuitable for scoring (Figure 5.1) despite undertaking optimisation experiments using FDH antibody at 1:50-1:600 with and without microwave antigen retrieval and avidin/biotin serum blocking. The author's experiments were repeated by a senior colleague in the laboratory, who manages the histochemistry facility (Mrs. W. Kennedy) who found similar results. We contacted Dr Krupenko, who kindly sent another antibody from a different batch. However, unfortunately this was also poorly specific for FDH in our hands.

5.2.2.3 Hep 27 immunohistochemistry

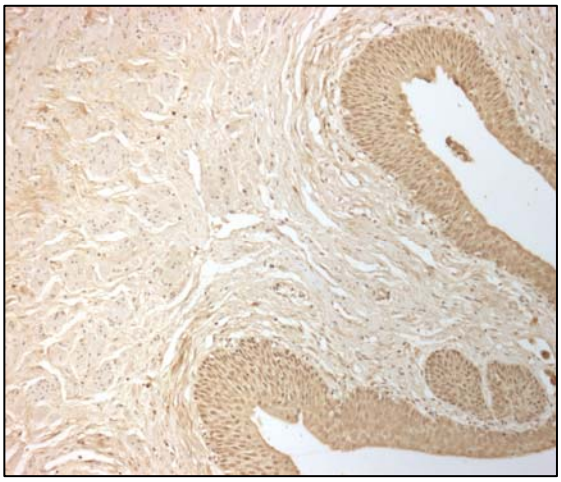
The Hep 27 antibody was optimised for use with the TCC sections. We selected Hep G2 cells as a known positive control and normal tissues such as lung, kidney and ureter as negative control tissues. These tissues were subjected to IHC using the primary antibody at 1:50-1:400 dilutions with and without microwave antigen retrieval, Avidin/biotin and serum blocking. The result of these experiments was that a 3-stage steptavidin-HRP immunolabelling protocol, incorporating avidin/biotin and serum blocking and microwave antigen retrieval, was optimal (Table 5.1) and was able to successfully stain the cytoplasm and cell membranes.

The assembled panel was subjected to IHC using this method and scored for staining intensity. Each section was examined by eye and scored by 2 investigators, myself and Dr Harnden, on a 5 point scale corresponding to 20% increments of stain intensity and coverage, ie. 1 corresponded to between zero and 20%, 2: 21-40%, 3: 41-60%, 4: 61-80%, 5: 81-100%. The identity and grade and stage of each tumour section were unknown to the investigators. The concordance of scores between both investigators was high with the greatest difference between a score of an individual tumour of 1 point (ie. 20%). Interestingly the scoring difference was consistent: I always scored the same or 1 point lower than Dr Harnden. Overall, the degree of Hep 27 IHC staining was increased in high vs. low-grade tumours in this panel of Ta TCC as predicted by our 2D PAGE findings and also in increasing amounts in more advanced tumours and the single sample of cis TCC. The Hep 27 staining pattern in a TCC is shown in Figure 5.2 and Figure 5.3. The scores for the panel and box and whisker plot of these scores are shown in Table 5.2 and Figure 5.4.

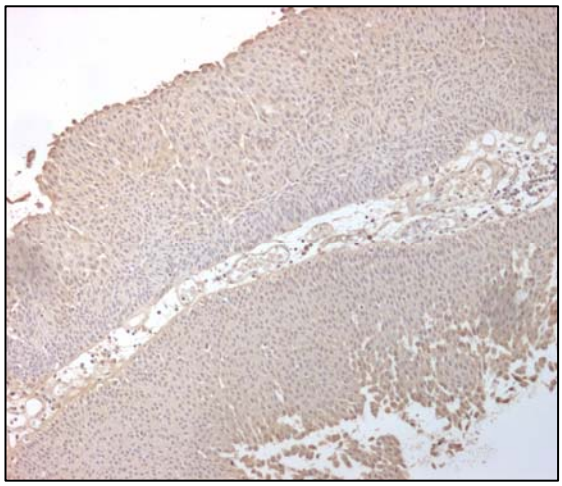
Figure 5.1 IHC images of FDH positive control (ureter) stained with FDH. Brown stain represents antibody binding that is non-specific



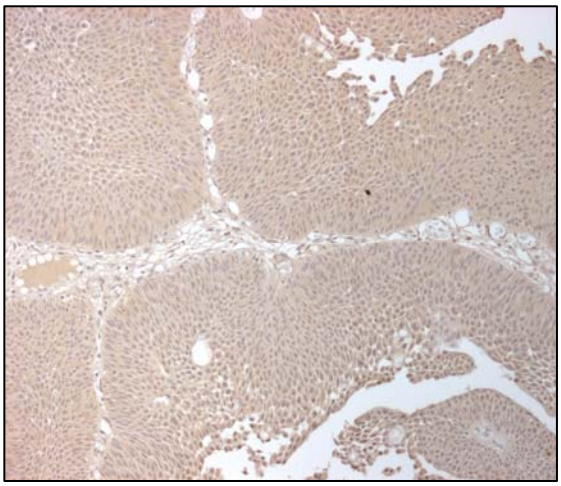
1. No primary Ab negative control



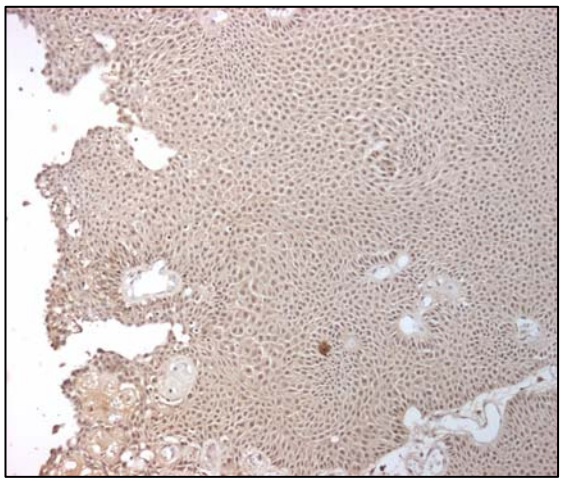
2. Positive control: normal ureter



3. Ta G2 TCC

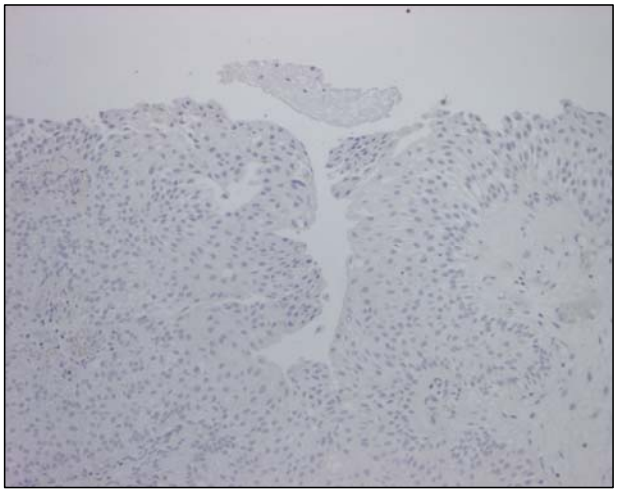


4. T1G3 TCC

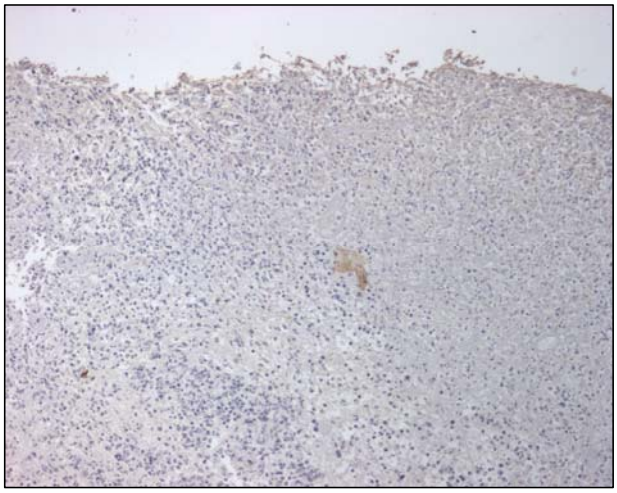


5. T1 G3 TCC

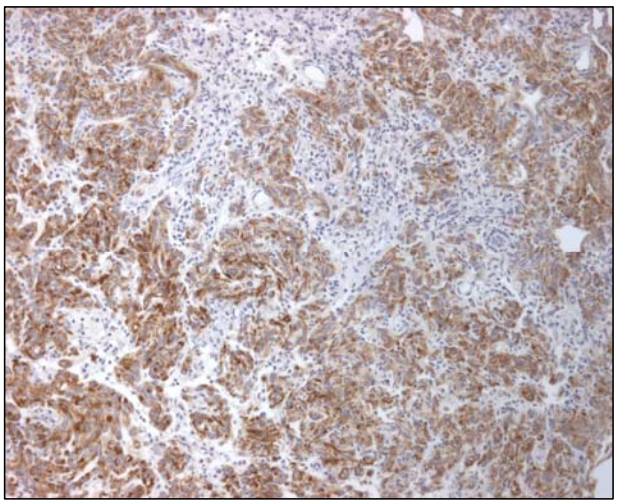
Figure 5.2 IHC images of 6 representative TCC sections (Ta-T2+)stained with Hep 27. Brown stain represents Hep27 protein



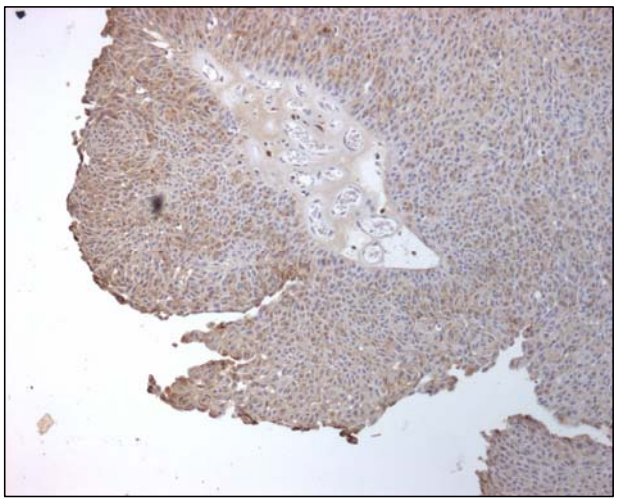
1. No primary Ab negative control



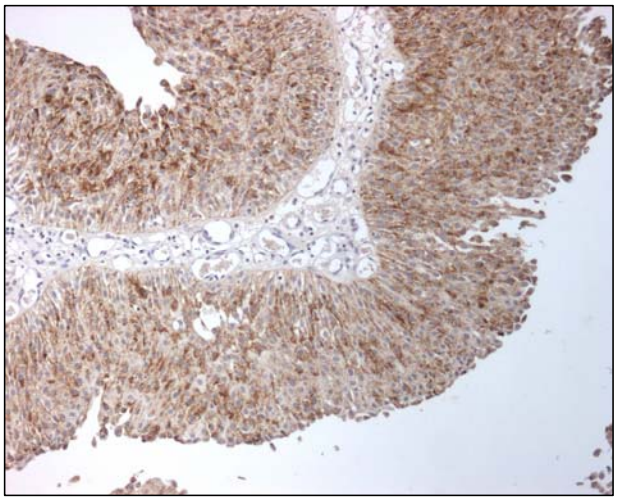
2. 0-20% staining: Ta G2 TCC



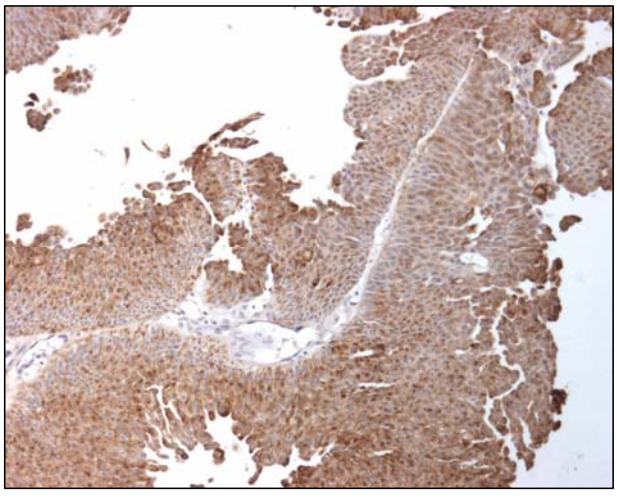
3. 20-40% staining: Ta G2 TCC



4. 40-60% staining: Ta G2 TCC



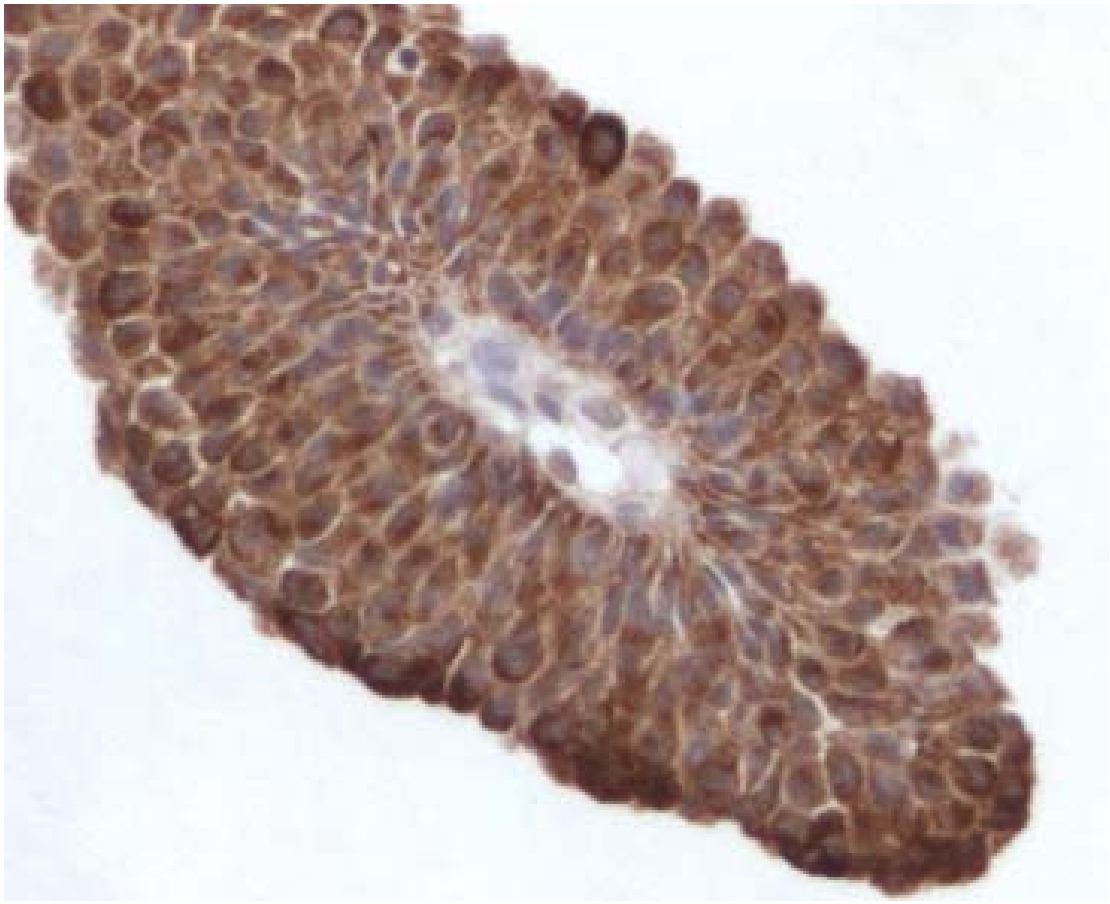
5. 60-80% staining: T1 G3 TCC



6. 80-100% staining: T2 G3 TCC

Figure 5.3 Detail of IHC images of a T2 G3 TCC (#23205) stained with Hep 27 antibody.

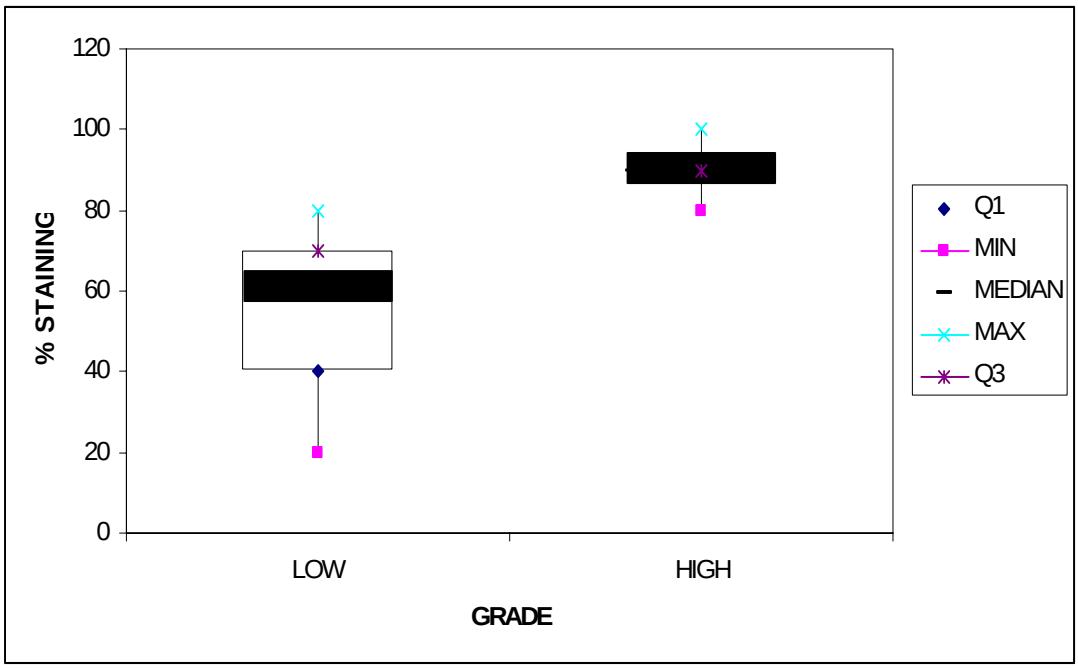
Brown stain represents Hep27 protein. The epithelial cells' cytoplasm stains strongly, with some variation between cells, the nuclei stain less so.

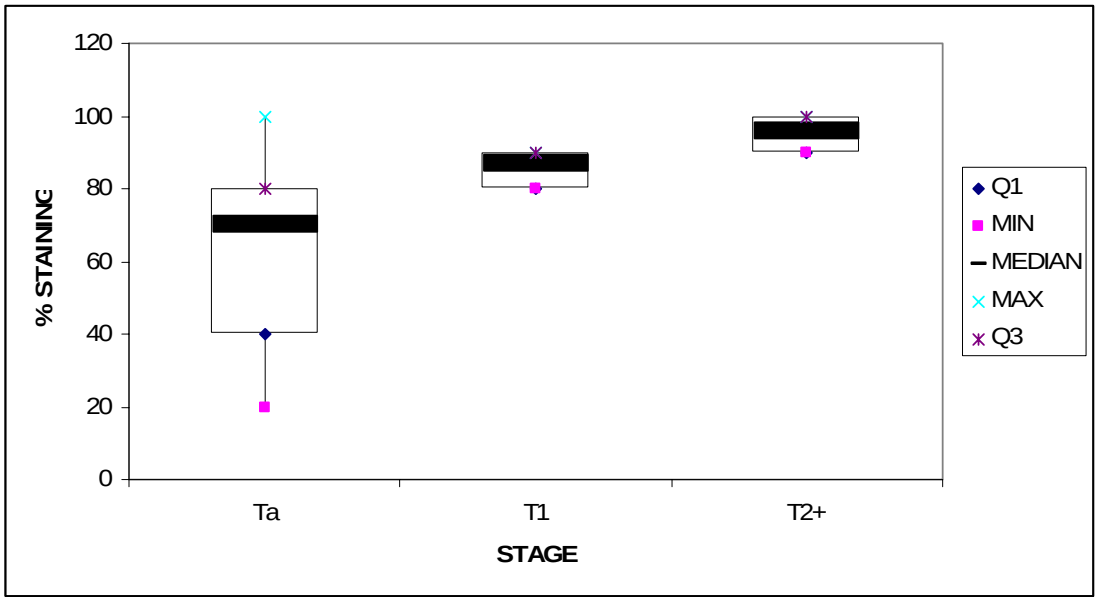


sample id	stage	grade	score(NM)	score(PH)	NM/PH difference	mean score	group mean score
2191/03/1	a	1	2	2	0	2	
12681/02	a	2	2	2	0	2	
17393/02	a	2	3	3	0	3	
18164/02	a	2	3	4	1	3.5	
18256/02	a	2	3	4	1	3.5	
18437/02	a	2	3	4	1	3.5	
20895/02	a	2	4	4	0	4	
23597/02	a	2	1	1	0	1	
24343/02/01	a	2	1	1	0	1	
24408/02	a	2	3	4	1	3.5	
2448/02	a	2	3	3	0	3	
25101/02	a	2	4	4	0	4	
6572/02	a	2	3	3	0	3	
6891/02	a	2	3	3	0	3	
6891/02	a	2	2	2	0	2	
6999/02	a	2	4	4	0	4	2.88
23201/02	a	3	4	4	0	4	
25716/02	a	3	4	4	0	4	4.0
15305/02	1	2	4	5	1	4.5	
17743/02	1	2	4	5	1	4.5	
1359/03/01	1	3	4	5	1	4.5	
15226/02/02	1	3	4	5	1	4.5	
18300/01	1	3	4	5	1	4.5	
19255/02	1	3	4	5	1	4.5	
20479/02	1	3	4	5	1	4.5	
21142/02	1	3	4	5	1	4.5	
22062/02	1	3	4	4	0	4	
22062/02b	1	3	4	4	0	4	
24968/02	1	3	4	5	1	4.5	
25708/02	1	3	4	5	1	4.5	
6120/02	1	3	4	4	0	4	
22062/02	1	3	4	5	1	4.5	4.4
23205/02	2	3	5	5	0	5	
23421/02	2	3	5	5	0	5	
25814/02	2	3	4	5	1	4.5	
25958/02	2	3	5	5	0	5	
26372/02/b3	2	3	5	5	0	5	
65731/02/01	2	3	4	5	1	4.5	
7007/02	2	3	5	5	0	5	
7452/02	2	3	4	5	1	4.5	4.8
262/03	cis	cis	5	5	0	5	5.0

Table 5.2 Hep 27 protein expression. IHC scores from 2 investigators (NM=Nic Munro, PH=Pat Harnden) for a panel of 41 TCC.

Figure 5.4 IHC findings for validation of TCC panel for Hep 27. A box and whisker plot showing distribution of TCC staining (Q1=1st quartile, Q3=3rd quartile.) a) Staining of high-grade Ta is consistently higher than low grade as predicted by 2D PAGE comparing G3/ G1&2 Ta TCC (n=19) and b) when expanded to compare Hep 27 expression by stage (n=41) this trend is maintained.





5.3 Real-time quantitative PCR (RT-QPCR)

This study investigated the tumour proteome to identify novel markers that had not been identified by nucleic acid levels. Indeed it is known that RNA and protein expression often correlate poorly (Anderson and Seilhamer, 1997). However, we only found it possible to examine the Hep 27 protein expression due to a paucity of antibodies. Therefore we decided to examine the RNA expression rather than the protein expression of our putative biomarker proteins.

In this study RNA was initially converted to cDNA by a reverse transcriptase reaction. The principle of the RT-QPCR used here is that SYBR Green fluorescence is generated as the dye intercalates in ds DNA produced in the PCR reaction. This is measured and analysed in real time as the PCR reaction progresses hence quantifying the amount of DNA produced. DNA production continues in an exponential manner until the reaction components become exhausted and the reaction reaches a plateau. Measuring total DNA upon reaction completion (such as the ethidium bromide staining intensity on a PCR-product gel) is therefore a poor method of quantifying initial template levels. RT-QPCR measures DNA during the exponential phase of the reaction when DNA levels reflect template abundance (for a given cycle of the PCR). A level of DNA product abundance is chosen as the threshold and the cycle number at which this threshold is reached (Ct) is recorded for each sample.

5.3.1 Materials and methods

5.3.1.1 RNA extraction and cDNA synthesis from TCC biopsies.

A new panel of TCC tissue samples was assembled as there was no remaining tissue from the original samples used in 2D PAGE experiments. The samples were taken with cold cup biopsy forceps before loop resection to avoid diathermy artefact, transported in RPMI medium, embedded in OCT and stored at –80ºC (as described in Chapter 2.1.3). In all work with biopsy sectioning, RNA extraction, first strand synthesis and PCR amplification an RNAse-free technique was undertaken to avoid sample degradation. Gloves were changed frequently, RNAse-free plasticware, tubes and pipette tips were used, and experiments were

performed in a laminar flow cabinet (Astec), wearing gowns. All reagents were RNAse free and regularly changed.

Pooled urothelial cells from normal human ureters were used as a normal control. Ureters were taken from radical nephrectomy specimens and viewed by a pathologist (Dr. P Harnden) to confirm their benign nature. They were rapidly transported to the laboratory in Transport medium, where they were dissected free of connective tissue, detubularised (incised to form a rectangle) and cut into 5mm squares. The tissue was incubated overnight at 4ºC in Hank's medium (modified by excluding calcium and magnesium and containing 10mM Hepes solution, 1000KIU Trasylol (Bayer) and 0.1% w/v ETDA). The urothelium was then easily peeled away from the underlying stroma into small clumps of cells that were centrifuged at 800g for 4 min, resuspended into freezing medium (Hank's medium with 10% DMSO and 10% fetal calf serum) and stored in liquid nitrogen. When 4 samples had been collected they were defrosted, mixed and incubated in 100µl of extraction buffer (PicoPure™, Arcturus) then split into 2 aliquots and subjected to RNA extraction as for TCC samples (see below).

The TCC biopsies were sectioned in a cryostat (Leica). An initial 5µm section was stained with hematoxylin and examined; samples whose sections demonstrated less than 80% tumour cell content were deemed of insufficient purity and discarded. An additional 5µm section was taken for hematoxylin and eosin (H&E) staining and then 10x 20µm sections were cut and transferred into an RNAse free 0.5ml microcentrifuge tube containing 50µl of extraction buffer (PicoPure™, Arcturus). A final 5µl section was taken for H&E staining and the tumour cell content inspected again to ensure purity throughout the sectioned tissue. The TCC sections in extraction buffer were incubated for 40 min at 42ºC and then frozen at –80ºC.

RNA was extracted using the PicoPure™ RNA isolation kit, (Arcturus). The manufacturers' instructions were followed exactly. The optional step of treating the sample with RNAse-free DNAse (Qiagen) was included. The extracted RNA

was eluted in 18µl in extraction buffer and divided into 3 aliquots: 12.9 µl was immediately taken for first strand synthesis, 1 µl for use as an RNA control and the remainder (~5µl) as a reserve stock were frozen at –80ºC.

RNA measurement was undertaken with a RiboGreen® RNA Quantitation Kit (Invitrogen). The manufacturer's instructions were followed exactly. A high range assay was performed to detect RNA between 20ng/ml and 1µg/ml concentration. Zero, 40, 200, 1000 and 2000ng/ml concentrations of ribosomal RNA standards and the test samples were added in duplicate to a 96-well plate and incubated with the Ribogreen reagent. The sample fluorescence was measured with a FLUOstar Galaxy microplate reader (BMG Labtechnologies) (excitation 480nm, emission 520nm). A standard curve was constructed and the concentration of RNA in the test samples determined.

First strand cDNA synthesis was undertaken using SuperScript™ II reverse transcription reagents (Invitrogen). To an RNAse free 0.2ml PCR tube, 12.9µl RNA/ PicoPure elution buffer and 2.5 µl of random primers (diluted to 250 ng/µl, Invitrogen) were mixed and heated to 70ºC for 10 min then snap chilled on ice for 5 min. The remaining reaction components were added: 6µl 5x first strand buffer, 3µl 0.1M DTT, 0.6 µl dNTP 10mM, 2.0µl SuperScript™ II reverse transcriptase and 3µl H_2O to give a final reaction volume of 30µl. The contents were spun at 800g for 2 min and then incubated at 42ºC for 120 min, 70 ºC for 20 min and then cooled to 4ºC in a GeneAmp 9700 thermal cycler (Applied Biosystems). Half (15µl) of the product was diluted with 195 µl of H_2O and divided into 3x 70µl aliquots and stored, with the remainder, at -80ºC.

5.3.1.2 Real time RT- QPCR

Real time quantitative RT-QPCR (RT-PCR) was performed using an ABI Prism™ 7700 Sequence Detector (Applied Biosystems) in conjunction with SYBR® Green reporter dye and ROX dye as the passive reference. The threshold at which the PCR demonstrated steady exponential growth (Cycle threshold: Ct)

was determined by Sequence Detection Software 1.9 (Applied Biosystems) using an algorithm to identify an exponential increase in fluorescence. Reactions were performed within a 96-well optical reaction plate. In outline, the various TCC cDNA samples were applied in duplicate rows across the plate (8 rows, 4 samples) and amplified by each gene-specific primer-pair arranged in columns (12 rows, 11 genes and controls).

Primers for all test genes and 2 reference genes were designed with Primer Express ™ software (Applied Biosystems). Selected cDNA sequences were acquired using the Genbank database via Ensembl! (www.ensembl.org). The Primer Express parameters were set to identify primer-pairs that produced an amplicon of 100-200 base pairs in length with a melting temperature (Tm) 20-60ºC. The primers were designed to be 10-30 residues long with a Tm range of 58-60ºC, and between 30-80% GC. The amplicons generated from the primer pairs were designed to span one or more exons to preferentially amplify cDNA (derived from RNA) rather than residual genomic DNA contamination (additionally, this contamination should have be avoided by the use of DNAse in the tissue extract preparation). The amplicon sequences were tested using the BLAST algorithm (www.sanger.ac.uk/HGP/blast_server.shtml) to ensure they matched the target gene alone and not other sequences (intronic or extronic) or pseudogenes. Details of the primer sequences used are tabulated in Appendix I.

A PCR master-mix was made for each test gene and 22.5 µl applied to the 8 wells of the appropriate column. This mix consisted of 50% SYBR Green Master Mix (Applied Biosystems) and forward and reverse primers made up to 22.5 µl/well volume with H_2O. Subsequently, 2.5µl of TCC cDNA was loaded into each well, (2 rows for each sample) to give a reaction volume of 25 µl. A non-template control, and a RT-negative control were included for each TCC sample. The plate was covered with optical caps, briefly spun at 800g and placed in the ABI Prism 7700.

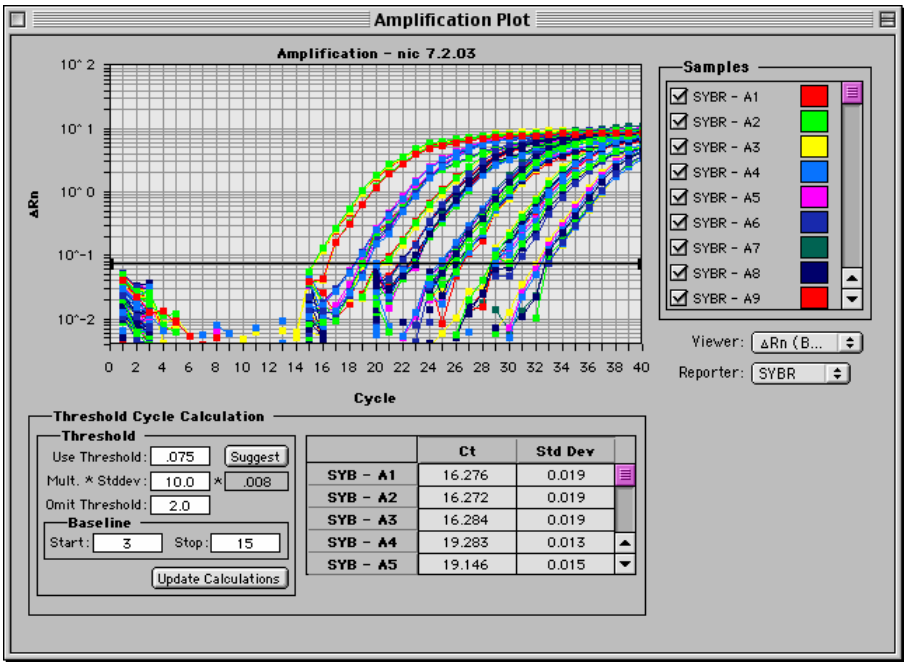
Analysis of the RT-QPCR was performed with Sequence Detection Software 1.9 (Applied Biosystems). The following PCR parameters were applied; initialisation at 50ºC for 2 min, incubation at 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 1 min, and finally 95ºC for 15 sec. After each run a melting curve analysis was performed for which the reaction temperature was raised from 60ºC (20 sec) to 95ºC (15 sec) at a rate of 0.029ºC/sec. The amplicons formed will melt at the temperature calculated using the Primer Express software. The temperature at which each component of the complete PCR melts is displayed. If the actual and expected melting temperatures match and only a single profile is obtained, this provides further evidence is provided that the PCR has amplified the intended sequence rather than non-specific products (Figure 5.5). Results files were exported into Excel (Microsoft) software for further analysis.

5.3.2 Results: RT-QPCR profiling of TCC

5.3.2.1 RNA retrieval from TCC tissue

The tissue panel of 41 TCC tissue samples used for the IHC analysis (Chapter 5.2) was to be subjected to RNA retrieval, cDNA synthesis and real-time quantitative PCR. We initially decided that as many of the muscle invasive TCC were less than 80% pure, TCC cells should be collected from the sample using laser capture micro-dissection (LCM). To aid this process a modified haematoxylin and eosin staining protocol was developed to visualise the tissue for LCM while minimising RNA degradation: all glassware was treated with 0.1% diethylpyrocarbonate (DEPC), DEPC water used to make up alcohol and eosin solutions, and a 1:50 dilution of Vanadyl-ribo-nucleoside complex (VRC) was added to Mayers's hamatoxylin. However after preliminary experimentation it became clear that it would be very difficult to capture enough tissue from the very small sections cut from the cold-cup biopsies.

A



B

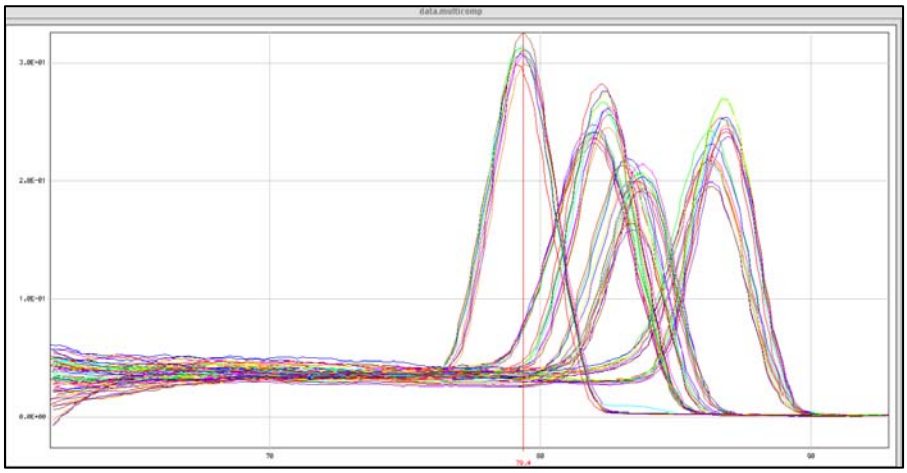


Figure 5.5 Screen view of a typical Quantitative RT-PCR experiment: assessing PCR efficiency for 6 primer-pairs; (A) Cycle number at which the recorded sample fluorescence (DNA quantity) is recorded (Ct ~10^1). Note the Ct values for replicates A1-A3 (aldolase) (16.27-16.28) are within a narrow range of error. (B) Melting curves for the same experiment confirm that 6 products were formed during the PCR reaction whose melting temperatures matched that calculated for the expected amplicons of the target genes.

The decision was made to abandon LCM to capture cells for RNA extraction and reassess the purity of biopsies. Approximately 1/3 of T2 and 1/5 of T1 TCC contained in excess of 20% stromal contamination and were excluded, so additional samples were collected to replace them. The resultant panel of 33 TCC is described in Table 5.3. It was also felt that although LCM promised greater TCC purity the process of staining and laser capture may have degraded the quality of RNA. All samples produced cDNA that was successfully amplified.

Normal human bladder urothelium was difficult to obtain so normal human ureteric tissue was collected as a substitute. Uncultured (P0) ureteric cells were harvested from stripped normal ureters, and these samples were mixed, divided into 2 groups and subjected to identical RNA extraction and cDNA synthesis.

5.3.2.2 TCC biopsy volume determination

Two representative TCC samples were selected (1 TaG2, 1 T1G3, with "average" cross sectional areas ~9mm^2). Sequential sections (20μm) were cut from both samples into separate tubes and RNA extracted with the Pico-pure Kit. The RNA amount for each tube was measured with the Ribo-green assay using a standard curve ranging from 0 to 2000ng/ml. The volume of extracted RNA was compared to the number of sections cut (Figure 5.6). It was calculated that 300ng of RNA would be in excess of the requirements for this study and that Pico pure RNA extraction of 10 x 20 μm sections would produce this amount for an average biopsy cross-sectional area (A volume of ~1800 μm^3). The cross-sectional area for each test biopsy was estimated before sectioning and the number of sections cut altered accordingly.

TCC stage	TCC grade	Unit id number	Sex	Mean %male	Age	Mean age	Date collected
Ta (n=12)	**2**	339	M	58%	74	76	Oct-01
		358	F		74		Mar-02
		359	F		76		Mar-02
		366	F		88		Apr-02
		395	M		73		Jul-02
		406	F		84		Aug-02
		451	M		89		Oct-02
		494	F		68		Nov-02
		518	M		66		Dec-02
		529	M		74		Dec-02
		536	M		79		Dec02
		540	M		64		Dec02
Ta (n=5)	**3**	467	M	80%	72	76	Oct-02
		482	F		81		Nov-02
		489	M		72		Nov-02
		490	M		71		Nov-02
		511	M		81		Nov-02
T1 (n=4)	**2**	385	F	75%	75	69	Jul-02
		457	M		67		Oct-02
		468	M		53		Oct-02
		554	M		79		Jan-03
T1 (n=4)	**3**	362	M	75%	81	70	Apr-02
		378	M		70		Jun-02
		418	M		74		Sep-02
		461	F		54		Oct-02
T2 (n=8)	**3**	137	M	25%	82	79	Dec-00
		169	F		90		Jan-01
		291	F		76		Sep-01
		329	M		67		Nov-01
		355	F		82		Mar-02
		417	F		89		Sep-02
		438	F		73		Sep-02
		498	F		71		Nov-02

Table 5.3 Details of TCC subjects for RT-QPCR analysis (n=33)

5.3.2.3 Optimising RT-QPCR

Primers were designed to all the putative protein markers except pyruvate kinase (detailed in Appendix I). Several primer pairs formed amplicons that were identical to areas in the genome that were not the location of the target gene when subjected to a BLAST search. It was found that aldolase and hnRNP K each have several pseudogenes. The sequence of these pseudogenes was aligned with the target genes and primer sequences chosen that were specific to the target genes alone when checked with a further BLAST search. This restricted the primer design to oligonucleotide pairs with sub-optimal characteristics for PCR, such as varying primer length, GC content and melting temperature between members of a pair. Hence 2 pairs were designed for aldolase and hnRNP K. All primer pairs amplified DNA successfully by RT-QPCR.

RT-QPCR was optimised with Universal-RNA (Stratagene) template, which comprised RNA pooled from 10 tumour cell lines. The stock was defrosted and 20µg in 200µl precipitated with ethanol and dried. The RNA was immediately resuspended in 20µl of water and 1µl added to a 20µl reverse transcriptase first strand synthesis reaction. The cDNA product was diluted 1:10 to 0.1mg/ml for use as template for optimisation PCR. The results of these experiments determined that each primer pair was diluted 1:10 from a stock concentration of 100pmol/µl and 1.5 or 4.0 µl added to the PCR reaction (total volume 25µl) to give primer concentrations of 0.6-1.6 pmol/µl in the reaction mix. For each primer combination (8) the reaction was performed in triplicate with and without template on the ABI Prism™ 7700 Sequence Detector. The lowest concentration of primer compatible with reproducible good amplification plots and producing a single product of the expected melting temperature (from the melting curve analysis see Figure 5.5) was selected for use with the TCC templates. All target and control gene pairs amplified cDNA successfully using these parameters.

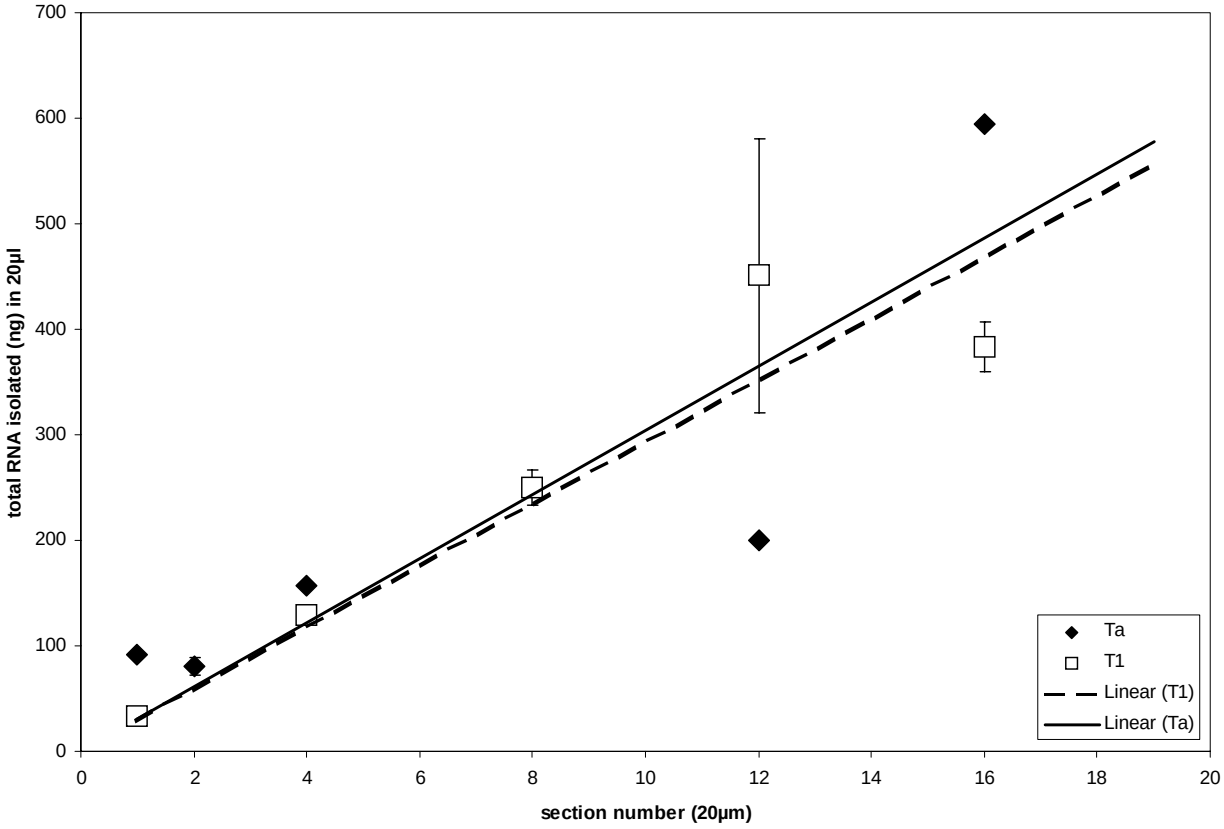


Figure 5.6 RNA isolated from Ta (black diamonds) and T1 (white square) TCC samples according to number of sections cut. Standard deviation error bars represent range of duplicates and mean, where there is no bar error was too small to plot. Linear regression curves were calculated for each sample (Ta solid line: R2= 0.75 T1 dashed line: R2=0.85). For ~300ng of RNA, an average of ~10x 20µm sections were required.

5.3.2.4 Assessment of RT-QPCR reaction efficiency

RT-QPCR measures the amount of DNA produced during each cycle of amplification. It is possible to include external DNA standards of known absolute amount and compare the test sample with this standard curve. However the most widely used and robust method is to relate expression of the target gene to one or more control gene(s) within the same sample- an internal standard.

Control genes are selected on the basis of constant expression in tumour and normal tissue. Thus the expression of the target gene may be expressed relative to this constant expression level. This allows comparison between different tissues where overall RNA extraction may vary. Selection of such genes is complex (see Chapter 5 Discussion). In this case, as proposed by other groups, we used 2 control genes in an attempt to improve the accuracy and assess any limitations of the technique: beta-2-microglobulin (B2M), a highly expressed gene and hypoxanthine phosphoribosyltransferase (HPRT), a moderately expressed gene (Rey et al., 2000; Vandesompele et al., 2002).

To allow direct comparison of test and control PCR reactions, the efficiency of each reaction must be known. Every optimised primer pair was used to amplify universal RNA template in triplicate for serial dilutions between 1-1000 fold. The cycle number at which the product accumulation reached the threshold level (Ct) was recorded. These values were entered into a PCR efficiency calculator Excel Macro (kindly supplied by Dr. J Louhelainen) that calculated the correlation of data onto a linear regression line and the absolute slope of that line. For a direct comparison between two PCR reactions, each PCR should amplify template in proportion to its abundance over the range of the experiment (1-1000 fold). Thus a plot of template concentration against ΔCt would produce a line of slope 0 with a high R^2 value. Based upon the manufacturer's recommendation, tolerance in efficiency variation was defined as a slope less than +/- 0.1. The majority of the reactions satisfied this condition excepting Hep 27 that became more efficient with reducing template concentration and hnRNP K (1) that became less efficient

with reducing template concentration, in comparison with both control genes. Such analysis is shown in Figure 5.7 a-d.

Each tumour cDNA (n=33) was amplified in duplicate on two separate occasions. The Ct value for each gene amplified and both control genes were recorded (Ct range 0-40) and exported into an Excel spreadsheet for analysis.

For samples with linear and comparable PCR efficiencies the Ct values generated for each PCR were directly converted into a value proportional to the absolute amount of RNA present. The ratio of this value to the control genes was calculated for each of the four samples and the mean ratio recorded. As expected there was some variation in the absolute values for gene expression between runs (minimal between wells on the same run) but this difference was generally reflected in both the control and test genes so the ratio of all 4 sample-runs was very consistent, with a small error around the mean value recorded.

For the Hep 27 assay that showed a non-linear PCR efficiency, a standard curve was used to convert the raw Ct data to comparable values. A first order natural log regression curve generated slope and intercept functions from the dilution series data for each gene using an Excel Macro (kindly supplied by Dr. J Louhelainen). These corrected values were used to generate comparable copy-equivalence numbers and subsequently expression ratios as described above.

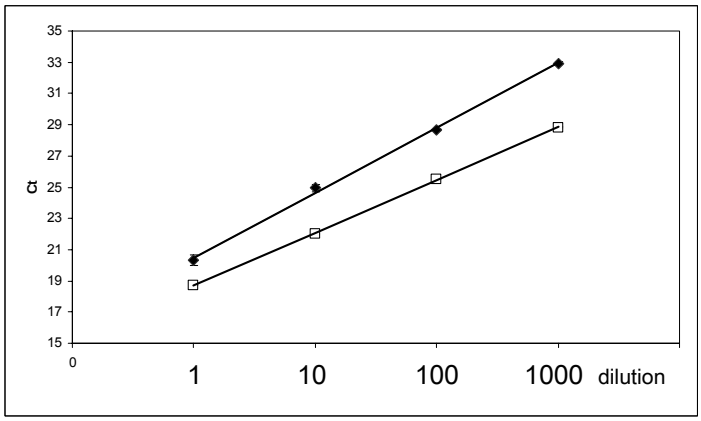


Figure 5.7a Increasing Ct for serial dilutions of universal cDNA template for B2M (white squares) and Hep 27 (black diamonds). Hep 27 loses PCR efficiency c.f. B2M with increasing dilution.

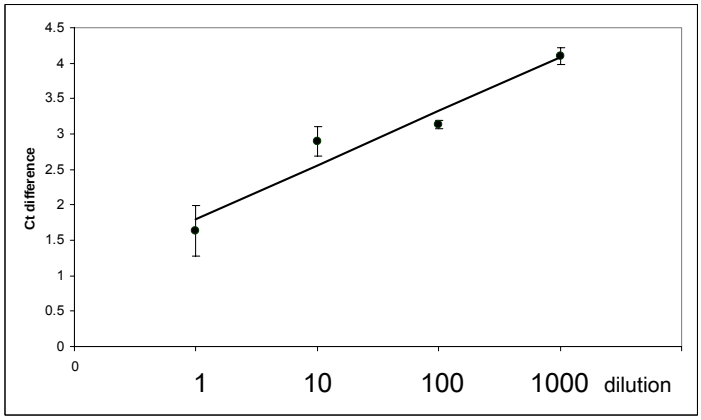


Figure 5.7b As shown in Figure 5.7a, ΔCt between B2M and Hep 27 increases with template dilution. The slope of this regression curve is 0.32ln(x) that exceeds the limit of 0.1ln(x) for direct comparison between PCR

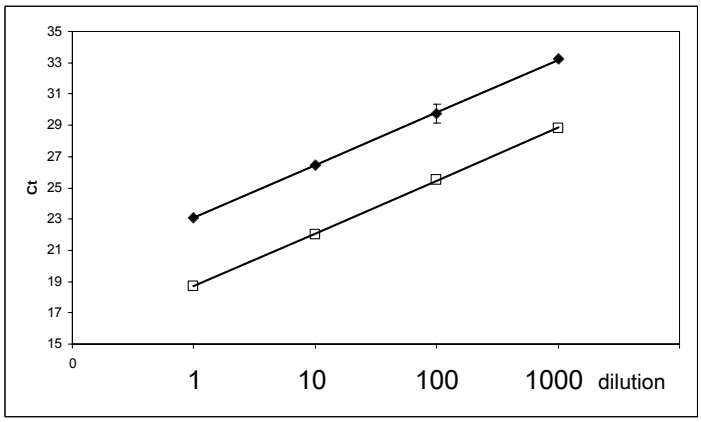


Figure 5.7c Increasing Ct for serial dilutions of universal cDNA template for B2M (white squares) and HPRT (black diamonds). Both PCR are of similar efficiency over a 1000-fold template-abundance range.

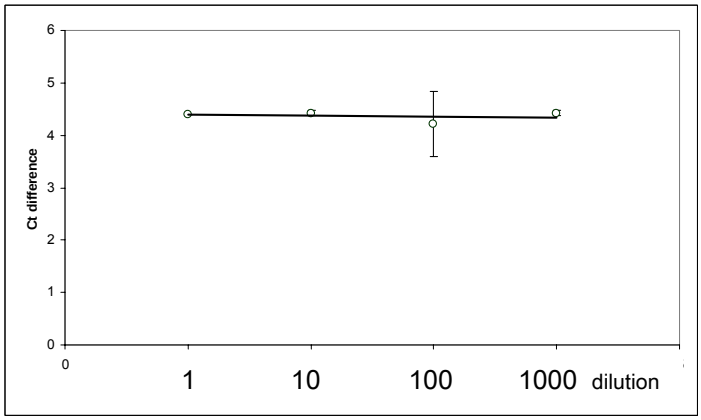


Figure 5.7d As shown in Figure 5.7c, ΔCt between B2M and HPRT remains constant. The slope of this regression curve is 0.008ln(x) that is within the limit of 0.1ln(x) for direct comparison between PCR

5.3.2.5 RNA expression of control genes (B2M and HPRT) in TCC and normal tissues

We compared the ratio of expression between the 2 selected control genes to see if our method of relative expression analysis was valid. If both control genes were expressed in a constant ratio throughout the test group it would suggest that these genes were expressed at a constant absolute level and could therefore be used as internal standards. It is possible of course that both independent genes were expressed at varying levels that happened to be identical in each sample, but this is less likely. In contrast, in analyses using a single reference/test gene ratio no such information is obtained. It can only be assumed that any change in ratio is due to the alteration of the test gene, rather than the control gene. We discovered that there is some variation in the individual sample ratios between the control genes, but minimal variation between median ratios for each group of samples stratified by grade and stage. In other words, it would appear that there was no bias between comparisons of gene expression according to disease type (Figure 5.8).

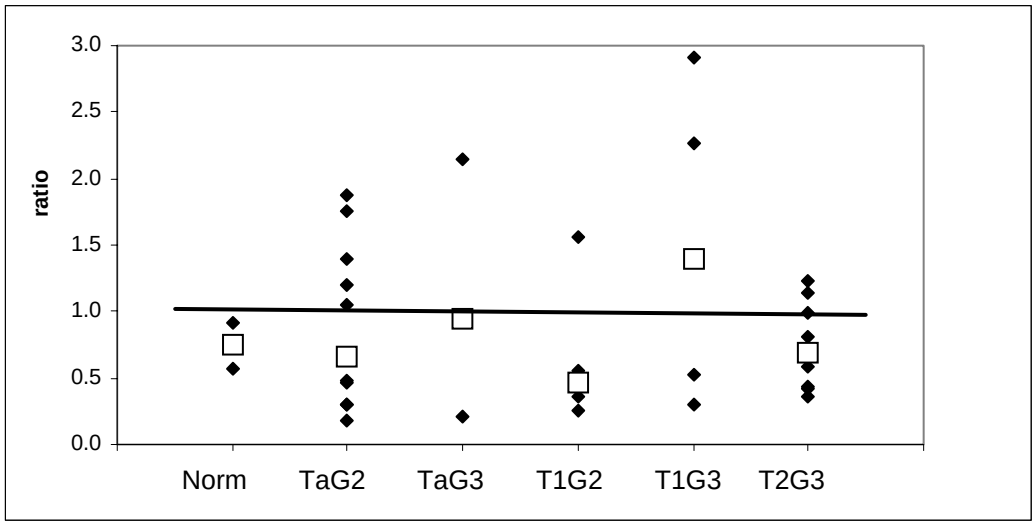


Figure 5.8 Ratios of expression of B2M and HPRT for 33 TCC and two pooled normal urothelium samples. Individual HPRT/B2M ratios (black diamonds) vary considerably, but group medians (white squares) are less variable. A linear regression line of individual ratios shows no trend to bias between sample groups (slope=0.0076).

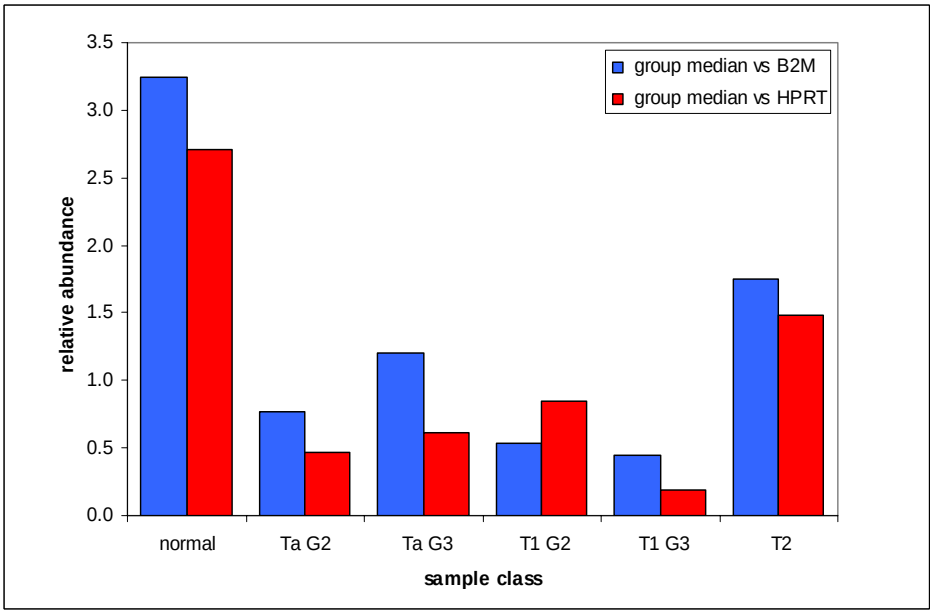
5.3.2.6 RNA expression of coronin, hnRNP K, aldolase, Hep27, and FDH in normal urothelium and TCC.

Expression ratios for 33 TCC and pooled normal urothelium were generated for the genes of interest. Some genes expressed similar RNA and protein expression patterns for Ta TCC and some did not. These findings are shown as Figure 5.9 a-e. We have chosen to show each gene's relative abundance for the median of each tumour class for both reference genes as Figures 5.9 a-e (i), and also as a box and whisker plot, Figures 5.9 a-e (ii), for a single reference gene (B2M). If plotted against the alternative reference gene, HPRT, there are small differences in the relative abundances but there is no alteration to the trends demonstrated. The RNA expression of each test gene relative to the control genes was higher in the pooled ureteric cells than any tumour sample (Chapter 5 Discussion).

2D PAGE of coronin demonstrated reduced protein expression in high vs. low-grade TCC. The opposite was seen with the RT-QPCR experiment, the median relative abundance of TaG3, T1 and T2 TCC samples was approximately 4 times that of the median of the TaG1/2 samples (Figure 5.9a). HnRNP K levels were predicted to be lowered in high-grade Ta TCC, although again this was not seen in RNA expression by RT-QPCR, which showed increased relative RNA abundance. More advanced tumours (T2+) did show a reduced level in comparison with Ta TCC, although this was based upon only 8 samples, showing significant variation in expression (Figure 5.9b).

In contrast, high-grade Ta TCC expressed FDH at approximately 20% compared with the low-grade TCC, in agreement with the 2D PAGE results. This trend was continued with tumours of increasing grade and stage (Figure 5.9c). Aldolase RNA expression was also increased in G3Ta TCC and T1 TCC compared with G1/2Ta TCC as predicted by the 2D PAGE experiments although this trend was not seen in T2TCC (Figure 5.9d). Hep 27 RNA expression did increase in G3Ta and more advanced tumours as predicted by 2D PAGE (Figure 5.9e).

i)



ii)

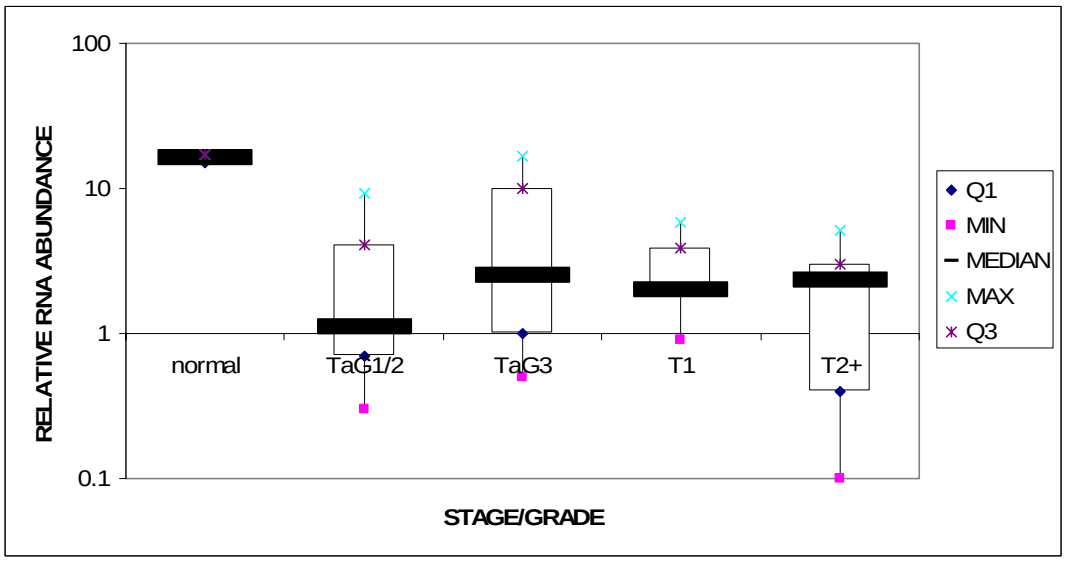
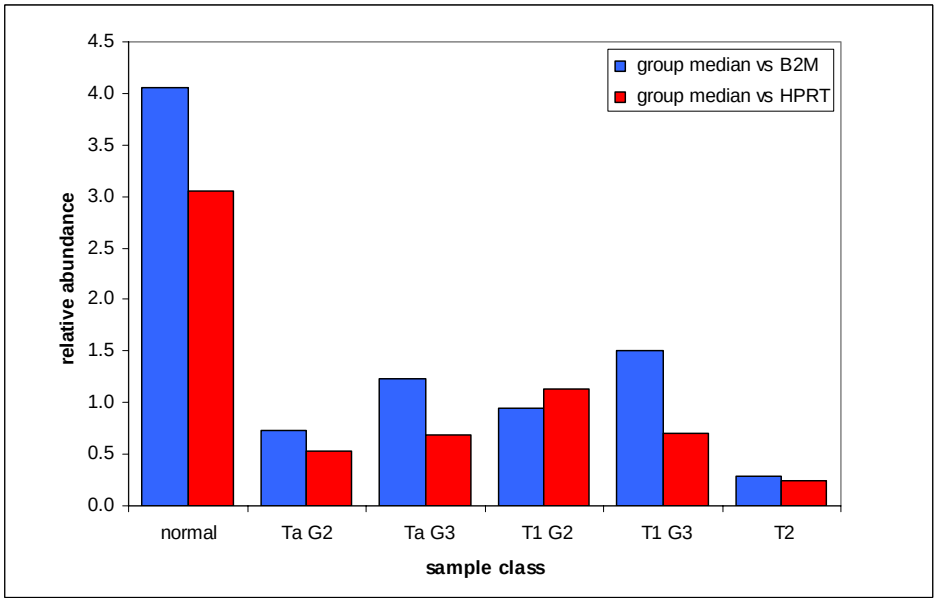


Figure 5.9a Coronin mRNA expression in TCC. (i) Median coronin expression by TCC types against B2M and HPRT reference genes. **(ii)** Box & Whisker plot of TCC coronin/B2M expression (relative abundance of 1= mean TaG2 coronin/B2M expression).

i)



ii)

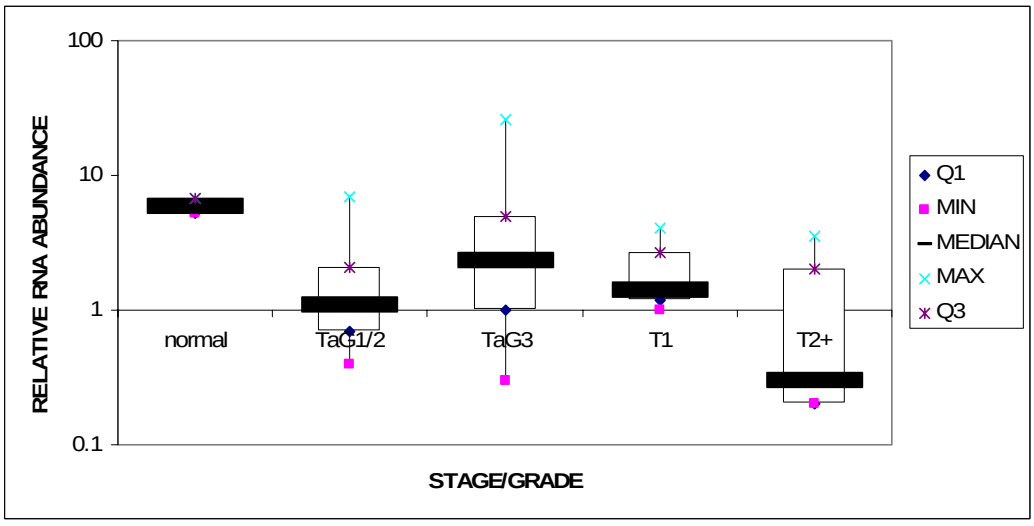
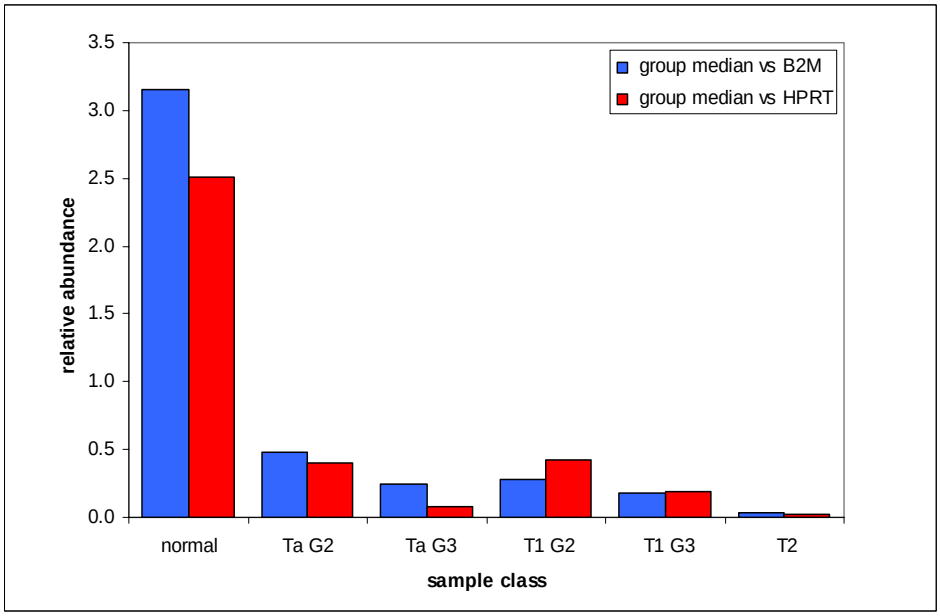


Figure 5.9b HnRNP K mRNA expression in TCC. (i) Median hnRNP K expression by TCC types against B2M and HPRT reference genes. **(ii)** Box & Whisker plot of TCC hnRNP K/B2M expression (relative abundance of 1= mean TaG2 hnRNP K/B2M expression).

i)



ii)

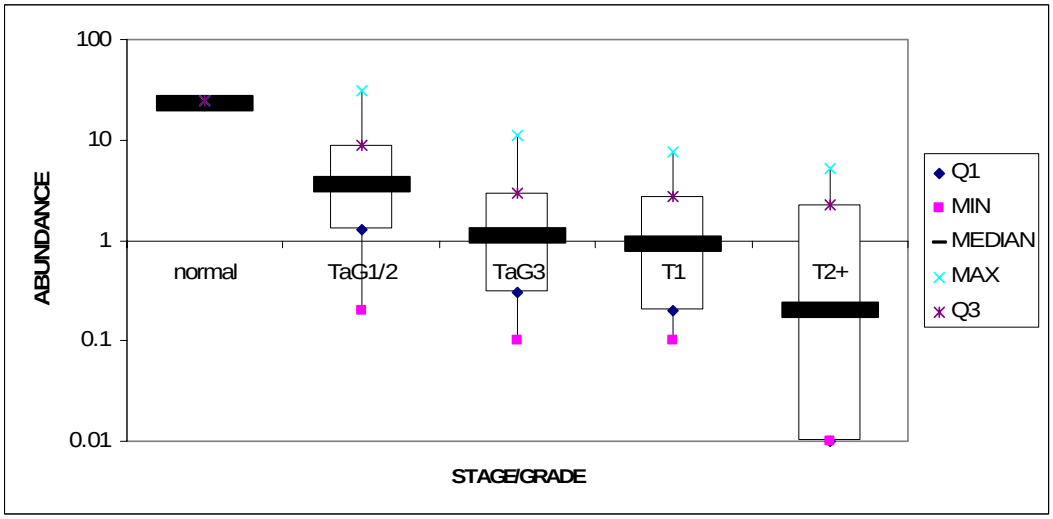
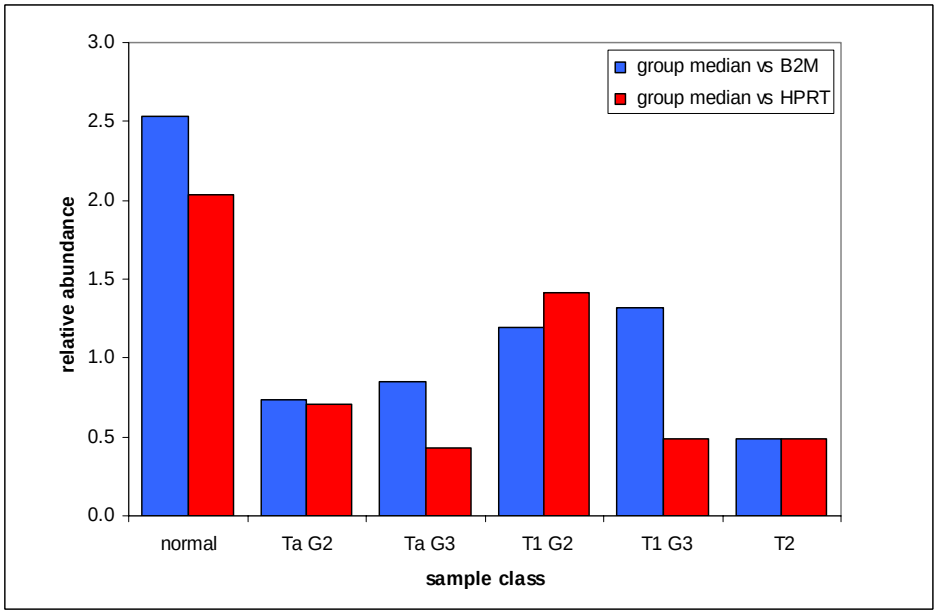


Figure 5.9c FDH mRNA expression in TCC. (i) Median FDH expression by TCC types against B2M and HPRT reference genes. **(ii)** Box and whisker plot of TCC FDH/B2M expression (relative abundance of 1= mean TaG3 FDH/B2M expression).

i)



ii)

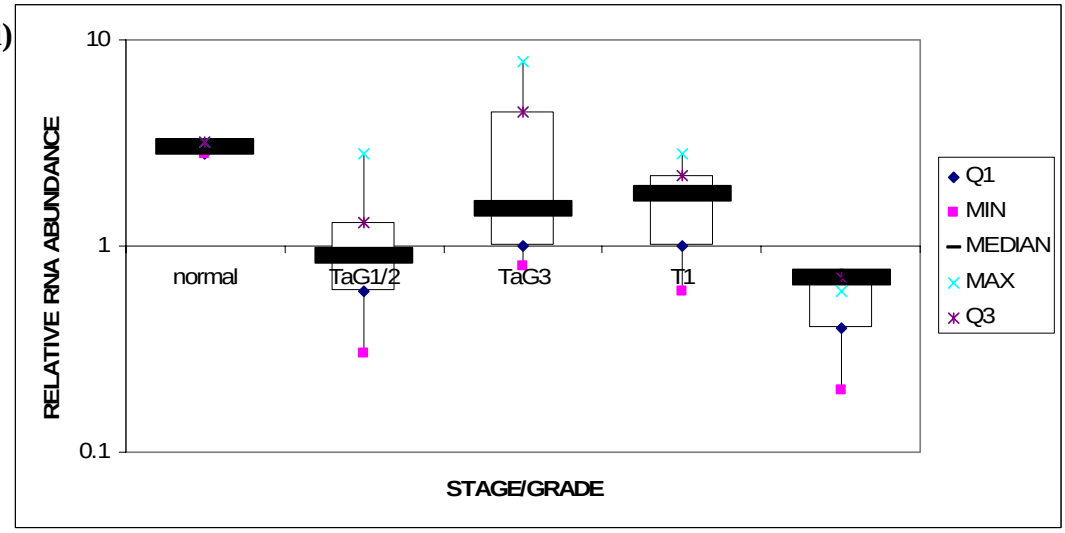
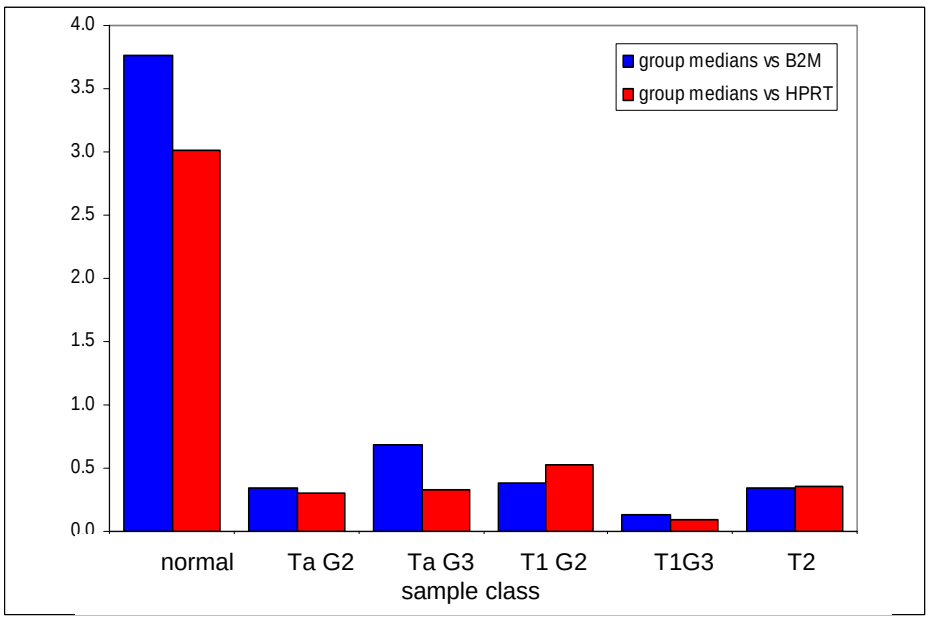


Figure 5.9d Aldolase mRNA expression in TCC. Primer 1 (similar to primer 2) (i) Median aldolase expression by TCC types against B2M and HPRT reference genes. **(ii)** Box & Whisker plot of TCC aldolase/B2M expression (relative abundance of 1= mean TaG2 aldolase/B2M expression).

i)



ii)

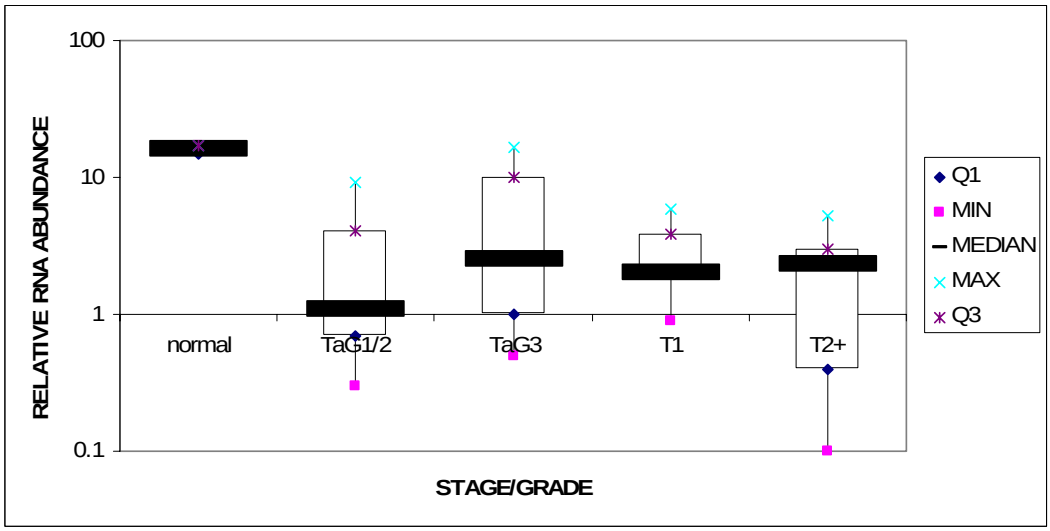


Figure 5.9e Hep 27 mRNA expression in TCC. (i) Median Hep 27 expression by TCC types against B2M and HPRT reference genes. **(ii)** Box & Whisker plot of TCC Hep 27/B2M expression (relative abundance of 1= mean TaG2 Hep 27/B2M expression).

5.4 Discussion

The aim of validating the 2D PAGE findings by using IHC on the initial samples and an additional panel of tumours was frustrated by a lack of functioning antibodies and limitations in the original tissue supplies. However despite these limitations substantial sized panels of TCC were assembled to test for Hep 27 protein expression (n=41) and RNA expression on all of the putative markers (n=33). We have not yet subjected the original tumours chosen for 2D PAGE to IHC to validate the protein expression. This experiment is awaiting supplies of antibody and will be completed in the near future.

In the tissue chosen to represent "normal urothelium": stripped normal pooled ureteric-urothelial cells, the test RNA was always more abundant (relative to the control genes) than in TCC samples. While this may be expected for some putative biomarker proteins such as FDH, coronin and hnRNP K, which showed reduced levels in high grade tumours, it was seen for all biomarkers including those which are presumed to be upregulated in advanced TCC. For example, aldolase protein has been shown to upregulated in renal and hepatic cancerous tissues compared to benign tissue (Castaldo et al., 2000; Unwin et al., 2003a) and it would it seem unlikely, though formally possible, that urothelium is unique in expressing much higher aldolase RNA in benign, compared to cancerous, tissue. The cells used in this study have not been cultured and so are not subject to stimulation by growth factors within culture media that are known to affect urothelial culture models (Southgate et al., 1994). However, the effect of stripping cells from the underlying stroma is unknown. Cell stripping may induce myriad cellular responses (eg. upregulate the glycolytic pathway) although the cells are kept cold throughout. An alternative explanation is that the expression of the control genes β2M and HPRT was affected by the collection of the primary urothelial cells, but the mean absolute amount of β2M and HPRT RNA recorded did not vary between the normal and TCC samples (data not shown).

The embryological origin of the bladder is from the chloaca, while the ureter derives from the ureteric bud, arising from the Wolffian duct (Baker and Gomez, 1998). It is known that TCC developing in the ureter and bladder show different molecular characteristics. For example, when Catto *et al* investigated the pattern of microsatellite alterations in upper and lower tract TCC, striking differences were seen between the microsatellite instability from analysis of 89 bladder and 71 upper-tract tumours (Catto et al., 2003). So does the urothelium of the normal ureter differ from that of the bladder? If so, to what extent, and does it prevent the use of ureteric tissue as a control for experiments investigating bladder pathology? The literature seems unable to answer these questions at present. So in conclusion, it was possible that the RNA levels recorded in the cells were accurate and all the test genes were more intensely expressed in the benign tissue. However, it is quite possible that these stripped, ureteric cells represent a poor model of normal urothelium *in vivo* for this RT-QPCR study.

To overcome these issues, the next experiment would be to collect cold-cup biopsy specimens of normal adult bladder mucosa and subject them to a process of DNA extraction and RT-QPCR exactly similar to the tumour biopsies. However, there are practical and ethical issues of obtaining such biopsies. Sources of “spare” normal bladder urothelium have previously been collected from patients undergoing reconstructive bladder surgery, typically clam enterocystoplasties for those with severe symptoms of an overactive bladder. However, it has been shown that these bladders are not truly “normal”. Although the development of an overactive bladder principally involves the detrusor muscle, molecular, histological or biochemical changes can be identified in all tissues of the overactive bladder (Brading, 1997). A possible source of normal adult urothelium may be collected from the reconstruction of the bladder neck during radical prostatectomy. Clearly such tissue collection would require ethical approval and patient consent. Some patients undergoing radical prostatectomy will display bladder outflow obstruction and may develop secondary bladder overactivity. It is equally possible that the bladder mucosa in these patients will be abnormal. Any such tissue collection should include screening of potential donors to eliminate such problems. The level of evidence required to determine

“normality” is debatable. While urodynamics may appear the most robust method of determining bladder function, a history and flow rate may prove a less involved and equally reliable tool.

The increased expression of Hep 27 in high grade Ta TCC seen in the 2D PAGE was confirmed in the 19 additional Ta tumours in the validation panel. This trend was also seen in 22 TCC of increasing stage. Hep 27 RNA expression in high grade Ta was higher than low grade TCC, and higher RNA expression was seen in T1 and T2+ TCC, albeit at a lower level than in Ta TCC. The survival and progression information for patients with TCC used in this study is immature and so we cannot directly relate Hep 27 protein expression to prognosis. However the clear relationship with stage and grade, chosen as surrogate markers, suggests that Hep 27 protein expression may relate to prognosis and survival.

The limited research undertaken to explore the function of Hep 27 suggests that it may play a role in maintaining the quiescence status of the cell (Donadel et al., 1991; Gabrielli et al., 1995). This would seem counter intuitive to the results obtained here; increased Hep 27 expression is seen in more advanced TCC; which is presumed to be associated with uncontrolled cell growth. Various human tissues subjected to Western blotting for Hep 27 show that while Hep 27 is expressed in normal human liver, placenta, ovary, testis and parotid tissues, it appears absent in human endometrium, lung, colon, brain, renal and adrenal tissue, as discussed in Chapter 4 (Pellegrini et al., 2002). These Western blots show 2 bands that correspond to different isoforms of Hep 27. In the liver and testis the 30kDa isoform predominates while in the human placenta, ovary and parotid, the 27kDa isoform is more abundant. Genetic analysis has identified an alternative upstream binding site and promotor in the Hep 27 gene to account for these isoforms (Pellegrini et al., 2002).

To explain our results, it may be proposed that the development of cancer is associated with a switching of Hep 27 isoforms, perhaps secondary to a mutation, and that the 2D PAGE and IHC experiments performed in this study only

recorded a cancer-specific isoform. However our 2D PAGE study independently identified both isoforms of Hep 27 at 27 and 30kDa that were both up-regulated in G3 vs. G1/2 Ta TCC (H2-4, Figure 4.4). In addition, the antibody used for IHC was identical to that used for the published Western blots (as it was a gift from Gabrielli's group) and has been demonstrated to bind to both isoforms.

So an understanding of the role of Hep 27 remains elusive. The independent identification of both isoforms up-regulated in 2D PAGE and clear IHC results is good evidence for its upregulation in more advanced bladder cancers. This molecule appears to have been little studied to date and further functional studies are required to elucidate its role. Other members of the SDR steroid dehydrogenases, have been shown to be intimately involved in cancer development, for example high expression of 17β hydroxysteroid dehydrogenase-1 is associated with estrogen metabolism and increased expresson is an indicator of adverse prognosis in breast cancer (Pasqualini and Chetrite, 2005).

We were unable to achieve specific binding for IHC with two FDH antibodies. In the published studies using one of these antibodies, IHC of tissue sections was not demonstrated although several successful immunoblots were shown (Krupenko and Oleinik, 2002; Krupenko, Vlasov, and Wagner, 2001; Krupenko and Wagner, 1999; Krupenko, Wagner, and Cook, 1997a; Krupenko, Wagner, and Cook, 1997b; Oleinik et al., 2005; Oleinik and Krupenko, 2003). The RT-QPCR experiment with FDH showed a clear trend: with increasing TCC stage FDH RNA expression was decreased. This is in agreement with the published IHC study (Krupenko and Oleinik, 2002). An alternative antibody of increased specificity (such as a monoclonal antibody) would allow further study of this molecule, both to allow IHC validation of the 2D PAGE, but also to undertake functional studies of FDH's role in TCC development.

The protein expression of coronin and hnRNP K was less in G3Ta compared with G1/G2Ta TCC in the 2D PAGE experiments. However, this was not mirrored by their RNA expression measured by RT-QPCR. Coronin was expressed in

increasing amounts in higher grade and stage TCC, although was expressed in the highest amounts in the normal urothelial samples. HnRNP K was also expressed in high levels in the normal tissues but was seen at its highest levels in TCC in the G3Ta group.

The expression of aldolase A RNA seems to be consistent with the 2D PAGE findings for Ta and T1 TCC. More-advanced TCC tend to have increased metabolic requirements and develop tissue hypoxia due to uncontrolled growth. This growth may induce glycolytic enzymes, previously described as the "Warburg Effect" (Denko et al., 2003). However, muscle invasive TCC appeared to express reduced levels of aldolase mRNA.

Muscle invasive tumours are usually solid lesions that have acquired significant genetic alterations compared with Ta TCC as described in Chapter 1. This study examined the relative expression of the test gene RNA in only 8 T2+ TCC. Conclusions about RNA expression in this group of TCC must be guarded due to the small number of samples analysed. Hep 27 RNA was seen at its highest abundance in T2+ TCC, in keeping with a steady trend to be up-regulated in TCC of increasing grade and stage. Coronin RNA expression was similar in T2+ TCC compared with other TCC groups while all the other proteins are all seen in relatively low levels in T2+ TCC. Such low levels might be expected in FDH and hnRNP K, but not aldolase. The explanation for this is unclear; the disrupted genome in advanced TCC may prevent some gene expression, although this seems unlikely.

Since 2000, RT-QPCR has been used to study several urological cancer types (reviewed by Schrader et al., 2003). In particular it has been used to quantify cytokeratin 20 expression in urine sediment (Inoue et al., 2001) and telomerase reverse transcriptase (hTERT) in TCC and benign tissues (De Kok et al., 2000) to diagnose bladder cancer. The RT-QPCR reaction is prone to technical problems and is characterised by significant variation and non-reproducibility, even with identical samples analysed in different laboratories (Bustin, 2000). However the

threshold cycle (Ct) variation reported in real time RT-QPCR is significantly less variable than standard PCR, with a co-efficient of variation of 14% for conventional, and 0.4-2.0% for real time assays (Zhang et al., 1997). In this study, the co-efficient of variation was typically 0.5% (data not shown) well within the expected parameters. The quality of the RNA template is arguably the most important determinant of the reproducibility and biological relevance of an RT-QPCR assay (Bustin and Nolan, 2004). RNA is prone to degradation, and this study was designed to handle the tissue as little as possible before extracting the RNA, (rather than subjecting the TCC to laser capture microdissection). Some RNA preparations contain inhibitors to the RT-PCR reaction (such as heme in mammalian blood) that must be excluded (Bustin and Nolan, 2004).

We chose to use SYBR green a non-specific DNA-binding dye. This has the advantage of simplicity and reduced cost compared with probe-based reporter assays. However, SYBR green will bind indiscriminately to any double-stranded DNA to give false positive readings and hence it is important to confirm that only the expected PCR product is produced. However, examination of the melting temperature of the amplicon: a melt curve analysis (Figure 5.5), gave reassurance that the product was specific to the selected primers. No indiscriminate binding was seen in this study. Measurement and analysis of the Ct is also open to error. The threshold is "chosen" by the experimental software based on an algorithm that measures background fluorescence. Where this background varies, for example between poorly-controlled comparison experiments, the chosen Ct differs preventing an accurate comparison (Bustin and Nolan, 2004).

Specific errors in the PCR of mRNA transcript are compounded by differing amounts of template between samples collected from different individuals. An accepted method is to measure the mRNA of an internal reference gene and to express the target gene expression level relative to that standard. These genes are often termed "housekeeping" or "control genes" and for this analysis to be meaningful the expression of these genes must not be influenced by molecular events associated with changes in the genes studied. Such invariability has been

called into question for many of the commonly used control genes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been used in numerous experiments to quantify cancer-associated genes, (Burger et al., 2003; Siraj et al., 2002). However there is increasing evidence that it's RNA expression varies with the development of breast cancer (Revillion et al., 2000) and melanoma cell models (Goidin et al., 2001) as well as with other cell-based disease models (Dheda et al., 2005; Wickert et al., 2002). Similar variation is seen with β-actin (Dheda et al., 2005; Goidin et al., 2001).

In response to these concerns, we chose to use two control genes, to assess if there was any difference in relative expression of the target genes between them. Beta-2-microglobulin (15q21) is a MHC class 1 receptor expressed at high levels in mammalian cells that has been used as the reference gene in numerous studies and hypoxanthine phosphoribosyltransferase (HPRT) (Xq26) a metabolic enzyme is expressed in lower levels. A published report suggested a benefit in using these two genes in combination (Rey et al., 2000) and others have recommended the use of multiple control genes in tumour profiling studies (Vandesompele et al., 2002). We hoped that the relative abundance of the target genes' RNA would not vary depending on the control gene used. In fact this was broadly true. Although there was some variation between individual tumours expression when referenced to either control gene, this was abrogated when group medians of TCC were compared, as seen in Figure 5.8.

The results of the RNA profiling for these genes demonstrate a variable correlation with the protein expression previously identified by 2D PAGE. It is unclear why FDH RNA/protein expression concurs, yet coronin and hnRNP RNA/protein expression conflict. It has been shown that there is variable concordance between RNA and protein abundance (Griffin et al., 2002), which is indeed one of the major reasons for exploring the global protein rather than mRNA levels in this study. One explanantion is that the variation in the stability of RNA and protein varies for each gene, possibly associated with post-translational modifications. Although most expression-profiling studies focus

upon transcriptional control, it is actually the mRNA steady-state levels that are measured, reflecting not only the production, but also stability of transcripts. It appears that mRNA levels are modulated via degradation via at least two exonucleolytic pathways and an endonucleolytic pathway. Additionally, the cell applies quality control mechanisms to destroy faulty transcripts (Mata, Marguerat, and Bahler, 2005). Transcripts encoding core metabolic proteins have longer half lives than those encoding transcription factors or members of the ribosome-biogenesis machinery (Yang et al., 2003). Furthermore RNA stability may be altered by subjecting the cell to stress inducing agents (Fan et al., 2002).

The level of validation achieved for the putative biomarker proteins identified by 2D PAGE varied for individual markers. The weakest evidence was generated by RNA study of hnRNP K, coronin and aldolase. It is important to recall that RNA and protein abundances are known to be independent and that real confirmation requires the development and use of robust antibodies to measure protein. FDH seems a very interesting molecule that may have a role as a marker in TCC (and other cancers). The RNA expression seems to strongly correlate to the 2D PAGE findings. However, only Hep 27 successfully underwent IHC to produce clear confirmation of the 2D PAGE findings.

6 Conclusions

Proteomics is a new approach to identify biomarkers and unravel the biology of tumours. It appears to offer additional, complementary information to genetic and cell-based approaches. It is perfectly suited to discover novel biomarkers that address the clinical problems of predicting TCC prognosis and identifying recurrence. This thesis describes two approaches, using established and innovatory proteomic platforms to identify such biomarkers. Particular attention has been paid to issues of methodological and analytical rigor, as these techniques are notorious for producing illusory results based upon error or confounding factors.

The novel markers discovered by 2D PAGE in this study require individual assessment, dependent on antibody production. FDH and Hep 27 seem most promising and have aroused significant interest and study amongst non-urological research groups. The fact that proteins, rather than gene or RNA change have been identified may enhance their chances of representing a real prognostic marker of benefit. We are continuing to collect outcome data on the original cohort of 12 Ta TCC to correlate their final outcomes with the Ta protein expression recorded, and we are considering prospectively assessing Hep 27 abundance by IHC and FDH by immunoblotting for new TCC collected at St James's. This further study will aim to discover if Hep 27 and FDH protein levels do add additional prognostic information as suggested by this work.

Molecular profiling of the expression of DNA, mRNA and protein of many human diseases has benefited from technological development, in particular the development of arrays. Complementary validation of mRNA levels of our putative prognostic markers using RNA arrays should be undertaken. Similarly we propose to subject a tissue array of many hundred TCC samples that is presently under construction in our laboratory to Hep 27 antibodies (and others as they become available, in particular FDH). This would give additional weight to this initial study based on a limited tumour panel.

Of all the putative prognostic markers, FDH has undergone the most study, principally by Dr Krupenko *et al.* It provides a model for further work that should be undertaken with the other prognostic markers. FDH has been expressed in a previously null cancer cell line and the effect on phenotype, including apoptosis examined. Subsequently FDH expression was undertaken in a cell line without functioning p53, where this effect was lost. Such studies could now be undertaken in bladder cancer cell lines.

SELDI-MS urine profiling appears to offer a non-invasive test for TCC superior to many current single-marker assays. However, at present its diagnostic accuracy does not attain the level required to offer as part of a surveillance program. However the rapidity of SELDI development, partly based upon the published experience in this unit, suggests that further advances are probable. The exact role for a proteomic assay is unclear: while cystoscopy approaches 100% sensitivity and is best suited to assess those at a high or medium risk of recurrence, a SELDI urine assay may be reserved for those with small, single low grade tumours after a period of disease-free cystoscopic surveillance. Of course further prospective trials are required, and the practical difficulties of maintaining the high quality control standards required may erode accuracy when applied more widely. However, the results so far were generated from a machine learning tool based upon a training set of 130. As additional samples are assimilated into an ever-larger training set, predictive accuracy is likely to improve.

SELDI studies have previously been limited by an inability to easily identify the proteins associated with critical features within a tumour profile. Technological advances detailed above now allow direct identification of such proteins by mass spectrometry. Such techniques are being introduced into our laboratory and will endeavour to identify the critical peaks selected by the Random Forrest classifier (Table 3.8). Development of protein chip proteomic platforms can only continue at an increased rate. It is critical that the attention to QC is continued to allow the identification of genuine biological changes rather than spurious effects based upon noisy or biased data. Our laboratory is continuing to develop new approaches to monitor and maintain QC in SELDI experiments, as well as undertaking new clinical projects in various diseases.

I would like to undertake a study of prognostic markers of TCC using the SELDI platform. The choice of tissue to profile is open to debate; serum and urine are relatively easy to collect and yet are less likely to contain critical markers reflecting TCC biology compared to the proteome of the tumour tissue itself. As discussed in the introduction, 2D PAGE and SELDI tend to capture different groups of proteins (based on their mass and chemical properties) so another examination of the proteome of Ta (as well as other TCC) should not duplicate this work. Of course, any putative protein biomarker identified in both studies would seem particularly worthy of further study. It is hoped that the methods developed in this thesis to generate sensitive, robust and reproducible protein profiles should contribute to the understanding and thus treatment of bladder cancer for the patient.

Appendix I. Primer sequences for RT-QPCR

Name	Strand	Primer sequence	Size(bp)	Primer GC (%)	Tm (ºC)	location	Predicted Amplicon length (bp)	Predicted Amplicon GC (%)	Predicted Amplicon Tm(ºC)	Exons spanned
Coronin	S	CTTCTGTGCTGTCAACCCTAAGTT	25	44.0	61.3	236	103	62	85	3-4
	A	CTTGTCCACACGTCCAGTCTTG	22	54.5	62.1	338				
Aldolase1	S	CCGTCACAGCGCTGCG	16	75.0	59.4	1765	136	64	86	12-13
	A	AGGGCCCAGGGCTTCA	16	68.8	56.9	1900				
Aldolase2	S	TGCCAATGTTCTGGCCC	17	58.8	55.2	1512	76	55	82	10-11
	A	GAGGATCTCAGGCTCCACG	19	63.2	61.0	1587				
HnRNPk 1	S	GCTGCTCTCATTCCACTGACAG	22	54.5	62.1	760	202	44	77	10-11
	A	ACTGGGCGTCCGCGA	15	73.3	56.0	961				

HnRNPk 2	S	GAATTACGCATTCTGCTTCAGAG	23	43.5	58.9	339	152	48	79	4-6
	A	ATATCAGCACTGATACTCAATATGCG	26	38.5	60.1	490				
FDH	S	TCCCCAAAGTCCTGGAGGTT	20	55.0	59.4	1127	107	56	82	8-9
	A	GCCATCACACAGCTCCTTCAC	21	57.1	61.8	1233				
Hep 27	S	AAGATCCTAAGTGTGAACGTGAAG	26	42.3	61.6	456	103	55	82	4-6
	A	CAATGGAAGAGACCAGGATGACA	23	47.8	60.6	559				
B2M	S	TGCTCGCGCTACTCTCTCTTT	21	52.4	59.8	38	147	47	79	1-2
	A	CAACTTCAATGTCGGATGGATG	22	45.5	58.4	184				
CDKAL1	S	ATGCTGAGGCTTGGCATGAC	20	55	58.0	964	76	43	77	10-11
	A	GGTGATTAAGGATTTTTGCCATTT	24	33	60.1	1039				
	S	GCCTGCAGCAAGGCC	15	73.3	56.0	1389				
HPRT	A	GACACTGGCAAAACAATGCA	20	45.0	55.3	475	101	49	79	6-7
	S	CTTCGTGGGGTCCTTTTCACC	21	57.1	61.8	575				

Appendix II. Patient information sheet:

A molecular genetic analysis of transitional cell carcinoma

Your doctor has decided that an operation on your bladder is needed. During this operation, tissue may be removed for diagnosis and treatment. We would like to invite you to take participate in a **RESEARCH** study of molecular changes found in bladder tumour tissues.

You are under no obligation to take part in the study and if you prefer not to do so this decision will be accepted without question and your treatment will not be affected in any way.

If you agree to take part, we ask you to consent to provide the following samples which will be used for research purposes:

1. A blood sample (10ml or 2 dessertspoonful). This may be taken while you are asleep at the time of surgery.

2. Urine samples. A sample will be collected at the time of surgery. On some occasions when you visit the clinic in the future you may be asked to provide a urine specimen.

We will carry out molecular tests on tumour tissue which is not required for diagnosis, on cells present in the urine samples and on the blood sample. Our aim is to identify common genetic alterations present in bladder tumours so that in the future new methods of diagnosis and treatment can be developed. This study will use samples from many patients treated in the Department of Urology and is intended to provide valuable information about molecular changes in a large group of patients. Our findings will not be disclosed to you at a later date and will not affect the treatment you receive.

Appendix III. Suppliers and manufacturers

Abcam Ltd.

332 Cambridge Science Park, Cambridge, CB4 0FW, UK.

www.abcam.com

Alpha Laboratories Ltd.

40 Parham Drive, Eastleigh, Hampshire, SO50 4NU, UK.

www.alphalabs.co.uk

Amersham Biosciences

Amersham Place, Little Chalfont, Bucks HP7 9NA, UK.
www.apbiotech.com

Anachem Ltd.

20 Charles Street, Luton, Bedfordshire, LU2 0EB, UK.
www.anachem.co.uk

Applied Biosystems

850 Lincoln Center drive, Foster City, CA 94404, USA

www.appledbiosystems.com

Arcturus Bioscience Inc.

400 Logue Avenue, Mountain View, CA 94043, UK.

www.arctur.com

Bayer

Bayer House, Strawberry Hill, Newbury RG14 1JA, UK.

www.bayer.co.uk

BDH Laboratory Supplies Ltd.

Broom Road, Poole, BH12 4NN, UK.
www.bdh.com

Beckman Coulter (UK) Ltd.

Oakley Court, Kingsmead Business Park, High Wycombe HP11 1JU, UK.

www.beckmancoulter.com

Becton Dickinson (UK) Ltd.

Between Towns Road, Cowley, Oxford, OX4 3LY, UK.

www.bd.com

Bio-Rad Laboratories (UK) Ltd.

Maylands Avenue, Hemel Hempstead, HP2 7TD, UK.

www.bio-rad.com

Biogenesis Ltd.

Technology Rd, Poole, BD17 7DA, UK.

www.biogenesis.co.uk

BMG Labtech (UK) Ltd.

PO Box 73, Aylesbury, HP20 2QJ, UK

www.bmglabtech.com

Ciphergen Biosystems Inc.

6611 Dumbarton Circle, Fremont, CA 94555, USA.

www.ciphergen.com

Clontech Laboratories Inc.

1020 East Meadow Circle, Palo Alto, CA 94303, USA.

www.clontech.com

Corning

Riverfront Plaza, Corning NY 14831, USA.

www.corning.com

Dako Cytomation Ltd.

Denmark House, Angel Drove, Ely, CB7 4ET, UK.
www.dakocytomation.co.uk

Decon Labs Ltd.

Conway St, Hove, BN3 3LY, UK.

www.decon.co.uk

Denville Scientific Inc.

3005 Hadley Rd. South Plainfield NJ 08840, USA

www.denvillescientific.com

Invitrogen Corporation

1600 Faraday Avenue, Carlsbad, CA 9200, USA
www.invitrogen.com

Leica Microsystems (UK) Ltd.

Davy Avenue, Knowlhill, Milton Keynes, MK5 8LB, UK.

www.leica-microsystems.com

Matritech Inc.

330 Nevada Street, Newton, MA, 02460, USA

www.matritech.com

Merck Biosciences (UK) Ltd.

Boulevard Industrial Park, Padge Road, Beeston, Nottingham, NG9 2JR, UK.

www.merckbiosciences.co.uk

Millipore (UK) Ltd.

Units 3&5 The Courtyards, Hatters Lane, Watford, WD18 8YH, UK.

www.millipore.com

Nalge (Europe) Ltd.

Unit 1a, Thorn Business Park, Hereford, HR2 6JT UK.
www.nalgenunc.com

National Diagnostics (Europe)Ltd.

Unit 4, Fleet Business Park Itlings Lane, Hull, HU13 9LX, UK.

www.nationaldiagnostics.com

New England Biolabs (UK) Limited

Knowl Piece, Wilbury Way, Hitchin, SG4 0TY, UK.

www.neb.com

Owl Separation Systems

55 Heritage Avenue, Portsmouth, NH 03801, USA.

www.owlsci.com

Pechiney Plastic Packaging

175 Western Avenue, Neenah, WI 54956, USA

www.parafilm.com

Perkin Elmer Applied Biosystems (UK) Ltd.

Chalfont Road, Seer Green, Beaconsfield HP9 2FX, UK.

www.lasperkinelmer.co.uk

Pierce Biotechnology Inc.

PO Box 117, Rockford, IL, 61105, USA.

www.piercenet.com

Promega Corporation

2800 Woods Hollow Road, Madison, WI 53711, USA

www.promega.com

Qiagen Ltd.

Boundary Court, Gatwick Road, Crawley, RH10 2AX, UK.

www.qiagen.com

R & D Systems (Europe) Ltd.

19 Barton Lane, Abingdon, OX14 3NB, UK.

www.mdsystems.com

Rainin Instrument, LLC

7500 Edgewater Drive, Oakland, CA, USA.

www.rainin.com

Roche Diagnostics Ltd

Bell Lane, Lewes, East Sussex, BN7 1LG, UK.

www.roche-diagnostics.com

Sanyo Biomedical Europe BV.

Monarch Way, Belton Park, Loughborough LE11 5XG, UK

www.sanyogallenkamp.com

Sigma-Aldrich Company Ltd.

Fancy Road, Poole, Dorset, BH12 4QH, England

www.sigmaaldrich.com

Thermo Electron Corperation (UK)

Unit 5, The Ringway Centre, Edison Rd, Basingstoke RG21 6YH, UK.

www.thermo.com

Valeant Pharmaceuticals International

Valeant plaza, 3300 Hyland Drive, Costa Mesa, CA 92626, USA

www.valeant.com

Vector Labs (UK) Ltd.

3, Accent Park, Bakewell Road, Peterborough, PE2 6XS, UK.

www.vectorlabs.com

Whatman Inc.

9 Bridewell Place, Clifton, NJ 07014, USA

www.whatman.com

Zeiss (UK) Ltd.

15-20 Woodfield Road, Welwyn Garden City, AL7 1LU, UK.

www.zeiss.co.uk

Appendix IV Associated publications, presentations and prizes.

Urinary biomarker profiling in transitional cell carcinoma

Nicholas P. Munro, David A. Cairns, Paul Clarke, Mark Rogers, Anthea J. Stanley, Jennifer H. Barrett, Patricia Harnden, Douglas Thompson, Ian Eardley, Rosamonde E. Banks and Margaret A. Knowles. International Journal of Cancer. *in press*

Surface Enhanced Laser Desorption/Ionisation Mass Spectrometry and Neural Network Modelling: A Proteomic Approach to the Identification of Urinary Biomarkers of Transitional Cell Carcinoma

NP Munro, M Rogers, R Craven, P Clarke, H Williams, P Harnden, I Eardley, J Noble, RE Banks, MA Knowles. American Association of Urology, poster Orlando 2002. *Awarded "Best Abstract- Bladder Cancer"*

A Proteomic Approach to the Identification of Urinary Biomarkers of Transitional Cell Carcinoma Using SELDI Mass Spectrometry and Neural Network-modelling

Nicholas Munro, Mark Rogers, Rachel Craven, Paul Clarke, Hywel Williams, Patricia Harnden, Ian Eardley, Jason Noble, Rosamonde Banks, Margaret Knowles. American Association of Cancer Research, Paper, San Francisco 2002

Proteomics-Based Approaches to the Identification of Bladder Tumour Markers

NP Munro, P Harnden, I Eardley, RE Banks, MA Knowles. British Association of Urological Surgeons, Poster, Manchester 2003

British Urological Foundation/ Glaxo SmithKline Scholarship 2002/3

Royal College of Surgeons of England Simpson Research Fellowship 2002/3

Bard Silver Medal, British Association of Urological Surgeons 2005

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