Biomarkers, July–August 2005; 10(4): 258–294

Biochemical and molecular markers in renal cell carcinoma: an update and future prospects

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Abstract

Cancer is a big problem in the developed world as well as in developing countries. Renal cell carcinoma (RCC) accounts for approximately 3% of adult malignancies and 90–95% of neoplasms arising from the kidney. RCC is more common in men than in women (2:1), and it most often occurs in patients between the ages of 50–70 years. In all cancers the cancerous cells release particular kind of proteins (called tumour markers) and blood tests are used to detect the presence of these markers. These tumour markers nowadays are an area of interest for oncologists who search for a possible solution in the detection and treatment of RCC. Different kinds of biochemical and molecular markers such as ferritin, MN/CA9, apoptotic index, p53, IL-2, gamma-enolase, CD44, CD95, chromosome instability and loss of heterozygosity have been tested in RCC, but so far no marker fulfils one or the other criteria to be considered as an ideal marker for RCC. This review gives basic and updated information about the different kinds of biomarkers studied in RCC and about the role implementation of genomics and proteomics in RCC.

Keywords: *Renal cell carcinoma, ferritin, prognostic marker, MN/CA9, kidney, prognostic value, p75, iNOS, reverse transcription polymerase chain reaction (RT-PCR), microsatellite markers*

(Received 2 February 2005; accepted 10 June 2005)

Introduction

The most common malignancy affecting the kidney is renal cell carcinoma (RCC), which is also known as hypernephroma or clear cell carcinoma. RCC includes several distinct entities with a range of biological and clinical behaviours from relatively

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ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500500218534

indolent to extremely aggressive. Although conventional prognostic factors such as stage and grade are quite useful, other clinical, laboratory and pathological findings are believed to have additional predictive value. In broad terms, the stages of RCC are as follows:

- Stage I: Patients have a small (<7 cm) tumour limited to one kidney. They usually do not have any evidence of lymph node involvement or metastatic disease.
- Stage II: Patients have a larger (>7 cm) tumour limited to the kidney. There is also no evidence of lymph node involvement or metastatic disease.
- Stage III: The tumour has invaded the adrenal gland (which sits atop each kidney), tissues surrounding the kidney or major nearby veins, such as the vena cava. Also includes patients with enlarged abdominal lymph nodes.
- Stage IV: Patients have large tumours that extend into surrounding tissues and/or metastasis to other distant locations.

Staging information is useful in determining treatment and prognosis. In general, patients with earlier stage disease have the most favourable prognosis or outcome. Potentially curative surgical treatment–surgery that attempts to cure the cancer is principally offered to the patients with early RCC (stages I–III disease). If cancer has not yet spread beyond the kidney, local surgery offers a reasonable likelihood for a cure, but several other types of surgery may also be considered.

RCC has four stages, with subclassifications according to the tumour location, lymph node involvement and the presence of metastases. Treatment for these stages (I–III) involves full or partial removal of an affected kidney. The prognosis for stage IV RCC is poor and treatment generally consists of palliative therapies, such as tumour embolization or external-beam irradiation. In select cases a radical nephrectomy can be performed. For patients with stages I–III with progressing, recurring or relapsing disease, management depends on prior treatment and the site of recurrence, as well as individual patient considerations. In most cases management follows the regimens described for stage IV disease (Gattinoni et al. 2003).

The most common subtypes of RCC are clear cell (70–80%), papillary (10%) and chromophobe (5%), with a variety of other types making up the rest (Pantuck et al. 2001, Motzer et al. 1996). In renal cell cancer there is a nearly complete expression of the multidrug-resistance gene MDR-1 and of its protein product P-glycoprotein. This makes RCC nearly impervious to chemotherapy. Molecular markers associated with each subtype of RCC continue to be elucidated, but at this point their usefulness as prognostic information or treatment guides is not yet determined (McCue & Gorstein 2001).

The distribution of different stages of tumour progression and percentage survival rate is shown in Figure 1, which is based on the method of Robson et al. (1969). This is an approximate 5-year survival rate for different stages of RCC.

Progress in the management of RCC is very slow because the disease tends to be present in several stages and it lacks a reliable tumour marker to detect this disease at an early stage. The tumour progression, number of patients and their percentage, according to the respective stage of tumour, are shown in Figure 2 (Kashyap et al. 2003).

RCC is known to release several hormones and biologically active substances that induce syndromes and produce metabolic damage in the host. The detection of

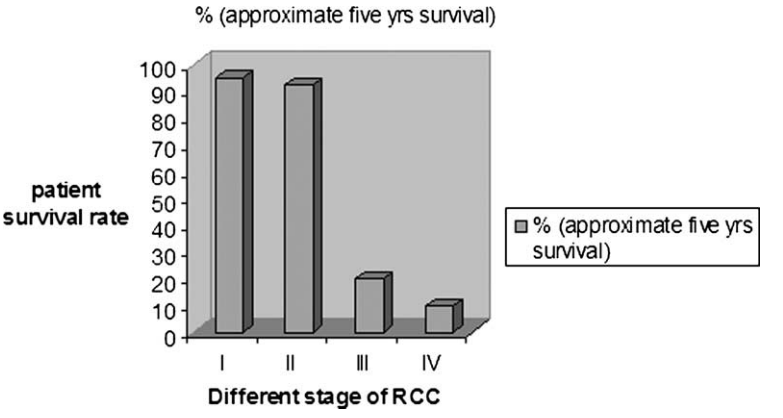

Figure 1. Percentage survival in different tumour stages.

elevated levels of these substances may be useful in the diagnosis and follow up of this malignancy (Kirkali et al. 1999).

RCC is a unique and challenging tumour because of the frequent occurrence of paraneoplastic syndromes, including hypercalcaemia, erythrocytosis and non-metastatic hepatic dysfunction (i.e. Stauffer syndrome). Polyneuromyopathy, amyloidosis, anaemia, fever, cachexia, weight loss, dermatomyositis, increased erythrocyte sedimentation rate and hypertension are associated with RCC. The different diseases and associated factors are noted in Table I.

Tumour markers are substances that may be found in the blood or urine of someone with RCC. According to the definition by Mejean et al. (2003, p. 821):

> A prognostic factor is a marker that can be used to determine progression of a disease or its arrest. The criteria to evaluate new prognostic factors are numerous and include feasibility, statistical significance in uni-variate analysis and subsequent independence in relation to other factors as assessed by multivariate analysis, clinical pertinence, reproducibility and confirmation by large series.

At present there is no known and accurate tumour marker for RCC. Most markers are connected with cell cycle/apoptosis/development regulation with cell–cell interaction

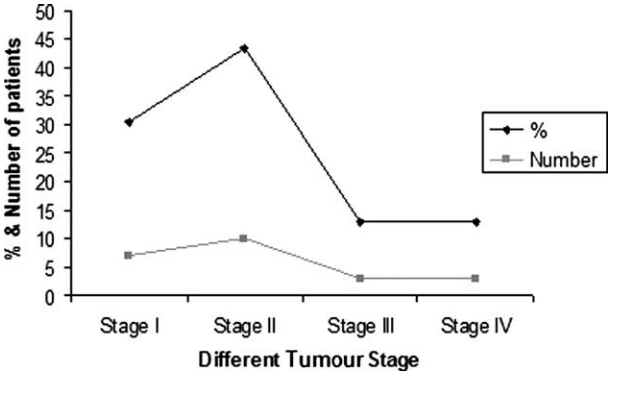

Figure 2. Number and percentage of patients according to their tumour stage.

Table I. Disease and the different types of factors associated with renal cell carcinoma (RCC).

Diseases and factors associated with RCC
Diseases associated with RCC:
1. Von Hippel-Lindau (VHL) disease in one-third to one-half of patients. Deletions of 3p occur commonly in RCC associated with VHL disease
2. Hereditary papillary renal carcinoma (HPRC)
3. Familial renal oncocytoma (FRO) associated with Birt–Hogg–Dube syndrome (BHDS)
4. Hereditary renal carcinoma (HRC)
5. Polyneuromyopathy, amyloidosis, anaemia, fever, cachexia, weight loss, dermatomyositis, increased erythrocyte sedimentation rate and hypertension
Cellular, environmental, genetic and hormonal factors:
1. Cigarette smoking doubles the risk of RCC and contributes to as many as one-third of all cases. The risk appears to increase with the amount of cigarette smoking in a dose-dependent fashion
2. Obesity is another risk factor, particularly in women; increasing body weight has a linear relationship with increasing risk
3. Additional factors associated with development of the disease include: hypertension, treatment for hypertension, unopposed oestrogen therapy, and occupational exposure to petroleum products, heavy metals, solvents, coke-oven emissions and/or asbestos
4. Risk of RCC is increased with the following: abuse of phenacetin-containing analgesics, acquired cystic kidney disease associated with chronic renal insufficiency, renal dialysis, and tuberous sclerosis
5. Renal transplantation: with its associated immunosuppression, it confers an 80-fold increase in the risk of renal cell cancer

motility and shape/biochemical metabolism of cell, tumour angiogenesis and plasma in the body. With the advent of molecular techniques, the field of molecular biology has been explored in recent years, resulting in detailed analysis of human cells at the DNA, RNA and protein levels. Important steps have been made in understanding RCC and metastasis. Systematically, three basic levels exist in RCC:

- Chromosomal alterations that represent the first 'hit'.
- Uncontrolled tumour cell proliferation.
- Metastasis of tumour cells to the distant sites due to angiogenic inducers and loss of adhesive molecules (Syrigos et al. 2004).

The characteristics of an ideal marker include the following:

- Must be secreted by malignant cells.
- Must be detected when a tumour becomes active, i.e. well differentiated.
- To be simple and detected by a simple method.
- Increased capability of diagnosis of tumours in the primary stage, mainly in *in situ* as well as *in vivo* cancers.
- Detect the recurrence of a tumour.
- Establish the success of the administered therapy.
- Have a positive correlation with the clinical stage of a tumour.
- Predict the outcome of the patients.
- Socially accepted with the least possible discomfort to patients.
- To be independent of subjective factors such as methodology and examiner experience.

Unfortunately, none of the presently available markers qualifies as an ideal marker for RCC. In RCCs, the prediction of metastasis via tumour prognostic markers remains a

major problem. Various conventional parameters such as tumour size, stage and grading have been studied to identify subsets of patients with better or worse prognosis. One of the most important challenges of cancer research is the prediction of the invasiveness and metastatic potential of a cancer at an early stage. A marker or subset of markers that would correlate with such kinds of behaviours would be of significant value in a prognosis. Therefore, much effort is needed towards the development of a new reliable tumour marker for RCC. Biological markers and factors are classified into four different categories, including the following:

- Patient-related factors.
- Tumour-related markers.
- Biochemical and molecular markers.

Patient-related factors

These factors include symptomatic presentation, significant weight loss, poor performance statistics, hypocalcaemia and an elevated alkaline phosphatase level in the patient.

Tumour-related markers

These include stage, nuclear grading, histological grade and metastasis. The stage measures an anatomic extent of disease and it is one archetypal prognostic factor for most malignant neoplasms. It is the most important and obvious prognostic factor for RCC. Survival based on Robson and tumour-node-metastasis (TNM) stage correlate very well. A perceived deficiency of the Robson staging is that it does not consider tumour size, while TNM staging takes it into account and it is also preferred on the grounds of being systemic and less ambiguous. It incorporates the most information for therapy and prognosis. In several modern studies, Robson staging unexpectedly proved superior to TNM staging (Robson et al. 1969, Storkel et al. 1989). The American Joint Committee on Cancer (AJCC 1997) has designated the staging of RCC by the TNM classification, with the following TNM definitions:

- Primary tumour (T):
  - TX: Primary tumour cannot be assessed.
  - T0: No evidence of primary tumour.
  - T1: Tumour 7 cm or less in greatest dimension limited to the kidney.
  - T2: Tumour more than 7 cm in greatest dimension limited to the kidney.
  - T3: Tumour extends into major veins or invades adrenal gland or perinephric tissues but not beyond Gerota's fascia.
  - T3a: Tumour invades the adrenal gland or perinephric tissues but not beyond Gerota's fascia.
  - T3b: Tumour grossly extends into the renal vein(s) or vena cava below the diaphragm.
  - T3c: Tumour grossly extends into the renal vein(s) or vena cava above the diaphragm.
  - T4: Tumour invades beyond Gerota's fascia.

- Regional lymph nodes (N):
  - NX: Regional lymph nodes cannot be assessed.
  - N0: No regional lymph node metastasis.
  - N1: Metastasis in a single regional lymph node.
  - N2: Metastasis in more than one regional lymph node.

Laterality does not affect the N classification.

- Distant metastasis (M):
  - MX: Distant metastasis cannot be assessed.
  - M0: No distant metastasis.
  - M1: Distant metastasis.

The classification based on TNM staging is shown in Table II.

Nuclear grading

The grading systems of RCC show independent prognostic value in various studies conducted previously. Nuclear grading is usually the second important and significant factor after tumour stage. Nuclear staging systems are more intensively in use as compared with histological ones. Feature based on nuclear size, shape and content proposed by Furhman et al. (1982) is the most frequently used method. However, a significant problem remains with the application and reproducibility of the nuclear grading system (Medeiros et al. 1988, Weiss et al. 1988).

Histochemical grading

Benign neoplasm such as papillary adenoma and renal oncocytoma are a different entity from RCC. Clear cell carcinoma is the most common carcinoma of the renal epithelium. Papillary carcinoma is the next most common type and is associated with variable outcome in comparison with the conventional RCC. Conventional RCC is associated with a relatively favourable outcome through the number of reported cases. Conventional RCC is an aggressive kind of neoplasm and tends to develop systemically. The haematoxylin-and-eosin staining pattern for three major types of RCC is shown in Figure 3.

Table II. TNM staging based on the classification of the American Joint Committee on Cancer (AJCC).

Stage I	Stage II	Stage III	Stage IV
T1, N0, M0	T2, N0, M0	T1, N1, M0	T4, N0, M0
		T2, N1, M0	T4, N1, M0
		T3a, N0, M0	any T, N2, M0
		T3a, N1, M0	any T, any N, M1
		T3b, N0, M0	
		T3b, N1, M0	
		T3c, N0, M0	
		T3c, N1, M0	

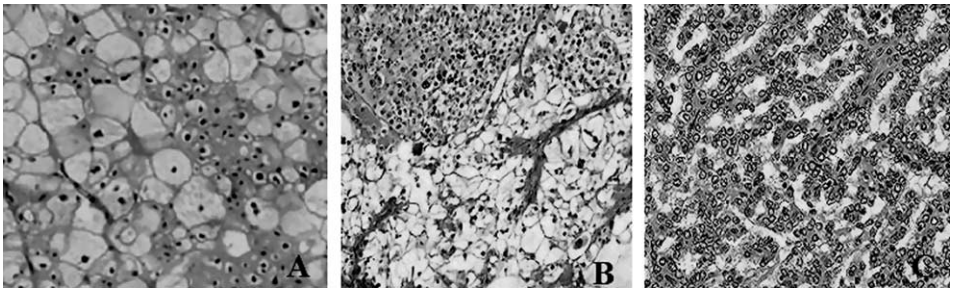

Figure 3. Haemotoxylin and eosin pattern in three major types of renal cell carcinoma: (1) chromophobe renal cell carcinoma, (2) clear cell carcinoma and (3) papillary renal cell carcinoma.

Metastasis

The presence of a metastasis lesion indicates poor survival. Patients without metastasis are known to recover better than those with metastasis.

Biochemical markers

Biochemical markers are further divided into several categories:

- Biomolecular markers: ferritin, nuclear matrix protein-22, ESR and neopterin.
- Cell cycle-based markers: Ki-67, p53, p21 and PCNA.
- Immunogens: PSA, tumour-associated trypsin inhibitor, tissue polypeptide-specific antigen, carcinoembryonic antigen and IL-2.
- Apoptosis-related markers: bcl-2, F7-26.
- Enzymatic markers: gamma-enolase, pyruvate kinase type M2, inducible nitric oxide (iNOS), receptor tyrosine kinase EphA2, thymidylate synthase (TS) and PTEN.
- ECF-related markers: P-selectin.
- Cluster designation of monoclonal antibodies: CD-10, CD154, CD44 and CD95.

Biomolecular markers

Ferritin. Iron is stored in the body as ferritin (a protein–iron complex) or hemosiderin. Ferritin is also present in hepatic parenchymal and reticuloendothelial cells of the bone marrow, liver and spleen. If the amount of apoferritin (a ferritin protein with almost no iron in it, and not in equilibrium with the body's stores) is insufficient to bind the remaining iron, it is usually deposited in tissues as small iron oxide granules known as hemosiderin (Herbert et al. 1997). The serum ferritin level is usually elevated in RCC. However, the exact mechanism of the increment is not well established. Various theories have been proposed for such kind of increments:

- Increase as an acute phase reactant.
- As a result of decreased clearance.
- Production by a tumour.

In 1982, there was the first report on the increased level of serum ferritin in RCC and a subsequent decrease in its level following nephrectomy (Mufti et al. 1982). The

progression of the tumour at different stages and the number of RCC patients with their percentage in the present study is shown in Figure 2. This study shows that the ferritin level was significantly different between patients and controls. There was no significant difference in the ferritin value between low (I and II) and high nuclear grades (III and IV) of tumour. This study shows a positive correlation between the volume of the tumour and serum ferritin level. The serum ferritin correlates with the maximum tumour dimension. This proves that it can still be used as a prognostic marker for RCC. The level of serum ferritin in different tumour stages is shown in Figure 4, where ferritin is expressed as ng ml^{-1} serum (Kashyap et al. 2003).

In the same study, when correlation analysis was applied between maximum tumour dimension and serum ferritin level, it was observed to be significant ($r = 0.5680$, $p < 0.05$). The scatter diagram in Figure 5 shows the correlation between the maximum tumour dimension and the serum ferritin level of patients.

Further, as currently there is no such reliable tumour marker for RCC, the value of ferritin as a marker should be investigated further before drawing any clinical conclusion.

Nuclear matrix protein-22 (NMP-22). NMP22 is a protein found in the urine of patients with bladder cancer. It is used for patient follow-up to avoid repeated cystoscopy (looking into the bladder). So far it has not been found to be sensitive enough to be used. Recently, the US Food and Drug Administration (FDA) approved nuclear matrix protein-22 for monitoring patients with transitional RCC instead of cystoscopy (Miyanaga et al. 1997, Zippe et al. 1999). In Ozer et al. (2002), the urinary NMP-22 level was strongly related to the presence of RCC, with a sensitivity rate of 87% and a specificity rate of 80% using the cut-off value of 4.9 U ml^{-1} as determined by receiver operating characteristic analysis. The urinary NMP-22 level may be taken into account when evaluating a patient at risk of having RCC. Urinary NMP-22 is currently used for monitoring patients with transitional cell carcinoma of the bladder. In a recent study, the level of preoperative urinary NMP-22 was significantly higher in the RCC group than in the control group. Ten days postoperation, the urinary NMP-22 level had decreased significantly. The post-operative urinary NMP-22 level was not different from that of the control group, which shows a relation between urinary NMP-22 and RCC. Urinary BTA, BFP and

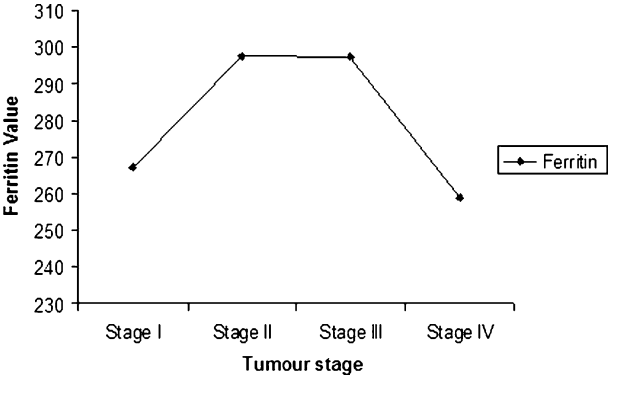

Figure 4. Serum ferritin level (ng ml^{-1}) and different tumour stages.

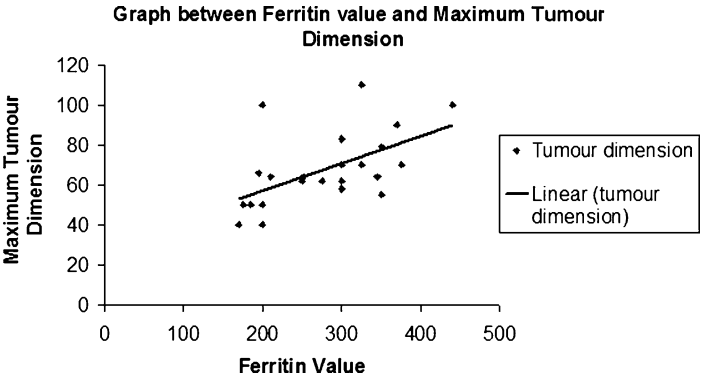

Figure 5. Adenocarcinoma tissue labelled with F7-26 monoclonal antibody. The arrow shows the labelling of single-stranded DNA.

NMP22 are used in Japan for the diagnosis of urothelial (transitional cell) carcinoma, mainly bladder cancer (Kikuchi 2004).

Erythrocyte sedimentation rate (ESR). The ESR is a non-specific screening test for various diseases. This 1-h test measures the distance (mm) between red blood cells (RBC) settled in unclotted blood towards the bottom of a specially marked test tube. ESR is also called 'sed' rate and sedimentation rate. ESR has been found to be a prognostic and predictive marker correlating with disease activity as well as survival in different types of malignancy including RCC (Ljunberg et al. 1997). According to Iversen et al. (1998), systematic ESR graphic recordings over time possibly enable a physician to determine each individual's baseline value and continuous rise in the ESR can help in further investigations.

Neopterin. Neopterin is a pteridine intermediate metabolite in the biopterin synthetic pathway. It is synthesized and secreted by activated monocytes and macrophages (Sheldon et al. 1991, Iwagaki et al. 1995). Neopterin has been recognized as a valid marker of cellular immune activation and therefore it has been investigated as a possible predictive marker for supportive immunotherapy as well as for the evaluation of the response to immunotherapy in RCC (Fumagalli et al. 1999).

Cell cycle-based markers.

Ki-67 (proliferative cell nuclear antigen). Ki-67 has been suggested as a marker for tumour aggressiveness in a lymph node-negative RCC. Hofmockel et al. (1995) found the Ki-67 labelling index to be predictive when used along with other factors. However, the results are not entirely consistent because these markers measure different aspects of the cell cycle and in some way may complement each other.

p53. p53 is a tumour suppressor gene whose normal activity prevents the formation of tumours. In the cell, p53 protein binds to DNA, which in turn stimulates another gene to produce p21. The p21 protein interacts with cdk2, a protein that stimulates cell division. When p21 binds cdk2, the cell cannot continue through the cell cycle and does not divide.

When the p53 gene is mutated or absent, p21 protein cannot be produced. As a result, there will be no stop signal for cell division and cells will divide uncontrollably, forming tumours. The p53 gene has also been implicated in the regulation of apoptosis, or programmed cell death. When the apoptosis machinery of a cell is disrupted, damaged cells that would normally die continue to grow, in some cases dividing to produce cancerous masses. Patients who inherit only one functional copy of the p53 gene are predisposed to cancer and often develop several independent tumours in a variety of tissues early in life. This condition, called Li–Fraumeni syndrome, is quite rare. It is clear that disruption of p53 is just one component of a network of events that contribute to tumour formation.

Mutations in the p53 gene are among the most commonly diagnosed genetic disorders, occurring in as many as 50% of cancer patients. For some types of cancer, most notably breast, lung and colon cancer, p53 mutations are the predominant genetic alternations found to date. These mutations are associated with genomic instability and thus an increased susceptibility to cancer. Some p53 lesions result in malignancies that are resistant to the most commonly used treatments. Researchers are studying p53 mutations and their correlations with specific therapeutic outcomes. Recently, the prognostic value of p53 has been evidenced by its immunoreactivity. p53 was over-expressed in papillary carcinoma as compared with conventional RCC (Zigeuner et al. 2004). These include p53, BCL-2 and p21 proteins. Over-expression of p53 protein would suggest occurrence of p53 gene mutation. However, an immunohistochemistry-based study on RCC could not find any independent association between p53 protein expression and survival (Richter et al. 2002).

p21. p21 is an inhibitor of cyclin-dependent kinases and a target of p53. Shalitin et al. (1994) described an enzyme-linked immunosorbent assay (ELISA) for p21 as a potential tumour marker for RCC because its changes appear to correlate with clinical outcome. In a recent study on an RCC cell line, over-expression of p21WAF1/CIP1 and p53 was observed. This study suggests that p21 (WAF1/CIP1) is a more potent growth suppressor than p53 of mouse tumour cells Renca. The divergent responses of tumour cells to p21 (WAF1/CIP1) over-expression could be due to various networks that differ between species (Kralj & Pavelic 2003).

Cyclin D1. Cyclin D1 is a protein with a molecular weight of 36 kDa and it is also known as PRAD-1 or bcl-1. Cyclins are regulatory subunits of the cyclin-dependent kinases (cdk), which control transition at different and specific phases of the cell cycle. Cyclin D1 is a putative proto-oncogene over-expressed in a wide variety of human neoplasm. It is a G1 cyclin that regulates the transition from G1 to S phase as its peak level and maximum activity is reached during the G1 phase of cell cycle (Handa et al. 1999).

A close association has been observed between expression of cyclin D1 in transitional cell carcinoma (TCCs) of the urinary bladder and tumour differentiation (Lee et al. 1997). Another study shows intense cyclin D1 staining within the adenocarcinoma elements, which demonstrated less proliferation than the surrounding TCC as assessed by PCNA staining. Therefore, the over-expression of cyclin D1 in the adenocarcinoma elements may have been linked to cellular transformation of the transitional epithelium of the renal pelvis rather than to a direct consequence of increased cell proliferation (Lee et al. 1998).

Immunogens as markers

Prostatic-specific antigen (PSA). In prostate cancer, PSA is a gold standard marker for screening and monitoring. Recent studies have revealed that PSA-related markers have additional information for patients with a grey zone PSA score. Horoszewicz et al. (1987) first reported this antigenic marker on prostate epithelial cells, which can be found in the serum. Prostrate-specific membrane antigen (PSMA) is a 100-kDa-type II membrane protein that is expressed in all types of prostatic tissues including normal epithelial cells, BPH, prostatic intraepithelial neoplasia and cancerous tissue. PSMA may provide new applications for the detection of high-grade cancer or microscopic circulating prostate cancer cells in the blood. In urothelial cancers, several urinary markers are available and may be helpful in the diagnosis of lower grade urothelial cancers, which have a low sensitivity of urinary cytology. In testicular cancer, lactate dehydrogenase (LDH), alpha-fetoprotein (AFP) and human chorionic gonadotropin (hCG) are essential markers not only for the determination of the tumour stage, but also for the prognosis of the patient (Shimazui & Akaza 2004).

Tumour-associated trypsin inhibitor. The evaluation of the tumour-associated trypsin antigen (TATI) in the serum of the patients with TCC was performed. In a study of 63 RCC patients and 24 patients with Nehing diseases, patients were radio-immunoassayed for TATI. All patients with RCC underwent radical nephrectomy and the value of TATI was assayed after 3–12 months. Fifteen patients with the benign disease and 44 patients with RCC had elevated TATI. TATI has been compared with other serum markers including CEA, CA15-3, CA125, CA19-9 and ferritin, and sensitivity rates were 5, 10, 13, 5 and 35%, respectively. The value of TATI in this study was correlated with the stage of the disease. Among 15 patients without metastasis, the mean preoperative value was 112 mg l^{-1}, which fell to 46 mg l^{-1} postoperation. In nine patients with metastasis, the preoperative means were 100 and 240 mg l^{-1} postoperation. TATI showed a better sensitivity rate (65%) than other markers for RCC, but its specificity is limited. Nevertheless, it can be useful for a postoperative follow-up. The group has also shown that TATI remains one of the most sensitive serum markers for RCC (Meria et al. 1995).

Tissue polypeptide-specific antigen (TPS). Tissue polypeptide antigen (TPA) is a heterogeneous combination of molecules with a molecular weight between 20 and 45 kDa. Bjorklund (1978) first defined it as a tumour associated antigen. Immunological TPA is defined as an aggregate of non-epidermal cytokines 8, 18 and 19 (Burt et al. 1987, Rydlander et al. 1996). TPS was characterized by the development of a monoclonal antibody against subgroups of TPA. Further studies have proved the similarity between M3 epitope of TPA and the second part of cytokine 18. TPS is the specific M3 epitope of TPA and also known as cytokine 18. It has been used as a prognostic parameter for some neoplasm since it was isolated in the serum or urine of some cancer patients (Bjorklund 1978).

TPS is a new tumour marker that indicates tumour proliferative rate rather than tumour burden. A recent study was carried out on RCC to check the role of TPS. The sensitivity rate of TPS for RCC was 60% in this study. This suggests that TPS may have a potential clinical role as a valuable tumour marker for RCC, especially in advanced disease and follow-up therapy response (Chang et al. 2002).

Carcinoembryonic antigen (CEA). CEA is an oncofoetal glycoprotein antigen. It is present in embryonic tissues and certain epithelial malignancies. Progressive elevations of CEA may herald tumour recurrence 3–36 months before clinical evidence of metastases. CEA is the preferred tumour marker in patients with colorectal cancer. A level above 5 U ml^{-1} is considered abnormal in the present study. Many clinicians use this marker to follow other cancers such as lung and breast cancers. The CEA level is also elevated in many other cancers such as thyroid, pancreas, liver, cervix and bladder cancers. It is also elevated in other diseases and in otherwise healthy smokers. It is not useful to screen for or to diagnose colorectal cancer. If it is elevated when colorectal cancer is detected, this may mean the cancer is more advanced. Elevated serum levels were found for all markers tested except for CEA. For CA-125 and CA-15-3, elevated serum levels were correlated with clinical stage and tumour grade (Grankvist et al. 1999). In a study conducted on 48 RCC patients, all the patients were negative for CEA (Ordonez 2004).

Autoantibodies to subunits of replication protein A (RPA). IL-12 was originally discovered as a product of Epstein–Barr virus-transformed B-cell lines. Subsequent studies have suggested that the major source of IL-12 consists of monocytes, macrophages and B-lymphocytes (Johnson & Brown 2000). IL-12 has an autoregulative feedback mechanism, as was shown recently in an experimental study on RCC, where long-term administration of IL-12 decreased its own serum level (Rakhit et al. 1999).

Apoptosis-based markers

Tumour growth attributes include increased cell proliferation, decreased cell death (apoptosis) or a combination of both.

Apoptotic index or TUNEL. Apoptosis or programmed cell death is a biological process that goes through systemic events and finally the cell commits suicide. It plays an important role in oncogenesis determining tumour growth and aggressiveness. Apoptotic cells in tissues can be detected by using a technique called terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL). The method uses terminal dideoxynucleotidyl transferase (TdT) to incorporate tagged/labelled nucleotides into the 3′-strand breaks that occur in DNA during apoptosis (Chapman et al. 1995). The apoptotic cell count can be considered as a legation of the percentage of tumoral cells present, i.e. the number of the apoptotic cells per square millimetre of neoplastic tissue, and it is usually described as the apoptotic index (AI) (Lipponen 1999).

Bcl-2. Bcl-2 proteins are a family of proteins involved in the response to apoptosis. Some of these proteins (such as bcl-2 and bcl-XL) are anti-apoptotic, while others (such as Bad or Bax) are pro-apoptotic. The pro-apoptotic bcl-2 proteins are often found in the cytosol where they act as sensors of cellular damage or stress. The level of various bcl-2 family proteins can significantly alter the apoptotic threshold of a cell. The apoptotic threshold is the point at which pro-apoptotic stimuli leads to irreversible cell death (Reed 1998). Over-expression of bcl-2 protein is associated with the inhibition of apoptosis and thus associated with prolongation of cell survival.

However, it has not been adequately studied as a prognostic factor (Gobe et al. 2002, Itoi et al. 2004).

Zhang and Takenaka (2000) revealed close associations between PI, tumour grade and stage, and also a significant relationship between AI and the tumour grade of RCC. The expression of bcl-2 was detected in 24 of 70 RCCs (34.3%), but it was not related to PI, AI or the clinicopathological factors of RCC. Bcl-2 over-expression was observed in 70% of the total RCCs, which suggests bcl-2 over-expression may have a role in tumorigenesis and may explain the relative resistance of RCC to chemotherapeutic agents and to radiation therapy (Huang et al. 1999).

F7-26. So far, apoptosis in RCC has been detected by using TUNEL assay. The independence of DNA breaks is critical as the techniques that detect double-stranded DNA breaks such as TUNEL are not specific for apoptosis (Charriaut-Marlangue & Ben-Ari 1995, Grasl-Kraipp et al. 1995, Didenko & Hornsby 1998). The detection of apoptosis with the ssDNA antibody clone F7-26 is based on the increased sensitivity of DNA in apoptotic cells to thermal denaturation. In this method, DNA is denatured by heat in the presence of formamide and stained with monoclonal antibody F7-26 specific to single-stranded DNA (ss-DNA). This antibody is very specific only for the apoptosis process as it avoids the detection of necrosis in the tissue system or cell line (Frankfurt et al. 2001a). Importantly, formamide induces DNA denaturation in the apoptotic cells but does not affect the stability of DNA with ss- or ds-DNA breaks in the absence of apoptosis (Frankfurt et al. 2001b).

The formamide-MAb procedure has the following advantages:

- Selective denaturation of DNA in apoptotic cells after the heating at a relatively low temperature (56–75°C instead of 100°C).
- Specific staining of apoptotic cells in thin sections of formalin-fixed tissues prepared with routine histological techniques (a previously described procedure produced the best results after methanol fixation). This new protocol produced the best and most stable results after formalin fixation. It specifically stains condensed chromatin of apoptotic cells.

The advantage of this detection method is that one can detect apoptosis by avoiding the necrosis-positive cells. This new detection method is superior to TUNEL as it can differentiate between necrotic and apoptotic cells. Thus, there is now a need to revise our concept about the role of apoptosis in RCC, as there is a possibility that we ignored any promising value of apoptosis in relation to RCC. The detection of apoptotic cells with the formamide-monoclonal antibody (MAb) procedure is based on the staining of condensed chromatin in apoptotic nuclei. Since chromatin condensation in compact masses is the most specific and definite hallmark of apoptosis, this method provides universal detection of apoptosis. The apoptotic events that occur without DNA breaks or without activation of specific caspases can be detected with MAb to ssDNA.

The following facts demonstrate that selective denaturation of DNA in apoptotic nuclei in the presence of formamide is not related to DNA breaks:

- Necrotic cells with a high level of ds-DNA breaks, demonstrated by TUNEL, do not react with the anti-ssDNA MAb.

- Apoptotic cells with very low and high levels of DNA fragmentation are stained by formamide-MAb technique with a similar intensity.
- Nuclei with ssDNA breaks induced by oxygen radicals do not bind the MAb after the heating in formamide, although alkaline denaturation detects DNA breaks in these nuclei (Frankfurt et al. 2001a,b).

Figure 6(A–D) shows the F7-26 labelling pattern in breast carcinoma. Apoptotic carcinoma cells stained with the F7-26 MAb were observed as clusters or single cells scattered among viable tissue in breast carcinoma (Figure 6A–C). Staining of fibroblast was observed in most carcinomas, including apoptotic death in stromal cells (Figure 6D) (Frankfurt et al. 1997).

Enzymatic markers

Gamma-enolase. Gamma-enolase or phosphopyruvate hydratase is a unique isoform composed of two gamma subunits and expressed specifically in mature neurons and neural-related cells, neuroendocrine cells, pituicytes and many tumour cells. Cell injury causes release of the enzyme into the blood and cerebrospinal fluid (CSF). Serum neuron-specific enolase (NSE) is used as a tumour marker of neuroendocrine cancers. NSE is of special diagnostic value as a panendocrine marker of neuroendocrine tumours that do not produce hormones and peptides (Honda et al. 2000).

Takashi et al. (1989) evaluated gamma-enolase as a tumour marker for RCC and suggested that it could be a useful tumour marker for the staging of disease and monitoring treatment in patients with RCC. The tissue levels of gamma-enolase in RCC were 34 and 15 times higher than in renal cortex and medulla. With regards to histological types of RCC, concentration of gamma-enolase in clear cell tumours was

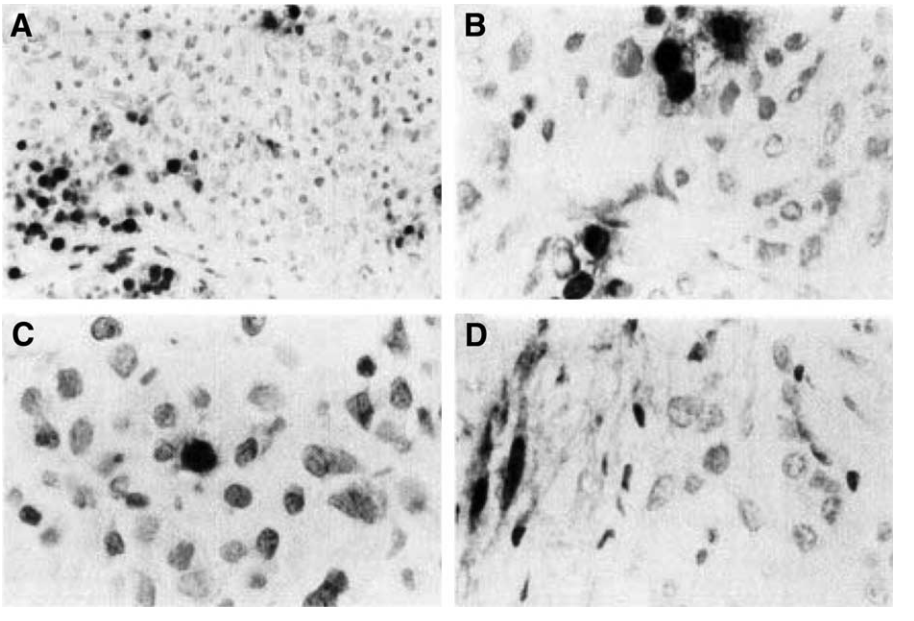

Figure 6. (A–C) Apoptotic carcinoma cells stained with F7-26 observed as clusters or single cells scattered among viable cells; (D) fibroblast staining in breast carcinoma. Courtesy and with permission from the American Association for Cancer Research.

significantly higher than in granular cell tumours. The mean serum gamma-enolase in patients with RCC was significantly higher than in controls. The mean serum gamma-enolase in patients with high stage tumours, i.e. III and IV, was significantly higher compared with low stage tumour I and II. Serum gamma-enolase in patients with urological tumours other than RCC was also evaluated and it was found that only testicular cancer showed a relatively high positive rate and high level of gamma-enolase. No patient with bladder cancer, prostrate cancer and renal angimyolipoma had an elevated serum gamma-enolase level.

Pyruvate kinase type M2. The dimeric form of pyruvate kinase type M2 is over-expressed in tumour cells (TuM2-PK). A recent study was carried out to clarify whether TuM2-PK works as a prognostic marker. Due to its low sensitivity and specificity, TuM2-PK is not a suitable marker for the diagnosis of RCC. Whether TuM2-PK may be useful in advanced RCC to control the success of palliative treatment regimens is still unclear (Hegle et al. 2003).

Inducible nitric oxide (iNOS). Nitric oxide is a highly reactive gaseous molecule. The NO molecule is a free radical, which makes it very reactive and unstable. NO is synthesized within cells by an enzyme NO synthase (NOS). NOS exists in three forms, which includes neuronal nitric oxide synthase (NOS1), inducible nitric oxide synthase (NOS2) and endothelium nitric oxide synthase (NOS3). Modification of NOS activity in tumours, and hence NO biosynthesis, may be regarded as a promising means for selective tumour blood flow modification. This modification provides a novel approach for reducing tumour oxygenation aimed at enhancing the efficiency of hypoxia-mediated, bio-reductively activated anti-cancer drugs (Alexandrova et al. 2001).

The iNOS form is a soluble enzyme that unlike eNOS and nNOS does not require elevated intracellular Ca^{2+} level for activation. NO is a pleiotropic signalling molecule that binds to cytochrome C oxidase (complex IV) reversibly and in competition with oxygen (Xu et al. 2004). The role of NO has been observed in RCC. Higher dose interleukin 2 (IL-2) therapy is known to induce the same cytokines in patients with advanced cancer. The study was carried out on RCC patients to observe the induced NO synthesis. The study demonstrates for the first time that a cytokine-inducible, high-output L-arginine/NO pathway exists in humans and shows increased endogenous nitrate synthesis in patients receiving IL-2 (Hibbs et al. 1992).

Recently iNOS was suggested as a molecule for RCC to acquire not only hypoxic adaptation, but also the ability to invade into veins and form tumour thrombi. It was found in six cases of 11 tumour thrombi. The cause-specific survival rate of patients with iNOS-positive tumour thrombi was lower than that of patients with iNOS-negative tumour thrombi and showed borderline significance. INOS-mRNA and protein were expressed in A498 and A704 RCC cells under hypoxic conditions (Hara et al. 2003).

Receptor tyrosine kinase EphA2. EphA2 is a tyrosine kinase receptor that plays an important role in different cancers and other biological processes such as axon guidance (Yamout et al. 2003). CD8+ and CD4+ T-cell respond to a new tumour-associated antigen, the receptor tyrosine kinase EphA2, which is broadly expressed in diverse cancer histology and is frequently over-expressed in advanced stage/metastatic

disease. ELISA analyses demonstrated prominent EphA2-restricted T-helper one-type CD4+ T-cell activity in patients with early stage disease, whereas T-helper two-type and T-regulatory-type responses predominated in patients with more advanced forms of RCC, implicating that the immune system of cancer patients actively monitors EphA2-derived epitomes, and that the magnitude and characteristics of T-cell responses to EphA2 epitopes may convey much-needed predictive information about disease stage and outcome (Tatsumi et al. 2003).

Thymidylate synthase (TS). TS catalyses the final step in the *de novo* synthesis of deoxythymidine monophosphate (dTMP) using the substrate dUMP and a cofactor, 5,10-methylene tetrahydrofolate (MHFR). TS is a critical target for fluoropyrimidines, an important group of antineoplastic drugs that are widely used in the treatment of solid tumours. Mizutani et al. (2003) suggest that TS activity may be associated with malignant potential of RCC, and it may be possible to use 5-FU as an antineoplastic drug for RCC with high TS activity.

Cluster designation (CD)-based marker.

CD10. CD10 is a cell surface enzyme with neutral metalloendopeptidase activity. It is also known as common acute lymphocytic leukaemia antigen (CALLA). It serves as a marker for the common form of acute lymphocytic leukaemia (ALL) as well as for Burkitt lymphoma and follicular germinal centre lymphoma. CD10 is normally present on the surface of early lymphoid cells as well as on a number of other types of normal cells, especially in the kidney. It is also expressed in some non-lymphoid tissues such as fibroblasts, breast myoepithelium and brush border of kidney. A recent study reported that CD10 is a better marker for distinguishing between hemangioblastoma and metastatic clear cell RCC (Jung & Kuo 2004).

CD154. CD154 is a ligand for CD40, a member of TNF-receptor family. CD40 activation by CD154 may trigger diverse cellular responses, ranging from proliferation and differentiation to growth suppression and cell death, in normal and malignant cells. However, the pathophysiological role of CD154 expressed by tumour cells remains unclear. The expression of the CD40-CD154 system in 24 primary cultures derived from RCC, its correlation with tumour stage and potential functional significance was studied. CD154, but not CD40 expression, significantly correlated with tumour stage. Moreover, RCC cell lines also released the soluble form of CD154 into the supernatant. CD40 engagement by CD154 did not affect apoptosis or survival. On the contrary, CD154 stimulated cell proliferation, motility and production of PAF, a phospholipid mediator of inflammation with angiogenic properties (Tong & Stone 2003). Furthermore, the RCC cell lines expressed PAF-R. Blockade of PAF-R by WEB-2170 (a PAF-R antagonist) abolished the CD154-dependent motility, indicating a role for PAF synthesized after CD154 stimulation in renal carcinoma cell motility. In conclusion, this study identifies new functional properties for CD154, which are potentially relevant for the growth and dissemination of RCC (Bussolati et al. 2002).

CD44. CD44 represents a large family of adhesion molecules that differ in primary structure with a predominant or standard form (CD44S) and various isoforms resulting from alternative splicing of ten exons (CD44v1–10) of a single gene mapped

on chromosome 11 (Screaton et al. 1992, Gilcrease et al. 1999). Differential expression of the CD44 molecule has been reported in different histological types of RCC (Heider et al. 1996, Terpe et al. 1996).

CD95 (APO-1/Fas). FAS belongs to TNF receptor family. The CD95 (Apo-1/Fas) receptor-ligand system is a key regulator of apoptosis. Down-regulation of CD95 receptor and up-regulation of CD95 ligand have been reported in a variety of human tumours and are thought to confer a selective survival advantage. FAS contains two isoforms (membrane anchored and soluble), both of which can mediate apoptosis through FAS-signalling process (Kamihira & Yamada 2001).

Disorders in negative growth control by CD95 (APO-1/Fas)-mediated apoptosis have been suggested to facilitate immune evasion of neoplastic cells and resistance to anticancer drugs. Cycloheximide-mediated inhibition of translation resulted in increased sensitivity to agonistic anti-CD95 antibodies, which suggests a role for short-lived apoptosis-protective proteins in the resistance of RCC to CD95-mediated apoptosis. Resistance to CD95-mediated apoptosis is a possible key feature of human RCC. This resistance might facilitate evasion from negative growth control and contribute to the failure of cytotoxic drugs in the treatment of human RCC (Gerharz et al. 1999).

Proteinacious marker

Erythropoietin. Erythropoietin is a glycoprotein produced by the renal cortex and is quantified by an enzyme immunoassay. Patients with high-grade tumours had more often a significantly increased erythropoietin level than those with low-grade tumours. However, no correlation was found between erythrocytosis and elevation of erythropoietin in serum. Erythropoietin as a tumour marker has a low sensitivity. However, it correlates with stage and grade and provides prognostic information (Westenfelder & Baranowski 2000).

Glycoprotein tumour markers. An increased level of glycoproteins has been reported in the serum of patients with RCC. These are carcinoembryonic antigen (CEA), CA-50, CA-19-9 and CA-15-3. Elevated serum levels were found for all markers tested except for CEA. In patients with elevated CA-125, survival time was significantly shorter than for patients with a normal CA-125 level. Thus, serum CA-125 and CA-15-3 may be useful as an adjunct in the staging of RCC. CA-125 also gives prognostic information and might be of predictive value in RCC (Meria et al. 1995).

Fibrinogen. Fibrinogen, also known as factor I, is a protein made in the liver and is essential for blood clotting. It is converted into fibrin as blood coagulates or clots. Acquired dysfibrinogenaemia has not been previously reported as a paraneoplastic marker for malignancy. Dawson et al. (1985) reported the clinical course of a patient who at the time of diagnosis of non-metastatic RCC had dysfibrinogenaemia characterized by prolongation of thrombin, reptilase and increased sialic acid content of the purified fibrinogen. The thrombin and reptilase times returned to normal after nephrectomy, but became abnormal with the development of non-hepatic metastases. It was concluded that acquired dysfibrinogenaemia can be a part of a paraneoplastic syndrome and is a sensitive plasma marker for tumour progression.

Vinculin. Vinculin is a large eukaryotic protein with a molecular weight of 116 kDa, involved in the attachment of the actin-based microfilaments to the plasma membrane. It is located at the cytoplasmic side of focal contacts or adhesion plaques. In addition to actin, vinculin interacts with other structural proteins such as talin and α-actinins (Rüdiger 1998). Kuroda et al. (2000) studied the possible role of vinculin as a tumour marker of the renal neoplasm in association with collecting duct phenotype. As is known, some oncogenes code for tyrosine kinases. Tony Hunter and colleagues from the Salk Institute have shown that one of their phosphorylation targets is vinculin, which is involved in maintaining normal cell shape (Cooper & Hunter 1983). Phosphorylation of vinculin and some other structural proteins may result in a spherical shape characteristic of cancer cells, but which is also seen in normal cells in early G1 stage. However, phosphorylation of vinculin may not be a necessary condition for cancer since some Rous sarcoma virus mutants cause cancer without phosphorylating vinculin (Keeton et al. 1986). To test the role of vinculin as a tumour marker, surgical materials obtained from 79 renal tumours from 78 patients were reviewed. The positive rate of vinculin in conventional-type of RCC (clear cell) was significantly different from that in the other types of renal tumours ($p < 0.01$) and it has been suggested as a useful marker of renal neoplasm with collecting duct phenotype and chromophobe type of RCC. Vinculin was positive in all cases of chromophobe and collecting duct carcinoma, whereas three of four sarcomatoid varieties and five of 12 papillary types were positive for vinculin. No clear cell type of RCC was positive for vinculin (Mejean et al. 2003).

AgNOR expression. AgNOR is the argyrophilic nucleolar organizer region. The argyrophil NOR-related proteins (RNA polymerase I, B23 protein, C23 protein or nucleolin) encoded by the genes located in the NORs can be identified as small black dots in the nucleus under a light microscope using the rapid one-step argyrophilic NOR (AgNOR) staining technique (Ploton et al. 1986). AgNOR counts have a strong prognostic value in RCC. AgNOR analysis is a simple and inexpensive technique that allows the simultaneous evaluation of cell proliferation and histology in the same specimen and may be regarded as a reliable diagnostic and prognostic indicator in tumour pathology (Pich et al. 1991). The role of AgNOR has been studied in different types of cancer including RCC and an association was found between AgNOR counts and pathological stage (Pich et al. 1995). An assessment of tumour growth kinetics showed significantly different mean silver-staining nucleolar organizer region (Ag-NOR) scores and Ki-67 indices. Multivariate analysis showed that tumour type, presence of metastases, AgNOR score and Ki-67 index were independently associated with survival in PRCC (Delahunt et al. 2001).

C-reactive protein (CRP). The major function of CRP by binding phosphocholine is to recognize some foreign pathogens and phospholipid constituents in damaged cells (Volankis 1997). CRP has been evaluated for potential use as a prognostic indicator in RCC and prostate cancer in a retrospective study. The serum level of CRP was measured by an ELISA-based kit and it was significantly higher in patients with RCC. The study suggests that there may be mild-to-moderate association between survival time and CRP in RCC (Mata et al. 2004).

Gp200. In normal kidney, gp200 is localized along the brush border of the pars convoluta and pars recta segments of the proximal tubule, as well as focally along the luminal surface of Bowman's capsule adjoining the outgoing proximal tubule. Gp200 is expressed by 93% of the primary and by 84% of the metastatic RCC (Yoshida & Imam 1989).

TFE3. TFE3 is a transcription factor binding to IGHM enhancer 3 (Xp11.22). It is a member of the basic helix–loop–helix family (b-HLH) of transcription factors primarily binding to the immunoglobulin enhancer muE3 motif (Clark et al. 1997). Previous analysis for sporadic type 1 and 2 PRCC cases for TFE3 rearrangement has not shown any kind of detection in the 22 PRCC cases examined (Hoffmann & Valencia 2004).

Adhesion molecules as markers

P-selectin. P-selectin is a leukocyte adhesion receptor expressed on the surface of activated platelets and endothelial cells. It is the largest known selectin with a molecular weight of 140 kDa. It contains nine consensus repeats (CR) and extends approximately 40 nm from the endothelial surface. Other names for P-selectin include CD62P, granule membrane protein 140 (GMP-140) and platelet activation-dependent granule to external membrane protein (PADGEM). P-selectin is expressed in alpha-granules of activated platelets and granules of endothelial cells. For expression of the P-selectin gene, venous blood was collected from 30 patients with RCC and 24 controls for cytometric analysis to evaluate platelet morphology. P-selectin as the CD62P receptor on blood platelets was marked by anti-CD61/62P MAb. Platelet activation was reflected by P-selectin expression was higher in the group of patients, compared with the controls. However, adenosine diphosphate (ADP)-stimulated platelet activity in RCC patients increased only by 0.24%, while following activation by thrombin, by 0.54%. Moreover, a higher statistical significant difference of platelets with P-selectin expression was found in patients with disseminated neoplastic changes in renal parenchyma as compared with patients with a single localized neoplastic lesion. A statistically significant difference was observed in the platelet count and anisocytosis in renal cancer patients. Renal cancer enhances P-selectin expression due to the presence of intensified thrombin genesis and other platelet agonists in the blood (Mantur et al. 2002). There is a need to investigate further the role of P-selectin in RCC to determine if it is an important marker for RCC.

Cadherin-6 (Cad-6). Cadherins are a family of calcium-dependent, cell–cell adhesion molecules that play an important morphoregulatory role in a wide variety of tissues. Alterations in cadherin function have been implicated in tumour progression in a number of adenocarcinoma. Despite the increasing number of new cadherins identified, little is known about cadherins in normal renal tissue and renal carcinomas. Paul et al. (1997) suggested that alteration of cadherin-6 expression is associated with progression of RCC.

Neovastat (Æ-941). Neovastat (Æ-941) is an aqueous extract of shark cartilage with antiangiogenic properties. It is also a naturally occurring multifunctional antiangiogenic agent (Boivin et al. 2002). It has reached phase III clinical trial evaluation for the treatment of solid tumours (non-small cell lung cancer and RCC) and a pivotal phase II

clinical trial in multiple myeloma was observed (Gingras et al. 2001). Æ-941 is an inhibitor of angiogenesis with an action mechanism that could be beneficial in the treatment of RCC. Recently there was a study report on the significant relationship between dose and survival observed without dose-limiting toxicity in RCC (Batist et al. 2002).

Cytological and DNA-based markers.

MN/CA9. The MN/CA9 antigen was first identified in 1992 in cervical cancer cell line HeLa. The MN/CA9 antigen was present virtually in all cervical cancer growths, although its function is unknown. In some cases, women whose cell samples looked completely normal but were positive for MN/CA9 were found to have precancerous or cancerous cervical lesions (Liao et al. 1997). Murakami et al. (1999) observed that MN/CA9 expression is a potential diagnostic biomarker of RCC, especially in the clear cell type, and it can be targeted using molecular biology techniques. The human MN/CA9 gene was isolated, characterized and sequenced in 1996. It is a novel member of the carbonic anhydrous (CA) family, which codes for widely distributed catalysts of the reversible conversion of carbon dioxide (CO_2) to carbonic acid (H_2CO_3). So far MN/CA IX is the only tumour-associated CA isoenzyme (Opavsky et al. 1996). In our study, MN/CA9 gene was positive in 84.2% of RCC samples, while in controls it was only 15.8%. The difference was statistically significant ($p < 0.05$) (Kashyap 2003).

Today there is an approach to using the immune system response using directly administered natural or altered antibodies in cancer patients. These antibodies attack a particular cell-surface protein. The G250 antigen, which is the same as the cellular protein MN/CA9, is specifically expressed in most renal cancers (Liao et al. 1997). G250 is one of the best tumour markers. Early phase clinical trials with unlabelled or ^{131}I-labelled radioactive G250 antibody, which is specific for this antigen, showed some stable disease responses (Steffens et al. 1999, Wiseman et al. 2001).

Mismatch repair gene mutations in RCC

DNA mismatch repair is one of the genome-based restoration mechanisms that repairs the mismatch of nucleotides during DNA replication (Modrich 1991). MMR deficiency was assessed using microsatellite instability (MSI) and genetic analyses of 25 cell lines derived from renal tumours. MMR gene alterations were detected using reverse transcription of RNA coupled with polymerase chain reaction (RT-PCR) and DNA sequencing. Three RCC lines with undetectable MLH1 were identified and investigated for MSI and inactivating mutations in the hMLH1 MMR gene. Genetic instability and hMLH1 mutations were identified in two RCC lines and their corresponding tumours. Genetic alterations affecting expression were limited to MLH1 since other MMR proteins (MSH2, MSH6 and PMS2) were detectable in these RCC lines. Complete inactivation of MMR was observed to be apparently uncommon in RCC and it occurs predominantly through inactivating mutations in the hMLH1 gene (Leach et al. 2002). HMLH1 and hMSH3 mRNA expression was significantly lower in RCC tissues than in normal tissues. Similarly, nuclear positivity of hMSH3 was significantly lower in RCC tissues than in normal tissues. Moreover, at the mRNA and protein level, hMSH3 expression in high-grade RCCs was significantly lower than in low-grade tumours. However, there was no significant

difference in hMSH2, hMSH6, hPMS1 or hPMS2 expression between RCC tissues and normal kidney tissues. In renal cancer cell lines demethylation with 5-aza-2′-deoxycytidine did not affect the expression of hMLH1 and hMSH3 genes. This study reports the down-regulation of mismatch repair genes in RCC and suggests that a selective defect in some mismatch repair genes can cause genomic instability and activate the malignant transformation as well as the progression of RCC (Deguchi et al. 2003).

Cyclin E and p27 protein. Aberrations in the G1–S transition have been observed in several malignancies, suggesting that cell cycle defects are linked to the activation of oncogenes and inactivation of suppressor genes involved in transformation process. The frequency of G1/S aberrations in human RCC has not been fully clarified. Cyclin E content was studied by using Western blotting in RCC and in corresponding kidney cortex tissues as well as the fraction of p27-positive renal carcinoma cells using immunohistochemistry technique (IHC). Cyclin E level was higher than the median and was associated with aneuploidy, high stage, grade and high erythrocyte sedimentation rate (ESR). Cyclin E inversely correlated with cyclin D1 and positively correlated with cyclin D3. Most tumours (76%) demonstrated a normal fraction of p27-positive cells. There was an inverse correlation between p27 positivity and tumour size, despite the lack of correlation between p27 and tumour cell proliferation. Patients with p27 low tumours had a poor survival rate. There was no correlation between p27 and cyclin E level. In summary, the results suggest that protein expression of cyclin E and/or p27 proteins was linked to tumour behaviour (Hedborg et al. 2002).

Microsatellite analysis and chromosomes instability. Microsatellite (sometimes referred to as a variable number of tandem repeats or VNTRs) are short segments of DNA with a repeated sequence such as CACACACA and they tend to occur in a non-coding DNA region. In some microsatellites, the repeated unit (e.g. CA) may occur four times; in others, it may be seven, two or 30 times. The most common way to detect a microsatellite is to design PCR primers that are unique to a particular locus of the genome and to the base pair on either side of the repeated portion. Therefore, a single pair of PCR primers can work for every individual in the species and produce different sized products for each of the different length microsatellite (Gupta & Varshney 2000).

Loss of various loci on chromosome 9 has been reported in various cancers. To determine the frequency of deletions at different loci of chromosome 9 in RCC, microdissected samples of normal renal epithelium and carcinoma from the same patients were analysed. Expression of p16 protein was absent or low in RCC samples, suggesting that loss of the p16 gene may be involved in RCC. A high incidence of loss of heterozygosity (LOH) on chromosome 9 was observed mainly at the 9p21 and 9p22–23 regions in RCC, suggesting several putative tumour suppressor genes in these regions. The identification of other tumour suppressor genes on the 9p21 and 9p22–23 regions warrants further studies (Grady et al. 2001).

Loss of (part of) the short arm chromosome 3. LOH at chromosome 3p and inactivation of the VHL gene are associated with the development of conventional RCCs. The study revealed allelic loss in 48.7% of informative microsatellite and a single case of

RER (replication error). Higher LOH frequency was observed in 3p25-26 regions, which represents the Von Hippel-Lindau (VHL) oncosuppressor gene. In addition, DNA hypermethylation, an alternative mechanism of VHL gene silencing, was evaluated by methylation-specific PCR. However, hypermethylation status was not detected in any tumour samples. The microsatellite analysis of chromosome 3 (short arm) was studied in sporadic RCC (Girolami et al. 2002). There is a need of studies based on large population so that an association between LOH, stage of disease, tumour size and histological grade can be validated. Analysis of a larger cohort of patients can allow a possibility of correlating the microsatellite instability with a clinical characteristic.

PTEN. PTEN is the phosphatase and tensin homologue deleted on chromosome 10. PTEN regulates cell cycle progression and cell survival *in vivo*. However, the role of PTEN alterations and its association with tumour growth and behaviour in patients with RCC has not been well established. Lee et al. (2003) showed that PTEN expression is frequently reduced in advanced RCC. The PTEN gene seems to be an important marker for the growth suppression of RCC, by inhibiting cell proliferation (Lee et al. 2003).

Multiple drug resistance (MDR) genes. As many as 40–45% of the cancer patients may have or develop resistance to anticancer drugs. Multidrug resistance (MDR) is a phenomenon whereby tumours become resistant to several commonly unrelated drugs, simultaneously. The *MDR*-1 gene encodes an ATP-dependent efflux pump called p-glycoprotein, which may become amplified in drug-resistant tumours. MDR activity may be reversed by drugs like calcium channel blockers (e.g. verapamil), cyclosporin and tamoxifen. Multidrug resistance occurs between several different structurally unrelated anti-tumour agents that apparently have different mechanisms of action. This resistance is obtained through systematic selection and it reflects the amplification of a gene that encodes a transmembrane protein that pumps the drugs out of the cell. Thus, the resistant cell maintains a lower intracellular drug level than the drug-sensitive parental cells. Ling et al. (1998) discovered that cell lines with a high level of resistance produced a large amount of glycoprotein with a glycoprotein of 170 kDa called P170 or P-glycoprotein. It is now clear that P170 is overproduced as a result of gene amplification. The degree of P-glycoprotein overproduction correlates well with the degree of drug resistance. RCC is a unique tumour that often develops a gene mutation that allows it to reject chemotherapy. This MDR gene is an ongoing target of research to determine how it can be deactivated. High levels of *MDR*-1 expression were found in primary tumours originating from tissues that normally express the protein, such as renal cell or adrenocortical cancer. Colon cancer is usually a chemoresistant form of tumour, and MDR-based mechanisms have been suspected to participate in the general unresponsiveness of colorectal tumours to cytotoxics. The most effective drugs in this setting, such as 5-fluorouracil, are not MDR dependent and results of clinical MDR-reversal trials were all disappointing. Now it's clearly apparent that MDR expression can be a marker of the aggressiveness of cells, which are inherently chemoresistant including non-MDR-dependent drugs (Weinstein et al. 1991).

Chromosomal aberrations. Chromosome abnormalities are frequently found in malignant cells. There is evidence that the increased frequency of chromosomal aberration

(CA) in peripheral blood lymphocytes is a predictor of cancer, but further data is needed to characterize better CA as a marker of cancer risk (Rossner et al. 2005).

Jiang et al. (1998) studied chromosomal alterations in 25 papillary RCCs by comparative genomic hybridization. Relative copy number gains were frequently detected at chromosomes 7p, 7q, 12q, 16q, 17p, 17q and 20q. Loss of chromosomal region was observed in 1p, 4q, 6q, 9p and 13q, and Xp, Xq and Y. There were clinical and genetic differences between the subtypes of papillary RCC. Type 2 tumours had higher nuclear grade, stage and a worse prognosis than type 1 tumours. The number of DNA copy gains per tumour at chromosomes 7p and 17p was significantly higher in type 1 than in type 2 tumours. The data suggest the existence of two distinct morphological and genetic subgroups of papillary RCC. Loss of chromosome Xp was associated with a short patient survival rate. Despite the small number of cases, this finding suggests that a gene on chromosome Xp may contribute to papillary RCC progression.

Chudek et al. (1997) studied chromosomal aberration and detected the smallest overlapping region between loci D3S3666 and D3S1560, which corresponds to an approximately 55 cM (recombination between loci) genetic distance.

VHL tumour suppressor gene. RCC develops in nearly 40% of patients with Von Hippel-Lindau (VHL) disease and is a major cause of death among those patients. The relationship between the VHL tumour suppressor gene and sporadic clear cell RCC has been reported (Yao et al. 2002).

LRIG1 and EGFR (epidermal growth factor receptor). LRIG1 is a leucine-rich repeat and immunoglobulin-like domain 1. A recently cloned gene LRIG1 (formerly Lig-1) had a homology with *Drosophila*'s Kek I gene. Kek I encodes a cell surface protein, Kekkon-1, which inhibits epidermal growth factor receptor-mediated signalling (Hedman et al. 2002). Thomasson et al. (2004) observed that the ratio between EGFR and LRIG1 by quantitative RT-PCR was at least two-fold higher than the mean normal ratio in more than 67% of cases.

Proto-oncogene Axl in RCC. Axl (a possible mitogenic factor), along with its ligand Gas-6, plays an important role in programmed cell death. A recent study showed that Axl expressed differentially in RCC and in normal human kidney (Chung et al. 2003).

P16INK4A and p15INK4B gene. A unidirectional change of state in which a cell performs one set of processes shifts to perform different activities from G1 to S, G2 to M, etc., and is essential for cell growth. Previous studies have been done on p16 and p15 gene alteration in association with oxidative stress markers, including inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2). The p16 gene can be altered by mutation loss or silencing of gene expression. The cell phenotype usually shows a reduced checkpoint control, a failure to senesce and predisposed to genomic alterations (Romanenko et al. 2002).

Chromosome 14q LOH. A relationship has been shown between non-papillary RCC and chromosome 14q LOH. Studies show a significant correlation between the chromosome arm 14q LOH, nuclear grade ($p < 0.001$) and stage ($p < 0.001$) of tumours. These observations indicate the presence of a tumour-suppressor gene at

chromosome segment 14q24.2-qter and demonstrate the usefulness of microsatellite analysis for assessing the possible clinical outcome in non-papillary RCC (Herbers et al. 1997).

TAP gene. The transporter associated with antigen processing (TAP) gene product is involved in the processing of endogenous peptides that bind to MHC class I molecules. TAP is a heterodimeric complex transmembrane pump consisting of TAP-1 and TAP-2 subunits, which translocate peptides from the cytoplasm into the lumen of the endoplasmic reticulum (ER), where they bind to nascent MHC class I molecules. Loss or reduced expression of TAP gene results in the synthesis of unstable peptide-free MHC class I molecules that are weakly expressed on the cell surface (Seliger et al. 1997). A possible alteration in the immune tolerance of RCC was shown in the study involving the altered TAP1 gene transfer (Kallfelz et al. 1999).

P16 tumour suppressor gene. Since its discovery as a CDKI (cyclin-dependent kinase inhibitor) in 1993, the tumour suppressor p16 (INK4A/MTS-1/CDKN2A) has gained widespread importance in cancer (Liggett & Sidransky 1998). The frequent mutations and deletions of p16 in human cancer cell lines first suggested an important role for p16 in carcinogenesis. Cairns et al. (1995) observed no additional cases of homozygous deletion or any rearrangements or point mutations of CDKN2/p16. This is the first report of 9p loss of heterozygosity, homozygous deletion of 9p21-22 and selective deletion of 9q in primary RCCs. Recently there was a study performed on the LOH and hypermethylation of p16 gene in which no correlation was found between hypermethylation of p16 gene promoter and LOH on 9p21. The study suggests that inactivation of p16 and the possibility of other unknown tumour suppressor genes located on other chromosomes could be involved in the pathogenesis of RCC (Sanz-Casla et al. 2003).

Loss of TGF-beta receptor in RCC. Genetic mutation or loss of activin/transforming growth factor-beta receptor function is shown in human lymphoid, breast, colorectal tumours, Hep2B and in Mv1Lu cell lines. Copland et al. (2003) observed an alteration in TGF-beta signalling due to the loss of TGF-beta receptor expression in human RCC.

Transcription factor TFEB. TFEB is a member of the MITF/TFE subfamily of basic helix–loop–helix leucine-zipper (bHLH-LZ), which can form both homo- and heterodimers. The previous study concluded that the up-regulation of TFEB was due to promoter substitution; thereby, it severely unbalances the nuclear ratios of the MITF/TFE subfamily members. Possibly this imbalance may lead to changes in the expression of downstream target genes, ultimately resulting in the development of RCC. Interestingly this is the second MITF/TFE transcription factor involved in RCC development and these findings indicate the possibility that the bHLH-LZ subfamily may play a critical role in the regulation of (aberrant) renal cellular growth (Kuiper et al. 2003).

Nuclear Factor-kB (NF-kB). Nuclear factor-kappa B (NF-kB) has been linked to all these cellular processes including regulation of inflammation, cell proliferation and apoptosis in terms of the critical step of gene regulation in different diseases including

cancer, hypertension, rheumatoid arthritis and AIDS (Ditsworth & Zong 2004). Nuclear factor-κB (NF-κB) is a heterodimeric complex, usually consisting of p50 and p65 (RelA) subunits, and it functions as a pleiotropic regulator of many genes modulating immunological and inflammatory processes (Thomas & Maniatis 1995). P50/p65 heterodimer associates with IκB to form an inactive cytoplasmic ternary complex. The p65 subunit may also associate with a precursor protein (p105) of p50 to form an inactive complex. Activation of NF-κB by LPS or cytokines requires either the degradation of its cytoplasmic inhibitor, IκB-α (Beg et al. 1993), or proteolytic cleavage of p105 through the ubiquitin-proteasome pathway after phosphorylation (Palombella et al. 1994). After degradation of IκB-α, an active heterodimer NF-κB translocates into the nucleus and activates gene expression, but in normal cells the NF-κB is maintained in the cytoplasm by protein–protein interaction with inhibitor IκB (Yoshida et al. 1999).

Expression of iNOS has the ability to produce NO at a toxic level and can induce tumour cell death via apoptosis (Xie & Huang 2003). NOS II in macrophages is regulated at the transcriptional level and its regulation requires several transcription factors such as IFN regulatory factor-1 and NF-kB (Adcock et al. 1994, Xie et al. 1994). Tumour cells also express NF-kB (Xie et al. 1996, Xie & Fidler 1998).

Activation of the nuclear transcription factor NF-kB is impaired in T-cells from RCC patients, a phenomenon that contributes to immune dysfunction. Two distinct mechanisms are responsible for the T-lymphocyte NF-kB defect and both are mediated by different kinds of tumour product (Ling et al. 1998). Malhi et al. (2002) observed abnormal expression of NF-kB in peripheral blood T-cells (T-PBL) of metastatic RCC patients.

Molecular markers

Role of genomics and proteomics in prognostic marker development

Proteomics and genomics are becoming a very important part of advanced biomedical sciences research. The advancement in such a branch can create some better outcomes in RCC research (Kashyap et al. 2003).

Role of genomics in RCC. There are few differences between genetics and genomics. This branch of biology combines biology and computer technology. Genetics focuses on the specific gene sequence (structure), mutation and variations to form a snapshot of the individual's potential for a clinical condition. At the same time, genomics takes into account a complex set of genes, identifying not only their sequences (structures), but also how they are expressed and interact with each other to affect how a condition develops.

The applications of genomics to cancer (oncogenomics) may seem straightforward, but the behaviour of cancer is dependent on many different genes, how they interact and the environment they create. It is possible to identify a single gene that may signal a more aggressive type of disease. However, the analysis of a key set of genes, which are expressed by the tumour, can provide far more specific and reliable information. With oncogenomics, it is anticipated that it will be possible to individualize cancer assessment and to improve dramatically the quality of a treatment decision for an individual patient.

Real-time PCR in RCC study. A technique such as real-time PCR proved valuable when studying cancer, as one can quantitate the expression of a gene at the mRNA level using this method. In contrast to regular reverse transcriptase-PCR and analysis by agarose gels, real-time PCR gives quantitative results. An additional advantage of real-time PCR is the relative ease and convenience of use compared with some older methods. Real-time PCR was developed due to following reasons:

- Need to quantitate differences in mRNA expression.
- Availability of only small amounts of mRNA in some procedures such as in the use of: (1) cells obtained by laser capture micro-dissection, (2) small amounts of tissue and (3) primary cells and precious reagents.

In real-time PCR using SYBR green to bind to amplified cDNA, one can simply measure the fluorescence, which increases as the dye binds to increasing amounts of DNA in the reaction tube. When it is bound to ds-DNA, it fluoresces brightly (brighter than ethidium bromide). The other reason to use SYBR green is that the ratio of fluorescence in the presence of ds-DNA to the fluorescence in the presence of ss-DNA is much higher than the ratio for ethidium bromide. An important thing that must be borne in mind when using this technique when studying any disease including RCC or any other type of cancer is the designing of the experiment such as a control versus a treatment group.

The criteria for making primers for real-time PCR are as follows:

- Design primers with a melting temperature (T_m) of 58–60°C. The T_m of both primers should be equal, and probes should have a $T_m = 10$°C higher.
- Primers should be 15–30 bases in length.
- The G+C content should ideally be 30–80%.
- The run of an identical nucleotide should be avoided. This is especially true for G, where runs of four or more Gs are not allowed.
- Total number of Gs and Cs in the last five nucleotides at the 3′ end of the primer should not exceed two (the newer version of the software has an option to do this automatically). This helps to introduce relative instability to the 3′ end of primers to reduce non-specific priming. The primer conditions are the same for SYBR green assays.
- Maximum amplicon size should not exceed 400 bp (ideally 50–150 bases). A smaller amplicon gives more consistent results because PCR is more efficient and more tolerant of reaction conditions (the short length requirement has nothing to do with the efficiency of 5′ nuclease activity).
- Probes should not have runs of identical nucleotides (especially four or more consecutive Gs), and the G+C content should be 30–80%. There should be more Cs than Gs, and G should not be at the 5′ end.
- To avoid false-positive results due to amplification of contaminating genomic DNA in the cDNA preparation, it is preferable to have primers spanning exon–exon junctions in the cDNA sequence. This way genomic DNA will not be amplified.

DNA microarray technology in RCC. There are two major types of DNA microarray technology: (1) the identification of sequence (gene–gene mutation) and (2) the determination of expression levels (abundance) of genes. For more information about

the microarray, see http://www.gene-chips.com. The microarray is an integration of a conventional technique and computer technology. It has been implemented in RCC and the expression of 7129 genes detected in both clear cell RCC tissue and cell lines using oligonucleotide arrays. Seventy-four commonly differentially expressed genes with more than five-fold changes in RCC tissues were identified. The expression alterations of selected genes from these 74 genes were further verified using a reverse transcription polymerase chain reaction (RT-PCR). Detailed comparisons of gene expression patterns in RCC tissue and RCC cell lines show significant differences between the two types of samples, but many important expression patterns were preserved. Most notably, genes involved in cell adhesion were up-regulated whereas genes involved in transport were down-regulated. This study reveals significant gene expression alterations in key biological pathways and provides potential insights into the understanding of the molecular mechanism of renal cell carcinogenesis (Liou et al. 2004).

More sophisticated multiplex panels have emerged from work with microarray. One such example is the the Netherlands breast cancer study (Van't Veer et al. 2002), which sought to distinguish between patients with the same stage of disease but a different response to treatment and overall outcome. The success of this initial study motivated a more extensive independent follow-up study involving 295 patients (Van de Vijver et al. 2002), which led to a nationwide clinical trial in the Netherlands in which gene expression profiles for 70 classifier genes were being collected on all breast cancer patients and used as an adjunct to classical clinical staging. The belief that this phenomenon will be general for both proteins and mRNA, and that combinations of markers can be found to identify and stage a wide range of diseases with useful specificity and sensitivity, is among the most important hypotheses of current biomedical research.

Thus, a major challenge is to decide when and upon whom these effective interventions should be carried out. A patient showing chest pain may have an acute myocardial infarction that requires immediate PCI or tPA treatment, stable angina requiring nitroglycerine and oesophageal spasm with no cardiovascular consequences, etc.

Role of proteomics

The key concept of proteomics (looking at many proteins at once) opens new avenues in the search for clinically useful biomarkers of disease, treatment response and ageing (Gorg et al. 2000). Only a few techniques are specially implemented and become the choice of the researchers in proteomics including two-dimensional PAGE and mass spectrometry.

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) is one of the main techniques used to separate hundreds to thousands of polypeptides from cultured cells and tissues. It is a method of protein separation by which proteins from a mixture are separated according to their isoelectric point (pI) in the horizontal direction (isoelectric focusing [IEF]) and molecular weight in the vertical direction (sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE]).

Recently, 2D PAGE and peptide mass fingerprinting with MALDI-TOF/MS were used together in RCC, and quantitative spot analysis showed a significant up-regulation of the tumour rejection antigen-1 (TRA1, grp94, gp96; 4.7-fold) and vimentin (4.0-fold) in the RCC lesions and a 3.9- and 3.7-fold down-regulation of the

ribosomal P0 protein and aminoacylase-1, respectively. Proteomweaver is a software solution used in the analysis of fast and precise analysis of 2D gel. It automatically detects significantly changed proteins, which is an important first step towards the understanding of pathological pathways in RCC. A better understanding of these pathways is necessary to focus more closely on promising targets in the drug development process. Identification of metabolic enzymes and markers for the selection of patients to undergo RCC-specific immunotherapy has been performed using the PROTEOMEX technique (Lichtenfels et al. 2003, Seliger et al. 2003).

Characterization of altered proteins in renal cancel cells can be very useful in providing insight into the changes in the functional pathways and thus the fundamental mechanisms of cancer development at the molecular level. A recent study in an RCC cell line was performed and 16 over-expressed and seven under-expressed proteins were identified. Over-expression of three proteins, alphabeta-crystallin, manganese superoxide dismutase (MnSOD) and annexin IV, most commonly observed in primary RCC cell cultures, was also observed by immunoblot analysis of proteins from the RCC tissues from which the primary cell cultures were derived (Shi et al. 2004).

Agmatinase is an enzyme that hydrolyses agmatine to putrescine and urea. Using mass spectrometry and a database search, it was shown that agmatinase was down-regulated in tumour cells and its decreased amount was also reported by real-time PCR. Immunohistochemical studies also revealed the heterogeneous distribution of agmatinase in the infected kidney (Dallmann et al. 2004).

Mass spectrometry (MS). MS is a powerful technique to identify proteins. As the number of proteins that can be detected in plasma or serum (the primary clinical diagnostic samples) increases to 1000, a paradoxical decline has occurred in the number of new protein markers approved for diagnostic use in clinical laboratories.

A set of 177 candidate biomarker proteins with reported associations to cardiovascular diseases and stroke are presented as a starting point for such a 'directed proteomics' approach.

The heterogeneity of disease processes and the genetic differences among individuals in the human population both tend to obscure what might otherwise be clear disease associations. However, if there are multiple markers affected by the disease which are not strongly correlated with each other, then a composite index combining these markers may provide a much more robust indication of disease. In measuring the acute phase response, e.g. a composite index summarizing a panel of weak acute-phase reactants (Doherty et al. 1998) can provide a more robust indicator of inflammation than a single marker (e.g. C-reactive protein or serum amyloid A). Similarly the relative risk of coronary heart disease (CHD) was better predicted by CRP (Rifai & Ridker 2003).

Future directions and conclusions

The recommendations for the prevention of RCC are therefore similar to those for the prevention of cardiovascular diseases and cancers, and should be disseminated to the general population. The high-risk groups identified are too large for a specific early-screening programme for RCC, but such screening might be appropriate if restricted to selected age groups. In the case of RCC, no definitive biomarker is available for its

Table III. Potential biomarkers and their significance in RCC.

Biomarkers	Significance in RCC	References
CA-125 and CA-15-3	Possible as an adjunct in the staging of RCC	Tsakalou et al. (1993)
Calretinin	Only marker that appears to have any utility in distinguishing between sarcomatoid mesotheliomas and sarcomatoid RCCs	Ordonez (2004)
CD10	It is a better marker for distinguishing between a hemangioblastoma and a metastatic clear cell RCC	Jung and Kou (2005)
CD44	More sensitive in serum compared with urine cytology samples and can be a potential a new tumour marker for early and non-invasive diagnosis of bladder cancer	Wu et al. (1997)
Gamma enolase	Tumour marker for the stage of disease and monitoring treatment in patients with RCC	Takashi et al. (1993)
IGF-IR	Expression is associated with CC-RCC survival and could potentially represent a molecular avenue for therapeutic intervention.	Parker et al. (2003)
Microsatellite marker analysis	More sensitive than conventional urine cytology in detecting bladder cancer cells in urine and represents a potential clinical tool for monitoring patients with low-grade/stage TCC	Seripa et al. (2001)
NMP-22	Food and Drug Administration (FDA)-approved urinary NMP-22 for monitoring the patients with transitional RCC	Zippe et al. (1999)
Nuclear grading	More sensitive method on histological methods	
TATI	TATI can be used as a serum marker (its more sensitive) for RCC	Meria et al. (1995)
TPS	TPS may have a potential clinical role as a valuable tumour marker for RCC, especially in advanced disease and follow-up therapy response	Chang et al. (2002)
TS	TS activity may be associated with malignant potential of RCC, and it may be possible to use 5-FU as an antineoplastic drug for RCC with high TS activity	Mizutani et al. (2003)

diagnosis and monitoring. Thus, we must find new specific biochemical and molecular markers that reflect the biological activities of RCC. The advantages of different potential markers for RCC are summarized in Table III.

In conclusion, the results with RCC tumour markers look very promising. Future directions could probably be a search for tumour markers, which have the best reliability and are expressed not only in RCC, but also in many different types of cancer. Creating novel approaches towards the early diagnosis of cancer and the development of cancer vaccines, such as dendritic cell vaccines (Engleman 2003), could bring scientists closer to eliminate a deadly disease. Modern techniques such as DNA microarray and mass spectrometry can help us identify the potential and promising molecules to detect RCC at an early stage. The proper use of gene microarray and the designing of experiments are critical factors, as identification of novel molecules fully depends on the experimental design between the controls versus treatments.

As science progresses, the system becomes increasingly more complex and therefore there is an increasing possibility of making a mistake in our judgement, which will be greatly amplified by modern means of communication. That is why in order to reduce mistakes, it is necessary to pay attention that the analysis of patient material for all known markers must be done, if at all possible, at the same time or immediately after patient examination by a medical doctor, and that markers are indeed related to the

dynamic of interaction of tumour with the host. It is better to find a marker that can be detected by methods of cell biology, but which also has a strong basis in medical/anatomical observations. The dynamics of metastasis is the most unpredictable factor. It is related rather not to the question of how normal cells become malignant, but to the evolution of cancer as a self-organized complex parasitic system inside the human organism. There is a large amount of data available, which is why a mathematical model of statistical analysis must be used by taking help from the theory of informatics. This will help not only in the checking about whether our up-to-date information about cancer is true and accurate, but also in obtaining new ideas by observing new connections between markers. Also, if we want to advance such a kind of research, we must understand complex diseases such as cancer in the context of its relation between nature, society and the individual to direct the research positively. This investigation must be done to help us finally win the battle with deadly diseases (Emelianenko 2005).

During the last decade, the application of advanced molecular biology techniques in medicine has allowed a deeper understanding of carcinogenic pathophysiological mechanisms. A large number of molecules involved in those mechanisms have been studied as potential markers for the diagnosis, recurrence and prognosis of RCC. It has been proved that enough of them give satisfactory rates for specificity, but not for sensitivity. Also, some of these markers still have to be confirmed in multivariate analysis. Research continues to seek possible combinations of molecular markers with diagnostic and prognostic applications aimed at identifying a group of patients that need a more intensive treatment and subsequently it can help the overall survival rate of RCC patients.

Acknowledgement

Part of the study was supported by a grant from PGIMER to S. K. S. The salary support from PGIMER, Chandigarh, to M. K. K. is greatly acknowledged. The authors thank Dr Eleni Karapanagiotou, Athens Medical School, Sotiria General Hospital, Athens, Greece, for providing the literature regarding molecular markers. They are also grateful to the American Association for Cancer Research (AACR) for giving permission to use images from *Clinical Cancer Research*. They also thank Dr Oscar S. Frankfurt, Cedars Medical Center, Miami, USA, for providing a reprint of his paper and for timely advice on figures for F7-26 labelling in carcinoma. They also thank Dr Ashok Chauhan, John Hopkins School of Medicine, Baltimore, USA, for critically reading the manuscript. They also thank their colleagues, especially Janus Lori and Christina Rothman, also for critically reading the manuscript. The authors apologize to their colleagues whose work could not be cited because of space constraints as it was tried to include the majority of references after 2000 only.

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