Immunomic analysis of human renal cell carcinoma

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Gerard Devitt

aus Cork, Irland

Dekan: Prof. Dr. Manfred Kappes
Referent: Prof. Dr. Margot Zöller
Korreferent: Prof. Dr. Jonathan Sleeman
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For James, Roseanne, Niall, Owen and Nadia

Das Nierenzellkarzinom ist der am häufigsten in den Nieren vorkommende Tumor. Er macht bis zu 3% aller malignen Erkrankungen bei Erwachsenen aus. Die Zahl der Nierenzellkarzinompatienten steigt jährlich. Bisher gibt es keine international standardisierte Therapie für das metastasierende Nierenzellkarzinom und die vollständige Entfernung der betroffenen Niere ist zur Zeit die einzige Behandlungsmöglichkeit. Das Nierenzellkarzinom ist sehr resistent gegen Bestrahlung und Chemotherapie, zeigt aber in manchen Untersuchungen eine spontane Regression, die auf Immunogenität des Tumors schließen lässt. Die Identifikation von nierenzellkarzinomassoziierten immunogenen Molekülen ist daher wichtig, um 1.) die grundlegenden Mechanismen der Immunogenität des Nierenzellkarzinoms zu verstehen, 2.) für mögliche neue Ansätze in Diagnose, Prognose und Therapie.

In dieser Arbeit wurden drei Techniken eingesetzt, um nierenzellkarzinomassoziierte Gene und Antigene zu erkennen: 1) Suppression substractive hybridization (SSH), 2) Serological analysis of recombinant cDNA expression libraries (SEREX), 3) Serum antibody detection array (SADA).

Durch die SSH-Methode konnte gezeigt werden, dass zwei Gene (BACE2 und SUPT5H) in 50% der getesteten Nierenzellkarzinompatienten stark überexprimiert sind. In 35 Nierenzellkarzinompatienten wurde das Expressionsmuster von neun anderen durch SSH identifizierten Genen (Semaphorin G (SemG), lysyl oxidase (LO), autotaxin (ATX), angiopoeitin-related protein (ARP2), ceruloplasmin (CP), vascular endothelial growth factor (VEGF), C1 respiratory complex (C1-RC), cyclin D1 und insulin-like growth factor binding protein-3 (IGFBP3) untersucht. Einige dieser Gene sind in 80-90% der untersuchten Nierenzellkarzinome überexprimiert, ein Hinweis auf die Wichtigkeit dieser Gene in der Tumorprogression. Hochregulierung von C1-RC, SUPT5H, VEGF, ARP2, ATX, LO und SemG korreliert mit der histologischen Untergruppe des „clear-cell“-Nierenzellkarzinoms, während BACE2 vermehrt im „mixed“- und „oxyphil“-Typ überexprimiert wird.

Der Differenzierungsgrad des Tumors steht in keinem Zusammenhang zur Überexpression der Gene, nur Hochregulierung von ATX und LO korreliert mit erhöhter Tumorprogression.

Die SEREX-Methode wurde angewandt, um Bibliotheken von Lambda-Phagen zu testen, die hergestellt wurden, aus 1) Nierenzellkarzinomgewebe, 2) Nierenzellkarzinom-Zelllinie (KTCTL-28) behandelt mit 5-aza-2´deoxycytodine, 3) Gewebe männlicher Keimzellen

Aus 4,5x 10^6 gestesteten Plaques resultierten 234 positive Klone, die 74 verschiedenen Genprodukten entsprechen. Anhand von Sequenzanalysen und Literaturrecherche konnten diese Antigene in folgende Kategorien eingeteilt werden: Cancer-Testis-Antigene, Antigene mit eingeschränkter Expression, Antigene mit heraufregulierter Expression, Antigene mit Chromosomentranslokationen, normale Autoantigene und unbekannte Antigene.

Seroreaktivität einige dieser Antigene war beschränkt auf Nierenzellkarzinompatienten. Dies kann als diagnostischer Marker oder potentiell in der Therapie eingesetzt werden.

Mit der SADA-Methode wurde Seroreaktivität der Nierenzellkarzinompatienten-Seren gegen 44 bekannte Antigene untersucht. 8 Antigene reagierten nur mit Seren von Nierenzellkarzinompatienten und nicht mit Seren von Personen der gesunden Kontrollgruppe.
Seroreaktivität gegen drei der Antigene (Par-3, se57-1 und HEXIM1) war in einem wesentlichen Anteil der Patienten (11-23%) zu verzeichnen und könnte als diagnostischer Marker Verwendung finden.

Renal cell carcinoma accounts for up to 3% of all adult malignancies and is the most common neoplasm in the adult kidney. The incidence of RCC is increasing yearly. There is no internationally standardized treament for metastatic RCC and the only curative option for localized RCC is radical nephrectomy. RCC is known to be highly chemoresistant and radioresistant. However, it is thought to be immunogenic, as there are frequent reports of spontaneous regression. Identification of RCC associated immunogenic molecules is therefore a priority in order to 1) understand the underlying mechanisms of immunogenicity of RCC and 2) to identify potential diagnostic, prognostic and therapeutic targets.

In this thesis, three techniques have been used to identify RCC associated genes and antigens: 1) Suppression subtractive hybridization (SSH) 2) Serological identification of antigens by recombinant expression cloning (SEREX) and 3) Serum antibody detection array (SADA) analysis.

Through SSH, two genes (BACE2 and SUPT5H) could be shown to be highly upregulated in the tumors of approximately 50% (for both) of all RCC patients tested. The expression profile of 9 other genes : semaphorin G (SemG), lysyl oxidase (LO), autotaxin (ATX), angiopoeitin-related protein (ARP2), ceruloplasmin (CP), vascular endothelial growth factor (VEGF), C1 respiratory complex (C1-RC), cyclin D1, and insulin-like growth factor-binding protein-3 (IGFBP3), previously identified by SSH was also examined in 35 RCC patients. Overexpression for some of these genes was found in 80-90% of RCC thus indicating the importance of these genes in tumor progression. Upregulation of C1-RC, SUPT5H, VEGF, ARP2, ATX, LO and SemG appear to correlate with the clear cell RCC histological subtype whereas upregulation of BACE2 has been found more in the mixed and oxyphil type. There was no correlation of gene overexpression with tumor grading and only ATX and LO showed a correlation with tumor progression.

The SEREX method has been used here to screen three λ-phage libraries derived from 1) an RCC tumor, 2) an RCC cell line (KTCTL-28) treated with 5-aza-2'-deoxycytidine and 3) testis tissue. Approximately 4.5 x 10^6 phage plaques were screened resulting in 234 positive clones. This corresponded to 74 different gene products. Through sequence analysis and examination of the literature, these antigens could be grouped into the following categories: Cancer/ Testis, Restricted expression, Upregulated antigens, Translocation antigens, Normal/ autoantigens and Unknown antigens. Seroreactivity to some of these antigens was restricted to RCC patients and may serve as diagnostic markers or potential therapeutic targets.

Finally, seroreactivity against a panel of 44 antigens was examined with RCC sera via the SADA method. In total, 14 antigens were found to react with RCC sera. Of these, 8 were reactive only in RCC patients and not in healthy controls. Seroreactivity to 3 of these antigens (Par-3, se57-1 and HEXIM1) was found in a significant proportion of RCC patients (11-23%) and may be suitable diagnostic markers.

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1. Introduction

1.1 Renal Cell Carcinoma

1.1.1 Etiology

Renal cell carcinoma (RCC) accounts for approximately 3% of all cancer related deaths. In Germany RCC is the eighth most common cause of male cancer. In the United States RCC accounted for over 31000 new cases and led to approximately 12000 deaths in 2001. The high mortality rate is due to the fact that over one third of RCC cases present with metastatic disease and 30% of those treated for localized disease eventually relapse. The causes of RCC have not been investigated as frequently as lung, breast and colon cancers. However, a number of risk factors have been identified.

1.1.2 Risk factors

Smoking. One-third of all cases of renal cancer are attributed to cigarette smoking (Motzer et al. 1996). Increased risks have been found in long-term or heavy smokers. As with other cancers, the risk declines within a few years after the cessation of smoking (McLaughlin et al. 1995).

Nutrition. Diet has been shown to be a major factor in the causes and prevention of kidney cancer. The influence of diet can best be appreciated by comparing international rates of kidney cancer. Asian populations have a 5-8 fold lower incidence than Western countries. Fried meats, especially poultry seem to be associated with an increased risk of RCC (Wolk et al 1996) while the consumption of fruit and cruciferous vegetables has been shown to have a protective effect (Wolk et al 1996, Yuan et al 1998).

Obesity. High body weight or body mass index has been shown to be a risk factor for renal cell carcinoma, particularly in females (Motzer et al. 1996). This may be due to the fact that obesity increases estrogen production which has been shown to promote kidney tumors in hamsters (Liehr 1997).

Drugs and alcohol. The use of antihypertensive drugs are considered a risk factor for RCC (Motzer et al. 1996; McLaughlin et al. 1995). However, it was reported that the independent effects of treatment for hypertension could not be distinguished from the effects of hypertension itself. Alcohol itself has not been demonstrated to be a risk factor for RCC despite the correlation of kidney cancer mortality rates with per capita intake of alcohol (Breslow et al 1974).

Occupation. A number of studies have demonstrated exposure to asbestos and cadmium as risk factors for RCC (Mandel et al. 1995, Pesch et al 2000). Increased risks have also been linked to exposures in coke production, the iron and steel industry, chemical cleaning solvents, engine fuels, and other petroleum products.

Hormonal factors. The IRCC study (Lindblad et al 1995) has demonstrated that risk of kidney cancer increases linearly with two or more births, compared to one birth. Unrelated factors were age at menopause and estrogen replacement therapy. An increased risk was observed for women having had both a hysterectomy and an oophorectomy. Use of oral contraceptives in non-smoking women reduced the risk of renal-cell cancer.

Genetic factors. There are four major hereditary types of renal cell carcinoma. The most studied being von Hippel Lindau disease (VHL). VHL is caused by mutations in the *VHL* gene located on chromosome 3p (Latif et al 1993). Less common is hereditary clear cell renal cell carcinoma (HCRC). This is caused by translocations of the short arm of chromosome 3p (Cohen et al 1979). The third and fourth hereditary forms are hereditary papillary renal carcinoma (HPRC), characterized by germline mutations in the *met* gene (Schmidt et al 1997), and hereditary renal oncocytoma (HRO) which has only relatively recently been described

(Weirich et al 1998). All of the hereditary forms appear to occur at a younger age than sporadic renal cancers (Linehan et al 1995) and are usually bilateral and multifocal.

1.1.3 Prognostic factors

Histology. There are four major histological classifications for RCC (Table 1). Clear cell RCC are the most common accounting for 70-80% of all RCC. They arise in the proximal tubules and on gross inspection have a yellowish appearance due to their high lipid content (Storkel et al 1997). Papillary RCC are the second most common at 10-15%. The architecture of these tumors may be papillary, tubular, tubulo-papillary, or solid (Zambrano et al 1999). These are also thought to arise from the proximal renal tubular epithelium. Chromophobe tumors account for 5% of RCC and arise from the intercalated cells of the collecting ducts. Genetically, chromophobe tumors are characterized by monosomy of multiple chromosomes, including, 1, 2, 6 and 10 (Pantuck et al 2001). Collecting duct carcinomas is a very aggressive form but are rare and account for less than 1% of RCC. Finally, unclassified RCC encompasses a heterogeneous group of tumors that includes tumors with extensive necrosis and minimal viable classifiable tumor, mucin-producing tumors, and tumors with sarcomatoid change in which the epithelial elements cannot be classified (Amin et al 2002).

Tumor stage. Tumor stage is considered one of the most important prognosticators for RCC. There are two forms of staging in current use, the Robson classification and the Tumor-Node-Metastasis (TNM) system (Table 2). According to the TNM system, the T value reflects the size of the tumor, N reflects the number of metastasis to lymph nodes, and M reflects the number of distant metastasis. From these three categories, RCC can be divided into four stages (Table 2).

Histological type	Occurrence (% of RCC)	Origin	Variants
Clear cell	70-80	Proximal tubules	A) Sporadic. Up to 60% with VHL mutation B) Hereditary. Up to 40% with VHL disease will develop clear cell RCC
Papillary	10-15	Proximal tubules	A) Type 1-basophilic cytoplasm B) Type 2- eosinophilic cytoplasm
Chromophobe	3-5	Intercelated cells of collecting ducts	A) Typical/Classical B) Eosinophilic/Granular
Collecting duct	< 1	Medulla of collecting ducts	Usually seen in African Americans with sickle cell disease or trait
Unclassified RCC			For any tumors not fitting the above histological types

Table 1. Summary of histological classification of RCC

T1a	Tumor less than or equal to 4cm and limited to the kidney
T1b	Tumor greater than 4cm but less than 7 cm and limited to the kidney
T2	Tumor greater than 7cm and limited to the kidney
T3	Tumor extends into major veins, or invades adrenal gland or perinephric tissues, but confined to Gerota's fascia
T3a	Tumor invades adrenal gland or perinephric tissues, but confined to Gerota's fascia
T3b	Tumor extends into renal vein or inferior vena cava below the level of diaphragm
T3c	Tumor extends into renal vein or inferior vena cava above the level of diaphragm
T4	Tumor extends to Gerota's fascia
N0	No regional lymph node metastasis
N1	Metastasis to a single regional lymph node
N2	Metastasis to more than one regional lymph node
M0	No evidence of distant metastasis
M1	Distant metastasis

Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1, T2	N1	M0
	T3	N0,N1	M0
Stage IV	T4	N0,N1	M0
	Any T	N2	M0
	Any T	Any N	M1

Table 2. TNM staging

Tumor grade. Tumor grade is the second most important prognosticator for RCC. The grade reflects the nuclear morphology. The current system in use is the Fuhrman system (Table 3). Unfortunately, controversy still exists concerning the inter-observer reproducibility of grading and the 1997 UICC/AJCC conference was unable to improve the system.

Grade	Nuclei Characteristics	Size
1	Round and uniform contour	10 μm nuclei
2	Irregular contour	15 μm nuclei
3	Very irregular contour	20 μm uclei
4	Bizzare and irregular	At least 20μm

Table 3. The Fuhrman grading system

Molecular markers. Molecular markers that have been shown to have some prognostic value in RCC include DNA ploidy (Ljungberg et al 1986)**,** cytogenetic changes such as deletions or translocations of chromosome 3p (Elfving et al 1997), increased levels of the proliferation markers proliferating cell nuclear antigen (PCNA) and Ki-67 (Onda 1999), and increased levels of apoptosis related molecules such as *bcl*-2 (Vasavada et al 1998).

1.1.4 Treatment

Surgical. Radical nephrectomy is the current established form of treatment for localized unilateral RCC. However recent studies have shown that nephron-sparing surgery (NSS) can be equally as beneficial for patients whose tumors are localized and no bigger than 4 cm. It has also been shown that NSS provides a long-term renal functional advantage over radical nephrectomy (Steinbach et al 1992). In the case of metastatic RCC, radical nephrectomy is only used in a palliative manner.

Chemotherapy and Radiotherapy. RCC has proven to be highly resistant to both chemotherapy and radiotherapy. The only cytotoxic agents that have proven to be someway effective are 5-fluorouracil (5-FU) and floxuridine. Both of these appear to be more active when used in conjunction with immunomodulators such as interleukinn-2 (IL-2) (Yagoda et al 1995)

Immunomodulators. Objective responses of 15% has lead to the approval by the USA Food and Drug Administration (FDA) of IL-2 for the treatment of metastatic RCC (Fyfe G et al 1995). However the high-dose bolus treatment is itself highly toxic with a treatment related mortality of up to 4%. Recent clinical trials have tried to reduce the toxicity via continuous high-dose intravenous infusion or subcutaneous infusion. A clear decrease in toxicity as well as comparable response rates were found (Yang et al 1997, Heinzer et al 2001). However, no randomized study of sufficient size has been published to date. The use of IFNα has been extensively examined as a treatment option for RCC by itself and in combination with other chemo- and immunotherapeutic agents. In two large trials, IFNα alone was reported to have a small but significant effect (Ritchie et al 1998, Pyrhonen et al 1996). However, to date, there has been no sufficiently large randomized Phase III trial showing a survival benefit for combination therapy compared to single-agent interferon.

Cellular therapy. Cell based therapies include the use of lymphokine activated killer (LAK) cells, tumor infiltrating lymphocytes (TIL), allogenic stem cells (allo-SCT) and dendritic cells. Despite promising *in vitro* results with LAK and TILs, randomized trials have shown no clinical benefit for these two approaches (Hoffman et al 2000). In two studies allo-SCT treatment after non-myeloablative chemotherapy has shown objective response rates of up to 53% (Childs et al 2000, Rini et al 2002). However, substantial toxicity due to graft-versus-host disease was encountered. This high toxicity may be reduced by first depleting the graft of T-cells and is currently under investigation. Studies involving dendritic cells are only at a relatively early stage and trials so far have only been able to show the safety and feasability of DC-based vaccines.

Monoclonal antibody therapy. The G250 antigen, also known as carbonic anhydrase 9, is expressed on more than 75% of primary and metastatic RCC (Oosterwijk et al 1986) but not on normal kidney tissue. This makes it a promising candidate for directed immunotherapy. Clinical trials using Iodine-131 labelled G250 antibodies have thus far demonstrated its specific antitumor effect (Divgi et al 1998).

1.2 Tumor immunology

1.2.1 Tumor Antigens

Rejection of tumors can only occur if the immune system recognizes the tumor as foreign. This recognition is mediated through antigens on the surface of the tumor. Different types of tumor antigen specificity exist (Table 4).

Antigen Type	Example	Reference
Cancer/Testis	MAGE-A, RAGE, NY-ESO-1	van der Bruggen et al 1991, Gaugler et al 1996, Jäger et al 1998
Differentiation	Tyrosinase,	Brichard et al 1993,
Mutation (point or frameshift)	p53, CDX2	Scanlan et al 1998, Ishikawa et al 2003
Translocation	DEK-CAN, BCR-ABL	Ohminami et al 1999, Berke et al 2000
Splice Variant	cTAGE	Usener et al 2003
Post-translational modification	Cytochrome-c	Mamula et al 1999
Post-translational protein splicing	Fibroblast growth factor 5	Hanada et al 2004
Gene amplification/overexpression	Her-2/neu	Hynes et al 1994
Cancer related autoantigen	CEBP	Tureci Ö et al 1999
Cancer independent autoantigen	U1-snRNP	Chen et al 1997
Viral	HERV-K10	Tureci et al 1997

Table 4. Classification of tumor antigens

Cancer/Testis antigens As the name suggests, Cancer/Testis antigens (CT-antigens) are expressed only in the testis and in various tumor types. Many of the CT-antigens are shared between different tumor types. For example, the MAGE-A genes have been found in malignant melanomas and carcinomas of the lung, head and neck as well as urinary bladder (Jungbluth et al 2000). The coincident expression of CT-antigens in cancer and testis is thought to be due to hypomethylation of CpG islands which is constitutive in testis but also occurs in tumors (De Smet et al 1996, Erickson et al 1993). Demethylation of CpG islands leads to the expression of genes that would normally be silent. With few exceptions (SCP-1 Kondoh et al 1997), most CT-antigens are localized to the X chromosome. Many of the CT-

antigens belong to multigene families. The MAGE A family contains 15 family members (van der Bruggen et al 1991) and the closely related MAGE-B family has 17 members (Muscatelli et al 1995). The natural function of most, again with the exception of SCP-1 (involved in the correct pairing of homologous chromosomes during meiosis, Meuwissen et al 1997), of the CT-antigens is unknown. However, they remain the prime candidates for immunotherapeutic strategies against cancer due to their restricted expression and the immune-privileged nature of the testis. Currently there are several clincal trials involving MAGE peptides for the treatment of malignant melanoma. In one trial with MAGE 3 peptides in HLA A1 patients, seven of 25 patients showed significant tumor regression, including three cases of complete remission (Marchand et al 1999).

Differentiation antigens Differentiation antigens are antigens derived from genes that show a lineage-specific expression in tumors and in the normal cells of the same origin. The tyrosinase gene is one example. It is expressed normally by melanocytes and is involved in the production of melanin. However, it has also been shown to induce both cellular and humoral immune responses in malignant melanoma patients (Coulie et al 1994, Sahin et al 1995).

Mutated antigens Mutations can arise from point mutations resulting in the change of a single amino acid or frameshifting where the open reading frame of the protein is shifted. Mutated antigens are generally unique and restricted to the patient in which the mutation arises. Therefore these antigens are unsuitable for immunotherapy purposes.

Translocation antigens Translocation between chromosomes is a common occurrence in neoplastic cells. New fusion proteins can result from these translocations and these are very often immunogenic. The BCR/ABL fusion protein is a result of a translocation between chromosomes 9 and 22. This translocation is particularly associated with Chronic Myelogenous Leukemia (CML). Recently, a clinical trial using a specific BCR/ABL inhibitor (Imatinib mesylate) has shown complete hematologic response in 98% of the patients treated and a complete cytogenetic response in 72% (Duffy 2003). Cancer related fusion proteins represent ideal targets for immunotherapy due to their expression solely on the tumor.

Splice variants Splice variants are also likely to be immunogenic as has been shown for the Hodgkin's disease associated antigen restin (Sahin et al 1995).

Post translational modifications Antigens resulting from aberrant post translational modification of proteins are also likely to be highly immunogenic. Post translational modifications include acetylation, glycosylation and isoaspartylation. For the latter case it has been demonstrated that isoaspartylation of cytochrome c peptides results in the induction of strong B and T cell autoimmune responses in mice (Mamula et al 1999). However the identification of such antigens is difficult due to the fact that antibodies tend to be cross reactive with both the wild type and modified forms (Mamula et al 1999).

Post-translational protein splicing Post translational protein splicing has only been recently described (Hanada et al 2004). It represents a completely new class of antigen. It also has major implications for the proteome and immunome alike. Hanada and colleagues found that a CTL directed against Fibroblast growth factor-5 (FGF-5) was specific for a nonapeptide that could only have been generated by splicing together two parts of the protein that are normally 60 amino acids apart. The question remains as to how often this occurs and to what purpose.

Gene amplification/overexpression Overexpression of gene products through gene amplification, increased induction or stability of mRNA or the protein product can result in the induction of an immune response due to the overriding of critical thresholds which are necessary for the maintenance of tolerance. Overexpression of genes is a common event in cancer and often these genes are involved in some aspect of tumor progression such as apoptosis inhibition, proliferation, adhesion or migration. Some examples of overexpressed genes acting as antigens include Aldolase A in lung carcinoma (Güre et al 1998), elF-4γ in small cell lung carcinoma (Brass et al 1997), carbonic anhydrase XII in renal cell carcinoma (Türeci et al 1998) and Her-2/neu in breast carcinoma (Disis et al 1994). The recently developed trastuzumab is a humanized monoclonal antibody against Her-2/neu. Clinical trials with trastuzumab alone or in combination with other chemotherapeutic drugs have shown substantial benefits for patients with metastatic breast cancer (Vogel et al 2003).

Cancer related autoantigen Cancer related autoantigens are genes that are ubiquitously expressed and at similar levels in both normal and neoplastic tissues. However, antibodies against these genes are only generated in cancer patients. The mechanism involved here is unclear but may be due to changes in antigen presentation and/or processing in tumor cells.

Cancer independent autoantigen The presence of antibodies against cancer independent autoantigens is not related to neoplastic disease and are found in equal proportions of healthy individuals and cancer patients. The role of these autoantigens in the immune system is unclear. One suggestion is that these autoantigens maintain and regulate immunological homeostasis via $CD4^+CD25^+$ regulatory T cells (Nishikawa et al 2003).

Viral antigens Viral antigens are ideal candidates for immunotherapy or preventative vaccination. One example identified so far is that of HERV-K10 in renal cell carcinoma (Türeci et al 1997).

1.2.2 Methods for the identification of tumor antigens

The repertoire of tumor antigens recognized by the immune system is referred to as the cancer immunome. The immunome comprises antigens defined by T cell epitope cloning, MHC peptide elution and serological methods such as SEREX (serological analysis of recombinant cDNA expression libraries) or SERPA (serological proteome analysis). Other techniques which may sometimes identify antigens include suppression subtractive hybridization (SSH), differential display RT-PCR (DD-RTPCR) and DNA microarray hybridization.

T cell epitope cloning T cell epitope cloning was pioneered by the group of Thierry Boon and led to the identification of the first human tumor antigen in 1991 (van der Bruggen et al 1991). Since then many more antigens recognized by cytotoxic T lymphotes (CTLs) have been identified. A database compiling all CTL identified antigens to date has been established and is located on the internet at www.cancerimmunity.org/peptidedatabase/Tcellepitopes. In this method a cDNA library is generated from a tumor and transfected into COS-7 or 293 cell line cotransfected with the MHC of the original tumor. The cDNA encoding the tumor antigen is then determined by the response of autologous CTLs against the transfected COS-7 or 293 cells. T cell epitopes can then be identified by generating synthetic peptides from the identified gene that fit the MHC of the original tumor and looking for responses of the CTLs to peptide loaded APCs. Some drawbacks to this method include the difficulty in establishing autologous CTLs in non melonoma tumors and the requirement for identifying the MHC of the patient in question.

MHC peptide elution This is a more direct approach to the identification of MHC restricted epitopes. It involves the acid elution of peptide-MHC complexes from the surface of tumor cells and subsequent seperation by reverse phase high performance liquid chromatography (RP-HPLC). Each fraction is then sequenced by mass spectrometry. Peptides identified by this manner still have to be tested for their ability to stimulate CTLs. One advantage however, is the fact that peptides identified are the naturally processed forms. On the other hand it is a highly complex methodology. The SYFPEITHI database of MHC ligands and peptide motifs can be found at www.syfpeithi.bmi-heidelberg.com

Serological techniques Serological techniques aim to identify the repertoire of circulating antibodies in cancer patients. As the production of antibodies by B cells is thought to require T cell help, this reflects the $CD4^+$ helper T cell repertoire. The SEREX method is by far the most successful method to date for the identification of circulating antibodies. This method was developed by Ugur Sahin and colleagues (Sahin et al 1995) as an improvement on the original method of autologous typing. In the SEREX approach (Figure 1), a cDNA library is generated from a tissue or cell line of choice (normally fresh tumor tissue). The cDNA is then cloned unidirectionally into a λ-phage vector. The resulting recombinant phages are then used to transfect *Escherichia coli*. Recombinant proteins, which are expressed during lytic infection of the bacteria, are transferred onto a nitrocellulose membrane which is then incubated with patient sera. Antibodies in the sera of the patient reacting with the recombinant proteins can then be detected with an enzyme-conjugated secondary antibody specific for human IgG. Positive clones are then subcloned to monoclonality and then converted to plasmids via *in vivo excision*. The insert can then be sequenced to identify the gene responsible for the antibody response. To date there are 2593 identified sequences in the SEREX database (www2.licr.org/CancerImmunomeDB/) derived from 2169 clones. Many of the genes have been isolated repeatedly from the same and/or various tumor types, thus indicating their high immunogenicity in the human host. One of the main advantages of SEREX is that cDNA libraries can be prepared from fresh tissue, thus obviating the need to culture tumor cells which are prone to loss of or neoexpression of antigens resulting in artifacts. Another advantage is that by using highly diluted patient sera (1:100 to 1:1000), only high-titer IgG antibodies are detected which reflects a strong immune response by the host. Some disadvantages of SEREX are that it cannot detect glycosylated epitopes or conformational epitopes. A development of SEREX is the SADA (serum antibody detection

array) method (Figure 2). This allows the screening of multiple previously identified antigens by multiple sera in one go. This method is used to define the seroreactivity of a group of patients against defined antigens. One method which addresses some of the shortcomings of SEREX is the SERPA (serological proteome analysis) approach (Klade et al 2001). In this method, protein lysates from normal tissue and tumor tissue are seperated by 2D gel electrophoresis. Proteins are then transferred to a membrane and screened with patient sera as in SEREX. Comparison of seroreactivity against normal tissue and tumor proteins identifies spots which are then sequenced by mass spectrometry. The advantage of SERPA over SEREX is that proteins are in their naturally processed forms with all the proper post-translational modifications. However the sensitivity is much less as compared to SEREX.

Molecular biological techniques Molecular biological techniques such as SSH, DD-RTPCR and hybridization of cDNA microarrays are very efficient at identifying differentially expressed genes. However the majority of genes identified by these techniques are not immunogens. In the SSH method one population of cDNA (driver e.g. normal kidney) is subtracted from a second population (tester e.g. renal cell carcinoma) via hybridization and subsequent PCR amplification from adaptors linked to the tester cDNA. Amplified products are then transformed into bacteria and subsequently sequenced. All identified clones have then to be checked for expression levels by Northern blotting or RT-PCR. In the DD-RTPCR method RT-PCR using a set of oligonucleotide primers is performed on two populations of mRNA and then compared on a DNA sequencing gel. A banding pattern is generated for each population and differentially expressed bands can be excised, gel-eluted, cloned into a vector and subsequently sequenced. Both up and down regulated genes can be identified by this method. Two major drawbacks of this method are the high incidence of false positives and the fact that there can be more than one cDNA species present within one amplified band. The hybridization of cDNA microarrays is a highly powerful technique for the identification of differentially expressed genes. Many thousands of cDNAs can be compared in one experiment. The only drawbacks being the high cost and the fact that unknown genes are not represented.

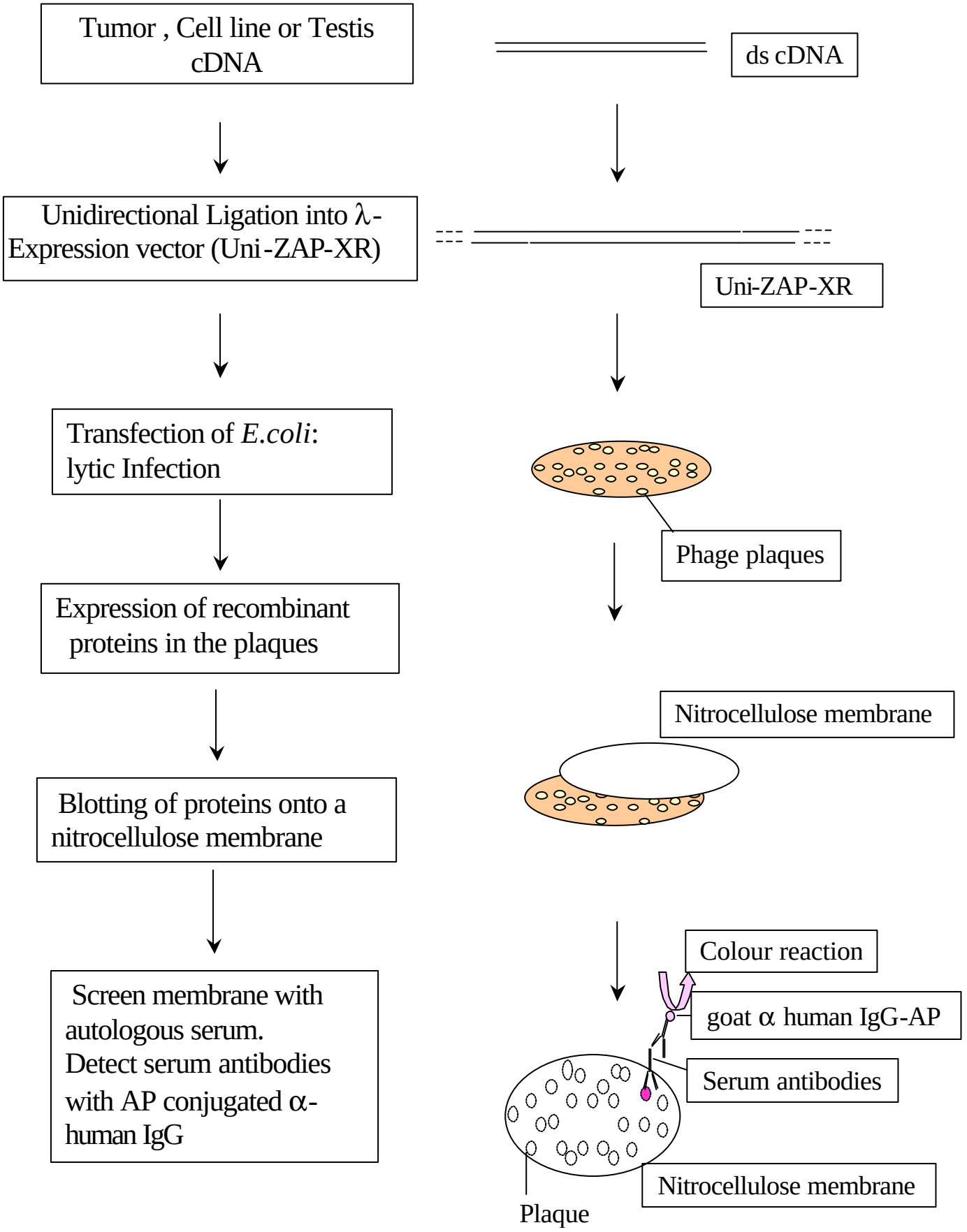


Figure 1 The SEREX method

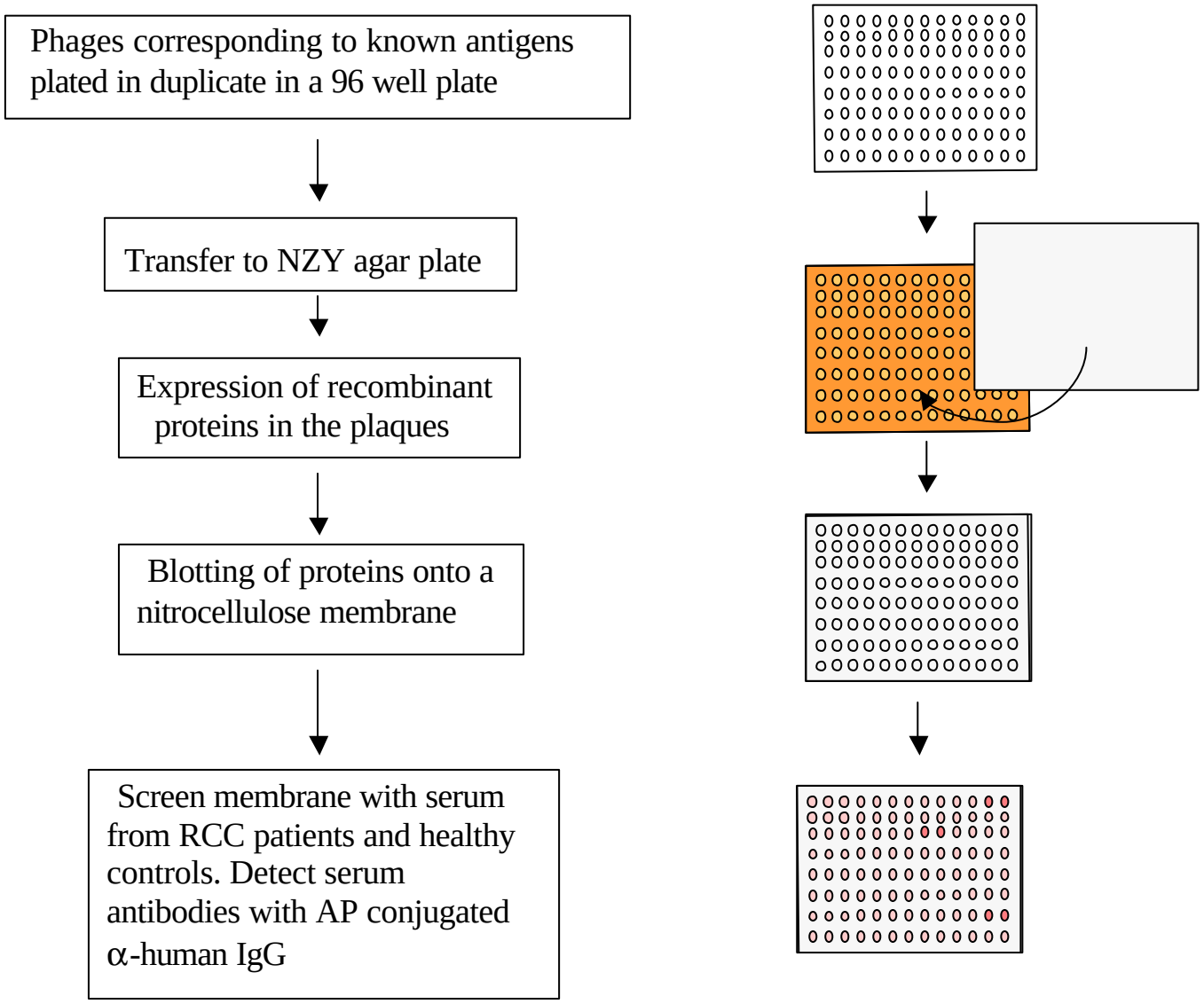


Figure 2. The SADA method

1.2.3 Renal cell carcinoma antigens

In comparison with other cancers such as melanoma, relatively few antigens have been identified to date in RCC. Table 5 lists the known RCC associated antigens to date. One of the RCC associated antigens identified by the T cell epitope cloning method is RAGE-1 (Gaugler et al 1996). RAGE-1 is expressed normally only in the retina. The frequency of its expression in RCC ranges from 2% to 36% depending on which study you read (Gaugler et al 1996, Neumann et al 1998, Oehlrich 2004, respectively). The study by Neumann et al also examined the expression of numerous other tumor associated antigens. By RT-PCR they could detect expression of PRAME (preferentially expressed antigen in melanoma) in 40% of RCC samples and gp75 in 11% whereas no expression could be found for MAGE-1, NY-ESO-1, tyrosinase, Melan-A/MART-1, gp100, beta-catenin, or MUM-1. Van den Eynde et al (1999) have identified another CTL epitope which they named RU2AS. The epitope is derived from the protein product of the antisense mRNA of RU2S. The antigen was found not to be tumor specific but to be a self-antigen with restricted tissue distribution.

The serological methods have had a little more success. Sahin et al performed the first SEREX on RCC in 1995. They identified one gene (carbonic anhydrase XII) which was found to be overexpressed in 10% of RCC. A subsequent study by Türeci et al in 1998 identified a new member of the cancer/testis antigen family, namely SCP-1(synaptonemal complex protein 1). It was found to be expressed in 3 of 36 RCC samples aswell as 40% of gliomas and 27% of breast carcinoma samples. Scanlan et al (1999) analyzed four additional cases of RCC and found 169 clones representing 65 different gene products. Of these, 36 were coded for by known genes and 29 were novel gene products. All the genes identified were found to be expressed in a range of normal tissues. With regard to the immunogenicity of these clones, 12 of the antigens were found to have cancer related seroreactivity i.e. antibodies were detected in 5-25% of patients but not in healthy controls. Koreleva et al (2002) used SEREX on two RCC specimens and identified a total of 96 clones corresponding to 66 different genes. Of these, 18 genes were of unknown function. Four of the genes were found to have a cancer related seroreactivity. However very little is known about these clones to date. The two proteomic studies of RCC (Klade et al 2001, Unwin et al 2003) have identified 12 antigens reacting with sera from RCC patients. Interestingly carbonic anhydrase I (CA I) was identified in both studies. Two other carbonic anhydrase members, CA IX/G250 (Oosterwijk et al 1986) and CA XII (Türeci et al 1998), have previously been shown to be RCC-tumor

antigens. The G250 antigen had been identified through a mouse monoclonal antibody specific for RCC and not normal tissue or other tumor tissues. It is the most promising candidate so far for immunotherapy of RCC.

Method of Identification	Antigen	Reference
CTL epitope cloning	RAGE-1	Gaugler et al 1996
	iCE	Ronsin et al 1999
	HSP70-2M	Gaudin et al 1999
	HLA-A2 R170	Brandle et al 1996
	RU1	Morel et al 2000
	RU2	Van den Eynde 1999
SSH	MAGE-9	Pitzer et al 1999
SEREX	SCP-1	Türeci et al 1998
	NY-REN-9	Scanlan et al 1999
	NY-REN-10	Scanlan et al 1999
	NY-REN-19	Scanlan et al 1999
	NY-REN-26	Scanlan et al 1999
	MO-REN-46	Koroleva et al 2002
	MO-REN-54	Koroleva et al 2002
	MO-REN-103	Koroleva et al 2002
	MO-REN-205	Koroleva et al 2002
	CA XII	Sahin et al 1995
SERPA/SPEAR	CA I	Klade et al 2001, Unwin et al 2003
	SM22α	Klade et al 2001
	Thymidine Phosphorylase	Unwin et al 2003
Monoclonal antibodies	MN/CA IX/G250	Oosterwijk et al 1986

Table 5. RCC associated antigens

1.2.4. Immunotherapy

1.2.4.1 Non specific Immunotherapy

Non-specific immunotherapy aims to boost the body's own immune system against cancer cells. In the normal course of a bacterial or viral infection, the body produces cytokines such as interleukin-2 (IL-2) or interferon-alpha (IFN-α) which then stimulate the growth and expansion of immune effector ells. However, non-specific immunotherapy is often toxic due to their lack of specificity.

Interleukin-2 Interleukin-2 (IL-2) is produced mainly by $CD4^+$ T cells but also $CD8^+$ T cells and large granular lymphocytes. It is thought to act by inducing the differentiation of lymphokine-activated killer (LAK) cells (Grimm et al 1992), maturation of antigen presenting cells (APCs) and enhanced ADCC (Eisenthal et al 1989). IL-2 can be given either alone or in combination with other treatment modalities. A high dose IL-2 bolus regimen has been approved by the FDA for the treatment of metastatic renal cell carcinoma. IL-2 has also been tested in combination with among others cyclophosphamide, interferon-α, histamine, and gp100 peptides. The combination of cyclophosphamide and low dose IL-2 used to treat melanoma patients was found to have an objective response rate of 25% (Mitchell et al 1988) as well as a lower toxicity than high dose IL-2 alone. Combinations of both IL-2 and IFN-α have been used to treat both RCC and melanoma with response rates up to 41% (Rosenberg et al 1989). However in a later comparison of IL-2 alone and IL-2 plus IFN-α, no difference in response rates could be observed (Marincola et al 1995). The combination of IL-2 plus histamine was found to benefit the subgroup of melanoma patients who had liver metastasis (Agarwala et al 2002). A 38% response rate was seen in a small group of melanoma patients receiving IL-2 in combination with a gp100 derived peptide (Rosenberg et al 1999).

Interferons Interferons can be subdivided into three subgroups (-α, -β, -γ). Their mechanisms of action are unclear but are thought to be stimulation of phagocytosis by macrophages, upregulation of MHC class I molecules and direct antitumor activities (Hancock et al 2000). To-date, the clinical relevance of IFN-β and IFN-γ for the treatment of metastatic RCC have not been proven. IFN-α, on the other hand has shown some benefit for patients. IFN-α may also be given alone or in combination with among others, vinblastine, 13-cis-retinoic acid, or IL-2. The combination of IFN-α and vinblastine was shown to be superior than vinblastine alone but not IFN-α alone (Pyrhonen et al 1999). In combination with 13-cis-retinoic acid (CRA) a higher response rate (12% versus 6%) was seen in those treated with the combination as compared to IFN-α alone (Motzer et al 2000). However no difference was seen in the median survival time.

1.2.4.2 Specific Immunotherapy

The first step in rational design of cancer immunotherapy is the identification of a suitable antigen. The more antigens known for a particular cancer the better. Targeting of a single antigen may lead to tumor escape by selection of antigen loss variants. Therefore, the aim should be to immunize not only with one or two but with a number of different antigens simultaneously. However, the quality of the antigen with regard to tumor rejection is also important. The potency of a tumor rejection antigen depends not only on the strength of immune response induced but also the vaccination protocol used. Weak tumor antigens can record as strong rejection antigens when used in protocols that favour the induction of CTL responses plus Th-1 $CD4^+$ T cell responses. Avidity and frequency of cognate T cells to a particular antigen are also important parameters. Several studies have shown that whereas low-avidity CTL can be readily detected by standard immunological assays, only high-avidity CTL exert biological function in vivo (Speiser et al 1992, Zeh et al 1999). A high frequency of responding T cells would conceivably be able to offset low avidity and thus result in an effective antitumor immune response. It is thought that the best tumor rejection antigens are those that are either patient-specific, tumor-specific or highly restricted shared antigens (such as the CT antigens) as these antigens are more likely to have been previously ignored by the immune system. However, there is a case for non-mutated self antigens when used in the correct context. Nishikawa et al (2001) have shown that combined vaccination of mice with a known tumor rejection antigen plus a non-mutated self antigen results in increased antitumoral activity as compared to vaccination with the tumor rejection antigen alone. Once a tumor rejection antigen has been identified and selected, the next question is what strategy to use to elicit an effective antitumor response. There are a number of different strategies such as loading of denritic cells (DCs) with peptides, lentiviral transduction of DCs, monoclonal antibodies, and gene therapy.

Dendritic cells Dendritic cells (DCs) have been shown to be the most potent antigen presenting cells of the immune system (Steinman 1991). DCs are uniquely able to prime both naïve CD4 or CD8 T cell responses making them central players in the activation of both cellular and humoral immune responses (see Figure 3). DCs also interact with natural killer cells (NK cells)(Fernandez et al 1999) and react to proinflammatory factors (Luft et al 2002), thus providing a link between the adaptive and innate immune systems.

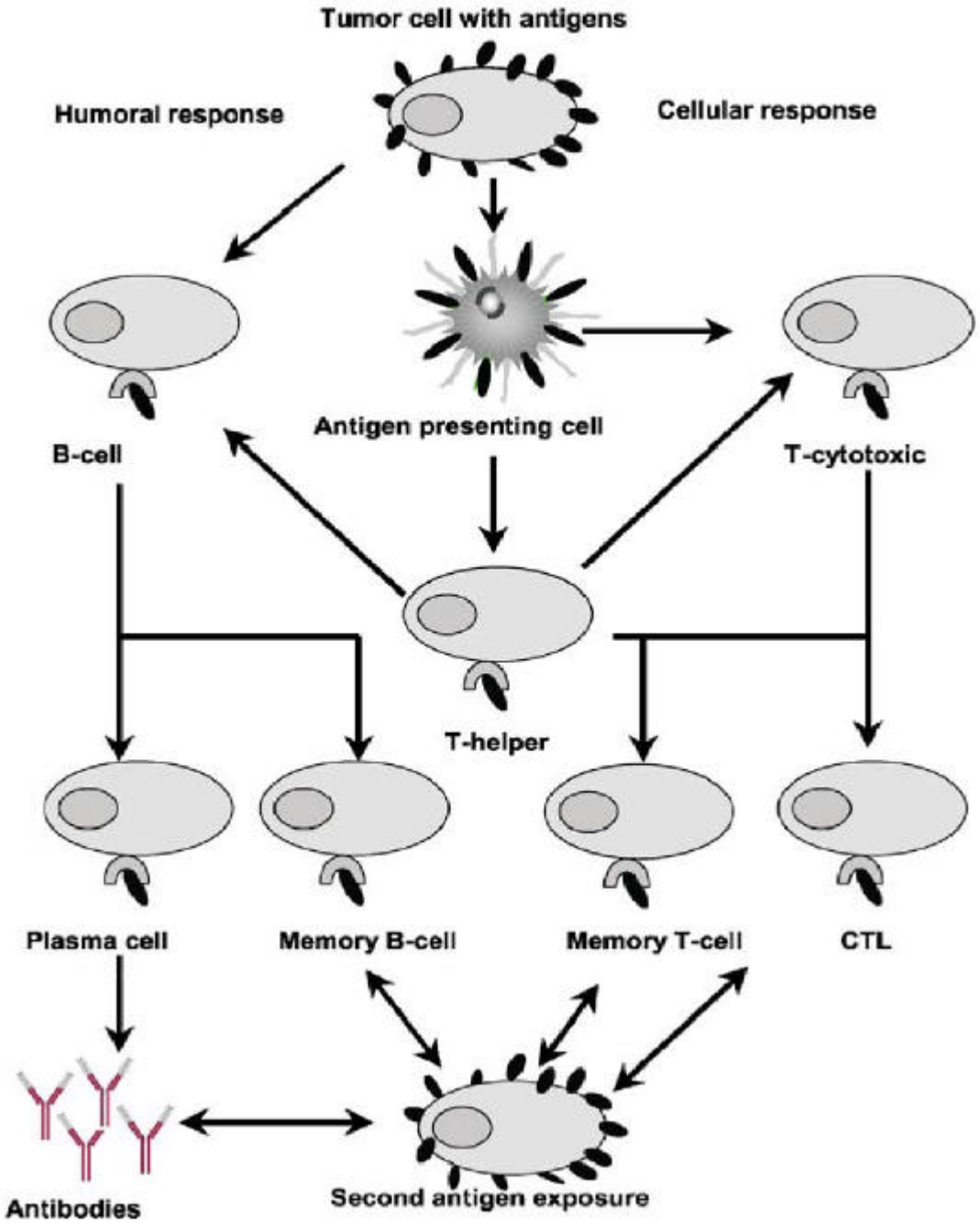


Figure 3. Central role of Antigen presenting cells in both humoral and cellular responses (Bleumer et al 2003).

DCs can be isolated from $CD34^+$ progenitor cells (Bernhard et al 1995) or from peripheral blood following attachment to plastic and maturation with cytokines such as GM-CSF, IFN-γ and IL-4 (Xu et al 1995).

All clinical trials involving DCs revolve around the loading of DCs with the antigen of interest followed by in vivo or ex vivo expansion of reactive $CD4^+$ or $CD8^+$ T cells. Antigen can be loaded onto DCs by a variety of techniques. Peptides, designed to fit in the appropriate MHC molecule, can be pulsed directly onto DCs. Whole proteins can be given to immature DCs which then process the protein and present peptide products on the MHC. DCs can also be transfected with tumor RNA (Boczowski et al 2000) or transduced with lentivirus containing the gene of interest (Rouas et al 2002).

Despite promising results in animal trials results from completed human DC trials have thus far only been able to demonstrate its safety. Conclusions about the efficacy and immunogenicity of DC vaccination have been hampered by the small scale of trials so far and by variables such as preparation of DCs, vaccine schedule and monitoring of the immune response. Efforts to standardize these procedures should give results that can be compared with other immunotherapy approaches. A complete review of clinical trials involving DCs has been published recently by Cranmer et al (2004).

Monoclonal antibodies Alone, monoclonal antibodies (mAbs) can target and kill cells via induction of antibody-dependent cellular cytotoxicity (ADCC) through the activation of complement. mAbs can also be conjugated to cellular toxins or radioisotopes (Scott et al 1997). Bi-specific mAbs target both tumor antigen and a receptor expressed by desired effector cell such as CD20 on B cells or CD3 on T cells.

There are currently several mAbs in clincal trials such as Rituximab for Non-Hodgkins lymphoma (McLaughlin 2001), Trastuzumab for breast carcinoma (Baselga 2001)and ^{131}I-WX-G250 for RCC (Steffens et al 1999). Clinical trials with Rituximab, which targets CD20 present on 90% of B cell lymphomas, have confirmed its therapeutic effect in low and intermediate grade CD20+ Non-Hodgkins lymphoma (McLaughlin et al 1998) and also in combination with radioisotopes (Witzig et al 2002). The anti HER2/neu monoclonal antibody, trastuzumab, has also shown promise in clinical trials, resulting in a higher rate of objective response, longer duration of response, improved 1-year survival, and longer overall survival (Slamon et al 2001). Trials of the RCC specific mAb are only at the stage of establishing maximal toleratable dose and safety.

Gene therapy Gene therapy can be divided into two strategies: corrective gene therapy and immunogene therapy. Corrective gene therapy aims to substitute defective genes with their functional counterparts. Most corrective gene therapy studies are aimed at tumor cell destruction with concomitant antitumor immune responses. Immunogene transfer is based on the delivery of DNA encoding immune modulators such as IL-2. Leuvectin® is a plasmid/lipid complex encoding human IL-2 (Figlin et al 1999). Clinical trials with Leuvectin have demonstrated a markedly reduced systemic toxicity as compared to systemic IL-2 treatment (Galanis et al 1999, Belldegrun et al 2001).

1.3 AIMS

The aims of this thesis are 1) to elucidate the repertoire of immunogenic molecules in RCC and 2) to evaluate the potential of identified molecules as diagnostic, prognostic or therapeutic targets with relation to RCC.

The first aim is to be achieved through the use of the following methods 1) Suppression Subtractive Hybridization (SSH), 2) Serological analysis of recombinant cDNA expression libraries (SEREX) and 3) Serum Antibody Detection Array (SADA) analysis.

The second aim is to be achieved by 1) examination of the literature and gene databases for known homologies to genes and possible relationship to cancer, 2) examination of the expression profiles of identified genes through Northern blotting and RTPCR, 3) examination of seroreactivity profiles through secondary SEREX, and 4) examination of immunogenicity of derived peptides in a mixed lymphocyte reaction.

2. Materials and Methods

2.1 RNA

2.1.1 Isolation of RNA

Total RNA was isolated from cell lines and tissues using Tri Reagent^{TM} (Sigma, Taufkirchen, Germany). Adherent cells were first trypsinized and immediately lysed in Tri Reagent^{TM} (1ml per 5-10x10^6 cells). Frozen tissue samples from human normal kidney and RCC were first pulverised with a membrane disruptor and immediately homogenized and lysed in Tri Reagent^{TM} (1ml per 50-100mg of tissue). After 5 minutes of incubation at room temperature, 200µl chloroform per ml Tri Reagent^{TM} was added. After mixing and incubating for 2-15 minutes at room temperature the samples were centrifuged for 15 minutes at 13000 rpm at 4^0C. The upper phase was transferred to a tube containing 0.5ml isopropanol per ml Tri Reagent^{TM}. After mixing and incubating for 5-10 minutes at room temperature, the samples were centrifuged for 10 minutes at 13000 rpm at 4^0C. The RNA pellet was washed with 1ml 70% ethanol and dried for 5 minutes in a speed-vac. The pellet was dissolved in 50µl diethylpyrocarbonate (DEPC)-treated water and stored at -80^0C. The amount of total RNA was determined by measuring the absorbance at 260nm. The total RNA was used for poly A^+ RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR), northern blotting or double strand (ds) cDNA synthesis.

DEPC-treated water: *200ml DEPC per litre distilled water*
Mix well and incubate overnight at room temperature
Autoclave

2.1.2 Poly A^+ mRNA preparation

Poly A^+ mRNA was isolated from total RNA samples with mini-oligo(dT) cellulose spin columns (Peqlab, Erlangen, Germany). A maximum of 1mg of total RNA from tumor tissue

or cell line in 0.5 M NaCl buffer was applied to a mini-oligo(dT) cellulose spin column. The column was then washed with 0.5 M NaCl and 0.1 M NaCl buffers by centrifugation to remove the unbound poly A^- RNA. Poly A^+ mRNA was spin eluted using a buffer without NaCl that was prewarmed to 65^0C. The amount of eluted poly A^+ mRNA wsa determined by measuring the absorbance at 260nm.

Elution buffer:	10 mM Tris-Cl *1mM EDTA* *pH7.5*
0.5 M NaCl buffer:	*0.5 M NaCl* *20 mM Tris-Cl* *5 mM EDTA* *pH7.5*
0.1 M NaCl Buffer:	*0.1 M NaCl* *20 mM Tris-Cl* *5 mM EDTA* *pH7.5*

2.1.3 RNA gel electrophoresis and Northern Blotting

As most RNAs are able to form secondary structures by intramolecular base pairing, RNA must be run under denaturing conditions. Denaturation is achieved by adding formaldehyde to the gel and loading buffer. Total RNA (20 µg) was loaded on a 1xMOPS/1.2% agarose gel containg ethidium bromide and 2.2M formaldehyde. The gel was run for 3 to 4 hours at 80 V in 1x MOPS electrophoresis buffer. The gel was examined on a UV transilluminator to visualize the RNA. The staining with ethidium bromide (25 µg/ml) causes rRNA molecules to appear as sharp bands that can be used as internal markers. In mammalian cells these molecules are 28S and 18S, corresponding to 4718 and 1874 nucleotides respectively.

Before blotting, the gel was rinsed in DEPC-treated water to remove formaldehyde. The gel was blotted upside down onto a positively charged nylon membrane (Hybond^{TM} –N^+, version 2.0; Amersham-Pharmacia, Freiburg, Germany) by overnight capillary blotting with 20xSSC. After blotting, the membrane was washed briefly in 2xSSC to remove any adhering agarose and air dried for up to an hour. To be able to reprobe the membrane several times, the

membrane was UV-crosslinked using the Stratalinker (Stratagene, Amsterdam, The Netherlands) and the auto-cross-link program. The membrane was stored dry between sheets of Whatmann^{R} 3MM filter paper at room temperature.

10xMOPS: *0.4 M MOPS*
0.1 M Sodium Acetate
0.01 M EDTA
pH 7.0

20xSSC: *3 M NaCl*
0.3 M Na_3 Citrate

2.1.4 Northern blot hybridisation

Northern blots were prewetted with DEPC-treated water and put into rollerbottles with the RNA side of the blot towards the inside of the bottle. Blots were prehybridized at 42^{0}C for at least 1 hour with 15 ml prehybridization solution containing denatured salmon sperm DNA. Probe DNA (~25 ng) was labelled with 50 µCi [α^{32}P]dCTP using Rediprime^{TM} II (Amersham). Before adding the denatured probe, 3 ml of 50% dextran sulphate was added to the prehybridization solution. Hybridization was performed overnight at 42^{0}C.

The next day the blots were washed for 15 minutes in 1xSSC/1%SDS at 42^{0}C, 15 minutes in 0.2xSSC/1%SDS at 42^{0}C and 15 minutes in 0.2xSSC/1%SDS at 55^{0}C. The blots were sealed in plastic wrap and signals were detectable on X-ray film (Hyperfilm^{TM} MP, Amersham-Pharmacia) after 1 hour to 14 days exposure at -80^{0}C with intensifying screens.

For reprobing, the blots were stripped in 40 mM Tris-HCL (pH7.5) with 0.1xSSC and 1% SDS. For this, the solution was boiled and the blots were washed in this solution for 15 minutes in a boiling waterbath.

Prehybridization solution: *50% Formamide*
1xHybridization buffer

10xDenhardt's
10 mM EDTA
0.1%SDS
0.2 mg/ml denatured salmon sperm DNA

4xHybridization buffer: *175.32 g/l NaCl*
73.08 g/l Tris
22.08 g/l NaH_2PO_4
64.32 g/l $Na_2H\ PO_4$
4 g/l Na-pyro PO_4
pH 7.5 with HCl

100xDenhardt's: *1% Ficoll (Type 400)*
1%polyvinylpyrrolidone
1%bovine serum albumin (BSA)(fraction V)

2.1.5 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed on total RNA extracted from either normal kidney tissue, tumor tissue or cell lines. Reverse transcription was carried out with the following components:

Component	Volume
RNA sample	1µg
Oligo dT (500ng/µl)	2µl
ImProm-II™ Reaction buffer 5x	4µl
$MgCl_2$, 25 mM	4.8µl
rRNasin® Ribonuclease Inhibitor (40U/µl)	0.5µl
ImProm-II™ Reverse Transcriptase (10U/µl)	1.0 µl
Nuclease-free water	Up to 20µl

All reagents from Promega Madison, USA, except Oligo dT.

The template RNA and the OligodT primer (Sigma) were first mixed and heated to 70^0C for 5 minutes then immediately chilled on ice. The remainder of the components were mixed

together on ice and then added to the primer and RNA sample mix. The mix was then incubated for 5 minutes at 25^0C followed by 1 hour at 42^0C. Finally the reverse transriptase was heat inactivated at 70^0C for 15 minutes. The resulting cDNA was then stored at -20^0C or used immediately for PCR.

2.2 DNA

2.2.1 DNA transformation of bacteria by heatshock

For one DNA transformation three tubes of competent bacteria were thawed on ice. About 100 ng of DNA (1-2 µl) was added to one tube of bacteria and mixed by stirring. As a negative control 1 µl of sterile distilled water was used and as a positive control 1 µl of Puc18 DNA (10 ng) was added to the other tubes. The bacteria were incubated on ice for 30 minutes. Every 5 minutes the bacteria were carefully mixed by flicking the tube. The bacteria were heat shocked for 1 minute at 42^0C and immediately after put on ice for 2 minutes. 250 µl SOC medium (room temperature) was added and the bacteria were incubated shaking at 37^0C for 30-45 minutes. 50-100µl of each transformation was spread on LB-agar plates with the appropriate selection antibiotic and incubated overnight at 37^0C.

SOC medium:

2% Tryptone
0.5% Yeast Extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$
20 mM glucose

LB medium:

10g/l Tryptone
5g/l Yeast Extract

10g/l NaCl

pH 7.0

LB agar plates: *LB medium with 1.5% Agar*

Selection antibiotics: *50 mg/ml ampicillin*

50 mg/ml kanamycin

12.5 mg/ml tetracyclin

2.2.2 Polymerase Chain Reaction (PCR)

All PCR reactions were carried out with the following components:

Component	Volume
10x PCR buffer	5 µl
10 mM dNTP mix	1 µl
50 mM $MgCl_2$	1.5 µl
Primer mix (10 µM each)	2.5 µl
Template DNA	10-100 ng
Taq DNA polymerase (5U/µl)	0.5 µl
Autoclaved distilled water	Up to 50 µl

All reagents from Invitrogen

PCR was carried out with the following primer pairs and conditions:

Primer pair	Sequence 5'-3'	Annealing conditions	Elongation conditions	Cycles	Acc. No & amplified bases
GAPDH FOR	ACCACAGTCCATGCCATCAC	52^{0}C x 30 sec	72^{0}C x 30 sec	25	BC029340
GAPDH REV	TCCACCACCCTGTTGCTGTA	52^{0}C x 30 sec	72^{0}C x 30 sec	25	572-1023
SCP-1 FOR	GTACAGCAGAAAGCAAGCAAC	55^{0}C x 30 sec	72^{0}C x 1 min	30	NM_003176
SCP-1 REV	GGAAATTGGATTCTAAAGCAG	55^{0}C x 30 sec	72^{0}C x 1 min	30	2095-2652
TSGA10 FOR	GTCTTCTACTAGGGAACTCT	58^{0}C x 30 sec	72^{0}C x 1 min	30	AF254756
TSGA10 REV	CTCGATTGGCCACCAGCAAA	58^{0}C x 30 sec	72^{0}C x 1 min	30	2151-2353
PLU-1 FOR	GACACTAAGGACAAGGAGT	58^{0}C x 30 sec	72^{0}C x 1 min	30	HSA132440
PLU-1 REV	GGACAACCCATTCGACGT	58^{0}C x 30 sec	72^{0}C x 1 min	30	723-877
RANBP2L1 FOR	GATTGGAAAGGAATCAAGAG	56^{0}C x 30 sec	72^{0}C x 1 min	30	NM_005054
RANBP2L1 REV	TGGTAACACACAGAACCAT	56^{0}C x 30 sec	72^{0}C x 1 min	30	5161-5340
STK31 FOR	ATGCAGGCAATCTTATAACA	54^{0}C x 30 sec	72^{0}C x 1 min	30	BC059374
STK31 REV	GGTCTTCAGAGTGTATTCTA	54^{0}C x 30 sec	72^{0}C x 1 min	30	841-1100
BRDT FOR	TCAAGAAAGGCACTCAACAG	56^{0}C x 30 sec	72^{0}C x 1 min	30	NM_001726
BRDT REV	TTCACTACTTGCTTTAACTGC	56^{0}C x 30 sec	72^{0}C x 1 min	30	544-797

2.2.3 Small scale DNA isolation from bacteria (miniprep)

Bacteria were grown overnight while shaking at 37^{0}C in LB medium with the appropriate selection antibiotic. 2ml of this bacterial suspension was centrifuged for 1 minute at 13000rpm. The pellet was resuspended in 250µl P1 solution. Then, 250µl P2 solution was added and the suspension was inverted to mix. After the addition of 350µl P3 and mixing by inverting, the tubes were centrifuged at 13000rpm for 15 minutes. 850µl of the supernantant was transferred to a tube containing 1 ml isopropanol, mixed and centrifuged for 30 minutes at 13000rpm. The DNA pellet was then washed with 1 ml 70% ethanol and air dried. The pellet was dissolved in 50µl distilled water and the DNA concentration was determined by measuring the absorbance at 260nm.

P1 solution: *50 mM Tris-Cl*
10 mM EDTA
pH 8.0

P2 solution : *0.2 M NaOH*
1% SDS

P3 solution : *2.55 M Kac*
pH 4.8

2.2.4 Cloning of PCR products

PCR products that were amplified with *Taq* polymerase have 3' ends with a single deoxyadenosine (A) overhang and were directly cloned into the plasmid vector PCR^{R}2.1-TOPO (Invitrogen, Groningen, Netherlands) which contains single 3' thymidine (T) overhangs. In short, 0.5 to 2 µl PCR product was ligated with 10ng of vector for 5 minutes at room temperature. The reaction was stopped with 1 µl stop solution and put on ice. 2 µl was added to one vial of One Shot^{TM} competent cells or 50 µl competent bacteria. After incubating for 30 minutes on ice, the bacteria were heat shocked (30 seconds at 42^{0}C), put on ice for 2 minutes and incubated for 30 minutes shaking at 37^{0}C in 250 µl SOC medium. 50-100 µl from each transformation was spread on ampicillin (50 µg/ml) containing agar-plates pre-incubated with IPTG (40 µl of 100mM solution) and X-gal (40 µl of 40 mg/ml solution) for blue-white screening. Plates were incubated overnight at 37^{0}C. The next day white colonies were picked and grown at 37^{0}C in LB medium containing 50 µg/ml ampicillin.

2.2.5 DNA digestion with restriction endonucleases

For DNA analysis 1-2µg DNA was digested with various restriction enzymes. The amount of enzyme used was according to the manufacturer's protocol. In general, the digestion was performed for 1 hour at 37^{0}C in a final volume of 20µl.

2.2.6 Cloning of BACE2 into the Uni-ZAP^{R} XR vector.

BACE2 cDNA was digested with the restriction enzymes *Apa I* and *Hind III*. The adaptors B2EcoApa and B2HindXho were then ligated to the digested product for 3 hours at room temperature with 1 unit of T4 DNA ligase (Stratagene). The ligated product was then ligated into the Uni-ZAP^{R} XR vector and packaged into λ phage as described in *Packaging and Titering* (2.3.1.1 Library Construction).

B2EcoApa *5'pAATTCGGCACGAGTG 3'*
3'GCCGTGCTCACCCGG 5'

B2HindXho *5'pAGCTTAGTTCGTCC 3'*
3'ATCAAGCAGGAGCT5'

2.2.7 Suppression Subtractive Hybridisation (SSH)

With subtractive hybridisation one is able to compare two populations of mRNA and obtain clones of genes that are expressed in one population but not in the other. In this case the mRNA of RCC tissue and normal kidney were compared. Clones that were differentially expressed by the RCC were further analysed by northern blotting and sequence analysis. For subtractive hybridisation the CLONTECH PCR-Select^{TM} cDNA subtraction kit was used. This method was originally described by Diatchenko et al in 1996.

1. First- and second- strand cDNA synthesis

From both RCC and normal kidney 2 µg of poly A^{+} RNA was incubated with 10 pmol cDNA synthesis primer for 2 minutes at 70^{0}C and cooled on ice. First-strand synthesis was performed at 42^{0}C for 90 minutes with 20 units AMV reverse transcriptase. Directly thereafter second-strand synthesis was performed by adding an enzyme cocktail consisting of DNA polymerase I (6 units/µl), Rnase H (0.25 units/µl), and *E.coli* DNA ligase (1.2 units/µl) and incubating for 2 hours at 16^{0}C. To create blunt ends, 6 units of T4 DNA polymerase were added and incubated for another 30 minutes at 16^{0}C. The second-strand synthesis was terminated by the addition of 1xEDTA/glycogen. The synthesised dscDNA was purified by

phenol:chloroform:isoamyl alcohol (25:24:1) extraction and chloroform:isoamyl alcohol (24:1) re-extraction and was precipitated by the addition of NH_4OAc (final conc. 400 mM) and 2 volumes of 100% ethanol. After centrifugation (13000 rpm; 20 min., 4^0C) the pellet was washed with 70% ethanol, air dried and dissolved in 50µl distilled water. To estimate the yield and size range of the ds cDNA products synthesized, 6µl of the sample was used for agarose gel analysis.

cDNA synthesis primer:	*5'TTTTGTACAAGCTT$_{30}$N$_{-1}$N3'*
	(N$_{-1}$=A, C or G;N = A, C, G or T)
20x EDTA/glycogen:	*0.2 M EDTA*
	1 mg/ml glycogen

2. RsaI digestion

The remaining 44 µl of the synthesised ds cDNA of both RCC and kidney were digested with 15 units Rsa I to generate shorter, blunt ended ds cDNA fragments which are of optimal size for subtraction and necessary for adaptor ligation. The reaction was terminated with 1xEDTA/glycogen and Rsa I digestion efficiency was analysed by agarose gel electrophoresis. The cDNA fragments were then purified as described for ds cDNA after second-strand synthesis.

3. Adaptor ligation

To be able to specifically amplify cDNA from RCC after the subtraction of normal kidney cDNA, adaptors were ligated only to the Rsa I digested cDNA but not to the normal kidney cDNA fragments. The digested cDNA was split into two and ligated to either adaptor 1 or adaptor 2R, which was performed overnight at 16^0C with 400 units T4 DNA ligase. The ligation reaction was terminated with 1xEDTA/glycogen and the ligase was heat-inactivated (5 min. 72^0C). Afterwards, ligation efficiency was analysed by a PCR reaction with primers to the ligated adaptors and GAPDH primers.

Adaptor 1R: *5'CTAATACGACTACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT 3'*
3'GGCCCGTCCA 5'

Adaptor 2R: *5'CTAATACGACTACTATAGGGCAGCGTGGTCGCGGCCGAGGT 3'*
3'GCCGGCTCCA 5'

4. First and second hybridisation

During this procedure, an excess of normal kidney cDNA was added to the RCC cDNA and the samples were heat denatured and allowed to anneal. During the first hybridisation the Rsa I digested normal kidney cDNA was mixed with either adaptor 1- or 2R-ligated RCC cDNA respectively. This hybridisation was performed at 68^0C for 8 hrs, after denaturation at 98^0C. The remaining ss cDNAs that were available for the second hybridisation were thus enriched for differential sequences, as non-target cDNAs present in the RCC and normal kidney cDNA form hybrids. The second hybridisation, in which the two samples from the first hybridisation were mixed together and to which freshly denatured kidney cDNA was added, was performed overnight at 68^0C. New hybrid molecules with different adaptors on each were formed during this step and represented the differentially expressed cDNAs in RCC.

5. PCR amplification

Differentially expressed cDNAs were selectively amplified by two PCR reactions. Before amplification the missing strands of the adaptors were filled in by a pre-incubation of 5 minutes at 75^0C in the presence of *Taq* polymerase. The first PCR reaction was perfromed with primer 1, which can bind to both adaptor 1 and 2R, with 27 cycles at 66^0C annealing temperature. A second PCR reaction with 12 cycles at 68^0C annealing temperature was performed with two nested primers (1 and 2R), which bind to adaptor 1 and 2R respectively. The PCR products after both the first and second PCR reaction were analysed on a 1xTAE/2% agarose gel containing ethidium bromide. The PCR mixture, containing enriched differentially expressed transcripts was then cloned into the PCR^R 2.1-TOPO vector (see cloning of PCR products).

PCR primer 1: *5'CTAATACGACTCACTATAGGGC 3'*

Nested PCR primer 1: *5' TCGAGCGGCCGCCCGGGCAGGT 3'*

Nested primer 2R: *5'AGCGTGGTCGCGGCCGAGGT 3'*

50xTAE: *242g Tris*
57.1 ml glacial acetic acid
100ml EDTA (pH 8.0)

6. Screening

PCR products were then transformed into bacteria (see 2.2.1 DNA transformation of bacteria by heatshock). DNA was then prepared (see 2.2.3) from randomly picked colonies and used for sequencing.

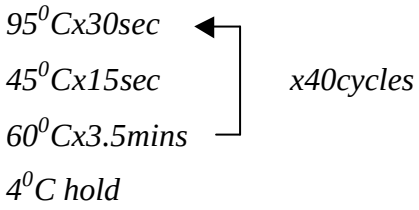
2.2.8 Sequencing

When sequencing was not performed commercially (MWG Biotech AG, Ebersberg, Germany) then it was performed with the ABI PRISM TM 310 GENETIC ANALYZER and the BIG DYE TERMINATOR CYCLE SEQUENCING READY REACTION KIT (Applied Biosystems). The following primers were used for sequencing:

Primer name	Sequence
M13 rev(-29)	CAG GAA ACA GCT ATG ACC
M13 For	GTA AAA CGA CGG CCA GT

For sequencing, 1-2 µg of plasmid DNA was used plus 1.25 µM primer and 2 µl BIG DYE mix in a total volume of 20 µl. The mix was then placed in a thermocycler with the following program:

$95^0Cx1min$



The DNA was then precipitated with 2 µl 3 M NaAc (pH 5) and 50 µl 100% ethanol. The reaction mix was placed on ice for 10 minutes and then centrifuged for 30 minutes at 13000 rpm. The DNA pellet was then washed with 70% ethanol and centrifuged again at 13000 rpm for 10 minutes. The supernatant was removed by aspiration and the pellet allowed to dry in air. The pellet was then resolved in 20 µl Template Suppression Reagent (Applied Biosystems). The sequuence reaction was placed in the ABI PRISM TM 310 GENETIC ANALYZER for sequencing. Sequences were analysed using various computer programs (see Computer analysis of DNA sequences).

2.2.9 Computer analysis of DNA sequences

The HUSAR program (Heidelberg Unix Sequence Analysis Resources) was used for analysis of DNA sequences (http://genome.dkfz-heidelberg.de/). The following programs within HUSAR were used.

Program	Use
Blastn	Comparison of nucleotide sequence with nucleotide database
BlastP	Comparison of peptide sequence with protein database
MAP	Finding open reading frames and restriction endonuclease sites
Translate	Translating nucleotide sequence to amino acid sequence
Malign/Clustal	Multiple alignments of sequences

Also the SEREX database was used for comparison with sequences identified in this thesis (http://www2.licr.org.CancerImmunomeDB) Ludwig Institute for Cancer Research.

The following program was used for virtual northern blot: Unigene Cluster (http://cgap.nci.nih.gov/Genes/GeneFinder) National Institute of Health, USA.

2.3 Protein

2.3.1 Serological analysis of recombinant cDNA expression libraries (SEREX)

The SEREX method was originally developed by Sahin et al. (1995) to identify antigens to which patients have a circulating antibody response. For this technique cDNA libraries were created from (i) human RCC tissue (U1607), (ii) RCC cell line KTCTL-28 treated with 5-aza deoxycytidine (see cells and tissue culture). A human testis library (generated in the same manner) was obtained from the lab of Dr. Stefan Eichmüller. The libraries were cloned into the Uni-ZAP® XR vector predigested with EcoRI and XhoI and treated with Calf Intestine Alkaline Phosphatase (CIAP) from Stratagene.

2.3.1.1 Library construction

First- and second-strand cDNA synthesis

The library construction started with the first strand cDNA synthesis of 5 µg poly A^+ RNA with 2.8 µg of the cDNA synthesis primer containing an XhoI site. The first strand reaction was performed for 1 hour at 37^0C with 75 units M-MuLV reverse transcriptase. The nucleotide mixture for the first strand synthesis contained normal dATP, dGTP and dTTP plus the analog 5-methyl dCTP to protect the cDNA from restriction enzymes in the subsequent cloning steps.

cDNA synthesis primer: *5'GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAG$(T)_{18}$3'*

Adaptors: *5' OH-AATTCGGCACGAG 3'*

3' GCCGTGCTCp 5'

EcoRI adaptor ligation:

The second-strand synthesis reaction was blunt ended by incubating 30 minutes at 72^0C with 5 units *Pfu* DNA polymerase, phenol-chloroform extracted and precipitated. The next day EcoRI adaptors were ligated to the blunt ended DNA with 4 units of T4 DNA ligase (overnight at 8^0C) and the adaptor ends were phosphorylated with 10 units T4 polynucleotide kinase (30 minutes 37^0C).

EcoRI adaptors: *5' OH-AATTCGGCACGAG 3'*
3' GCCGTGCTCp 5'

Ligating cDNA into the Uni-ZAP® XR vector

The adaptor-ligated cDNA was digested with 120 unit XhoI for 1.5 hours at 37^0C and precipitated. Large cDNA molecules were separated from smaller fragments (<0.1kb) by CHROMA SPIN-200 DEPC-H_2O column chromatography (Clontech). The resulting cDNA was phenol-chloroform extracted and precipitated. The next day, 100ng cDNA was cloned into 1 µg Uni-ZAP^R XR vector with 2 units T4 DNA ligase (overnight, 16^0C).

Packaging and titering

0.1 to 1.0 µg of ligated DNA was added to one Gigapack III Gold Packaging Extract (Stratagene) and incubated for 2 hours at room temperature. Then, 500 µl SM buffer and 20 µl chloroform were added and the phage suspension was titered. For this purpose, host bacteria (XL1-Blue MRF') were prepared by growing the bacteria to an OD_{600} of 0.7 to 0.8, centrifuging the bacteria for 10 minutes at 500xg and resuspending the bacteria with 10 mM $MgSO_4$ to an OD_{600} of 0.5. Then, 1 µl of undiluted and 1:10 diluted phage suspension was added to 200 µl host bacteria and incubated for 15 minutes at 37^0C to allow the phage to attach to the bacteria. 4 ml NZY topagar with 15 µl of 0.5 M IPTG (topagar/IPTG) was added

to the bacteria and immediately poured onto NZY agar plates (80 mm). The plates were dried for 15 minutes at room temperature and incubated inverted overnight at 37^0C. The next day the number of plaques were counted.

SM buffer:	*5.8 g/l NaCl*
	2 g/l $MgSO_4.7H_2O$
	50 ml 2% gelatin
NZY:	*5 g/l NaCl*
	2 g/l $MgSO_4.7H_2O$
	5 g/l yeast extract
	10 g/l NZ amine (casein hydrolysate)
	pH 7.5
NZY agar plates :	*NZY medium with 1.5% agar*
NZY topagar:	*NZY medium with 0.7% agarose*

Amplifying the Uni-ZAP® XR library

To make a large, stable quantity of a high titre stock of the library, the library was amplified in one round. For this, aliquots of the phage suspension containing $5x10^4$ plaque forming units (pfu) was combined with 600µl of host cells (XL1-Blue MRF'; prepared as described previously) and incubated for 15 minutes at 37^0C. For the amplification of $1x10^6$ plaques, a total of 20 aliquots were used. After incubation 8 ml NZY topagar/IPTG was added and poured onto 150 mm plates. The plates were dried for 15 minutes at room temperature and incubated inverted overnight at 37^0C.

The next day 8 ml SM buffer was put onto the agar plates and the plates were incubated overnight at 4^0C with gentle rocking. This allowed the phages to diffuse into the SM buffer. Then, the bacteriophage suspension was recovered and chloroform was added to a 5% (v/v) final concentration. After mixing, the suspension was incubated for 15 minutes at room temperature, centrifuged for 10 minutes at 500xg and transferred to a fresh tube. Chloroform

was added to a 0.3% (v/v) final concentration and the phage suspension was stored at 4^0C. Small aliquots of the amplified library were stored with 7% (v/v) DMSO at -80^0C. The titre of the amplified library was checked as described previously.

Performing plaque lifts

The amplified library was plated on 150 mm NZY agar plates (at least 2 days old) in a concentration that the plaques were subconfluent after overnight incubation at 37^0C. The plates were chilled for at least 2 hours to prevent the NZY topagar from sticking to the membrane.

For protein screening nitrocellulose membranes (Sartorius AG, Göttingen, Germany) were used that were placed onto the plates after 4 hours at 37^0C. After lifting, the membranes were washed in TBST to remove remaining topagar and used immediately for screening.

TBST:	*10 mM Tris-HCl (pH 8.0)*
	150 mM NaCl
	0.05% Tween 20

2.3.1.2 Preabsorption of human serum

Preabsorption of the sera is necessary to remove any antibodies that would recognise proteins derived from the λ phage or from *E.coli*. The preabsorption was carried out twice for every serum for each of the following methods i) preabsorption on a sepharose column ii) preabsorption on a nitrocellulose filter.

i) Preabsorption on a sepharose column

Protein lysate from *E.coli* XL1-Blue MRF' was coupled to CNBr activated Sepharose 4B (Pharmacia) according to the manufacturers' instructions. Initially, host bacteria (XL1-Blue MRF') were prepared by growing the bacteria to an OD_{600} of 0.7 to 0.8, centrifuging the bacteria for 10 minutes at 500xg and resuspending the bacteria with 10 mM $MgSO_4$ to an OD_{600} of 0.5. The bacteria were centrifuged again and resuspended in 20 ml LB medium with 0.2% maltose and 10 mM $MgSO_4$. 10 ml of this culture was then infected with 5 µl λ phage without insert (SIST λ phage 3.5 x 10^9 pfu/ml). The other 10 ml was not infected and served as a control for the efficiency of the lysis. Both cultures were incubated for 7 hours at 37 ^{0}C shaking. The cultures were then placed in collodium dialysis bags (Sartorius) and dialysed for 2 days against 0.1 M $NaHCO_3$ buffer (pH 8.3). Dialysis buffer was changed regularly during the 2 days. The dialysate was treated with 0.05% NaN_3 to prevent growth of bacteria. Protein concentration was measured by absorbance at 280 nm. The efficiency of the lysis could be determined by comparing the absorbance of both cultures.

Protein from the dialysate of the infected *E.coli* XL1-Blue MRF' was then used for coupling to the CNBr-Sepharose matrix (> 5 mg potein /ml gel) according to the manufacturers' instructions.

For preabsorption, sera were diluted 1:1 with PBS and then allowed to flow through the Sepharose column at 4 ^{0}C with a speed of 0.6ml per minute. The preabsorbed sera were then stored for further use at -20 ^{0}C with 0.05% NaN_3. This preadsorption step was carried out twice for every serum.

ii) Preabsorption on a nitrocellulose filter

200 µl *E.coli* XL1-Blue MRF' (OD_{600}=0.5) was infected with 1 µl λ phage without insert (SIST λ phage 3.5 x 10^9 pfu/ml). After 15 minutes at 37^{0}C, 7 ml of NZY topagar was added and then plated on a 145 mm petri dish. Plates were then incubated for 4-6 hours at 37^{0}C. Then nitrocellulose filters were placed on top and incubated overnight at 37^{0}C. The next day the filters were removed and then washed 3 x 10 minutes with TBST. The serum to be preabsorbed was then added at a dilution of 1:100 or 1:250 and incubated overnight at 4^{0}C. Finally, the serum was removed and stored at 4^{0}C with 0.05% NaN_3. This procedure was carried out at least twice for each serum.

Before reusing the column, reacting antibodies were washed off with 100% solution B for 20 minutes. This was followed with a wash containing a mix of 31% solution A/69% solution B. Then the column was washed with 100% solution A for 10 minutes. Finally the column was equilibrated with 100% solution B for storage at 4^0C.

Solution A: *0.1 M Glycine, pH 2.7*
0.1% Sodium Azide

Soultion B: *20 mM Na_2HPO_4, pH 7.2*
0.1% Sodium Azide

2.3.1.3 Screening of membranes with human serum

Nitrocellulose membranes were blocked by incubating for 1 hour with TBST/5% milk powder at room temperature and subsequently washed three times with TBST. Membranes were incubated overnight at 4^0C with 12.5 ml human serum diluted in TBST/5% milk. The different libraries were screened with the following sera and dilutions:

Serum: / Library:	Autologous 1:100	Pooled 35 patients 1:100 each	35 sera individually 1:100	Patient 3 1:100	Patient 7 1:100	Patient 20 1:100	Total no. of plaques screened
U160799	1,500,000	300,000					1,800,000
KTCTL-28/dC		2,000,000		50,000	50,000	50,000	2,150,000
Testis			20,000	200,000	200,000	200,000	620,000
							4,570,000

After washing three times with TBST, the membranes were incubated with 12.5 ml goat-anti-human IgG (whole molecule)-alkaline phosphatase antibody (Dianova, Hamburg, Germany; 1: 20,000 in TBST/5% milk) for 2 hours at room temperature. After three washes with TBST, antibody binding was visualised by incubating the membranes for at least 15 minutes with BCIP/NBT soultion. The color reaction was stopped by washing the membranes with

tapwater. Positive plaques corresponding to positive signals on the membrane were cut out of the agar plate, transferred to a microcentrifuge tube containing 500 µl SM buffer and 20 µl chloroform, mixed and incubated overnight at 4^0C. The SM buffer containing the phage particles was titered (see *Packaging and titering*), a plaque lift was performed and the new membrane was rescreened with the same serum. If the new membrane still contained positive plaques after this rescreening procedure, one plaque was cut out of the agar as described above, which was then used for a) screening with serum from normal donors or other RCC patients and b) *in vivo* excision and subsequent sequence analysis.

BCIP/NBT solution:	*35 ml of 10 ml BCIP (0.5 g in 10 ml 100% dimethylformamide)*
	45 ml of 10 ml NBT (0.5 g in 10 ml 70% dimethylformamide)
	in Tris-pH 9.5
Tris-pH 9.5	*100 mM Tris*
	100 mM NaCl
	pH 9.5
	50 mM $MgCl_2$

In vivo excision using ExAssist helper phage with E.coli strain XLOLR

From the isolated positive phages the pBluescript phagemid was excised from the Uni-ZAP® XR vector using the ExAssist helper phage and the *E.coli* strain XLOLR. The ExAssist helper phage contains an amber mutation that prevents replication of the phage genome in a

nonsuppressing *E.coli* strain such as XLOLR cells. Therefore, only the excised phagemid is allowed to replicate in the host. In short, host cells (XL1-Blue MRF' and XLOLR) were prepared as described before, but this time the cells were resuspended in 10 mM $MgSO_4$ to an OD_{600} of 1.0. Of these cells, 200 µl XL1-Blue MRF' cells were combined with 250 µl phage stock and 1 µl ExAssist helper phage and incubated for 15 minutes at 37^0C. Then, 3 ml LB medium was added and the tube was incubated for 2.5-3 hours at 37^0C with shaking. The tube was heated to 65-70^0C for 20 minutes and centrifuged at 1000xg for 15 minutes. The supernatant, containing the excised pBluescript phagemid packaged as filamentous phage particles, was stored at 4^0C for up to 2 months.

To plate the excised phagemids, 200 µl of XLOLR cells (OD_{600} of 1.0) were incubated with 10 and 100 µl of phage supernatant respectively for 15 minutes at 37 ^{0}C. 200 µl of this cell mixture was plated on LB-ampicillin (50 µg/ml) agar plates and incubated overnight at 37 ^{0}C. From these bacteria, DNA was isolated (see DNA isolation from bacteria) and sequence analysis was performed (see Sequence analysis). The expression pattern of the isolated genes was determined by Northern blot analysis (see RNA gel electrophoresis and Northern blotting).

2.3.2 Serum antibody detection array (SADA)

300 µl of previously identified phage (at 2000 pfu) were placed in duplicate into wells of a 96-well microtitre plate. 24-26 different phages were used per 96-well plate. The phages were then transferred to a rectangle shape agar plate covered 3 hours previously with *E.coli* XL1-Blue MRF plus topagar plus 10 mM IPTG. The plates were then incubated overnight at 37 ^{0}C. The next day the agar plates were incubated with nitrocellulose membranes for 3-4 hours. Membranes were then either used immediately for screening with human serum (see Screening of membranes with human serum) or stored at -20^0C.

2.3.3 Peptide synthesis

Purified peptides were obtained as lyophilized powder from Dr. R. Pipkorn (Peptide synthesis lab, DKFZ, Heidelberg). Peptides were reconstituted in DMSO to a concentration of 10mg/ml

and then stored in aliquots of 100µl at –20^{0}C. Peptides were diluted to the required concentrations with PBS.

The following peptides were predicted to contain the strongest HLA-A2.1 binding motifs from the BACE2 protein, by the SYFPEITHI program (http://www.syfpeithi.de).

Peptide Name	Peptide Sequence
BACE2-1	ILLVLIVLL
BACE2-2	SLSEPILWI
BACE2-3	LLVLIVLLL
BACE2-4	ILGLAYATL
BACE2-5	VLGGIEPSL
BACE2-6	AILLVLIVL
BACE2-7	WLLRAAPEL

2.4 Cells and tissue culture

2.4.1 Cell lines

Human renal cell carcinoma cell lines, Caki-1, Caki-2, KTCTL-2, KTCTL-28, KTCTL-84, KTCTL-128, A-498, 769-p and 786-O were obtained from the tumour bank of the German Cancer Research Center. All cell lines were cultured and maintained in RPMI-medium supplemented with 10% fetal calf serum (FCS) and antibiotics/L-glutamin (hereafter called culture medium) at 37 ^{0}C, 5% CO_2 in a humidified atmosphere.

Antibiotics/L-glutamine: *100 mg/ml streptomycin sulfate (749 U/mg; Serva, München, Germany)*

58 mg/ml penicillin G (1730 U/mg; Sigma)

245.7 mg/ml L-glutamine (Sigma)

2.4.2 Treatment of KTCTL-28 with 5-AZA-CdR

In order to increase the chances of identifying cancer-testis related antigens, the RCC cell line KTCTL-28 was treated with 5-AZA-CdR. 5-AZA-CdR is a demethylating agent. Genome wide demethylation often occurs in cancer cells as well as in testis and results in the expression of genes that are normally turned off due to methylation of their promoters. Treatment with 5-AZA-CdR (Sigma), was performed as described previously (Coral et al 1999). Briefly, cells were seeded at a density of 3–4 x 10^5 cells/ml in a T175 tissue culture flask. When cells became firmly adherent to plastic, the medium was replaced with fresh medium containing 1 µM 5-AZA-CdR, every 12 h for 2 days (four pulses). At the end of treatment, the medium was replaced with fresh culture medium without 5-AZA-CdR, cells were cultured for an additional 48 h. mRNA was then isolated (see 2.1.1 isolation of mRNA) and used for the creation of cDNA for the construction of a λ-phage library or stored at –80^0C for future use in northern blotting or RT-PCR.

2.4.3 Generation of human dendritic cells (DCs) from peripheral blood monocytes

50 ml fresh human blood was diluted 1:1 with sterile PBS, carefully layered on Ficoll (Histopaque®-1077, Sigma) and centrifuged for 20 minutes at 2000 rpm at room temperature. The peripheral blood lymphocytes (PBL)-containing interphase was collected and washed twice with sterile PBS. Cells were finally resuspended in Iscove's medium supplemented with 10% autologous serum, and incubated for 1.5 hours in an incubator (37 ^{0}C, 5% CO_2 in a humidified atmosphere). Nonadherent cells were removed by carefully washing the culture medium. Remaining adherent cells were incubated for 10 days in Iscove's medium supplemented with 10% autologous serum, 150 U/ml rhGM-CSF (Strathmann Biotech, Hannover, Germany), 50 U/ml rhIL-4 (Strathmann), 50 U/ml rhIFNγ and antibiotics/L-glutamine. Every 2-3 days, the culture medium was refreshed. Maturity of DCs after 9-11 days of culture was confirmed by microscopy (veiled cells) and/or flourescent activated cell sorter (FACS) analysis.

2.4.4 Proliferation assay: PBL activated by peptide loaded DCs

Mature DCs were loaded with varying concentrations of synthetic MHC class I-binding peptides of the BACE2 proteins for 1.5 hours in 24 well plates at 37^0C and subsequently washed. Autologous PBL, put into culture 2 days before with 10 U/ml rhIL-2 (Strathmann), were added to the loaded DCs at ratios between 10:1 and 100:1 (PBL:DC) and cocultured for 3 days. 150 µCi [^{3}H]-Thymidine (Amersham-Pharmacia) was added to each well and incubated overnight at 37^0C. The next day cells were harvested using a cell harvester (Wallac, Freiburg, Germany) and radioactivity was measured in a liquid-scintillation β-counter (Pharmacia).

2.4.5 FACS analysis

For flow cytometry, 3-5x10^5 cells per well (96 well round bottom microtiter plate) were stained with primary antibody in a volume of 50 µl for 1 hour at 4^0C, then washed three times with PBS. Afterwards, cells were incubated with flourescent- labelled secondary antibody (50 µl) for 30 minutes at 4^0C in the dark and subsequently washed 3 times in PBS. The following primary antibodies were used; OKT4 (anti-human CD4), OKT8 (anti-human CD8), BB7.2 (anti-human HLA-A2.1), W6/ 32 (anti-human MHC class I), anti-human CD80 (BD Biosciences, Heidelberg, Germany) and anti-human CD86 (BD Biosciences). Secondary antibodies used were; Flourescein (FITC) labelled rat anti-mouse IgG or FITC labelled rabbit anti-rat IgG. Analysis was performed using a FACSCalibur and CellQuest software (BD Biosciences).

3. Results

3.1 Suppression Subtractive Hybridization

Suppression subtractive hybridization (SSH) is a technique used for the identification of differentially expressed genes. Because upregulation of genes can induce antibody responses it was thought that SSH would be a suitable technique for the identification of RCC associated antigens. A previous SSH performed on a single RCC patient in this lab had identified the MAGE-9 gene. In this study, a pool of 6 RCC versus the matched pool of normal kidney tissue was used for SSH. This second SSH served two purposes 1) to confirm the findings and reliability of the first SSH and 2) to increase the chances of identifying more upregulated genes.

Over 100 clones were initially picked and sequenced. After eliminating duplicated sequences and sequences too short for a significant match in the database, 47 clones were left. Six of the 47 had already been identified by a previous SSH in this laboratory i.e. insulin-like growth factor binding protein-3 (IGFBP3), ceruloplasmin, angiopoietin-related protein 2 (ARP2), autotaxin (ATX), vascular endothelial growth factor (VEGF) and cyclin D1. The remaining 41 genes were examined for their expression in the 6 RCC samples and corresponding normal kidneys by Northern blotting. Differential expression of 12 of the 41 genes could be confirmed by Northern blotting (Table 6).

Clone no.	Identity	Expression pattern
4	Suppressor of Ty (SUPT5H)	Strong upregulation in 2/6
9	Ribosomal protein S2	Weak upregulation in 1/6
10	Unknown/ BAC RP11-674J14	Weak downregulation in 1/6
16	Unkown/ homologous to mouse clone an01-h01	Weak downregulation in 1/6
18	Okadoic acid-inducble and cAMP regulated phosphoprotein 19 (ARPP-19)	Strong upregulation in 2/6 Strong downregulation in 2/6
22	Ribosomal protein L8	Weak upregulation in 1/6
36	Adenosine Deaminase (ADA)	Weak upregulation in 1/6
47	Laminin receptor homolog	Weak upregulation in 1/6
48	Beta-site APP-cleaving enzyme (BACE2)	Strong upregulation in 2/6
49	Diubiquitin (UBD)	Weak upregulation in 1/6
80	Unknown/ KIAA1253	Weak upregulation in 1/6
89	Mitochondrial DNA	Weak downregulation in 1/6

Table 6. Differential expression of SSH clones.

As only minor differences could be seen for 9 of the 12 genes these genes were excluded from further analyses. Expression levels of the ARPP-19 gene varied widely between normal kidney tissues and was also eliminated from further study. The two remaining genes, suppressor of Ty (SUPT5H) and beta-site APP-cleaving enzyme (BACE2) were found to be strongly upregulated in 2 of the 6 patients each (Figure 4).

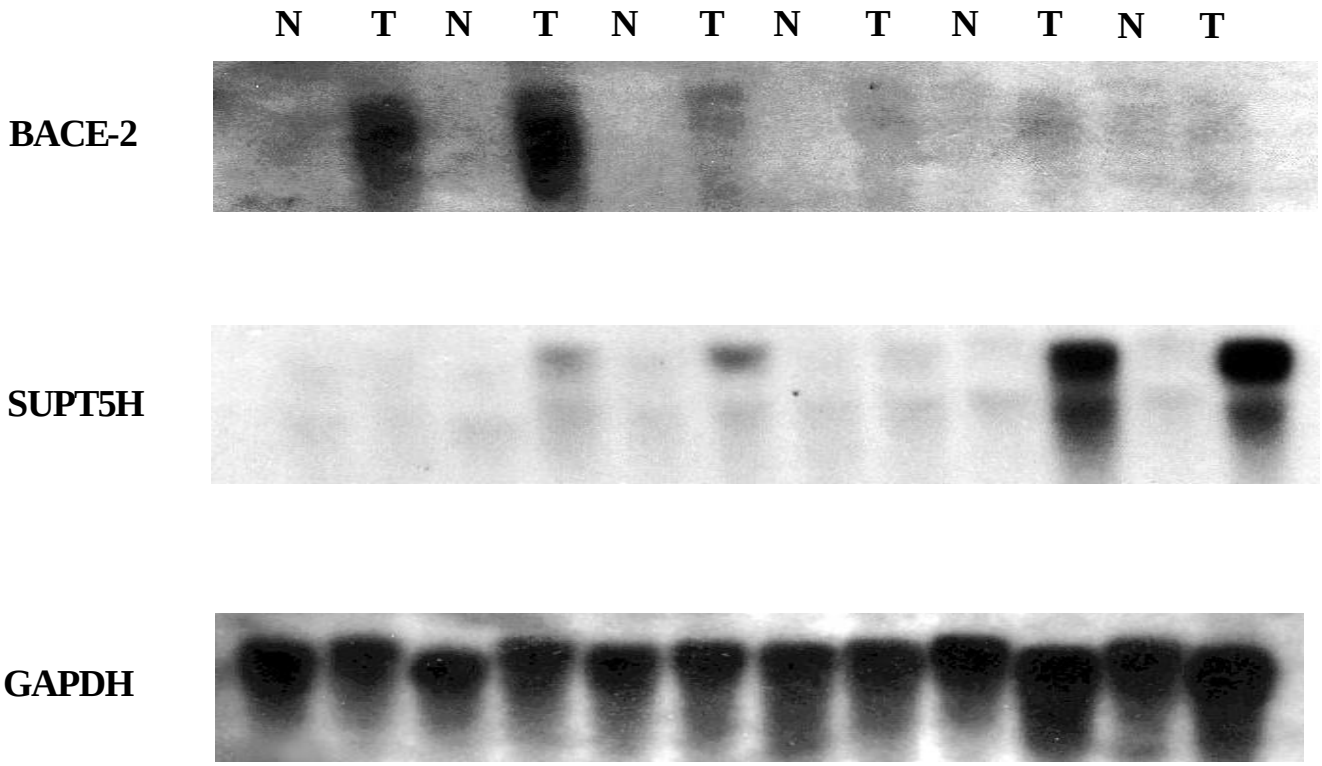


Figure 4. Expression by Northern blot analysis of BACE-2 and SUPT5H.

Shown are the 6 patients from which the SSH library was derived; (N) = normal kidney, (T) = RCC tissue. Northern blots were stripped and reprobed with GAPDH as a loading control.

3.1.1 Correlation of expression with histology, grading and staging

To be useful as a prognostic or diagnostic marker, overexpression of a gene would have to correlate with patient outcome or the histology, grading or staging of the tumor. It was therefore decided to examine the expression of the two overexpressed genes (BACE2 and SUPT5H) as well as nine other differentially expressed genes previously found by an SSH in this laboratory in a panel of 35 RCC patients of which the tumor histology, grading and

staging is known. The nine other genes previously identified are: semaphorin G (SemG), lysyl oxidase (LO), autotaxin (ATX), angiopoeitin-related protein (ARP2), ceruloplasmin (CP), vascular endothelial growth factor (VEGF), C1 respiratory complex (C1-RC), cyclin D1, and insulin-like growth factor-binding protein-3 (IGFBP3). Samples of the Northern blots can be seen in Figure 5. The resulting expression profile of the 11 genes in 35 patients is depicted in Figure 6 which groups the patients according to histological subtype.

Statistical analysis of the correlation of differential gene expression with tumor histology, grading and staging is shown in Table 7. Apart from the clear-cell group, the number of patients per group in the other histological types was too small to allow a statistical analysis between these groups. However, they could be compared to the group of clear-cell RCC. The clear-cell RCC group could be compared to all non-clear-cell RCC. For some of the genes, namely C1-RC, SUPT5H, VEGF, ARP2, ATX, LO and Sem G, there appears to be a correlation with histological subtype. Overexpression of these genes has been found mainly in clear cell RCC. BACE2 on the other hand is preferentially overexpressed in mixed (clear and granular) and oxyphil. There was no correlation of gene overexpression with tumor grading and only ATX and LO showed a correlation with tumor progression. Correlation with patient outcome/ survival will be performed after a 5 year period to determine if any of the genes have prognostic significance.

3.1.2 Testing the immunogenicity of BACE2

Immune responses of the T helper ($CD4^+$) and T cytotoxic ($CD8^+$) type were examined for the BACE2 protein. The existence of T helper responses was assayed indirectly by looking for the presence of anti-BACE2 antibodies in the sera of RCC patients. This was achieved by expressing the BACE2 protein in λ-phage and performing a secondary SEREX with this phage. No antibodies could be detected in the sera of 35 RCC patients nor in 15 healthy controls

Seven peptides derived from BACE2 and predicted to bind HLA-A2.1 were assayed in a mixed lymphocyte culture test for their ability to induce $CD8^+$ T cell proliferation. None of the peptides examined were found to induce proliferation in 3 donors.

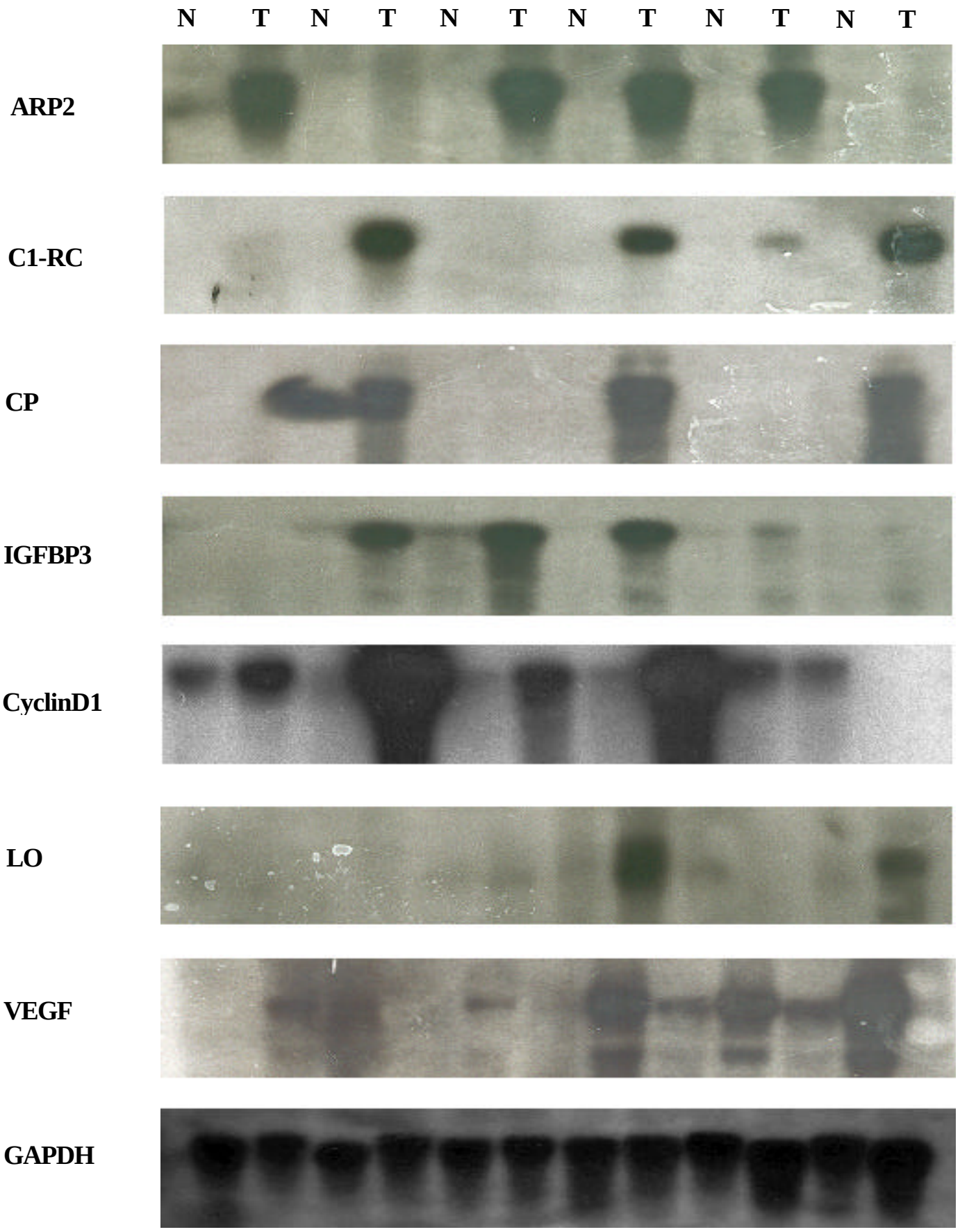


Figure 5. Expression by Northern blot analysis of ARP2, C1-RC, CP, CycD1, IGFBP3, LO, and VEGF.

Shown are the 6 patients from which the SSH library was derived; (N) = normal kidney, (T) = RCC tissue. Northern blots were stripped and reprobed with GAPDH as a loading

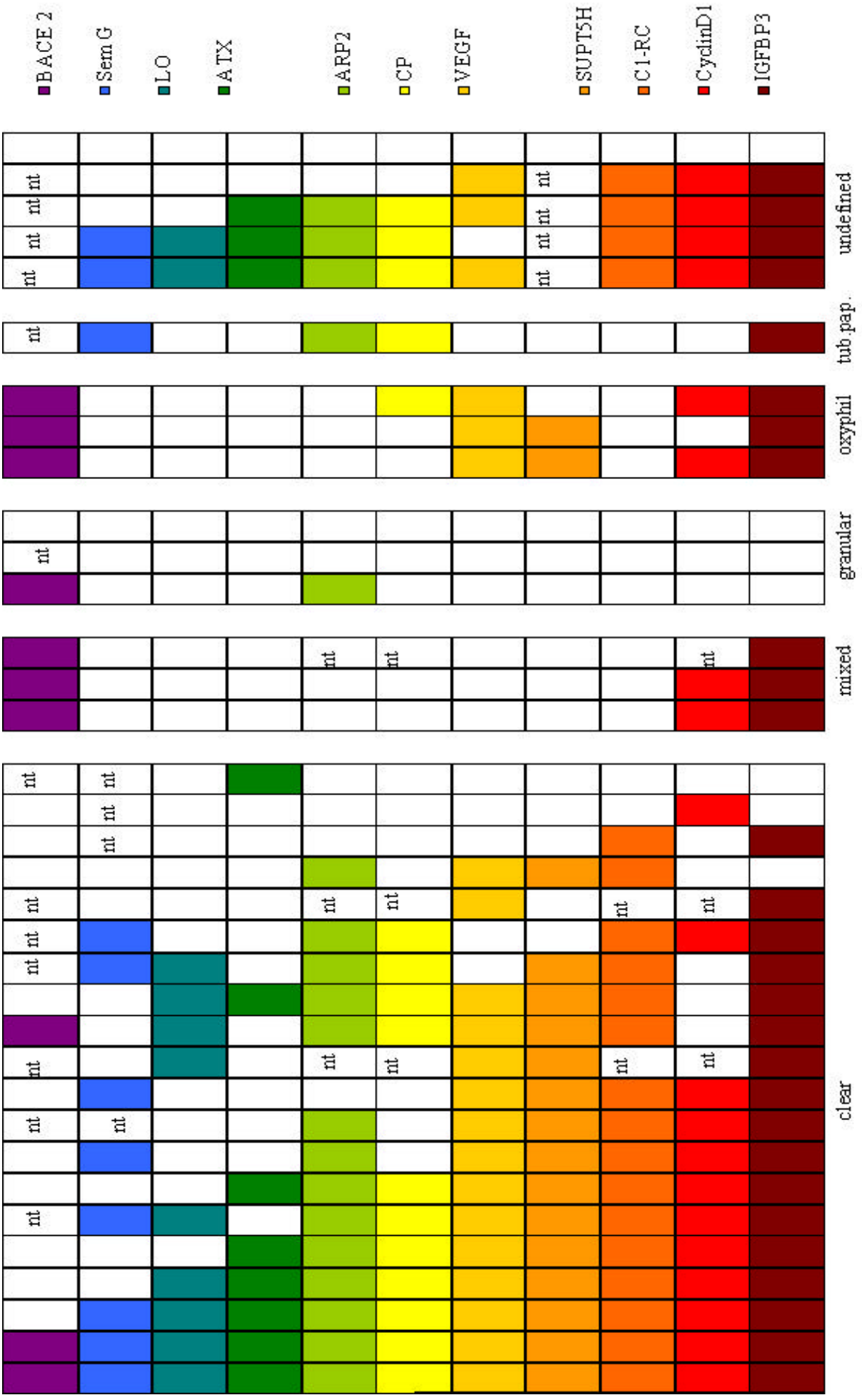


Figure 6. Expression profile of the 11 genes in 35 patients.
Patients are grouped according to histological type. White squares indicate no differential expression. nt: not tested.

Clinical features	IGFBP3	Cyc.D1	C1-RC	SUPT5H	VEGF	CP	ARP2	ATX	LO	SemG	BACE2
Histology											
Clear (20)	17/20	12/18	16/18	15/20	15/20	11/18	14/18	8/20	9/20	8/16	3/13
P value (vs all other types)[1]	ns[2]	ns	<0.0001	0.007	0.02	ns	0.005	0.02	0.01	0.04	0.002
Clear and granular (3)	3/3	2/2	0/3	0/3	0/3	0/2	0/2	0/3	0/3	0/3	3/3
P values (vs clear)[1]	ns	ns	0.007	0.03	0.03	ns	ns	ns	ns	ns	0.02
Granular (3)	0/3	0/3	0/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	1/2
P values (vs clear)[1]	0.01	ns	0.007	0.03	0.03	ns	ns	ns	ns	ns	ns
Oxyphil (3)	2/3	2/3	0/3	2/3	3/3	1/3	0/3	0/3	0/3	0/3	3/3
P values (vs clear)[1]	ns	ns	0.007	ns	ns	ns	0.03	ns	ns	ns	0.02
Tubulopapillary (1)	1/1	0/1	0/1	0/1	0/1	1/1	1/1	0/1	0/1	1/1	
Grading[3]											
GI (13)	9/13	6/10	5/11	7/13	9/13	4/10	6/10	1/13	3/13	2/10	7/12
GII (13)	11/13	8/13	8/13	7/13	6/13	7/13	7/13	4/13	4/13	5/12	2/8
GIII (5)	3/5	2/5	3/5	3/5	3/5	2/5	3/5	3/5	2/5	2/5	1/1
Staging											
T1,N0, M0 (17)	12/17	8/14	7/15	8/17	10/17	5/14	8/14	1/17	3/17	5/14	7/12
T2, N0, M0 (6)	4/6	3/6	2/6	3/6	2/6	2/6	2/6	1/6	1/6	1/6	2/3
T3a/3b, N0, M0 (5)	4/5	3/5	4/5	3/5	3/5	3/5	3/5	3/5	2/5	1/4	0/4
T3a/t4, N2, M0 (3)	3/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	1/2
P value[4]	ns	ns	ns	ns	ns	ns	ns	0.002	0.03	ns	ns

Table 7. Correlation of gene expression with tumor histology, grading and staging.

[1]*P* values are derived from Fisher's exact test, clear cell RCC were compared against all non-clear cell RCC, the other groups were compared against clear-cell RCC as far as the minimal number of 3 samples had been tested.
[2]ns: not significant.
[3]no statistically significant differences were found in correlation with tumor grading.
[4]*P* values are derived from the exact Jonckheere test, which describes a trend from T1, N0, M0 towards T3/T4, N2, M0

3.2 SEREX

As none of the genes identified by SSH could be classed as antigens (with the exception of MAGE-9), it was decided to screen for antigens by the SEREX method. Clones identified by the SEREX method are by definition immunogens as they have already induced a B-cell response which implies T-cell help. Three different libraries were screened in this study; i) a library derived from a tumor of a patient, ii) a library from a RCC cell line treated with 5-aza deoxycytidine and iii) a testis library. The libraries were screened either with autologous serum (in the case of the patient tumor library), pooled serum (in the case of the patient tumor library and the cell line library), or with various individual sera (in the case of the testis library. All positive phages were tested twice to ensure no false positives. Plaques were then subcloned to monoclonality and in vivo excised. The resulting phagemid was sequenced and each sequence was compared to the genome database (www.ncbi.nlm.nih.gov) and to the SEREX database (www2.licr.org/CancerImmunomeDB). Selected clones were then analyzed for mRNA expression and seroreactivity.

3.2.1 Screening of a patient tumor library with autologous and pooled sera

A phagemid library was constructed from RNA isolated from the tumor of a 72 year old female with Renal Cell Carcinoma. The tumor was of the mixed type and the staging of the tumor was defined as T1G1. The mRNA for the genes IGFBP3 and BACE 2 were found to be upregulated in this patient but not SemG, SUPT5H, ATX, LO, C.1., VEGF, ARP2, Ceruloplasmin or CyclinD1.

Approximately 1.5 $x10^6$ plaques from this library were screened initially with serum from the same patient. 83 clones were identified as potential positives. 23 of these could be discounted immediately as they were directly interacting with the secondary antibody. The remaining 60 could not be verified by rescreening.

Pooled serum from 33 patients with RCC was then used to screen $3x10^5$ clones. 17 clones were initially identified. However only 5 of these could be verified after rescreening. The clones were named HD-RCC-1 to HD-RCC-5 according to SEREX terminology (Table 8).

HD-RCC- 1, 2, 3 and 5 all represent novel genes. HD-RCC-4 contains part of the sequence of NADH Ubiquinone Oxidoreductase chain5. Examples of SEREX membranes are shown in Figure 7.

Clone	Accession no.	Identity	SEREX homologs
HD-RCC-1	AC008009	DNAJ domain containing protein	None
HD-RCC-2	HS1056L3	No homologies	None
HD-RCC-3	AC005383	Actin associated filament protein	None
HD-RCC-4	BQ614315	NADH Ubiquinone Oxidoreductase chain 5	None
HD-RCC-5	BC012607/ AC006559	Translocation between Chr.19q13.2 and Chr.12p12	None

Table 8. Clones identified from screening of a tumor library with pooled patient sera.

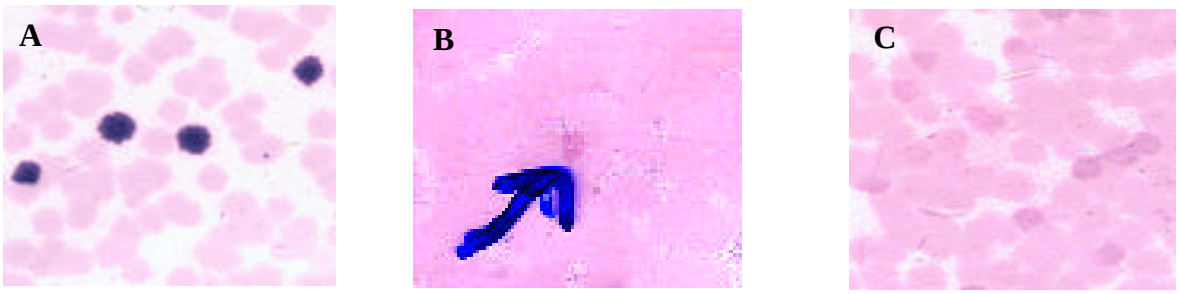


Figure 7. Developed SEREX membranes.
A) positive/negative control, B) Initial identification of clone HD-RCC-1, C) Secondary SEREX of HD-RCC-1 with ~50% negative phages.

HD-RCC-1 has a sequence of 1270 bp and can be localised to chromosome 3q26.2-27 (AC008009). The gene consists of at least 5 exons. Analysis of the sequence reveals an open reading frame of 345 bp which results in an a.a. seq of 115 aa (Figure 8). No translation

initiation site could be identified but it cannot be far from the 5 prime end as Northern blot analysis reveals a transcript of between 1300 and 1400 bp. Comparison of the a.a. sequence with sequences in the database reveals a 60% homology to DNA J domain containing proteins. These proteins belong to the family of molecular chaperones of which heat shock proteins are a large part. It has been shown that hsp27 is upregulated in RCC. Also 4 different molecular chaperones have previously been identified by the SEREX method in RCC.

HD-RCC-2 has a sequence of 800bp and is localised to chromosome 1p35-36 (HS1056L3). Hybridization of this clone to Northern blots reveals a transcript of approximately 4 kb. Sequencing of an IMAGE clone of 3kb which overlaps the clone we have isolated reveals an ORF of at least 2 kb which terminates prior to the beginning of the clone isolated by SEREX. Therefore the clone isolated lies in the 3 prime untranslated region of the gene. Therefore the immunogenicity of this clone cannot be related to the gene from which it comes. However the sequence of the clone shares some homology with another unknown sequence on chromosome 3 and this sequence may somehow be immunogenic.

HD-RCC-3 has a sequence of 3660 bp and is localized to chromosome 10q25.1 (AC005383). It consists of 19 exons with 2 possible alternative splicing sites. Hybridization to Northern blots reveals a transcript of about 4 kb. Depending on the splicing the open reading frame is either 2451bp or 2652bp resulting in an aa sequence of 817/884 aa's (Figure 9). Sequencing of this clone revealed that the 2451 splice variant is the one expressed in the tumor from which the library was derived. Comparison of the aa sequence with the databases reveals a 40% homology to actin associated filament proteins.

HD-RCC-4 was identified as NADH Ubiquinone Oxidoreductase chain 5. However the sequence isolated belongs to the 3 prime untranslated region of the gene. Analysis of the aa sequence of the clone reveals a 54% homolgy to Epstein Barr Virus and this homology may be the cause of the positive reaction found. It is quite possible that one of the 33 patients had an EBV infection and antibodies toward this in the patients serum may have reacted with the clone we have isolated.

Sequencing of HD-RCC-5 reveals a different sequence at the 5 prime end than at the 3 prime end. The sequence at the 5 prime end belongs to chromosome 19 and the 3 prime end is from chromosome 12. Therefore this clone could be from a translocation between chromosome 19 and 12. The 5 prime end corresponds to a clone (BC012607) which is similar to pregnancy

specific beta-1-glycoprotein 5. However, in the SEREX clone, the gene is in the negative orientation. There are no significant open reading frames of the reverse strand of the pregnancy specific clone. The 3 prime end, which corresponds to a region of chromosome 12p12 (AC006559), contains an open reading frame of 126 æs. There were no open reading frames found as a result of the translocation. Therefore, the antibodies detected against this clone must be directed against the 12p12 part of the clone.

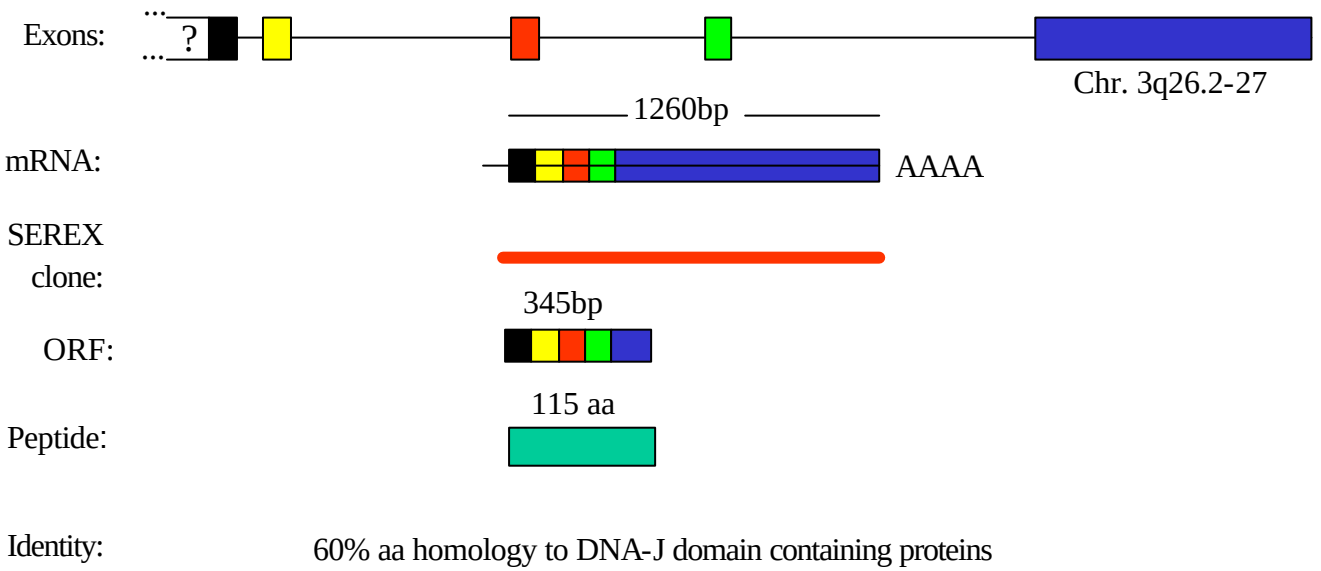


Figure 8. Localisation and Characterisation of HD-RCC-1

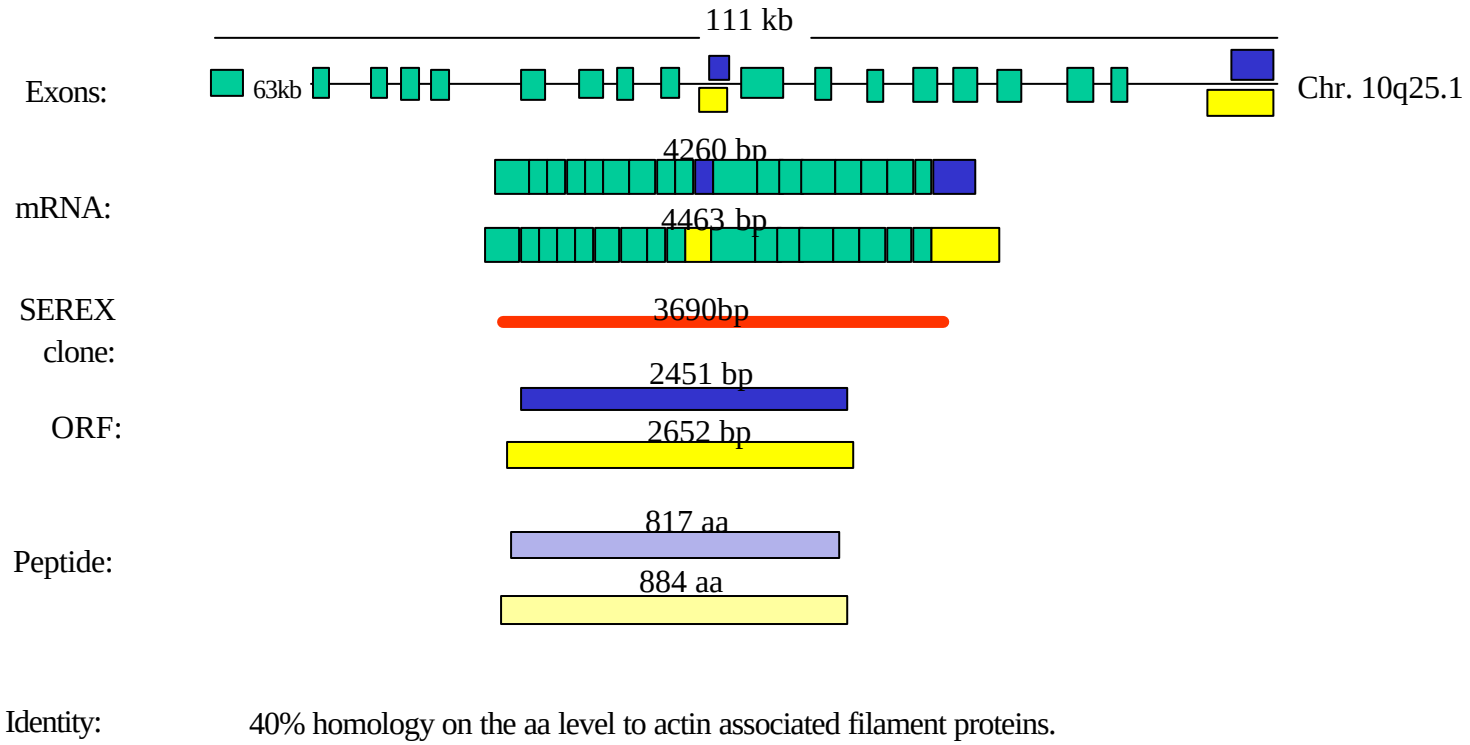


Figure 9. Localisation and Characterisation of HD-RCC-3

3.2.1.1 mRNA expression and seroreactivity of HD-RCC-1 to 5

The mRNA expression of each clone was examined by Northern blotting or RT-PCR. Seroreactivity was examined by secondary SEREX i.e. the isolated clones were plated ~50:50 with negative clones followed by incubation of the membrane with individual sera. Table 9 summarizes the results of the mRNA expression and seroreactivity of each of the clones.

Clone	mRNA expression	Seroreactivity	
		Patient	Normal
HD-RCC-1	n.d.	1/35	0/15
HD-RCC-2	n.d.	1/35	0/15
HD-RCC-3	n.d.	1/35	0/15
HD-RCC-4	n.d.	1/35	0/15
HD-RCC-5	Restricted to the tumor from which the library was derived	1/35	0/15

Table 9. mRNA expression and seroreactivity of HD-RCC-1 to 5. n.d. No differential expression between normal kidney and RCC.

For clones HD-RCC-1,to –4, no difference in mRNA expression could be seen between normal kidney and RCC tumor. For HD-RCC-5, only the patient from which the library was derived expressed the translocation product. For all cases, only 1 in 35 patients had reactive antibodies (in each case a different patient). Serum from 15 healthy donors did not react to any of the clones.

None of the above clones are therefore suited to immunotherapy as they are either uniformly expressed or restricted to an individual patient. Also, only 1 out of 35 patients reacted to any of these clones. It was decided not to analyse these clones any further.

3.2.2 Screening of a RCC cell line library with pooled sera

A λ-phage library was constructed from the RNA from the RCC cell line KTCTL-28 treated with 5-aza-2'-deoxycytidine (5-AZA-CdR). 5-AZA-CdR is a hypomethylating agent. Treatment with 5-AZA-CdR has been shown to increase the expression of cancer testis antigens (Coral et al 2002). Approximately $2x10^6$ plaques from this library were screened with pooled sera. A total of 20 positives were initially identified. However, only 6 of these could be confirmed after rescreening. These 6 clones are named HD-RCC-6 to HD-RCC-11 (Table 10).

Clone	Accession no.	Identity	SEREX homologs
HD-RCC-6	NM_152233	Sorting nexin 6 (SNX6)	NY-BR-48
HD-RCC-7	NM_002078	Golgin subfamily a, 4 (GOLGA4)	Hom-Br2-54 HOM-MA1-12 HOM-MA1-8 MO-CO-134 MO-CO-77 NGO-St-67 se20-7
HD-RCC-8	NM_003472	DEK proto-oncogene (DEK)	None
HD-RCC-9	BC013724	Human ferritin, heavy chain	None
HD-RCC-10	NM_018003	Uveal autoantigen (UACA)	MO-BC-423; MO-BC-440; NY-ESO-12; MO-CO-80; MO-TES-217
HD-RCC-11	NM_152255	Proteosome subunit, alpha type 7 (PSMA7)	None

Table 10. Clones identified from screening of the KTCTL-28/5-AZA-CdR library with pooled patient sera.

HD-RCC-6 encodes a member of the sorting nexin family. Members of this family contain a phox (PX) domain, which is a phosphoinositide binding domain, and are involved in intracellular trafficking. Sorting nexin 6 (SNX6) associates with the long isoform of the leptin receptor, the transforming growth factor-beta family of receptor serine-threonine kinases, and with receptor tyrosine kinases for platelet-derived growth factor, insulin, and epidermal

growth factor. SNX6 may form oligomeric complexes with family member proteins through interactions of both the PX domain and the coiled coil regions of the molecules (Parks et al 2001). Translocation of this protein from the cytoplasm to the nucleus occurs after binding to proviral integration site 1 protein (Ishibashi et al 2001). This gene results in two transcripts encoding two distinct isoforms. Sequencing of the SEREX clone revealed that HD-RCC-6 corresponds to the shorter isoform. SNX6 has been identified previously as an antigen in the screening of a breast cancer sample with autologous serum. No mutations could be identified in the sequencing of the clone.

Golgin subfamily a, 4 (GOLGA4) is a member of the golgin family of proteins which are localized to the golgi apparatus. GOLGA4 is thought to play a role in Rab6-regulated membrane-tethering events in the Golgi apparatus (Barr,F.A 1999). Alternative splice variants have been described but their full-length nature has not been determined. GOLGA4 is also known to be an autoantigen (Kooy et al 1992) and has been identified in seven other SEREX studies to date. There were no mutations in the HD-RCC-7 clone as compared to the database.
DEK is a 43-kDa phosphoprotein that was first isolated as part of a fusion protein expressed in a subtype of acute myeloid leukemia (AML). The fusion protein is a result of a (6;9) chromosomal translocation resulting in the chimeric *dek-can* fusion protein (von Lindern et al 1992). Its role in leukemia, however, is unclear. DEK itself has been identified as an autoimmune antigen in patients with pauciarticular onset juvenile rheumatoid arthritis, systemic lupus erythematosus, and other autoimmune diseases (Szer et al 1994, Dong et al 1998, Wichmann et al 2000). DEK has been reported to be a site-specific DNA binding factor, which recognizes a specific DNA element in the HIV-2 enhancer (Fu et al 1997). Again, no mutations could be identified in the HD-RCC-8 clone.

Human ferritin is a major tissue iron-binding protein (Hann et al 1980) and, in its native form, is approximately 500 kDa. It is composed of 24 subunits consisting of acid/heavy (H) and basic/light (L) chains (Arosio et al 1979, Theil et al 1987). The genes encoding H- and L-ferritin are found in different chromosomes and are transcriptionally independent (McGill et al 1987). The 24-subunit polymer may form isoferritins, which are either more acidic (H-rich) or more basic (L-rich), depending on the relative proportions of H and L chains. It has been shown that H-ferritin from melanoma cells may suppress immune responses and that this immunosuppression is attributable to changes in antigen presenting cells, which resulted in the preferential activation of regulatory T cells that produce interleukin 10 (IL-10) (Gray et al

2001, Gray et al 2002). The human ferritin heavy chain has not been previously identified by SEREX. The HD-RCC-9 clone does not contain any mutations as compared to the database.

The human uveal autoantigen (UACA: uveal autoantigen with coiled coil domains and ankyrin repeats) has only recently been identified (Yamada et al 2001). Its function is unknown. However, autoantibodies against UACA appear to be more prevalent in people with panuveitis (Yamada et al 2001). Several other SEREX studies have identified UACA with seroreactivity in normal healthy controls ranging from 50% to 100% depending on the study. No mutations were identifed in the sequence of the HD-RCC-10 clone.

The proteasome is a multicatalytic proteinase complex with a highly ordered ring-shaped 20S core structure. The core structure is composed of 4 rings of 28 non-identical subunits; 2 rings are composed of 7 alpha subunits and 2 rings are composed of 7 beta subunits. Proteasomes are distributed throughout eukaryotic cells at a high concentration and cleave peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway. An essential function of a modified proteasome, the immunoproteasome, is the processing of class I MHC peptides. The proteosome subunit, alpha type 7 (PSMA7)gene encodes a member of the peptidase T1A family, that is a 20S core alpha subunit. This particular subunit has been shown to interact specifically with the hepatitis B virus X protein, a protein critical to viral replication (Huang et al 1996). In addition, this subunit is involved in regulating hepatitis virus C internal ribosome entry site (IRES) activity, an activity essential for viral replication (Kruger et al 2001). This core alpha subunit is also involved in regulating the hypoxia-inducible factor-1alpha, a transcription factor important for cellular responses to oxygen tension (Cho et al 2001). Multiple isoforms of this subunit arising from alternative splicing may exist but alternative transcripts for only two isoforms have been defined. PSMA7 itself has not been previously identified by SEREX, however another subunit (PSMA6) has been found 3 times with seroreactivity against PSMA6 two to three times as frequent in patients with malignant stomach cancer as in normal healthy controls. No mutations were identified in the HD-RCC-11 clone.

3.2.2.1 mRNA expression and seroreactivity of HD-RCC-6 to 11

Northern blotting of the 6 clones showed no differences between normal kidney and RCC tumors of 10 patients. However, mRNA for the human ferritin heavy chain was upregulated in the 5-AZA-CdR treated KTCTL-28 line as compared to non-treated KTCTL-28. Results of seroreactivity and mRNA expression are summarized in Table 11.

Clone	mRNA expression	Seroreactivity	
		Patient	Normal
HD-RCC-6	n.d.	1/35	0/15
HD-RCC-7	n.d.	35/35	15/15
HD-RCC-8	n.d.	1/35	0/15
HD-RCC-9	n.d. Upregulated in 5-AZA-CdR treated KTCTL-28	1/35	0/15
HD-RCC-10	n.d.	35/35	15/15
HD-RCC-11	n.d.	1/35	0/15

Table 11. mRNA expression and seroreactivity of HD-RCC-6 to 11. n.d. No differential expression between normal kidney and RCC.

Apart from HD-RCC-7 (GOLGA4) and HD-RCC-10 (UACA), the seroreactivity of the clones identified is restricted to 1/35 patients (in each case a different patient). GOLGA4 and UACA appear to be strong autoantigens and antibodies against these genes has no correlation with disease state.

3.2.3 Screening of a RCC cell line library with individual sera

The 5-AZA-CdR treated KTCTL-28 cell line was then screened with sera from 3 individuals. $0.5x10^5$ plaques were screened with each of the 3 sera. 10 clones were initially identified of which only 5 could be confirmed after rescreening. Of these 5, 2 clones corresponded to the same gene. Table 12 lists the clones identified in this screening.

Clone	Accession no.	Identity	SEREX homologs
HD-RCC-12	NM_006793	Peroxiredoxin 3 (PRDX3)	None
HD-RCC-13	BC012423	Superoxide dismutase 2 (MnSOD)	None-
HD-RCC-14	NM_005751	A kinase anchor protein 9 (AKAP9)	MO-REN-1
HD-RCC-15	BC012579	KIAA1229/ unknown protein	None

Table 12. Clones identified from screening of the KTCTL-28/5-AZA-CdR library with 3 individual patient sera.

Peroxiredoxin 3 (Prdx3) expression is induced by oxidants in the cardiovascular system and is thought to play a role in the antioxidant defense system and homeostasis within the mitochondria (Araki et al 1999, Wonsey et al 2002). Prdx3 is a c-*myc* target gene, and antisense experiments have shown that its expression is required for neoplastic transformation by c-*myc* (Wonsey et al 2002). Prdx3 has been shown to be overexpressed in hepatocellular carcinomas (Choi et al 2002). Prdx3 has not been previously identified by SEREX, however another family (Prdx1) member has been identified in the screening of a melanoma cell line with serum from melanoma patients.

Superoxide dismutase 2 (SOD2), also known as manganese-dependent superoxide dismutase (MnSOD), is the mitochondrial scavenger of O_2^-. Its activity has been shown to be dramatically reduced in several classes of malignancies, including melanoma, hepatocarcinoma and breast cancer (Sun 19990). On the other hand, overexpression of MnSOD has been shown for neuro-epithelial (Landriscina et al 1996), ovarian (Ishikawa et al 1990), cervical (Nakano et al 1996) and thyroid tumors (Nishida et al 1993), with MnSOD levels correlating with tumor severity. These differences in response to MnSOD may be due to different constitutive levels of MnSOD and may also be dependent upon the cell type. Neither MnSOD nor any of its family members have been identified before by SEREX. An analysis of RCC patients using the SERPA technique has identified increased levels of anti-MnSOD antibodies in 4 out of 6 RCC patients (Unwin et al 2003).

The A-kinase anchor protein 9 (AKAP9) is a member of a group of structurally diverse proteins which have the common function of binding to the regulatory subunit of protein kinase A (PKA) and confining the holoenzyme to discrete locations within the cell. Alternate

splicing of this gene results in many isoforms that localize to the centrosome and the Golgi apparatus, and interact with numerous signaling proteins from multiple signal transduction pathways. AKAP9 also known as yotiao is a scaffold protein that physically attaches type I protein phosphatase (PP1) and PKA to N-methyl-D-aspartate (NMDA) receptors to regulate channel activity (Westphal et al 1999). AKAP9 has been identified before by SEREX in the screening of a RCC tumor with autologous serum.

HD-RCC-15 corresponds to a protein of unknown function (KIAA 1229). The KIAA 1229 is located on chromosome 1p22.3 and has 57% sequence similarity on the amino acid level with a human reverse transcriptase homolog (accession no. S65824). Virtual Northern blotting of KIAA 1229 shows that it is expressed in many normal tissues (http://cgap.nci.nih.gov/Genes/GeneFinder). KIAA 1229 has not been identified previously by SEREX.

3.2.3.1 mRNA expression and seroreactivity of HD-RCC-12 to 15

The mRNA expression of the four clones was analysed by Northern blotting. No differential expression could be found for any of the clones in 24 RCC patients. The seroreactivity of the clones was then analyzed with 35 patient sera and 15 healthy controls. The results are summarized in Table 13.

Clone	Identity	Seroreactivity	
		Patient	Normal
HD-RCC-12	PRDX3	4/35	1/15
HD-RCC-13	MnSOD	5/35	0/15
HD-RCC-14	AKAP9	12/35	9/15
HD-RCC-15	KIAA1229	3/35	1/15

Table 13. Seroreactivity of HD-RCC-12 to 15 in RCC patients and healthy controls.

For 3 of the above antigens (PRDX3, AKAP9, KIAA1229), there is little difference in seroreactivity between RCC patients and healthy controls. Therefore these antigens are unsuitable as prognostic, or diagnostic markers.

In the case of MnSOD, 5 out of 35 patients have antibody responses against this protein whereas none of the healthy controls are reactive. This makes MnSOD a good candidate for prognostic or diagnostic purposes.

3.2.4 Screening of a Testis library with individual sera

A testis library was obtained from the laboratory of Stefan Eichmüller (Skin Cancer Unit, DKFZ, Heidelberg) and screened with individual sera from 35 RCC patients. Initially $2x10^4$ plaques were screened with each of the 35 sera. Thereafter the top 3 responding patients (top 3 sera with the most reactive clones) were used to screen the testis library further. $2x10^5$ plaques were screened with each of the 3 sera. In total, 104 clones were identified; 15 from serum no.3, 30 from serum no. 7, 33 from serum no. 20, and 26 from all others combined. The 104 clones corresponded to 59 different transcripts, 20 of which belong to unknown genes. Table 14 lists the 59 different transcripts. Clones are ordered according to the serum with which they were identified (3, 7, 20, all others, respectively).

Table 14. Clones identified from screening of a testis library with 35 individual patient sera.

Clone no.	Accession no.	No. of times isolated	Identity	SEREX homologs
HD-TES-1	NM_006267	2	RAN binding protein 2	MO-BC-1083
HD-TES-2	BC002362	2	Lactate dehydrogenase B	NY-REN-46, NW-TWe 43, TC45
HD-TES-3	AC109815	1	BAC clone	None
HD-TES-4	NM_005054	5	RAN binding protein 2 like-1	Hom-Gliom, GT 39, HOM-Ts-PMR1-11, Hom-TSMa5-58, MO-BC-1083
HD-TES-5	AF093415	1	Cell division protein	None
HD-TES-6	BC036109	1	SECIS binding protein 2	MO-BC-204, NGO-Br-48
HD-TES-7	AC112246	1	BAC clone	None
HD-TES-8	AC007899	1	RPII-53	None
HD-TES-9	AC129915	1	Chromosome 8 clone	None
HD-TES-10	AP_000354_1	1	Chromosome 22 clone	None
HD-TES-11	AF097485	1	Transducin beta like-2 gene	None
HD-TES-12	BC008881	3	Kinesin 2	Hom-TSSemA-65, NGO-St-51, NY-BR-45, MO-REN-2, MO-REN-49
HD-TES-13	BC047764	2	Syntaxin binding protein 3	None
HD-TES-14	AF254756	3	TSGA10	None
HD-TES-15	NM_002078	6	Golgin subfamily a, 4	Hom-Br2-54, HOM-MA1-12, HOM-MA1-8, MO-CO-134, MO-CO-77, NGO-St-67, se20-7
HD-TES-16	NM_016343	2	Centromere protein F	NGO-Pr-24, NGO-Br-7, NY-BR-69, NY-ESO-11, MO-CO-139, MO-CO-18, MO-CO-151, MO-TES-148
HD-TES-17	ALI 57387	1	RP11-20F24	None
HD-TES-18	BC012498	1	Valosin containing protein	None
HD-TES-19	Z77885	1	flow-sorted chromosome 6 TaqI fragment	None
HD-TES-20	AF109135	1	Archivillin	cl#11

Table 14. Continued.

Clone no.	Accession no.	No. of times isolated	Identity	SEREX homologs
HD-TES-21	BC013835	1	Beta-actin	NY-SAR-52, KM-PA-3
HD-TES-22	NM_005751	1	A kinase anchor protein 9	MO-REN-1
HD-TES-23	NM_030906	3	Serine threonine kinase 33	None
HD-TES-24	AK097129	2	cDNA FLJ 39810	None
HD-TES-25	NM_006633	1	RAS-GAP-related protein	TE2-35a
HD-TES-26	AY014284	1	Nucleoporin NYD-SP7	None
HD-TES-27	HSA132440	1	PLU-1	None
HD-TES-28	BX161471	1	DNA clone (CSODI069YD09) of placenta	None
HD-TES-29	AF432211	1	CLL assiciated antigen KW11	KW11
HD-TES-30	BC059374	17	Serine threonine kinase 31	None
HD-TES-31	NM_001726	2	Bromodomain testis specific protein	None
HD-TES-32	AC114486	1	RP11-1217.3	None
HD-TES-33	AC008088.8	1	Chr. 17 clone	None
HD-TES-34	BC048287	1	SEC63-like protein	None
HD-TES-35	NM_025009	1	Hypothetical protein FLJ13621	None
HD-TES-36	NM_014648	1	Zinc finger DAZ interacting protein 3	HOM-TSMa4-10, Lu22.2, MO-OVA-102
HD-TES-37	AC097467	1	RPII-27G13	None
HD-TES-38	NM_002154	1	Heat shock 70kDa protein 4	KM-PA-1, NGO-Br-43, NGO-St-81, NY-CO-32, NY-CO-40
HD-TES-39	M18112	1	Human poly(ADP-ribose) polymerase	NY-BR-59
HD-TES-40	BC012003	1	KIAA0635	NW-TK 190

Table 14. Continued.

Clone no.	Accession no.	No. of times isolated	Identity	SEREX homologs
HD-TES-41	NM_001813	1	Centromere protein E	None
HD-TES-42	AL831917	1	Thyroid hormone receptor interactor 8	None
HD-TES-43	NM_003472	1	DEK proto-oncogene	None
HD-TES-44	AB014543	1	KIAA0643	MO-OVA-68, MO-CO-98
HD-TES-45	AC090768	1	RPII-722P15	None
HD-TES-46	NM_203292	1	Retinoblastoma-interacting protein 8	None
HD-TES-47	NM_016061	1	Yippee protein	None
HD-TES-48	NM_024581	1	Chr. 6 ORF 60	MO-TES-14, NGO-St-98
HD-TES-49	BC058921	1	Glutamyl prolyl tRNA synthetase	None
HD-TES-50	AK001695	1	Strawberry notch homolog 1	None
HD-TES-51	AF458591	1	Hypothetical protein	None
HD-TES-52	BC012846	1	Isocitrate dehydrogenase 1	None
HD-TES-53	BC001282	1	high mobility group nuceosomal binding domain 4	None
HD-TES-54	NM_152233	1	Sorting nexin 6	NY-BR-48
HD-TES-55	NM_003176	1	SCP-1 meiosis specific protein	Hom-TSRCC1-7, se2-1, se5-1, se20-8, se33-2, NW-TK 119
HD-TES-56	AY092062	1	Breast cancer antigen U1717	None
HD-TES-57	BC015815	1	Butyrophilin subfamiliy 3, member A3	None
HD-TES-58	AK025531	1	cDNA FLJ 21878 fis	None
HD-TES-59	AC112246	1	BAC clone	None

3.2.4.1 Sequence analysis of HD-TES-1 to 59

The PubMed database was examined for literature on all the known clones. From this, the antigens are predicted to belong to the following categories according to their predicted expression: Cancer-Testis antigens, Restricted expression antigens, Upregulated antigens, Normal/ ubiquitous antigens (autoantigens), and Unknown antigens.

Cancer-Testis antigens Six of the clones identified here are predicted to be expressed only in testis tissue. The six clones (HD-TES-4, 14, 27, 30, 31 and 55) correspond to RAN binding protein 2 like-1 (RANBP2L1), TSGA10, PLU-1, serine/ threonine kinase 31 (STK31), Bromodomain testis specific protein (BRDT), and SCP-1 meiosis specific protein, respectively. RANBP2L1 has been identified 5 times in this screening, four times from one serum and once with another. It has also been identified in other SEREX studies. TSGA10 has been isolated here 3 times from the same serum. No other SEREX studies have identified TSGA10 as an antigen before. PLU-1 has already been suggested to be a cancer-testis antigen and has been shown to be upregulated in breast cancer (Barret et al 2002). It has not been identified before as an antigen for RCC. STK31 has been identified 17 times with the one serum in this screening possibly indicating high immunogenicity of this protein in this patient. It has not been found by other SEREX studies. BRDT has been identified as a cancer-testis antigen in lung cancer (Scanlan et al 2000). It has been isolated twice from the same serum in this screening but not in any other SEREX screening to date. The synaptonemal complex protein 1 (SCP-1) gene was originally identified through SEREX screening of a testis library with serum from an RCC patient (Tureci et al 1998).

Restricted expression antigens Restricted expression antigens are those that are expressed in only a limited number of tissues, or they may have low level expression in many tissues but high expression in a select few. Three of the clones identified in this screening have restricted expression. HD-TES-6, 11, and 23 correspond to SECIS binding protein 2 (SBP2), Transducin betra like-2 gene (TBL2), and serine/ threonine kinase 33 (STK33), respectively. SBP2 has been shown to be overexpressed in testis (Lescure et al 2002). It has been identified twice in other SEREX studies. TBL2 is expressed as a 2. 4-kb transcript predominantly in testis, skeletal muscle, heart and some endocrine tissues, with a larger approximately 5-kb transcript detected ubiquitously at lower levels (Perez et al 1999). TBL2 has not been

identified before by SEREX. STK33 is expressed in many but not all tissues (Mujica et al 2001). In this study, STK33 has been isolated 3 times by two different sera. It has not been identified previously by other SEREX studies.

Upregulated antigens The antigens in this category are normally ubiquitously expressed but have been found to be upregulated in cancer. Three of the known genes fall into this category. HD-TES-2, 18, and 46 correspond to lactate dehydrogenase B (LDHB), Valosin containing protein (VCIP), and Retinoblastoma interacting protein 8 (RBBP8), respectively. Lactate dehydrogenases are metabolic enzymes which catalyze the conversion of L-lactate to pyruvate, the last step in anaerobic glycolysis. Overexpression of LDHB has been found in breast cancer cell lines as compared to normal tissues (Forti et al 2002). Serum antibodies against LDHB have been identified in four other SEREX studies. VCIP is a member of the "ATPases associated with various cellular activities" superfamily and plays a key role in the ubiquitin-dependent proteasome degradation pathway (Dai et al 1998). Increased expression of VCIP has been found in pancreatic ductal adenocarcinoma, colorectal carcinoma and gastric carcinoma and has been shown to correlate with lymph node metastasis and with disease progression (Yamamoto et al 2004, 2004, 2003). No other SEREX studies have identified VCIP as an antigen before. RBBP8 is a binding partner for the retinoblastoma (RB) protein, which is a known tumor suppressor. RBBP8 is expressed ubiquitously at low levels and has been found to be upregulated in many tumor cell lines (Fusco et al 1998). RBBP8 itself has not been identified previously by SEREX but two other family members (Rbbp6 and Rbbp7) have.

Normal ubiquitous antigens Antigens in this group are ubiquitously expressed and to date have not been shown to be overexpressed in cancer. The greatest proportion of antigens identified in this screening belong to this group. This is also the case for most other SEREX studies. The majority of entries in the SEREX database belong to normal ubiquitously expressed genes. For most of these antigens, seroreactivity is expected to be equivalent in both normal controls and cancer patients. Therefore, presence of antibodies against these antigens is not related to disease.

Unknown antigens The sequences from all of the unknown antigens were virtually translated in all 3 frames to identify possible open reading frames (ORFs). The most likely ORFs were then compared to the protein databases using BlastP. Many of the antigens did not contain any

significant ORFs and were therefore not studied futher. Three more sequences were found to contain ALU repeat sequences. Alu repetitive sequences are interspersed in the human genome with an average spacing of 4 kb. Some of them are actively transcribed by pol III. Normal transcripts may contain Alu-derived sequences in 5' or 3' untranslated regions. However, cDNA libraries also contain partial and/or rearranged cDNAs ligated with Alu-derived sequence in any orientation. Although Alu elements (especially situated on the complementary strand) have a great potential to create additional/alternative exons, consideration should be given to the possibility that the presence of an Alu in an open reading frame may have resulted from a cloning artifact or may be due to misinterpretation of sequencing data. Therefore these clones were not examined any further. Only 8 of the known antigens were found to have significant open reading frames which also do not correspond to Alu repeat sequences. Table 15 lists the 8 antigens and their homologies to other proteins.

Clone HD-TES	**Chr.**	**Protein homology**	**Predicted Expression (Virtual Northern)**
9	8	No homologies / 1 possible ORF	unknown
10	22q11.2	No homologies / 3 possible ORFs	unknown
28	14q32.32	Hypothetical protein FLJ23027	Low ubiquitous Increased in retina and spine
35	4q12	Hypothetical protein FLJ13621	Highly ubiquitously expressed
40	4q12	KIAA 0635 protein	Low ubiquitous Increased in lung, heart and stomach
44	16p13.3	KIAA 0643 protein	Low ubiquitous
51	17q24.3	Hypothetical protein MGC33887	Low ubiquitous
58	22q11.23	KIAA0376 protein	Low ubiquitous. Increased in pancreatic and lung carcinoma

Table 15. Protein homologies and predicted expression of 8 of the unknown clones

Expression of the clones was predicted by virtual Northern. No predictions could be made for clones HD-TES-9 and 10. The other clones appear to be expressed ubiquitously at low levels with the exception of HD-TES-35 which is predicted to be expressed ubiquitously at high

levels. Both HD-TES-40 and 58 are predicted to be upregulated in cancer. HD-TES-40 and 44 have previously been identified by SEREX but not any of the other unknown clones.

3.2.4.2 mRNA expression and seroreactivity of HD-TES-1 to 59

The mRNA expression of 23 of the 59 clones was examined by Northern blotting of 24 RCC patients (both normal and RCC tissue). Expression of the CT antigens were also examined by RT-PCR. For the CT antigens, no signals could be detected on Northern blots nor by RT-PCR. This could be due to the low level of expression and the low frequency of expression of CT antigens. For all other antigens, signals could be detected on Northern blots. However, no differential expression could be detected except in the case of HD-TES-46 (RBBP8) (Figure 10). It is possible that some of the antigens are post transcriptionally regulated. This would not be detected by Northern or RT-PCR. Altered post-translational modifications or mutations may also cause induction of antibodies.

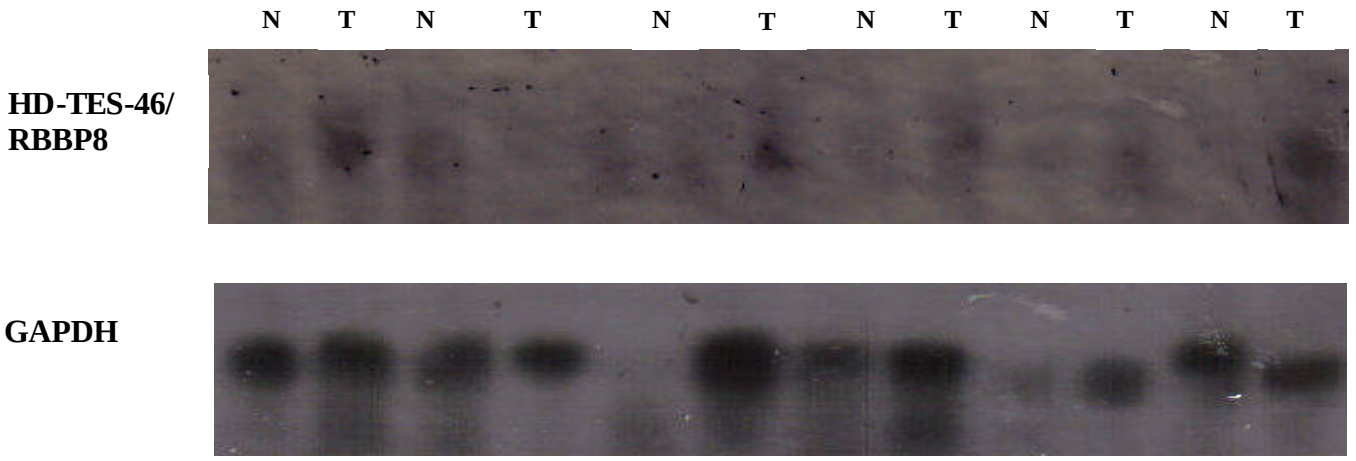


Figure 10. Expression of HD-TES-46 (RBBP8) in 6 samples of RCC.
N: Normal kidney tissue. T: Tumor kidney tissue.

While the loading of mRNA is not equal for all samples (as judged by the GAPDH control), it can be clearly seen that RBBP8 is upregulated in the tumor of two of the six patients (First and last patients in Figure 10). Analysis of a further 18 patients did not show upregulation of RBBP8 in any more RCC patients.

The seroreactivity of 34 of the clones was determined by secondary SEREX with 35 RCC sera and 15 sera from healthy controls. The results of this screening are summarized in Table 16.

The majority of the antigens screened here seem to elicit a similar frequency of antibody responses in both normal controls and healthy patients. HD-TES-2, 5, 11, 13, 22, 23, 25, 28, 31, 32, 35, 37, 39, 42, and 44 all appear to fall into the category of non-cancer related autoantigens. The majority of the antigens in this group (10/15) have not been identified before by SEREX and thus represent novel autoantigens.

Eight of the antigens elicit antibody responses more frequently in RCC patients than in healthy controls. HD-TES-4, 10, 12, 24, 41, 46, 47 and 50 belong to this category. The difference between seroreactivity in RCC patients and normal controls varies for each clone. The frequency difference ranges from 1.4 (HD-TES-4) to 4.8 (HD-TES-47) times higher in cancer patients. Whether the increase in antibody responses is related to cancer is unclear. Only 2 of the 8 antigens in this group (HD-TES-4 and 12) have previously been identified before by SEREX.

The remaining antigens elicit antibody responses only in cancer patients and not in healthy controls. Seroreactivity against the antigens HD-TES-9, 14, 18, 27, 30, 48 and 55 are found at frequencies of 3% (1 of 35) to 17% (6 of 35). Interestingly, 4 of the 7 antigens in this category belong to the cancer testis classification of antigens. HD-TES-14, 27, 30 and 55 have all been described to be expressed only in testis tissue. HD-TES-18, which corresponds to valosin containing protein is known to be upregulated in some cancers. The other two antigens are of unknown function. Four of the antigens (HD-TES-9, 14, 18 and 30) have not been identified by other SEREX screenings

Clone HD-TES	RCC serum n=35	Normal serum n=15	Clone HD-TES	RCC serum n=35	Normal serum n=15
2	60%	53%	30	11%	0%
4	57%	40%	31	20%	27%
5	23%	27%	32	6%	7%
6	23%	0%	34	31%	7%
9	11%	0%	35	26%	20%
10	46%	27%	37	6%	7%
11	9%	7%	39	20%	20%
12	20%	7%	41	20%	7%
13	11%	13%	42	30%	27%
14	11%	0%	44	17%	13%
18	17%	0%	46	40%	13%
22	34%	27%	47	34%	7%
23	17%	13%	48	3%	0%
24	29%	13%	50	40%	20%
25	14%	7%	55	9%	0%
27	11%	0%	57	6%	0%
28	17%	7%	58	31%	20%

Table 16. Seroreactivity of RCC patients versus healthy controls for 34 of the 59 antigens.

3.3 SADA

SADA (Serum Antibody Detection Array) is based on the SEREX method and is used to determine seroreactivity against known antigens. In this method, phages containing the gene of interest are spotted at high concentrations on agar plates. Up to 96 different phages can be plated on one plate. Once the plaques are transferred to nitrocellulose membranes, the procedure is the same as for SEREX. In this study we have analysed the seroreactivity of RCC patients versus normal healthy controls for 44 different known antigens. These known antigens have been identified in previous SEREX screenings of melanoma and cutaneous T-cell lymphoma (CTCL) libraries (Stefan Eichmüller: Skin Cancer Unit, DKFZ, Heidelberg). Table 17 lists the phages used here. The phages are arranged on the SADA membrane according to Figure 11. Figure 11 also shows examples of normal and RCC sera on the SADA membranes.

The 44 antigens used here were divided into 2 membranes with each antigen spotted twice on each membrane. Screening with each serum was repeated twice and antigens were only considered positive if the duplicates were positive on both occasions. A spot was scored as positive if it was clearly darker than the spots corresponding to the negative phage. Table 18 summarizes the results of the SADA screening. Only antigens that showed reactivity with sera are shown.

Table 17. List of the phages used in the screening of RCC sera by the SADA method

No	Identity	Accession no.	No.	Identity	Accession no.
1	Pinch	U09284	23	RP11_157D8	
2	Retinoblastoma binding protein 2 homolog 1a (Rbbp21a)	AJ243706	24	Mitogen-activated protein kinase 6 (MAPK6)	NM_002748
3	Retinal scDH reductase (RscDHr)	AF273056	25	Ubiquitin protein ligase E3A (UBE3A)	NM_000462
4	cTAGE-5a	NM_022663	26	Squamous cell carcinoma antigen recognized by T cells 3 (SART 3)	NM_014706
5	Par-3 partitioning defective 3 homolog (Par-3ta)	NM_019619	27	InsP3 phospatase (InsP3)	
6	Negative phage/ No insert		28	Protein Tyrosine phospatase (PTp)	
7	Retinoblastoma-associated protein 140 (RAP140)	NM_015224	29	Nuclear distribution gene C homolog (NDCH)	
8	PLU-1	AJ132440	30	NP220 nuclear protein (NP220)	NM_014497
9	KIAA0555	NM_014790	31	Ribosomal protein	
10	cTAGE-1	NM_022663	32	LAR-interacting protein 1a/b (Lari 1a/b)	U22815/6
11	Synaptonemal complex protein 1 (SCP-1)	NM_003176	33	Hexamethylene-bis-acetamide-inducible transcript (HEXIM-1)	AB021179
12	Guanylate-binding protein5-ta (GBP-5ta)	AF328727	34	Hypothetical protein BM-009 (BM-009)	BC017297
13	CTCL tumor antigen se57-1 (se57-1)	AF273051	35	Serine/ threonine kinase 17b (apoptosis inducing) (STK 17b)	NM_004226
14	Dynein heavy chain (DNEL-2)	AJ000522	36	Cell division autoantigen 1 (CDA1)	AY040871
15	Cytoskeleton associated protein 2 (CKAP2)	NM_018204	37	Unknown/ chromosomal sequence (9Ka)	
16	MAGE-A9	NM_005365	38	Zinc finger protein 133 (ZNF 133)	BC001887
17	GRIP and coiled-coil domain-containing 2 (LTA 1-1)	NM_014635	39	Splicing factor 3b, subunit 2 (Sp3b)	BC000401
18	Guanylate-binding protein-5a (GBP-5a)	AF430643	40	Putative S1 RNA binding domain protein (PS1)	
19	Serine/threonine kinase (KDS)	AF181985	41	MAGE-A3	NM_005362
20	Golgin subfamily a, 4 (GOLGA4)	NM_002078	42	Chromosome 8 est sequence (8EST)	
21	Zinc finger protein 195 (ZNF 195)	NM_007152	43	Elongation factor 1 alpha (EF-1 alpha)	BC019669
22	GAGE-3	U19144	44	Humanin	AY029066

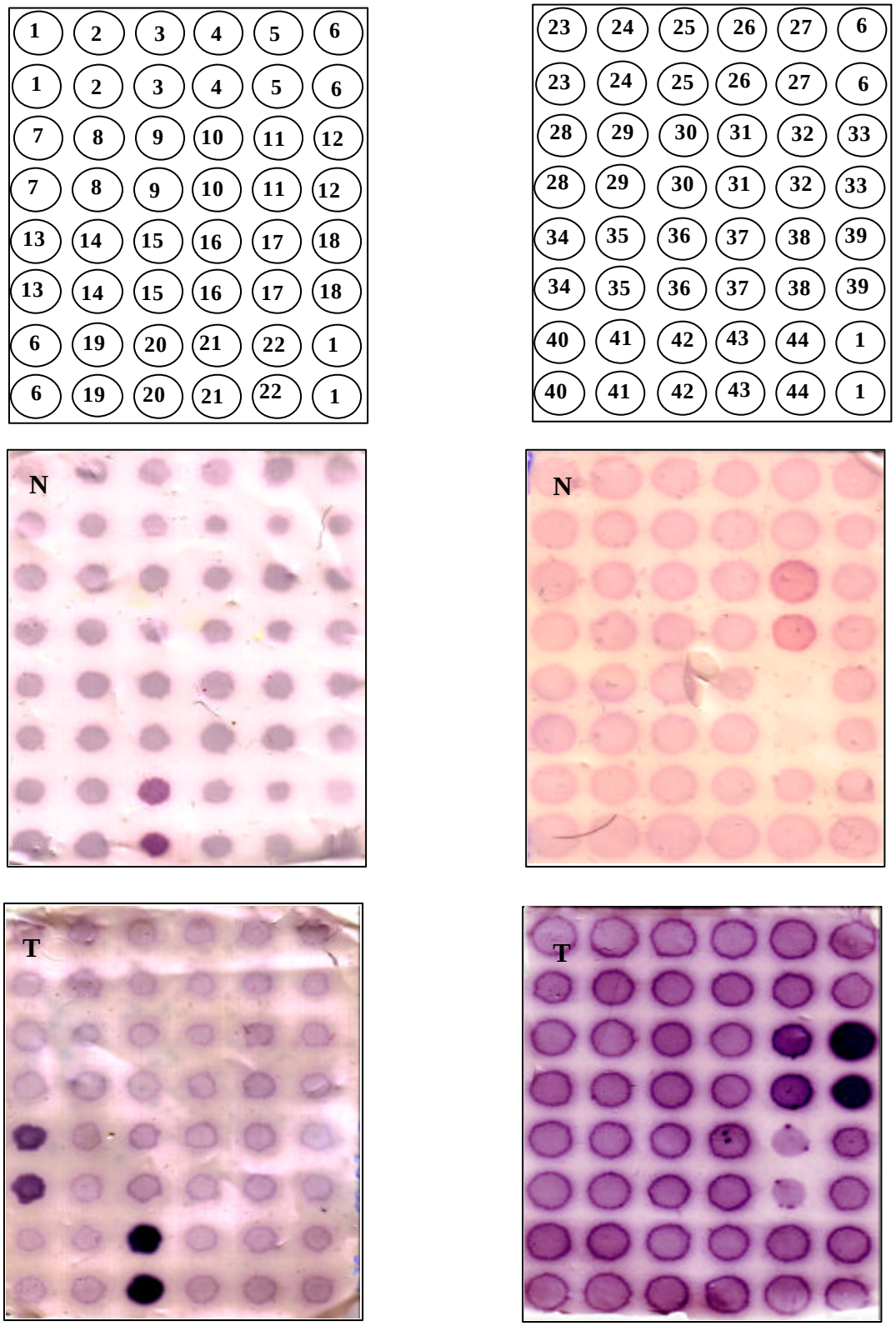


Figure 11. Examples of seroreactivity of normal and RCC sera on the SADA membranes.

N; normal serum. T; RCC serum.

No.	Clone	RCC serum n = 35	Normal serum n = 15
2	Rbbp21a	3%	0%
4	cTAGE-5a	3%	0%
5	Par-3ta	23%	0%
7	RAP140	3%	0%
11	SCP-1	29%	7%
13	se57-1	17%	0%
15	CKAP2	6%	0%
17	LTA 1-1	37%	20%
20	GOLGA4	94%	87%
22	GAGE-3	3%	0%
32	Lari 1a/b	97%	100%
33	HEXIM-1	11%	0%
36	CDA1	26%	7%
38	ZNF 133	40%	7%

Table 18. Seroreactivity of RCC patients versus healthy controls.

Antigens not displaying reactivity with either RCC sera or healthy controls are not shown.

Reactivity against the above antigens can be divided into 3 categories; 1) antigens to which the majority of both healthy and RCC patients are reactive (autoantigens), 2) antigens to which RCC patients are more reactive towards, and 3) antigens to which RCC patients are exclusively reactive (i.e. no reactivity in healthy controls).

The GOLGA4 antigen and the Lar interacting protein both fall into the category of autoantigens according to this screening. It is already known that the GOLGA4 is an autoantigen. The Lar interacting protein, on the other hand, has not been identified before as an autoantigen. While it was not the original intention, the presence of these autoantigens on the SADA membranes served as a positive control.

Four of the antigens screened fall into the second category. SCP-1, LTA 1-1, CDA1 and ZNF 133 all show a higher percent reactivity in RCC patients than in normal healthy controls. However, the difference between the level of reactivity between healthy controls and RCC patients varies e.g. 29% versus 7% for SCP-1, 40% versus 7% for ZNF 133.

The 3rd category contains the most antigens. Seroreactivity against Rbbp21a, cTAGE5a, Par-3, RAP 140, se57-1, CKAP2, GAGE3, and HEXIM-1 was only found in RCC patients and not in healthy controls. This, however does not mean that only RCC patients have antibodies against these antigens. Patients with other tumor types are reactive against these antigens (these antigens have previously been identified by SEREX). For some of the antigens, where only 3% of patients are reactive (Rbbp21a, cTAGE-5a, RAP140, GAGE-3), the size of the groups are too small to make any conclusions. For the other 3 antigens, Par-3, se57-1 and HEXIM-1, significant numbers of patients have antibodies against these antigens (23%, 17% and 11% respectively).

4. Discussion

Molecular biological and serological techniques have been used here to identify genes and antigens associated with RCC. Through SSH, 2 genes (BACE2 and SUPT5H) could be shown to be upregulated in RCC. The frequency of upregulation of 9 other genes and their relation to tumor progression was also examined. Through the SEREX method, 74 antigens could be identified. These antigens have been examined for their expression profile and seroreactivity in RCC patients. Finally, a further 44 antigens have been examined by the SADA method to determine seroreactivity in RCC. The relationship of the genes and antigens identified here to RCC, the RCC Immunome and cancer in general is discussed below.

4.1 Genes identified by SSH

The identification of MAGE-9, a cancer/ testis antigen, by SSH in RCC (Pitzer et al 1999) led to the further use of this technique in the hope of identifying further RCC associated antigens. Screening of a pooled library resulted in the identification of 12 genes with differential expression in RCC. Two of these genes (BACE2 and SUPT5H) could be shown to be highly upregulated in RCC.

BACE2. Beta-site amyloid precursor protein cleaving enzyme 2 (BACE2) is a glycosylated transmembrane protein of the aspartyl protease family (Bennet et al 2000). It is highly homologous to BACE1 and both are involved in the processing of amyloid precursor protein (APP) to amyloid beta peptide (Aβ) (Farzan et al 2000). Cerebral deposition of amyloid beta peptide is an early and critical feature of Alzheimer's disease and a frequent complication of Down syndrome. Accumulation of Aβ is also found in the muscle of patients with sporadic inclusion-body myositis (s-IBM) and hereditary inclusion-body myopathy (h-IBM) (Askanas et al 2001). Indeed, BACE2 localizes to the 'Down critical region' of chromosome 21and is thus thought to play a major role in the progression of this disease. Montanaga et al (2002) have detected increased expression of BACE2 in the brains of Down syndrome patients displaying alzheimer neuropathology but not in brains without alzheimer neuropathology.

BACE2 mRNA is expressed at low levels in most human peripheral tissues and a higher levels in colon, kidney, pancreas, placenta, prostate and stomach. Increased expression has been found in breast cancer and BACE2 is suggested to contribute to the proteolytic cascade in neoplastic cells (Xin et al 2000). Also worth noting is the fact that APP, the target of BACE2, is involved in the growth of human colon carcinoma cells in vitro and in vivo (Meng et al 2001).

In this study, BACE2 was found to be upregulated in 10 out of 22 RCC patients tested (45%). It was also found to be highly expressed in 5 of 10 RCC cell lines and 9 of 22 lung carcinoma cell lines. Statistical analysis showed a preferential expression in mixed and oxyphil RCC. BACE2, therefore, seems to play a significant role in RCC and other cancers. Targeting BACE2 with specific inhibitors (as suggested for the treatment of Alzheimers) may be of benefit in controlling tumor growth and progression.

BACE2 does not appear to be immunogenic. Loading of dendritic cells with BACE2 derived peptides did not induce proliferation of T cells in a mixed lymphocyte reaction. Also, no antibodies could be detected in RCC patients against BACE2 in a secondary serex screening of this molecule.

SUPT5H. SUPT5H is the human homolog of the yeast gene SPT5 (Suppressor of Ty 5). The SPT genes were identified through a genetic screening for mutations in the yeast *Saccharomyces cerevisiae* that restore gene expression disrupted by the insertion of the transposon Ty. SUPT5H is mapped to chromosome 19q13 and encodes a 1087aa protein that is expressed in all tissues. Together with SUPT4H and SUPT6H, SUPT5H is known to play a critical role in transcription elongation and activation of transcription (Wen and Shatkin 1999, Kaplan et al 2000, Yamaguchi et al 2001).

Overexpression of SUPT5H has not been shown before for any cancer. In this study it was shown to be overexpressed in 17 of 31 RCC patients (55%). It was mainly seen in clear cell RCC. As SUPT5H is critical for transcription initiation and elongation, it is not surprising that this gene is upregulated. Upregulation of this gene in cancer probably enhances cell proliferation. However, this also makes SUPT5H an unlikely target for immunotherapy as to interfere with this molecule would be lethal for both normal and cancer tissue.

4.1.1 Expression of previously identified SSH genes in RCC

The expression of 9 other genes in 35 RCC patients, previously identified by SSH, was examined in order to determine if there is a correlation between upregulation of the gene and tumor histology, grading or staging. All of the genes examined here have some relation to the tumorigenic process. Three of the genes, (Cyclin D1 C1-RC and IGFBP3) are related to tumor growth/ survival. Three more are related to angiogenesis (VEGF, CP and ARP-2). The final three are involved in cell adhesion/ motility (ATX, LO, SemG). The overexpression of these genes in RCC is therefore no surprise. The fact that some of these genes are overexpressed in 80-90% of RCC suggests that they are important for tumor progression. Although no correlation could be made between gene upregulation and grading, overexpression of 6 of the genes (VEGF, ARP2, ATX, LO and SemG) was found mainly in clear cell RCC. The overexpression of ATX and LO was found to correlate with tumor progression. Whether this is of prognostic relevance remains to be seen pending the outcome of a follow-up study. As the overexpression of these genes is clearly important for tumor progression, direct inhibition of these gene products may therefore help in controlling the growth of the tumor. Thus, the genes examined here are potential therapeutic targets.

4.2 Antigens identified by SEREX

Genes identified by the SEREX method are by definition antigens, as they have elicited an antibody response which is thought to reflect T cell help. In this study, 3 cDNA libraries (RCC tumor, RCC cell line and Testis) have been screened with serum from RCC patients in an attempt to elucidate, at least in part, the RCC Immunome. 74 antigens have been identified in total which can be grouped according to the following antigen categories: Cancer/Testis, Restricted expression, Upregulated antigens, Translocation antigens, Normal/autoantigens and Unknown antigens.

Cancer/Testis antigens Cancer/Testis antigens are considered to be ideal candidates for immunotherapy due to their restricted expression. In this study 6 CT antigens have been

identified. The six antigens (RANBP2L1, TSGA10, PLU-1, STK31, BRDT and SCP-1) have been examined for their mRNA expression and seroreactivity in RCC.

RANBP2L1 is identical on its 5' end to RANBP2 and is thought to have resulted from a duplication of the RANBP2 gene. RANBP2 is a large 358-kDa protein which is localized at the cytoplasmic side of the nuclear pore complex and likely constitutes the RAN-GTP binding site at the cytoplasmic face of the complex (Melchior et al 1995). RANBP2L1 is a testis-specific nucleoporin and possibly acts as a docking site and a cotransporter of Ran and transportin (Cai et al 2002). It has not been associated before with cancer and unfortunately expression of this gene could not be confirmed in RCC patients. There does however seem to be an increase in seroreactivity in RCC patients as compared to healthy controls (57% and 40% respectively). The question remains however, whether the antibody against RANBP2L1 is specific or whether it is a crossreacting antibody which reacts with the highly related RANBP2. Indeed, RANBP2 itself was also isolated in this study. Experiments to prove the specificity of the anti-RANBP2L1 antibody would need to be conducted to confirm this genes role as an RCC antigen.

TSGA10 is predicted to play a role in the sperm tail fibrous sheath (Modarressi et al 2004). TSGA10 mRNA transcripts could not be detected in RCC patients. It may be that it is expressed at a very low level in tumors and thus difficult to detect. It is also likely to be expressed at a low frequency. Many CT antigens are expressed at frequencies between 5 and 20%. Indeed the seroreactivity against TSGA10 in RCC patients reflects this (11%). Healthy controls did not possess antibodies against this gene. This makes TSGA10 a possible candidate as a diagnostic or prognostic marker.

PLU-1 is a large nuclear protein that is proposed to function as a regulator of gene expression and interacts with the developmental transcription factors BF-1 and PAX9 (Tan et al 2003). Barret et al (2002) have shown that PLU-1 is expressed at high levels in testis but not any other human adult tissue except for ovaries. They also found PLU-1 to be upregulated in breast cancer. Again mRNA for this gene was not detectable in RCC patients but the seroreactivity profile was similar to that of TSGA10 and other CT antigens (11%). Presence of anti-PLU-1 antibodies in serum is thus a potential marker for cancer.

STK31 has only been identified recently in a systematic search for genes expressed in mouse spermatogonia but not in somatic tissues (Wang et al 2001). Nothing is known about this protein to date except that it is expressed only in testis tissue. Like the other CT antigens identified here, mRNA expression from this gene could not be confirmed. The restricted seroreactivity (11% of patients) makes this another potential candidate as a diagnostic or prognostic marker for RCC.

BRDT is a testis-restricted member of the RING3 protein family (Jones et al 1997). It possesses 2 bromodomain motifs and a PEST sequence (a cluster of proline, glutamic acid, serine and threonine residues), characteristic of proteins that undergo rapid cellular degradation. The bromodomain is found in proteins that regulate transcription. BRDT has been shown to play a role in the large-scale reorganization of acetylated chromatin (Pivot-Pajot et al 2003). Expression of BRDT was found in 12 of 47 cases of non-small sell lung cancer (Scanlan et al 2000). It has not been found previously in RCC. BRDT expression could not be confirmed in this study by RTPCR or Northern blotting. There was no significant difference in seroreactivity between RCC patients and healthy controls (20% versus 27%). BRDT is thus not a good candidate as a potential prognostic, diagnostic or therapeutic target.

As well as RCC, SCP-1 has been found to be expressed in melanoma, breast cancer, cutaneous T-cell lymphoma, stomach cancer and hepatocellular carcinoma (Tureci et al 1998, Eichmüller et al 2002, Jager et al 2002, Eichmüller et al 2001, Mashino et al 2001, Chen et al 2001). SCP-1 is expressed during the meiotic prophase of spermatocytes and is involved in the pairing of homologous chromosomes (Tureci et al 1998). In this study 9% of RCC patients were found to have antibodies against SCP-1. This would be consistent with the expression pattern found by Tureci et al (1998) of 3 out of 36 RCC patients. Unfortunately this could not be confirmed for the RCC patients used here. The expression of SCP-1 and the presence of antibodies against it seem to be strongly associated with cancer. SCP-1 is therefore a strong candidate as an immunotherapeutic target, not only on RCC but also many other cancers.

Restricted expression antigens Antigens showing restricted expression may not be suitable candidates for immunotherapy but their overexpression in a tumor may serve as diagnostic or prognostic markers. Three antigens (SBP2, TBL2, STK33) with restricted expression were

identified in this study. Their expression in RCC and the frequency of antibody responses against each of these antigens was examined.

SBP2 is one of the key players in the synthesis of selenocysteine and selenoproteins. Selenoproteins are normally enzymes with selenium in the form of selenocysteine at their active site. Most of these enzymes are involved in redox reactions. Thioredoxin reductase and glutathione peroxidase are just two examples. Another example is the phospholipid hydroperoxide glutathione peroxidase which is involved in sperm maturation (Ursini et al 1999). It is therefore no surprise to find SBP2 highly expressed in testis tissue. What role SBP2 might play in cancer, if any, is unclear. There was no differential expression of SBP2 between normal and tumor kidney for 24 RCC patients. However, 23% of patients have generated an antibody response against this protein in comparison to 0% of controls. The induction of anti-SBP2 antibodies must therefore be related to the tumorigenic process. Whether this is specific for RCC remains to be seen.

TBL2 is a member of the transducin family which have regulatory functions. TBL2 encodes a protein with four putative WD40-repeats. WD40 domains are found in a number of eukaryotic proteins that cover a wide variety of functions including adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly. TBL2 has been shown to be deleted in Williams-Beuren syndrome (WBS), which is a developmental disorder (Perez et al 1999). There is no known association of TBL2 with cancer. In this study, mRNA levels were similar in both normal and tumor kidney. Also, seroreactivity was similar in both RCC patients and healthy controls (9% versus 7%, respectively).TBL2 is therefore not suitable as a diagnostic or prognostic marker.

STK33 belongs to the family of serine/ threonine kinasess. Protein kinases represent a large diverse family which play major roles in signal transduction, DNA replication, metabolic pathways and cell growth, differentiation, proliferation and cell death (Hanks and Hunter 1995, Wilmanns et al 2000). Very little is known so far about STK33 apart from its' chromosomal location. It is localized to chromosomal region 11p15 which is known to be associated with several diseases including predispositions to develop various tumor types Bepler and Koehler 1995, Redeker et al 1995). STK33 may belong to the calcium/calmodulin-dependent protein kinase group. If expression of STK33 itself predisposes to certain cancers is not known. It was not found to be differentially expressed in RCC patients. Neither was

there a significant difference in seroreactivity between RCC patients and controls (175 versus 13%, respectively). STK33 is therefore also not suitable as a diagnostic or prognostic marker.

Upregulated antigens Ubiquitously expressed genes that are upregulated in cancer often lead to the induction of an antibody response and many such antigens have been identified by SEREX. The identification of upregulated gene products can also give insight into the tumorigenic process. While upregulation of some genes may only be a result of increased proliferation, the upregulation of other genes may confer a distinct growth advantage for proliferating cells. As discussed earlier, most of the SSH identified genes that were screened here are related to tumour growth/survival, angiogenesis, and cell adhesion/motility. Four antigens (LDHB, VCIP, RBBP8and MnSOD) were identified that have previously been shown to be upregulated in other cancers. All four have been checked for their mRNA expression and seroreactivity in RCC.

Lactate dehydrogenases are metabolic enzymes which catalyze the conversion of L-lactate to pyruvate, the last step in anaerobic glycolysis. Malignant cells tend to use 5-10 fold more glucose than do normal tissues, deriving energy for active proliferation mainly from anaerobic glycolysis. Although It is not clear whether the increased serum levels of LDHs commonly found in cancer patients reflect greater production and release of the enzymes by malignant cells, it is well known that increased serum LDH activity has diagnostic and prognostic significance in patients with various tumors (Dumontet et al 1999, Buamah et al 1990, Finck et al 1983). Levels of LDH were found not to differ in the 24 RCC patients examined here. There was no significant difference in the seroreactivity of RCC patients and healthy controls to the LDHB antigen. Thus, at least for RCC, LDH is not a suitable marker or therapeutic target.

VCIP is a member of the "ATPases associated with various cellular activities" superfamily and plays a key role in the ubiquitin-dependent proteasome degradation pathway (Dai et al 1998). VCIP is known to inhibit apoptosis after stimulation with cytokines such as tumor necrosis factor via degradation of inhibitor ΝBα, an inhibitor of nuclear factor-ΝB (Dai et al 1998). Although it is found to be upregulated in other cancers, it could not be confirmed here in this study for RCC. However, 17% of RCC patients had antibodies against the VCIP protein in contrast to 0% of controls. The presence of anti-VCIP antibodies in serum may

therefore be a marker cancer. Whether this is specific for RCC or whether it has prognostic significance remains to be seen.

The retinoblastoma binding protein 8 (RBBP8) is localized on chromosome 18q11.2. Very little is known about this gene apart from the fact that it can bind to the retinoblastoma (RB) protein (Fusco et al 1998). The RB protein is a nuclear polypeptide that is phosporylated in a cell cycle-dependent manner (Taya, 1997). Unphosphorylated RB represents the active form, which binds other nuclear proteins, including the E2F transcription factor (Bandara and La Thangue 1991, Chellappan et al 1991). The RB-E2F complex leads to repression of transcription (Adnane et al 1995). Phosphorylation or sequestration of RB by other proteins releases E2F, which is then able to activate transcription (Buchkovich et al 1989, Bandara and La Thangue 1991). Molecular alterations of the Rb gene are infrequent (<2%) in renal cancers, and Rb protein is present in the majority of primary (92%) and metastatic (100%) renal tumors (Presti et al 1996). The interaction of RBBP8 with RB may allow transcription of genes that promote cell proliferation. It is no surprise to see that RBBP8 is overexpressed in many tumor lines (Fusco et al 1998). In this study, RBBP8 was found to be upregulated in 2 of 24 RCC patients. It was also found to be highly expressed in 4 RCC cell lines. The seroreactivity profile also suggests a strong correlation with cancer. 40% of RCC patients have antibodies against RBBP8 whereas only 13% of healthy controls react to this protein. This is the first demonstration of the upregulation of a retinoblastoma binding protein in RCC. The upregulation of RBBP8 and the presence of anti-RBBP8 may be of diagnostic or prognostic significance.

Both upregulation and downregulation of MnSOD have been demonstrated in different cancers. The differences are thought to depend on the cell type. In this study the mRNA expression level of MnSOD was found to be equivalent in both normal kidney and RCC tissue. However, seroreactivity against MnSOD was found to be differential between healthy controls and RCC patients. 14% of RCC patients were found to have anti-MnSOD antibodies in contrast to 0% of controls. An increase in anti-MnSOD antibodies was also found by Unwin et al (2003) in their SERPA analysis of RCC. Presence of Anti-MnSOD antibodies may therefore be of diagnostic or prognostic significance for RCC.

Translocation antigens Both development and progression of cancer are associated with nonrandom chromosomal abnormalities (Wada et al 1997). Several cytogenetic studies describe correlations between various cytological subtypes or RCC and specific chromosomal aberrations (Zhao et al 1995, van den Berg et al 1993 van der Hout et al 1993). Loss of chromosome 3p or translocations between 3p13 and 5q22 are frequent in RCC (Kovacs G 1993). The VHL gene, which is mutated in 57% of sporadic RCC, is located on 3p25-26. The VHL gene is also associated with hereditary RCC (Gnarra et al 1995). A translocation between chromosome 19 and 12p12 has been identified here in this study. Such a translocation has not been identified before for RCC. However, gains of chromosomes 12 and 19 have been shown for RCC (Zhao et al 1995, van den Berg et al 1993 van der Hout et al 1993). It is likely to have been a unique event, occurring only in 1 of 35 patients. The seroreactivity also reflects this. Only this patient had antibodies against the phage produced protein. This antigen is therefore not useful for diagnostic or prognostic purposes.

Normal autoantigens Approximately 50% of the antigens identified in this study can be considered to be normal ubiquitously expressed autoantigens. This is also the case for most other SEREX studies, as demonstrated by the sheer number of such antigens in the SEREX database. The majority of these autoantigens elicit antibodies at a similar rate in both healthy controls and cancer patients. Increased seroreactivity was found in this study for 4 supposedly normal autoantigens. Expression analysis found no difference in mRNA levels for each of the 4 antigens between controls and RCC patients. Four of the antigens (HD-TES-34/ SEC63, 41/ CENPE, 47/ Yipee, 50/ SBNO1) were found during the screening of the testis library.

SEC63-like is the human homolog of the yeast Sec63 gene (Sec63). In yeast, the Sec63 protein (Sec63p) forms a complex with Sec61p, Sec62p, Sec71p and Sec 72p which together form the Sec complex (Deshaies and Schekman 1987, Hartmann et al 1994, Deshaies et al 1991). This complex is essential and sufficient for the post translational protein translocation into the endoplasmatic reticulum (ER) (Panzner et al 1995). Although it does not appear to be upregulated in RCC, 31% of RCC patients have antibodies against the SEC63-like protein. For comparison, only 7% of healthy controls have anti-SEC63-like antibodies. Presence of these antibodies may therefore be an indicator of disease progression.

The kinetochore-associated microtubule motor protein CENP-E (centromere associated protein-E) is required for establishing and maintaining the mitotic checkpoint, the major cell cycle control pathway in which unattached kinetochores prevent anaphase onset (Abrieu et al 2000). This checkpoint is essential for accurate seperation of chromosomes during mitosis. CENP-E has been shown to interact with other kinetochore proteins such as CENP-F. Antibodies against CENP-F have also been identified in this study aswell as many other SEREX studies. However, anti-CENP-F antibodies have been found at similar frequencies in both cancer patients and healthy controls in all of these studies. Anti-CENP-E antibodies have not been identified by SEREX but have been found to be associated with a particular form of systemic sclerosis (SSc) (Rattner et al 1996). In this study 20% of RCC patients had antibodies against CENP-E compared to 7% of controls. As CENP-E is important for cell division, the increase in autoantibodies against CENP-E could reflect the increased proliferation of RCC cells. Alternatively, mutations in CENP-E, which may result in chromosomal aberrations, could be the trigger for these autoantibodies.

The Yippee protein has only been identified recently in a yeast interaction trap screen for interactions with *Hyalophora cecropia* Hemolin (Roxstom-Lindquist and Faye 2001). Hemolin is a member of the immunoglobulin superfamily which is constitutively expressed and strongly upregulated upon bacterial infection (Andersson and Steiner 1987, Lindstrom-Dinnetz et al 1995). It is the first member of a new family of proteins highly conserved among eukaryotes. Yippee appears to be a ubiquitous intracellular protein with a potential to bind zinc and a capacity to interact with itself. In this study, expression levels of Yippee were found to be the same for normal kidney and RCC tissue. However there was a large difference in seroreactivity between healthy controls and RCC patients (7% versus 34% respectively). As there is so little known about this protein, to speculate on its role in cancer would be unwise.

The strawberry notch homolog-1 (SBNO1) gene is the human equivalent of the strawberry notch gene originally identified in Drosophila. It participates with members of the Notch pathway in facilitating developmentally relevant cell-cell communications (Coyle-Thompson and Banerjee 1993). Analysis by virtual northern shows that it is expressed in many normal tissues. In this study, equivalent amounts of SBNO1 were found in both normal kidney and tumor tissue. However, antibodies against SBNO1 are twice as frequent in RCC patients as healthy controls (40% versus 20%). No association with cancer has been identified todate for

SBNO1. However, many developmentally associated proteins are found to be associated with cancer. Whether SBNO1 can be considered a true developmental gene is unclear.

Unknown antigens In total, 22 unkown antigens have been identified in this study. However, only 12 of these were found to contain significant ORFs that do not correspond to ALU repeat sequences. Of these 12, 10 are predicted to be expressed ubiquitously. The expression of the other two (HD-TES-9, 10) could not be predicted. Subsequent analysis by northern blotting showed equivalent levels of these two genes in both normal kidney and RCC tissue. Three of the 12 antigens (HD-RCC-1, 2, 3) elicited antibodies in only 1 patient out of 35 and not in healthy controls. These antigens are therefore patient specific and not suitable as diagnostic, prognostic or therapeutic targets. Of the remaining 9 antigens only 3 (HD-TES-9, 10, 24) displayed differential seroreactivity between RCC patients and healthy controls (11% versus 0%, 46% versus 27%, 29% versus 13%, respectively). The role of these 3 antigens in cancer cannot be speculated. However, upregulation at the mRNA level can be ruled out.

4.3 RCC associated antigens identified by SADA

The SADA (serum antibody detection array) technique is an extension of the SEREX method. It is used for determining the frequency of antibody responses in patients against many known antigens simultaneously. In this study 44 previously identified antigens were studied for their seroreactivity in 35 RCC patients versus 15 healthy controls. Of the 44 antigens only 14 displayed seroreactivity in RCC patients. Eight of the antigens were found to exclusively react with RCC sera and not healthy controls. Four other clones displayed increased seroreactivity in RCC patients as compared to controls. The last two antigens displayed almost 100% seroreactivity in both RCC patients and healthy controls. Of the eight antigens showing exclusive seroreactivity in RCC patients, four (Rbbp21a, cTAGE-5a, RAP140, CKAP2, GAGE) showed seroreactivity in only 1-2 patients. Interestingly two of these antigens (cTAGE-5a and GAGE) are cancer/ testis antigens. The other three in this group (Par-3, se57-1 and HEXIM1) are discussed in more detail below. The antigens showing increased levels of seroreactivity (SCP-1, LTA1-1, SREB-3, ZNF133) are also discussed briefly.

The Par-3 gene belongs to the family of partitioning-defective genes which are involved in asymmetric cell division and polarized growth. Studies in *Caenorhabditis elegans* have demonstrated the essential role of these proteins in the establishment of anterior/ posterior polarity (Nelson and Grindstaff 1997). Par-3 encodes a large protein with three PDZ (PSD-95/Dlg/ZO-1) domains (Etemad-Moghadam et al 1995). These domains mediate protein–protein interactions, usually with other PDZ domains or with specific carboxy-terminal motifs (Fanning and Anderson 1996, Ponting et al 1997). PAR-3 localizes to the cell periphery at the anterior end of the zygote. An atypical PKC, PKC-3, co-localizes with PAR-3 and is also required for asymmetric cell division (Tabuse et al 1998), and another par gene product, PAR-6, which contains a single PDZ domain, co-localizes with PAR-3 and with PKC-3 (Hung and Kemphue 1999). The clone used here (Par-3ta) is lacking the first of the three PDZ domains. Par-3 is known to be expressed as different splicing products. In normal tissue, 3 splicing products have been identified whereas in tumor tissue only one intense band is seen (Usener 2000). The Par-3ta clone thus appears to be a tumor specific splice variant of the Par-3 gene. Usener (2000) also found that this variant is serologically specific for tumor patients. In this study 23% of RCC patients were reactive to this clone. It remains to be seen if the apparent tumor specific transcript is also present in RCC.

The se57-1 clone was identified in the screening of cutaneous T cell lymphoma (CTCL) by SEREX (Eichmüller et al 2001). Its expression was found in normal tissues to be limited to bone marrow, colon, small intestine, spleen, testis and trachea. In CTCL it was found to be expressed in 6% of patients while seroreactivity was found to be at 33%. Nothing else is known about this gene. In this study 17%of patients were found to have antibodies against the corresponding protein. No healthy controls were seroreactive for se57-1. The expression of se57-1 could not be confirmed in those patients which were reactive. It is thought that the se57-1 gene is expressed only at a very low level. This may explain the inability to confirm its expression in RCC patients.

The HEXIM1 (Hexamethylene-bis-acetamide-inducible-1) gene was originally identified by differential display of vascular smooth muscle cells (VSMC) after treatment with hexamethylene-bis-acetamide (HMBA) (Kusuhara et al 1999). It was found to play an inhibitory role in NF-κB-dependent gene expression in VSMC (Ouchida et al 2003). Two SEREX studies have identified HEXIM1 as an antigen. Seroreactivity in both cases was limited to cancer patients (Ehlken et al 2004, Stone et al 2003). Here it was found to elicit

antibody responses in 11% of RCC patients but not in any of the controls. The presence of anti-HEXIM1 antibodies therefore appears to be a marker cancer in general.

The SCP-1 gene has already been discussed, however it is worth noting that the seroreactivity here differs from that obtained earlier with the testis identified clone (HD-TES-55). Secondary serex analysis with the HD-TES-55 clone showed 9% seroreactivity against this clone in RCC patients only. The SADA analysis on the other hand indicates that 29% of patients have antibodies against the SCP-1 protein along with 7% of controls. It may be possible that in this case the SADA approach was more sensitive in detecting anti-SCP-1 antibodies than the secondary serex. Nevertheless, seroreactivity against SCP-1 remains an apparent marker for cancer.

LTA1-1 is a ubiquitously expressed peripheral membrane protein localized to the trans-Golgi network. The encoded protein contains a GRIP domain which is thought to be used in targeting (Luke et al 2003). Previous SEREX analysis has shown that 50% of CTCL patients and 0% of healthy controls are reactive against this protein (Eichmüller et al 2001). In this study, 37% of RCC patients were seroreactive in comparison to 20% of controls. The discrepancy between the two studies with regard to seroreactivity in healthy controls could lie in the fact that only 5 controls were taken in the study of CTCL. It may also lie in the sensitivity of the SADA method. However, increases in anti-LTA1-1 antibodies may be a marker for cancer.

The cell division autoantigen 1 (CDA1) gene was identified in a screening of a testis library with serum from a patient with discoid lupus erythematosus (Chai et al 2001). It was found to be localized to the nucleus. It is proposed to be a negative regulator of cell growth whose activity is regulated by its expression and phosphorylation. Eichmüller et al have found anti-CDA1 antibodies in 30% of CTCL patients and not in controls. In this study, 26% of RCC patients displayed seroreactivity against CDA1 in contrast to only 7% of controls. Presence of anti-CDA1 antibodies seems therefore to correlate strongly with cancer.

The ZNF133 gene belongs to the zinc finger *Krüppel* family (Tommerup and Vissing 1995). Zinc finger proteins are important transcriptional activators and repressors. Several observations have implicated ZNF genes with developmental and malignant disorders. For example, mutations of the zinc finger gene WT1 predispose to the development of Wilms

tumor (Call et al 1990) as well as to congenital malformations of the urogenital tract (Pelletier et al 1991). The ZNF133 gene was found to be amplified in a neuroblastoma cell line (Heiskanen et al 2000). A large number of zinc finger proteins have been identified by SEREX. In this study 40% of RCC patients were reactive against the ZNF133 protein compared with only 7% of controls. It seems as if seroreactivity to zinc finger proteins is also associated with cancer.

4.4 Review of the methods used in this study

The SSH technique is a powerful method for the identification of upregulated genes. One particular advantage over cDNA array is that it can identify unknown genes. However, there are some inherent drawbacks with this method. A false positive rate of up to 50% can be expected, possibly due to inefficient hybridisation during the subtraction process. It is essential to test all clones by northern blotting to confirm differential expression. When the goal is to identify antigens, then SSH is not a very suitable technique as shown by this study. Also only short transcripts are produced which are unsuitable for expression cloning.

SEREX, on the other hand is ideally suited for antigen identification. For a successful screening, a number of crucial steps need to be performed. Firstly, to eliminate antibodies in human sera which react with bacterial or phage components, extensive preabsorption of diluted serum is essential. When the cDNA library is derived from a tumor which may harbour B cells, it is essential either to eliminate IgG clones or to retest all identified clones without the test sera (i.e. only with secondary antibody). To determine the relevance of identified clones to cancer, the initial step is to sequence the clone and compare this to what is in the database. The next step is to determine if the gene has a restricted or non-restricted expression in normal tissues and in the tumor being investigated. This is achieved by RTPCR or Northern blotting. Finally it is important to determine whether the seroreactivity against the identified clone relates to cancer or is non-specific. On a small scale this can be achieved by secondary SEREX. This is where the clone is plated at a ~50:50 ratio with empty phage. The membrane can then be cut up and used to examine multiple sera. On a larger scale, the SADA technique is more suitable, especially if multiple clones are to be analyzed. Alternatively, purified recombinant protein can be used in an ELISA. This eliminates the need for

preabsorption of sera. It also eliminates the grey area of trying to distinguish between a weakly positive signal and a high background which is often arbitrary if not impossible.

A number of modifications to the SEREX method have been established in order to enhance its effectiveness. The screening of testis libraries instead of tumor libraries enhances the likelihood of identifying cancer/ testis antigens. Another modification is the use of subtracted libraries. Clones identified in this manner are much more likely to be differentially expressed in the target tumor. One modification that has been proposed is to use different sources of antibody. Instead of using autologous or allogenic sera, human oligoclonal or mAb generated from B cells from peripheral blood, tumor, or draining lymph nodes of cancer patients could be used.

One of the main shortcomings of SEREX is that it is a prokaryotic system. Therefore, posttranslational modifications such as glycosylation, lipidation, phosphorylation, methylation, etc, are not represented. Folding of proteins is also different in prokaryotes and eukaryotes. Such modifications generate confirmational epitopes which are missed by conventional SEREX. Recently, Mischo et al (2003) have developed a yeast based system to circumvent these problems.

In comparison with other SEREX studies, this study is no exception. The vast majority of clones identified are ubiquitously expressed genes that do not show differential expression in the tumor. The lack of clones identified in the screening of the tumor library and the RCC cell line was however surprising. In the case of the tumor, it may be that this patient's tumor is not very immunogenic. The high dilution (1:3000) of the pooled sera may be also to blame. This is also the case for the initial screening of the cell line. However, this also means that the clones identified have elicited very high titer antibodies. Indeed, three of the clones represent known autoantigens (GOLGA4, UACA and DEK). Two of these autoantigens elicit antibodies in the vast majority of both normal and cancer patients. The effective dilution of specific antibody would then only be 1:100. In the case of the SNX6 clone, only one patient had reactive antibodies. This clone was identified repeatedly in the screening of the cell line library and also the testis library indicating a very high titer of anti-SNX6 antibodies in this particular patient. However there was no differential expression of SNX6 between the normal kidney and the tumor from this patient. Perhaps, SNX6 is posttranslationally upregulated or alternatively modified in this patient.

The screening of the testis library initially with each individual sera, followed by the top 3 responding sera allowed for the identification of a significant number of antigens. As well as the known ubiquitously expressed genes, a significant number of unknown genes were identified. A decent number of upregulated and cancer/ testis antigens were also identified. It was however disappointing not to be able to confirm the expression of the cancer/ testis antigens in RCC patients. This may be due to the infrequency and low expression level of these antigens. However, this should be repeated before making any final conclusions.

As mentioned earlier, one of the drawbacks of the SADA method is trying to distinguish high background from weakly positive signals. This may explain some of the discrepancies between equivalent clones screened by both secondary SEREX and SADA. The use of the GOLGA4 and/ or the UACA autoantigens as positive controls may help to alleviate some of these problems and is therefore put forward by this work as a recommendation for future SADA studies. Still, the SADA method was found in general to be less sensitive than secondary SEREX. Of the 44 antigens screened by SADA, only 14 showed any reactivity in either normal or RCC patients. In comparison, all of the clones identified in the testis screen were found to be reactive in at least one person when examined by secondary SEREX. However, this lack of sensitivity may also be an advantage for identifying cancer related clones. Indeed, 57% of clones that were reactive in the SADA screening were shown to react exclusively with RCC sera. This compares with 26% of the clones reactive in the secondary SEREX screening.

4.5 The RCC Immunome

The cancer immunome refers to the complete repetoire of immunogenic products in human cancer. The goals being 1) to understand better the generation of immune reactions against cancer and 2) to identify potential targets for diagnostic, prognostic or therapeutic use. These goals are now more acheivable thanks to the SEREX method. While the identification of cancer specific antigens is of utmost importance, the identification of antibodies that are equally present in both cancer patients and healthy controls as well as antibodies that are more frequently found in cancer patients should not be neglected.

Natural autoantibodies (na-Ab) were first suggested to have a 'sewage' role whereby na-Ab are responsible for inactivating any biologically active molecules that are overproduced (Grabar, 1975). Since then na-Ab have been shown to have a variety of functions. Some na-Ab provide transportation of molecules-ligands as well as protection of ligands from proteolysis (Wang et al 1992). The finding that some antibodies can modulate functions of intranuclear proteins in vivo, suggests that antibodies are also capable of translocation across cell membranes (Zaitchik and Churilov, 2001). Antibodies have also been shown to possess enzymatic activity (abzymes) (Poletaev and Morozov, 2000).

The fact that autoantibodies do not automatically result in autoimmune disease suggests that the source of antigen is less important than the context in which it is presented. This observation has led to the concept of the 'danger' model which supersedes the previous 'self/ nonself' model (Matzinger 1994, Fuchs and Matzinger 1996). In this model, any antigen can potentially be seen as 'dangerous' and stimulate an immune response with the appropriate stimuli. These appropriate stimuli are thought to come from proinflammatory cytokines and chemokines elaborated by cells of the innate immune system such as naural killer (NK) cells and dendritic cells (DC).

Recent work by Nishikawa et al (2001, 2003) using SEREX defined autoantigens, suggests a role for these autoantigens in maintaining and regulating immunological homeostasis and that this is achieved via $CD4^+$ $CD25^+$ regulatory T cells. Specifically, they found that coimmunization of mice bearing a tumor with DNA from a SEREX defined autoantigen plus DNA from a tumor-specific CTL epitope leads to heightened $CD8^+$ T cell responses and increased resistance to tumor challenge in a $CD4^+$ dependent manner. They also found that immunization with DNA from the SEREX autoantigen alone leads to heightened sensitivity to tumor challenge and that this was mediated by $CD4^+$ $CD25^+$ regulatory T cells.

The term Immunculus (immunological homunculus) has been proposed recently by Poletaev and Osipenko (2003) to describe the network of constitutively expressed na-Ab. They suggest that the Immunculus mirrors the organism's physiological state. In healthy people the Immunculus is relatively constant and is characterized by minimal individual quantitative variations. However, abnormal metabolic deviations, which precede or accompany different diseases result in quantitative rather than qualtitative changes in this network of na-Ab. The mapping of this network in healthy individuals should identify the normal state of na-Ab in

the healthy condition. Quantitative differences to this norm may then be used for the early detection of potentially pathogenic metabolic changes.

Concerning this work, the majority of the antigens found elicit antibodies at equivalent rates in both normal and cancer patients. These antibodies can be considered as part of the normal repertoire of na-Ab. Those antigens eliciting antibodies at higher frequencies in RCC patients may reflect the metabolic changes induced by the presence of the tumor. Therefore, this study has identified a portion of the 'Immunculus', some of which are indicators of cancer.

With regard to the RCC immunome, a total of 26 antigens were identified that react solely with RCC sera. They may also react with other cancer sera but this has yet to be tested. Sixteen of the antibodies were found in only 1 to 2 of the 35 patients. These may represent tumor specific antigens and are thus not suitable for inclusion in a panel of antibodies identifying RCC. Ten antibodies however were found in 11-23% of RCC patients and are suitable for such a diagnostic panel. As can be seen from Figure 12, up to 77% of RCC patients have antibodies to at least one of these antigens. Whether these autoantibodies are of prognostic remains to be seen. The three cancer/ testis antigens (TSGA10, PLU-1 and STK31) may also be of immunotherapeutic value. This will depend on the expression profile of these antigens in RCC.

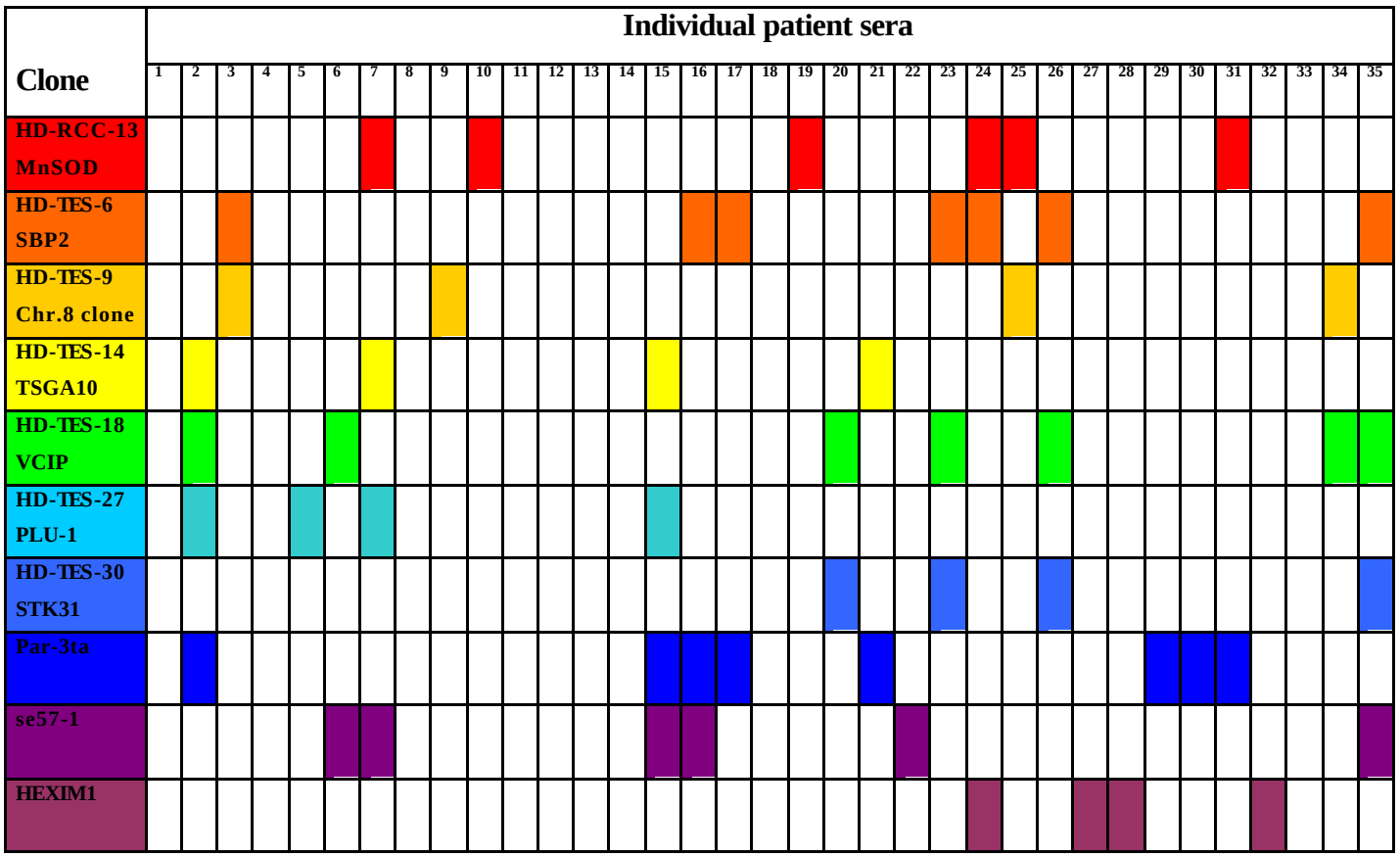


Figure 12. Seroreactivity of the 35 RCC patients against the 10 antigens showing exclusive reactivity in RCC.

Colored boxes represent positive seroreactivity.

5 Conclusions

This work has contributed to the identification of RCC associated genes and the elucidation of the RCC immunome and the Immunculus.

Through the use of SSH and Northern blotting a panel of genes has been identified which are significantly upregulated in high proportions of RCC patients. These genes are related to the main features of malignancy, i.e. growth dysregulation, angiogenesis and motility. These molecules may serve as diagnostic or therapeutic targets.

The combination of SEREX and SADA has identified a total of 88 antigens which are reactive with RCC sera. The majority of these antigens (62) elicit antibodies also in healthy controls. These antibodies can be considered part of the natural antibody repertoire, disturbances to which may indicate disease. Of the remaining 26 antigens which do not elicit antibody responses in healthy controls, 10 may be used together as a panel of antigens identifying up to 77% of RCC. These antigens/ antibodies may also serve as diagnostic, prognostic or therapeutic markers.

Remaining questions are 1) the immunogenicity of these antigens in other cancers, 2) expression profile of the cancer/ testis antigens in RCC and 3) the full cloning and identification of the unknown antigens.

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List of Abbreviations

Weights and measures:

bp	base pairs
Ci	Curie
G	gram
hrs	hours
kb	kilobases
kD	kilodalton
l	liter
M	Molar
mg	milligram
min.	minutes
ml	milliliter
mM	millimolar
µg	microgram
ng	nanogram
sec.	seconds
U	unit
V	Volt
^{0}C	degrees Celsius

Amino Acids:

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

[α^{32}P]dCTP	Deoxycytidine 5'-[α ^{32}P]-triphosphate, triethylammonium salt
5-AZA-CdR	5-aza-2'-deoxycytidine
5-FU	5-fluorouracil
A	Adenine
a.a	Amino acid
Ab	Antibody
ADCC	Antibody dependent cellular cytotoxicity
AICD	Activation-induced cell death
AJCC	American Joint Committee on Cancer
AKAP9	A kinase anchor protein 9
allo-SCT	allogenic stem cells
AML	Acute Myeloid Leukemia
AMV	Avian Myeloblastosis Virus
AP	Alkaline Phosphatase
AP1 or 2	Adaptor primer 1 or 2
APC	Antigen presenting cell
ARP	Angiopoietin-related protein
ATP	Adenosine triphosphate
ATX	Autotaxin
BACE	Beta site amyloid precursor protein cleaving enzyme
BCIP	5-bromo-4-chloro-3-indoyl phosphate p-toluidene salt
BRDT	Bromodomain testis specific
BSA	Bovine serum albumin
C	Cytosine
C1-RC	C1 respiratory complex
CA	Carbonic anhydrase
CD	Cluster of differentiation
CDA1	Cell division autoantigen 1
cDNA	Complementary DNA
CENP	Centromere protein
CIAP	Calf Intestinal alkaline Phosphatase
CKAP2	Cytoskeleton associated protein 2
CML	Chronic Myelogenous Leukemia
CP	Ceruloplasmin
cpm	Counts per minute
CRA	13-cis retinoic acid
CT	Cancer/ testis (antigen)
cTAGE	CTCL tumor associated antigen
CTCL	Cutaneous T-cell lymphoma
CTL	Cytotoxic T lymphocyte
dATP	2-Deoxyadenosine 5' triphosphate
DC	Dendritic cell
dCTP	2-Deoxycytidine 5' triphosphate
DD RTPCR	Differential display RTPCR
DEPC	Diethylpyrocarbonate
dGTP	2-Deoxyguanosine 5' triphosphate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Desoxyribonuclease
DNEL-2	Dynein heavy chain

dNTP	Desoxyribonucleoside triphosphate
ds	Double strand
DTT	Dithiothreitol
dTTP	2-Deoxythymidinetriphosphate
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid
EF	Elongation factor
EGFR	Epidermal growth factor
ELISA	Enzyme Linked Immunosorbent Assay
EPAS	Endothelial PAS domain protein
ER	Endoplasmic Reticulum
EST	Expressed sequence tag
et al.	And others (et aliqui)
FACS	Flourescence-activted cell sorter
FCS	Fetal calf serum
FDA	Food and Drug Administration (USA)
FGF-5	Fibroblast growth factor 5
FITC	Flourescein-Iso-Thio-Cyanate
G	Guanine
GAPDH	Glycerinaldehydephosphate dehydrogenase
GBP	Guanylate binding protein
GMCSF	Granulocyte Macrophage Colony Stimulating Factor
GOLGA4	Golgin subfamily a,4
HCRC	Hereditary clear cell renal cell carcinoma
HEXIM1	HMBA inducible transcript 1
HIF	Hypoxia inducble factor
HLA	Human Leukocyte Antigen
HMBA	Hexamethylene-bis-acetamide
HPRC	Hereditary papillary renal carcinoma
HRO	Hereditary renal oncocytoma
i.v.	Intravenous
IFNα	Interferon alpha
IFNβ	Interferon beta
IFNγ	Interferon gamma
Ig	Immunoglobulin
IGFBP3	Insulin like growth factor binding protein 3
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-2	Interleukin 2
IL-4	Interleukin 4
IPTG	Isopropyl-β-D-thiogalactoside
IRCC	Institute for Cancer Research and Treatment
IRES	Internal ribosome entry site
LAK	Lymphokine activated killer cells
LB	Luria and Bertani culture medium
LDH	Lactate dehydrogenase
LO	Lysyl oxidase
mAb	monoclonal antibody
MAGE	Melanoma associated antigen
MAPK	Mitogen-activated protein kinase
MHC	Major Histocompatability Complex

MnSOD	Superoxide dismutase 2
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
n.t.	Not tested
na-Ab	Natural autoantibody
NADH	Nicotinamide adenine dinucleotide
NBT	p-nitroblue tetrazolium chloride
NDCH	Nuclear distribution gene C homolog
NF-? B	Nuclear factor ? B
NK	Natural killer cells
NMDA	N-methyl-D-aspartate
NSS	Nephron sparing surgery
OD	Optical density
ORF	Open reading frame
Par-3	Partitioning defective homolog 3
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
Pcdh	Protocadherin
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC	Protein kinase C
PP1	Protein phosphatase type 1
PRDX3	Peroxiredoxin 3
PS1	Putative S1 RNA binding domain protein
PSA	Prostate specific antigen
PSMA7	Proteosome subunit, alpha type 7
PTp	Protein Tyrosine phosphatase
PX	Phox
RACE	Rapid amplification of cDNA ends
RAGE	RCC associated antigen
RANBP2L1	RAN binding protein 2-like 1
RAP140	Retinoblastoma-associated protein 140
RB	Retinoblastoma protein
RBBP	Retinoblastoma binding protein
RCC	Renal Cell Carcinoma
rhIFNγ	Recombinant human IFNγ
RNA	Ribonucleic acid
RNase	Ribonuclease
RPHPLC	Reverse phase high performance liquid chromatography
rpm	Revolutions per minute
RTPCR	Reverse transcriptase polymerase chain reaction
SADA	Serum antibody detection array
SART3	Squamous cell carcinoma antigen recognized by T cells 3
SBNO1	Strawberry notch homolog-1
SCID	Severe combined immunodeficiency
SCP-1	Synaptonemal complex protein 1
SDS	Sodium Dodecylsulfate
SemG	SemaphorinG
SEREX	Serological analysis of recombinant cDNA expression libraries
SERPA	Serological proteome analysis

SNX6	Sorting nexin 6
Sp3b	Splicing factor 3b
SPEAR	Serological and proteomic evaluation of antibody responses
ss	Single strand
SSC	Sodium saline citrate
SSc	Systemic sclerosis
SSH	Suppression subtractive hybridization
STK	Serine threonine kinase
SUPT5H	Suppressor of Ty
T	Thymidine
TAE	Tris-Acetate-EDTA
TBST	Tris buffered saline with 0.5% Tween
Tc	T cytotoxic cell
Th	T helper cells
TILs	Tumor infiltrating Lymphocytes
TNM	Tumor, node, metastases (staging system)
Tris	Tris[hydroxymethyl]aminomethane
UACA	Uveal autoantigen
UBD	Diubiquitin
UICC	Union Internationale Contre le Cancer
UV	Ultraviolet
VCIP	Valosin containing protein
VEGF	Vascular endothelial growth factor
VHL	von Hippel Lindau disease
VLB	Vinblastine
VSMC	Vascular smooth muscle cells
WBS	William-Beuren syndrome
X-gal	5-bromo-4-chloro-3-indolyl bd-galactopyrano-side
ZNF	Zinc finger protein

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Curriculum Vitae

Personal Data:

Name:	Gerard Devitt
Address:	Schlossberg 2, 69117 Heidelberg
Date of birth:	03.11.1975
Place of birth:	Cork, Ireland
Nationality:	Irish

Education and work experience:

1987-1993:	Christ the King secondary school, Cork, Ireland
1993-1997:	BSc Microbiology, University College Cork, Ireland
1997-1999:	MSc Biotechnology, (equivalent to German Diplom) University College Cork, Ireland
1999-2000:	Industry work experience at Novo Nordisk, Denmark.
2000-2004:	PhD-student in the Department of Tumor Progression and Immune Defence (Head: Prof. Dr. M.. Zöller), German Cancer Research Center (DKFZ), Heidelberg, Germany

List of Publications:

1. Devitt GP, Creagh EM, Cotter TG (1999)The Antioxidant 4b, 5, 9b,10-Tetrahyroindeno[1,2-b] inhibits apoptosis by preventing Caspase activation following mitochondrial depolarization. Biochem Biophys Res Commun **264** 622-629

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