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# Identification of *CD70* as a diagnostic biomarker for clear cell renal cell carcinoma by gene expression profiling, real-time RT-PCR and immunohistochemistry

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**Abstract**

The underlying molecular mechanisms of renal cell carcinoma (RCC) are poorly understood and more reliable markers for early diagnosis are needed. Hence, alternative strategies for biomarker discovery with appropriate validation technologies have to be performed. To elucidate genesis and progression of RCC we used high parallel chip based gene expression profiling comparing normal and tumour tissues. We compared corresponding control and tumour tissue samples from 10 patients with clear cell RCC. We isolated RNA from histologically well characterised tissue sections and performed reverse transcription, labelling and linear RNA amplification. Samples were hybridised on microarrays containing 642 human cDNAs. Of the 352 differentially expressed genes found, *CD70* and *FRA2* were selected for further evaluation by real-time RT-PCR. The analysis all showed a high potential to discriminate between normal and tumour tissue. Moreover, increased *CD70* mRNA expression in tumour cells could be correlated to its expression at the protein level. Immunohistochemistry (IHC) showed very strong expression of *CD70* in all tumour samples but no expression in adjacent normal kidney tissue. With our combined approach we were able to identify *CD70* as a new marker for RCC, which may be useful in the future for improved immunohistochemical diagnosis.


*Keywords:* cDNA-arrays; LightCycler; Kidney; Microarray; Marker gene

## 1. Introduction

Renal cell carcinomas (RCC) comprise 3% of all human neoplasms and are increasing in incidence. RCCs are characterised by high resistance to chemo-, radio- and immunotherapy. Important prognostic factors for RCCs are both tumour stage and surgical resectability, although knowledge about the histological subtype and grade provides additional prognostic information.

RCCs can be classified into clear cell, papillary and chromophobe carcinomas based on their histological appearance [1,2]. The clear-cell variant is the most frequent type of renal cancer, accounting for 80–85% of adult renal neoplasms.

Although, at the moment, a small number of possible marker genes to predict more accurately RCC behaviour is known, a reliable marker for early diagnosis of RCC is missing [3–5].

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Cancer development and progression are accompanied by profound changes at the cellular and subcellular level, involving RNA, DNA and protein structure and function. A combined analysis of those levels by cDNA-microarrays, real-time RT-PCR and immunohistochemistry could lead to the discovery of additional biomarkers for early diagnosis.

Up to now, microarray technology has supplied comprehensive insights into the underlying molecular mechanisms of many cancer types. The generated gene expression profiles can serve as a molecular signature of cancer probably leading to novel clinical subtypes such as those related to drug response and reflecting the heterogeneity in transformation mechanisms, cell types, and behaviour of the tumours. For instance, several studies have identified distinct gene sets that distinguish serous and mucinous ovarian cancer, acute myeloid and acute lymphoblastic leukemias, *BRCA1* and *BRCA2* hereditary breast cancer, hepatitis-B and hepatitis-C hepatocellular carcinomas, and diffuse large B-cell lymphomas with good and poor prognosis [6–8]. In kidney tumours, several gene expression studies were performed combining microarray experiments with real-time PCR identifying several prognostic gene sets [8–10].

In this study we used a human specific PIQOR™ Immuno/Oncoarray consisting of 642 carefully selected cDNA fragments to identify differentially expressed genes in RCC. We validated some candidate genes with real-time RT-PCR using LightCycler techniques. In addition, we performed immunohistochemical analysis of *CD70* to examine whether and where the proteins are located within the tumour tissue and of *CXCR4* as an available immunohistochemical standard marker.

## 2. Materials and methods

### 2.1. Tissue samples

Samples from 10 patients with clear cell RCC, containing control non-tumourous (“normal”) and tumour tissue were obtained from fresh nephrectomy specimens. After surgical resection the samples were immediately snap frozen in liquid nitrogen and stored at −80 °C at the Department of Urology of the Friedrich-Schiller-University, Jena. Paraffin sections from each specimen were reviewed by a pathologist and classified histologically according to UICC-TNM classification (1997). All Fuhrmann grading spectra [1–3] are represented.

### 2.2. Total RNA extraction

Total RNA was isolated by standard methods using a commercially available RNA Isolation Kit (Qiagen, Hilden, Germany). Qualitative integrity test of purified total RNA was done with capillary electrophoresis using a Bioanalyser 2100 (Agilent Technologies, Palo Alto, CA, USA). The amount of RNA was measured with a UV and spectrophotometer.

### 2.3. Microarray experiments

Linear amplification of RNA was done using a modified protocol of a previously described method [11]. Amplified RNA (aRNA) samples were quantified by spectrophotometry and quality was assessed by capillary electrophoresis (Bioanalyser 2001, Agilent).

Two micrograms of aRNA from normal and tumour tissue were then reverse-transcribed by adding it to a mix consisting in 8 μl 5× First Strand Buffer (Invitrogen, Carlsbad, CA, USA), 2 μl Primer-Mix (oligo-dT and randomeres, Memorec Biotech GmbH, Cologne, Germany), 2 μl low C-dNTPs (10 mM dATP, 10 mM dGTP, 10 mM dTTP, 4 mM dCTP), 2 μl FluoroLink Cy3/5-dCTP (Amersham Pharmacia Biotech, Freiburg, Germany), respectively, 4 μl 0.1 M DTT and 1 μl RNasin (20–40 U) (Promega, Madison, WI, USA). Two hundred units of SuperScript™ II Reverse Transcriptase (Invitrogen) was added, incubated at 42 °C for 30 min followed by the addition of 1 μl of SuperScript™ II Reverse Transcriptase (Invitrogen) and incubated under the same conditions as detailed above. About 0.5 μl of RNaseH (Invitrogen) was added and incubated at 37 °C for 20 min to hydrolyse RNA. Cy3- and Cy5-labeled samples were combined and cleaned up using QIAquick™ (Qiagen). Eluents were diluted to a volume of 50 μl. Fifty microliters of 2× hybridisation solution (Memorec Biotech GmbH), pre-warmed to 42 °C, was added. The samples were then subjected to a predefined PIQOR™ microarray consisting of 642 human cDNAs and hybridisation was performed according to manufacturers guidelines (Memorec Biotech, GmbH) using a GeneTAC hybridisation station (Perkin–Elmer, Wellesly, MA, USA). Slides were fixed in the GeneTAC hybridisation station.

One hundred microliters of prehybridisation solution were added and slides were prehybridised at 65 °C for 30 min. Thereafter, 100 μl purified Cy3- and Cy5-labeled probes in 2× hybridisation solution were pipetted onto the slides thereby displacing the prehybridisation solution. Hybridisation was then performed for 14 h at 65 °C, followed by four washing steps carried out at 50 °C [12]. The slides were produced and scanned by Memorec [12]. Image capture and signal quantification of hybridised PIQOR™ cDNA arrays were done with the ScanArray 4000 Lite (Perkin–Elmer) and ImaGene software version 4.1 (BioDiscovery, Los Angeles, CA, USA). For each spot, the local signal was measured inside a circle adjusted to the individual spot (160–230 μm diameter), and background was measured outside the circle within specified rings 30 μm distant to the signal and 100 μm wide. Signal and background was taken to

be the average of pixels between defined low and high percentages of maximum intensity with percentage parameter settings for low/high being 0/97% for signal and 2/97% for background. Local background was subtracted from the signal to obtain the net signal intensity and the ratio of Cy5/Cy3. Subsequently, the mean of the ratios of 2 corresponding spots representing the same cDNA was computed. The mean ratios were normalised to the median of all mean ratios by using only those spots for which the fluorescent intensity in 1 of the 2 channels was two times the negative control. The negative control for each array was computed as the mean of the signal intensity of two spots representing herring sperm and two spots representing spotting buffer only. Only genes displaying net signal intensity twofold higher than the negative controls in the control sample or treatment sample were used for further analysis. Normalised ratios are shown as Cy5 signal intensity divided by Cy3 signal intensity of the respective gene. Unsupervised cluster analysis (Average Linkage Clustering) was carried out using "Cluster" and "Tree View" programs according to Eisen [13]. Microarray experiments were performed according to the MIAME guidelines [14].

### 2.4. Real-time RT-PCR

For real-time PCR, 2 μg aRNA from both tumour and normal tissue were reverse transcribed with SuperScript™ II Reverse Transcriptase (Invitrogen) using 100 μM random hexamer primers according to the manufactures protocol.

Two gene primer pairs for *CD70* and *FRA2* as well as one reference gene primer pair for *MPC* (Table 1) were designed such that the corresponding amplified cDNA fragment fulfils several selection criteria with respect to, *e.g.*, homology to other known genes (85%) and uniformity of the fragment length.

Primers were designed from the published sequence of human SCC (SCC A1: DDBJ/EMBL/GenBank; HSU19556) antigen cDNA. Selection criteria are summarised in detail elsewhere [14]. The primer design was performed with the software OLIGO (version 5.0). Specificity of the desired RT-PCR products was documented using gel electrophoresis and melting curve analysis (LightCyler Software Version 3.5, 2001, Roche Molecular Biochemicals). The product specific melting curves showed only single peaks and no primerdimer peaks or artifacts.

Real-time RT–PCR was performed with a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) in capillaries using a commercially available master mix containing HotStartTaq DNA polymerase and SYBR-Green I deoxyribonucleoside triphosphates (QuantiTect SYBRGreen PCR-Kit, Qiagen). The following real-time PCR protocol was used for all genes: initial denaturation program for activating the HotStart enzyme (15 min @ 95 °C), amplification and quantification program repeated 45 times (15 s @ 95 °C; 10 s @ 55 °C; 30 s @ 72 °C with a single fluorescence measurement). After the completion of PCR amplification, a melting curve analysis was carried out.

The *"delta-delta CP method"* for comparing relative expression results between treatments in real-time PCR was applied [15].

### 2.5. Immunohistochemistry

Immunohistochemistry was performed on frozen sections (8 μm). The panel of antibodies used is compiled in Table 2. Frozen sections were fixed in acetone for 10 min. Slides were washed in Tris Buffer/Tween (0.1% w/v Tween, TBS-T) for 5 min. Blocking of biotin was performed using the biotin blocking system from DAKO (DAKO, Glostrup, Denmark). After washing with TBS-T twice for 1 min and incubation for 10 min, protein blocking was carried out using a blocking solution containing 25 ml serum from goat in 250 ml TBS, 25 ml RPMI and 250 mg sodium azide for 10 min. Following washing 3× for 1 min and incubation for 10 min with TBS-T sections primary antibody–antigen reaction was performed with the antibody against *CD70* (dilution 1:250) overnight at 4–8 °C. After washing with TBS-T ($3 \times 1$ min, $1 \times 10$ min), slices were incubated with the secondary antibody coupled with biotin (DAKO kit 5005, Germany) for 35 min according to manufactures instructions. Slides were washed again with TBS-T ($3 \times 1$ min, $1 \times 10$ min) and Avidin–alkaline-phosphatase

Table 2
List of antibodies used with clones and sources

| Antibody | Clone | Company |
|---|---|---|
| CD 70 | HNE.51 | DAKO, Hamburg, Germany |
| CXCR-4 | C 20 | Santa Cruz Biotechnology, CA, USA |
| | A17 | Santa Cruz Biotechnology, CA, USA |
| FRA-2 | Q 20 | Santa Cruz Biotechnology, CA, USA |

Table 1
Primer sequences for real-time RT-PCR

| Candidate genes | Sense primer 5′–3′ | Antisense primer 5′–3′ |
|---|---|---|
| *CD70*: tumour necrosis factor (ligand) superfamily, member 7 | AATCACACAGGACCTCAGCAGGACC | AGCAGATGGCCAGCGTCACC |
| *FRA2*: FOS-related antigen 2 | CCCTGCACACCCCCATCGTG | TGATTGGTCCCCGCTGCTACTGCTT |
| *MCP*: membrane cofactor protein | ACTACAAAATCTCCAGCGTCCAG | CAACTATGGCAATAACAATCACAGC |

(DAKO kit 5005, Germany) solution was added for 30 min. After washing with TBS-T (3 × 1 min, 1 × 10 min), the enzyme reaction was carried out adding substrate (DAKO kit 5005, Germany) solution for 7 min. Slides were washed with water (3 × 1 min, 1 × 10 min) and 1 min with destilled water. Finally, sections were stained with Haemalaun solution.

## 3. Results

### 3.1. cDNA microarray analysis

From the 642 available genes present on the microarray, 325 (50.1%) genes were found to be more than twofold differentially expressed in at least one microarray experiment. Cluster analysis was performed to look for commonly up- or down-regulated gene clusters in RCC (Fig. 1).

Previously described regulated genes were found through the analysis as well as possible new marker genes for RCC (the entire gene list with all results can be seen in 'supplementary material'). *CXCR4* was recently described as a potential biomarker for RCC [16]. *CXCR4* was also found to be up-regulated in seven out of 10 tumour samples in the course of this study. Furthermore, several previously identified genes (*e.g.*, genes with relevance to the extracellular matrix) were confirmed to be differentially expressed such as collagen type II and IV as well as integrin, type alpha, 1 and 5 and beta, type 2. A list comparing the regulated genes found in this study with genes identified by other groups is shown in Table 3. Two additional novel genes that so far had not been identified by previous studies, *CD70* and *FRA2*, were further studied by real-time RT-PCR.

### 3.2. Real-time RT-PCR

The two candidate genes that had significantly altered expression in RCC from microarray experiments were verified via real-time RT-PCR (Table 4).

In order to compare the quantified mRNA molecules of the candidate genes in the relative expression ratio model, *MCP* was chosen as the reference gene, as it showed no significant regulation on all hybridised microarrays.

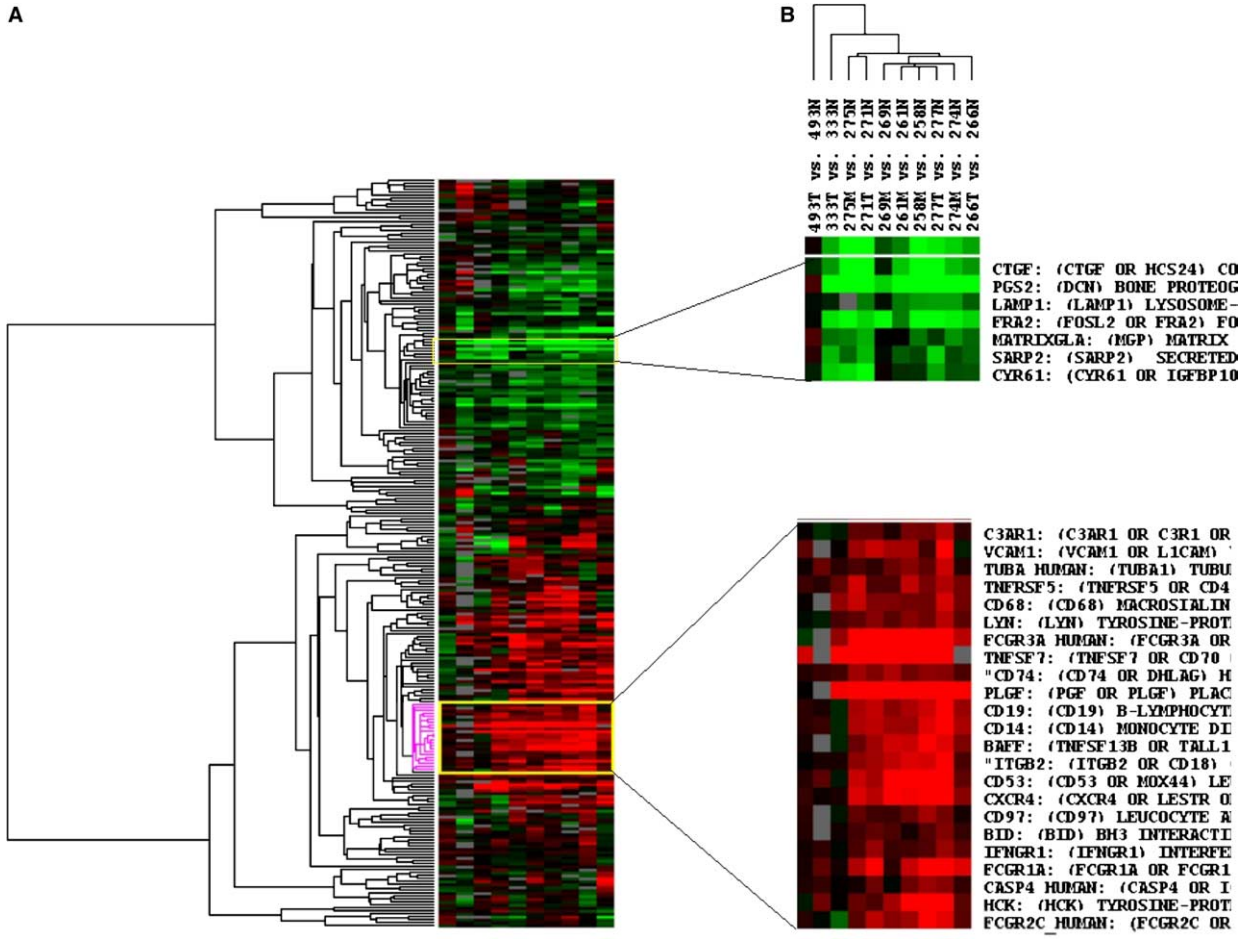


Fig. 1. (A) Cluster analysis of all genes, detectable in all microarray experiments. The yellow border marks the area of cluster analysis. (B) Enlargement of the area of interest (T and M, tumour tissue, N, normal tissue).

Table 3
Genes found differentially expressed from this study and those previously described by other groups

| Gene name | Unigene number | Number of patients showing ⩾ twofold expression (10/10) | Previously described |
|---|---|---|---|
| VWF | HS 440848 | 6/10 | Braybrooke and colleagues (2000) |
| VEGF | HS 135039 | 8/10 | Gerritsen and colleagues (2002), Takahashi and colleagues (1999) |
| VCAM1 | HS 109225 | 5/10 | Droz and colleagues (1994) |
| PAI1 | HS 356427 | 6/10 | Swiercz and colleagues (1998) |
| HEVIN | HS 75445 | 8/10 | Gerritsen and colleagues (2002) |
| CCND1 | HS 371468 | 7/10 | Stassar and colleagues (2001) |
| CALB1 | HS 65425 | 5/10 | Martignoni and colleagues (2001) |
| *CXCR4* | HS 421986 | 7/10 | Schrader and colleagues (2002) |
| Collagen type II | HS 408182 | 5/10 | Droz and colleagues (1994) |
| Collagen type IV | HS 407912 | 5/10 | Droz and colleagues (1994) |
| Integrin type alpha I | HS 519305 | 5/10 | Droz and colleagues (1994) |
| Integrin type alpha 5 | HS 149609 | 5/10 | Droz and colleagues (1994) |
| Integrin type beta 2 | HS 375957 | 6/10 | Droz and colleagues (1994) |
| LINK | HS 2799 | 3/10 | Boer and colleagues (2001) |
| LOX | HS 102267 | 3/10 | Lenburg and colleagues (2003) |

Table 4
Qualitative correlation of microarray and real-time RT-PCR data for two selected genes that appeared to allow discrimination between normal *vs.* tumour tissue

| Regulated gene | UniGene Accession number | Number of patients showing ⩾ twofold expression on microarray | Number of patients showing ⩾ twofold expression on LightCycler |
|---|---|---|---|
| *CD70* | HS 99899 | 8/10 | 8/10 |
| *FRA2* | HS 170253 | 9/10 | 5/10 |

The number of patients in which the respective gene is >twofold regulated is shown for both expression profiling platforms.

The real-time RT-PCR efficiency was set to 2 ($E = 2.0$) for all factors. Relative expression data derived from microarrays and LightCycler experiments for the gene *CD70* is shown (Fig. 2) as an example. The standard deviation in all experiments performed was between 0.02% and 1.9%.

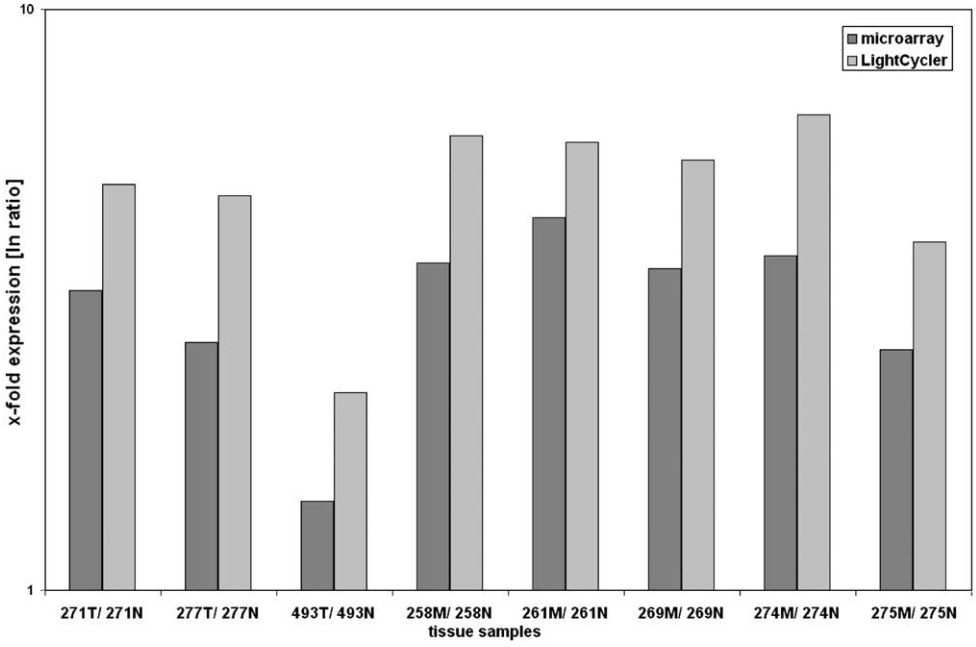


Fig. 2. Comparison of *CD70* expression of tumour *vs.* normal tissue analysed either by microarray or real-time RT-PCR technology (the real-time RT-PCR experiments were performed in triplicate and the deviation within the standard error lies between 0.02% and 1.9%; T and M, tumour tissue, N, normal tissue).

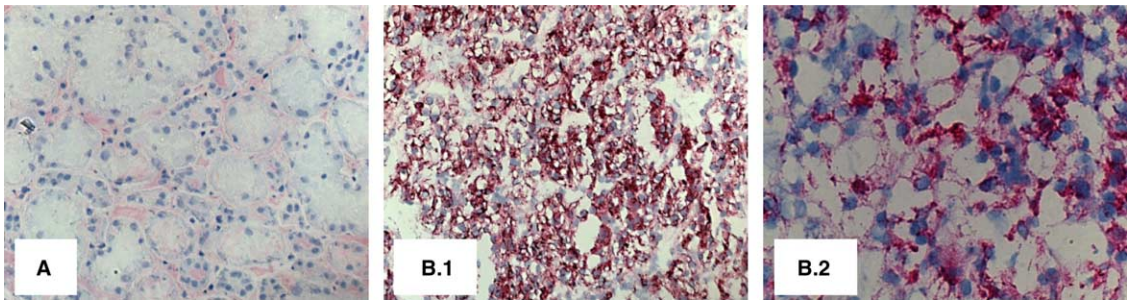


Fig. 3. Immunostaining for *CD70* on frozen sections of (A) normal tissue (200×) and corresponding (B.1) tumour tissue 200× and (B.2) 350× magnification.

The induction of *CD70* gene expression was among the strongest found over all 642 genes that had been analysed. In two microarray experiments in which *CD70* was not detectable, a modest repression of the gene was found via real-time RT-PCR.

### 3.3. Immunohistochemistry

To test if the strong induction detected at the mRNA level in tumour samples was also reflected on the protein level, we examined the localisation of the protein by immunohistochemistry in the same 10 RCC tissue sections that were analysed with cDNA microarray technology and real-time RT-PCR. Immunohistochemistry was performed for *CD70*, *CXCR4* and *FRA2*. Negative controls demonstrated negative results for all three antibodies. In each tissue section more than 50% tumour cells were stained.

In all stained tumour tissue sections, a positive result could be found for *CD70* whereas the normal tissue sections showed no reactivity (Fig. 3). Staining was accentuated in the tumour cell membrane and a weak intracellular positivity could be observed (not shown). No immunoreactivity could be detected in the surrounding stroma (not shown). In addition, we found the specific staining for *CD70* in 30 more cases, which were not previously examined by cDNA-microarray experiments (Junker and colleagues, personal communication). The immunohistochemical staining for *CXCR4* and *FRA2* showed no conclusive discrimination between normal and tumour tissue as positive staining for both proteins could only be observed in two out of 10 samples.

## 4. Discussion

The molecular mechanisms involved in tumour development and progression in RCC are not well understood although several genetic alterations have been identified. In fact, only one gene has so far been found as being involved in the development of distinct types of RCC. The Von-Hippel-Lindau tumour suppressor gene (VHL) located on 3p25-26 is altered in up to 75% by mutations or hypermethylation in clear cell RCC [17]. Many other known tumour suppressor genes and oncogenes seem not to be crucial for RCC genesis. Clearly, the discovery of more gene markers for RCC could lead to more accurate and sensitive diagnoses and treatments.

Microarray technology has already been applied successfully in biomarker discovery [6,7]. Nevertheless a general need has been shown for validation of microarray data by other independent technologies like real-time RT-PCR, which is used to quantify physiological changes in gene expression and to verify gene expression results derived from microarrays [12,18]. Up to now, at least seven different microarray studies, have compared normal and tumour tissue using different microarray platforms and confirmed their results by real-time RT-PCR or immunohistochemistry (*e.g.*, Lenburg and colleagues, Gerritsen and colleagues [19,20]).

In the present study, we analysed the expression of 642 genes by cDNA arrays in tissue samples from patients with RCC to find differences in gene expression between normal and tumour tissue. Additionally, we validated our results by a combination of real-time RT-PCR and immunohistochemistry. Overall, results derived from this microarray study correlated with already published gene expression data from other microarray analyses. However, our microarrays revealed two genes, *CD70* and *FRA2*, which were differentially expressed in normal and tumour tissue in at least half of the patient samples (Table 4).

The FOS protein FRA2 forms transcription factor complexes and has been implicated as a regulator of cell proliferation, differentiation, and transformation. In some cases, expression of the *FOS* gene has also been associated with apoptotic cell death [21]. Despite promising results on microarrays, the discriminating potential of *FRA2* could not be confirmed by real-time RT-PCR and it could only be detected focally in two cases by IHC.

The differential expression of *CD70* between normal and tumour tissues revealed by the mircroarrays in

eight of 10 patient samples was not only confirmed by RT-PCR but found to be true by IHC for all 10 patient samples. This difference between the two techniques is not surprising given the higher sensitivity of RT-PCR compared to microarrays. The strong induction of *CD70* observed with microarrays and confirmed through RT-PCR has not been described for clear cell RCC until now. The gene could be considered as a potential new biomarker for early diagnosis in clear cell RCC. However, more research into its function is necessary.

Although some information about *CD70* is available, the role of this gene in tumour development and progression remains unclear. It is a Type II transmembrane glycoprotein known to mediate the interaction between T and B-lymphocytes; and natural killer-cell activation. It has also been implicated in processes like cell proliferation, signal transduction and cell-to-cell signalling, and can also induce apoptosis by binding to its receptor CD27 [22,23]. In addition, several studies have shown that *CD70* can be overexpressed in malignant lymphomas and nasopharyngeal tumours which are both associated with Epstein–Barr virus [22]. Nevertheless, in other genome wide studies performed so far, with the exception of the study of Lenburg and colleagues [10,19] the up regulation of *CD70* has not been detected in clear cell RCC.

In our study, high expression of CD70 was found by IHC in all examined clear cell tumours, whereas no expression occurred in the related normal epithelial cells (Fig. 3). In addition, we show in a parallel study that CD70 is not found by IHC neither in papillary nor in chromophobe RCC and is therefore a very specific biomarker for clear cell RCC (Junker and colleagues, 2005, in press). Given that RCC can be considered as an immunogenic tumour, it is tempting to speculate that a *CD70* overexpression might be a possible immune escape for clear cell RCC. Interestingly, the role for CD70/CD27 interactions in T- and B-cell activation has already led to promising treatments for cancer [23].

Another recently described marker, *CXCR4*, was found to be overexpressed in clear cell RCC on the RNA-level but showed heterogeneous staining in IHC [24]. We could confirm the expression of *CXCR4* on cDNA-microarrays in seven out of 10 tumour samples. We compared CD70 and CXCR4 specificity for tumour cells in IHC experiments and found more conclusive results for CD70.

In conclusion, both cDNA arrays and real-time PCR are related to the ''rnomic'' level of gene expression and may lead to biased results due to the fact that protein expression was disregarded. We showed that expression profiling of tumours in combination with IHC provides novel insights into the pathogenesis of the disease and is a powerful method for the identification of new clinical biomarkers.

## Conflict of interest statement

None declared.

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