# Cell-free Circulating DNA: Diagnostic Value in Patients with Renal Cell Cancer

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**Abstract.** *Objectives: To analyse the diagnostic and prognostic value of cell-free DNA in patients with renal cell carcinoma (RCC). Patients and Methods: Cell-free DNA was measured in 35 patients with RCC and 54 healthy individuals using quantitative real-time PCR. ACTB-106 detects fragmented cell-free DNA due to apoptosis and ACTB-384 detects long DNA fragments by necrosis. DNA-Integrity (ACTB-384/ACTB-106 ratio) served as measure of DNA fragmentation. Results: Levels of both DNA fragments were increased in RCC patients compared to healthy individuals (ACTB-384: 1.77 vs. 0.61ng/ml, p=0.0003; ACTB-106: 1.31ng/ml vs. 0.77 ng/ml p=0.003). Receiver operator characteristic analysis (ROC) showed at a threshold level of 1.03 ng/ml for ACTB-106 68.6%, sensitivity and 70.4% specificity (AUC: 0.69). ROC analysis showed at a threshold level of 1.70 ng/ml for ACTB-384 57.1%, sensitivity and 81.5% specificity (AUC: 0.73). DNA integrity was increased in RCC (1.07 vs. 0.72 p=0.04). In vascular invasion the DNA integrity was reduced (p=0.003). Conclusion: Cell-free-DNA levels are increased in RCC. The DNA integrity indicates mostly necrotic origin in RCC.*

In 1977, Leon *et al*. described increased levels of circulating cell-free DNA in plasma of patients with various malignancies in comparison to healthy individuals (1). The potential of cell-free DNA as diagnostic tumour marker has been confirmed by various groups for different malignancies (2-5). Cell-free DNA levels are useful in distinguishing cancer patients from healthy individuals as well as patients with various non-malignant diseases (*e.g.* autoimmune disease, inflammation, benign prostate hyperplasia, HIV (2, 3)). In addition to diagnostic purposes, cell-free DNA levels seem also to be useful for prognostic purposes: higher cell-free DNA levels have been observed in cancer patients with poor outcome (3, 4, 6). In addition to quantitative differences between cancer patients and non-malignant controls, several studies have reported qualitative differences: cell-free DNA in patients with breast cancer, colon cancer and head and neck cancer has an increased DNA integrity (*i.e.* an increased ratio of large DNA fragments) (5, 7, 8). In contrast, cell-free DNA is more fragmented in patients with prostate cancer in comparison to controls (3). The different fragmentation pattern in cancer patients and controls is suspected to be caused by different underlying cell-death mechanisms. High molecular DNA has been shown to be derived from necrotic cells, whereas small (<200 bp) DNA fragments were largely derived from apoptotic cells (9). Similar to quantitative changes, fragmentation patterns are useful for diagnostic as well as prognostic approaches (3, 5).

Despite many attempts to find a serum biomarker that fulfils the criteria to be considered an ideal marker for renal cell cancer (RCC), none have been identified to date (10). Circulating DNA fragment levels and fragmentation patterns have not been studied in patients with RCC. In this study, it was hypothesised that cell-free DNA may also represent a useful biomarker for patients with RCC. Circulating DNA levels and DNA fragmentation patterns were examined in serum of patients with renal cell carcinoma and compared them to a healthy control group.

## Patients and Methods

*Patients, sample collection and DNA isolation*. The present work is a prospective multicenter study. Thirty-five patients with renal cell cancer treated at the Departments of Urology at the Universitätsklinikum Bonn, the St. Josef Hospital Troisdorf and the Herz-Jesu-Krankenhaus Lindlar (all Germany) were included.

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Table I. *Clinicopathological parameters of patients with kidney cancer and healthy individuals.*

| | Renal cancer patients N=35 | Healthy controls N=54 |
|---|---|---|
| Age, years; median (Min-Max) | 66 (31-80) | 28.5 (18-56) |
| Male/female | 28/7 | 37/17 |
| Histology | | |
| Clear cell | 29 | 82.8% |
| Papillary | 4 | 11.4% |
| Chromophobe | 2 | 5.5% |
| TNM staging | | |
| pT1 a | 14 | 40.0% |
| pT1 b | 7 | 20.0% |
| pT2 | 1 | 2.8% |
| pT3a | 4 | 11.4% |
| pT3b | 6 | 17.1% |
| pT3c | 1 | 2.8% |
| n.a. | 2 | 5.7% |
| N+ | 2 | 5.7% |
| M+ | 3 | 8.5% |
| R1 | 3 | 8.5% |
| Necrosis | 10 | 28.6% |
| Surgery | | |
| Radical nephrectomy | 15 | 43% |
| Nephron-sparing | 20 | 57% |
| Grading | | |
| G1 | 5 | 14.3% |
| G2 | 27 | 42.8% |
| G3 | 2 | 5.7% |
| N.A. | 1 | 2.8% |

Among these patients, 15 underwent open radical nephrectomy and 20 underwent open nephron-sparing surgery. Twenty-nine patients had clear cell histology, papillary tumour was found in 4 patients and chromophobe type was found in 2 specimens; 54 healthy individuals served as control subjects. The control group was recruited from nurses, students and laboratory staff; they gave blood samples on a voluntary basis and none of them had relevant comorbidities. The collection of the blood samples was approved by the local Ethics Committee. All subjects gave written informed consent according to the institutional guidelines. The clinical information of the study patients is listed in Table I. Serum samples were collected one day before surgery in Serum-S monovette with clotting activator (Sarstedt, Nürnbrecht, Germany). Clotting occurred for 30-240 minutes prior centrifugation at 1800×*g* (10 min). Serum samples were stored at –20˚C before shipping on dry ice to the University Hospital. Shipping occurred within one week following collection of the samples. Thereafter, the serum samples were stored at –80˚C until DNA isolation. Cell-free DNA was isolated from 1 ml serum using the ChargeSwitch gDNA Kit (Invitrogen, Paisley, Scotland) according the manufacturer's recommendations.

*Quantitative real-time PCR*. Two primer sets amplifying a sequence of the actin-beta gene (*ACTB*) were used to quantify the levels of a 106 bp DNA amplicon ($ACTB^{106}$ amplifying both short and long DNA fragments), and a 384 bp amplicon ($ACTB^{384}$, amplifying only large DNA fragments) as published before 3 (11). The $ACTB^{106}$ results represent total cell-free DNA including DNA of apoptotic origin, whereas the $ACTB^{384}$ results represent DNA from non-apoptotic cells. The annealing sites of the $ACTB^{106}$ are within the $ACTB^{384}$ annealing sites, thus the ratio of $ACTB^{384}$ to $ACTB^{106}$ termed as DNA integrity characterizes the fragmentation pattern of cell-free serum DNA (*i.e.* the DNA integrity is 1 if template DNA is not fragmented and 0 if DNA is completely truncated to fragments smaller than 384 bp). Quantitative real-time PCR was carried out in triplicate on an ABIPrism 7900HT (Applied Biosystems, Foster City, CA, USA). Each 10 μl reaction consisted of 1× SYBRGreenER Mix (Invitrogen), 200 nM forward and reverse primer and 1 μl of DNA sample. PCRs were conducted at 90˚C for 10 min, followed by 40 cycles at 95˚C for 15 s and 60˚C for 60 s. Melting curve analysis was performed to confirm specificity of the PCR products. Each run included 5-fold dilutions of an external standard, negative control (healthy leukocyte DNA) and water blanks.

Table II. *Distribution of circulating serum DNA fragments in patients with renal cancer and healthy individuals.*

| | Renal cancer patients | Healthy control | *p*-Value* |
|---|---|---|---|
| Median $ACTB^{106}$ (ng/ml) | 1.31 | 0.77 | 0.003 |
| Median $ACTB^{384}$ (ng/ml) | 1.76 | 0.61 | 0.0003 |
| $ACTB^{384}/ACTB^{106}$§ | 1.074 | 0.716 | 0.0396 |

*$p$-value: (Mann-Whitney $t$-test) compared to healthy individuals; §Median of all calculated ratios.

*Statistical analysis*. Cell-free DNA levels and DNA integrity were analysed using the Mann-Whitney test. The area under the curve (AUC), sensitivity and specificity were determined by receiver operating characteristic (ROC) analysis. Correlations between clinic pathological parameters and serum DNA fragment levels and the DNA integrity were assessed using the Mann-Whitney test. Statistical tests were performed using SPSS (Chicago, IL, USA). Significance was concluded at $p<0.05$.

## Results

*Serum levels of cell-free DNA fragments*. Median $ACTB^{384}$ serum DNA levels were approximately three-fold higher in patients with renal cell cancer in comparison to healthy individuals (1.76 ng/ml *vs*. 0.61 ng/ml; $p=0.0003$), for $ACTB^{106}$ DNA levels were two-fold fold higher (1.31ng/ml *vs*. 0.77 ng/ml; $p=0.003$).

The significant higher level of $ACTB^{384}$ in cancer patients indicates that cell-free serum DNA is fragmented to a higher degree in cancer patients. The ratio $ACTB^{384}/ACTB^{106}$ describes the fragmentation pattern of serum DNA. Renal cancer and control subjects showed distinctly different fragmentation patterns. This is emphasised by the fact that cell-free DNA was moderately fragmented in the control group ($ACTB^{384}/ACTB^{106}=0.716$), whereas a significantly

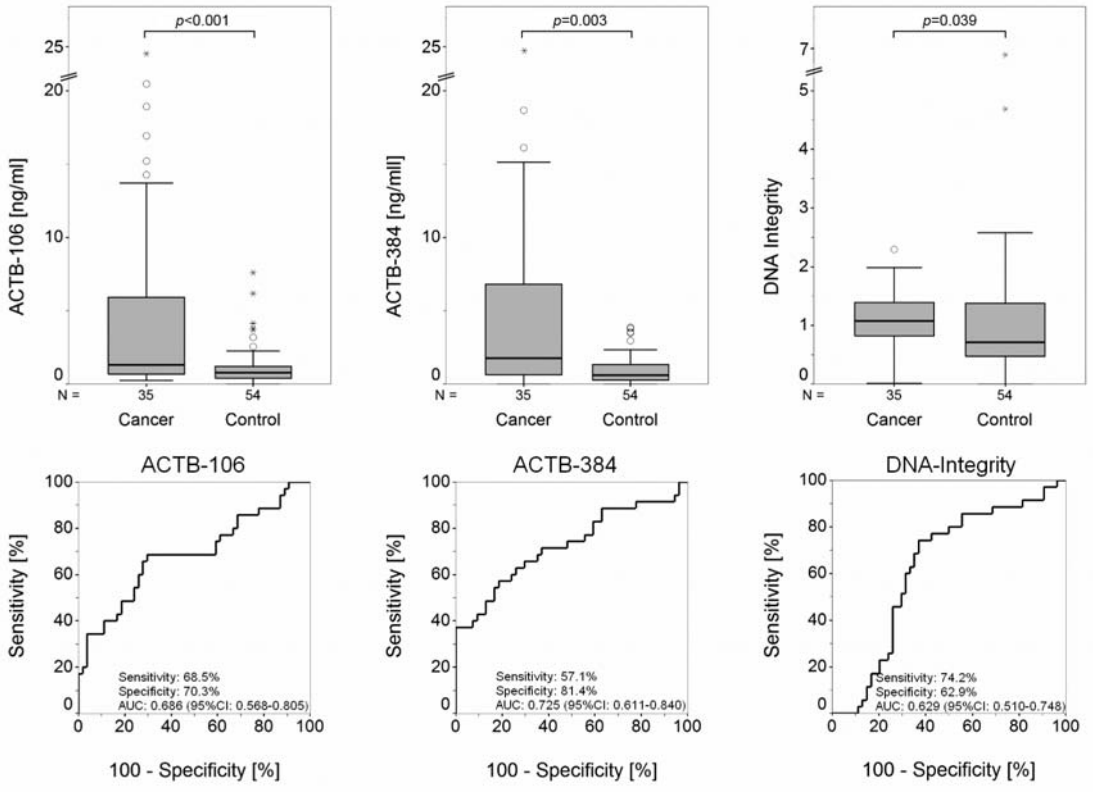


Figure 1. *Box-plot: cfDNA levels for ACTB$^{106}$, ACTB$^{384}$ and DNA integrity. ROC analyses: Diagnostic information of cell-free ACTB-DNA fragments in patients with kidney cancer.*

increased DNA fragmentation was observed in renal cancer patients (ACTB$^{384}$/ACTB$^{106}$=1.074, $p$=0.0396). See Table II for details. In a biologically exact model, the quotient cannot be greater than 1. When the data were reviewed, it was found that many values of ACTB$^{106}$ fell in the range of ACTB$^{384}$ values, supporting the thesis of necrotic origin. A quotient value greater 1 may be explained by technical reasons, such as heterogeneous aspiration of serum or inconsistent pipette handling. These circumstances can potentially cause values minimally higher values for ACTB$^{106}$ than for ACTB$^{384}$ especially when the concentration is low.

*Diagnostic information, specificity and sensitivity.* ROC analysis demonstrated that cell-free DNA levels distinguished between renal cancer patients and healthy controls. ACTB$^{384}$ showed the greatest AUC (0.725) (Figure 1). The sensitivity, specificity and AUC for ACTB$^{384}$ (cut-off: 1.70 ng/ml; sensitivity: 57%; specificity: 81%; AUC:0.72), ACTB$^{106}$ (cut-off: 1.03 ng/ml; sensitivity:68%; specificity: 70%; AUC: 0.68) and ACTB$^{384}$/ACTB$^{106}$ (cut-off: 0.83; sensitivity: 74%; specificity: 62%; AUC: 0.62), are not in the range of an 'ideal' marker but due to the lack of biomarker in renal cell carcinoma they are noteworthy (*i.e.* the AUC for total-PSA for cancer detection is 0.569 (12)).

*Prognostic relevance of serum DNA fragments.* No significant correlation of cell-free DNA levels (ACTB$^{384}$, ACTB$^{106}$) and DNA integrity with pT-stage, grading or histological subtype was observed. Furthermore, the influence of cigarette smoking on DNA levels was evaluated. There was no significant difference between smokers (n=10) and non-smokers (n=21) ($p$>0.05). There was no difference in DNA levels between symptomatic (n=12) and asymptomatic (n=20) patients. No significant difference was found between patients with tumour necrosis and those without histologically confirmed tumour necrosis (ACTB$^{106}$: $p$=0.880; ACTB$^{384}$: $p$=0.734; DNA integrity: $p$=0.678). In cases of metastatic disease compared to non-metastatic disease, a significant correlation of ACTB$^{106}$ ($p$=0.034) was found, whereas ACTB$^{384}$ and DNA integrity did not reach statistical significance. The only other prognostic parameter which correlated with DNA integrity was venous vessel infiltration ($p$=0.0029, n=5).

## Discussion

No definitive biomarker is available for diagnosing and monitoring treatment response in RCC (10). The development of reliable marker would help to facilitate the clinical management of patients with RCC. Cell-free DNA

levels in serum/plasma seem to be an interesting universal marker of malignancy, and numerous studies have been performed to evaluate its value in various tumour entities (3, 6, 7, 11, 13).

Due to the limited follow-up and only three patients with preoperative metastatic disease, a correlation of preoperatively cell-free DNA and outcome was not possible. Nevertheless in lung and prostate cancer cell-free DNA levels have been shown to be prognostically relevant. In patients with PSA recurrence following prostatectomy, cell-free DNA levels are preoperatively increased (3, 6). The survival period of patients with non-small cell lung cancer is shorter with high cell-free DNA levels at baseline (4).

The qualitative changes in circulating cell-free DNA in cancer are an important issue. Cell-free DNA is of apoptotic or necrotic origin (9). In case of apoptotic origin the cell-free DNA is fragmented to 180-200 bp, whereas cell-free DNA from necrotic origin is high molecular with more than 10,000 bp.

The potency of cell-free DNA as a biomarker is supported by findings of these previous studies. Cell-free DNA quantification is repeatable, and cell-free DNA levels are characterized by a high stability. Even if blood processing is delayed up to 6 hours following blood withdrawal, the DNA levels in serum and especially in plasma do not change significantly. Nevertheless the comparison of different studies is difficult: Serum has approximately six-fold higher cell-free DNA levels than plasma, and for a long time it was assumed that higher levels are due to cell lyses during clotting. However, it was shown that higher levels of cell-free DNA in serum are not caused by extraneous contamination (14). Therefore serum was used in the current study. The use of different DNA isolation kits also complicates the comparison of results because DNA extraction efficiencies of these kits are quite variable (15). Finally, even though real-time PCR is now the gold standard for the analysis of cell-free DNA, the use of different primer sets as well as the analysis of genomic, retroviral or mitochondrial DNA makes comparison difficult. The influence of surgery on cell free DNA levels is unclear and was not investigated in the present study. Elevated cell-free DNA levels due to trauma reached normal levels within 2 hours after traumatic injury and the half-life of cell-free DNA is approximately 16 minutes (16, 17). Therefore in this study, it was assumed that cell-free DNA levels would decrease to normal values in limited disease, whereas cell-free DNA levels in metastatic disease would remain high.

A few biochemical markers and molecular markers have been tested in RCC. Ferritin is likely to show increased serum levels in RCC patients. Singh *et al*. showed a correlation between serum ferritin levels and tumour size and grade (18). In the current patient cohort, cell-free DNA was not correlated to tumour size or grade. NMP-22 showed higher levels in the preoperative RCC group compared to the control group. The levels in the RCC group decreased to normal values within 10 days after surgery. Schips *et al*. showed a significant higher level of serum VEGF, a potent angiogenesis stimulator, in RCC patients compared to a control group (19). VEGF is a dimeric glycoprotein and the expression of VEGF in RCC is caused by the inactivation of the von Hippel-Lindau gene (VHL) which acts as a tumour suppressor gene. There was no difference in preoperative VEGF levels in the histological subtypes. In the statistical analyses, VEGF failed to be prognostic (19). In contrast, $ACTB^{106}$ showed significant correlation with metastases and DNA integrity showed correlation with venous vessel involvement, discrimination between histological subtypes was not possible. The tumour-associated trypsin inhibitor (TATI) was evaluated in patients with RCC (20). Elevated serum levels of TATI were found in 57% of the RCC patients (21). TATI is expressed in some renal tumours and also found in cancer cell lines. Several cancers have ability to express TATI *e.g*. ovarian, colonic, gastric and hepatocellular carcinomas (22-25). TATI was compared to other serum markers (CEA 5%, CA 15-3 10%, CA 125 13%, CA 19-9 5%, ferritin 35%) and was found to have highest sensitivity (69%) of the investigated markers (20). The sensitivity of $ACTB^{106}$ (68.5%) was as high as that for TATI; DNA integrity showed a higher sensitivity (74.2%) compared with TATI and $ACTB^{106}$. In a recent review, Gang *et al*. investigated the integrity of cfDNA in kidney cancer patients, they investigated long and short fragments of a housekeeping gene (26). As in the current cohort, significantly higher levels of long fragments (>397 bp) compared to those on the healthy control group were found. The only clinical parameters which reached significant association with DNA-integrity were tumour stage and size. This association was not found in the current cohort.

Radiological criteria frequently do not reflect the biological response to targeted therapy (27). For this setting, cell-free circulating DNA levels may be a potential biomarker of treatment response. Further investigation will be necessary to evaluate cfDNA as a marker of biological response to systemic cancer therapy.

## Conclusion

Cell-free circulating DNA levels in serum of renal cancer patients may be a promising biomarker to distinguish patients from healthy individuals. Consequently, standardization as well as larger, prospective studies including follow up information is necessary before cell-free DNA analysis can be implemented in clinical routine investigations.

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