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Diagnostic and prognostic potential of circulating cell-free genomic and mitochondrial DNA fragments in clear cell renal cell carcinoma patients

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ABSTRACT

Background: There is inconsistent information about the clinical usefulness of circulating cell-free DNA (cfDNA) in plasma from clear cell renal cell cancer (RCC) patients. This is attributed to preanalytical, analytical, and clinical factors that were considered as far as possible in this study.
Methods: cfDNA was extracted from EDTA plasma of healthy people (n = 40), non-metastatic (n = 145) and metastatic (n = 84) RCC patients using the QIAamp Circulating Nucleic Acid Kit. Genomic and mitochondrial cfDNA concentrations were determined using qPCR of different cfDNA fragments (67–306 bp). Their diagnostic and prognostic potential was estimated using receiver operating characteristics (ROC) and Cox regression analyses.
Results: The 67 bp and 180 bp genomic cfDNA fragments did not differ between the three study groups while the 306 bp fragment was lower in RCC patients than in controls. The mitochondrial cfDNA was higher in metastatic than in non-metastatic patients and controls. The cfDNA integrity indices decreased from controls to metastatic patients. Models built by logistic regression and Cox regression resulted in area under the ROC curves >0.75 and concordance indices of >0.800 in predicting recurrence-free survival and overall survival.
Conclusion: The study suggests that combinations of cfDNA markers have promising diagnostic and prognostic potential in RCC patients and are worth for further validation in future prospective multicenter studies.
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1. Introduction

Renal cell cancer (RCC) is with 2.4% of all adult malignancies one of the ten most frequent cancers worldwide. In 2012, RCC accounted for 338,000 new cases and 144,000 deaths [1]. Three main histological RCC morphotypes are distinguished, while the clear cell RCC is with approximately of 80–90% the most frequent one, followed by the papillary and chromophobe RCC with 15 and 5%, respectively. For localized, early-stage tumors, partial or total nephrectomy in curative intent represents the gold standard. However, approximately 30% of the RCC patients present evidence of distant metastasis, which is associated with poor prognosis [2]. After nephrectomy, one-third of the patients with initially localized RCC develop metastasis [3].

The diagnostic and prognostic indicators for patients with RCC are mainly based on traditionally clinicopathological and radiological examinations. The lack of non-invasive blood or urine markers and the inherent limitation of the diagnostic and prognostic models, which were built with the help of these conventional data, are essential short-comings in the management of these patients [4]. Therefore, new molecular markers are urgently needed for higher diagnostic accuracy and predicting the clinical outcome of patients with RCC.

In this respect, circulating cell-free DNA (cfDNA) might be a promising option. Most cfDNAs are double-stranded molecules (approximately 0.18 kB to 21 kB), which circulate as nucleoprotein complexes in blood. Detection of cfDNAs in plasma or serum is possible not only in cancer

Abbreviations: APP-1, APP-2, and APP-3, fragments of 67, 180, and 306 bp of the gene amyloid beta (A4) precursor protein (APP); AUC, area under the ROC curve; bp, base pairs; ccRCC, clear cell renal cell carcinoma; cfDNA, circulating cell-free DNA; CI, confidence interval; C-index, concordance index; DCA, decision curve analysis; G, histopathological grading according to Fuhrman; Mito-1 and Mito-2, mitochondrial DNA sequences of 65 and 175 bp; pT, pathological tumor classification; R, surgical margin classification; ROC, receiver operating characteristics; r_S, Spearman rank correlation coefficient; SINE-1 and SINE-2, Alu sequences with of 79 and 248 bp.

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patients but also in healthy individuals [5]. DNA released from tumor cells by different molecular processes like cell apoptosis, necrosis, micrometastasis, and secretion has been considered to be an important cancer biomarker [6]. The size distribution of cfDNA from cell apoptosis and necrosis is different. Apoptotic cells release cfDNAs of 180 to 200 bp or multiples of this size, while necrotic cells generally generate longer DNA fragments [7,8]. The ratio of long to short DNA fragments, termed as integrity index, was reported to be a useful tool to differentiate these processes [9].

Increased concentrations of cfDNA and altered integrity indices were reported in patients with breast, liver, lung cancer, ovarian, prostate, and gastric cancer (reviewed in [10]). In rare reports about RCC, the level of plasma cfDNA has also been demonstrated as a useful diagnostic and prognostic marker [11–14]. However, as already criticized by de Martino et al. [12], the studies included generally few patients and were therefore underpowered and limited in predicting the outcome of patients. In addition, most studies used serum as sample. However, serum is generally considered a confounder variable as part of serum DNA does not correspond to the cfDNA because of the released DNA from blood cells during the clotting process [15]. Moreover, new data on cfDNA proved that fragments <100 base pairs (bp) and also mitochondrial DNA were more relevant for tumor cfDNA [16–19]. These new aspects, in addition with a higher analytical sensitivity using Alu sequences as new measurement tools [20], have prompted us to re-assess these factors of cfDNA measurements in RCC patients with regard to their diagnostic and prognostic validity. Alu sequences are repetitive DNA sequences that occur in multiple copies in the human genome. These elements have approximately 300 bp and belong to the group of the short interspersed elements (SINEs) with less than 500 bp [21].

Therefore, the aims of our study including controls and patients with non-metastatic and metastatic clear cell RCC were (a) to quantify plasma cfDNA with different fragments based on the determination of the gene amyloid beta (A4) precursor protein (APP), Alu sequences, and mitochondrial DNA, (b) to estimate the association between cfDNA and clinicopathological variables, (c) to evaluate the diagnostic usefulness of these single markers, their ratios, and combinations regarding their discrimination ability between the study groups, and (d) to assess the validity of these markers in predicting the outcome of patients regarding recurrence-free survival and overall survival after nephrectomy.

2. Materials and methods

2.1. Patients and samples

The study was approved by the local University Hospital Ethics committee and informed patients consent was obtained. The study was carried out in accordance with the Declaration of Helsinki. The REMARK and STARD guidelines were correspondingly applied [22,23].

In this retrospective study, a total of 269 subjects were investigated during 2005–2012 and followed up to 2014. The control group consisted of 40 healthy subjects with no evidence of malignancies, infections or gastrointestinal, hepatic, immunologic, renal or other serious diseases. Only patients suffering from a clear cell RCC as the most frequent RCC was included in this study. This RCC cohort was subdivided into 145 patients without metastases at the time of partial or radical nephrectomy and 84 patients with metastases at the time of nephrectomy, at initiation or during targeted therapy. The clinical and clinicopathological data of these study groups are summarized in Table 1.

Blood samples of the RCC patients were collected in K_2EDTA Vacutainer (Becton Dickinson, Heidelberg, Germany) one to 24 days before nephrectomy or before/during targeted therapy and centrifuged (2000 × g, 10 min, 4–8 °C) within 30 min after venipuncture. To prevent a cellular DNA contamination [24] the plasma supernatants were carefully removed and re-centrifuged (16,000 × g; 10 min at 4–8 °C). The prepared plasma samples were archived at −80 °C until analysis.

2.2. DNA extraction and quantitative real-time PCR

All methods were performed according to the MIQE guidelines and were documented in a corresponding checklist found in Supplementary Data [25].

Total DNA was isolated with QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) according to the Qiagen Blood and fluids protocol. Each column was loaded with 1 ml plasma and the extracted DNA was eluted with AVE buffer in final volumes of 30 μl. Aliquots were stored at −20 °C until analysis. Two plasma pools were used for intra-run and between-run precision controls.

DNA was quantified using Quant-iT™ PicoGreen® dsDNA Reagent (ThermoFisher, Invitrogen, Darmstadt, Germany) in Greiner 384 well plates (Sigma-Aldrich, Taufkirchen, Germany) on the microplate reader Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany).

Quantitative real-time PCR (qPCR) was performed on the Light-Cycler 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany). Human Genomic DNA (Roche; cat.no. 11691112001; 200 μg/ml) was used as traceable standard for all measurements. APP (HGNC id: 620) was used as target for the quantification of cfDNA fragments with the amplicons of 67, 180, and 306 bp according to Pinzani et al. [26]. These assays use the same forward primer and hydrolysis probe but specific reverse primers. Final reaction volumes of 10 μl were used consisting of 5 μl of LightCycler® 480 Probes Master, 1 μl DNA sample, 100 pmol/ml of the 5′-FAM-and 3′-TAMRA labeled probe ACCCCAgAggAgCgCCACCTg, and 250 pmol/ml forward and reverse primers (forward APP F: 5′-TCAggTTgACgCCgCTgT; reverse APP R1: 5′-TTCgTAgCCgTTCTgCTgC, size of PCR product is 67 bp, in the following termed APP-1; reverse APP R2: 5′-TCTATAAATggACACCgATgggTAgT, size of PCR product is 180 bp, in the following termed APP-2; reverse APP R3: 5′-gAgAgATAgAATACATTACTgATgTgTggAT, size of PCR product is 306 bp, in the following termed APP-3). The preincubation step was at 95 °C for 10 min, followed by 45 cycles of the amplification program (95 °C for 10 s, 57 °C for 20 s, 72 °C for 40 s), and then 1 min cooling to 40 °C. The qPCRs of SINEs and mitochondrial DNA were performed using Human SINE Screen 3plex Kits and Human Mitochondrion Screen 3plex Kits (CONGEN Biotechnologie, Berlin-Buch, Germany) according to the manufacturer's protocol on the same Instrument. As determined by gel electrophoresis, the lengths of the fragments for SINE-1 and SINE-2 were 79 and 248 bp while the mitochondrial DNA fragments Mito-1 and Mito-2 had lengths of 65 and 175 bp, respectively (Supplementary Data, Fig. S1). The kits contain the ready reaction solutions with all components and a separate Taq Polymerase without giving further details on the primer sequences etc. Before analysis, a reagent mixture of one volume fraction of Taq Polymerase with 199 volume fractions of the ready reaction solution was prepared. The final reaction mixture consisted of 20 μl of this reagent mixture and 5 μl of 1:10 diluted DNA sample. Different detection channels (FAM and CY5) were used in each reaction for the long and short fragments.

Calibrators, controls, and samples were analyzed in triplicate using LightCycler® 480 software, release 1.5.0; and water blanks were included in every run. Standard curves were performed using serial dilutions of DNA. PCR product specificities were confirmed by gel electrophoretic separation of the PCR products. All further methodical details are compiled within the Supplementary Data.

2.3. Data analysis and statistical analysis

The differences between runs were normalized with the inter-plate calibrators by qbase^{PLUS} software (Biogazelle, Gent, Belgium). Statistical analyses were performed using SPSS 23 (SPSS Inc., Chicago, IL, USA), GraphPad Prism 6.07 (GraphPad Software, La Jolla, CA, USA), and MedCalc 15.8 (MedCalc Software bvba, Ostend, Belgium). Non-

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