available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Concentration and microsatellite status of plasma DNA for monitoring patients with renal carcinoma

Roberto A. Perego[a,b,*], *Matteo Corizzato*[a], *Paola Brambilla*[a], *Stefano Ferrero*[c], *Cristina Bianchi*[a,b], *Ester Fasoli*[c], *Stefano Signorini*[d], *Barbara Torsello*[a], *Lara Invernizzi*[a], *Silvia Bombelli*[a], *Valentina Angeloni*[a], *Marina Pitto*[a,b], *Cristina Battaglia*[e], *Vanessa Proserpio*[a,d], *Fulvio Magni*[a,b], *Giacomo Galasso*[f], *Paolo Mocarelli*[a,b,d]

[a]*Department of Experimental Medicine, School of Medicine, University of Milano-Bicocca, Via Cadore 48, 20052 Monza (MI), Italy*
[b]*Interdepartmental Center for Proteomics, University of Milano-Bicocca, Milan, Italy*
[c]*Department of Medicine, Surgery and Dentistry, Pathological Anatomy Unit, San Paolo Hospital, University of Milano, Milan, Italy*
[d]*Department of Laboratory Medicine, Desio Hospital, University of Milano-Bicocca, Milan, Italy*
[e]*Department of Biomedical Sciences and Technologies and CISI, University of Milano, Milan, Italy*
[f]*Institute of Urology, IRCCS Policlinico, University of Milano, Milan, Italy*

ARTICLE INFO

Article history:
Received 2 January 2008
Received in revised form
10 March 2008
Accepted 11 March 2008
Available online 7 April 2008

Keywords:
Renal cell carcinoma
Plasma DNA concentration
Microsatellite LOH
Tumour markers
Follow-up

ABSTRACT

We verified the feasibility of plasma bound method for detecting renal cell carcinoma (RCC) combining the study of plasma DNA concentration and microsatellite alterations (LOH). Plasma DNA concentration was evaluated with real-time PCR in 54 patients with renal neoplasm before surgery and in 20 of these patients during a 26–64 month follow-up. Microsatellite study was performed on tumour tissue DNA of 33 RCC clear cell (RCCcc) and on plasma DNA of 14 RCCcc patients during preoperative and/or follow-up period. Patients had a significantly high (26.4 ± 48.3 ng/ml versus controls 3.2 ± 1.5 ng/ml; $p = 0.003$) preoperative plasma DNA concentration that decreased after nephrectomy. During follow-up, plasma DNA increased in 12 patients without evidence of neoplasia; 3 patients successively relapsed. Tumour tissue DNA of 25 RCCcc patients (75.8%) displayed microsatellite LOH. Preoperative plasma DNA of 9 patients harboured LOH in 5 cases (55.6%). Augmented plasma DNA of 7 patients displayed LOH in 3 cases (42.9%) at follow-up, and in 1 case preceded the recurrence of disease. Plasma DNA concentration combined with microsatellite LOH in plasma DNA may predict disease recurrence in RCC patients.

1. Introduction

Renal cell carcinoma (RCC) accounts for 2–3% of adult malignancies in humans. The most prevalent form of RCC is the clear cell (RCCcc) subtype that accounts for 75% of all renal carcinoma, whilst papillary (RCCpap) and chromophobe subtypes account, respectively, for about 12% and 4%.[1] The only beneficial therapy is surgical resection in early stages, in fact, RCC is chemotherapy resistant, immunotherapy has limited efficacy,[1] and recent kinase inhibitors improve progression-free survival but do not seem to have a significant effect on overall survival.[2] Clinically approved serological tumour markers for the early detection of renal carcinoma, that has an insidious onset without early symptoms, are not available,

* *Corresponding author:* Address: Department of Experimental Medicine, School of Medicine, University of Milano-Bicocca, Via Cadore 48, 20052 Monza (MI), Italy. Tel.: +39 02 6448 8303; fax: +39 02 6448 8068.
E-mail address: roberto.perego@unimib.it (R.A. Perego).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2008.03.008

therefore, new biomarkers are necessary for detecting early signs of disease or recurrence or predicting response to therapy.

The presence of DNA in the plasma of cancer patients, with a level greater than in normal subjects, has been described and tumour-related alterations have been detected in the plasma DNA of patients affected by tumours.[3] The presence of tumour DNA in plasma is the result of different mechanisms such as apoptosis, cellular necrosis, active spontaneous release of DNA by tumour cells,[3,4] that may also account for the different fragment lengths of plasma DNA.[5,6] All these characteristics make plasma DNA a useful tool on the basis of its concentration[7–11] and integrity[12,13] but also as a source in which to detect tumour-related genetic and epigenetic alterations[14–17] as molecular signatures of tumour presence.[3] The subchromosomal losses on chromosome 3p, also at the von Hippel-Lindau (VHL) locus, provoking microsatellite loss of heterozygosity (LOH) are characteristically present in RCCcc, even in the sporadic forms.[18] These complex genetic modifications not only have shed light on some pathogenetic events of RCC but these genetic events might also be exploited for clinical use in helping the diagnostic process.[1] In this study, we quantified plasma DNA levels and studied microsatellite alterations in primary tumour and in plasma DNA evaluating the feasibility of plasma bound methods for detecting RCC and for monitoring follow-up in patients after surgical treatment.

2. Materials and methods

2.1. Subjects

Fifty-four consecutive non-selected patients, affected by renal tumours diagnosed by standard procedures, were enrolled, after written consent, from 2002 to 2006. The patients, who had not received any previous chemotherapeutic treatment, underwent partial or total nephrectomy. Thirty-one patients were males, (mean ± SD age 63.7 ± 9.5 years, range 44–84) and 23 were females, (age 65.8 ± 11.7 years, range 49–88). 41 healthy individuals without diseases and with negative inflammatory indices were also enrolled. All procedures were approved by the Local Ethics Committee on human experimentation.

2.2. Histological analysis

Histological type determined according to the WHO classification[19] included 39 RCCcc, 5 RCCpap, 4 mixed type (RCCcc and RCCpap), 1 urothelial carcinoma, 5 oncocytoma. Fuhrman tumour grading was 2 G1, 34 G2, 9 G3, 1 G4. The pathological tumour stage (pT) was 25 pT1, 8 pT2, 15 pT3, 1 pT4; the nodal status (pN) in 13 patients: 12 pN0, 1 pN2. Microvessel density and proliferating cells, using monoclonal antibodies (Dako A/S, Glostrup, Denmark) against CD34 (antibodies dilution 1:20; clone QB-END/10) and Ki67 (antibodies dilution 1:2000; clone MIB-1), respectively, have been evaluated in 44 of these patients (23 males, 21 females) classified as 35 RCCcc, 3 RCCpap, 2 mixed type, 1 RCC urothelial and 3 oncocitoma. The reaction was detected with the Dako ChemMate EnVision Detection Kit. The histological sections, stained for CD34 or Ki67, were examined at 100× magnification and three areas with the highest number of positive cells were randomly selected. Microvessels were counted at 400× magnification in each of these areas, and were identified as endothelial cells if positive for CD34, either single or clustered in nests or tubes, clearly separated from one another, and either with or without a lumen. Vessels with muscular walls were excluded.[20] The final microvessel density was expressed as the average of the three separate visual counts. For proliferating cells 2000 cells were counted at 400× magnification in the three selected areas, the number of Ki67 positive cells was calculated and expressed as a percentage of the total counted cells.[21]

2.3. Blood sample collection, DNA extraction

Peripheral blood (7.5 ml in EDTA tubes) was drawn from healthy donors, and from patients the day before surgery and, when possible, during follow-up. Plasma was separated after two centrifugation at 2500 rpm for 10 min at 4 °C within 2 h and stored at –80 °C until usage. DNA was extracted from fresh tumour tissue, density gradient separated lymphocytes and plasma samples, by using QIAamp DNA Mini kit (Qiagen, Italy) according to 'tissue protocol' and 'blood and body fluids protocol'. Plasma (1 ml) was passed five times through the same column, and the retained DNA was eluted in 50 μl of sterile bidistilled water and stored at –80 °C. Tumour and lymphocyte DNA concentrations were estimated by spectrophotometry.

2.4. Quantification of plasma DNA

Quantification of plasma DNA was performed by real-time PCR (reagents and apparatus from Applied Biosystem) amplifying the β-*globin* single copy gene[22] as frequently reported in the literature,[3] the amplicon size was 73 bp. The following are the sequences of primers forward 5′-GTGCACCTGACTC CTGAGGAGA-3′, reverse 5′-CACCAACTTCATCCACGTTCA-3′ and probe (VIC)5′-TCTGCCGTTACTGCCCT-3′(MGB) used. Fluorogenic PCRs were carried out in a reaction volume of 25 μl on a GeneAmp 7900HT Sequence Detection System. Each PCR reaction mixture consisted of 2×TaqMan Universal Master Mix, a final concentration of 0.2 μM of probe and 0.3 μM of primer forward and reverse, 5 μl of plasma DNA solution and sterile water. Each sample was analysed in triplicate. Thermal cycling steps were 2 min at 50 °C then 10 min at 95 °C and (15 s 95 °C, 1 min 60 °C) × 40 cycles. A standard curve using scalar amount (100, 10, 1, 0.1, 0.01 ng) of TaqMan Control Human Genomic DNA was generated. The amounts of DNA in the experimental sample, expressed as ng/ml, were obtained interpolating the standard curve of DNA at a known quantity with amplification cycle threshold of the unknown target sample.

2.5. Microsatellite analysis

Microsatellite LOH were evaluated only in RCCcc by studying the microsatellite markers D3S1566, D3S1285, D3S1300, D3S1289, D3S1597 located on chromosome 3p that is characteristically and frequently involved in RCCcc[18] subtype. Twenty nanograms of tumour tissue and lymphocyte DNA

were analysed and, when available, plasma DNA was analysed in the range of 6–10 ng per reaction. The microsatellite analysis in plasma DNA has been done with the same plasma samples used to determine the plasma DNA concentration. The PCR reaction mixture (reagents and apparatus from Applied Biosystem) in a final volume of 45 μl was: 4.5 μl of 10×Buffer II Gold PE, 4.5 μl of $MgCl_2$ 25 mM, 1 μl of dNTP mix 2.5 mM, 3 μl of fluorescent labelled primer pairs 10 mM, 0.5 μl of Ampli*Taq*Gold 5 U/μl and water. The thermal cycling, in a GeneAmp PCR system 2700 Thermal Cycler, was first (15 s 94 °C, 15 s 55 °C, 30 s 72 °C) × 10 cycles and then (15 s 89 °C, 15 s 55 °C, 30 s 72 °C) × 20 cycles. The fluorescent PCR products were analysed using ABI Prism DNA Sequencer 3100 Avant, equipped with GeneScan 2.1 software. Microsatellite alterations were detected comparing the profiles obtained with tumour tissue DNA or with plasma DNA and normal DNA from peripheral lymphocytes. Microsatellite LOH was obtained as allelic imbalance that was calculated by the ratio of the peak area of two alleles for each tumour sample and normalised with respect to the ratio of the peak area of the same two alleles in the corresponding normal lymphocytes.[23] Allelic imbalance was present if the normalised ratio of the two alleles in the pathologic samples was greater than or equal to 1.30 or less than or equal to 0.70, whereas ratios 0.71–1.29 were regarded as maintenance of heterozygosity. In the case the amount of plasma DNA available for microsatellite analysis was below 6 ng, 2–5 ng of plasma DNA were pre amplified with a PCR using up to three fluorescent labelled primer pairs chosen from those specific for the five microsatellites of interest. The primer pair mix of each microsatellite to a final concentration of 22.5 nM, plus 2×Amplification Gold PCR Master Mix and sterile water were added to reach a final volume of 50 μl. The thermocycling profile for pre-amplification PCR reaction was as follows: 1 cycle of 95 °C for 10 min, (30 s 95 °C, 30 s 55 °C, 30 s 72 °C) × 14 cycles, 72 °C for 7 min. After that 5–10 μl of the pre-amplified samples were processed for microsatellite analysis as described above. Preliminary tests in lymphocyte and plasma DNA of normal subjects did not show for the single microsatellites false positive LOH.

2.6. Statistical analysis

SAS software (SAS Inc., Cary, NC) was used to perform the two-tailed Student's *t*-test, Kruskal–Wallis test and the receiver operating characteristics (ROC) curve. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Quantification of circulating DNA in healthy donors and in preoperative patients

Table 1 summarises the results of quantification of circulating DNA in control subjects and in preoperative patients. The difference of mean plasma DNA concentration between controls (3.2 ± 1.5 ng/ml) and patients (26.4 ± 48.3 ng/ml) is significant

Table 1 – Plasma DNA concentrations in healthy controls, in patients before surgical treatment and stratified according to clinical and pathological characteristics

Variable	Subjects, *n*	Concentration of plasma DNA (ng/ml), mean ± SD (range)	Statistical analysis	*p*-Value
Healthy controls	41	3.2 ± 1.5 (1.3–7.1)		
Patients	54	26.4 ± 48.3 (0.2–299.7)	*t*-Test	$p = 0.003$*
Sex			*t*-Test	$p = 0.76$
Male	31	24.4 ± 34.3 (0.2–154.1)		
Female	23	28.4 ± 62.6 (0.2–299.7)		
Age (years)			Kruskal–Wallis	$p = 0.32$
44–53	12	18.0 ± 23.7 (0.2–64.7)		
54–63	14	32.3 ± 78.3 (0.2–299.7)		
64–73	17	39.3 ± 44.4 (0.3–154.1)		
>74	11	9.8 ± 5.9 (0.7–19.9)		
Histological type			*t*-Test	$p = 0.61$
RCCcc	42	24,5 ± 51.6 (0.2–299.7)		
Other hist. types	12	32.4 ± 36.5 (0.5–111.6)		
Tumour size (cm)			*t*-Test	$p = 0.71$
<5 cm	23	30.6 ± 62.3 (0.3–299.7)		
>5 cm	28	25.4 ± 37.7 (0.2–154.1)		
Tumour grade (all types)			*t*-Test	$p = 0.71$
G1+G2	36	25.2 ± 55.9 (0.2–299.7)		
G3+G4	10	32.2 ± 36.7 (1.7–111.6)		
pT Category (all types)			Kruskal–Wallis	$p = 0.91$
pT1	25	22.9 ± 34.1 (0.2–154.1)		
pT2	8	47.2 ± 102.8 (1.2–299.7)		
pT3–pT4	16	22.3 ± 29.7 (0.2–111.6)		

* Compare with healthy controls.

($p = 0.003$), the significance in respect to the controls persists even when patients are stratified by sex, age, histology, tumour size, grading and pathologic TNM. However, there are no significant differences into the stratified groups (Table 1). The percentile distribution of plasma DNA concentration is reported in Fig. 1 and Table 2. ROC curve analysis showed that the area under curve (AUC) assessing plasma DNA concentration was 0.761 (95% confidence interval 0.655–0.848) (Fig. 2) suggesting a moderate careful discriminating power of the DNA quantification assay and that plasma DNA levels might be a potential biomarker in renal cancer. The elaboration of the ROC curve provided also the values of the best sensitivity combined with the best specificity (Table 3). It was possible to evidence two groups in preoperative patients using a cut off value of 6.2 ng/ml (mean + 2SD of plasma DNA concentration in healthy donors): one with high plasma DNA concentration and the other with a low concentration. Thirty-four out of 54 patients (62.9%) had a concentration higher than 6.2 ng/ml, whilst only 1 out of 41 controls (2.4%) had the level of DNA up at 6.2 ng/ml.

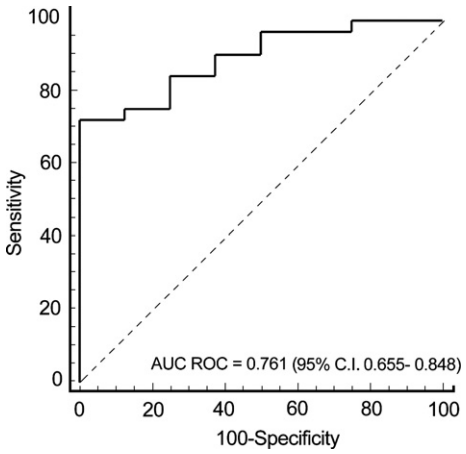

Fig. 2 – Receiver-operating characteristics (ROC) curve. Evaluation of the performance of plasma DNA quantification in 54 patients with kidney neoplasms versus 41 healthy controls. The area under the curve (AUC) and the 95% confidence interval (CI) are reported.

3.2. *Microvessel density and proliferation fraction*

We investigated if plasma DNA level was influenced by the rate of microvessel density and proliferating cells in the primary tumour. The CD34 endothelial marker and the Ki67 proliferating marker were determined by immunohistochemistry. Microvessel density was 84.6 ± 43.8 CD34+ cells/high-power field (mean ± SD), range was 14.3–178.7 and median 83.2 in the patients studied. The proliferating cells were 9.1 ± 6.0 percentage of Ki67+ cells (mean percentage ± SD), the range was 2.3–24.8 and the median 8.3. It was not possible to evidence any correlation between these two parameters and plasma DNA concentration (Fig. 3) in our series, even when considering only RCCcc. However, in several cases of RCCcc, the low or the high concentration of circulating DNA matches, respectively, a lower or higher endothelial density or proliferating fraction. Furthermore, it seems that there is a trend between the Ki67 cell positivity and the level of plasma DNA (Fig. 3).

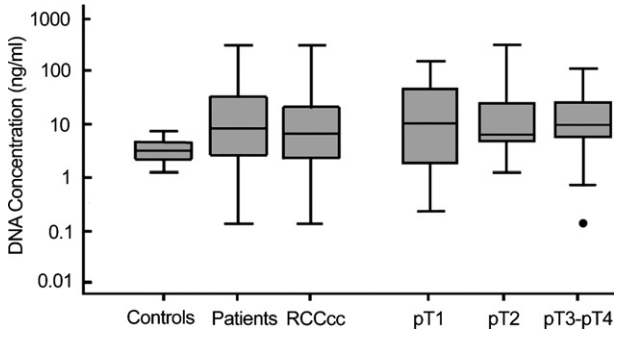

Fig. 1 – Box-plots of percentile distribution of plasma DNA concentration in healthy controls, patients, only patients affected by RCCcc type and different pT categories of all RCC types.

3.3. *Quantification of circulating DNA in patients during follow-up*

Post-surgery follow-up (range 26–64 months; median 51 months) was possible only for twenty patients (19 RCCcc, 1 RCCpap) whose preoperative plasma DNA was evaluated. Fig. 4 shows the clinical outcome and the pattern of follow-up plasma DNA levels evaluated with respect to the cut-off value of 6.2 ng/ml. It is of note that, except for one patient, all those with preoperative plasma DNA higher than the cut-off value at the first time point after surgery showed a concentration lower than the cut-off value. Patient 24MO affected by RCCpap with a concentration higher than the cut-off level had, however, a drastic reduction with respect to the preoperative level (Fig. 4).

Eighty-two plasma samples were collected at time points ranging between 1 and 54 months during follow-up; in 21 of

Table 2 – Percentile distribution of plasma DNA concentration values (ng/ml) for healthy controls, patients, only patients affected by RCC clear cell type and different pT categories of all RCC types

	Min	10th	25th	Median	75th	90th	Max	Mean	SD
Healthy controls	1.3	1.5	2.1	3.0	4.0	5.2	7.1	3.2	1.5
Patients	0.2	1.2	2.6	8.3	31.6	59.2	299.7	26.4	48.3
RCCcc	0.2	1.1	2.3	6.6	20.8	52.8	299.7	24.5	51.6
pT1	0.2	0.5	1.8	9.9	45.8	55.0	154.1	22.9	34.1
pT2	1.2	2.1	4.6	5.9	27.9	221.5	299.7	47.2	102.8
pT3–pT4	0.2	0.8	5.4	9.4	27.0	62.7	111.6	22.3	29.7

Table 3 – Sensitivity and specificity of plasma DNA quantification assay (with 95% confidence interval)

Cut point (ng/ml)	Sensitivity (95% CI)	Specificity (95% CI)
2.5	77.4 (63.8–87.7)	30.0 (14.8–49.4)
3.5	73.6 (59.7–84.7)	66.7 (47.2–82.7)
4.4	69.8 (55.7–81.7)	80.0 (61.4–92.2)
5.6	66.0 (51.7–78.5)	96.7 (82.7–99.4)
6.1	62.9 (47.9 -75.2)	96.7 (82.7–99.4)
6.7	58.5 (44.1–71.9)	96.7 (82.7–99.4)
7.3	52.8 (38.6–66.7)	100.0 (88.3–100.0)

these samples the circulating DNA level was over the cut-off limit. During follow-up, 8 patients had a plasma DNA level persistently under the cut-off value and they did not show recurrence of disease. The other 12 patients showed an increment of plasma DNA concentration over the cut-off value. Until today, nine of them still do not show clinical evidence of neoplastic disease and in some cases subsequent decrement below the cut-off value has been demonstrated. In 3 cases, the increment preceded the recurrence of disease. The outcome of these 20 patients, keeping into account the limited number of cases, does not seem to be related to the preoperative level of plasma DNA.

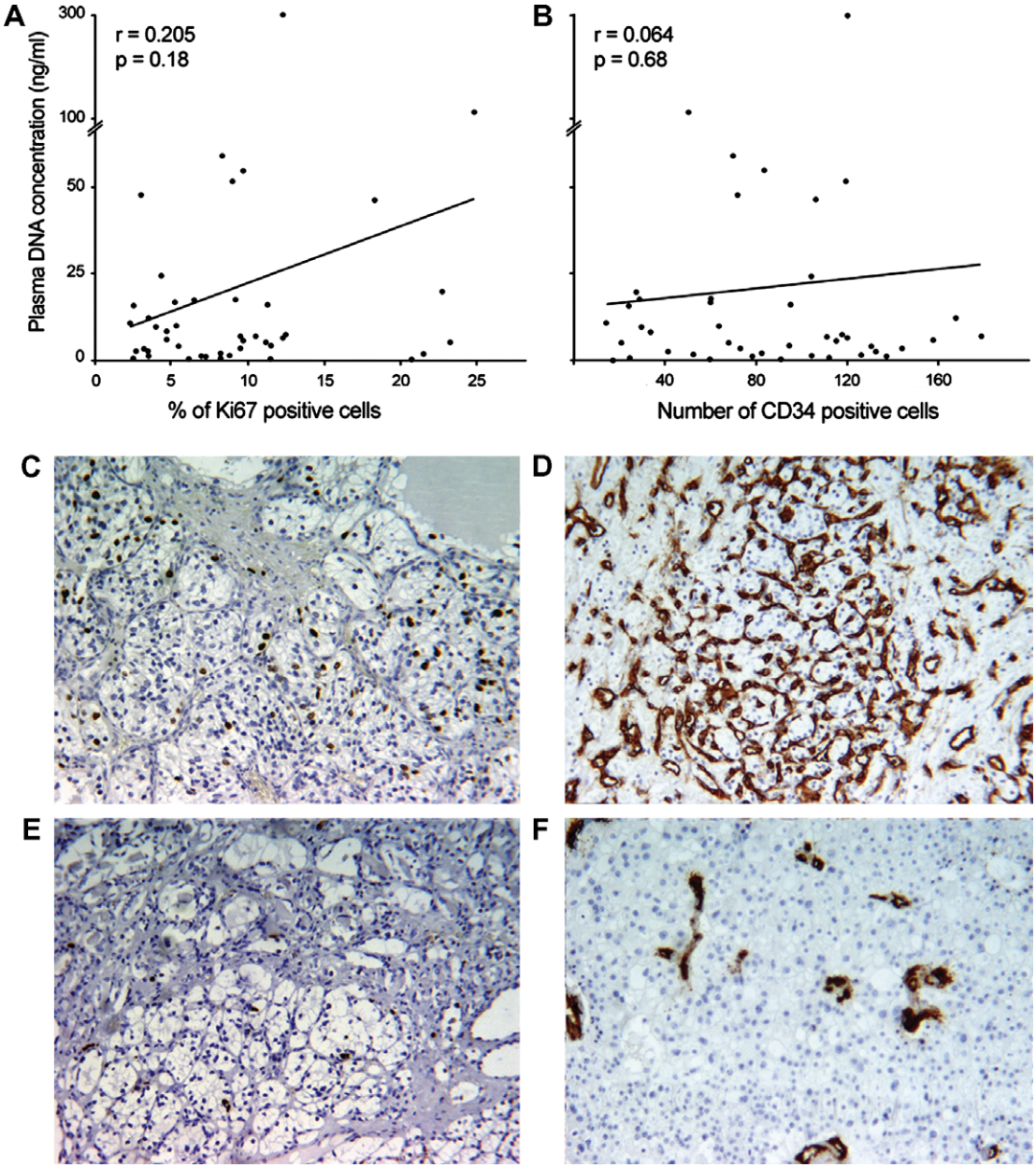

Fig. 3 – Distribution of plasma DNA concentration and (A) Ki67 and (B) CD34 expression. Representative cases of high plasma DNA concentration and (C) high Ki67+ cells, (D) high CD34+ cells. Low plasma DNA concentration and (E) low Ki67+ cells, (F) low CD34+ cells. Tumour sections at 100× magnification, the brown stain identifies the Ki67 and CD34 positive cells.

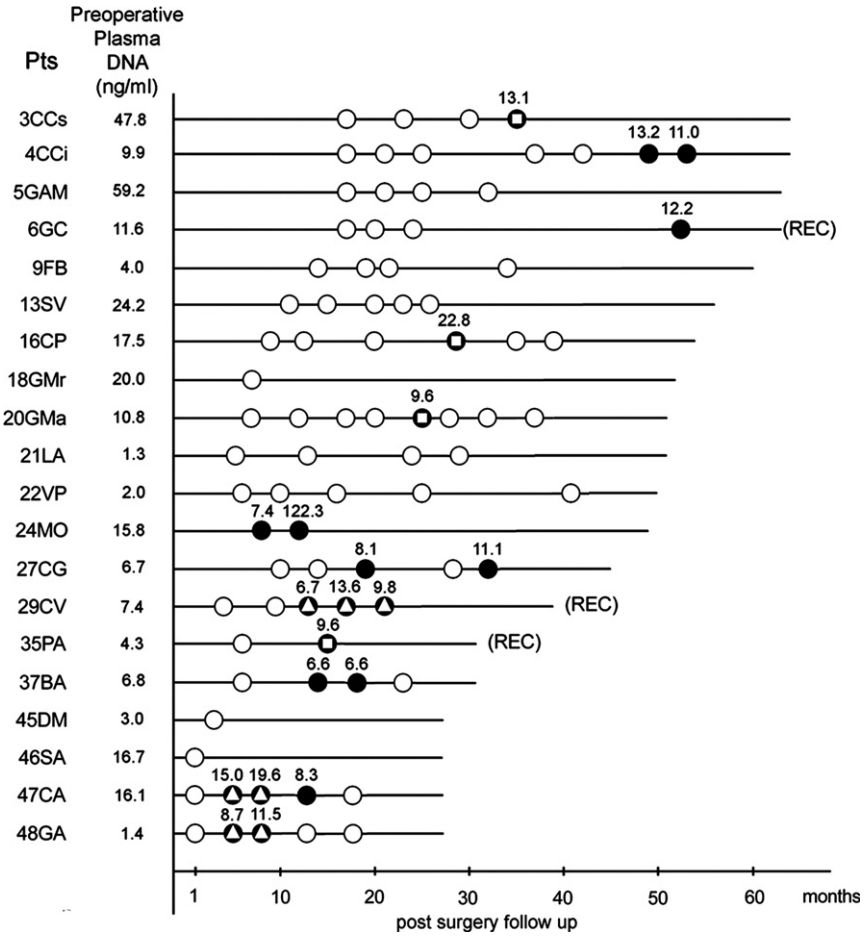

Fig. 4 – Serial plasma DNA quantification during follow-up of surgically treated RCC patients. ○ and ● denote the time points with plasma DNA concentrations, respectively, lower or higher than the cut-off value of 6.2 ng/ml. ◘ and ◭ denote the absence or the presence of microsatellite LOH in plasma DNA with concentration higher than the cut-off value. Plasma DNA concentration values of preoperative samples and of follow-up samples with values above the cut-off are reported. The patients (Pts) identified by acronyms had RCCcc except patient 24MO that had RCCpap. REC: recurrence of disease.

3.4. *Analysis of microsatellites in RCC clear cell tumour DNA*

The genetic events characteristically present on chromosome 3p in RCCcc were exploited to make a more comprehensive evaluation on the variation of plasma DNA concentration. In order to study the microsatellite alterations in plasma DNA, the status of 5 microsatellites was established in tumour tissue of 33 RCCcc patients whose DNA extracted from fresh tumour tissue was available (Fig. 5). Twenty-five of 33 patients (75.8%) displayed microsatellite LOH in tumour specimens at least at one of the five loci studied on 3p, and 24 patients had more than one LOH. A total of 163 microsatellites were analysed and 45.4% had LOH. Amongst the five markers studied, the most frequently altered was D3S1597 (LOH in 20 of 33 patients; 60.6%); the least involved was D3S1285 (LOH in 11 of 33 patients; 33.3%). Neither a significant relation between detected DNA alterations of the investigated loci and tumour characteristics (staging, grading, size and circulating DNA level) were found. The presence of microsatellite LOH was only accompanied by a higher vasculature density (mean CD34+ cells/high-power field: 103.7 ± 36.3 versus 77.0 ± 50.4) and higher proliferating fraction (mean percentage of Ki67+ cells: 9.3 ± 5.2 versus 8.6 ± 6.6) although not significant.

3.5. *Analysis of microsatellites in RCC clear cell preoperative and follow-up plasma DNA*

In 14 of 33 RCCcc patients 52 microsatellites in plasma DNA at the same five loci considered for the primary tumour DNA were studied (Fig. 6).

In preoperative plasma DNA, the study of microsatellite alterations was possible for 9 patients whose preoperative plasma DNA was available, and in which one or more microsatellites were found altered in tumour tissue DNA as shown in Fig. 5. In particular, of the 23 microsatellites studied in preoperative plasma DNA, 12 showed the same LOH found in the primary tumour DNA, 8 microsatellites lost the LOH that were instead present in tumour tissue DNA and 3 microsatellites maintained without changes the same absence of LOH or not informative status present in tumour tissue DNA (Fig. 6). On the whole, five of the 9 patients (55.6%) harboured, in preoperative plasma DNA, at least one microsatellite alteration corresponding to that present in the tumour. These

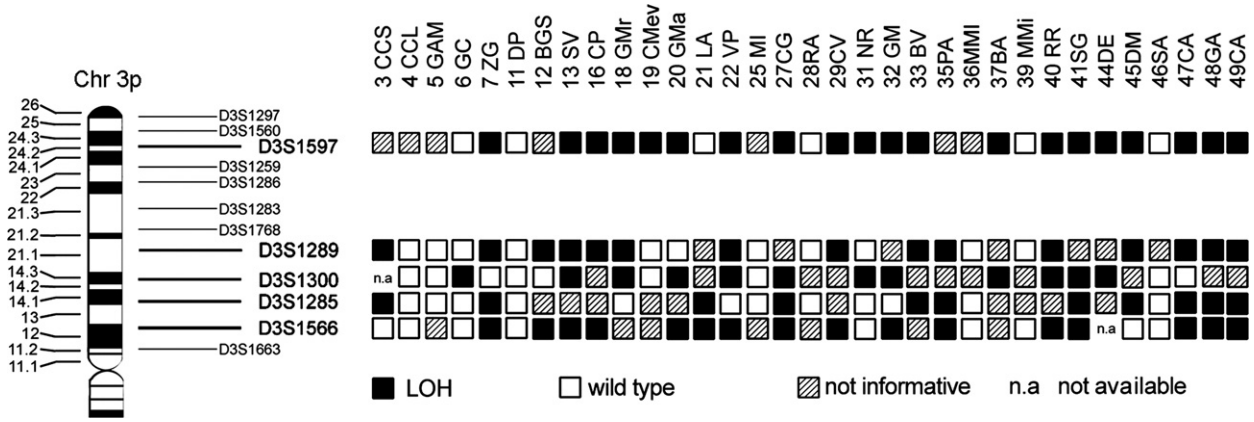

Fig. 5 – Microsatellite analysis in the tumour tissue DNA of 33 RCC clear cell patients. On the left are shown the 5 microsatellites studied (larger characters) and their reciprocal positions on chromosome 3p. The single patients are identified by acronyms.

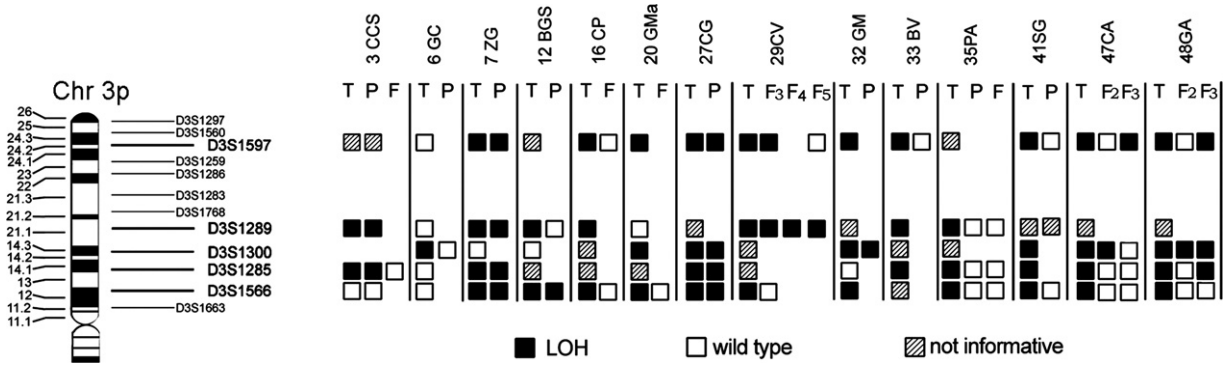

Fig. 6 – Microsatellite analysis in plasma DNA. Comparison of tumour tissue DNA (T) with preoperative plasma DNA (P) and with follow-up plasma DNA (F). The follow-up time points analysed are those specified in Fig. 4. F_2, F_3, F_4, F_5 denote the specific time points analysed in the specific patients. Preoperative plasma DNA concentration (ng/ml) of the following patients was: 7ZC, 45.4; 12BGS, 17.7; 32GM, 6.1; 33BV, 6.4; 41SG, 46.5. Microsatellite analysis and the quantification of plasma DNA were performed on the same plasma samples. The single patients are identified by acronyms.

findings were not related to preoperative plasma DNA concentration.

Eleven follow-up time points of 7 patients in which the plasma DNA concentration was higher than the cut-off limit were also analysed (Fig. 4). In 4 of these patients, the plasma DNA did not evidence the LOH present in primary tumour DNA. In the other 3 patients, we found the same LOH detected in primary tumour DNA (Fig. 6). Amongst the 29 microsatellites studied in plasma DNA during follow-up, 10 showed the same LOH present in tumour DNA whereas 19 lacked the LOH (Fig. 6). In patient 29CV the plasma DNA of 3 different follow-up time points showed microsatellite LOH and it is of note that this patient at the third time point analysed had recurrence of disease. In patient 35PA the metastatic disease was diagnosed after an increment of plasma DNA concentration that was not accompanied by the LOH seen in tumour tissue DNA, however, these LOH were not present even in the preoperative plasma. In the other patients, the increment during follow-up of plasma DNA concentration without LOH was correlated with the movement of inflammatory indices.

4. Discussion

The level of circulating DNA, measured by real-time PCR, was threefold different between healthy controls and preoperative patients. The control group has a homogeneous distribution of plasma DNA value, whilst the patient group has a heterogeneous distribution that varies from levels of about 300 ng/ml to levels like those present in the healthy control group. Even though levels of plasma DNA may be related to the tumour type, steps like plasma sample collection and storage, DNA extraction and storage are critical and can influence the values of plasma DNA concentration[4,24]; it must be noted that some of our plasma samples were processed about 2 years after storage. In any case, the AUC obtained with ROC curve analysis (0.761) demonstrated a moderate careful discrimination power in accordance with some reports.[7,13,14] The levels of circulating DNA had no correlation with clinical status, tumour size, histological type, Ki67 and CD34 markers. One significant aspect of our study is the quantitative plasma DNA analysis during follow-up (26–64 months) that permitted to observe, for the first time, longitudinal changes after surgical

resection. Our data on 20 kidney carcinoma patients showed a rapid decrease of circulating DNA values after nephrectomy. However, during the subsequent follow-up 12 patients had an increment in the quantity of plasma DNA, and not one of these patients had clinical evidence of tumour recurrence at the moment of circulating DNA increment, but 3 of them successively relapsed.

Although, it seems possible that the level of plasma DNA might be used as a marker in patients previously diagnosed and treated for RCC, this molecular assay in plasma still remains to be improved for routine use in clinical practice.[14] An improvement could be achieved with a multimodal approach that exploits the combination of an increased plasma DNA level with the presence of tumour genetic alterations. Interestingly, we show the applicability of this approach for a plasma bound RCC detection, in fact, it was possible to study microsatellite alterations not only in preoperative plasma DNA in accordance with others,[25–28] but also for the first time, in follow-up plasma DNA obtaining results concordant with those obtained in primary tumour DNA. However, unaltered normal genomic DNA is always present in the plasma samples and this makes a complete LOH in microsatellite analysis almost impossible, therefore, microsatellite alterations are referred as allelic imbalance. To reduce the risk that PCR artefacts can be misinterpreted as plasma DNA allelic imbalance, a microsatellite analysis on DNA of primary tumours must always be performed. It has to be underlined that by using only a limited number of microsatellites, our data on DNA of primary tumour tissues were concordant with the literature, in fact, 25 out of 33 (76%) of the patients analysed displayed LOH at least at one locus on 3p.[25–27] In our series we never detected microsatellite alterations in plasma DNA that were not first evidenced in primary tumour; during follow-up, the LOH, present in circulating DNA, was able in one case to anticipate the relapse proving the tumour origin of the released plasma DNA.

In conclusion, we show that the use of the combination of plasma DNA level variations with the presence of tumour genetic changes in plasma DNA is feasible for studying RCC patients and has been able to anticipate the recurrence of the disease. The use of nucleic acids as molecular biomarkers might become an important tool in oncology, as already routinely performed in oncohaematology for detecting minimal residual disease and for stratifying patients on the basis of the presence of molecular alterations.[29] However, efforts should be made to encourage the standardisation of sample collection and molecular procedures and to schedule the best time to perform serial molecular follow-up for monitoring patients.

Conflict of interest statement

None declared.

Acknowledgements

This study was funded by grants from Italian Ministry for Research: COFIN 2001 (no. 58539), FIRB 2001 (no. RBNE01HCKF), PRIN 2004 (no. 61903_004), PRIN 2006 (no. 69373_004). We thank Dr. Rosanna Falbo from the Dept. of Laboratory Medicine Desio Hospital for the revision of the manuscript.

REFERENCES

1. Cohen HT, McGovern FJ. Renal cell carcinoma. *N Engl J Med* 2005;**353**:2477–90.
2. Brugarolas J. Renal cell carcinoma – molecular pathways and therapies. *N Engl J Med* 2007;**356**:185–7.
3. Fleischhacker M, Schmidt B. Circulating nucleic acids (CNAs) and cancer – a survey. *Biochim Biophys Acta* 2007;**1775**:181–232.
4. Gormally E, Caboux E, Vineis P, Hainaut P. Circulating free DNA in plasma or serum as biomarker of carcinogenesis: practical aspects and biological significance. *Mutat Res* 2007;**635**:105–17.
5. Jahr S, Hentze H, Englisch S, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res* 2001;**61**:1659–65.
6. Wang BG, Huang HY, Chen YC, et al. Increased plasma DNA integrity in cancer patients. *Cancer Res* 2003;**63**:3966–8.
7. Sozzi G, Conte D, Mariani L, et al. Analysis of circulating tumor DNA in plasma at diagnosis and during follow-up of lung cancer patients. *Cancer Res* 2001;**61**:4675–8.
8. Sozzi G, Conte D, Leon M, et al. Quantification of free circulating DNA as a diagnostic marker in lung cancer. *J Clin Oncol* 2003;**21**:3902–8.
9. Gormally E, Hainaut P, Caboux E, et al. Amount of DNA in plasma and cancer risk: a prospective study. *Int J Cancer* 2004;**111**:746–9.
10. Ren N, Ye QH, Qin LX, Zhang BH, Liu YK, Tang ZY. Circulating DNA level is negatively associated with the long-term survival of hepatocellular carcinoma patients. *World J Gastroenterol* 2006;**12**:3911–4.
11. Chun FK, Muller I, Lange I, et al. Circulating tumour-associated plasma DNA represents an independent and informative predictor of prostate cancer. *BJU Int* 2006;**98**:544–8.
12. Hanley R, Rieger-Christ KM, Canes D, et al. DNA integrity assay: a plasma-based screening tool for the detection of prostate cancer. *Clin Cancer Res* 2006;**12**:4569–74.
13. Umetani N, Kim J, Hiramatsu S, et al. Increased integrity of free circulating DNA in sera of patients with colorectal or periampullary cancer: direct quantitative PCR for ALU repeats. *Clin Chem* 2006;**52**:1062–9.
14. Chang HW, Lee SM, Goodman SN, et al. Assessment of plasma DNA levels, allelic imbalance, and CA 125 as diagnostic tests for cancer. *J Natl Cancer Inst* 2002;**94**:1697–703.
15. Andriani F, Conte D, Mastrangelo T, et al. Detecting lung cancer in plasma with the use of multiple genetic markers. *Int J Cancer* 2004;**108**:91–6.
16. Diehl F, Li M, Dressman D, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. *Proc Natl Acad Sci USA* 2005;**102**:16368–73.
17. Urakami S, Shiina H, Enokida H, et al. Wnt antagonist family genes as biomarkers for diagnosis, staging, and prognosis of renal cell carcinoma using tumor and serum DNA. *Clin Cancer Res* 2006;**12**:6989–97.
18. Chudek J, Wilhelm M, Bugert P, Herbers J, Kovacs G. Detailed microsatellite analysis of chromosome 3p region in non-papillary renal cell carcinomas. *Int J Cancer* 1997;**73**:225–9.
19. Eble JN, Sauter G, Epstein JI, Sesterhenn IA. *WHO pathology and genetics of tumours of the urinary system and male genital organs*. Lyon: IARC Press; 2004.

20. Panteli K, Zagorianakou N, Bai M, Katsaraki A, Agnantis NJ, Bourantas K. Angiogenesis in chronic myeloproliferative diseases detected by CD34 expression. *Eur J Haematol* 2004;**72**:410–5.
21. Florena AM, Tripodo C, Iannitto E, Porcasi R, Ingrao S, Franco V. Value of bone marrow biopsy in the diagnosis of essential thrombocythemia. *Haematologica* 2004;**89**:911–9.
22. Poncz M, Schwartz E, Ballantine M, Surrey S. Nucleotide sequence analysis of the delta beta-globin gene region in humans. *J Biol Chem* 1983;**258**:11599–609.
23. Larson PS, Schlechter BL, de las Morenas A, Garber JE, Cupples CL, Rosenberg CL. Allele imbalance, or loss of heterozygosity, in normal breast epithelium of sporadic breast cancer cases and BRCA1 gene mutation carriers is increased compared with reduction mammoplasty tissues. *J Clin Oncol* 2005;**23**:8613–9.
24. Sozzi G, Roz L, Conte D, et al. Effects of prolonged storage of whole plasma or isolated plasma DNA on the results of circulating DNA quantification assays. *J Natl Cancer Inst* 2005;**97**:1848–50.
25. Goessl C, Heicappell R, Münker R, et al. Microsatellite analysis of plasma DNA from patients with clear cell renal carcinoma. *Cancer Res* 1998;**58**:4728–32.
26. Eisenberger CF, Schoenberg M, Enger C, et al. Diagnosis of renal cancer by molecular urinalysis. *J Natl Cancer Inst* 1999;**91**:2028–32.
27. von Knobloch R, Hegele A, Brandt H, et al. High frequency of serum DNA alterations in renal cell carcinoma detected by fluorescent microsatellite analysis. *Int J Cancer* 2002;**98**:889–94.
28. Gonzalgo ML, Eisenberger CF, Lee SM, et al. Prognostic significance of preoperative molecular serum analysis in renal cancer. *Clin Cancer Res* 2002;**8**:1878–81.
29. Brüggemann M, Raff T, Flohr T, et al. Clinical significance of minimal residual disease quantification in adult patients with standard-risk acute lymphoblastic leukemia. *Blood* 2006;**107**:1116–23.