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Circulating cell-free DNA in plasma of melanoma patients: Qualitative and quantitative considerations

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ABSTRACT

DNA integrity in blood is an emerging biomarker in cancer. Here we report a real time PCR approach for the absolute quantification of four amplicons of 67, 180, 306 and 476 bp in cutaneous melanoma. Three different integrity indexes (180/67, 306/67 and 476/67 ratios) were tested for their ability to reflect differences in plasma cell-free DNA (cfDNA) fragmentation in 79 patients affected by cutaneous melanoma and 34 healthy subjects. All the three integrity indexes showed higher values in melanoma patients in comparison with healthy subjects.

According to ROC curve analysis, the ratio 180/67 is the most suitable index to be used in cancer patient selection, even if the combination of the 3 indexes gives the best performance in terms of clinical sensitivity. The most represented fragments in plasma of melanoma patients are those comprised between 181 and 307 bp, while in healthy subjects there is a prevalence of shorter fragments (67–180 bp).

In conclusion, DNA integrity indexes can be considered suitable parameters for monitoring cfDNA fragmentation in melanoma patients.

1. Introduction

The hypotheses on the origin of cell-free DNA (cfDNA) are still controversial and details on the exact mechanisms of release are not completely disclosed [1].

Previous studies demonstrated an increase of circulating cfDNA in different types of cancer (see Ref. [2] for a review) in comparison to healthy population. In affected patients, DNA concentration in plasma can be influenced by tumor stage, size and location [3].

However, these values may also be altered in patients with various diseases such as trauma, stroke, burns, sepsis, and autoimmune diseases, thus limiting their value for diagnosis of cancer [1].

Therefore quantitative analyses limited to cfDNA concentration cannot provide the expected clinical specificity, unless combined with qualitative alterations of DNA, such as mutations, loss of heterozigosity (LOH), microsatellite instability and epigenetic changes [3]. The detection of these biomarkers entails sophisticated techniques due to their scarcity in plasma if compared to wild type sequences and the high sensitivity requested for their detection makes these approaches challenging for immediate clinical applications.

Circulating cfDNA is released from apoptotic or necrotic cells, reflecting a differential DNA origin, as well as from living cells through a mechanism of active release [1,4]. Necrosis is common in solid malignant tumors and generates a spectrum of DNA fragments with variable size, due to random digestion by DNases. In contrast, cell death in normal blood nucleated cells occurs mostly via apoptosis that generates small and uniform DNA fragments. In support to this hypothesis, recent studies demonstrated increased DNA length in plasma from patients with breast [5] and gynecologic cancers in comparison to healthy subjects [6]. Moreover, DNA characterized by higher integrity could be evidenced in cancer of the head and neck [7], prostate [8], kidney [9,10], esophagus [11], nasopharynx [12] and colon [13]. The test is based on the hypothesis that DNA fragments in plasma of cancer patients are longer than those detectable in healthy individuals.

Nonetheless, a limited number of studies reported decreased DNA integrity in cancer patients compared to the healthy population [14–16] or were unable to demonstrate a difference between cancer and non-cancer patients [17,18]. This could be due to distinctive features of different cancer types or to preanalytical and analytical factors [3].

Our attention was focused on the qualitative and quantitative characterization of cfDNA in patients with cutaneous melanoma.

We adopted a quantitative real time PCR (qPCR) approach based on the measurement of amplicons of increasing length to determine

Abbreviations: cfDNA, cell-free DNA; qPCR, quantitative real time PCR.

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three different integrity indexes, able to reflect plasma cfDNA fragmentation.

The method was evaluated by comparing the integrity indexes in healthy subjects and melanoma patients as well as in pre and post surgical samples from the same patient.

2. Material and methods

2.1. Patients

Seventy-nine consecutive patients treated at the Department of Dermatological Sciences of University of Florence, were evaluated for plasma DNA integrity. Blood samples were collected before primary tumor resection or, for metastatic patients, before lymph node or subcutaneous metastases excision.

The group included patients undergoing surgery for in situ melanoma (n = 12) (median: 59.5 range: 39–80 yrs), primary invasive melanoma (n = 57) (median: 67 range: 23–88) and metastatic melanomas (n = 10) (median: 48.5 range: 28–94). In addition, 34 healthy subjects (median: 59 range: 29–85), who voluntarily donated their blood to be submitted to plasma DNA integrity evaluation, were chosen as the control population. The clinical pathological parameters of primary cutaneous invasive melanomas are reported in Table 1.

A subgroup of 21 patients was submitted to a second blood drawing after 15 days from surgery.

The research protocol was approved by the local ethic board and all the patients signed an informed consent.

2.2. DNA extraction

A 5 ml aliquot of peripheral blood was collected in EDTA tubes, transported within 1 h to the laboratory and centrifuged at 4 °C for 10 min (1600 rcf); supernatant was recovered and centrifuged at 14,000 rcf for 10 min at 4 °C. Plasma aliquots (600 μl) were stored at −80 °C before use. DNA was extracted from 500 μl of plasma, using the QIAamp DSP Virus Kit (Qiagen, Italy) and RNAse digestion to prevent RNA interference during assay reaction. Elution volume was 20 μl.

Table 1
Description of the clinical pathological parameters of primary cutaneous invasive melanomas (n = 57).

Parameter	Cases, n (%)
Thickness	
≤1 mm	35 (61.4)
1.01–2.0 mm	12 (21.0)
2.01–4.0 mm	7 (12.3)
>4 mm	3 (5.3)
Level	
II	12 (21.0)
III	21(36.8)
IV	24 (42.1)
Ulceration	
Absent	47 (82.5)
Present	10 (17.5)
Anatomic site	
Head and neck	4 (7.0)
Trunk	32 (56.1)
Extremities	17 (29.8)
Acral sites	3 (5.2)
Genital regions	1 (1.7)
Histotype	
Superficial spreading melanoma	48 (84.2)
Nodular melanoma	6 (12.8)
Lentigo maligna melanoma	2 (3.5)
Acral-lentiginous melanoma	2 (3.5)
Regression	
Absent	40 (70)
Present	17 (30)

2.3. Plasma DNA integrity indexes by qPCR

The quantity and integrity of the cfDNA circulating in plasma was evaluated by qPCR targeting the human APP gene (Amyloid Precursor protein, chr. 21q21.2, accession NM_000484). The assays were designed in a way that the forward primer and the probe were the same for all amplicons, whereas the reverse primer varied (see Table 2 for sequences). The length of the amplicons were 67, 180, 306 and 476 bp respectively.

Absolute quantification of the shortest amplicon (67 bp) was performed in plasma samples to accurately measure the amount of free circulating DNA per ml plasma [19]. This assay was assumed to be able to measure the total amount of circulating plasma DNA, including fragments down to 67 bp in length. Quantification of DNA concentration was obtained by interpolation on an external reference curve of genomic DNA ranging from 10 to 10^5 pg/reaction.

DNA extracted from a blood pool of healthy donors, measured spectrophotometrically (Nanodrop ND1000, Nanodrop, USA) and evaluated for integrity characteristics by gel electrophoresis on agarose gel was employed as the standard.

All measurements were performed from a 500 μl-aliquot of patient's plasma.

qPCR assays were run in the 7900HT Fast Instrument (Applied Biosystems). Thermal cycling setting was performed in order to achieve a comparable qPCR efficiency among the four different amplicons. In particular thermal conditions included a denaturation step at 95 °C for 10 min and 50 cycles of PCR as follows: i) 15 s at 95 °C and 60 s at 60 °C for both 67 and 180 bps amplicons, ii) 15 s at 95 °C, 60 s at 56 °C and 60 s at 72 °C for the longer amplicons of 306 and 476 bps. All the measurements were performed in triplicate on 1 μl of DNA.

Each integrity index was calculated as the ratio between the absolute concentration of the longer amplicons 180 bp, 306 bp and 476 bp and the total cf DNA amount (amplicon 67 bp).

Alternatively the results were used to evaluate the fraction of plasma DNA fragments with length varying from 67 to 180 bp, from 181 to 306 bp, from 307 to 476 by subtracting the absolute concentration of the longer fragment to that of the shorter one. These results were normalized on the total cfDNA amount and expressed as a percentage of it.

2.4. Statistical analysis

Statistical analysis was carried out using the SPSS software package 17.0 (SPSS, Chicago, USA). Statistical differences between quantitative data were evaluated by Student's *t* test for unpaired or paired samples. A p value lower than 0.05 was considered statistically significant. All the results were reported as mean ± standard error (mean ± S.E.).

3. Results

3.1. qPCR efficiency for size specific amplicons

The efficiency each qPCR assay was evaluated in 5 different runs using five ten-fold dilutions of genomic DNA, ranging from 100 ng to 10 pg. For the 67 bp amplicon the slope = −3.37 ± 0.06 (mean efficiency = 98%) and Y-intercept = 38.25 ± 1.8, with coefficient of correlation always higher than 0.99. Analogously the analysis was repeated for the other amplicons (Fig. 1) obtaining a perfect parallelism.

Table 2
Primers and probe sequences.

Amplicon length	Hydrolysis probe (5′-FAM, 3′-TAMRA)	Forward primer (5′–3′)	Reverse primers (5′–3′)
67 bp	ACCCCAGAGGAGCGCCACCTG	TCAGGTTGACGCCGCTGT	TTCGTAGCCGTTCTGCTGC
180 bp			TCTATAAATGGACACCGATGGGTAGT
306 bp			GAGAGATAGAATACATTACTGATGTGTGGAT
476 bp			TAAAGTAGGACTTAATTGGGTCACAAAC

The assays showed a sensitivity suitable to the measurement of cfDNA using a sample volume equivalent to 25 µl of plasma.

3.2. Plasma cfDNA concentration and fragment-size composition in pre-surgery melanoma and control samples

cfDNA concentrations (mean ± SE; ng/ml plasma) in plasma of healthy subjects are reported in Table 3. The results show a decrease of cfDNA concentrations as the amplicon dimensions increase in both healthy and melanoma subjects (Fig. 2A).

Moreover, for each amplicon we found a statistically significant difference between healthy individuals and patients, with constant higher values in the melanoma group (Table 3).

Analyzing the integrity indexes 180/67, 306/67 and 476/67, invariably higher values were found in melanoma patients than in control subjects (Table 3), (Fig. 2B). Statistically significant differences can be noticed for all the considered ratios (Table 3).

Within the group of invasive melanomas, no significant correlation was found between each DNA integrity index and clinical–pathological parameters, such as Breslow thickness, Clark level, presence of ulceration, anatomic site, histotype and regression (data not shown).

Calculating the amount of fragments whose dimension is comprised between each amplicon sizes (see Section 2.3), we were able to evidence that around 50% of the total cfDNA composition in healthy subjects is related to the presence of fragments of 67–180 bp of length. On the contrary, almost 50% of DNA fragments encountered in melanoma patients before surgery were comprised between 181 and 306. Both DNA fragment categories showed concentrations significantly different in the two groups ($p<0.0001$ and $p=0.039$, respectively). On the contrary, the percentage of fragments between 307 and 476 bp (20% in melanoma patients) was not statistically different between the two groups (Fig. 2C).

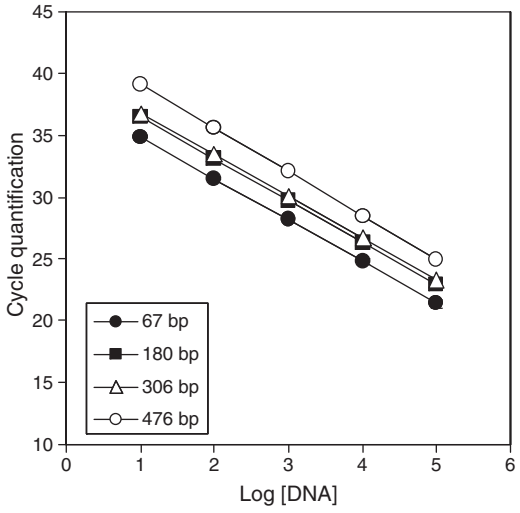

Fig. 1. Standard curve plots for the qPCR assays targeting 67, 180, 306, 476 bp amplicons. Quantification cycle plotted versus log DNA concentration.

3.3. Plasma DNA integrity in post surgery blood samples

We studied total plasma DNA concentration 15 days after melanoma resection in a subgroup of 21 patients (2 in situ, 17 invasive and 2 metastatic melanomas).

No statistical differences were evidenced between pre-surgery (9.6 ± 2.5, 8.6 ± 2.7, 3.0 ± 0.5 respectively for the 67, 180 and 306 bp amplicon) and post-surgery (10.8 ± 0.9, 4.0 ± 0.4, 2.4 ± 0.3 respectively for the 67, 180 and 306 bp amplicon) total cfDNA concentration, with the exception of the longest amplicon resulting 1.2 ± 0.3 pre-surgery and 0.4 ± 0.1 post-surgery ($p=0.025$).

A reduction of all the considered integrity indexes could be noticed after 15 days from tumor removal ($p<0.05$). In fact, integrity index 180/67 resulted 0.8 ± 0.1 pre-surgery and was reduced to 0.4 ± 0.05 after 15 days ($p=0.003$). Similarly, the index 306/67 showed a reduction of about 50% (from 0.4 ± 0.06 to 0.2 ± 0.03; $p=0.011$) and the index 476/67 was even more strongly reduced (80% mean reduction; $p=0.012$) changing from 0.2 ± 0.06 to 0.05 ± 0.01 (Fig. 3A).

The percentage of fragments between 67 and 180 bp was significantly enhanced 15 days after surgery (60.5 ± 5.6% versus 37.0 ± 5.8, $p=0.004$). On the contrary, the percentage of fragments between 180 and 306 bp was significantly reduced after surgery (23.6 ± 5.2% vs 65.7 ± 12.5%, $p=0.016$).

A non statistically significant decrease in the percentage of fragments between 307 and 476 bp was observed after surgery (45.5 ± 18.9% versus 22.5 ± 3.3, $p=0.259$) (Fig. 3B).

3.4. Clinical sensitivity and specificity

ROC curve analysis was used to assess the performances of our evaluation of DNA integrity in melanoma patients and controls. The area under the ROC curves for melanoma versus healthy patients, the clinical sensitivities and specificities at chosen cut-off values are reported in Table 4 for each integrity index. By evaluating the case study in terms of positivity/negativity on the basis of the chosen cut-offs, no integrity index is capable by itself to identify more than 68% of melanoma patients, while combining the 3 parameters we can reach a higher sensitivity. In fact 74/79 melanoma patients result above the reported cut-off for at least one parameter, with a total sensitivity of 93.7%.

Table 3
cfDNA concentration and integrity indexes (mean ± SE) with corresponding p values.

Amplicon size	Melanoma patients (n = 67)	Controls (n = 34)	Probability (P)
67 bp	12.4 ± 1..4	8.2 ± 0.9	0.015
180 bp	9.1 ± 0.9	4.4 ± 0.7	<0.001
306 bp	2.9 ± 0.3	1.8 ± 0.2	0.001
476 bp	1.9 ± 0.2	1.0 ± 0.2	0.006
180/67 bp ratio	0.8 ± 0.05	0.5 ± 0.04	<0.001
306/67 bp ratio	0.3 ± 0.03	0.2 ± 0.02	0.008
476/67 bp ratio	0.2 ± 0.03	0.1 ± 0.01	0.002

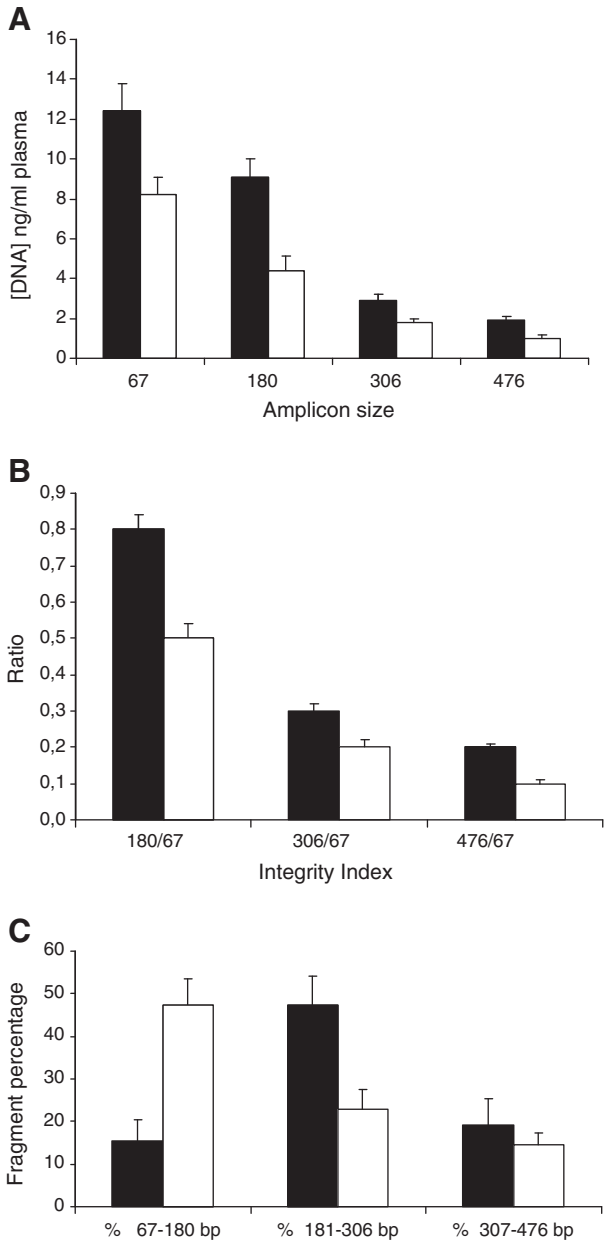

Fig. 2. cfDNA concentration (A), integrity indexes (B) and percentages of DNA fragments of a defined length range (C) in melanoma patients (n = 79, black columns) and healthy subjects (n = 34, white columns). Data are reported as mean ± S.E.

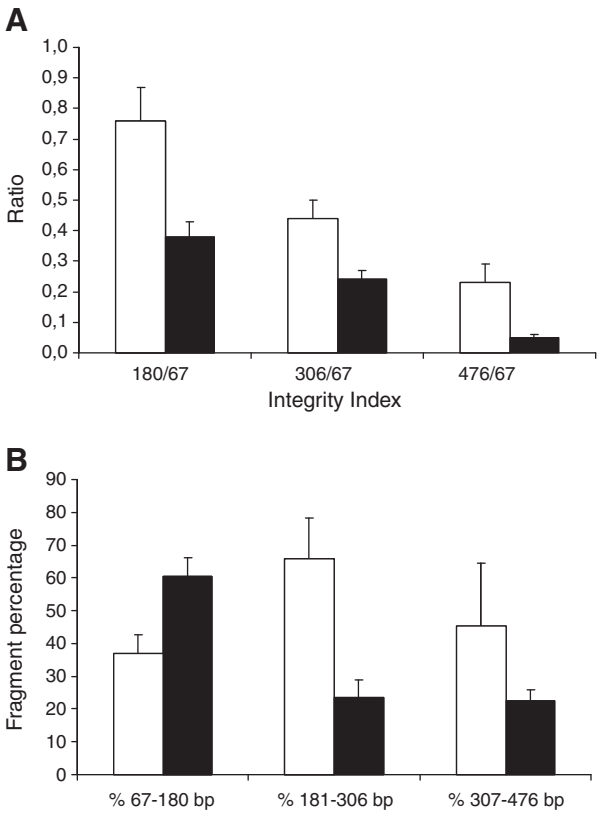

Fig. 3. (A) Integrity indexes (mean ± S.E.) in melanoma patients (n = 21) pre-surgery (white columns) and 15 days after surgery (black columns). (B) Plasma cell-free DNA fragment composition. Percentages of DNA fragments of a defined length range pre-surgery (white columns) and 15 days after surgery (black columns).

4. Discussion

The aim of the present work was to study cfDNA integrity in cutaneous melanoma, so far not investigated about this aspect.

The study was directed to the quantitative determination of circulating DNA by means of four qPCR assays differing for the amplicon length (67, 180, 306 and 476 bp respectively).

Using this approach we confirmed the potential diagnostic ability of the cfDNA circulating in plasma to discriminate cancer patients from healthy control subjects. Melanoma patients had higher levels of cfDNA than healthy subjects (independently from amplicon length), similarly to already published results for different types of cancer [2,3].

A statistically significant increase could be evidenced in melanoma patients when compared to healthy subjects for the three integrity indexes calculated in this study (180/67, 306/67 and 476/67), in agreement to already reported data. In fact, notwithstanding a great heterogeneity in the pre analytical and analytical steps, most of the papers, based on qPCR approaches to measure DNA fragmentation in plasma, report an increase of integrity index in tumor patients in comparison to the healthy population [5–7,11,13].

The construction of a ROC curve allowed the identification of the ratio 180/67 as the most suitable index to be used in melanoma patient selection. Nevertheless, the combination of the 3 indexes (180/67, 306/67 and 476/67) gives the best performance in terms of clinical sensitivity.

On the basis of our results the most represented fragments in plasma of melanoma patients are those comprised between 181 and 306 bp, while in healthy subjects there is a prevalence of shorter fragments (67–180 bp). It is generally accepted that the 180 bp-fragment reflects apoptosis, which is the prevalent mechanism of cell death in normal cells, while necrosis, producing much longer DNA fragments, seems to occur more frequently in tumor cells [20,21].

Post-surgical melanoma samples show a higher percentage of small DNA fragments (67–180 bp) together with a lower percentage of long DNA fragments (181–306 bp) in comparison to the pre-surgery condition.

In conclusion, DNA integrity indexes resulted suitable parameters for monitoring cfDNA fragmentation in melanoma patients. As a perspective in melanoma patients, further studies using DNA integrity

Table 4
Area under ROC curves and clinical specificity/sensitivity for the 3 integrity indexes.

Integrity index	Area under the curve	Cut-off	Sensitivity	Specificity
180/67	0.75	0.625	68%	72%
306/67	0.56	0.175	68%	40%
476/67	0.59	0.105	62%	56%

index to monitor patients' outcome and the effect of therapy are advisable.

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