Detection of Plasma Tumor DNA in Head and Neck Squamous Cell Carcinoma by Microsatellite Typing and p53 Mutation Analysis

Florence Coulet, Helene Blons, Arnaud Cabelguenne, Thierry Lecomte, Olivier Laccourreye, Daniel Brasnu, Philippe Beause, Jessica Zucman, and Pierre Laurent-Puig

ABSTRACT
Recent arguments have suggested that tumor DNA in cancer patients could be found in plasma, but different points remain unclear. Using a series of 117 head and neck squamous cell carcinoma tumors, our goals for this study were: (a) to quantify the amount of plasma DNA; (b) to evaluate the presence of plasma tumor DNA; and (c) to analyze the clinical relevance of tests based on plasma DNA analyses. Low levels of plasma DNA were found in most samples, but all were successfully amplified. Two different methods were used to detect tumor-specific genetic alterations: (a) microsatellite instability assay at UT5085 with an established sensitivity of 1:500; and (b) p53 mutation screening. Of the 117 tumors typed at UT5085, 65 demonstrated bandshifts (55%). Plasma and tumor DNA showed similar alteration in only one case among these samples, and the prevalence of tumor DNA in plasma was estimated to be <2% using microsatellite analysis. Tumor DNA was detected in plasma at a higher prevalence of 11% (2 of 18 cases) when using p53 mutant allele-specific amplification. These results showed that in plasma, tumor DNA is largely diluted by normal DNA. By comparison with previously published studies, the prevalence of microsatellite alterations in plasma in this series of head and neck squamous cell carcinomas is very low, despite the fact that a large series of tumors was analyzed. To explain this discrepancy, we analyzed the possibility of PCR artifacts as suspected by the presence of loss of heterozygosity in two plasma DNA samples with a similar tumor DNA alteration. When DNA concentrations were under the threshold of detection (<100 ng/ml), we demonstrated that PCR artifacts could occur at random, and, if misinterpreted, these false genetic alterations could artificially enhance the frequency of plasma DNA alterations. This may have been suspected in previously published series, but it has never been discussed before. Microsatellite analysis on plasma DNA is difficult to interpret and can frequently be misleading. Plasma DNA should be analyzed with very sensitive and specific methods such as mutant allele-specific amplification, which excludes artifacts but requires specific optimization that is probably not compatible with routine and clinical use.

INTRODUCTION
Recently, arguments suggested that in cancer patients, tumor DNA could be found in plasma and could stand as evidence for the presence of a tumor (1–3). Testing this suggestion could be an interesting and seemed easy to undertake by the development of molecular analysis. Different detection methods have been used, such as microsatellite typing to show the presence of LOH1 or MSI and specific mutation analysis (p53 or K-ras), and more recent methods have used detection of aberrant methylation of the p16 gene promoter (4–12).

However, two issues need to be addressed. The first one concerns the technical aspects of plasma DNA analyses, and the second one concerns the clinical relevance of such an approach in the management of the cancer patients. Indeed, discrepancies have appeared between studies. In some series, LOH or MSI in plasma indicated the presence of a large excess of tumor DNA (4, 6, 7), and in others, the necessity of developing very sensitive mutation detection methods tends to prove that tumor DNA is largely diluted in normal DNA (8, 9). In several studies, LOH or MSI was observed in plasma DNA but not in corresponding tumor DNA, without any clear or well-stated explanations. This fact needs to be investigated because obviously the long-term goal of such research is to analyze serum or plasma samples in the absence of tumor DNA. Furthermore, tumors that were screened previously were not large enough to allow clinical evaluation of plasma DNA analyses. To find answers to these questions, a large series of head and neck cancer patients was screened for DNA plasma alterations. Tumor-specific serum markers could contribute with the clinical evaluation to the follow-up of patients; therefore, it seemed interesting to look for tumor DNA in the plasma of head and neck cancer patients (6). In this work, plasma DNA was screened using microsatellite marker UT5085, which demonstrates a high rate of instability. This test was chosen to enhance efficiency by comparison with LOH analysis and allowed rapid tumor screening. Different arguments in this series as well as in previously published studies have suggested the existence of PCR artifacts in plasma amplified microsatellites. This last point was analyzed by studying the influence of PCR conditions on microsatellite patterns.

PATIENTS AND METHODS
Patients. One hundred and seventeen patients with HNSCC treated by the department of surgery of Laennec Hospital (Paris, France) were recruited in a prospective study, and informed consent was obtained from each patient. Tumors were located in the oral cavity (n = 16), the oropharynx (n = 49), the hypopharynx (n = 32), and the larynx (n = 20). Tumors were grouped according to the Tumor-Node-Metastasis (TNM) classification and staged as recommended by the American Joint Committee on Cancer (13). The initial tumor staging was as follows: (a) 6 stage I patients (3%); (b) 21 stage II patients (18%); (c) 30 stage III patients (26%); and (d) 62 stage 4 patients (53%).

Sample Preparation and Storage. Sample collection and storage were done according to standard methods. Tumor tissues and 10 ml of peripheral blood were collected at the time of initial diagnosis, during endoscopy under general anesthesia. Tumors were immediately snap-frozen in liquid nitrogen, and blood samples in EDTA tubes were centrifuged for 10 min at 3000 × g to separate buffy coats and plasma. Buffy coats were stored, whereas plasma was carefully removed from the EDTA-containing tubes, transferred into propylene tubes for a second centrifugation under identical conditions, and stored in fresh tubes. All samples were stored at −80°C until further processing.

The presence of neoplastic tissue was confirmed by histopathological analysis before DNA extraction. All samples were diagnosed as invasive squamous cell carcinomas. Tissue DNA was extracted using a standard method, and matching lymphocyte DNA was extracted using the WIZARD extraction kit (Promega, Charbonnières, France).

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Plasma DNA Extraction. DNA was extracted from plasma by using the QIAamp Blood Kit (Qiagen, Courtaboeuf, France) according to the blood and body fluid protocol recommended by the manufacturer. One ml of plasma was treated, and a DNA elution volume of 50 µl was obtained per extraction. The extraction yield has been established to 70% using dilutions of known quantities of SW480 cell line DNA (2, 20, and 200 ng/ml); this result is in accordance with previously published data (14).

DNA Quantification. Quantification of plasma DNA was performed for all samples on a volume of 3 µl by fluorescence emission after intercalation of Hoechst dye. The fluorescence was read by a DyNa Quant 200 fluorometer, using the cuve and capillary DyNa Quant Capillary Cuvette Adaptor Kit (Pharmacia Biotech, Orsay, France). The threshold of DNA detection established by the manufacturer is 2 ng/µl, which corresponds to 100 ng/ml plasma in our extraction protocol.

Microsatellite Analysis. Microsatellite UT5085 was amplified with primers referenced in the GenBank sequence database (accession number GDB309286): (a) UT5085A, 5'-AAAGGGGATAAGGCGAC-3'; and (b) UT5085B, 5'-AGATGACAACTACGAC-3'. Amplification was performed in a 50-µl reaction volume with 1.25 units of AmpliTaq Gold polymerase Cetus (PE Applied Biosystems, Inc., Les Ulis, France), 200 mM deoxynucleotide triphosphate, 7.5 pmol of each forward and reverse primer, 1.5 mM MgCl₂, and 5 µl of 10× AmpliTaq buffer. A total of 50 ng of DNA was used as a template for tumors and lymphocytes. In the case of plasma, 5 µl of the DNA elution volume obtained after Qiagen extraction was used as a template. The mixes were denatured for 12 min at 95°C followed by cycles of 95°C for 30 s, 54°C for 45 s, and 72°C for 90 s. A final elongation was performed for 10 min at 72°C. The number of cycles performed was 35 for tumor and lymphocyte and 45 for plasma. Amplified products diluted in a 1:3 ratio with loading buffer were run on 6% polyacrylamide sequencing gels [7M urea, 8% acrylamide gel and visualized after ethidium bromide staining.

To demonstrate the existence of DNA in plasma at concentrations lower than the threshold value of 2 ng/µl, an amplification of the HLA locus was performed with the following primers: (a) GH26, GTTGGTCAGGTTGTA- AACTTGATACCAG; and (b) GH27, CACGGGTCCGGTACGCAGCTT- GAGTT. The PCR protocol was similar to that used with UT5085 for plasma DNA.

Microsatellite Typing on an Automated Sequencer. This method was used to analyze the influence of PCR conditions on microsatellite patterns using normal DNA as a template. Four microsatellite markers were selected for this analysis (D13S1293, D13S1248, D13S152, and D13S1319). Amplifications were run with sets of primers, one of which was labeled with a fluorescent dye and different amounts of DNA (40, 20, 10, 4, 2, 1, 0.5, 0.25, 0.125, and 0.065 ng). Amplifications were performed with AmpliTaq polymerase Cetus (PE Applied Biosystems, Inc.) according to the manufacturer’s instructions. PCR products were detected by laser fluorescence on ABI373 (PE Applied Biosystems, Inc.). Data were analyzed by Genescan analysis and genotyper software (PE Applied Biosystems, Inc.).

TP53 Mutation Detection in Tumor DNA. Exons 5–8 were screened for mutations using denaturing gradient gel electrophoresis in accordance with the method described by Hamelin (15). Tumors showing an electrophoretic variant pattern were amplified and sequenced for each variant exon. PCR products were purified with QIAquick PCR Purification Kit (Qiagen) and sequenced on both strands using an ABI 310 genetic analyzer (Applied Biosystems, Inc., Courtaboeuf, France). We used the Big Dye Terminator sequencing kit (PE Applied Biosystems) according to the manufacturer’s instructions. Sequences were analyzed by Sequence Analysis 3.0 software (PE Applied Biosystems).

RESULTS

A series of 117 patients with primary HNSCC were included in this study. Plasma DNA concentrations were quantified by fluorimetry for all 117 patients. DNA concentrations were measurable in 41 patients (35%; >2 ng/ml, corresponding to DNA concentrations over 100 ng/ml plasma) and ranged from 100–688 ng/ml. No significant correlation was observed between gender, tumor stage, localization, and plasma DNA concentration (Table 2). An amplification test was performed for all plasma on the HLA locus using 5 µl of the elution volume of the Qiagen column as DNA template, although the DNA concentration was below the threshold of detection. Amplification products were visualized in all cases by ethidium bromide staining, showing the presence of DNA in all samples.

Polymeric tetranucleotide microsatellite UT5085 was analyzed in tumor, lymphocyte, and plasma DNA by PCR. The threshold of

<table>
<thead>
<tr>
<th>Exon</th>
<th>Codon</th>
<th>wt*</th>
<th>Mut</th>
<th>Primer sense 5’-3’</th>
<th>Primer antisense 5’-3’</th>
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<tr>
<td>5</td>
<td>175</td>
<td>CCG</td>
<td>CAC</td>
<td>GCTCATGTGGGGGCAGT</td>
<td>CACTGTGACGCTGTGTTT</td>
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<tr>
<td>6</td>
<td>193</td>
<td>CAT</td>
<td>CTT</td>
<td>CTGGCCTCTCTCAGCC</td>
<td>CACTGACAAACACCCTTA</td>
</tr>
<tr>
<td>6</td>
<td>205</td>
<td>TAT</td>
<td>TGT</td>
<td>GAAATTTGCTGTGGAGTG</td>
<td>CACTGACAAACACCCTTA</td>
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<tr>
<td>7</td>
<td>242</td>
<td>TGC</td>
<td>TCG</td>
<td>CTACATGGTACACACACCTT</td>
<td>TGTCAGGAGGTGGCAAGT</td>
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<tr>
<td>7</td>
<td>247</td>
<td>CGG</td>
<td>TGA</td>
<td>GCATGGGGGCATGACCT =</td>
<td>TGTCAGGAGGTGGCAAGT</td>
</tr>
<tr>
<td>7</td>
<td>248</td>
<td>CGG</td>
<td>CAG</td>
<td>CATGGGGGCATGACCTA =</td>
<td>TGTCAGGAGGTGGCAAGT</td>
</tr>
<tr>
<td>7</td>
<td>273</td>
<td>CGT</td>
<td>TGT</td>
<td>CGGACACGGTTTAGGCTT</td>
<td>CTGCTTATGTCCTCCCTG</td>
</tr>
</tbody>
</table>

*a wt, wild-type codon; Mut, mutated codon.

Table 2 Relationship between plasma DNA concentration and gender, stage, and localization

<table>
<thead>
<tr>
<th>Gender</th>
<th>M</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>69 (91%)</td>
<td>28 (87.5%)</td>
<td>8 (80%)</td>
</tr>
<tr>
<td>I + II</td>
<td>7 (9%)</td>
<td>3 (12.5%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>Stage</td>
<td>19 (25%)</td>
<td>5 (16%)</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>III + IV</td>
<td>57 (75%)</td>
<td>26 (84%)</td>
<td>9 (93%)</td>
</tr>
<tr>
<td>Localization</td>
<td>11 (15%)</td>
<td>4 (13%)</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>Oral cavity</td>
<td>31 (41%)</td>
<td>13 (42%)</td>
<td>5 (50%)</td>
</tr>
<tr>
<td>Oropharynx</td>
<td>20 (26%)</td>
<td>9 (29%)</td>
<td>3 (30%)</td>
</tr>
<tr>
<td>Hypopharynx</td>
<td>14 (18%)</td>
<td>5 (16%)</td>
<td>1 (10%)</td>
</tr>
</tbody>
</table>

*NS, non-significant.
bandshift detection was determined for this marker by a 32P-labeled probe, and a ratio (tumor DNA:normal DNA) of 0.5% was allowed to detect tumor DNA. Microsatellite UT5085 typing showed that 65 of 117 tumors demonstrated bandshifts (55%), and the corresponding patients were selected for further analysis. The concomitant analysis of UT5085 patterns in tumor, lymphocyte, and plasma DNA from the same patient led to the following results (Fig. 1A). In 62 of 65 cases, plasma patterns were comparable to lymphocyte patterns; in 1 case, the plasma pattern showed a shift similar to the tumor pattern (Fig. 1B); and in 2 cases, plasma DNA demonstrated LOH, but no such alteration was detected in tumor DNA (Fig. 1, C and D). In these two cases, plasma DNA could not be quantified and was therefore estimated to be <100 ng/ml plasma. The discordant results between tumor and plasma DNA could not be explained by contamination of the tumor tissue by normal cells because at least one tumor demonstrated clear LOH on other chromosome arms. Therefore, we suspect the existence of PCR artifacts.

To investigate this point, four microsatellite loci were amplified with different amounts of normal DNA (40, 20, 10, 4, 2, 1, 0.5, 0.25, 0.125, and 0.065 ng) as a template. Amplifications were obtained for all DNA concentrations used as shown by clear bands on 1% agarose gels. As shown in Fig. 2, allelic imbalance appeared in PCR products run with 1 ng of DNA, and complete allelic losses were observed for ≤0.25 ng of DNA (Fig. 2, A–C). In the last part of this study, the characterization of plasma DNA was done in a subgroup of samples, using a method with enhanced sensitivity. Eleven patients with tumors demonstrating eight different p53 mutations were selected for plasma analysis. We searched for mutations in plasma by a mutant allele-specific amplification method adapted to each type of mutation. The method was shown to be highly sensitive because 1 mutant allele could be detected among 10,000 wild-type alleles. In 11 plasma samples tested, two p53 mutations were retrieved, demonstrating the existence, in a few cases, of tumor DNA in plasma collected from cancer patients.

DISCUSSION

It has been suggested in different series of the literature that cancer patient plasma may contain a large excess of tumor DNA, but it may not be so evident. Several groups looked for MSI or LOH in plasma collected from patients with different cancer types (Refs. 4, 6, 8, and 17; Table 3). In this series, plasma DNA concentrations were studied in 117 untreated primary HNSCC patients. The results demonstrated that in the vast majority of cases, plasma DNA concentrations were <100 ng/ml (n = 76), whereas 41 cases had plasma DNA concentrations of >100 ng/ml. This represents low levels of circulating DNA, although >50% of tumors were high stages (stage III and stage IV). As far as is possible to compare plasma DNA concentrations between different series, this result seems comparable to those published previously (2, 6). No correlation was seen between tumor stage and the level of plasma DNA. It is known that the choice of microsatellite markers can influence results, depending on the abilities of markers to exhibit shifts or LOH. To analyze all tumors under the same condition, a single tetranucleotide microsatellite UT5085 that
was particularly prone to replication errors was selected for plasma analysis, and it allowed the screening of 55% of the tumors. Surprisingly, only one patient (1.5%) showed a similar pattern in both tumor and plasma DNA, which differs greatly from the percentage of 26% observed by Nawroz et al. (6). The sensitivity of our method has been tested and has shown that a 0.5% tumor: normal ratio could be detected. These data are in accordance with the usual sensitivity level described in the literature for microsatellite shift detection (18). This first result led to the conclusion that plasma tumor DNA, if present in head and neck cancer patients, is largely diluted in normal DNA. In previously published series, the finding of LOH in plasma suggested, on the contrary, that tumor DNA is enriched in plasma DNA (4, 6, 7, 17). Tumor type is not likely to be the only explanation for this discrepancy. In plasma DNA studies, arguments suggesting the existence of PCR artifacts, probably due to low DNA concentrations, and leading to overinterpretations of LOH have to be put forward. Indeed in some cases, LOHs are found in plasma, but the alteration is not found in tumor tissue (as reported for two samples in our series). When this was the case, the alteration in plasma was always interpreted as true LOH (Refs. 4 and 7; Table 3), and the discordance between plasma and tumor was discussed using arguments that failed to arrive at a totally satisfactory conclusion. The different possible explanations forwarded included: (a) the characterization of LOH in the tumor failed due to excessive contamination of tumor tissue by normal cells; or (b) a subclone of tumor cells with the alteration could release DNA in the plasma at higher concentrations compared to other clones in the tumor. The possibility of technical artifacts was not even discussed by the authors. Another point that needs to be discussed is the fact that for some tumors exhibiting LOH and MSI, LOH was retrieved in plasma, whereas instabilities were not. This is inexplicable according to the sensitivities of both methods, and, again, it suggests that LOH in the plasma could occasionally be due to artifacts (Ref. 7; Table 3). Finally, most LOHs in plasma that are shown in studies appeared as total allelic losses, suggesting a very large excess of tumor plasma DNA (4, 6, 7). Different arguments are in favor of a low ratio of tumor DNA normal DNA in plasma: (a) the impossibility of detecting gene mutations by standard methods; (b) the necessity of developing highly specific and sensitive techniques (5, 8); and (c) the finding that plasma DNA concentrations, in healthy individuals, range up to 100 ng/ml (2, 3).

Low quantities of DNA used as template in PCR reactions have been linked to unreliable genotyping (19), even using nested PCR. To explore the existence of PCR artifacts leading to false LOH in plasma, we tested the influence of different quantities of DNA on dinucleotide microsatellite profiles on the ABI373. This work demonstrated that allelic imbalance and total LOH appeared in normal DNA when amplifications were run with DNA quantities < 2 ng as template. The exact amount of DNA used in each PCR was never clearly specified in the different published series, and levels of DNA around 2 ng by PCR reactions may have sometimes been used to perform the amplifications. Furthermore, the use of microsatellite sequences located in chromosome regions frequently deleted in tumors reinforced the risk of coincidental similar allelic loss in tumor and plasma DNA. The repetition of the PCR does not render it safe from such an artifact because it was reproducible in our case (data not shown). It is worth noting that nonquantifiable plasma DNAs could always be amplified; however, this is not synonymous with a reliable genotyping.

The observation of the low ratio of tumor DNA: normal DNA has already been suggested by others (5, 8, 9). Our mutation analysis was performed using a high sensitivity mutant allele-specific amplification method, and 2 of 11 plasma samples were shown to contain tumor DNA. This method, which allows the detection of 1 mutated copy among 10,000 copies of the wild-type allele, seems to be more efficient for the detection of tumor plasma DNA, as reported previously (8).

In conclusion, we do not deny the existence of tumor plasma DNA as demonstration was done by mutation analysis, but certainly not in large concentrations or in a large excess as compared to normal DNA, at least in head and neck cancer patients. It is necessary to emphasize that the interpretation of microsatellite profiles in plasma DNA amplified products is particularly prone to errors due to PCR artifacts. Interpretation of LOH or instability in plasma DNA needs to be carefully stated, especially when tumor cell DNA is not available for analysis. One recommendation would be that the quantities of DNA templates used in PCR amplification need to be precisely known. New approaches to detecting alterations such as methylated CpG islands in the promoter of p16 (12, 20) need to be evaluated for the detection of tumor DNA in plasma. In the case of head and neck cancer, the clinical usefulness of a test based on plasma DNA remains uncertain.

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