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Correspondence re: F. Coulet *et al.*, Detection of Plasma Tumor DNA in Head and Neck Squamous Cell Carcinoma by Microsatellite Typing and p53 Mutation Analysis. Cancer Res., *60:* 707–709, 2000.

Letter

In September of 1996, Chen *et al.* (1) and Nawroz *et al.* (2) described microsatellite alterations in plasma DNA similar to those found in DNA of primary tumors of each respective patient. Since then, numerous authors have reported this circumstance in several types of tumors. Using different markers, they have characterized the circulating DNA, assessing its correlation with tumor DNA, microsatellite changes (3–5), mutations in *TP53* and *K-ras* genes (6–9), methylation of gene promoters (10, 11), oncogene amplification (12), and gene rearrangement (13).

Recently, Coulet *et al.* (14) described an analysis of MI[1] and TP53 mutations in a series of 117 head and neck tumors. Their main findings were:

1. Although a high proportion of cases [65 (55%) of 117] exhibited microsatellite alterations (MI) in DNA from tumor compared with circulating lymphocytes, only in three cases were alterations found in plasma DNA.
2. In only one of these three cases, the same alterations were present in both plasma and tumor DNA. In two cases, LOH observed in plasma DNA was absent in tumor DNA.
3. By carrying out PCR amplification with increasing dilutions of normal DNA, they demonstrated artifactual LOH in samples with low amounts of template DNA.

Coulet *et al.* concluded that "Plasma tumor DNA is largely diluted in normal DNA, at least in head and neck cancer patients, and this low plasma DNA concentration can induce PCR artifacts that can be misinterpreted as microsatellite alterations."

We would like to comment on some discrepancies between these findings and data available in the literature and from our own experience, and propose possible alternatives to the conclusions reached by Coulet *et al.*

1. The high MI rate these authors report, 55%, with the only tetranucleotide marker utilized, has not been previously observed even when 9 and 12 different tri- and tetrarepeat markers were used concomitantly in head and neck tumors (2, 15). This suggests that some of the tumor alterations were false positives because of PCR artifacts. Some of the bands shown in Figure 1 of Coulet *et al.* are doubtful, because their mobilities were so divergent from the constitutive allele pattern. On the other hand, the low incidence of alterations in plasma DNA observed by Coulet *et al.* may have been influenced by the use of this tetranucleotide marker. One of the critical points in microsatellite analysis from plasma DNA is the selection of suitable microsatellite markers. Long microsatellites are difficult to amplify correctly (16). We have also experienced this problem (3). The marker used by Coulet *et al.* is quite long (>350 bp) and is probably not an ideal marker for plasma DNA analysis. In addition, they centrifuged the blood at 3000 × *g*, which is three times faster than usual. At such a speed, some buffy-coat cells might be lysed, which will dilute the tumor DNA in the plasma and lower the possibility of detection. This, in combination with the low amounts of DNA detected in plasma (see below), could also explain the low frequency of tumor-specific alterations in plasma DNA observed by Coulet *et al.*
2. Coulet *et al.* found only one case that matched the same alteration in tumor and plasma DNA. The amount of DNA detected in plasma was low, inasmuch as only 35% of the cases had detectable concentrations (>100 ng/ml); if their extraction protocol yielded 70%, for 1 ml of plasma they would recover in most of the samples no more than 70 ng of DNA. Because they eluted each extraction in 50 μl and used 5 μl as a template, the final DNA quantity for a 50-μl reaction would be ~7 ng. It is possible that this low plasma DNA concentration led to the disappearance of the mobility shift present in the tumor, especially if the DNA was diluted with DNA from buffy-coat cells (see above). In colon carcinomas, we have observed occasionally the absence of faint new bands in tumor samples with instability when the template concentration ranged between 6 and 10 ng, or less. Previous reports of plasma DNA analysis have stressed the presence of false microsatellite alterations using a DNA template lower than 20 ng in a 50-μl reaction volume (16, 17).
3. Changes, mainly LOH, that occur in plasma DNA but not in tumor DNA, which Coulet *et al.* suggest as artifact, are detected in a small proportion of case studies. Some authors have attempted to attribute this to heterogeneous tumor clones (1), although we agree with Coulet *et al.* that this explanation is unsatisfactory and needs to be demonstrated. Nevertheless, it is interesting to note that LOH in plasma DNA matches LOH in tumor DNA in more than 90% of the cases described, and if false LOH were a frequent and random artifact, the expected discordance rates would be higher.

In summary, plasma DNA analysis is a process under investigation and consolidation and it is necessary to know the current limitations. There is a general consensus concerning the improvement of some aspects, such as the extraction procedures and identification of the best markers for each tumor, which can optimize and introduce this molecular procedure into clinical oncology.

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[1] The abbreviations used are: MI, microsatellite instability; LOH, loss of heterozygosity.

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Reply

We would like to respond to the different remarks of Silva *et al.* to avoid specific dispute and to suggest a few guidelines in the validation of the identification of tumor plasma DNA by microsatellite typing.

We do not deny the existence of tumor plasma DNA, but we would like to underline a few points to avoid misinterpretation of results and to reinforce the potential clinical use of such markers in cancers. Concerning our work, Silva *et al.* misinterpreted Table 2, because 35% and not 9% of our patients have a DNA concentration superior to 100 ng/ml range (100–688 ng/ml). It limits the possibility of the systematic disappearance of the mobility shift of the large PCR amplicon UT5085 (1) as suggested. As underlined by Silva *et al.*, the concentration of DNA used as a template for PCR is a major point to validate these studies. The absence of precise DNA quantification in many studies leads to questionable results. An interesting paper has been published by Nunes *et al.* (2) in head and neck cancer. Plasma samples from healthy individuals and cancer patients were tested in triplicate PCR reactions. Concerning plasma DNA, allelic ratios were calculated and varied from 0.4 to 0.6 in one case and from 0.33 to 0.66 in the other. Furthermore, only 30% of their controls allowed a reproducible PCR amplification of the different microsatellite loci, and random variations in allelic ratio were observed in some cases. Our results from diluted DNA showed clearly similar variability and indicate the limits of microsatellite genotyping with a low amount of DNA. Most figures illustrating allelic losses in plasma showed larger allele losses (3–6). We believe that preferential amplification of the smaller allele in PCR reactions is amplified when small amounts of DNA are used. It leads to amplification profiles resembling large allelic loss profiles. According to Nunes *et al.*, among the 21 tumors demonstrating concordant allelic loss in tumor and plasma, 15 cases concerned large allele loss *versus* 6 cases for small allele loss. Equal proportion of losses for each allele is expected from a random distribution of allelic losses. The observed distribution (15/21) can occur by chance in $<10\%$ of the cases. Therefore, it highly suggests that a subgroup of the observed larger allele losses could be caused by PCR artifacts.

As guidelines to validate studies, we suggested considering results as discordant when genetic alterations were found only in plasma or when tumor genetic alterations were retrieved in plasma only in a subgroup of markers. Using these criteria, $\leq 50\%$ (2) of the results should not have been considered discordant. Moreover, we suggest that plasma or serum DNA be quantified systematically to avoid amplification of <10 ng of DNA, to verify the absence of distortion in the rate of allelic losses between the small and the large allele in concordant samples, to point out discordance between plasma and tumor DNA, and to analyze these cases independently.

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