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Letter to the editor

Two distinct TP53 mutations in HNSCC primary tumor: Only one circulates in the blood

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Dear Editor,

Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cancer worldwide and comprises 5,5% of all cancer incidence. Altogether intratumoral clonal heterogeneity, late stage diagnosis, local invasiveness and high rate of relapse result in a poor survival rate [1,2]. Unluckily, advances in the surgical and medical treatment of HNSCC over the past decades have not significantly improved overall diseases outcomes. The insurgence of recurrences after seemingly complete surgical resection, probably due either to the existence of pre-neoplastic processes at multiple sites in the mucosa ("field cancerization" hypothesis) or tumor cells histologically not-detected is a key feature of HNSCC [3,4]. To gap the lack of cost-effective diagnostic tools for patient's monitoring and for identifying either minimal residual disease (MRD) or early local recurrence is an urgent and unmet clinical need in HNSCC.

Somatic genomic testing on tumor DNA (tDNA) from tissue biopsy and circulating tumor DNA (ctDNA) in liquid biopsies (LB) have emerged as a promising molecular tool for diagnostic refinement, risk stratification, and therapeutic approach in a variety of cancers, including HNSCC [5,6]. Mutational analysis in HNSCC is challenging due to its high intratumoral heterogeneity. Indeed, the variant allelic frequency (VAF) is frequently very low (<1%) [7,8].

Here we reported a case in which unpaired detection between tissues

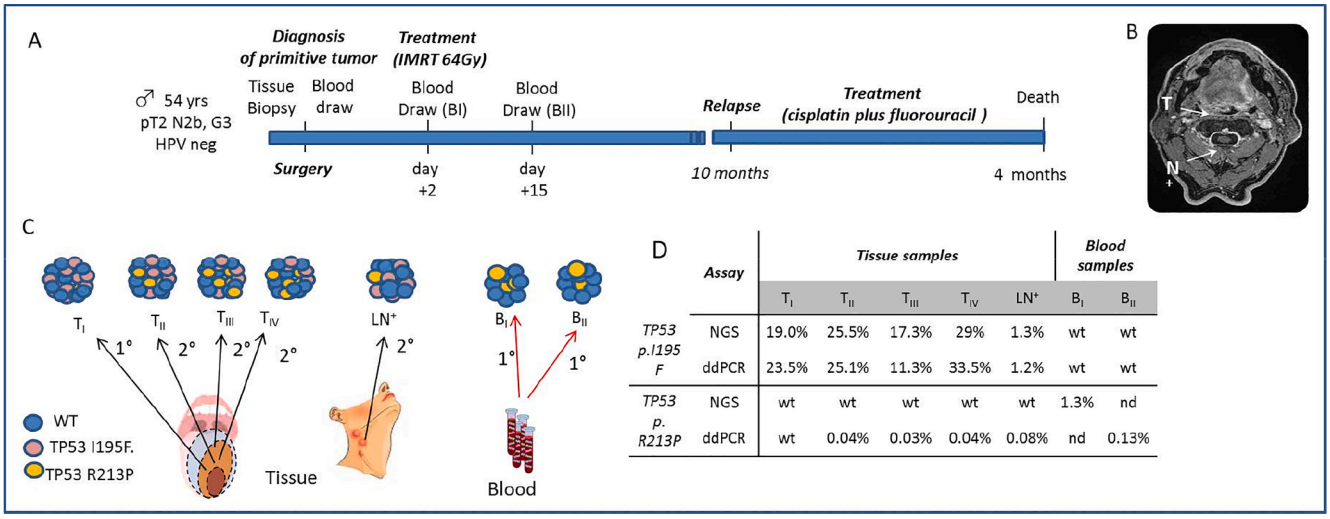

Fig. 1. Patient clinical features and molecular overview of tumor and blood samples. (A) Patient medical history timeline and clinical case presentation. (B) Representative MRI showing tumor extension and cervical lymph-nodes localization before surgery (see text). (C) A two-step analysis (1° and 2°) was applied for mutational profiling of samples (see text). All samples were analyzed by NGS and validated by ddPCR. Tumor tissues (TI-TIV) were positive for TP53 p.I195F mutation (Pink circle) and/or for TP53 p.R213P, while blood showed the TP53 p.R213P mutation (yellow circle); Tissues from histologically positive lymph node, (LN+) were positive for both mutations. (D) Longitudinal NGS and ddPCR data. VAF = variant allele frequency; WT = Wild type; nd = not performed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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and matched plasma of two distinct *TP53* mutations was deeply investigated. The aforementioned patient is a 54-year-old Caucasian men, heavy smoker with history of alcohol abuse and HPV negative, who was referred to our hospital because of a painful ulcerated lesion of 4 cm of maximum diameter of the left tongue border (DOI 8 mm at MRI), with homolateral (level IIb) multiple lymph nodes enlargement (cT2N2bM0) (Fig. 1A-B). Under local anaesthesia, an incisional biopsy was performed with histological detection of poorly differentiated squamous cell carcinoma (G3). Upfront treatment consisted in transoral hemiglossectomy with concomitant homolateral neck dissection of level I-V. The pathological examination confirmed the initial diagnosis and stage (pT2N2b).

Despite adjuvant radiotherapy (IMRT 64 Gy) was administered starting 48 days after surgery, 10 months later the patient developed an unresectable local recurrence that was treated with cisplatin (100 mg/m^2 of body-surface area on day 1) plus fluorouracil (1000 mg/m^2 per day for 4 days) every 3 weeks for 3 cycles and plus cetuximab 300 mg/m^2 per week). Plasma samples were collected for routine analysis the day before, 1 and 15 days after surgery. Four months later the patient died because of disease progression.

We aim to assess the mutational status of *TP53*, *FAT1* and *CDKN2A*, the three most frequently mutated genes in HNSCC in both primary tumoral tissue and blood tumoral cell free DNA of this patient. Next Generation Sequencing (NGS) and digital droplet PCR (ddPCR) analyses were performed. An amplicon-based method called IonAmpliSeq (Thermo Fisher Scientific) based on customized panel containing the entire coding regions of *TP53, FAT1 and CDKN2A* genes was used. ddPCR Mutation Detection Assays by QX200 ddPCR™ System (Bio-Rad Laboratories) was used. As a first step, we sequenced DNA derived from: (i) frozen primary tumor tissue (TI) and matched peritumor (histologically tumor free tissue taken at least 1 cm from primary tumor) (ii) plasma samples collected at 1 and 15 days after surgery. *TP53* p.I195F mutation was detected in frozen tumor tissue (TI) by both NGS and ddPCR analyses (Fig. 1C). Surprisingly, we found a *TP53* p.R213P mutation in cell free DNA plasma samples collected at 1 (BI) and 15 (BII) days after surgery. We hypothesized that the observed discordance between tissues and matched plasma might be due to intratumoral heterogeneity. To verify this hypothesis, we analyzed DNA derived from: (i) 3 different regions of FFPE primary tumor tissues (TII, TIII, TIV); (ii) 1 histologic positive lymph node (LN+) (Fig. 1C). The use of H&N-based NGS chip allowed identifying *TP53* p.R213P mutation, previously detected only in plasma, also in tumor tissues as well as in lymph node. This was also confirmed by ddPCR. The peritumoral tissue carries *TP53* intact gene (Fig. 1C-D).

The VAF of *TP53* p.R213P mutation (~0,04%) was significantly lower than that of p.I195F (~ 25%). This might suggest that the release of a given mutation in the bloodstream occurs irrespectively of its specific VAF. Of note, the VAF of *TP53* p.R213P detected in plasma collected 1 day after surgery and before radiotherapy (BI;VAF = 1,3%) was higher than that detected in the second blood draw (BII; VAF = 0,13%) (Fig. 1D). As BII was collected 13 days after the administration of adjuvant radiotherapy, the reduction of VAF in this sample might be attributed to the treatment.

Collectively, we found that the sequencing of the entire coding regions of *TP53*, *FAT1* and *CDKN2A* with NGS technology was capable to detect multiple mutations with very low allelic frequencies (<1%) in different tissue samples thereby contributing to decipher intratumoral heterogeneity. In addition, the approach is able to detect somatic mutations in plasma ctDNA revealing potential tumor cell spreading.

Since the high rate of recurrence is detrimental for HNSCC patient survival the use of chip-based NGS and dPCR approach to deeply evaluate tumor genotype in both tissue and plasma samples might greatly improve the tracing of preneoplastic cells. The ability of the chip-based NGS and ddPCR approach to identify mutations in the plasma, which apparently are different from those identified in the primary tumor tissue, might represent a tool to trace molecularly HNSCC intratumoral heterogeneity with prognostic and therapeutic implications.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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