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Liquid biopsy for mutational profiling of locoregional recurrent and/or metastatic head and neck squamous cell carcinoma

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ABSTRACT

Objectives: The molecular landscape of head and neck squamous cell carcinoma (HNSCC) harbors potentially actionable genomic alterations. We aimed to study the utility of liquid biopsy to (i) characterize the mutational landscape of recurrent/metastatic HNSCC using a comprehensive gene panel and (ii) estimate the concordance between DNA mutations identified from circulating tumor DNA (ctDNA) and matched tumor tissues.

Materials and methods: Targeted next-generation sequencing (NGS) was performed on cell-free DNA (cfDNA) of 39 patients with locoregional recurrent (n = 19) and/or metastatic (n = 20) HNSCC. Tumor biopsy (n = 18) was sequenced using the same technique.

Results: ctDNA was detected in 51% of patients (20/39) with a higher probability of detection in metastatic than locoregional recurrent disease (70% versus 30%, p = 0.025). 81% and 58% of the tissue tumor variants were not detected in plasma when considering all patients and only metastatic patients with detectable ctDNA, respectively. In a multivariate analysis, the likelihood of detecting the tissue tumor variant in plasma was related to metastatic status (p = 0.012), tumor variant allele frequency (p < 0.001) and ctDNA quantity (p < 0.001). 26% of the variants were detected only in liquid and not in the solid biopsy. Three patients without an available tumor sample had plasma containing three different potentially actionable *PIK3CA* mutations.

Conclusion: CtDNA detection and characterization using targeted NGS is feasible in metastatic HNSCC. Liquid biopsies do not reflect the complete mutation profile of the tumor but have the potential to identify actionable mutations when tumor biopsies are not available as well as variants not found in matched tumor tissue.

Introduction

Head and neck squamous cell carcinoma (HNSCC) represents more than 700.000 new cases per year worldwide, ranking it as the seventh most common cancer. Treatment strategies for HNSCC are still based on disease localization and tumor staging and do not incorporate molecular characteristics. Despite multimodal treatment strategies that include surgery and/or (chemo)radiation, less than 60% of patients with locally advanced disease remain free of disease at three years. Patients with recurrent and/or metastatic (R/M) disease have a poor prognosis with a median survival of between 10 and 15 months. Recently, the characterization of the molecular landscape of HNSCC has identified potential therapeutic targets, opening the door to new treatment opportunities [1]. These are currently under investigation in clinical trials [2].

HNSCC is, as other tumors, an evolutionary and heterogeneous disease [3]. The characterization of the active molecular landscape and the application of precision medicine in HNSCC thus requires to obtain fresh tumor biopsies multiple times over the course of the disease. In advanced HNSCC, obtaining sequential tumor biopsies might be

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challenging. Therefore, the ability to identify potential therapeutic targets using a liquid biopsy is an interesting alternative to tumor biopsy. Different studies have explored the feasibility of identifying somatic alterations using liquid biopsy in HNSCC. However, these were mainly single-mutation approaches and/or based on alterations previously identified in the tumor biopsy [4–8]. The use of liquid biopsy to characterize the mutational landscape of HNSCC using a large comprehensive gene panel is underexplored [9].

Using next-generation sequencing (NGS) on a custom panel of 604 genes, we investigated the feasibility of detecting ctDNA in a prospective cohort of R/M HNSCC patients using a tissue-agnostic approach (without prior knowledge of somatic mutations in the solid tumor). As a second step, we assessed how ctDNA reflects the mutational landscape of the tumor.

Materials and methods

Patient cohort and study design

The two endpoints of our study were to determine: (i) the value of targeted NGS in detecting ctDNA in two different groups of R/M HNSCC: patients with locoregional recurrent HNSCC without distant metastasis, and patients with metastatic disease with or without locoregional relapse; and (ii) the concordance of the mutational landscape between ctDNA and the matched tumor.

Patients with incurable locoregional recurrent and/or metastatic HNSCC treated with standard of care at our institution were prospectively enrolled in the UCL-ONCO-2013 trial, a non-interventional trial collecting whole blood, plasma samples and, whenever possible, tumor tissue biopsies (NCT02139020). The study was approved by our independent ethics committee and conducted in accordance with the Declaration of Helsinki (October 2000). Written informed consent was obtained for each patient. Details on sample collection and storage can be found in the supplementary data.

For this work, we selected 39 patients out of this patient cohort, of which 20 patients with metastatic disease and 19 with locoregional recurrent disease. The plasma samples used in this analysis were the baseline samples obtained at the first incurable relapse or at diagnosis of upfront incurable disease. When available (n = 18), the matching tumor tissue was also sequenced.

Targeted cfDNA sequencing

Germline DNA from whole blood, cfDNA from plasma and DNA from formalin-fixed paraffin-embedded (FFPE) tumor tissues was extracted using the corresponding extraction kit (supplementary data). For the FFPE sample, at least 40% of tumor cells was required on the hematoxylin slide to make the sample eligible for DNA extraction. Targeted next-generation sequencing (NGS) of the germline, cell-free and tumor tissue biopsy-derived DNA at a median coverage depth of 1000x was performed using a custom panel of 604 genes, covering a list of frequently mutated tumor suppressor genes and oncogenes in HNSCC (supplementary Table 1). Library preparation, sequence capture and sequencing (Illumina HiSeq®4000, paired-end 75 bp reads) were carried out by IntegraGen (Evry, France) (supplementary data).

A variant was considered if it was (i) supported by at least 10 reads, (ii) passed MuTect2 quality filters, and (iii) was present in less than 10 individuals in ascertained public sequence databases (ExAC, GoNL, 1000G). All retained variants were manually inspected for alignment. Thereafter, two additional statistical filters were applied to ascertain the somatic nature of the variant and distinguish it from background noise (supplementary data). Only the variants fulfilling these criteria were considered to be true somatic mutations.

Statistics

Tumor tissue biopsy and cfDNA samples were paired to estimate the concordance of the findings. Results were correlated with clinic-pathological features (disease status). Mann-Whitney and Fisher's exact tests were used for inferential statistics depending on the type of data, with a two-sided level of significance of 0.05.

A logistic regression model was created to analyze the relationship between the detection of a tumor tissue variant in plasma (dependent binary variable) and five independent variables (metastatic status, the variant AF in the tumor tissue, ctDNA quantity, classification of the variant as a driver or passenger based on the TCGA data [1], and the time between tumor tissue biopsy and plasma sample collection).

Digital droplet PCR (ddPCR)

Mutation detection by ddPCR (Bio-rad Laboratories, QX200™ ddPCR system, California, USA) was used to confirm results obtained by NGS (supplementary data). Data analysis was performed on QuantaSoft™ v1.7.4 software according to the manufacturer's instructions (Bio-rad Laboratories, California, USA).

Results

ctDNA detection

Targeted sequencing was performed on the cfDNA of 39 HNSCC patients; Patient characteristics are listed in supplementary Table 2. 20 had metastatic disease and 19 had locoregional recurrent HNSCC without distant metastasis. The most frequent subtype was oropharyngeal cancer (22/39, 56%) of which 5 were p16 positive (23%). In total, 285 variants were called in the cfDNA of 27 patients. Of these, 146 unique variants (51%) in 20 cfDNA samples passed our filtering criteria. ctDNA was thus detected in 20 patients out of 39 (51%) (Fig. 1). These patients carried a median of four variants (range 1–55 variants; Table 1). The plasma sample of patient HNSCC-62, who had p16-positive oropharyngeal cancer (p16 + OPC), was the only outlier, with a high mutation rate (55 variants). This patient had a history of heavy smoking (> 10 pack-years) and a somatic mutation in a mismatch-repair gene, *MSH2*, that might explain the higher mutation rate [10]. The probability of ctDNA detection was higher for patients with metastatic disease (14/20, 70%) compared to locoregional recurrent (6/19, 30%) (p = 0.025; Fig. 2). The quantity of ctDNA, estimated as

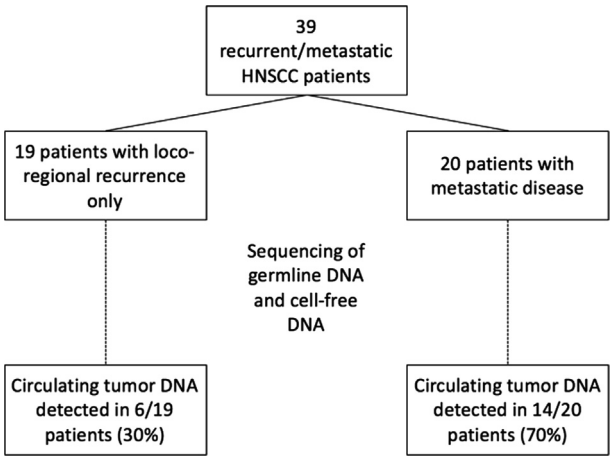


Fig. 1. Flow diagram of the study. In total, 39 patients were analyzed, 19 patients with locoregional recurrence and 20 with metastatic disease. For each patient, the germline DNA and the cell-free DNA was sequenced. Circulating tumor DNA was detected in 30% of patients with locoregional recurrence only, and in 70% of patients with metastatic disease.

Table 1
cfDNA samples with detectable ctDNA.

Patient ID	Disease status	estimated ctDNA (%)	Min AF (%)	Max AF (%)	Nb of variants
HNSCC-45	M	20.25	5.66	39.9	4
HNSCC-27	M	14.25	1.33	60.18	8
HNSCC-54	M	13.31	7.23	27.68	10
HNSCC-59	M	12.36	10.82	15.81	4
HNSCC-56	LR	5.97	2.03	11.82	6
HNSCC-73	LR	5.05	5.05	5.05	1
HNSCC-47	M	4.32	1.32	10.24	17
HNSCC-62	M	3.43	1.86	27.08	55
HNSCC-30	LR	2.87	2.87	2.87	1
HNSCC-64	M	2.46	1.31	3.19	6
HNSCC-31	LR	2.32	2.32	2.32	1
HNSCC-34	M	2.22	0.98	4.74	7
HNSCC-71	LR	2.21	1.67	2.75	2
HNSCC-39	M	2.16	2.16	2.16	1
HNSCC-68	M	1.99	1.98	4.75	3
HNSCC-38	M	1.86	0.88	2.88	5
HNSCC-44	M	1.86	1.15	3.1	4
HNSCC-51	M	1.78	1.2	2.67	4
HNSCC-60	M	1.55	1.49	1.6	2
HNSCC-67	LR	1.16	0.81	1.32	5

AF, allele frequency; cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; LR, locoregional; M, metastatic; Nb, number.

the median allele frequency (AF) of the somatic variants (passing filtering criteria) detected in the cfDNA, ranged from 1.16% to 20.25% (Table 1, Fig. 2).

Mutational landscape of ctDNA

Fig. 3 represents the somatic mutational landscape of the 20 cfDNA samples in which ctDNA was detected. Only non-synonymous mutations are shown. As expected, the most frequently mutated gene in oral cavity, hypopharynx, larynx, and p16-negative oropharyngeal (p16-OPC) cancers was *TP53* (n = 8/16; 50%). This was followed by mutations in genes implicated in the phosphoinositide 3-kinase (PI3K)-pathway, which were present in both groups (n = 3/20; 15%).

Other less frequent mutations were detected in multiple pathways that might be implicated in HNSCC oncogenesis. Mutations in different genes coding for tyrosine kinase receptors (TKR) were detected (*EGFR*, *ERBB3*, *AXL*, *CSF1R* and *RET*; each gene was mutated in one patient). Although we did not detect any *HRAS* mutation, the mitogen-activated protein kinase (MAPK) signaling pathway was altered by mutations in *MAPK1* or *NF1* (a negative regulator of RAS activation), in two other patients. Genes implicated in chromatin regulation are known to be altered in HPV-negative HNSCC [1] and in up to 33% of HPV-positive OPC [11]. In our cohort, we detected mutations in some of these genes (*KMT2C, KMT2B*, *NSD1, CREBBP, SMARCA2* and *SMARCA4*), in six patients. Mutations were detected in genes of the Notch, Hedgehog (HH) and Wnt pathways, as well as in the apoptosis-related gene CASP8, in six and one patient, respectively. Finally, four patients harbored mutations in genes implicated in DNA repair. Two mutations were seen in homologous recombination-related genes (*ARID1A, BRCA1*), and three mutations in mismatch repair genes (*MSH2, MLH1*).

Concordance of variants between liquid and solid biopsy

A matched tumor biopsy was available for 18 patients (8 locoregional recurrent and 10 metastatic). These biopsies were taken at the same time point as the sequenced plasma sample in all but two patients (HNSCC-34 and HNSCC-56, for which we used an archival biopsy).

In total, 209 variants were detected across these 18 tumor tissue biopsies (range: 4–21). In this group of 18 patients, ctDNA was detected in 12. The concordance rates were highly dependent on the ctDNA quantity harbored by the patient. Considering the 18 patients, 81% of the variants (169/209) identified in solid tumors were not detected in plasma. Conversely, 26% of the plasma variants (14/54) were not detected in the matched tumors, possibly reflecting tumor heterogeneity. Fig. 1 of the supplementary data shows the individual concordance for the 12 patients with detectable ctDNA and available paired tumor-biopsies. Considering only metastatic patients with detectable ctDNA, the rate of solid tumor variants also detected in plasma increased to 42%. For patients HNSCC-27, -54 and -59 harboring the highest ctDNA

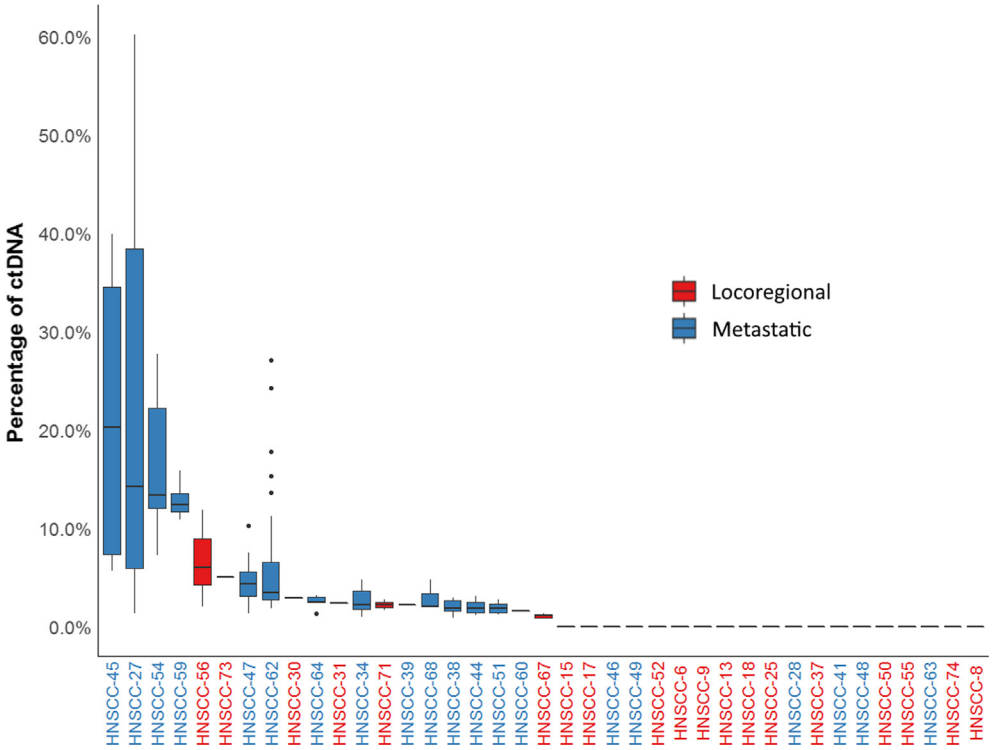
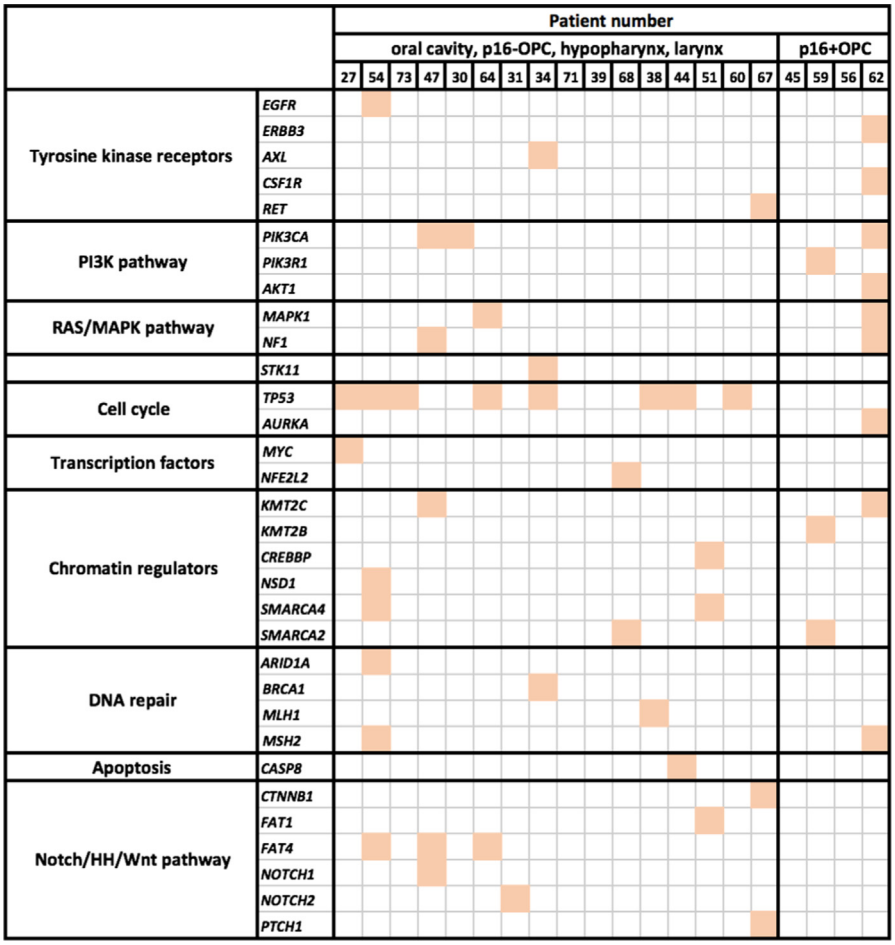


Fig. 2. ctDNA detection in 39 cfDNA samples from patients with HNSCC. Box-plot showing the allele frequencies of all the somatic plasma variants (y-axis) for each cfDNA sample (x-axis). The quantity of ctDNA was estimated as the median of the allele frequencies of all the somatic variants retained in the plasma sample (indicated by the horizontal black bar in the box plot). Patients with metastatic disease and locoregional recurrence are indicated in blue and red, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



p16- OPC, p16 negative oropharyngeal cancer; p16+ OPC, p16 positive oropharyngeal cancer; PI3K, phosphoinositide 3-kinase; RAS/MAPK, rat sarcoma/ mitogen-activated protein kinase; HH, hedgehog pathway

Fig. 3. Mutational landscape of the 20 cfDNA samples in wich ctDNA is detected. The mutational landscape of the 20 ctDNA-positive cfDNA samples is presented. Genes with potential roles in HNSCC oncogenesis that have non-synonymous mutations in at least one sample are shown, sorted by pathway.

quantities (> 10%), the amount of tumor variants detected in plasma was greater than 80%. However, for samples with a ctDNA quantity below 10%, the detection rate of tumor variants in plasma was lower (median: 14%, range: 0–55%).

In a logistic regression model, the likelihood of detecting tumor tissue variants in plasma was significantly impacted by the patient's metastatic status (odds ratio (OR) 4.50, $p = 0.012$), the variant AF in the solid biopsy (OR = 5.31, $p < 0.001$), and the quantity of ctDNA (OR = 4.13, $p < 0.001$). Classification of the variant as a driver or passenger based on The Cancer Genome Atlas (TCGA) and the Catalogue of Somatic Mutations in Cancer (COSMIC) was, however, not relevant (OR = 1.05, $p = 0.948$), nor was the concordance of time between tissue biopsy and plasma sample collection (OR = 1.39, $p = 0.7$).

Concordance of driver events between liquid and solid biopsy

We performed a specific analysis focused on potential driver events. A driver event was defined as one of the following types of somatic alterations in a gene considered to be a HNSCC oncogenesis driver by TCGA [1]: (i) missense variants or in-frame indels predicted as pathogenic by at least four types of predictive software, (ii) frameshift or nonsense variant in genes for which loss of function is expected to be pathogenic, (iii) mutations predicted to affect splicing or (iv) known COSMIC mutations. In total, 23 driver events were detected in the cfDNA and/or FFPE samples of 17 patients (Table 2).

Fig. 4 depicts the landscape of potential driver events in paired samples of both cfDNA and tumor tissue. As expected, *TP53* alterations were the most frequent driver events, both in the liquid and solid biopsies. We also detected other frequently reported driver alterations, including mutations in the PI3K pathway and one *EGFR* mutation. Forty-one percent (9/22) of solid tumor driver events were detected in plasma. After manual inspection, nine additional events that did not pass our background-noise threshold were found in plasma, underlining the limited sensitivity of the technique. Patient HNSCC-34 harbored one *TP53* mutation in the solid tumor but had a different *TP53* mutation in plasma.

Of clinical interest, the plasma of three patients for whom no tumor was available carried three different potentially actionable *PIK3CA* mutations, including the p.E545K hotspot mutation.

Lowering thresholds only revealed more background-noise

As our retrospective manual analysis revealed the presence of variants that did not pass our background-noise threshold, we performed an analysis with less stringent criteria. We lowered the cut-off for variant detection in plasma to ≥5 reads while retaining the downstream criteria. This resulted in the detection of four driver events that were not retained in our first analysis, enhancing the rate of detection of driver events to 60%. This less stringent analysis also revealed new and potentially interesting variants. We detected the somatic *ERBB22* S310Y variant (a hotspot activating mutation in breast cancer) in the

Table 2
Driver events in paired samples.

Patient	Disease status	ctDNA (%)	Gene	SNP effect	COSMIC ID	Detected in the solid tumor and cfDNA sample	AF in tumor (%)	AF in plasma (%)
HNSCC-27	M	14.25	*TP53* c.919 + 1G > C	Splice-site	COSM13585	Yes	49%	59%
HNSCC-27	M	14.25	*MYC* c.1096G > A	Non-synonymous	NA	Yes	21.8%	9.2%
HNSCC-54	M	13.31	*TP53* c.711G > A	Non-synonymous	COSM3378348	Yes	36%	11.4%
HNSCC-54	M	13.31	*EGFR* c.2584C > G	Non-synonymous	NA	Yes	34.4%	19.4%
HNSCC-59	M	12.36	*PIK3R1* c.1699A > G	Non-synonymous	COSM1154507	Yes	26%	11.9%
HNSCC-73	LR	5.05	*TP53 c.731G > T*	Non-synonymous	COSM43652	Yes	60.6%	5.1%
HNSCC-73	LR	5.05	*CDKN2A* c.243delC	Frame-shift	NA	No	62.5%	/
HNSCC-64	M	2.46	*TP53 c.892G > T*	Stop-gain	COSM10710	Yes	58.6%	2.4%
HNSCC-31	LR	2.32	*TP53* c.559 + 1G > A	Splice-site	COSM131536	No	26.9%	/
HNSCC-34	M	2.22	*TP53* c.949C > T	Stop-gain	COSM1709728	No	/	4%
HNSCC-34	M	2.22	*TP53* c.919 + 1G > C	Splice-site	COSM13585		27.3%	/
HNSCC-68	M	1.99	*TP53* c.520A > T	Non-synonymous	COSM3773316	No	37.2%	/
HNSCC-68	M	1.99	*TP53* c.993G > T	Non-synonymous	COSM1158345	No	20.6%	/
HNSCC-68	M	1.99	*MYC* c.629 T > G	Non-synonymous	NA	No	31.2%	/
HNSCC-44	M	1.86	*TP53* c.818G > A	Non-synonymous	COSM1645335	Yes	19.7%	2.3%
HNSCC-60	M	1.55	*TP53 c.844C > T*	Non-synonymous	COSM1636702	Yes	71.7%	1.6%
HNSCC-13	LR	0	*TP53* c.892G > T	Stop-gain	COSM10710	No	77%	/
HNSCC-18	LR	0	*TP53* c.581_585delTTATC	Frame-shift	NA	No	5.6%	/
HNSCC-18	LR	0	*PIK3CA* c.1034A > T	Non-synonymous	COSM94978	No	3.2%	/
HNSCC-41	M	0	*TP53* c.517G > A	Non-synonymous	COSM2744864	No	26.6%	/
HNSCC-49	M	0	*TP53* c.454C > T	Non-synonymous	COSM43582	No	31.4%	/
HNSCC-52	LR	0	*TP53* c.637C > T	Stop-gain	COSM3378350	No	51%	/
HNSCC-55	LR	0	*TP53* c.329G > T	Non-synonymous	COSM99929	No	4.5%	/

AF, allele frequency; cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; LR, locoregional; M, metastatic; SNP, single-nucleotide polymorphism.

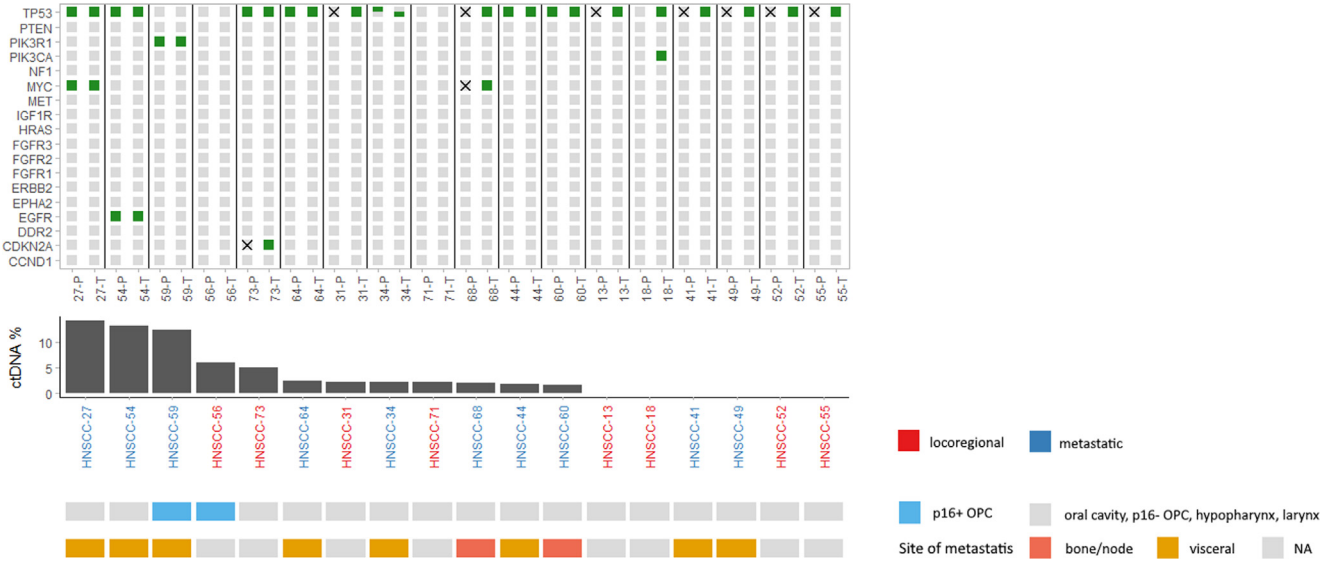


Fig. 4. Concordance of driver events between ctDNA and the matched tumor. The concordance of driver events detected in ctDNA and matched tumor tissue of 18 patients is shown according to the ctDNA quantity, with clinical characteristics indicated at the bottom. Concordant driver events are shown in green. Black crosses indicate the driver events that were not retained by our classical filters but that were detected upon manual inspection of the sequencing data. HNSCC-34 harbors two different *TP53* mutations in the solid tumor versus the cfDNA sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cfDNA of three different patients. However, we considered them as false positive findings, as the ddPCR analysis of this variant in the three plasma samples was negative.

ddPCR to detect mutations with higher sensitivity

As shown in Fig. 4 and as previously discussed, manual inspection of the data from plasma samples revealed nine driver events that were initially detected in the matched tumor sample but which failed to pass our background-noise threshold in plasma. We used ddPCR to screen these mutations in the tumor tissue biopsies and plasma samples of five patients for whom cfDNA was still available (Supplementary Table 3). All five variants were detected in the matching plasma samples, one with an AF below 0.1%. ddPCR confirmed the variants in all five of the solid tumors tested and yielded a similar AF as that obtained by NGS. Slight variations in AF may be due to spatial tumor heterogeneity as new DNA extractions from the same FFPE block were required for ddPCR testing.

Discussion

We explored the relevance of liquid biopsy to characterize the mutational landscape of R/M HNSCC using a panel of 604 cancer-related genes at deep coverage. We report ctDNA detection in 20/39 patients (51%) with a significantly higher probability for ctDNA detection in patients with metastatic disease compared to patients with

only locoregional recurrence (70% vs 30%). This finding suggests a potential limitation of this technique given that around one third of recurrent HNSCC patients will have locoregional relapse without distant metastases [12,13]. The difficulty to detect ctDNA in localized disease using a similar sequencing approach has been also shown in other tumor types [14]. In HNSCC, Wang and colleagues reported a higher rate of ctDNA detection in early stage disease [4] using a PCR-based technique with detection of allele frequencies that are below our threshold of detection with targeted NGS. In addition, PCR-based approaches are restricted to a low number of genes.

Overall, the mutational landscape characterized by ctDNA in our study reflects the mutational spectrum that has been reported in the literature by tissue tumor sequencing, with *TP53* mutations and genomic alterations in the PI3K pathway being among the most frequent events. We also detected a large amount of different mutations at low frequencies in pathways possibly implicated in HNSCC oncogenesis. These included the tyrosine kinase receptors [1,15,16], MAPK [1,15,17], chromatin regulation [1,11], Notch/HH/Wnt [1,18,19], and DNA repair pathways [20]. Surprisingly, we did not detect any *HRAS* or *CDKN2A* mutations in the cfDNA. This might be linked to the limited sensitivity of our technique and/or to low sample size. Moreover, p16-negative status is not only linked with *CDKN2A* mutations, but also with copy number variations (deletions) or epigenetic alterations, which were not analyzed in this work. Interestingly, three patients without available tumor harbored a potentially targetable *PIK3CA* mutation, supporting the diagnostic role of liquid biopsy when tumor biopsy is neither feasible nor available. Furthermore, 26% of the plasma variants were not detected in the matched tumors. This could be linked to variants originating from tumor clones that are not represented in the single tumor biopsy that has been sequenced. This last finding suggests that liquid biopsies might also complement the tumor biopsy by identifying other variants that can be missed due to tumor heterogeneity, or in the absence of tumor biopsy.

Concordance between the variants, as determined by sequencing the tumor tissue and the cfDNA, was low in our cohort, with only 19% of tumor variants detected in the corresponding cfDNA sample. In line with our results, other studies have reported low concordance rates (8.6% − 22%) for broad gene-panel testing [21–23]. Series focusing on single gene analysis (using PCR-based platforms for specific mutation detection) have shown more encouraging results with a 93% agreement rate for *RAS* status in colon cancer [24], or a 94.3% concordance rate with a sensitivity of 65.7% for the detection of *EGFR* mutation in lung cancer [25]. Testing for recurrent hotspot mutations in one specific gene with a highly sensitive amplification-based method performs better than broad panel testing but fails to capture the full molecular landscape and tumor heterogeneity. Nonetheless, higher concordance rates have also been described with more comprehensive gene panels. Using a hybrid capture-based panel of 62 genes in patients with non-small cell lung cancer, 78% of short variants from solid tumor were also detected in plasma (n = 33) [26]. This may be attributable to the high median unique exon coverage depth achieved (6873x), which was much deeper than with our technique. In a study dedicated to prostate cancer, 93.7% of the solid tumor genomic alterations were detected in plasma. However, different sequencing techniques were applied to the tumor tissue and cfDNA, with a lower sequencing depth on the solid tumor, possibly resulting in underestimation of the number of mutations in the former as compared to the latter [27]. In contrast, a strength of our work is the application of the same sequencing technique and coverage depth to the germline samples, tumor tissues and the cfDNA. The high sequencing depth should capture a greater diversity of tumor subclones, not all of which are shed into circulation. Finally, two other studies have reported a 82–97% detection rate of tumor variants in cfDNA of advanced cancer patients using a panel of 50 and 46 genes [28,29]. Our larger panel of 604 genes may have led to the detection of more subclonal genomic alterations and passenger events, contributing also to a subsequent decrease in the concordance rate.

Beside the number of genes included in the panel and the sequencing technology that is used, a patient's clinical status can also influence the concordance rate between liquid and solid biopsies. All previous studies contained only patients with metastatic disease and, in many, concordance was reported only for patients in whom ctDNA was detected [26,27,29]. In our logistic multivariate regression model, the detection of a tumor tissue variant in plasma was significantly related to the patient's metastatic status and the quantity of ctDNA. For ctDNA-positive metastatic patients, our concordance for the detection of solid tumor variants in plasma increased to 42%.

For two patients, the liquid biopsy was not taken at the same time as the solid biopsy. This may be an important factor given that the samples from one of these patients (HNSCC-34) showed no molecular concordance. Two different *TP53* mutations were detected in this same patient's plasma and tumor tissue sample. As the FFPE sample was taken at the time of primary disease and the plasma sample at recurrence, molecular evolution of the disease could explain this result. Another confounding factor in HNSCC is that it is almost impossible at times to clinically distinguish between disease recurrence or the presence of a second primary tumor. This outlines the importance of developing non-invasive techniques to characterize the molecular landscape of HNSCC throughout the disease course.

One limitation of our study is the sensitivity of our targeted sequencing approach (median coverage 1000x), which was restricted to mutations with an allele frequency ≥ 1%. We have shown that lowering the thresholds for variant calling seemed to inflate false positives by retaining more background noise, illustrating the biases that can be introduced by the variant-filtering criteria applied. Similarly, a recent paper compared four different plasma NGS assays to matched tissue sequencing results. The investigators observed an increased proportion of false positive or negative results when the AF dropped below 1%, and these were mainly linked to technical variations (nonspecific variant calling and sequencing noise) rather than often-discussed biological factors, such as tumor heterogeneity [30]. Variants detected by NGS with an AF < 1% should be interpreted with caution and stringent criteria should be applied to avoid false positive results. ddPCR allowed us to confirm the presence of variants detected by manual inspection that did not pass our background-noise threshold, further highlighting the limited sensitivity of this NGS approach. Using ddPCR, we detected mutations with an AF < 0.1% and enhanced our concordance rate between solid and liquid biopsies for driver events up to 80% (18/22).

Another limitation is the small sample size of this patient cohort, hampering us to correlate our findings with the clinical outcome.

In conclusion, our findings show that ctDNA detection using a large targeted sequencing gene panel is technically feasible for patients with HNSCC, especially those with metastatic disease. Although the molecular landscape characterized by cfDNA is in line with the literature, it does not reflect the global picture of a particular tumor, preventing us from confidently using this sequencing technique for liquid biopsies in a tissue-agnostic approach. Importantly, actionable mutations were detected in three samples without an available tumor biopsy, supporting the complementary role that liquid biopsy can play in molecular diagnosis when a new tumor biopsy is not feasible.

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Declaration of Competing Interest

None declared.

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Appendix A. Supplementary material

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