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Head and Neck Carcinoma and biomarker profiling: searching tumor identity card.

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Summary

Background

Head and Neck Tumors

Head and neck tumors represent the seventh most frequent oncological pathology in the world, with an incidence of 930,000 cases per year and 470,000 deaths in 2020. This figure appears to be growing considering that in 2018 these numbers were 890,000 and 430,000 respectively [1]. In Italy, 13,000 new cases are diagnosed every year, with an incidence of 18/100,000 inhabitants, higher in northern Italy, lower in the south. People around the age of 50 are mostly affected, with a preference for men for each different histology and location, although this difference is clear in laryngeal tumors (>10 times) and minimal in salivary gland tumors (1,4 times) [2].

Among head and neck tumors, 93% are represented by histologies of epithelial origin and 90% are of the squamous cell carcinoma type (SCC) with localizations in the larynx, oropharynx, hypopharynx, nasopharynx, tongue and oral cavity. The other histologies are represented by adenocarcinomas, sarcomas and lymphomas [3].

The etiology of this type of tumor is multifactorial. Smoking, alcohol, EBV or HPV-16 and 18 viral infections represent the main causes that lead to the genesis of tumors. In particular, HPV is related to oropharyngeal tumors that strike at a younger age and often without a history of tobacco use [4]. Finally, these tumors may correlate to genetic syndromes such as Li Fraumeni or Fanconi Anemia [5]

Currently, the main treatments available are surgical, radiotherapy and chemo-immunotherapy, based on the location and invasiveness of the tumor. An earlier diagnosis improves the chances of disease control [6]. The greater the invasiveness of the tumor, the greater the cost of the surgical for some locations or beyond certain stages, surgical treatment is directly replaced by combined or sequential chemo-radiotherapy protocols.

Despite the consistent progress being made in oncology with target therapies and molecular analyzes of tumors, for this pathology the advances have been very limited, with the inclusion of Cetuximab (an anti-EGFR IgG1 antibody) as the only target

therapy applied until 2013. Recently, immunotherapy has established itself as a new therapeutic possibility, thanks to the PD-1 checkpoint inhibitors, such as Nivolumab and Pembrolizumab. Thanks to these advances in therapy, 1, 3 and 5 year overall survival (OS) in Italy is respectively 80, 63 and 57% [7]. These results have improved in recent decades, but are still far from those obtained for other pathologies. The reasons for this relatively reduced disease control are multiple:

• The locally advanced diagnosis in over half of cases

• The risk of local or distant recurrence in the first 3 years of up to 50%, often with outcomes that preclude further local and sometimes even systemic treatments

• The onset of multiple primary tumors linked to the non-interruption of exposure to the triggering risk factors or the damage already accumulated in previous times.

To date, therefore, the patient with head and neck cancer represents an important challenge for oncology, in search of the key to find in order to have real, effective and prolonged disease control over time for a greater percentage of cases.

Liquid biopsy in head and neck tumors

Given its minimal invasiveness and ease of sample collection, liquid biopsy is emerging as a promising low-cost platform for screening, diagnosis and monitoring of HNSCC. Circulating Tumor DNA (ctDNA), Circulating Tumor Cells (CTCs) and exosomal microRNAs (miRNAs) have been found not only in the patient's blood, but also in saliva and can be used as surrogates for biopsy [8-10].

In recent years, liquid biopsy studies in oncology have increased, compared to previous decades and are rapidly gaining importance with promising non-invasive tumor biomarkers. To the main techniques for identifying circulating nucleic acids or intact tumor cells, further techniques are now being added such as the isolation of extracellular vesicles or proteomic targets in fluids. Head and neck tumors have also found themselves involved in research in this area and today we are starting to have evidence of the feasibility of various methods.

Somatic DNA mutations

Somatic DNA mutations represent the simplest elements to distinguish the presence of a tumor when isolated in plasma. The exclusive nature of their finding makes their isolation in peripheral blood a practically 100% specific indicator. Head and neck tumors are in nature very heterogeneous and if we take even only those of non-viral etiology, the presence of mutations affecting TP53 is recognized in 80% of cases, followed by FAT1, CDKN2A, NOTCH1 and PIK3CA in around 20%, which becomes 80% in HPV-negative cases [11-14]. These mutations have been identified in plasma and saliva in several studies and represent potential diagnostic and predictive biomarkers [10,15,16]. In particular, the use of multiplex PCR in the saliva of patients with oral cavity cancer made it possible to detect such mutations in 100% of cases, a percentage that dropped in other tumors such as the hypopharynx, larynx and oropharynx, while circulating DNA was found in the plasma only in the 80% of cases. In the same study, in 3 out of 9 cases who relapsed, tumor DNA with its target mutations was present in the patient's saliva before there was clinical evidence of disease recurrence [10]. Biomarkers based on DNA mutations currently represent a very important resource with increasingly solid evidence of sensitivity and specificity [15,17] and by its very nature DNA appears more stable and amplifiable than RNA or protein markers.

Methylation-based biomarkers

DNA methylation is an important epigenetic mechanism that for years we have recognized as, in some cases, absolutely tumor specific and therefore part of the tumor heritage in an oncogenic sense. Liu MC et al. in 2020 published an important study with more than 6500 patients with 50 different types of cancer to identify target methylations in circulating free DNA (cfDNA) that would allow the tumor to be recognized and its origin to be identified. By analyzing in parallel the entire genome and more than 100,000 different methylation sites, the tissue of origin was identified with accuracy greater than 90% [18]. As an alternative to blood sampling, a search for biomarkers based on DNA methylations was also performed on saliva samples, confirming this method of tumor DNA sampling as valid also in this case as in the case of mutations with regards to tumors of the oral cavity and oropharynx [19-21]. Although these studies confirm the possible identification of the methylative as well as

mutational structure of tumor DNA in plasma and saliva, the meaning of the most frequently identified methylations still remains unclear.

Biomarkers based on mitochondrial DNA

Mitochondrial DNA (mtDNA) mutations have been identified as potentially useful in a prognostic and predictive sense in head and neck cancers [22-26]. The characteristics of this DNA, with a clonal nature and a high number of copies available, can transform it into a potentially useful tool for the identification of tumor cells at the margin level intra-operatively or at the lymph node level or in the search for the primitive in CUP (Carcinoma Unknown Primary) Syndrome. The characteristics of this DNA make it more stable and a more sensitive marker than genomic tumor DNA. Demonstrations of the presence of mutations at the mtDNA level in head and neck tumors have already reported [27,28], but the potential use as a tumor marker in plasma or saliva is still to be clarified, also because the sequencing platforms have low sensitivity in identifying low prevalence heteroplasmids.

Extracellular vesicles and exosomes

Extracellular vesicles (EVs) represent the entity composed of exosomes (40-100 nm), microvesicles (50-1000 nm) and apoptotic bodies (50-2000 nm) which are released in good quantities into the tumor microenvironment [29]. Inside them you can find DNA, mRNA, miRNAs, circulating RNA, proteins, metabolites, all of tumor origin. These substances are often biologically active and play an important role in tumor progression [30]. In head and neck tumors, exosomes and vesicles have been searched for and studied in the peripheral blood, also observing their pre- and post-therapy variations [31-35]. Alongside these, in particular with regard to the exosomal miRNA, other studies have examined its identification at the salivary level and the progression after therapy [36,37]. Although the role of this type of markers both at plasma and salivary levels may now be demonstrated, the technologies for their isolation, purification and quantification do not currently make them widely usable.

Transcriptome biomarkers

The genetic and epigenetic mutations of the tumor cell, before manifesting themselves with the specific phenotype, present transcription products which in themselves already have all the characteristics to be considered potential biomarkers identifiable in the plasma of patients with various types of tumors, including those of the head and neck district [38,39]. Li Y et al had already managed to identify tumor mRNA at salivary level in 2004, proposing it as a potential diagnostic marker for oral cavity tumors [40]. Today, studies for the sequencing of tumor RNA on plasma or saliva have multiplied and demonstrated its potential as a marker to evaluate the progress of the disease and the response to treatments [41-44], but the platforms used are not sufficiently validated, do not make these techniques exportable into clinical practice.

Circulating tumor cells

Circulating tumor cells (CTCs) are cells that passively separate from the tumor and enter the circulation. They represent a marker of tumor invasiveness and potential aggressiveness and in particular in head and neck tumors they have been shown to be an indicator of a tumor with a bad prognosis [45,46]. The trend in the levels of these circulating cells in locally advanced or metastatic tumors treated with chemotherapy and Cetuximab appears to be correlated with the response to therapy and PFS and thus the partial or complete clearance of these cells from peripheral blood compared to the response after treatment of tumors in stages I-III [45,47-50]. However, despite this evidence on the potential routine use of CTCs dosage in head and neck tumors, the technologies required for these dosages are still poorly available and it limits the use they could have.

Viral biomarkers

In oropharynx tumors, HPV infection represents an important prognostic and predictive factor for response to treatment [51-54]. Consequently, the search for HPV-DNA circulating in the patient's plasma or saliva conceptually represents a possible non-invasive marker, in particular for monitoring the progress of the disease. Unfortunately, until now the technologies based on quantitative PCR that can be used for these assays

do not achieve sufficient sensitivity for a routine use. The NGS technique, by adding viral genes to the panel of genes to be identified, could represent a resource with repercussions in this area too, but at the moment we are in a very early phase and need to be confirmed [55,56].

Regarding nasopharyngeal tumors and related EBV infection, PCR techniques are able to identify, analyze and somehow sequence the viral DNA, making this marker a potential indicator for the early diagnosis and monitoring of these patients, additionally acquiring a prognostic value [57-63]. Different methylation profiles and concentrations have also recently been demonstrated between patients diagnosed with cancer and those with infection alone [64]. Precisely in these areas, clear variations in the oncopromoter viral DNA correlated with the presence of tumor and its prognosis are beginning to be identified [65].

Applications of liquid biopsy in head and neck tumors

Among all the markers seen above, due to the technologies available and specificity of the marker, the most used for analysis on liquid biopsies today are somatic DNA alterations, circulating tumor cells and the use of HPV-DNA [66].

Early diagnosis and screening

If the search for CTCs in plasma is more complex and more correlated to advanced or metastatic stages, as we have seen previously, in the early stages of the disease the identification of tumor DNA with specific somatic mutations or methylations in plasma or saliva can allow identifying head and neck tumors early in populations at risk. This data reveal an important correlation between the finding of mutations in the DNA using liquid biopsy and that on tissue biopsy. The same can be said between the ctDNA identification in plasma and saliva, in particular with regard to oral cavity cancer. In a prospective observational study, specific alterations in particular of the PMAIP1 and PTPN1 genes detected in salivary ctDNA discriminated 100% between the presence or absence of primary oral cavity cancer in the patients analyzed [67].

Research studies on plasma EBV-DNA for nasopharyngeal tumors and HPV-DNA in plasma or saliva for patients with HPV-related oral heteroplasia also fit into this setting.

Response to therapy or relapse

An important paradigm for the use of circulating biomarkers of any type for monitoring response to treatment and/or possible recurrence over time is that the levels found are correlated with changes in tumor volume. Several studies have confirmed this characteristic of both ctDNA and CTCs [68,69]. An example is the aforementioned study by Garrel et al where the levels of CTCs could predict the PFS and OS of patients after systemic treatment according to the Extreme scheme [45]. In another study, Hilke et al. analyzed the trend of plasma ctDNA compared to the clinical trend of the patient with locally advanced HNSCC, demonstrating a significant correlation between tumor volume and basal ctDNA [70]. Furthermore, Bai J et al. in 2020 he published a study in which the non-identification in NGS of mutations affecting the TP53 and PIK3CA genes in post-surgical follow-up correlated with a time to relapse of 20 months longer [71].

Monitoring of cellular mutations under therapeutic pressure

Among the therapies available for the systemic treatment of head and neck tumors, the only registered drug to be considered target therapy is Cetuximab, an anti-EGFR IgG1 monoclonal antibody. The mechanism of action of this drug and the signaling cascade related to its action is well known. Braig et al were the first group to use ctDNA to study tumor evolution under the pressure of this targeted therapy and the resulting resistance at the time of progression. NGS on liquid biopsy revealed that 46% of these patients at progression had an additional mutation in the RAS gene, closely related to resistance to Cetuximab.

With the advent of anti PD-1 antibodies Nivolumab and Pembrolizumab, Abbot et al analyzed NGS analysis on liquid biopsy on 13 patients at the beginning of treatment and at progression to immunotherapy with Nivolumab. Post-therapy analysis identified mutational changes in numerous different oncogenes on different signaling pathways, in particular the ERK1/2/MAPK chain, not observed on the tissue biopsy of the primary tumor, potentially responsible for treatment resistance [72].

The number of patients analyzed in both of these studies represents only a stimulus to expand the case series. However, they present data that certainly see the liquid biopsy as a potential analytical tool for mutational resistance.

Immunotherapeutic biomarker

Several tumor type have seen the use of PDL-1 expression scores, tumoral or combined, as a predictive factor for response to treatment. On this principle, studies analyzing the expression of circulating PDL-1 mRNA and the expression of PDL-1 on CTCs have begun, combined with the already developed prognostic importance of plasma CTCs in head and neck tumors [73]. The trend of PDL-1, if overexpressed at the start of therapy, seems to be directly related with the response to treatment. In locally advanced tumors subjected to chemoradiotherapy, it has been seen that the elimination in NGS analysis of PDL-1 and IDO1 after treatment correlates with a longer PFS [74]. We consider that the expression of PDL-1 in CTCs does not correspond exactly with its expression on tumor tissue.

Applications on viral DNA

As previously mentioned, the potential importance of using cfHPV-DNA as a serum or salivary marker for tumors of the oral cavity and oropharynx linked to this virus is undoubted. In addition to the known prognostic element of the detection of HPV-16 infection on histology, this DNA also represents a possibility for monitoring the response to treatment and any appearance of relapses during follow-up. However, PCR-based technologies do not allow it to be used routinely and effectively today, but by using NGS technology to search for viral genes, the possibilities of standardization and therefore use for HPV-related tumors become more real [56,75]. Studies are currently underway which, with the use of cfHPV-DNA trends, aim to define therapy de-escalation paths, evaluating the possible serum response after induction chemotherapy and the consequent aggressiveness of radical radiotherapy, so as to spare the patient from sometimes even persistent toxicities [76,77].

Aim of the study

The aim of the present study is to identify higher frequency target mutations or prognostic mutational factors by identifying them with NGS analysis of ctDNA, in patients affected by head and neck heteroplasia with a histopathological examination positive for squamous cell carcinoma (HNSCC), in a large and diverse real world population.

Matherials and Methods

Study design

From January 2021 to June 2023, all patients with a histologically confirmed diagnosis of HNSCC belonging to the Medical Oncology Unit were enrolled, in collaboration with the Medical Genetics Unit. Upon diagnosis of locally advanced or metastatic disease, the patients underwent a family oncological history and circulating DNA sampling, then analyzing the mutational expression in NGS according to the liquid biopsy technique. When possible correlations with hereditary syndromes emerged in the family history or in the case of multiple tumors in the same subject, the presence of constitutive mutations on the genomic DNA (Genomic DNA, gDNA) was investigated by means of clinical exome sequencing (Clinic Exome Sequencing, CES). An informed consent at the Genetics Unit has been obtained for each patient enrolled, as well as all clinical data and disease progression in response to various systemic and possibly local treatments were monitored, in compliance with privacy regulations and processed through correlation analysis.

The study did not include any interventional element from a therapeutic point of view in light of the response of the NGS analysis. Patients were then treated according to national guidelines and regional indications regarding treatment lines and therapeutic appropriateness. Staging, restaging and follow-up of the disease followed the criteria of the eighth edition of the American Joint Commission on Cancer AJCC/TNM of 2018 and was carried out according to the times and techniques established by the guidelines.

Patients

Enrolled patients had to meet the following criteria:

Inclusion criteria:

- Age over 18 years

- Ability to express informed consent

- Histopathologic diagnosis of HNSCC

- Treatment and monitoring performed according to national and regional guidelines

Exclusion criteria:

- Refusal or inability of the patient to provide consent to genetic analysis

- Loss to follow-up or transfer of reference center for treatment

- Histopatholoic diagnosis negative for SCC

Liquid biopsy: NGS sequencing of plasma ctDNA

The enrolled patients underwent molecular characterization via liquid biopsy on ctDNA isolated from plasma, through peripheral blood sampling performed during genetic counseling at the Medical Genetics Unit of the Siena University Hospital. Peripheral blood (10 ml) was collected in cell-free DNA (cfDNA) BCT tubes (STRECK tubes).

Isolation of cfDNA

Plasma was obtained by a double centrifuge at 1900 g, the first for 15 minutes and the second for 10 minutes. Subsequently cfDNA was extracted from 4 ml of plasma using the AVENIO cfDNA Isolation kit.

Once extracted, the cfDNA was subjected to quantification and quality control of the fragments obtained. Sample quantification and recording were performed using the Qubit dsDNA HS Assay Kit. The quality of the cfDNA was verified thanks to the Agilent bioanalyzer (Agilent Bioanalyzer – High Sensitivity DNA chip): typically the cfDNA is found in the form of short fragments less than 1000 bp in size, unlike genomic DNA (gDNA) which occurs in fragments with highest molecular weight >2000bp. A quality cfDNA therefore appears between 160 and 200bp. Finally, the mass of cfDNA isolated from plasma was calculated, to allow AVENIO to calculate the number of mutant molecules per ml of plasma in the sample report.

Preparation of the library

Using the AVENIO cfDNA Library Prep kit, each sample is linked to a unique adapter that allows multiple samples to be sequentially multiplexed, i.e. in parallel. Each library uses an input range of cfDNA between 10 and 50 ng. This is then followed by PCR amplification of the DNA strands linked to adapters and, again, the evaluation of the concentrations in the pre-enriched libraries using the Qubit dsDNA HS Assay kit and of their quality with the Agilent bioanalyzer.

Enrichment protocol

Using the AVENIO ctDNA Enrichment Kit, Post-Hybridization kit and Expanded Kit for the identification of genes associated with solid tumors using targeted probes, any mutations are identified on a panel of 77 tumor-related genes **(see figure 1),** providing a profile complete genomic analysis on four classes of mutations: SNVs, insertions or deletions, gene fusions and CNVs.

Analyzed genes: ABL1, AKT1, AKT2, **ALK**, APC, AR, ARAF, BRAF, BRCA1, BRCA2, CCND1, CCND2, CCND3, CD274, CDK4, CDK5, CDKN2A, CSF1R, CTNNB1, DDR2, DPYD, **EGFR**, ERBB2, ESR1, EZH2, FBXW7, FGFR1, **FGFR2, FGFR3**, FLT1, FLT3, FLT4, GATA3, GNA11, GNAQ, GNAS, IDH1, IDH2, JAK2, JAK3, KDR, KEAP1, KIT, KRAS, MAP2K1, **MET**, MLH1, MSH2, MSH6, MTOR, NF2, NFE2L2, NRAS, **NTRK1**, PDCD1LG2, PDGFRA, PDGFRB, PIK3CA, PIK3R1, PMS2, PTCH1, PTEN, RAF1, RB1, **RET**, RNF43, **ROS1**, SMAD4, SMO, STK11, TERT, TP53, TSC1, TSC2, UGT1A1, VHL

Figure 1: Panel of 77 tumor-related genes from Avenio ctDNA Expanded Kit (Roche). Genes highlighted in bold are also analyzed for copy number variations (CNVs). Coverage: 100% for 29 genes. Reading depth: >=1000x. Annotations: the following are not reported: variants reported as polymorphisms or as having no clinical relevance in the databases; the nuceloride variants which, on the basis of literature data or data produced by the Medical Genetics laboratory, can be classified as having little or no clinical significance; silent variants and intronic variants, with the exception of those that alter, or are potentially capable of altering, the normal processing of messenger RNA.

The libraries thus constituted and enriched are first denatured into single-stranded DNA, and then hybridized by biotinylated oligonucleotide probes. These probes are then linked to magnetic microspheres conjugated with streptavidin (Streptavidin Magnetic Beads, SMB). These conjugated fragments are then magnetically extracted from the solution and subjected to elution from the microspheres and washed. The PCR amplification phase of the enriched and isolated ctDNA fragments then begins

with the quantification of the concentration of the individual enriched libraries using the Qubit dsDNA HS Assay kit and evaluated for quality using Agilent.

NGS sequencing of ctDNA

Once the quality and quantity control of the "enriched" libraries has been carried out, the samples are sequenced using the Illumina NextSeq550 sequencer. The protocol involves the sequencing of up to 12 samples using Hight Output flowcells (Illumina) for multiplex analysis, ensuring a reading depth always greater than 1000x (coverage).

Analysis and reports

Data analysis was performed by analyzing the AVENIO Oncology Analysis software which identifies various types of alterations. In fact, it is possible to find from insertions and deletions to single nuvleoride variants (SNVs to gene fusions and copy number aberrations (CNVs) of six 77 genes associated with cancer with a Limit of Detection (LOD) up to 0.5 %.The Oncology Analysis v2.0.0 software then examines the primary sequencing data and provides secondary analysis results, i.e. identifying any variants.

The reporting tool provides a variant report that includes both sample and variant information (genomic location, protein location, coding codon location, allele fraction) accompanied by data from common annotation databases for each gene. The quality control report obtained provides information on the quality of the sequential execution and the overall performance of the test, and includes parameters such as coverage uniformity.

Germline mutations: NGS sequencing of the Clinical Exome (CES)

If at the time of genetic counseling the patient had a family history suspicious for a hereditary syndrome, the individual genetic susceptibility for multiple tumors was also investigated through clinical exome sequencing.

Exons are the coding regions of DNA and represent approximately 1% of the entire genome. The clinical exome test searches coding regions for variants with known clinical associations with disease. A panel of genes of interest associated with multiple malignancies was used in our study.

For sequencing, a target approach similar to the previous one of liquid biopsy with NGS technology was therefore used.

Collection and isolation of gDNA

Genomic DNA (gDNA) was extracted from the cell-containing phase, the buffy coat, of peripheral blood collected in EDTS tubes, using MagCore Super Automated (Diatech Lab Line). The amount of gDNA was estimated with the Qubit 3.0 Fluorometer.

Preparation of the library

The Nexter DNA Flex Library Preparation kit integrates the fragmentation and library preparation phases in a single step with the addition of the adapters necessary for the subsequent sample indexing phase, making the workflow faster.

Sample preparation was performed following the Nexter Flex for Enrichment manufacturer's protocol. It is characterized by the use of a transposome complex linked to microspheres (Enrichment Bead-Linked Transposome eBLT) which mediates the simultaneous fragmentation and labeling of the gDNA (tagmentation phase).

After this phase, a first amplification of the DNA follows by PCR which adds the index primers to the ends of the DNA fragments creating an indexed library.

Enrichment protocol

Up to 12 libraries are grouped together. The pool is then concentrated and the libraries denatured into single-stranded DNA (sDNA). Hybridization reactions then follow in which biotinylated oligonucleotide probes are hybridized to the denatured library fragments, corresponding to the regions of interest. We then proceed to enrichment with SMBs that bind to the probes and the DNA fragments are then magnetically extracted from the solution. Then, the enriched and indexed libraries are eluted from the beads and further amplified before final sequencing.

Clinical exome sequencing

Clinical exome sequencing was performed by the Illumina NovaSeq6000 system.

Analysis and reports

The reads are aligned to the hg19 human reference genome, using the Burrow-Wheeler BWA aligner. Once a series of quality controls have been passed, we proceed to the secondary analysis to search for any variants which for our study involved the use of the Genome Analysis Toolkit Best Practices tools. Sequencing data were filtered by eVai (enGenome) software

Definition of study endpoints

For statistical analysis, numerical variables were expressed as median and range, and were compared using non-parametric tests (Mann-Whitney U-Test). The qualitative variables were expressed as frequencies, and organized in contingency tables; associations between categorical variables were studied through Fischer's exact test or Person Chi-Square test. A p-value <0.05 was considered statistically significant; Stata software was used for the analysis.

The primary end-point was:

- identify any potentially predictive or prognostic correlations relating to the finding of specific mutations highlighted with NGS technique on liquid biopsy.

For this purpose, progression free survival (PFS) was calculated from the date of start of therapy to disease progression and/or death for any cause. The Kaplan-Meyer method was used to estimate PFS. The statistical differences between the various groups according to the covariates of interest were calculated using the Long-Rank test.

Secondary endpoints are:

- identify any specific mutations, gene alterations or alterations of specific signaling cascades that are repeated more frequently and any possible targets for targeted therapies

- identify any genomic variations related to phenotypes presenting multiple primary tumors or particular cohorts.

Results

Patient characteristics

From January 2021 to June 2023, 47 patients, 36 men and 11 women, with an average age of 68.5 years (41-85), all diagnosed with HNSCC underwent liquid biopsy. Among these patients, 20 had locally advanced or metastatic disease at diagnosis, without indication for radical local treatment and 13 presented a recurrence after surgical treatment (11 patients) and/or radical radiotherapy of the primary treatment (4 patients). Fourteen patients instead presented locally advanced disease, without distant spread, and then underwent radical local treatment.

The primary tumor was located in 15 cases in the oral cavity, in 9 cases at the level of the larynx, 5 patients had a tumor of the nasopharynx, 4 of the hypopharynx, 3 of the tonsil, in 2 cases at the level of the maxillary sinuses and the same number of the tongue. Finally, 3 patients presented with CUP Syndrome, therefore with laterocervical lymph node localization due to SCC without identification of the primary. Positivity to HPV viral infection was documented on histology in 6 cases of oral cavity cancer, 2 cases of tonsil cancer and EBV infection was found in all patients with nasopharyngeal neoplasia. The expression of CPS PDL-1 was evaluated in 22 patients, all with locally advanced or metastatic neoplasia; 17 patients were found to have CPS PDL-1 greater than 20, of which 6 were greater than 50 and among these 5 were greater than 80. Among the patients with moderate or weak expression of the CPS PDL-1, 3 patients with values between 5 and 20 and 2 patients with values lower than 5, but still with minimal expression, were found; no patient tested negative for CPS PDL-1 expression.

All patients underwent systemic medical therapies; in 9 cases exclusively pre-radiotherapy induction treatment and in a further 8 cases also concomitant radical chemo-radiotherapy treatment, in 1 case further adjuvant chemotherapy was followed after salvage surgery for a single lymph node recurrence. Of the total patients, 28 underwent systemic treatment in an advanced stage, 10 underwent a single line of treatment, and the other 18 underwent 2 to 4 lines in total. Among the drugs used, 25 underwent immunotherapy treatment with inhibitors of the related PDL-1 immune

checkpoint (Pembrolizumab or Nivolumab), among these, 17 used these drugs in the first line and 8 in the second line of treatment. The chemotherapy treatments saw the use of Platinum, FluoroUracil and Taxanes according to guidelines, associated with Cetuximab in 8 cases in the first line, and in 5 cases in subsequent lines.

Liquid biopsy results

All patients evaluated in the study underwent liquid biopsy and NGS analysis of ctDNA. Among the 47 patients, 7 of them (14.9%) presented a negative result for the search for mutations in the genes investigated in the panel; 11 patients presented only one mutation (23,4%), 10 presented 2 (21,3%) and 15 patients presented from 3 to 9 mutations affecting the target genes analyzed (31,9%); finally, in 4 cases (8,5%) constitutive mutations were found, 2 of which were in the BRCA2 gene, 1 in MSH6 and 1 in DICER1, all with uncertain pathogenic potential. In 8 cases (17%), a liquid re-biopsy was performed after clinical and radiological progression to treatment to monitor the preservation of the variety of mutations involved and in all cases, the NGS result in ctDNA gave a modified response compared to the previous biopsy. (**See Table 1**)

Pt.	First liquid biopsy	Second liquid biopsy
1	GNAS, FBXW7, APC	GNAS
2	neg	TP53
3	MET, TP53	TP53
4	PIK3CA, TP53, RNF43	PIK3CA, TP53, TP53, RNF43, PTEN
5	ERBB2ampl, TP53, MYC	ERBB2ampl, TP53
6	neg	CD274
7	PIK3CA, TP53, BRCA2	TP53, BRCA2
8	PIK3CA, TP53	neg

Table 1: Variations between liquid biopsy at the beginning of systemic treatments and liquid biopsy at progression

Among the mutated genes found, with various expression in the ctDNA, the most represented was TP53 with mutations in 17 cases (36.2%), followed by PIK3CA with 12 mutations (25.53%)

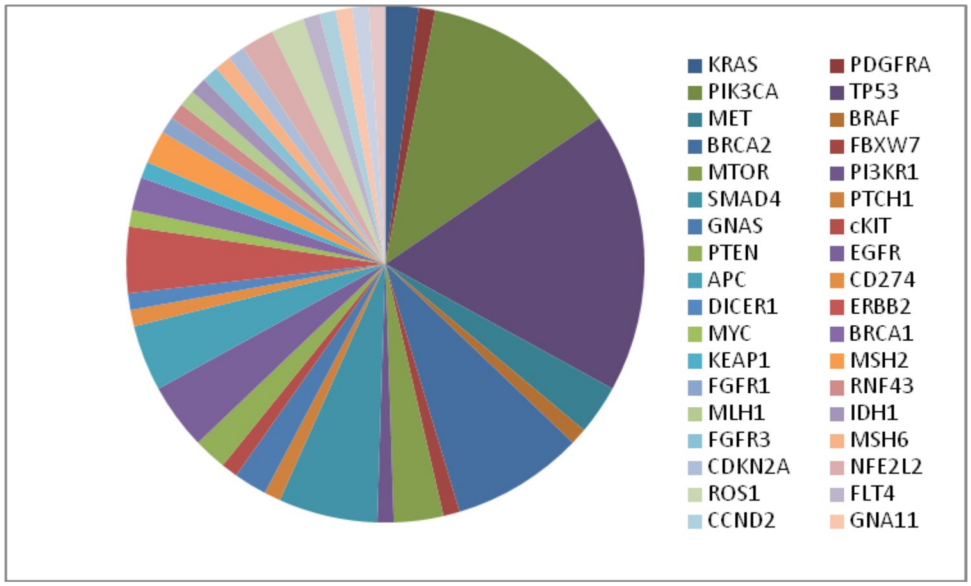
(see Table 2), BRCA2 with 8 mutations (17.0%) (**See Table 3**), SMAD4 with 6 (12.8%) and EGFR, ERBB2 and APC with 4 mutations each (8.5%). The other 31 genes found presented from 1 to 3 different mutations in the various patients (**See Graph 1**).

Pt.	Mutation PIK3CA	Canonic mutation (Y/N)
1	P539R	N
2	E542K	Y
3	E545K	Y
4	E542K	Y
5	Q546L	N
6	E542K	Y
7	P539R	N
8	H1047Y	Y
9	E542K	Y
10	G118D	N
11	E545K	Y
12	Q546L	N

Table 2: Mutations on PIK3CA

Pt.	Mutation *BRCA1*/BRCA2
1	L246S
2	L2587I
3	A1668T
4	K3083E
5	I1583F
6	Y2013del
7	*N243S*
8	*L246S*

Table 3: Mutations on BRCA1 and BRCA2



Graph 1: Mutated genes and their frequency

The variety of mutated genes detected involves genes that encode proteins of different types and with different roles in the regulation of cellular functions and replication.

DNA and RNA regulatory molecules

In the sample of patients analyzed, TP53 appeared as the gene with the highest presence of mutations, 17, in 3 cases even with multiple mutations in the same patient. Alongside this gene, however, many mutations were also found in other DNA regulatory genes such as BRCA2 in 8 cases and BRCA1 in 2 cases, GNAS and GNA11 in 2 patients and 1 respectively; The MYC, DICER1, AR genes and those involved in the mismatch repair system MSH2 (2 cases), MSH6, MLH1 were also mutated in only 1 case. Further genes involved were those that encode components of cell cycle regulation, i.e. CDKN2A, CCND2, FBXW7, NF2.

Transmembrane Tyrosine Kinase Receptors

Nine different genes have been identified that encode transmembrane tyrosine kinase receptors, involved in numerous signal transmission pathways for cell proliferation. Among these, the most represented were EGFR and ERBB2 in 4 cases and MET in 3 cases, followed by ROS1 with 2. In only 1 case for each gene were mutations in PDGFRA, cKIT, FGFR1, FGFR3, FLT4 identified.

Components of signal transmissions

A further group of multiple molecules represented as mutated in the liquid biopsies to which the patients were subjected is the group of genes that code for molecules involved in the various signal transmission cascades that connect membrane receptors to the nuclear regulation of growth, differentiation and proliferation mobile phone. Among these, the most represented with 12 mutations found in our patients, in 2 cases even with multiple mutations in the same patient, was PIK3CA. Followed by SMAD4 with 6 mutations, APC with 4 mutations, 3 mutations detected in MTOR and 2 for KRAS and PTEN. In only 1 case were the BRAF, PI3KR1, RNF43, PTCH1 genes mutated. The signal cascades most involved are therefore those of PI3K/AKT, KRAS/MPAK and beta-catenins.

Control of oxidative stress and immune response

Among the genes analyzed, in 3 cases the complex composed of NRF2/KEAP1, regulator of the immune response against the cell under oxidative stress, was involved. In one case the CD274 gene encoding PDL-1 was directly involved and in another case IDH1.

Special populations

Among analyzed patients, it was possible to identify some groups that have particular clinical characteristics due to the course of the disease or anamnesis or the result of a liquid biopsy.

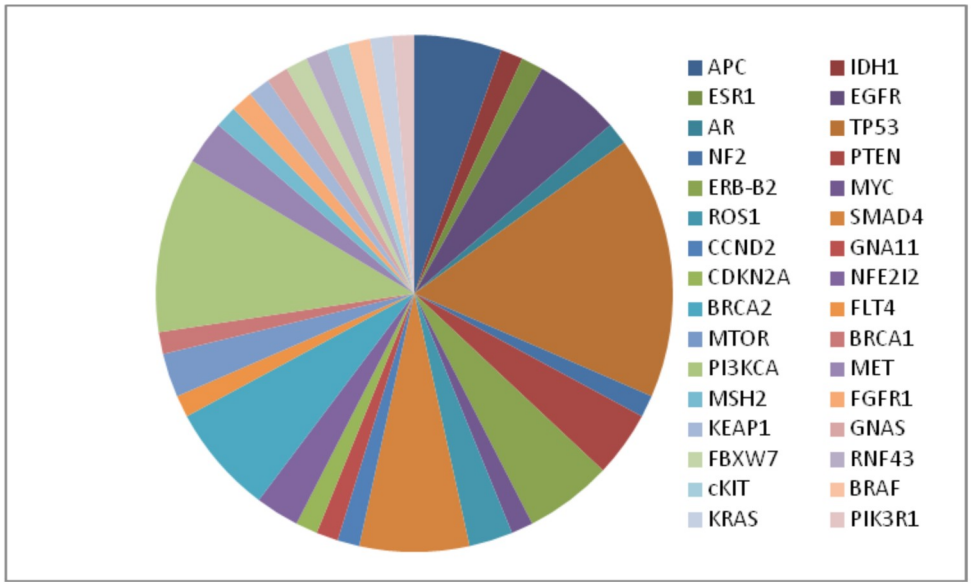
Patients with multiple primary tumors (MPTs)

Among enrolled patients, 20 had multiple primary tumors, of which 10 in the head and neck area, 7 in other organs or systems, 3 had both in the head and neck area and in other systems (**see Table 4**). If diagnosed metachronously, such tumors were considered already resolved with local healing treatment at the time of taking charge, but if diagnosed synchronously, they were of secondary clinical importance compared to the tumor under active treatment.

	Liquid Biopsy from index tumor	MPTs		Liquid Biopsy from index tumor	MPTs
Pt. 1	TP53, SMAD4, ROS1	1 CUP Syndrome	Pt. 11	PIK3CA, MET, MSH2, APC, EGFR, FGFR1	1 Oral cavity
Pt. 2	TP53	1 Breast	Pt. 12	ERB-B2, TP53, MYC	3 Larynx, 1Hypopharinx, 1 Esophagus
Pt. 3	GNAS, FBXW7, APC	1 Oropharynx	Pt. 13	DICER1	1 NSCLC, 1 SCLC
Pt. 4	MET, TP53	1 Oral cavity	Pt. 14	CD274 (PDL-1)	1 Oral cavity
Pt. 5	CCND2, Her-2, GNA11	1 colon, 1 gastric	Pt. 15	EGFR	1 Breast
Pt. 6	NFE2L2, TP53(x2), PIK3CA(x3)	1 Larynx, 2 Lung	Pt. 16	TP53	2 Oral cavity, 3 Larynx
Pt. 7	/	1 Lung	Pt. 17	APC, TP53, NF2, PTEN	1 CUP Syndrome
Pt. 8	MSH6	1 Oral cavity, 1 colon, 1 gastric, 2 cutaneus, 1 Lung	Pt.18	cKIT, PTEN, TP53	4 Oral Cavity
Pt. 9	FGFR3	1 parotid, 1 colon	Pt. 19	TP53, PTCH1	1 Oral Cavity
Pt. 10	…	1 Lung	Pt. 20	BRCA2	1 Nasopharinx

able 4: Patients and their NGS analysis in Multiple Primary Tumors

The results of the liquid biopsy in these patients highlighted in all cases the finding of mutated genes among the 77 analyzed **(see Graph 2).** No gene or gene family prevailed over the others, thus respecting the frequency in the general population.



Graph 2: Mutated genes and their frequency in TMPs population

Poor prognosis patients

In the clinical history of head and neck tumors, some patients have rapidly evolving diseases, with poor response to radiotherapy and the proposed pharmacological schemes. By identifying these patients as those with progression in less than 6 months after radical or first line metastatic chemo-radiotherapy treatment, we isolate a cohort of 15 patients with a demographic distribution and origin of head and neck heteroplasia comparable to the general population of the study patients. Among these, only 6 had multiple tumors.

The results of the liquid biopsy in these patients showed 3 patients with absence of mutations in the analyzed genes, 7 patients with mutation of the TP53 gene, 4 mutations of the PIK3CA gene, 6 patients with mutations of tyrosine kinase receptor genes (FGFR1, FGFR3, MET, 2 EGFR, PDGFRA). Also in this case, no particular profile attributable a priori to a rapidly evolving disease was identified.

Hypermutated patients

Looking at the results of the liquid biopsy, a group of 15 patients with 3 or more mutations is identified. These patients manifest something that can be defined as a "fragility" of the DNA with mutations in multiple genes, also involved in different signal transmissions and sometimes

even with multiple mutations in the same gene. However, the percentage of expression of these mutations is variable in the results of the liquid biopsy. This data is obviously evident from circulating DNA which may be evidence of multiple mutations in the same neoplastic monoclonal cell, but also of cellular polyclonality.

Among these 15 patients, 8 had multiple primary tumors and 5 had rapidly evolving pathology. No statistical differences by site of the primary or demographic data compared to the general study population.

As regards the response to therapies, the PFS data of all 47 patients were analyzed beyond the mutational structure, identifying an average PFS of response to platinum and flurouracil-based chemotherapy of 6.79 months. This average, for patients who had at least one mutation, was 5.33 months, little different from the average PFS of hypermutated patients of 5.4.

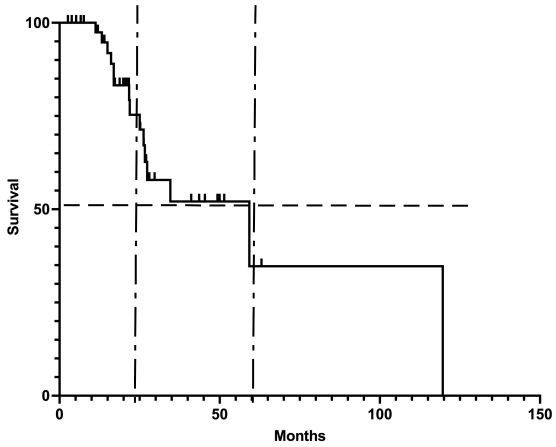
Compared to the use of immunotherapy treatments, the average PFS of 5.77 months in 1st line and 10.22 months in 2nd line for the general population becomes 5.66 months in 1st line and 9 months in 2nd line in patients with mutations in NGS and 6 and 4.3 months respectively in "hypermutated" patients.

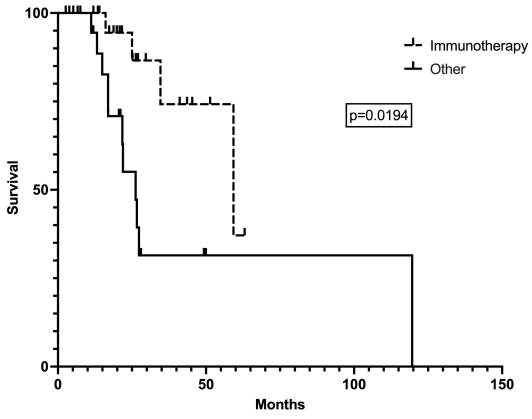
Survival analysis

General population

Considering the data collected, we studied the survival curve of our patient population (graph 3) and tried to process this survival data based on some aspects:

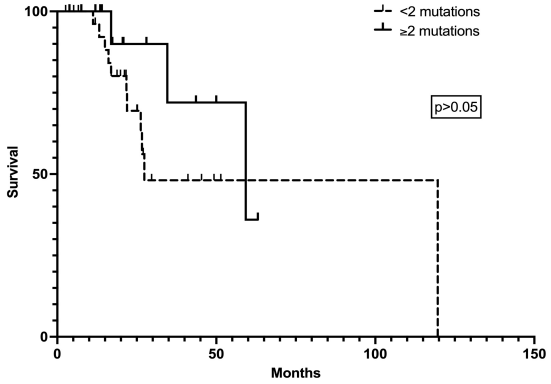
- Having received immunotherapy or not during the course of treatment (graph 4)
- The number of mutations found in liquid biopsy, setting as cut off 0 or 1 mutation vs. 2 mutations or more (graph 5)
- Patients with locally advanced cancer versus metastatic patients (graph 6)

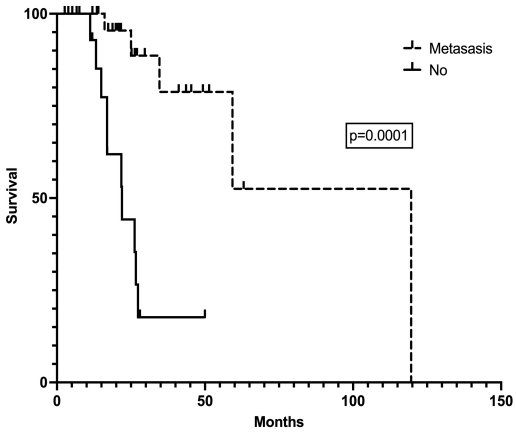




Graph 3

Graph 4





Graph 5

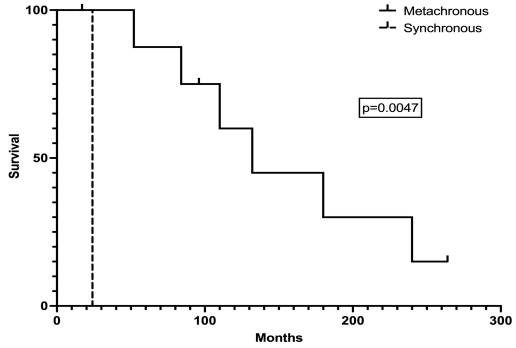
Graph 6

Taking into account the number of patients analyzed, the median survival from cancer diagnosis is 60 months, demonstrating a significant *p* for patients who underwent immunotherapy during their treatment process and with metastatic cancer compared to locally advanced cancer.

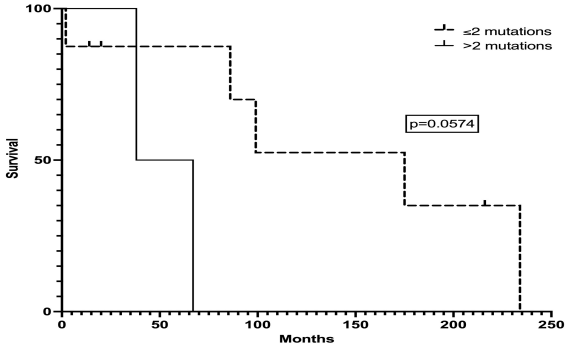
Survival in MPTs

By analyzing the population affected by multiple tumors, we can further highlight some survival data. In cases of metachronous tumors, the times are marked not by the tumor being treated and analyzed in the study period, but from the time of diagnosis of the first tumor. First, we analyzed the population in general, differentiating between diagnoses of synchronous or metachronous multiple tumors (graph 7), and then focusing on various specific aspects:

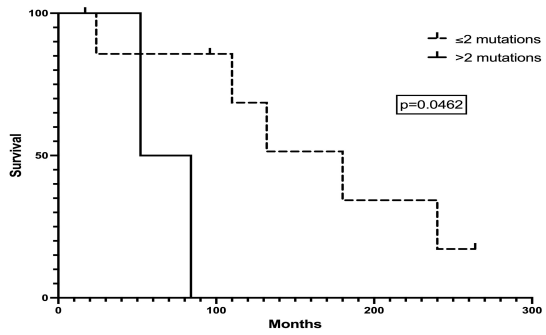
- PFS and OS based on the number of mutations, again keeping the cut off at 2 mutations (graph 8 and graph 9)
- Having undergone immunotherapy (graph 10), also compared with the number of mutations (graph 11)



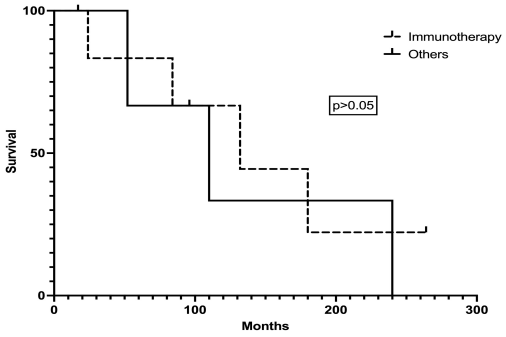
Graph 7



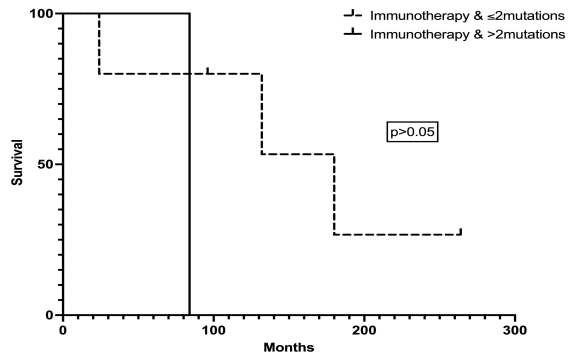
Graph 8



Graph 9



Graph 10


Immunotherapy & ≤2mutations
Immunotherapy & >2mutations
p>0.05
Survival
100
50
0
0
100
200
300
Months

Graph 11

Discussion

Considerations on liquid biopsy application

Our data, with the detection of mutations detected in NGS in 85.1% of cases, appear consistent with the literature which reports that the liquid biopsy has a sensitivity of uptake of tumor DNA in 80% of cases of tumors in the district head-neck [10]. It would therefore seem that in cases in which no mutations were found with the NGS technique in liquid biopsy, it is actually a problem of the sensitivity of the method rather than the presence of mutations itself.

This data confirms the possibility of detecting tumor DNA at plasma level, analyzing it in NGS, confirming the possible diagnostic and potentially monitoring indication. The importance of the method acquires relevance in all stages of neoplasia and the data collected, both in locally advanced and metastatic patients allow a possible use in both of these disease settings. A re-biopsy at the end of the radical chemo-radiotherapy treatment or serial re-biopsies at each progression of the disease could allow studying the trend of resistance to the various treatments in line with other studies already cited [45-47,70,71,78].

Considerations on detected mutations

Although the representativeness of each mutation is highly variable, some genes were more represented and in some cases represent possible targets on which it might be worth spending some considerations.

TP53

As previously mentioned, the gene represents an important tumor suppressor which, when mutated, loses its function allowing the development of tumors with a certain aggressiveness. Its mutation rate in head and neck patients, particularly those HPV-negative, certainly makes it an interesting target for a therapeutic strategy that "compensates" for its reduction or lack of function. The strategies to do this have been

and are still being investigated on multiple fronts, also linked to the complexity of the consequences that these mutations bring with them in the various differences between them [79]. Given the ubiquity of TP53 mutations in oncological pathologies, in-depth studies in the field of head and neck tumors have nevertheless been carried out [80], albeit without reaching precise indications or identification of drugs that can be used in clinical practice.

PIK3CA

The presence of various mutations of this gene in head and neck tumors makes it a very interesting target for possible therapeutic strategies today. PIK3CA is the gene that encodes p110α, the protein that represents the class 1A catalytic subunit of PI3K; its mutation leads to aberrant alterations in the functioning of various signal transmissions [81]. Among the mutations that this gene can present, in 63% of cases they are represented by the same three hotspots E542, E545 and H1047. These three mutations are called canonical and, when mutated, we know the consequences in cellular balance and in particular on the PI3K/AKT/mTOR transmission pathway [82]. The remaining 37% is represented in head and neck cancer by 32 different mutations, all with different percentages of appearance, of which 10 identified only in tumors of this district. The significance of the alteration of function resulting from these mutations is not currently known [83].

In clinical practice, a PI3K inhibitor, Alpelisib, is now available in breast tumors, indicated in association with Fulvestrant in the second line of HR +, Her-2 negative metastatic tumors. In the registration study of this drug, in addition to identifying its efficacy in canonical mutations, it also presented results in 3 other mutations (C420R, Q546E and Q546K) [84]

Studies on the use of Alpelisib in head and neck tumors are currently underway, both in monotherapy and in combination therapies, with anti-HRAS such as Tipfarnib, anti-cyclins such as Palbociclib, anti-EGFR such as Cetuximab and associated with concomitant with RT [85-87]. However, it is still early to talk about new target therapy available in this type of disease.

BRCA1/2

Although in our study we found 8 different mutations of BRCA2 and 2 of BRCA1, none of these represented mutations with a certain pathogenic character registered for the indication of anti-PARP therapies in other pathologies of HBOC syndromes (breast, ovary and prostate). Nonetheless, the use of Olaparib and anti-PARPs within clinical studies is a reality, mainly linked to a radio-sensitizing role of these molecules [88-90]. More recently, the effects of anti-PARPs on the cellular microenvironment and its immunostimulatory effects have been evaluated, suggesting a possible synergy with both chemo- and immunotherapeutic treatments [91]. However, none of these studies uses liquid biopsy and the identification of any mutations to guide the therapeutic choice.

As regards the other mutations found during our study, all with a percentage lower than 10%, therefore practically only in single or almost single cases, it is not possible to outline a clinical-therapeutic consequence even in the long term. The mutation of receptors such as FGFR or PDGFRA or FLT4 for which drugs are used or are starting to be used in other oncological diseases certainly represents an invitation to move forward in this direction of delineation and analysis of tumor identity cards.

Considerations on survival analysis

The study presents data characterized by the presence in the cohort of some long-surviving patients. From this perspective, the diagnosis of metastatic disease appears advantageous compared to that of a tumor with recurrent loco-regional growth. This data is consistent with the patient's history of illness who often dies from bleeding or infections of the ulcerated tumor rather than from organ failure due to metastatic spread.

On the same level we can consider the finding of synchronous or metachronous tumors which see the treatment possibilities very differentiated based on whether or not multiple tumors are diagnosed simultaneously. Even in this case, the survival disadvantage is understandable because the multidisciplinary choices faced with two

localized tumors diagnosed at the same time are understandably projected on systemic rather than local treatment.

The data also present an apparent advantage although not significant in the case of multiple tumors with low mutational load found in NGS analysis. This data requires expanding the case history before giving interpretations as the result is so borderline.

Limitations of the study

The results of the study were certainly characterized by the difficult comparability linked to the reduced representativeness of the individual mutations and by the small number of patients linked to its monocentricity. The investigative observational approach to evaluate what emerged from the analyzes is nevertheless part of current research for this type of tumor and adds its portion of information to the scientific literature data

Conclusions

Since our study began, research on the application of liquid biopsy in head and neck tumors has become an increasingly studied field of interest all over the world, in search of the best use of the method for these patients. Precisely, the liquid biopsy and the analysis of circulating DNA represent a valid diagnostic method to be used and investigated in this pathology. Therefore, the aim of using this innovative translational oncology technique is to identify potential target mutations, useful for any targeted treatments, from a prognostic or even predictive point of view of response to the therapeutic strategies available to us today.

From the data emerging from the mutational analyses, it would seem useful to investigate the possible use of some target drugs for mutations such as PIK3CA and BRCA1/2 also in head and neck tumors.

Circulating DNA appears as a highly specific marker of the tumor which also tells us about its variations during the course of therapies and in the various stages of the disease. If we associate this element with the possibility of using liquid biopsy on DNA that can be isolated from a salivary sample [8-10], we can recognize its even simpler sampling role. Our study is therefore part of these lines of research, demonstrating how much remains to be explored in depth, but also confirming the first certainties we have.

Just a thought about the circulating tumor DNA of head and neck patients which is interesting not so much for one or more mutations, which always change, but precisely for the uniqueness that each patient presents in NGS, as it also presents in the clinic and in his personality and relationships. We are talking about a complex DNA, with one or more fragilities, with many ways of "self-harm", accompanied by an equally fragile immune system, but potentially all of this can become the real key on which to leverage to fight the pathology. We can say the same about people affected by this type of cancer, who come from histories of alcoholism, strong addiction to nicotine or other substances, often fragile and accompanied by even more fragile people who take care of them or at least think of trying.

In the end all the research should never lose sight of patients' faces, to find the motivation to continue investing time, money and energy in asking questions and not stop searching until the answers are found... and new questions too....

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