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Oncogenic drivers in 11q13 associated with prognosis and response to therapy in advanced oropharyngeal carcinomas

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ABSTRACT

Objectives: To identify potential molecular drivers associated with prognosis and response to treatment in advanced oropharyngeal squamous cell carcinomas (OPSCC).
Materials and methods: Thirty-three OPSCC biopsies from untreated Brazilian patients were evaluated for human papilloma virus genotyping, genome wide copy number alterations and gene expression profiling. Data were integrated using CONEXIC algorithm. Validation with TCGA dataset and confirmation by RT-qPCR of candidate genes were performed.
Results: High-risk HPV positive cases, detected in 55% of advanced OPSCC, were associated with better outcome. Losses of 8p11.23-p11.22, 14q11.1-q11.2 and 15q11.2, and gains of 11q13.2 and 11q13.2-q13.3 were detected as recurrent alterations. Gains of 3q26.31 and 11q13.2 and losses of 9p21.3 were exclusively detected in HPV-negative tumors. Two clusters of expression profiles were observed, being one composed mostly by HPV positive cases (83%). HPV-positive enriched cluster showed predominantly immune response-related pathways. Integrative analysis identified 10 modulators mapped in 11q13, which were frequently cancer-related. These 10 genes showed copy number gains, overexpression and an association with worse survival, further validated by TCGA database analyses. Overexpression of four genes (*ORAOV1, CPT1A, SHANK2* and *PPFIA1)* evaluated by RT-qPCR confirmed their association with poor survival. Multivariate analysis showed that *PPFIA1* overexpression and HPV status are independent prognostic markers. Moreover, *SHANK2* overexpression was significantly associated with incomplete response to treatment.
Conclusion: The integrative genomic and transcriptomic data revealed potential driver genes mapped in 11q13 associated with worse prognosis and response to treatment, giving fundamentals for the identification of novel therapeutic targets in OPSCC.

Introduction

In the last decades, several epidemiological studies have revealed decreased incidence of head and neck squamous cell carcinomas (HNSCC) in oral cavity and larynx as a consequence of lower exposure to the tobacco products. Nevertheless, an increasing incidence of

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oropharyngeal squamous cell carcinomas (OPSCC) mainly associated with oncogenic human papillomavirus (HPV) has been reported [1–6].

In general, HPV-positive OPSCC is associated with good prognosis, presenting better survival in comparison with HPV-negative cases [7–11]. De-escalation of radiation and chemotherapy for HPV-positive cases has been proposed and tested in different clinical trials aiming to avoid overtreatment and long-term toxicities [8,12,13]. However, accurate identification of cases with good prognosis and treatment-responsive tumors are critical findings, since distant metastasis may occur in a set of HPV-positive OPSCC [8,14]. In contrast, few advances have been made for treatment of HPV-negative OPSCC patients and a large number of them will present loco-regional recurrence [9].

The molecular mechanisms underlying oropharyngeal carcinogenesis have been investigated and potential biomarkers were reported, however the data are still unclear and controversial [15–19]. The integration of genomic and transcriptomic analysis can be used to identify cancer-driver genes and disrupted pathways, which can be drug targetable [20]. This strategy has revealed functionally relevant drivers involved in the carcinogenic process in different tumor types, including oral carcinoma [21], ovarian cancer [22], penile carcinoma [23,24], uterine leiomyoma [25] and leiomyosarcoma [26].

Integration of genomic, transcriptomic and epigenomic data of 279 HNSCC, including oral (n = 172, 62%), oropharyngeal (n = 33, 12%) and laryngeal (n = 72, 26%) carcinomas was reported by The Cancer Genome Atlas (TCGA) [27]. Distinct genetic alterations were observed between HPV-positive HNSCCs (68% in oropharynx) and HPV-negative cases. Recurrent deletions and truncating mutations of *TRAF3* found in HPV-positive tumors were associated with anti-viral immune response. Conversely, HPV-negative HNSCCs presented loss of 9p21.3 (including *CDKN2A* gene) and co-amplifications of 11q13 and 11q22, which contain genes implicated in cell death/NF-kB and Hippo pathways [27]. A distinct genetic subgroup of HPV-negative tumors is also being reported, characterized by low frequency of copy number alterations (CNA), wild-type *TP53*, mutation in *HRAS* and *CASP8* and more favorable prognosis [13].

Nevertheless, prognostic and predictive biomarkers in advanced OPSCC are still limited and need to be further investigated. In this study, we integrated DNA CNA and gene expression analyses to identify drivers in advanced OPSCC according to HPV status. *In silico* functional analysis was performed to identify genes and pathways associated with oropharyngeal carcinogenesis, which can reveal potential drug targets.

Patients and methods

Patients and samples

Fresh-frozen tumor biopsy samples from 40 OPSCC patients naive of treatment were obtained from A.C. Camargo Cancer Center and Barretos Cancer Hospital, Brazil. Eligibility criteria included patients harboring locally advanced clinical stages III, IVA and IVB according to AJCC (7^{Th} Edition). The follow-up time ranged from 0.5 to 190 months (mean of 53 months). The study was approved by the Human Research Ethics Committee from both Institutions (A.C. Camargo Cancer Center #1249/09 and Barretos Cancer Hospital #139/2008). All patients provided written informed consent. Patients underwent curative therapy according to standard clinical protocol taking into account the medical decisions for each patient, which included induction chemotherapy (IC) followed by radiotherapy with concurrent chemotherapy (CT-RT), and upfront surgical resection followed by radiotherapy with or without chemotherapy. Pretreatment risk stratification was defined as low, intermediate and high, based on HPV status, smoking history and tumor/node stage [7].

Treatment and response assessment

The majority of patients included in this study were treated by IC followed by CT-RT (n = 24). Patients were treated with a combination of docetaxel, cisplatin and 5-fluorouracil (TPF) as IC followed by radiotherapy and weekly carboplatin or cisplatin concurrent to radiotherapy. Cetuximab was employed in eight cases as concurrent therapy to radiation (one case received IC before). Six patients were treated by surgery followed by adjuvant therapy (RT or RT + CT); one patient deceased surgery and one patient lost the follow-up before completing the treatment (supplementary Table S1). Six patients were treated only by surgery followed by adjuvant therapy (RT and or CT), one patient deceased soon after surgery and one patient lost the follow-up before treatment begins.

Nucleic acids extraction and HPV genotyping

OPSCC samples (80% of tumor cells) and surrounding normal tissues were macrodissected for DNA (Qiagen DNeasy Blood & Tissue Kit; Qiagen, Valencia, CA) and total RNA extraction (RNeasy MiniKit; Qiagen, Valencia, CA). The Linear Array HPV Genotyping Test Kit (Roche Molecular Systems, Inc., Branchburg, NJ, USA) was used for HPV detection.

CNA analysis by array-based Comparative genomic Hybridization

OPSCC (n = 33) and normal commercial DNA (Promega) samples were differentially labeled (Genomic DNA Enzymatic Labeling Kit; Agilent Technologies) and the hybridized on Agilent Human CGH 180K Oligo Microarrays. Genomic data were extracted by Feature Extraction 10.1.1.1 software (Agilent Technologies) and analyzed using the Nexus Copy Number software (v.6.0, Biodiscovery, El Segundo, CA, USA). CNA was defined as exceeding the significance threshold of 1×10^{-6} and containing at least five consecutive altered probes per segment. The thresholds were defined as the average $\log_2$ CGH fluorescence ratio for copy gains ≥ 0.6, high copy number gains ≥ 1.4, losses ≤ -0.6 and homozygous losses ≤ -1.25. Genomic variants detected in control individuals from worldwide populations and classified as common (> 1%) according to DGV database (http://dgv.tcag.ca/dgv/app/home) were excluded. Alterations detected in at least 20% of the cases were selected for further analysis. The unsupervised hierarchical clustering analysis was performed using complete linkage and Euclidian distance.

Gene expression microarray

Total RNA from OPSCC (n = 33) and surrounding non-neoplastic oropharyngeal tissues (n = 3) were labeled and hybridized using the Two-Color Human GE 4x44K microarray platform (Agilent Technologies), following the manufacturer instructions. Data processing, quality control filtering and normalization (Lowess) were performed using the Feature Extraction v.10.1.1.1 software (Agilent Technologies) and an in-house pipeline. Gene expression analysis was performed using R *version* 2.15 (http://www.bioconductor.org/) and BRB ArrayTools software (v.4.4.0). An unsupervised hierarchical clustering analysis was employed with the most variable probes (interquartile range > 0.1) using complete linkage and Euclidian distance. Transcriptomic variations among clusters were identified by significance analysis of microarray (SAM) (false discovery ratio < 1%).

The CNA and expression microarray data are available at the Gene Expression Omnibus (GEO) (GSE111395).

Integrative analysis

Paired CNA and gene expression data of 33 OPSCC was integrated using COpy Number and EXpression In Cancer (CONEXIC) algorithm to identify drivers, which results in a ranked list with high scores modulators [28]. In this analysis, unbalanced expressed genes are correlated with the expression of group of genes (modules), and genomic regions