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Low prevalence of HPV-driven head and neck squamous cell carcinoma in North-East Italy

Lorena Baboci [a,b], Dana Holzinger [a], Paolo Boscolo-Rizzo [c], Giancarlo Tirelli [d], Roberto Spinato [e], Valentina Lupato [c], Roberto Fuson [e], Markus Schmitt [a], Angelika Michel [a], Gordana Halec [a], Maria Cristina Da Mosto [c], Michael Pawlita [a], Annarosa Del Mistro [f,*]

[a] Division of Molecular Diagnostics of Oncogenic Infections, Research Program Infection and Cancer, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 242, 69120 Heidelberg, Germany
[b] Department of Surgery, Oncology and Gastroenterology, University of Padua, Oncology and Immunology Section, Via Gattamelata 64, 35128 Padua, Italy
[c] Department of Neurosciences, ENT Clinic and Regional Center for Head and Neck Cancer, University of Padua, School of Medicine, Treviso Regional Hospital, P.le Ospedale 1, 31100 Treviso, Italy
[d] Head and Neck Department, Hospital of Cattinara, University of Trieste, Strada di Fiume 447, 34149 Trieste, Italy
[e] Venetian Department of Otolaryngology, Ospedale dell'Angelo, Via Paccagnella 11, 30174 Venezia Mestre, Italy
[f] Veneto Institute of Oncology IOV - IRCCS, Immunology and Molecular Oncology Unit, Via Gattamelata, 64, 35128 Padua, Italy

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ABSTRACT

Objectives: To investigate the frequency of Human papillomavirus (HPV)-driven head and neck squamous cell carcinoma (HNSCC) among patients living in North-East Italy, by assessing HPV-DNA positivity in all tumors and additional markers whenever possible.
Material and methods: HPV types, viral load, viral RNA, HPV16/18 E6 protein and $p16^{INK4a}$ and pRb expression were determined in primary tumor tissues from 247 HNSCC patients. Tumor-specific HPV seropositivity was analyzed in 102 patients.
Results: Tumor HPV-DNA prevalence was 8.5% overall (21/247) and 27% in oropharynx (17/63). HPV16 accounted for 95% of all HPV types found. Among HPV-DNA+ tumors, type-concordant HPV $E6^*I$ RNA prevalence was 79%. HPV DNA+ RNA+ tumors showed high viral load, up-regulated $p16^{INK4a}$, down-regulated pRb and presence of HPV16 E6 protein. Eight cases showed tumor-specific HPV seropositivity, all type-concordant with the tumor. Tumors were defined as HPV-driven when positive for HPV-DNA plus 2 additional HPV transformation-related markers.
Conclusion: Relative prevalence of HPV-driven tumors (14 HPV16, 1 HPV58) was 6% overall and 20% among oropharyngeal cancers. In the oropharynx the HPV-driven group showed a trend for better survival versus the HPV-negative group.

The relative prevalence of HPV-driven oropharyngeal cancer is low in North-East Italy as compared to Western and Northern Europe.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) accounts for approximately 5% of all cancers worldwide [1]. Globally, human papillomavirus (HPV) DNA was found in 30% of HNSCC, with a higher prevalence in oropharyngeal SCC (OPSCC) (46%) than oral SCC (24%) or laryngeal and hypopharyngeal SCC (22%) [2].

Among the 12 HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) defined as carcinogenic in cervical carcinoma (CxCa) by the International Agency for Research on Cancer (IARC), only type 16 is currently recognized as carcinogenic in OPSCC [3].

A wide variation of HPV DNA prevalence in OPSCC has been reported in the literature between regions and countries and within regions and countries, with overall figures higher in North

Abbreviations: HNSCC, head and neck squamous cell carcinoma; OPSCC, oropharyngeal squamous cell carcinoma; HPV, Human Papilloma Virus; CxCa, cervical carcinoma; FF, fresh-frozen; FFPE, formalin-fixed paraffin-embedded; PCR, polymerase chain reaction; MS, multiplex serology; GST, glutathione S-transferase; OS, overall survival; PFS, progression free survival

* Corresponding author.
E-mail addresses: lorena.baboci@gmail.com (L. Baboci), d.holzinger@dkfz.de (D. Holzinger), paolo.boscolorizzo@unipd.it (P. Boscolo-Rizzo), tirelli@units.it (G. Tirelli), robertospinato@hotmail.com (R. Spinato), valentinalupato@gmail.com (V. Lupato), roberto.fuson@gmail.com (R. Fuson), markus-schmitt@dkfz-heidelberg.de (M. Schmitt), a.michel@dkfz-heidelberg.de (A. Michel), g.halec@dkfz.de (G. Halec), mariacristina.damosto@unipd.it (M.C. Da Mosto), m.pawlita@dkfz-heidelberg.de (M. Pawlita), annarosa.delmistro@ioveneto.it (A. Del Mistro).

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America (60.4%) than in Europe (41.4%), and ranging from 56.5% for Northern to 24.2% for Southern countries within Europe [2].

While in the last decades HNSCC showed declining or unchanged incidence, an increase of HPV-associated HNSCC, particularly for the oropharynx, was observed [4]. In countries like USA, Denmark, Norway and Spain a 5% yearly increase (not statistically significant in Spain) of new OPSCC or tonsillar carcinoma cases was reported [5–8]. In other surveys the increase was not observed [9], while for several areas data are still scarce.

However, only a subset of HPV DNA positive HNSCC display HPV carcinogenic activity in the tumor tissue, i.e. are HPV-driven. Thus, the presence of HPV DNA alone is not a sufficient proof for causal involvement of HPV in HNSCC tissue [10]. The HPV carcinogenic model derived from CxCa [11] was also recently confirmed for OPSCC [12].

Truly HPV-driven tumor cells contain at least one viral genome copy per cell and express the viral oncogenes E6 and E7 [11]. Interaction of the viral E7 and E6 proteins with key regulators of cell cycle and apoptosis leads to up-regulation of cellular protein $p16^{INK4a}$ [13] and down-regulation of tumor suppressor proteins pRb and p53. Patients with invasive HPV-driven cervical [14], penile [15] and oropharyngeal [16] SCC frequently show strong antibody responses against HPV E6 and E7 proteins while such antibodies are rare in tumor-free populations. Clinically, patients with HPV DNA positive HNSCC in comparison to those with HPV DNA negative HNSCC present a smaller tumor size, early nodal involvement, and are more frequently nonsmokers and non drinkers [17]. Controversial data were reported regarding younger age of HPV-positive HNSCC patients [18].

In Italy, HNSCC accounts for approximately 5% of all cancers [19]. The prevalence of HPV-DNA positive HNSCC cases reported so far has ranged from 10% [20] to 46% [21]. Most of the studies relied on qualitative HPV DNA markers alone, only a few included detection of viral transcripts [20,22,23] or the expression of the $p16^{INK4a}$ protein [24,25].

In the present study, we provide new data on prevalence of HPV-driven HNSCC in North-East Italy, evaluate their clinical outcome and suggest a combination of markers to define the HPV-driven cases.

2. Patients and methods

2.1. Patients and samples

A total of 247 patients diagnosed with primary HNSCC located in oral cavity (International Classification of Diseases for Oncology (ICD-O): C02–04, C05.0, C06), oropharynx (ICD-O: C01, C05.1, C05.2, C09, C10), larynx (ICD-O: C32, C10.1) and hypopharynx (ICD-O C12–13) from 2003 to 2012 in the ENT (Ear Nose and Throat) Units of Treviso Regional Hospital, Hospital of Mirano and Hospital of Cattinara (Trieste), North-East Italy, were enrolled in the study. The first 77 enrolled patients have been already reported [20]. The whole study was approved by the ethic committee for clinical experimentation (CEP) of Treviso (Ethic votes: 345/AULSS9 and 421/AULSS9). All patients signed an informed consent.

Of the enrolled patients, 75% were males, 36% never tobacco smokers and 30% never alcohol drinkers. Of the tumors, 39% were in the larynx, 26% each in the oropharynx and the oral cavity and 10% in the hypopharynx. Small-sized T category (T1-T2) was present in 50% of the patients, with regional lymph nodes being involved in 52%. Only 1% of patients had distant metastases at diagnosis date.

From all the enrolled patients we obtained fresh frozen (FF) specimens of the neoplastic lesions. Formalin-fixed paraffin-embedded (FFPE) tumor tissues of 54 (22%) cases were retrieved from one of the three participating centres (Treviso), and used to complete molecular analyses of HPV-DNA-positive cases with small FF samples, and to perform immunohistochemistry. Sera were collected starting in 2010 from a total of 102 (41%) patients. Anatomical and clinical data were collected and available from the clinical database for each patient. No differences were observed between the above-mentioned subgroups and the whole case series. Histological diagnoses were determined by the local pathologists. Management decisions for the patients were not guided by knowledge of HPV status or other tested markers.

2.2. Tissues sectioning and DNA analyses

HPV analyses were carried out partly at the Veneto Institute of Oncology and partly at the German Cancer Research Center; homogenization in liquid nitrogen of the cryosections [26] was performed only in the German Center. The mean tumor content on adjacent hematoxylin-and-eosin stained sections was 55% (range 25–80%).

DNA was isolated from all 247 FF tumor tissues by Phenol-Chlorophorm (PC9) method as described in [27,28]. One-hundred-fourteen samples were re-sectioned and DNA was re-extracted by MagNA Pure 96 DNA and viral NA Large Volume Kit (Roche, Penzberg, Germany) according to the manufacturer´s recommendations [26,29].

FFPE sectioning and genomic DNA extraction were performed as described in [29].

PC9-extracted DNA was tested by MY09/MY11 PCR and restriction fragment length polymorphism (RFLP) analysis of the amplification products [30].

MagNA Pure-extracted DNA was analyzed by BSGP5+/6+-PCR/MPG_{51} assay, a broad-spectrum PCR for 51 mucosal HPV types followed by hybridization to type-specific probes on Luminex beads [31,32].

DNA quality of samples tested by MY09/MY11 was verified by amplification of the β-globin gene; the analytical sensitivity ranges from 10^2 to 10^4 HPV copies for any of the 49 HPV mucosal types [33]. All samples were also analyzed by HPV16 type-specific PCR [20].

The analytical sensitivity for any of the 51 HPV types detected by the BSGP5+/6+-PCR/MPG_{51} assay is equal or less than 100 viral genome copies [29].

HPV16 viral loads were determined by quantitative (q)PCR targeting E6 gene sequences (Schmitt et al. submitted) and expressed as HPV16 genome copy-number per cell. A cut-off of 0.1 copies per cell defined high ($HPV16_{high} \geq 0.1$) or low ($HPV16_{low} < 0.1$) viral load. Real-time qPCR for β-globin was used to verify the DNA quality and to measure the amount of input DNA. The analytical sensitivity for both HPV16 and β-globin detection is below 100 plasmid copies.

HPV58 viral load was estimated from the quantitative BSGP5+/6+- PCR/MPG_{51} data as described in [32].

2.3. RNA isolation and E6*I mRNA reverse transcription (RT)-PCR

RNA was isolated from FF sections using the RNeasy Mini kit (Qiagen, Hilden, Germany) and from FFPE sections using the Pure Link FFPE Total RNA Isolation Kit (Invitrogen, Carlsbad, CA). DNase I digestion (RNase-free DNase Set, Qiagen) was included to ensure an exclusive amplification of RNA, and was carried out on the RNA purifying columns during sample processing.

All samples HPV DNA positive by either method and a group of HPV DNA negatives were analyzed for ubiquitin C and HPV16 E6*I RNA and for E6*I RNA of additional HPV types positive by HPV genotyping [29].