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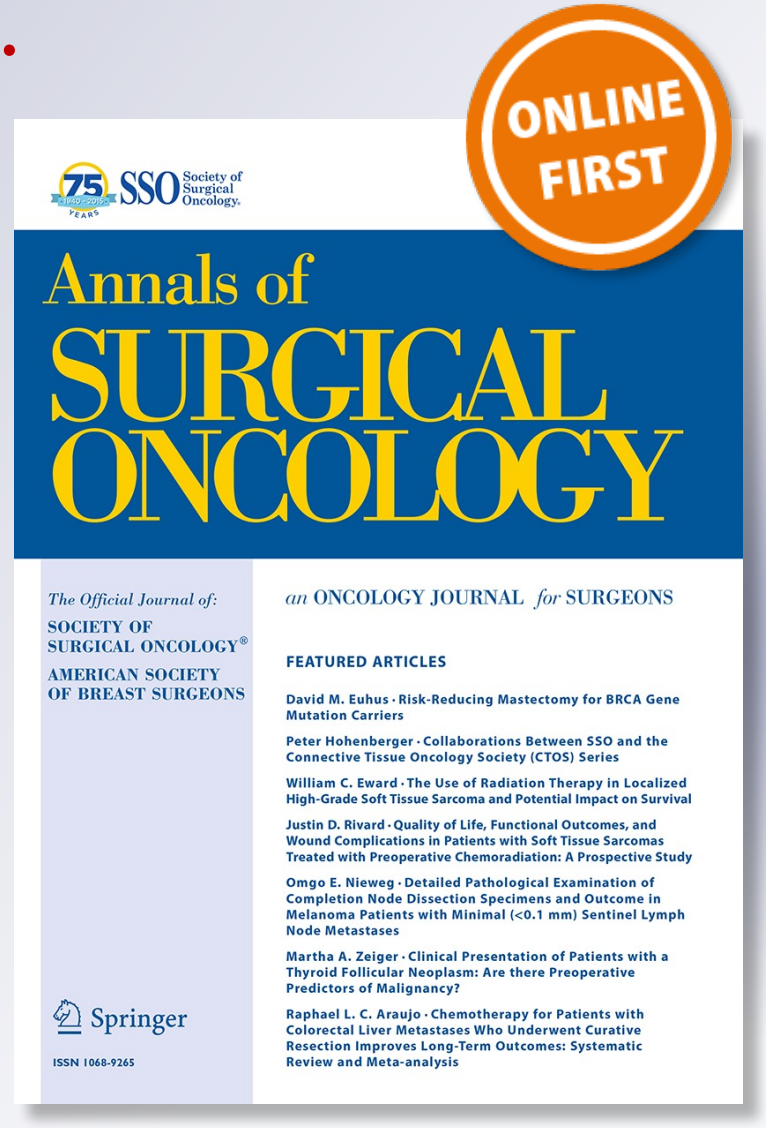

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HPV and EBV Infections in Neck Metastases from Occult Primary Squamous Cell Carcinoma: Another Virus-Related Neoplastic Disease in the Head and Neck Region

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ABSTRACT

Background. Approximately 1–9 % of all head and neck squamous cell carcinomas are neck metastases from clinically undetectable primary tumors. Human papillomavirus (HPV) and Epstein–Barr virus (EBV) are proven carcinogenic factors that are associated with oropharyngeal squamous cell carcinoma and nasopharyngeal carcinoma, respectively. In the present study, we evaluated the prevalence of these viruses in neck metastases from unknown primary squamous cell carcinoma.

Methods. We evaluated fresh samples from a consecutive series of 22 neck dissections for metastases from unknown primary squamous cell carcinoma obtained between 2010 and 2012 at a single institution. The samples were tested for the presence of HPV E6 and E7 mRNA and EBV DNA.

Results. Oncogenic viral infections were detected in 12 cases (54 % total; 2 HPV18, 5 HPV16, 2 EBV infection, and 3 EBV/HPV16 coinfections). The most frequent primarily involved neck level in our series was IIA (70 %), which had the highest prevalence of viral infection (66 %). We did not find any other significant correlations between virus detection and clinicopathologic parameters or prognosis.

Discussion. Neck metastasis from unknown primary squamous cell carcinoma could be another virus-related malignancy in the head and neck region, along with nasopharyngeal and oropharyngeal carcinoma. An evaluation of the impact of viral infection on patient prognosis and sensitivities to different treatment modalities could modify our prognostic assessments and treatment planning. Furthermore, virus detection would have a decisive impact on diagnostic/decisional algorithms, especially if detection methods are implemented on cytologic samples (e.g., thin prep).

Metastatic cancers from unknown primary lesions represent a heterogeneous group that is estimated to account for 5–10 % of all malignancies. Approximately 90 % of these neoplasms are squamous cell carcinomas (SCC), and the remainder includes adenocarcinomas, melanomas, and other rare histologic variants.[1–3]

In the head and neck, cervical lymph node metastasis is often the first clinical manifestation of SCC. An appropriate head and neck examination coupled with endoscopy and imaging usually allows the detection of the primary SCC in the head and neck mucosal areas. Very small tonsil and tongue base SCCs that are radiologically and endoscopically undetectable and that present with enlarged neck nodes can be adequately diagnosed by base-of-tongue biopsies and tonsillectomies, as indicated in many guidelines.[4] Nevertheless, in approximately 1–9 % of all head and neck malignancies, which are usually characterized by unilateral metastases at level IIa (jugulodigastric), the primary SCC remains clinically undetectable (unknown primary tumor, UP) after a thorough and appropriate assessment and rarely becomes apparent on follow-up.[5–7]

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Such neck nodal metastases from unknown primary tumors, even if rare, are considered a distinct clinical entity in the National Comprehensive Cancer Network (NCCN) head and neck guidelines, and they present peculiar issues to the clinician in both the diagnostic and therapeutic phases.[4]

Notoriously, cancers that arise from mucosal head and neck sites can be etiologically linked with oncogenic viral infections, namely nasopharyngeal carcinoma with the Epstein–Barr virus (EBV) and oropharyngeal SCC with high-risk human papillomavirus (HPV) genotypes.[8–12] Knowledge of virus-driven carcinogenesis and experimental evidence indicate that in a very high proportion, approaching 100 %, of metastases from primary virus-induced head and neck SCCs, viral nucleic acid can still be detected.[8,13–16]

The evaluation in neck metastatic nodes of both EBV and HPV is therefore rational and has been proposed as a tool in the search for the primary tumor site, with promising preliminary results.[15,17–21]

EBV infection is typically associated with nonkeratinizing nasopharyngeal carcinomas (World Health Organization type 2), which clearly have better survival and are markedly more radiosensitive than differentiated cases (World Health Organization type 1).[9–11,22,23]

Also HPV-associated oropharyngeal SCC has a better prognosis; HPV16 is the only proven carcinogenic HPV genotype.[8,12,24–26]

A hypothesis that suggests the involvement of any of these oncogenic viruses in the development of metastases from UPs through the regression of immunogenic primary tumors and the concomitant progression of regional metastases appears intriguing and somewhat rational. Such a hypothesis has been tested in a few HPV studies using a small number of human specimens and different methods, including the poorly reliable $p16^{ink4a}$ immunochemistry and fluorescence in-situ hybridization assays.[26–34] Most of these studies described a significant prevalence of HPV infection, with rates as high as 66.7 % in two small series of 6 and 3 cases.[31,32]

To our knowledge, the etiologic role of EBV in metastases from UPs has not been specifically and systematically addressed by any study, even if occasional EBV detection has been reported in metastases from undetectable primary in some studies.[13,14] Additionally, EBV positivity has been reported in neck metastases without any evident primary tumor at diagnosis, which successively developed overt nasopharyngeal carcinomas.[13,18]

The aim of the present study was to systematically evaluate the prevalence of oncogenic viral infections (both HPV and EBV) in neck nodal metastases for which the primary site remains undetected.

MATERIALS AND METHODS

In the present study, we enrolled 22 consecutive cases of neck metastases from SCC for which the primary site was not detected after complete head and neck examination and assessment. Patients were evaluated and treated at the Institute of Otolaryngology, Università Cattolica del Sacro Cuore, Rome, Italy, between January 2010 and January 2012. Cases for which a primary SCC was detected in any phase of the described assessment were excluded from the present study.

Assessment

In all patients, endoscopy of the upper airways and imaging of the head and neck region [contrast-enhanced magnetic resonance imaging and computed tomography (CT)] and the chest (contrast-enhanced CT) were negative for primary tumors. A fine-needle aspiration biopsy (FNAB) was then performed with either positive or suspicious result for SCC. Neither the subsequent positron emission tomography (PET)/CT scans, also used to refine the tumor staging and detect possible positive contralateral nodes, nor the multiple nasopharyngeal and base-of-tongue biopsies and bilateral tonsillectomies allowed the identification of the primary tumor. Personal data were collected, and particular attention was given to environmental risk factors (smoking, alcohol consumption). Patients with a history of malignancy and prior excision, destruction, or regression of cutaneous lesions were excluded from the study.

After all the above-cited steps, a comprehensive neck dissection was performed (bilateral in 1 case, monolateral in the others) to obtain a definite histologic report, pathologic staging, and gross disease removal. We recorded the number and level (according to the classification of the American Academy of Otolaryngology) of positive neck nodes and collected fresh samples from the bulkiest node.[35,36]

The histologic report confirmed the presence of metastatic SCC in one or more neck nodes. After confirmation of squamous histology, the patients signed informed consent for their participation in the present study, which had been previously approved by the ethical committee.

Adjuvant Treatment

After neck dissection, we always administered an adjuvant treatment that encompassed the tumor bed (60–66 Gy) as well as potential primary sites, as determined by the neck node levels involved (50–66 Gy), and the contralateral neck (44–64 Gy to uninvolved nodal stations) for

TABLE 1 Descriptive statistics of the main variables concerning patients and tumor parameters

Characteristic	22 patients
Age at diagnosis	
Median	62
Range	41–83
Follow-up period in months	
Median	18.5
Range	4–38.17
Pack-years of cigarette smoking	
Median	12
Range	0–31
Sex no. (%)	
Male	19 (86 %)
Female	3 (14 %)
N classification no. (%)	
N1	2 (9.1 %)
N2a	4 (18.2 %)
N2b	14 (63.7 %)
N2c	1 (4.5 %)
N3	1 (4.5 %)
Histopathological grade no. (%)	
I	1 (4.5 %)
II	8 (36.4 %)
III	13 (59.1 %)
Level of the largest metastatic node no. (%)	
IIA	13 (59.1 %)
III	4 (18.2 %)
IV	3 (13.6 %)
VA	2 (9.1 %)
Number of positive nodes	
Median	2
Range	1–5
Detection of viruses no.(%)	
No viruses	10 (45.5 %)
EBV (alone)	2 (9.1 %)
HPV16 (alone)	5 (22.7 %)
HPV18	2 (9.1 %)
EBV-HPV16 (coinfection)	3 (13.6 %)
Detection of distant metastases (at follow up) no.(%)	
No	18 (81.8 %)
Yes	4 (18.2 %)
Cause of death no.(%)	4 deaths
Neck relapse	2 (50 %)
Distant metastasis	2 (50 %)

N1 disease without extracapsular spreading.[37] We also administered concomitant cisplatin (100 mg/m^2 every 3 weeks) to N2–3 cases and any cases of extracapsular spreading.[4]

Detection of Viral Infection

Fresh tumor samples were collected from the neck dissection samples and were immediately stored in RNA*later* RNA Stabilization Reagent (Qiagen, Valencia, CA, USA) at −80 °C until tested. DNA and RNA were extracted from each sample. Nucleic acid was extracted from the tissues with the NucliSens easyMAG platform (bioMérieux, Rome, Italy), according to the manufacturer's instructions.

The Q-EBV Alert Kit (Nanogen Advanced Diagnostics, Milan, Italy) was used to detect EBV-DNA. Briefly, 5 µl of the extracted specimen were added to 20 µl of the PCR master mix, which contained the AmpliMIX, AmpliMASTER, and AmpliPROBE, and the reactions were processed under the following conditions: 50 °C for 2 min, an initial denaturation step at 95 °C for 10 min, and 45 cycles at 95 °C for 15 s, 60 °C for 45 s, and 72 °C for 15 s. The cycle thresholds (Ct) for a tenfold dilution series of plasmid DNA were plotted to yield a standard curve. Amplification data were analyzed by SDS Software (Life Technologies, Carlsbad, CA).

RNA samples were analyzed for HPV E6 and E7 mRNA with the NucliSENS EasyQ HPV v1 test (bioMérieux), which is based on the NASBA technique. This test specifically detects the E6/E7 mRNA from five high-risk HPV types (HPV16/18/31/33/45). Briefly, three premixes were produced by reconstituting reagent spheres in reagent diluent and then adding primer/molecular beacon mixes (U1A/HPV16, HPV18/31, or HPV33/45). Ten microliters of premix was added to each well, followed by 5 µl of sample, and these mixtures were incubated for 4 min at 65 °C and 2 min at 41 °C. The reactions were initiated by adding enzymes and were measured in real time with a NucliSENS EasyQ analyzer at 41 °C. Data analysis was performed with NucliSENS EasyQ Director software.

Statistical Analysis

Statistical analysis was performed by JMP 7.0.1 (SAS Institute, Cary, NC).

The α level was fixed at 0.05 for all statistical tests. Correlations between the different nominal variables were evaluated by the Chi square test. For a more thorough evaluation of the impact of viral infections on neck metastases, we transformed the nominal pN parameters to numerical variables (numeric N score; pN2a = 2, pN2b = 3, pN2c = 4, pN3 = 5). Relapse-free survival and disease-specific survival were calculated from the date of the neck dissection until the date of death, relapse, or last follow-up, and survival curves were plotted according to the Kaplan–Maier method and compared according to different parameters with the Wilcoxon test.

RESULTS

The patient and tumor characteristics are shown in Table 1. None of the patients who agreed to participate in the present study was lost to follow-up.

The most frequently involved nodal level was IIA. Only one patient showed bilateral nodes in a PET/CT scan, which was also confirmed in the pathologic sample.

We recorded four deaths, two of which were due to distant metastasis, despite locoregional control in 1 case.

Approximately 55 % of the analyzed metastatic nodes were positive for at least one of the viruses under investigation. The most frequently detected virus was HPV16 (8 cases; 36 %), followed by EBV (5 cases; 23 %); notably, in 3 cases (14 %), both viruses were detected in the tumor samples. Finally, 2 cases (9 %) were positive for HPV18.

The localization of the bulkiest metastatic node at level IIA was associated with an oncogenic viral infection ($p = 0.05$, χ^2 test), and in particular with a high-risk HPV genotype ($p = 0.0276$, χ^2 test; Fig. 1). None of the other personal and tumor specific parameters, including age, sex, nominal and numerical N classifications, number of involved nodes, histopathologic grading, and distant metastases in the follow-up, was significantly modified by viral infection.

Disease-specific and relapse-free survival at 2 years were 86 and 83 %, respectively, when patients were grouped according to any of the viral infections under study, and no statistically significant differences were detected with respect to survival.

DISCUSSION

The proportion of human cancers caused by infectious agents was recently estimated to exceed 20 %. The identification of new cancer sites that can be attributed to infectious (particularly viral) agents indicates that more cancers are potentially preventable. On the other hand, many infection-associated malignancies show a better prognosis than their non-virus-related counterparts and might respond to specific therapies against the etiologic infectious agents (such as hepatitis C virus and *Helicobacter pylori*–associated lymphomas).[8]

To our knowledge, this is the first study to systematically evaluate the prevalence of oncogenic viral infection (both high-risk HPV and EBV) in neck nodal metastases from UPs. We detected at least one oncogenic virus in 55 % of the samples from the overall series, and this rate increased to 70 % among cases in which the bulkiest node was observed at level IIA. Notably, a 55 % viral infection rate is comparable to the rates observed among nasopharyngeal and oropharyngeal carcinomas.[9–12,26,38] Therefore, if we agree with NCCN and consider UP to be a distinct pathologic entity, the present findings lead us to consider such disease, particularly when in the presence of a bulky IIA node, to be another virus-related malignancy, similar to oropharyngeal and nasopharyngeal carcinoma.

The immunogenicity of the oncogenic virus could be a key factor in the pathogenesis of this condition and might determine the host immune system-mediated eradication of a hypothetical primary tumor localized somewhere within

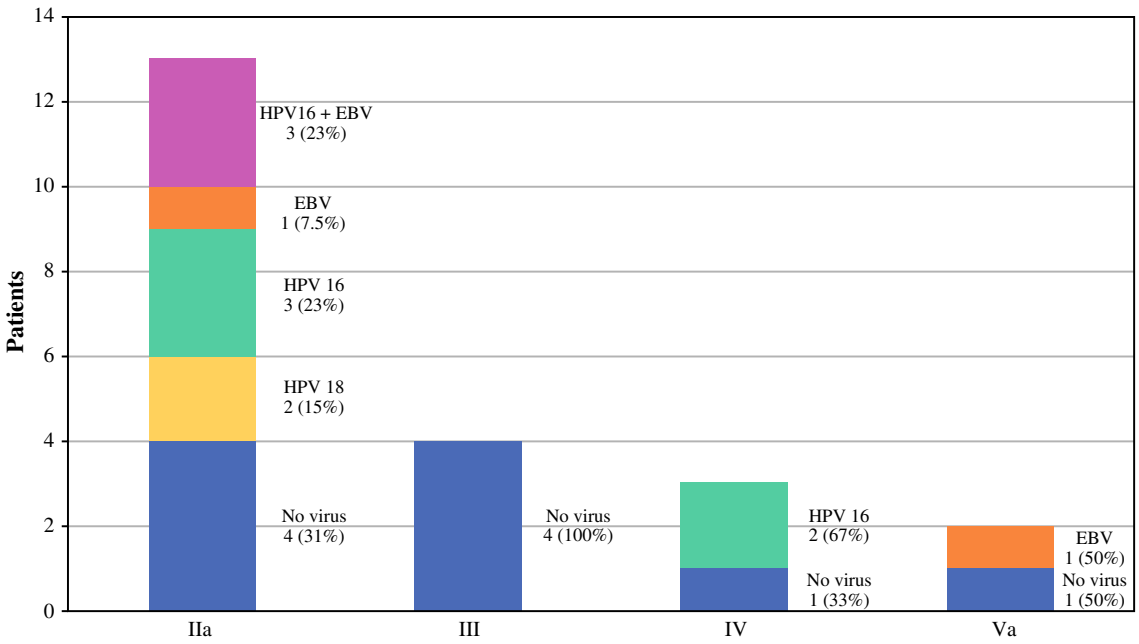

FIG. 1 Prevalence of viral infection according to most involved neck level

the head and neck region, while regional metastasis, which would be somehow less immunogenic, progresses to clinical manifestation. The detection of HPV18, which is not a proven oncogenic virus in the oropharynx, confirms this hypothesis.[8,26] In fact, HPV18 might be too immunogenic to give rise to a clinically evident primary SCC in the oropharynx, but a subclinical HPV18-related mucosal lesion could cause early nodal metastases before regressing. This would explain why, when using the same diagnostic tools, we never detected HPV18 in oropharyngeal SCC.[26] This genotype would be therefore specifically associated with metastasis from an unknown primary SCC.

The relatively high incidence of distant metastases is not very surprising, as these are usually associated with regional spreading. Nevertheless, this phenomenon does not display any statistical correlation with viral infection.

In general, the prognosis does not seem to be affected by the presence of any oncogenic virus, in contrast to the expectations based on data from the nasopharynx and oropharynx, and also to a recent study on metastases from unknown primaries, in which virus-associated cancers generally have a better prognosis.[12,22,24,25,39] Nevertheless, the small number of patients and the short follow-up period did not allow us to draw definitive conclusions about this matter, and the hypothesis that a better prognosis is associated with viral detection remains rational and deserves to be tested again in a wider series with a longer follow-up time because, if confirmed, this finding could facilitate treatment selection in clinical practice.

The main objectives of diagnostic evaluations of patient with metastatic cancer from an UP are to determine the histology and to exclude the presence of a primary tumor.[3] These goals are pursued through different sequential steps in an algorithm that the NCCN guidelines attempted to standardize and that might be partly modified by the present findings.[4] The pathologic diagnosis in the absence of a primary site should rely on a FNAB; the excision of a single node is acceptable only if frozen sections are available and the surgeon is ready to immediately perform a neck dissection when a solid malignancy is detected in the frozen sections. The diagnosis of HPV infection from neck node FNAB has been described.[40] According to our experience and to preliminary reports in the literature, thinprep samples from a FNAB permit the extraction of adequate amounts of nucleic acids to diagnose both high-risk HPV and EBV according to the methods described in this study.[20,21] The detection of one of these viruses (particularly HPV) would be an indirect confirmation of the squamous histology, although it should always be noted that EBV has been associated also with several hematologic malignancies, as well as gastric cancer.[8,11,41,42] In any case, the detection of an oncogenic virus in a FNAB or histologic sample compatible with SCC would certainly be helpful when searching for the primary tumor, would guide the biopsy site choice and would indicate a bilateral tonsillectomy if high-risk HPV is detected.

DISCLOSURE The authors declare no conflict of interest.

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