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Original contribution

Human papillomavirus infection is not involved in esophageal verrucous carcinoma☆,☆☆

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Summary Verrucous carcinoma of the esophagus (VCE) is a rare variant of squamous cell cancer, with a puzzling clinical, etiological, and molecular profile. The etiological involvement of human papillomavirus (HPV) in the cancer's natural history is controversial. This study considers 9 cases of VCE, focusing on patients' clinical history before surgery, histologic phenotype, immunophenotype (epidermal growth factor receptor [EGFR], E-cadherin, cyclin D1, p16, and p53 expression), HPV infection, and *TP53* gene mutational status (exons 5-8). Using 3 different molecular test methods, not one of these cases of VCE featured HPV infection. The only case with synchronous nodal metastasis was characterized by a *TP53* missense point mutation in association with high EGFR and low E-cadherin expression levels. In conclusion, HPV infection is probably not involved with VCE, while *TP53* gene mutation, EGFR overexpression, and E-cadherin loss might fuel the tumor's proliferation and lend it a metastatic potential.

1. Introduction

Verrucous carcinoma of the esophagus (VCE) is a rare epithelial malignancy characterized by distinctive histologic features, including an exophytic growth of well-differentiated and keratinized squamous epithelium, and a pushing (often lymphocyte-rich) invasive front [1,2]. Since its first description by Minielly and colleagues [3], about 80 cases of VCE have been reported [3-40]. The natural history includes a slow local invasiveness with rare nodal metastases; morbidity and

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mortality have been mainly associated with direct infiltration of nearby structures in advanced local disease or with complication after surgery [27,32]. Like other esophageal cancers, VCE triggers no early alarm symptoms, and its puzzling endoscopic appearance, the limited diagnostic value of (superficial) biopsy sampling, and an equivocal histologic phenotype all contribute to delaying a patient's assessment for cancer [27].

Possible risk factors for VCE include smoking, alcohol consumption, hiatal hernia, achalasia, chronic or reflux esophagitis, esophageal diverticula, lye ingestion, radiations, and human papillomavirus (HPV) infection [27]. The etiological role of HPV has been explored by applying both direct (e.g., polymerase chain reaction [PCR] analysis and hybridization) and indirect (e.g., immunohistochemistry) methods, neither of which have so far generated conclusive results [5,7,11,14,17,18,22,25-27,32,33].

As for the disease's molecular background, most studies have focused on *TP53* mutational status and p53-protein expression [14]. We still do not know whether the biological profile of VCE also includes some of the molecular disruptions associated with the family of esophageal squamous cancers [41], and particularly overexpression of epidermal growth factor receptor (EGFR), and cyclin D1 [42-45]; *p16INK4A (CDKN2A)* mutation and promoter hypermethylation [46]; and *CDH1* promoter hypermethylation [47].

This study concerns the clinical history, histologic features, HPV status, and molecular profiles of a monoinstitutional series of 9 Italian patients with VCE.

2. Materials and methods

2.1. Materials

The study concerns 9 white Italian patients with a histology-based diagnosis of VCE. The patients' demographics and clinical history are shown in Table 1. None of the considered cases had medical history of esophageal motility issues.

In all the cases considered, the specimens were fixed (in neutral formalin) immediately after the surgical procedure, and multiple cancer and noncancer tissue samples were obtained after appropriate fixation, according to the local protocol. The histologic assessment was based on the 2010 World Health Organization criteria [2]. All cases were jointly assessed by 3 pathologists with elective expertise in gastrointestinal diseases (M. F., R. C., and I. C.), distinguishing between papillary-type and warty-type VCEs.

Immunohistochemical staining was done automatically with the Bond Polymer Refine Detection kit (Leica Biosystems, Newcastle upon Tyne, United Kingdom) in the BOND-MAX system (Leica Biosystems). Formalin-fixed and paraffin-embedded (FFPE) sections 4 μm thick were stained with the primary antibodies for EGFR (clone H11; Dako Cytomation, Glostrup, Denmark; dilution 1:400), E-cadherin (clone NCH-38; Dako Cytomation; dilution 1:200), cyclin D1 (clone EP12; Dako Cytomation; dilution 1:20), p16INK4A (p16; clone JC8; Santa Cruz Biotechnology, Santa Cruz, CA; prediluted), and p53 (clone DO-7; Dako Cytomation; prediluted), as described elsewhere [48-50].

Only membrane immunoreactions were considered when assessing EGFR and E-cadherin expression, whereas for cyclin D1, both nuclear and cytoplasmic staining were measured. Immunohistochemical expression was scored on a 4-point scale: 0, 0 to 5%; 1, 6% to 30%; 2, 31% to 60%; and 3, 61% to 100%. The intensity of the immunoreaction was distinguished as follows: 0, absent; 1, weak; 2, moderate; and 3, strong. The resulting values were combined to obtain an overall score ranging from 0 to 6, which was then classified as a low (score 0-2), moderate (score 3-4), or high (score 5-6) expression. The immunohistochemical expression of p16 was assessed by applying a 4-tiered scale, as proposed by Roncalli et al [43]. P53 was considered positive if more than 75% of neoplastic cells showed strong nuclear immunostaining. All immunoreactions were assessed independently by 2 pathologists (R. C. and I. C.), and where their opinions diverged, a third expert gastrointestinal pathologist (M. F.) was also involved.

2.2. Handling of FFPE tissue samples and DNA extraction

To prevent cross-contamination, a dedicated protocol was established for sectioning the FFPE tissue samples. Only disposable supplies were used, and a new sterile microtome blade was used for each specimen. Five consecutive 10-μm-thick FFPE sections were obtained from each paraffin block. The first and last sections (both 5 μm thick) obtained from the paraffin block were used for histologic confirmation (hematoxylin and eosin staining) of the presence of the target lesions. To enrich their neoplastic cellularity (>70% of tumor cells), cancer areas were microdissected manually (under direct microscopic visualization) by a pathologist (I. C.).

DNA was extracted and purified using the QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions [51]. DNA concentration and integrity were assessed using 1 μL of sample and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA) on a Qubit 3.0 fluorometer (Thermo Fisher Scientific). All extracts were stored at −80°C until further use.

2.3. HPV detection and genotyping

Different detection methods were used for HPV detection/genotyping. DNA from vulvar condyloma with known HPV-6 and HPV-11 infections and DNA from normal cervix resected for uterine leiomyoma were used as positive and negative controls, respectively, and were run along with each test.

2.3.1. Technique number 1

Ten microliters of DNA template was used for real-time PCR with target-specific primers, and TaqMan fluorescent probes with the Realquality RQ-HPV HR/LR Multiplex (AB Analitica, Padua, Italy) to detect high-risk (International Agency

Table 1 Clinicopathological features of our series of verrucous carcinomas of the esophagus

Case	Sex	Age (y)	Risk factors	Medical history	Family medical history	Tumor site	T	N	pTNM stage	P16	RQ-HPV HR/LR	Inno-LIPA	Ampliquality HPV-type	ISH	*TP53* gene status
1	♂	67	–	–		M	3	1	IIIA	–	–	–	–	–	M246I
2	♀	61	–	Hypertension, arteriopathy, liver disease, COPD		U	1	0	IA	–	–	–	–	–	WT
3	♀	60	—	Colorectal cancer	—	L	1	0	IA	–	–	–	–	–	WT
4	♂	61	Smoking, alcohol consumption	Polycystic liver	Brother: brain cancer	L	1	0	IB	+	–	–	–	–	WT
5	♀	71	–	–	Son: testicular cancer	L	1	0	IB	–	–	–	–	–	WT
6	♂	30	Smoking, alcohol consumption	–	–	L	3	0	IIA	–	–	–	–	–	WT
7	♀	68	–	Tonsillectomy, chronic gastritis, eosinophilic esophagitis	–	U	1	0	IA	+	–	–	–	–	WT
8	♀	70	–	Tonsillectomy, *Candida* esophagitis	Sisters: ORL cancer and leukemia; daughter: breast cancer	M	1	0	IA	–	–	–	–	–	WT
9	♀	60	–	Kidney cancer	Aunt: uterine cancer	M	1	0	IB	–	–	–	–	–	WT

Abbreviations: ♂, male; ♀, female; ISH, in situ hybridization; L, lower part of the esophagus; M, middle part of the esophagus; N, lymph node; T, primary tumor; U, upper part of the esophagus; WT, wild type.

Fig. 1 Representative pictures of papillary-type (A) and warty-type (B) esophageal verrucous carcinoma. Papillary-type VCE is characterized by complex and arborescent papillae with irregular fibrovascular cores, whereas the warty type shows long, undulating, and rounded papillae lined with acanthotic and markedly hyperkeratotic epithelium (hematoxylin and eosin stain, original magnification ×10).

for Research on Cancer [IARC] groups 1 and 2A) HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 and genotype for HPV-16 and HPV-18 and to detect low-risk (IARC group 2B and others) HPV types 26, 53, 66, 67, 70, 73, 82, 6, and 11 and genotype for HPV-6 and HPV-11.

2.3.2. Technique number 2

Ten microliters of the DNA solution from each tumor sample was used for PCR amplification and reverse hybridization with the INNO-LiPA HPV Genotyping Extra II line probe assay (Fujirebio Europe, Ghent, Belgium) to detect and identify the following HPV genotypes: high-risk (IARC groups 1 and 2A) HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, and low-risk (IARC group 2B and others) HPV types 26, 53, 66, 70, 73, 82, 6, 11, 40, 42, 43, 44, 54, 61, 62, 67, 81, 83, and 89 [52,53]. Another 10 μL of DNA template was used with another line probe assay, the Ampliquality HPV-type Express v3.0 kit (AB Analitica), again based on the PCR amplification and reverse hybridization principle, following the

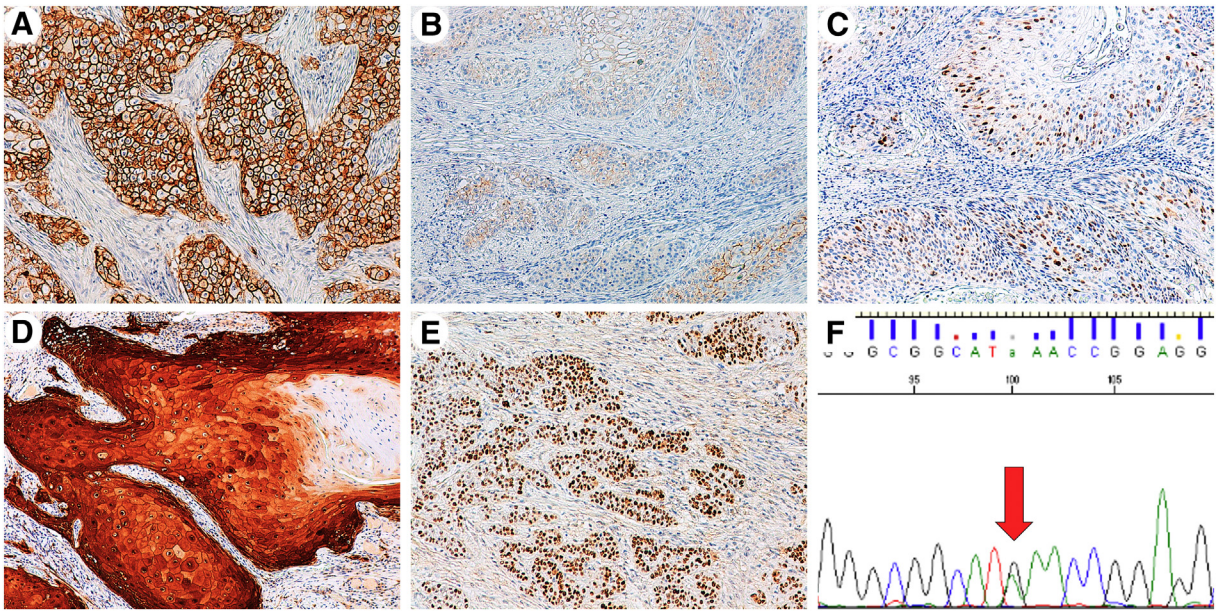

Fig. 2 Representative examples of positive EGFR (A), E-cadherin (B), cyclin D1 (C), p16INK4a (D), and p53 (E) immunoreactions in VCE. Original magnification ×100 for all. F, Electropherogram of the c.738G >A *TP53* point mutation determining the p.M246I substitution observed in the metastatic tumor.

manufacturer's instructions. This method can detect and typify the following HPV genotypes: high-risk (IARC groups 1 and 2A) HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, and low-risk (IARC group 2B and others) HPV types 26, 53, 66, 70, 73, 82, 6, 11, 40, 42, 43, 44, 54, 61, 62, 67, 81, 83, 89, 55, 64, 69, 71, 72, 84, 87, and 90.

2.3.3. Technique number 3

In situ PCR amplification and chromogenic (nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate [NBT/BCIP]) hybridization were performed on an FFPE section 4 to 5 μm thick from each tumor sample using full-length locked nucleic acid genomic probes for the high-risk (IARC groups 1 and 2A) HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, and 68, and for the low-risk (IARC group 2B and others) HPV genotypes 6, 11, 30, 42, 43, 44, and 70, as explained elsewhere [54]. Treated sections were counterstained with nuclear fast red.

2.4. *TP53* mutational analysis

The mutational status of exons 5, 6, 7, and 8 of the *TP53* gene was assessed by PCR amplification of appropriate fragments and conventional Sanger sequencing. PCR products were purified using the ChargeSwitch Pro PCR Clean-up Kit (Invitrogen, Carlsbad, CA). Sequence analyses were performed on the 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Exon 5 primers: forward *5′-CAC TTG TGC CCT GAC TTT CA-3′,* reverse *5′-AAC CAG CCC TGT CGT*

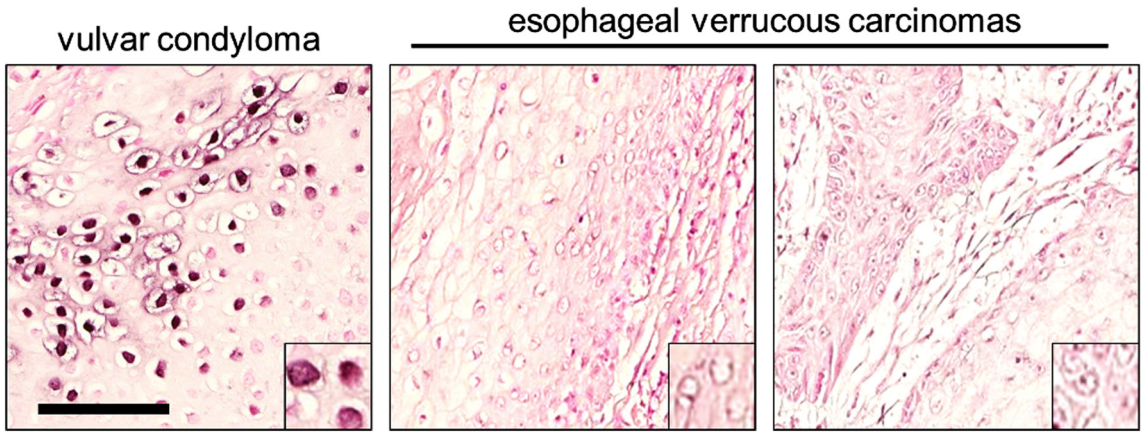

Fig. 3 Representative pictures of in situ PCR and chromogenic hybridization for HPV. A vulvar condyloma with known HPV-6 and HPV-11 infection was used as a positive control and displayed an evident nuclear signal (nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate [NBT/BCIP] stain). All VCEs resulted negative. Original magnification ×400; scale bar, 100 μm.

Table 2 Literature data on verrucous squamous cell carcinoma of the esophagus

Reference	No. of cases	Sex (no. of cases)	Age (y), mean ± SD	Tumor site (no. of cases)	N status (no. of cases)	HPV infection	Detection method	Molecular alteration
Minielly et al [3]	5	♂ (3); ♀ (2)	58.2 ± 13.9	U (3); L (2)	−	NE	–	NE
Parkinson et al [24]	1	♂	76	M	−	NE	–	NE
Meyerowitz and Shea [19]	1	♂	45	L	−	NE	–	NE
Sakurai et al [29]	1	♀	78	U	−	NE	–	NE
Agha et al [4]	1	♂	66	U	−	NE	–	NE
Koerfgen et al [16]	2	♂	64.5 ± 14.8	L (2)	−	NE	–	NE
Jasim and Bateson [13]	1	♂	79	L	−	NE	–	NE
Biemond et al [7]	1	♀	76	M	−	Absent	Hybridization and Southern blot	NE
Roach and Barr [28]	1	♂	67	U	−	NE	–	NE
Poljak and Cerar [26]	1	Unknown	Unknown	Unknown	−	Absent	Hybridization and PCR	
Garrard et al [12]	1	♀	51	L	−	NE	–	NE
Mori et al [20]	1	♂	75	M-L	−	NE	–	NE
Malik et al [18]	1	♂	66	L	−	Absent	IHC	NE
Kavin et al [14]	1	♀	60	M	+	Absent	Hybridization and PCR	WT *TP53* gene
Devlin et al [11]	1	♀	56	L	−	Absent	IHC	NE
Pfitzmann et al [25]	1	♀	66	M	−	Absent	PCR	NE
Odze [23]	1	♂	73	U	−	NE	–	NE
De Petris et al [10]	2	♂ (2)	65.5 ± 10.6	L (2)	−	Absent	Hybridization and IHC	NE
Chryssostalis et al [9]	1	♀	73	U	Unknown	NE	–	NE
Na et al [22]	1	♂	50	L	−	Absent	Hybridization	NE
Tonna et al [32]	1	♂	61	U	−	HPV 51	PCR	NE
Munson et al [21]	1	♀	63	M	+	NE	–	NE
Lagos et al [17]	1	♂	74	L	+	Absent	Hybridization	NE
Vieira et al [33]	1	♂	58	U	−	HPV 11	Hybridization	NE
Ahmed et al [5]	1	♀	58	L	−	HPV (not genotyped)	IHC	NE
Ramani et al [27]	1	♂	78	M-L	+	Absent	Hybridization	NE
Sweetser et al [31]	11	♂ (7); ♀ (4)	66.2 ± 5.7	L (4); M (2); U-M-L (5)	−	NE	–	NE
Kulemann et al [55]	1	♂	45	U	−	NE	–	NE
Cox et al [36]	1	♂	62	M	−	Absent (in the gross specimen)	IHC	NE
Abe et al [35]	1	♂	68	Unknown	−	NE	–	NE
Ogawa et al [56]	1	♂	77	M-L	Unknown	NE	–	NE
Hoffmann et al [37]	2	♂ (1); ♀ (1)	56.5 ± 4.5	L (2)	−	HPV (not genotyped)	IHC	NE
Behrens et al [38]	15	♂ (11); ♀ (4)	68.6 ± 10.6	Unknown	+ (1)	NE	–	NE
Brandalise et al [39]	1	♂	64	U-M-L	−	NE	–	NE
Egeland et al [40]	2	♂ (2)	63.0 ± 4.0	L (2)	−	Absent	Unknown	NE

Abbreviations: ♂, male; ♀, female; IHC, immunohistochemistry; L, lower part of the esophagus; M, middle part of the esophagus; N, lymph node; NE, not evaluated; U, upper part of the esophagus; WT, wild type.

CTC T-3′. Exon 6 primers: forward *5′-CAG GCC TCT GAT CCT CAC T-3′*, reverse *5′-CTT AAC CCC TCC TCC CAG AG-3′*. Exon 7 primers: forward *5′-CCA CAG GTC TCC CCA AGG-3′*, reverse *5′-CAG CAG GCC AGT GTG CAG-3′*. Exon 8 primers: forward *5′-GCC TCT TGC TTC TCT TTT CC-3′*, reverse *5′-TAA CTG CAC CCT TGG TCT CC-3′*.

3. Results

3.1. Clinical findings

The clinicopathological features of the 9 cases of VCE are summarized in Table 1. The patients' mean age was 60.9 ± 12.4 years (range, 30-71 years), and the male-to-female ratio was 1:0.5. Two patients had a history of alcohol consumption and smoking, one of eosinophilic esophagitis and one of esophageal *Candida* infection. Two patients had previously had surgery for carcinoma (colorectal cancer and renal cell carcinoma), but none of the patients had a positive family history of tumors of the digestive system.

Two of 9 tumors were located in the upper third of the esophagus, 3 in the middle, and 4 in the lower third. The cancer ranged in largest diameter from 2 to 8 cm (mean, 5.0 ± 2.8 cm). Eight patients underwent total esophagectomy with regional lymph node resection, whereas a cervical esophagectomy with regional lymph node excision was performed in 1 patient. Neoadjuvant chemotherapy and radiotherapy were administered to 3 patients.

3.2. Histologic findings

The maximum level of neoplastic infiltration was into the periesophageal fat (pT3) in 2 cases. The mean number of lymph nodes isolated was 31.3 ± 11.3 (range, 13-51). Nodal metastases were detected in 1 case (in 7/13 nodes). The pathological stage (pTNM) of the tumors is listed in Table 1.

Seven of 9 cases (including the one with metastases) featured the warty phenotype, whereas the other 2 cases were classified as papillary (Fig. 1). All specimens showed the typical exophytic growth consisting of well-differentiated squamous epithelium (with marked keratinization in 6 cases). Three cases revealed comedo-like necrosis (none of these patients had received neoadjuvant treatments). Pseudokoilocytotic keratinocytes were observed (at least focally) in all tumors. Seven of 9 cases (including the metastatic VCE) featured deep pushing margins, whereas the other 2 cases showed a jagged invasion front. Perineural invasion was seen in 3 of 9 cases. Vascular invasion was only found in association with nodal metastases.

3.3. Immunohistochemical profile

Five of 9 tumors showed a low EGFR expression (whereas immune expression was moderate or high in 2/9, one of which was the metastatic VCE; Fig. 2). E-cadherin expression was high in 6 of 9 tumors, moderate in 2 tumors, and only low in the 1 metastatic tumor (Fig. 2). Cyclin D1 expression was low in 3 cases (including the metastatic tumor), moderate in 4 cases, and high in 2 cases (Fig. 2). Overexpression of p16 was found in 2 of 9 VCEs (Fig. 2). In none of the tumors, p53 immunoreaction was completely absent, and 4 of 9 VCEs showed a significant p53 nuclear immunostaining in more than 75% of neoplastic cells (Fig. 2).

3.4. HPV status

With all the methods used, all the VCEs were always negative for HPV infection (Fig. 3).

3.5. *TP53* mutational status

Only one cancer featured mutations in *TP53* exons 5, 6, and 8. The *TP53*-mutated VCE harbored a heterozygous point mutation in position c.738G >A of exon 7 of *TP53*, resulting in the mutant variant p.M246I of p53 (Fig. 2). The mutation was associated with immunohistochemical p53 overexpression.

4. Discussion

VCE is a rare variant of esophageal squamous cancer, and its clinical features and molecular profile have yet to be definitively established [1]. Table 2 lists all cases of VCE reported in the available scientific literature (cases with significant inconsistencies vis-à-vis currently adopted diagnostic criteria are not included) [6,8,30,34]. The present monoinstitutional series of 9 white (Italian) patients largely reflects the typical clinicopathological picture of VCE in terms of patient's age at presentation, cancer site within the esophageal tract, and histologic features [3-34,36,37,39,40,56].

None of our patients' relatives had a history of gastrointestinal malignancies. One patient had already undergone surgery for renal cancer, and another for colon cancer. Three of our 9 patients had risk factors for tumor development (alcohol consumption, smoking, and chronic esophagitis). One patient had a history of *Candida*, and another had experienced eosinophilic esophagitis (both conditions are known to be associated with an increased risk of esophageal neoplasms [57,58].

In keeping with the available information, the present series of VCE featured local invasiveness, with a low potential for nodal involvement. Among the 67 tumors classifiable as VCE according to current criteria in the literature, lymph node metastases were reported in just 5 cases (7.5%), with an overall prevalence basically consistent with that of the present series (1/9 tumors) [14,17,21,27,38]. It is noteworthy that this one case of nodal involvement was associated with vascular invasion at the primary esophageal site.

The molecular profile of this metastatic case featured a high EGFR protein expression (oncogenic stimulus) and a *TP53*

missense mutation (loss of tumor suppressor control), a combination promoting tumor proliferation. It was also the only case showing a significant downregulation of E-cadherin expression, potentially leading to the acquisition of metastatic potential through epithelial-mesenchymal transition [59,60].

Based on the morphologic similarities between HPV-related neoplastic lesions of the uterine cervix and esophageal squamous malignancies, Syrjänen and colleagues [61] initially suggested a potential involvement of HPV in esophageal carcinogenesis. In the present series, however, 4 different molecular methods failed to detect any evidence of HPV infection despite the presence of the pseudo-koilocytotic changes. From 1987 to 2014, HPV infection has been tested in 18 VCEs; only 3 of them (16.7%) featured HPV infection (one for the low-risk HPV-11, one for high-risk HPV-51, and no genotyping was available for the last case) [5,7,11,14,17,18,22,25-27,32,33,36,37,40].

In the HPV-positive cases reported by Tonna and colleagues [32], the high-risk HPV 51 was initially assessed on presurgical biopsy samples, but the infection was not confirmed on the tissue samples obtained from the surgical specimen. In such cases, the discordant results were tentatively attributed to DNA degradation or low viral load in the tissue samples obtained from the surgical specimens. A false-positive result in the biopsy material cannot be ruled out, however.

HPV infection was tested by p16-IHC in 7 of the cases reported in the literature, and the result was considered consistent with HPV infection in only one of them (no other tests were applied) [5,10,11,18,36,37]. In our study, 2 of the VCEs might have been considered HPV related if the p16 threshold usually applied in the cervical cancer setting had been adopted (ie, strong/diffuse nuclear/cytoplasmic immunoreaction in all tumor cells). Most VCEs in the present series featured p16 expression in isolated/microclustered cancer cells. Based on previous evidence and the present findings, this feature should be critically reconsidered: instead of HPV infection being responsible for the p16-positive immunophenotype, it might be that this represents an attempt by the cellular tumor-suppressor mechanisms to block uncontrolled proliferation.

Because different molecular HPV detection methods, such as PCR of the L1 region or hybridization for different genotypes, were used in the 11 cases described in the literature, there is room for doubt concerning their outcomes [5,7,11,14,17,18,22,25-27,32,33]. For a start, the sensitivity of each approach is different. Some studies are also rather dated, and the reliability of the tests performed may have been suboptimal at the time. To avoid the risk of false-negative results, we used 4 different, sensitive molecular techniques for HPV detection and typing, so we are confident of the absence of HPV infection in our 9 cases [62].

Another strength of our study lies in that only resected specimens were analyzed, preventing the risk of false-negative results due to any uneven distribution of the virus in the tumor tissue. The extracted DNA was also checked for integrity before testing.

In conclusion, VCE is a rare esophageal tumor that occurs more frequently in the sixth decade of life and in the lower third of the esophagus. Local cancer invasion is associated with a low prevalence of nodal involvement. The only case with synchronous nodal metastasis in our series was associated with a *TP53* missense point mutation, EGFR overexpression, and E-cadherin downregulation. In the present series of 9 Italian patients, there was not a single case associated with HPV infection.

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Authors' contributions: All authors of this research article participated directly in the planning and execution of the study, and in the analysis of the results.

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