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ORIGINAL ARTICLE

Clinicopathologic characterization of cervical metastasis from an unknown primary tumor: a multicenter study in Korea

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Background: Research regarding cervical metastasis from an unknown primary tumor (CUP) according to human papillomavirus (HPV) and Epstein-Barr virus (EBV) status in Korea has been sporadic and small-scale. This study aims to analyze and understand the characteristics of CUP in Korea according to viral and p16 and p53 status through a multicenter study. **Methods:** Ninety-five cases of CUP retrieved from six hospitals in Korea between January 2006 and December 2016 were subjected to high-risk HPV detection (DNA in situ hybridization [ISH] or real-time polymerase chain reaction), EBV detection (ISH), and immunohistochemistry for p16 and p53. **Results:** CUP was HPV-related in 37 cases (38.9%), EBV-related in five cases (5.3%), and unrelated to HPV or EBV in 46 cases (48.4%). HPV-related CUP cases had the best overall survival (OS) ($p = .004$). According to the multivariate analysis, virus-unrelated disease ($p = .023$) and longer smoking duration ($p < .005$) were prognostic factors for poor OS. Cystic change ($p = .016$) and basaloid pattern ($p < .001$) were more frequent in HPV-related cases, and lymphoepithelial lesion was frequent in EBV-related cases ($p = .010$). There was no significant association between viral status and p53 positivity ($p = .341$), smoking status ($p = .728$), or smoking duration ($p = .187$). Korean data differ from Western data in the absence of an association among HPV, p53 positivity, and smoking history. **Conclusions:** Virus-unrelated CUP in Korea had the highest frequency among all CUP cases. HPV-related CUP is similar to HPV-mediated oropharyngeal cancer and EBV-related CUP is similar to nasopharyngeal cancer in terms of characteristics, respectively.

Key Words: Unknown primary neoplasms; Lymph node metastasis; Human papillomavirus virus; Epstein-Barr virus infections

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Cervical metastasis from an unknown primary tumor (CUP) in the head and neck region is not rare. Reasons that primary tumors can avoid detection include small size, slow growth, hidden location, or involution [1,2]. The incidence of CUP in the West is approximately 1%–9% of all head and neck carcinomas [3-5]. Histologically, the most common type is squamous cell carcinoma, accounting for 75%–90% of all cases [6-8].

Human papillomavirus (HPV) [9,10] and Epstein-Barr virus (EBV) [11,12] are well-established carcinogenic viral agents in head and neck cancers and are increasingly associated with HPV infection [13,14]. HPV-mediated oropharyngeal cancer cases have better survival rates than HPV-unrelated cases. The unique biologic behavior and natural history of diseases caused by these viruses necessitated the development of a new staging system [15]. The eighth edition of the American Joint Committee on Cancer (AJCC) introduced separate classification systems for unknown primary head and neck carcinomas, as follows: (1) EBV-positive, (2) HPV-positive, and (3) EBV-negative and HPV-negative [13,16].

A meta-analysis of 17 studies on head and neck squamous cell carcinomas of unknown primary cause reported that the HPV-positivity rate of CUP was 49%, which is 10% lower than the

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rate of oropharyngeal carcinoma. The survival benefit of HPV positivity was also favorable [17]. In contrast, a recent Korean study revealed that HPV status did not significantly affect the survival rate in unknown primary head and neck cancer [18]. Previous studies in Korea offered similar results, reporting no significant survival benefit of HPV in oropharyngeal carcinoma [19,20]. These results may be attributed to the high smoking rate in Korea.

HPV infection and disruptive *TP53* mutations are considered non-overlapping events, so HPV infection has shown an inverse relationship with *TP53* mutations in various studies [21,22]. The *TP53* mutation rate in CUP or the relationship between the *TP53* mutation and HPV infection may also influence the behavior of CUP in Koreans.

To date, research in Korea has been sporadic and small-scale. We therefore sought to analyze and understand the clinico-pathological characteristics of CUP in Korea through a multicenter study. We intended to investigate the role of HPV and EBV in CUP in Korea with p16 or p53 expression.

MATERIALS AND METHODS

Patient selection and patient inclusion as CUP

Between January 2006 and December 2016, 159 patients diagnosed with metastatic carcinoma in cervical lymph nodes from the unknown primary site were analyzed across six hospitals (Asan Medical Center, Seoul St. Mary's Hospital, Sanggye Paik Hospital, Kangbuk Samsung Hospital, Chungbuk National University Hospital, and Severance Hospital, Yonsei University College of Medicine) in South Korea.

Among 159 cases, the primary sites in 64 cases were located after pathologic diagnosis of lymph node metastasis. The remaining 95 cases were categorized as CUP, wherein the primary sites were not identified at the time of study initiation.

Hematoxylin and eosin–stained slides and formalin-fixed paraffin-embedded tissue were used for the analysis. Tissue microarrays (TMA) were constructed from representative parts of the tumor.

The research ethics committee of each institution deliberated on this process.

Clinicopathologic characteristics

Clinical data were collected through medical records, including age at diagnosis, sex, smoking history, follow-up duration, and clinical outcomes. Pathologists at each hospital reviewed the hematoxylin-and-eosin–stained slides of corresponding hospital cases; confirmed the lymph node location of the metastatic tumor; and determined the size of the largest metastasis, extranodal extension, and N category. Histological findings were also analyzed, including keratinization, cystic change, basaloid pattern, and lymphoepithelial lesions.

Immunohistochemistry

Immunohistochemical (IHC) staining was performed on 4-μm sections of TMA using the Ventana autostainer and UltraView DAB detection kit (Ventana Medical Systems, Tucson, AZ, USA), according to the manufacturer's instructions. The antibodies we used were p16INK4a (1:6, clone E6H4, mouse mAb, Ventana Medical Systems) and p53 (1:1,500, clone M7001, mouse mAb, Dako, Glostrup, Denmark). According to the eighth edition of the AJCC cancer staging manual, p16 immunostaining was positive when it showed greater than a +2/+3 intensity in > 75% of tumor cells. Separately, the result of p53 was positive if nuclear staining was present in > 10% of tumor cells.

In situ hybridization

EBV infection was evaluated by RNA in situ hybridization (ISH) (INFORM EBER, Ventana Medical Systems) and HPV infection was evaluated by DNA ISH (INFORM HPV III Family 16 Probe (B), Ventana Medical Systems). The INFORM HPV III Family 16 Probe (B) detects the following high-risk HPV types: 16, 18, 31, 33, 35, 45, 52, 56, 58, and 66. ISH was considered positive when > 70% of tumor cells showed nuclear staining.

Real-time polymerase chain reaction

For cases wherein HPV ISH was unavailable, real-time polymerase chain reaction (RT-PCR) was performed. Nucleic acids were extracted from 10-μm (×5) paraffin tissue sections, and the CFX96TM RT-PCR system (Bio-Rad Laboratories, Hercules, CA, USA) and Anyplex II HPV28 Detection system (31744024, Seegene, Seoul, Korea) were used. Anyplex II HPV28 detection (A) detects the following high-risk HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68.

Grouping of cervical metastases according to HPV and EBV status

Patient cases were divided into three groups according to HPV and EBV status, as follows: HPV-related CUP, EBV-related CUP, and CUP unrelated to both HPV and EBV. HPV-related CUP was defined by results of p16 overexpression via IHC, positive high-risk HPV via HPV ISH, or positive high-risk HPV via RT-PCR analysis. EBV-related CUP was defined by EBV

confirmation via EBV ISH. HPV-unrelated and EBV-unrelated CUP was defined by results negative for p16, HPV ISH, HPV RT-PCR, and EBV ISH. Cases were categorized as "not determined" when the HPV or EBV ISH test finding was unavailable.

N category

According to the eighth edition of the AJCC cancer staging manual, three different approaches were applied to cases with unknown primary tumors. As the primary T category is T0, the N caegory was determined by different staging systems according to EBV and HPV status, i.e., "nasopharynx" staging for EBV-related CUP, "HPV-mediated (p16+) oropharyngeal cancer" staging for HPV-related CUP, or "cervical lymph nodes and unknown primary tumors of the head and neck" staging for EBV-unrelated and HPV-unrelated CUP.

Statistical analysis

Fisher's exact test analyzed the variance between the three groups, which was then compared between them. Overall survival (OS) was counted from the first diagnosis of CUP to the date of death or final follow-up. Univariate and multivariate Cox proportional hazard regression models were used to identify a significant factor in predicting OS. Kaplan-Meier assessment was used to analyze OS, and the effect of groups on OS was investigated using the log-rank test. The variance ($p < .05$) significantly affecting OS in the univariate analysis was further tested through multivariate analysis. $p < .05$ was considered to be statistically significant. In the statistical comparison among groups according to viral status, cases that were "not determined" (n = 7) were excluded.

RESULTS

Clinicopathologic and immunohistochemical factors of CUP cases

The histologic type of all 95 CUP cases was squamous cell carcinoma. Fifty-two patients (54.8%) were aged ≥60 years. Among the 95 CUP cases, 77 were male (81.1%) and 18 were female (18.9%). Thirty patients were non-smokers (31.6%), 16 were past smokers (16.8%), 32 were current smokers (33.7%), and smoking status was not available for 17 patients (17.9%). Additionally, 19 patients (20.0%) had smoked for 1–20 pack-years, 16 patients (16.8%) had smoked for 21–40 pack-years, 10 (10.5%) had smoked for >40 pack-years, and smoking duration data were not available for 20 patients (21.1%). The most frequent size of the largest metastatic lymph node was ≤3 cm (n = 53, 55.8%). Cervical level II lymph node involvement was identified in 68 patients (71.6%), with the highest frequency. Extranodal extension was identified in 35 patients (36.8%). Stage N1 (n=39, 41.1%) was the most common stage. Keratinization was identified in 36 patients (37.9%), cystic changes were identified in 29 patients (30.5%), a basaloid pattern was identified in 37 patients (38.9%), and lymphoepithelial lesions were identified in 20 patients (21.1%). The p16 IHC finding was positive in 34 patients (35.8%) and negative in 54 patients (56.8%). The p53 IHC finding was positive in 51 patients (53.7%) and negative in 34 patients (35.8%) (Table 1).

High-risk HPV and EBV status by DNA ISH or RT-PCR and comparison with p16 and p53 positivity

EBV ISH was available in 86 cases and was positive in five cases (5.8%). HPV ISH or RT-PCR was available in 82 cases, and high-risk HPV was detected in 22 cases (26.8%).

Among the five EBV-positive cases, four (80%) were p53 positive, one (20%) was p53 negative, and none of the five cases showed p16 overexpression or identified high-risk HPV in RT-PCR or HPV ISH. A p16 overexpression status was significantly associated with high-risk HPV status. Among the 22 HPV-positive cases, 19 showed p16 overexpression, while 13 among the 60 HPV-negative cases showed p16 overexpression (90.5% vs. 21.7%, $p < .001$) (Table 2).

There was no significant relationship between p16 overexpression and p53 positivity ($p = .113$) nor between high-risk HPV infection and p53 positivity ($p = .203$).

Clinicopathologic comparison among three groups based on viral status

According to the IHC and ISH results, 37 cases (38.9%) were in the HPV-related group; five (5.3%) were in the EBV-related group; and 46 (48.45%) were in the HPV- and EBV-unrelated group, which displayed the greatest frequency of cases (Table 1).

The frequency of those <60 years of age was high in the HPV-related CUP group (n = 24, 64.8%). Meanwhile, the frequency of patients aged ≥60 years was high in the HPV- and EBV-unrelated CUP group (n = 33, 71.7%), which showed a significant difference between groups ($p = .013$) (Table 1).

There was no significant difference in smoking status ($p = .738$) or smoking duration ($p = .187$) between groups divided by HPV/EBV status.

The ≤3-cm cases showed the greatest frequency of the largest lymph node size across all three groups. In the EBV-related group, the largest lymph node size was ≤3 cm in all five cases. In the

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Table 1. Characteristics according to the HPV and EBV status of cervical metastasis from an unknown primary tumor

	Total (n=95)	HPV-related (n=37, 38.9%)	EBV-related (n=5, 5.3%)	HPV and EBV-unrelated (n=46, 48.4%)	Not determined (n=7, 7.4%)	p-value
Age (yr)						.013
<50	12 (12.6)	7 (18.9)	1 (20.0)	4 (8.7)	0	
50–59	31 (32.6)	17 (45.9)	2 (40.0)	9 (19.6)	3 (42.9)	
60–69	26 (27.4)	8 (21.6)	2 (40.0)	15 (32.6)	1 (14.3)	
≥70	26 (27.4)	5 (13.5)	0	18 (39.1)	3 (42.9)	
Sex						.907
Male	77 (81.1)	31 (83.8)	4 (80.0)	37 (80.4)	5 (71.4)	
Female	18 (18.9)	6 (16.2)	1 (20.0)	9 (19.6)	2 (28.6)	
Smoking status						.738
Non-smoker	30 (31.6)	11 (29.7)	1 (20.0)	16 (34.8)	2 (28.6)	
Past smoker	16 (16.8)	7 (18.9)	0	9 (19.6)	0	
Current smoker	32 (33.7)	11 (29.7)	1 (20.0)	15 (32.6)	5 (71.4)	
NA	17 (17.9)	8 (21.6)	3 (60.0)	6 (13.0)	0	
Smoking duration (pack-years)						.187
Never-smoker	30 (31.6)	11 (29.7)	1 (20.0)	16 (34.8)	2 (28.6)	
1–20	19 (20.0)	9 (24.3)	0	7 (15.2)	2 (28.6)	
21–40	16 (16.8)	8 (16.2)	0	11 (19.5)	1 (14.3)	
≥41	10 (10.5)	1 (2.7)	1 (20.0)	7 (15.2)	1 (14.3)	
NA	20 (21.1)	10 (27.0)	3 (60.0)	7 (15.2)	0	
Lymph node size (cm)						.030
≤3.0	53 (55.8)	23 (62.2)	5 (100)	24 (52.2)	1 (14.3)	
>3.0, ≤6.0	28 (29.5)	6 (16.2)	0	20 (43.5)	2 (28.6)	
>6.0	7 (7.4)	5 (13.5)	0	2 (4.3)	0	
NA	7 (7.4)	3 (8.1)	0	0	4 (57.1)	
Lymph node level						
Level I	11 (11.6)	4 (10.8)	1 (20.0)	6 (13.0)	0	
Level II	68 (71.6)	30 (81.1)	4 (80.0)	28 (60.9)	6 (85.7)	
Level III	35 (36.8)	12 (32.4)	1 (20.0)	21 (45.7)	1 (14.3)	
Level IV	16 (16.8)	3 (8.1)	1 (20.0)	12 (26.1)	0	
Level V	9 (9.5)	1 (2.7)	2 (40.0)	5 (10.9)	1 (14.3)	
Level VI	0	0	0	0 (0)	0	
Retropharyngeal	2 (2.1)	0	1 (20.0)	1 (2.2)	0	
Axillary	1 (1.1)	0	0	1 (2.2)	0	
Supraclavicular	3 (3.2)	1 (2.7)	0	2 (4.3)	0	
Extranodal extension						.046
Positive	35 (36.8)	10 (27.0)	3 (60.0)	20 (43.5)	2 (28.6)	
Negative	44 (46.3)	25 (67.6)	2 (40.0)	15 (32.6)	2 (28.6)	
NA	16 (16.8)	2 (5.4)	0	11 (23.9)	3 (42.9)	
N category						.001
1	39 (41.1)	28 (75.7)	5 (100)	5 (10.9)	1 (14.3)	
2	1 (1.1)	1 (2.7)	0	0	0	
2a	6 (6.3)	0	0	6 (13.0)	0	
2b	5 (5.3)	0	0	5 (10.9)	0	
2c	2 (2.1)	0	0	2 (4.3)	0	
3	5 (5.3)	5 (13.5)	0	0	0	
3a	0	0	0	0	0	
3c	22 (23.2)	0	0	20 (43.5)	2 (28.6)	
NA	15 (15.8)	3 (8.1)	0	8 (17.4)	4 (57.1)	
Keratinization						.023
Present	36 (37.9)	11 (29.7)	0	24 (52.2)	1 (14.3)	
Absent	59 (62.1)	26 (70.3)	5 (100)	22 (47.8)	6 (85.7)	

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Table 1. Continued

	Total (n=95)	HPV-related (n=37, 38.9%)	EBV-related (n=5, 5.3%)	HPV and EBV-unrelated (n=46, 48.4%)	Not determined (n=7, 7.4%)	p-value
Cystic change						.016
Present	29 (30.5)	18 (48.6)	0	11 (23.9)	0	
Absent	66 (69.5)	19 (51.4)	5 (100)	35 (76.1)	7 (100)	
Basaloid pattern						<.001
Present	37 (38.9)	23 (62.2)	2 (40.0)	11 (23.9)	1 (14.3)	
Absent	58 (61.1)	14 (37.8)	3 (60.0)	35 (76.1)	6 (85.7)	
Lymphoepithelial lesion						.010
Present	20 (21.1)	8 (21.6)	4 (80.0)	7 (15.2)	1 (14.3)	
Absent	75 (78.9)	29 (78.4)	1 (20.0)	39 (84.8)	6 (85.7)	
p16 IHC						<.001
Positive	34 (35.8)	34 (91.9)	0	0	0	
Negative	54 (56.8)	2 (5.4)	5 (100)	46 (100)	1 (14.3)	
NA	7 (7.4)	1 (2.7)	0	0	6 (85.7)	
p53 IHC						.341
Positive	51 (53.7)	18 (48.6)	4 (80.0)	29 (63.0)	0	
Negative	34 (35.8)	17 (45.9)	1 (20.0)	16 (34.8)	0	
NA	10 (10.5)	2 (5.4)	0	1 (2.2)	7 (100)	
Follow-up duration (mo)						
Median	23.0	47.63	6.0	16.7	52.8	
Range	0.0–163.0	0–154	1–67	0–163	4–113	
Clinical outcome						
NED	56 (58.9)	27 (73.0)	2 (40.0)	22 (47.8)	5 (71.4)	.011
AWD	16 (16.8)	7 (18.9)	2 (40.0)	7 (15.2)	0	
DOD/DOC	23 (24.3)	3 (8.1)	1 (20.0)	17 (37.0)	2 (28.6)	

Values are presented as number (%) unless otherwise indicated.
In the statistical comparison among groups according to viral status, cases of 'not determined (n=7)' are excluded.
HPV, human papillomavirus; EBV, Epstein-Barr virus; NA, not assessed; IHC, immunohistochemistry; NED, no evidence of disease; AWD, alive with disease, DOD, death of disease, DOC, death of other cause.

Table 2. Comparison of p16 overexpression and high-risk HPV detection

	HPV ISH or RT-PCR			Total
	Positive	Negative	ND	
p16				
Positive	19	13	2	34
Negative	2	47	5	54
ND	1	0	6	7
Total	22	60	13	95

HPV, human papillomavirus; ISH, in situ hybridization; RT-PCR, real-time-polymerase chain reaction; ND, not determined.

HPV- and EBV-unrelated group, the frequency of lymph nodes >3 cm was highest (n=22, 47.8%) among the three groups, and the frequency of cases with lymph nodes measuring 3–6 cm was particularly high (n=20, 43.5%).

Level II lymph node involvement was most frequently observed across all three groups. Extranodal extension was infrequent in the HPV-related group (n=10, 27.0%), while the HPV- and EBV-unrelated group (n=20, 43.5%) and the EBV-related group (n=4, 80%) showed a significantly greater frequency (p=.046) (Table 1).

All five patients in the EBV-related group were stage N1, which was the most frequent stage in the HPV-related group (n=28, 75.7%). In the HPV- and EBV-unrelated group, N3c (n=20, 43.5%) was the most frequent stage, and the differences were statistically significant (p<.001) (Table 1).

Among histologic factors, cystic changes and the basaloid pattern were significantly frequently observed in the HPV-related group (n=18, 48.6%, and n=23, 62.2%, respectively). Lymphoepithelial lesions were significantly common in the EBV-related group (n=4, 80%, p=.010). The histologic features in a representative case for the three groups are shown in Fig. 1.

The results of the p53 IHC were not significantly different between the groups based on viral status (p=.341) (Table 1).

Regarding the clinical outcomes, the proportion of patients with no evidence of disease (NED) was 73.0% (27/37) in the HPV-related group, representing the highest frequency among the groups. In the HPV- and EBV-unrelated group, the rate of

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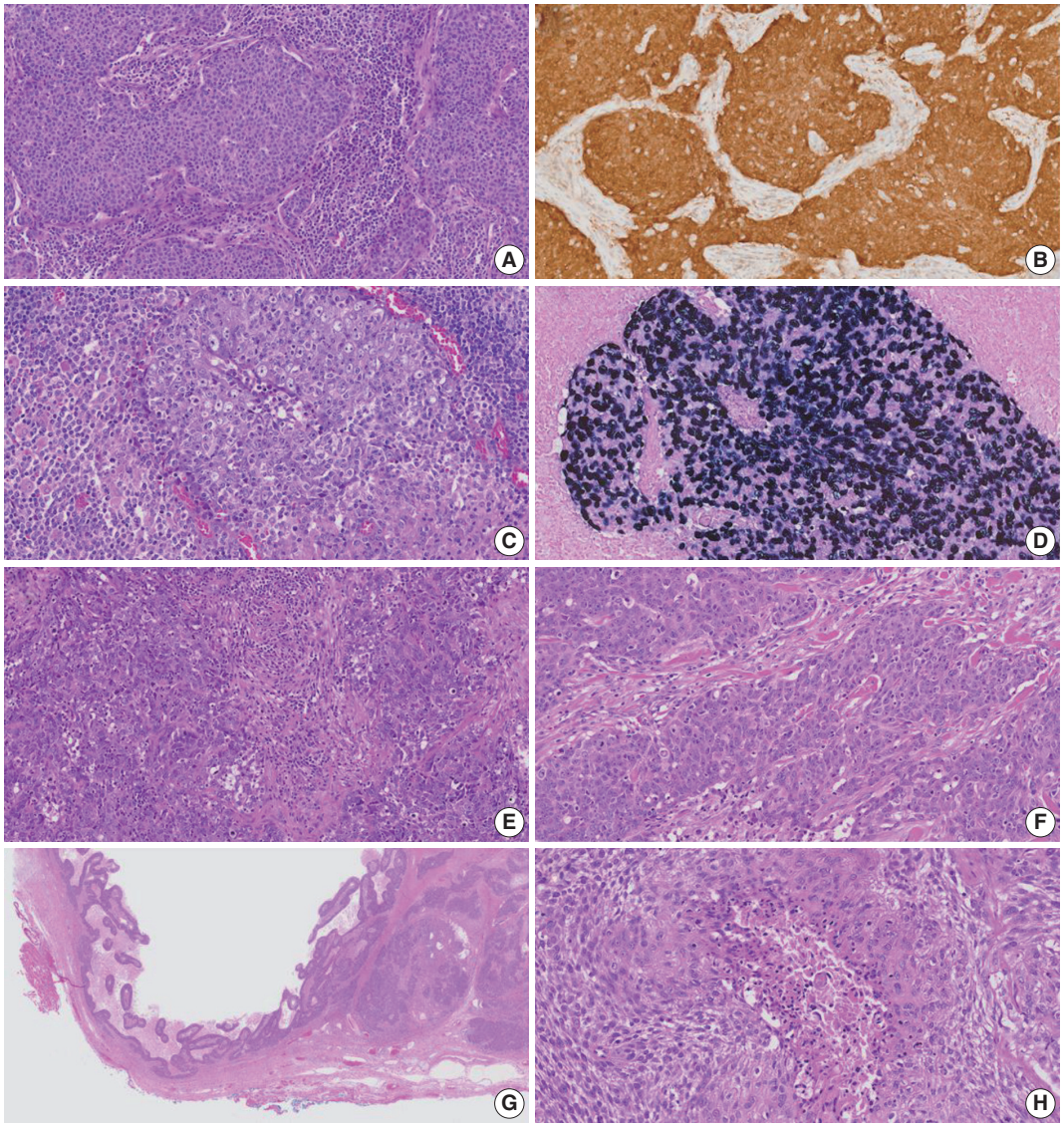

Fig. 1. Cervical lymph node metastasis from an unknown primary tumor (CUP). Human papillomavirus (HPV)–related CUP shows a basaloid pattern (A) and is positive for p16 immunohistochemistry (B). Epstein-Barr virus (EBV)–related CUP (C) was confirmed by EBV in situ hybridization (ISH) (D). HPV- and EBV-unrelated CUP (E, F) was defined as cases that are negative for p16, HPV ISH, HPV real-time polymerase chain reaction, and EBV ISH results. (G) Cystic change in the HPV-related CUP. (H) Keratinization in the HPV- and EBV-unrelated CUP.

death from disease (DOD)/death from other disease (DOC) was 37% (17/46), which was higher than that of the two virus-related groups. There was a significant difference in the clinical outcomes among the three groups (p = .011) (Table 1).

OS estimates in CUP

The univariate analysis revealed that ≥60 years of age (p < .001), current smoker (p = .024), >40 pack-years (p = .002), presence of extranodal extension (p = .001), and HPV- and EBV-unrelated group status (p = .005) were significant factors for a poor prognosis. The presence of the basaloid pattern (p = .042) and p16 IHC positivity (p = .007) were significant prognostic factors for good outcomes. Sex, largest lymph node size, the presence of keratinization, cystic changes, lymphoepithelial lesions, and p53 IHC positivity did not significantly affect the OS in the univariate analysis (Table 3).

In the multivariate analysis, long-term smoking (21–40 vs. ≤20 pack-years, p = .014; >40 vs. ≤20 pack-years, p = .038) and HPV- and EBV-unrelated group vs. HPV-related group status (hazard ratio, 13.238; 95% confidence interval, 1.427 to 122.820; p = .023) were significant prognostic factors for poor outcomes (Table 3).

Table 3. Univariate and multivariate analyses on overall survival in cervical metastasis from an unknown primary tumor

Variable	Univariate		Multivariate	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Age (≥60 yr vs. <60 yr)	13.532 (3.137–58.374)	<.001	0.000 (0.000–5.590e128)	.929
Sex (male vs. female)	0.878 (0.256–2.960)	.825		
Smoking (current smoker vs. non- or past smoker)	2.990 (1.152–7.760)	.024	1.552 (0.209–11.545)	.668
Smoking				
Non-smoker, or 1–20 pack-years	1 (reference)			
21–40 pack-years	2.558 (0.894–7.315)	.080	11.893 (1.638–86.333)	.014
>40 pack-years	5.362 (1.852–15.525)	.002	9.742 (1.131–83.944)	.038
Lymph node size (cm)	1 (reference)			
≤3.0				
>3.0, ≤6.0	1.471 (0.585–3.703)	.412		
>6.0	0.457 (0.060–3.506)	.451		
Keratinization (present vs. absent)	1.529 (0.634–3.683)	.344		
Cystic change (present vs. absent)	0.438 (0.169–1.138)	.090		
Basaloid pattern (present vs. absent)	0.375 (0.146–0.965)	.042	3.130 (0.482–20.328)	.898
Lymphoepithelial lesion (present vs. absent)	0.512 (0.172–1.527)	.230		
Extranodal extension (present vs. absent)	9.017 (2.509–32.412)	.001	0.440 (0.055–3.488)	.470
Group				
HPV-related	1 (reference)			
EBV-related	6.608 (0.596–73.322)	.124	1.213e7 (0.000–1.018e142)	.918
HPV and EBV-unrelated	8.078 (1.859–35.106)	.005	13.238 (1.427–122.820)	.023
p16 IHC (positive vs. negative)	0.135 (0.031–0.585)	.007	-	
p53 IHC (positive vs. negative)	0.930 (0.375–2.304)	.875		

HR, hazard ratio; CI, confidence interval; HPV, human papillomavirus; EBV, Epstein-Barr virus; IHC, immunohistochemistry.

The Kaplan-Meier survival curves also estimated that OS was significantly better in non-smokers or past smokers than in current smokers (p = .018). Furthermore, groups who had smoked for < 20 pack-years, including non-smokers, showed the best OS, followed by those who had smoked for 21–40 pack-years, while those who had smoked for > 40 pack-years showed the worst OS (p = .003) (Fig. 2). However, in the analysis of individual groups, there was no significant OS difference in HPV-related CUP according to smoking status (non-smokers or past smokers vs. current smokers, p = .160) (non-smoker or < 20 vs. 21–40 vs. > 40 pack-years, p = .340). Among HPV- and EBV-unrelated CUP cases, non-smokers or past smokers tended to show better OS times than current smokers, but there was no significant difference (p = .064). There was also no significant OS difference in smoking duration (non-smoker or < 20 vs. 21–40 vs. > 40 pack-years, p = .400) among HPV- and EBV-unrelated CUP cases.

With the exception of HPV status, p16 alone was associated with a better OS (p = .002); however, there was no significant difference in OS between p53-positive and p53-negative patients (p = .875) (Fig. 2). The HPV-related CUP cases had the longest OS, and the HPV- and EBV-unrelated CUP patients had the worst prognosis, with a significant difference among the three groups (p = .004) (Fig. 3).

Cervical metastasis with subsequent confirmation of the primary tumors

Among the patients initially presenting with cervical metastasis with unknown primary tumors, 64 cases were found at the primary sites. Primary tumors were most frequently found at the oropharynx (n = 25, 89.3%), followed by at the hypopharynx and nasopharynx (n = 5, 7.8%, each). There were four cases (6.3%) of the esophagus; three cases (4.7%) of the oral cavity; and one case each (1.6%) of the pharynx, not specified, retropharynx, larynx, anus, and uterine cervix. Among them, 28 cases (43.8%) were identified as HPV-related tumors through p16 IHC or HPV-PCR tests. HPV-related primary tumors originated at the oropharynx, pharynx, not specified, anus, and uterine cervix. There were three cases of EBV-related tumors confirmed by EBV ISH, and the primary sites of all cases were the nasopharynx (Table 4). There was no significant difference in OS among the three groups according to viral status (p = .073) (Fig. 3). Clinical and pathologic characteristics according to viral status are presented with detailed tables in Supplementary Table S1.

DISCUSSION

This is the first multicenter study in Korea on CUP and has

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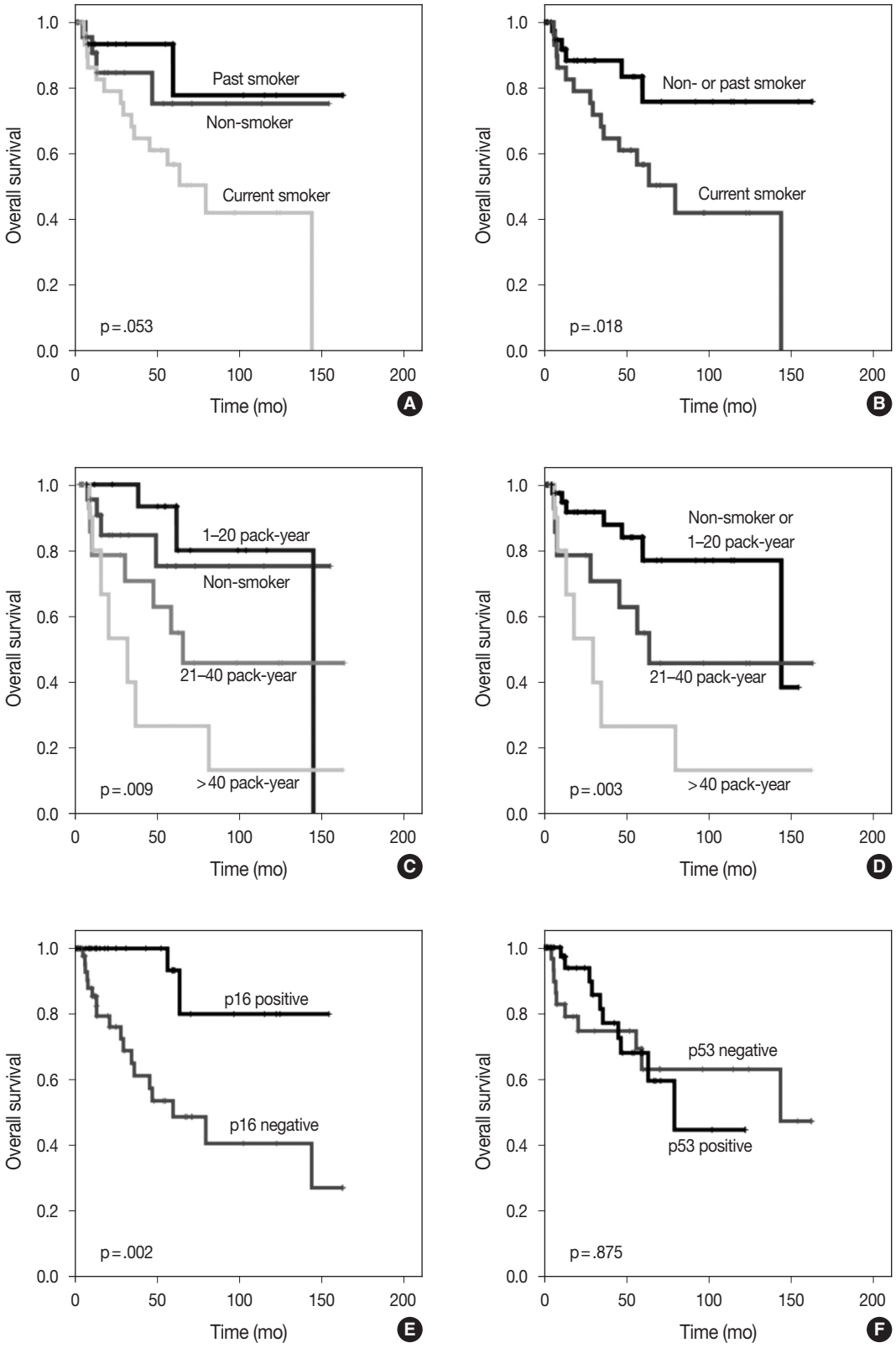

Fig. 2. Comparison of overall survival in cervical metastasis from an unknown primary tumor according to smoking status (A,B), smoking duration (C, D), and p16 (E) and p53 (F).

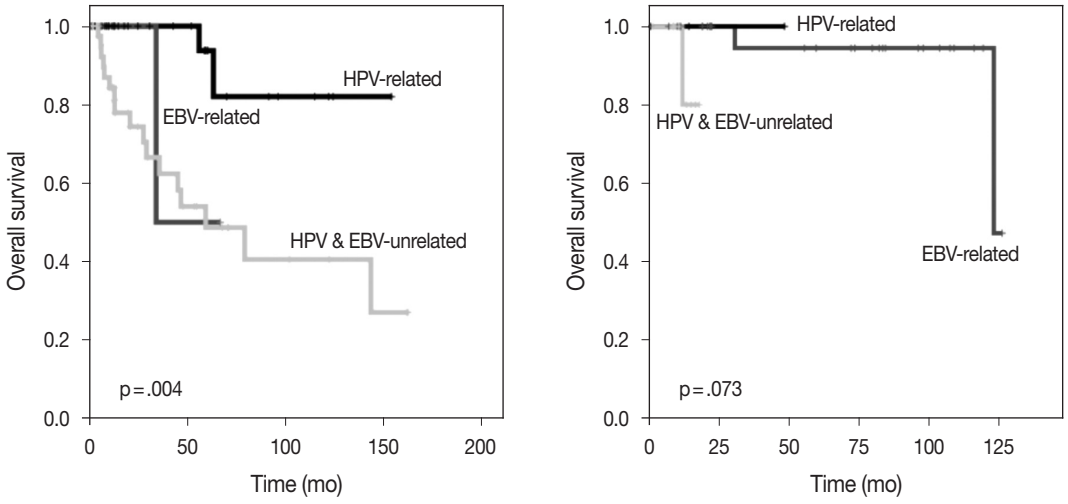

Fig. 3. Comparison of overall survival according to viral status in cervical metastasis from an unknown primary tumor and cervical metastasis with proven primary sites. (A) The human papillomavirus (HPV)–related cervical metastasis from an unknown primary tumor (CUP) cases had the longest overall survival, and the HPV- and Epstein-Barr virus (EBV)–unrelated CUP patients had the worst prognosis, with a significant difference among the three groups. (B) In cervical metastasis with proven primary sites, there was no difference in overall survival according to viral status.

Table 4. HPV and EBV status of cervical metastasis with proven primary sites

Primary site (n=64)	HPV-related (n=28, 43.8%)	EBV-related (n=3, 4.7%)	HPV & EBV-unrelated (n=10, 15.6%)	Not determined (n=23, 35.9%)
Oropharynx (n=41, 64.1%)	25 (89.3)	0	1 (10)	15 (65.2)
Hypopharynx (n=5, 7.8%)	0	0	1 (10)	4 (17.4)
Nasopharynx (n=5, 7.8%)	0	3 (100)	1 (10)	1 (4.3)
Oral cavity (n=3, 4.7%)	0	0	2 (20)	1 (4.3)
Pharynx, not specific (n=2, 3.1%)	1 (3.6)	0	0	1 (4.3)
Retropharynx (n=1, 1.6%)	0	0	0	1 (4.3)
Esophagus (n=4, 6.3%)	0	0	4 (40)	0
Larynx (n=1, 1.6%)	0	0	1 (10)	0
Anus (n=1, 1.6%)	1 (3.6)	0	0	0
Uterine cervix (n=1, 1.6%)	1 (3.6)	0	0	0

Values are presented as number (%).
HPV, human papillomavirus; EBV, Epstein-Barr virus.

noted several important findings. First, HPV-related cases constituted 38.9% of all CUP cases, which is approximately 10% lower than the frequency (49%) reported in a meta-analysis of 17 studies [17]. Second, HPV-related CUP in Korea showed better survival outcomes than HPV-unrelated CUP, per the studies in Western countries, including 978 cases in the United States [5] and 68 cases in the National Cancer Database [6]. Interestingly, a previous Korean study [18] reported opposing findings, a finding which might have been attributed to the small number of cases. A multicenter study is considered to have merits of case collection and reduction of bias due to the hospital size.

Although only 5 EBV-related CUP cases were analyzed in this study, virus-related CUP had a better prognosis than the virus-unrelated group, and HPV-related CUP showed the best OS (p = .004) and a high NED status frequency (73.0%). Our results were consistent with the eighth edition of the AJCC staging system that accepted the unique biologic behavior and natural history of EBV- and HPV-related tumors. In addition, our results supported a unique staging system for cervical lymphadenopathy with an unknown primary tumor to apply to oropharynx and nasopharynx staging according to HPV and EBV status.

Characteristics of viral-related tumors were also well organized among CUP cases in Korea. HPV- and EBV-unrelated CUP cases showed the worst OS and a high DOD/DOC status frequency (37.0%, p = .011) among the three groups, with a high rate of extranodal extension (p = .046) and N staging (p = .001). HPV-

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related CUP patients were younger than HPV-unrelated CUP patients (p = .013), and their lymph nodes showed a higher frequency of cystic changes (p = .016) and the basaloid pattern (p < .001) as seen in HPV-mediated oropharynx cancer. EBV-related CUP showed a high frequency of lymphoepithelial lesions (p = .010), as with nasopharynx cancer associated with EBV.

Dixon et al. [23] found no significant differences in OS (p = .85 and p = .42) and disease-free survival (p = .87 and p = .58) in CUP through the univariate analysis of smoking duration (≤ 10 vs. > 10 pack-years) and smoking status (current smoker vs. ex-smoker vs. never-smoker). Tribius et al. [7] found that a smoking history of > 10 pack-years showed a worse prognosis than that of ≤ 10 pack-years in HPV-DNA-positive and p16-positive CUP. In addition, HPV-DNA–positive and p16-positive CUP with a smoking history of > 10 pack-years showed a similar survival curve to HPV-DNA–negative or p16-negative groups (p = .02) [7]. In our study, smoking duration was a significantly worse prognostic factor for OS in the multivariate analysis of total CUP cases. The Kaplan-Meier survival curves also showed significant differences in OS according to smoking status (non-smokers or past smokers vs. current smokers, p = .018) and smoking duration (non-smoker or < 20 vs. 21–40 vs. > 40 pack-years, p = .003). However, there was no significant difference in OS in the HPV-related CUP or virus-unrelated CUP groups according to smoking status or duration.

As a limitation of our study, HPV-related CUP was defined by three methods of RT-PCR, DNA ISH, and p16 IHC. RT-PCR is stable and reproducible. However, as the sensitivity is high, there is a possibility of contamination by surrounding HPV-infected normal epithelium or other samples [24]. DNA ISH is widely used in research due to its low price but shows different sensitivity and specificity values according to the type of probe for the target HPV [25]. The Ventana system was used in this study, but even with Ventana, different performances were achieved [26,27] owing to the varying quality-control procedures, laboratory experience, and techniques [28]. p16 positivity in IHC is used as a surrogate marker for high-risk HPV-associated tumorigenesis because p16 can be overexpressed by the loss of inhibitory feedback of the phosphorylated Rb protein, degradated by the E7 protein of high-risk HPV [29]. However, other processes, such as inflammation, regeneration, and *TP53* mutation, contribute to p16 overexpression [30,31]. The choice of one of the three methods varied across institutions and even within the same institution. In this study, 90.5% (19/21) of high-risk HPV-positive cases showed p16 overexpression, and 21.7% (13/60) of high-risk HPV-negative cases showed p16 overexpression. At Johns Hopkins Hospital, which routinely uses both the HPV DNA ISH test and p16 IHC, they found p16-positive/HPV-DNA–negative cases in 18% of oropharyngeal squamous cell carcinoma [32], similar to the 16% identified in our study. They performed an additional RNA ISH assay for high-risk E6/E7 mRNA and confirmed the presence of active transcriptional active HPV in 84% of these cases. Judging from the characteristics of each method and the results of this study, the cause of the discrepancy in the cases showing p16 overexpression but DNA ISH negativity may be due to the false-negative result of DNA ISH from the background signal or due to the overexpression of p16 by another non-viral mechanism. HPV type 16 infection and disruptive *TP53* mutations did not seem to overlap, so HPV infection showed an inverse relationship with *TP53* mutations [21,22]. This study showed no inverse relationship between p53 and HPV infection. In this study, tumors showing nuclear staining in ≥ 10% of the tumor cells are considered positive for p53 immunostaining. The reason for the cutoff of 10% was based on the analysis of multiple studies that found significant correlations between p53 overexpression and higher tumor grade [33,34], *TP53* gene mutations [35-37], or worse prognosis [38,39] when the threshold was set at 10%. However, the p53 immunostaining results in this study may not represent the entirety of the tumor due to being performed in the TMA. Different interpretations of p53 positivity among institutions may have resulted in different results in previous studies. Additionally, > 10% of p53 nuclear expression may not represent the *TP53* mutation of the tumor with rarity in this study. We lacked enough follow-up data and scale because we could only secure OS data. Although this is a multicenter study, its statistical power to understand CUP remains insufficient due to the small collection of data, which is the peculiarity of the low incidence of this entity.

In conclusion, virus-unrelated CUP in Korea had the highest frequency among CUP cases. Virus-related CUP had a better prognosis than the virus-unrelated group, and patients with HPV-related CUP showed the best OS. HPV-related CUP was similar to HPV-mediated oropharyngeal cancer and EBV-related CUP was similar to nasopharyngeal cancer in terms of clinicopathologic characteristics. In total CUP cases, longer smoking duration and virus-unrelated CUP were significant factors for poor prognosis.

Supplementary Information

The Data Supplement is available with this article at https://doi.org/10.4132/jptm.2023.04.12.

Ethics Statement

This study was approved by the Institutional Review Board of Asan Medical Center (2020-1722), and patient informed consent was waived, given the retrospective nature of the study. All procedures were performed in accordance with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Availability of Data and Material

All data generated or analyzed during the study are included in this published article (and its supplementary information files).

Code Availability

Not applicable.

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Conflicts of Interest

J.S.S., a contributing editor of the *Journal of Pathology and Translational Medicine*, was not involved in the editorial evaluation or decision to publish this article. All remaining authors have declared no conflicts of interest.

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Supplementary Table S1. Characteristics and survival according to the viral status of cervical metastasis with proven primary tumor

Total (n=64)	HPV-related (n=28, 43.8%)	EBV-related (n=3, 4.7%)	HPV & EBV-unrelated (n=10, 15.6%)	Not determined (n=23, 35.9%)	p-value
Time to the discovery of primary tumor					
Median	0.35	0	0	0.65	
Range	0 ~24.4	0	0	0–105.7	
Age (yr)					.035
<50	8 (28.6)	1 (33.3)	0	6 (26.1)	
50–59	12(42.9)	2 (66.6)	2 (20.0)	9 (39.1)	
60–69	6 (21.)	0	4 (40.0)	8 (34.8)	
≥70	2 (7.1)	0	4 (40.0)	0	
Sex					.033
Male	15 (53.6)	1 (33.3)	1 (10.0)	19 (82.6)	
Female	13 (46.4)	2 (66.7)	9 (90.0)	4 (17.4)	
Smoking status					.978
Non-smoker	13 (46.4)	2 (66.7)	5 (50.0)	6 (26.1)	
Past smoker	7 (25.0)	1 (33.3)	2 (20.0)	7 (30.4)	
Current smoker	8 (28.6)	0	3 (30.)	10 (43.5)	
Smoking duration (pack-years)					.159
Never-smoker	13 (46.4)	2 (66.7)	5 (50.0)	6 (26.1)	
1–20	12 (42.9)	1 (33.3)	2 (20.0)	7 (30.4)	
21–40	3 (10.7)	0	3 (30.0)	6 (26.1)	
≥41	0	0	0	4 (17.4)	
NA					
Lymph node size (cm)					
≤3.0	19 (67.9)	3 (100)	5 (50.0)	12 (52.2)	.563
>3.0, ≤6.0	5 (17.9)	0	4 (40.0)	8 (34.8)	
>6.0	2 (7.1)	0	1 (10.0)	1 (4.3)	
NA	2 (7.1)	0	0	2 (8.7)	
Lymph node level					
Level Ⅰ	0	0	1 (10.0)	6 (26.1)	
Level Ⅱ	24 (85.7)	3 (100)	2 (20.0)	21 (91.3)	
Level Ⅲ	6 (21.4)	1 (33.3)	4 (40.0)	7 (30.4)	
Level Ⅳ	4 (14.3)	2 (66.7)	2 (20.0)	3 (13.0)	
Level Ⅴ	1 (3.6)	1 (33.3)	2 (20.0)	3 (13.0)	
Level Ⅵ	0	0	0	0	
Retropharyngeal	0	0	0	0	
Axillary	0	0	0	0	
Supraclavicular	2 (7.1)	0	1 (10.0)	0	
Extranodal extension					.047
positive	4 (14.3)	1 (33.3)	2 (20.0)	7 (30.4)	
negative	18 (64.3)	0	1(10.0)	13 (56.5)	
NA	6 (21.4)	2 (66.7)	7 (70.0)	3 (13.0)	
N category					>.99
1	21 (75.0)	3 (100)	7 (70.0)	15 (65.2)	
2, 2a, 2b	2 (7.1)	0	1 (10.0)	1 (4.3)	
3, 3a, 3b	2 (7.1)	0	1 (10.0)	4 (17.4)	
NA	3 (10.7)	0	1 (10.0)	3 (13.0)	
Keratinization					.293
present	5 (17.9)	0	4 (40.0)	6 (26.1)	
absent	22 (78.6)	3 (100)	6 (60.0)	16 (69.6)	
Cystic change					.003
Present	14 (50.0)	0	0	10 (43.5)	
Absent	13 (46.4)	3 (100)	10 (100)	12 (52.2)	

NA	1 (3.6)	0	0	1 (4.3)	
Basaloid pattern					<.001
Present	25 (89.3)	0	0	12 (52.2)	
Absent	2 (7.1)	3 (100)	10 (100)	10 (43.5)	
NA	1 (3.6)	0	0	1 (4.3)	
Lymphoepithelial lesion					.009
Present	7 (25.0)	3 (100)	1 (10.0)	4 (17.4)	
Absent	20 (71.4)	0	9 (90.0)	18 (78.3)	
NA	1 (3.6)	0	0	1 (4.3)	
p16 IHC					<.001
Positive	11 (100.0)	0	0	0	
Negative	0	3 (100)	10 (100)	1 (4.3)	
NA	17 (60.7)	17 (60.7)	0	22 (95.7)	
p53 IHC					.002
Positive	2 (7.1)	2 (66.7)	9 (90.0)	1 (4.3)	
Negative	9 (32.1)	1 (33.3)	1 (10.0)	0	
NA	17 (60.7)	0	0	22 (95.7)	
T category					.415
1	13 (46.4)	0	2 (20.0)	14 (60.9)	
2	10 (35.7)	0	2 (20.0)	4 (17.4)	
3	1 (3.6)	0	1 (10.0)	0	
NA	4 (14.3)	3 (100)	5 (50.0)	5 (21.7)	
Follow-up duration (mo)					
Median,	72.7	14.3	11.4	55.6	
Range	7–126	1–48	1–18	0–142	
Last status					.014
NED	22 (78.6)	1 (33.3)	3 (30.0)	13 (56.5)	
AWD	4 (14.3)	2 (66.7)	6 (60.0)	2 (8.7)	
DOD/DOC	2 (7.1)	0	1 (10.0)	8 (34.8)	
NA					

HPV, human papillomavirus; EBV, Epstein-Barr virus; NA, not assessed; IHC, immunohistochemistry; NED, no evidence of disease; AWD, alive with disease; DOD, death of disease; DOC, death of other cause.