Relationship between cervical esophageal squamous cell carcinoma and human papilloma virus infection and gene mutations

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Abstract. Cervical esophageal squamous cell carcinoma (CESCC) is rare, accounting for 5% of all esophageal carcinomas. Several diagnostic and predictive markers have been studied. However, to the best of our knowledge, no biomarker is known to determine patient management except the clinical stage. The present study aimed to evaluate whether human papilloma virus (HPV) infection, epidermal growth factor receptor (EGFR) and its pathway-related gene mutations, known to be sensitive biomarkers of oropharyngeal carcinomas, could be used as biomarkers for the prediction of the prognosis of patients with CESCC. The present retrospective study included patients with CESCC who received chemoradiotherapy or surgery. HPV infection and the genomic status of *EGFR*, *KRAS*, *BRAF*, *NRAS* and *PIK3CA* of each tumor sample from patients with CESCC were analyzed by *in situ* hybridizations (ISH) and PCR methods, respectively. The present study included 33 patients with CESCC (male/female, 29/4; median age, 62 years; age range, 41-86 years; clinical stage I/II/III/IV, 2/6/10/15). The present study detected HPV in one patient (3.0%) by ISH and PCR. Concerning the investigation of *EGFR* and its pathway-related gene mutations, the present study detected 15.1% of *EGFR*, 6.0% of *NRAS*, 3.5% of *BRAF*, 3.0% of *KRAS* and 3.0% for *PIK3CA* mutations, with no significant relationship between any gene mutations and the clinical prognostic factors. The HPV-infected patient did not exhibit any gene mutations. The present study indicated that HPV infection, EGFR and its pathway-related gene mutations rarely exist in patients with CESCC. The relationship between these biomarkers and the prognosis in patients with CESCC is still unclear.

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Introduction

The cervical esophagus is the short part of the esophagus between the lower border of the cricoid cartilage and the thoracic inlet, 18 cm from the incisor teeth (1). Cervical esophageal carcinoma (CESCC) is less common than thoracic esophageal carcinoma (ESCC), accounting for less than 5% of all esophageal carcinoma (2). The management of CESCC differs from cancers of the other parts of the esophagus in that CESCCs are often locally advanced at the time of diagnosis infiltrating nearby anatomical structures including the thyroid, carotid artery, and trachea. Moreover, patients with CESCC often present with lymph node metastases[1]. Some of CESCC are not treatable by surgery even when diagnosed at an early stage, as this would involve mutilating resections including pharyngo-laryngo-esophagectomy. Therefore, definitive chemoradiation (dCRT) is the standard treatment modality for CESCC recommended by the National Comprehensive Cancer Network and European Society for Medical Oncology (ESMO) guidelines (3,4). Several diagnostic and predictive markers have been studied, but no biomarker to clearly determine patient treatment. Recently, many new biomarkers related to carcinogenesis, prognosis or response to therapies

for several carcinomas are known. In addition, HPV infection is known to be a major etiologic factor in oropharyngeal squamous cell carcinomas, as a strong and independent prognostic marker (5-7). Indeed, patients with HPV-positive oropharyngeal carcinomas have a significantly decreased mortality risk compared with HPV-negative oropharyngeal carcinoma patients (7). While some articles have described the HPV status of esophageal carcinoma patients, HPV status as a prognostic or predictive biomarker of ESCC is not yet established. We therefore explored HPV infection status in patients with CESCC. CESCC is suspected to demonstrate more similar characteristics with oropharyngeal carcinomas than ESCCs at other sites. Genomic analyses of *EGFR*, *KRAS*, *NRAS,* and *PIK3CA*, also seem to be potent biomarkers for targeted therapy, such as anti-*EGFR* therapy or *PIK3CA* inhibitor therapy. The present study aimed at determining how many CESCC patients are HPV-infected and/or *EGFR*- and SCC-related gene mutations by analyzing each patient's tumor sample.

Patients and methods

Patients. This retrospective study enrolled 33 CESCC patients who received CRT or surgery at National Cancer Center Hospital from March 2001 to September 2006. The clinical criteria for enrollment were as follows: Archive tissue available, no other malignancies, written informed consent given and a primary lesion existing in the cervical esophagus. The cervical esophagus is defined as the upper side without extension to the inferior margin of the hyoid bone or the lower side of the primary without extension to the superior margin of the carina. The institutional review board of the NCCH (no. 2008-119) approved this study and it was performed in accordance with the Declaration of Helsinki.

Treatment: dCRT regimens and surgical procedures for CESCC patients. The dCRT regimen for CESCC patients comprised of 70 mg/m^2 of cisplatin (CDDP) administered intravenously for 120 min on days 1 and 29, 700 mg/m^2 of 5-FU administered continuously on days 1-4 and 29 to 32, and radiation therapy at a dose of 60 Gy irradiated concurrently. If the therapeutic effect was observed, 2 repeated cycles of 80 mg/m^2 of CDDP and 800 mg/m^2 of 5-FU were administered on day 1 and days 1 to 5, respectively, every 4 weeks. Concerning radical surgical resection, cervical esophagus resection preserving the larynx or pharyngo-laryngo-esophagectomy was performed. The patients who underwent curative resection did not receive preoperative irradiation.

Tumor sample collection and tissue processing procedure
Polymerase chain reaction. We briefly stained deparaffinized sections with hematoxylin and used them for DNA extraction. The carcinoma components were separately dissected using sterilized toothpicks under a microscope. The proper muscle tissue distant from the tumor was used as a non-tumor sample. The dissected samples were incubated in 100 μl of DNA extraction buffer [50 mmol/l Tris-HCl, pH 8.0, 1 mmol/l ethylenediaminetetraacetic acid, 0.5% (v/v) Tween-20, 200 μg/ml proteinase K] at 55˚C overnight. We then heated the samples at 100˚C for 10 min to inactivate proteinase K and directly subjected them to polymerase chain reaction (PCR). DNA samples were obtained from cervical carcinomas with known human papillomavirus 16 (HPV16) infection and the HeLa cell line positive for HPV18 were used as positive controls. PCR was performed using HPV consensus primers GP5$^+$/6$^+$ (8) and two pairs of genotype-specific primers for HPV16 and HPV18, as previously described (9,10). Primer sequences of the elongated 23-mer GP5 (named GP5$^+$) and 25-mer GP6 (named GP6$^+$) are indicated (10). ACTB was amplified to ensure proper DNA extraction. The PCR products were electrophoresed in a 2% (w/v) agarose gel and visualized under ultraviolet light with ethidium bromide staining.

In situ hybridization. DNA ISH was performed on formalin-fixed, paraffin-embedded (FFPE) tissue blocks from each case using the ISH I View Blue Plus Detection Kit (Ventana Medical System, Inc.) in accordance with the manufacturer's instructions. The assay used the Ventana HPV III Family 16, Probe B, a cocktail recognizing the HPV types 16, 18, 31, 33, 35, 45, 51, 52, 56, 59, 68 and 70. Ventana Red Counterstain II (Ventana Medical System, Inc.) was used (Fig. 1).

Controls in each run included a known HPV 16-positive HeLa cell line. A pathologist read the cases, and blue nuclear dots were considered positive staining. Any definitive nuclear staining in the tumor cells was considered positive. Cases were classified in a binary manner as either positive or negative.

Mutation analyses of EGFR, KRAS, BRAF, PIK3CA and NRAS. We collected FFPE tissue, and the DNA of samples were extracted from FFPE tumor tissue sections. The tumor cell-rich areas in the hematoxylin and eosin section were marked under a microscope, and the tissue was scratched from the area of another deparaffinized unstained section. The EGFR mutation statuses were evaluated by the PCR-invader method (BML, Inc.) analysis. DNA from pieces of the scratched tissue sample was isolated using the QIAamp DNA FFPE Tissue Kit (Qiagen KK). Exon 2 (codon 12, 13), exon 3 (codon 61) and exon 4 (codon 146) of the *KRAS* gene; exon 15 (codon 600) of the *BRAF* gene; exon 9 (codon 542, 545) and exon 20 (exon) of the *PIK3CA* gene and; exon 2 (codon 12, 13) and exon 3 (codon 61) of the *NRAS* gene were amplified by PCR (GeneAmp PCR System 9700 Thermal Cycler). We visualized the PCR products using agarose gel electrophoresis with ethidium bromide staining and directly sequenced using an ABI 3130x Genetic Analyzer [Life Technologies Japan (Applied Biosystems), Tokyo, Japan] in accordance with the manufacturer's instructions.

Statistical analysis. All enrolled patients were divided into two groups: i) Patients who have some mutations and ii) did not have any mutation. The median OS was statistically compared between the two groups using the log-rank test. The statistical analyses were performed with SAS 9.4 (SAS Institute Inc.).

Results

Patient characteristics. We enrolled 33 CESCC patients in this study according to the present criteria. The background characteristics of the CESCC patients are shown in Table I. Most patients (88%) were males, with only 4 were females (12%).

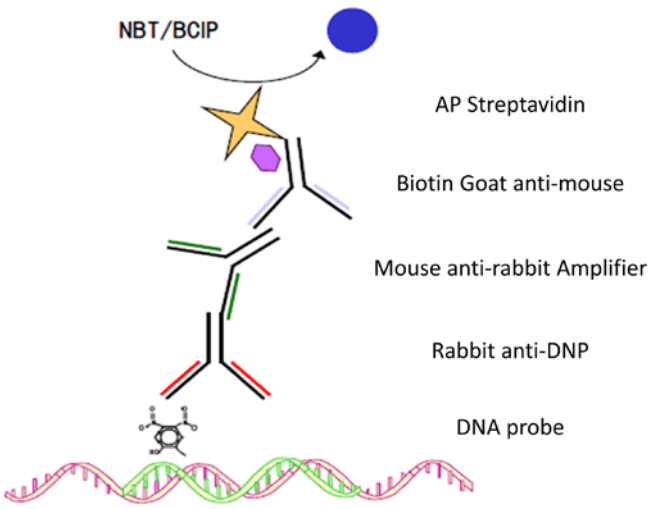

Figure 1. Ventana inform human papilloma virus probe. NBT/BCIP, nitrotetrazolium blue chloride/5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt; AP, alkaline phosphatase.

25 patients (75%) were diagnosed with clinical Stage III or IV (lymph node) (UICC-TNM 6th) (11), while 8 patients (25%) were clinical Stage I or II. Surgery was performed in 13 patients (35%), and dCRT in 20 patients (65%).

HPV analyses. Only one patient was HPV 16-positive (3.0%) on ISH and PCR. This patient was a 41-year-old male with a light alcoholic and non-smoking history who had been diagnosed as clinical T1N0M1 Stage IV. He received CRT and achieved a complete response. He later underwent salvage resection of the esophagus for local recurrence and is presently alive.

Mutations of EGFR, KRAS, BRAF, PIK3CA and NRAS. Direct sequencing of the tissue samples in CESCC patients determined the proportion of mutations of *EGFR*, *KRAS*, *BRAF*, *PIK3CA*, and *NRAS* (Table II). *EGFR* mutations were observed in T790 (9.0%, n=3/33), G719S and L858R (3.0%, n=1/33), *KRAS* mutations in codon 13 (3.0%, n=1/33), *BRAF* mutations in V600E (3.5%, n=1/28), *PIK3CA* mutations in exon 9 (3.0%, n=1/33), and *NRAS* mutations in codons 12 (3.3%, n=1/30) and 13 (3.3%, n=1/30). No mutations were observed in *KRAS* codon 61, *KRAS* codon 146 or other *NRAS* codons. No patient among the CESCC patients with gene mutations in their tumor tissue had multiple mutations. The tumor cell in the HPV 16-positive patient did not show any gene mutation.

Given these findings, no significant relationship was noted between HPV infection and *EGFR*, *KRAS*, *BRAF*, *PIK3CA* and *NRAS* gene mutations with clinical prognostic factors in CESCC patients.

Discussion

CESCC is a less common disease and often locally advanced at the time of diagnosis resulting in limited locoregional disease control and poor survival. Due to the presence of locally advanced disease at the time of diagnosis and the carcinoma being close to larynx, spinal cord, and upper airway, sometimes non-surgical treatment seems to be the appropriate option. Several different CRT schedules and techniques have been investigated in the past, but no consensus has been reached concerning the optimal treatment for CESCC patients. Many institutions used CDDP/5-FU regimen and a concurrent radiation dose of 60 Gy, which was the community standard treatment. There is no indication to choose the treatment and its response or prognosis in patients with CESCC.

Researches investigating the potential association between HPV infection and ESCC show contradicting results. In the present study, the HPV infection rate in CESCC patients was only 3.0%. Geographical locations with a high incidence of ESCC tend to have a higher incidence of HPV infection; it is more frequent in Asia (26.3%) and less frequent in other

Table I. Baseline patient characteristics (n=33).

Characteristics	N (%)
Sex	
Male	29 (88)
Female	4 (12)
Tumor stage	
1	5 (16)
2	3 (9)
3	19 (57)
4	6 (18)
Nodal stage	
0	21 (63)
1	12 (37)
CStage	
I	2 (6)
II	6 (18)
III	10 (31)
IV (lymph node)	15 (45)
Treatment	
Surgery	13 (35)
Chemoradiation	20 (65)

Staging (TNM classification) was diagnosed based on the Japanese Classification of Esophageal Carcinoma (6th Edition).

Table II. Proportion of mutations in the EGFR-related genes in the tissue samples of the patients with cervical esophageal squamous cell carcinoma.

Gene mutations	Proportion (%)
EGFR mutations	5/33 (15.1)
KRAS mutations Codon 13	1/33 (3.0)
BRAF mutations V600E	1/28 (3.5)
PIK3CA mutations exon 9	1/33 (3.0)
NRAS mutations Codon 12	1/30 (3.3)
NRAS mutations Codon 13	1/30 (3.3)

EGFR, epidermal growth factor receptor.

Table III. Comparison between CESCC and ESCC gene mutations.

Gene	CESCC cases (n=33), %	ESCC cases (n=71), %
EGFR	15.1	8.0
PIK3CA	3.0	24.0
KRAS	3.0	6.0
NRAS	6.6	3.0
BRAF	3.5	N.E.

N.E., not evaluated; CESCC, cervical esophageal squamous cell carcinoma; ESCC, esophageal carcinoma.

Western countries (14.0%) (12). A few reports have shown that the rate of HPV infection in ESCC patients range from 9.4 to 24.1% in Japan (12). HPV-related esophageal cancer may correlate with lifestyle, culture, economic conditions, and may be an epidemiological theme in the future. The HPV-positive CESCC patients who underwent CRT achieved cure and had a good long-term survival. HPV-infected CESCC can be a predictive biomarker in sampling and analyzing the survival and efficacy of HPV-positive CESCC cases who underwent CRT.

Though some studies on genetic mutations in ESCC were done, to the best of our knowledge, there are no studies investigating only genetic mutations in patients with CESCC only. The most common genetic mutations consist of *p53*, *RB1 (retinoblastoma protein)*, *ALDH1 (Aldehyde dehydrogenase-2 gene)*, *MTHFR (methylene tetrahydrofolate reductase gene)*, *EGR1 (early growth response gene-1)*, *CCND1 (cycline D1)* and *cMYC* (13-17). *MAPK* signaling pathways are one of the upregulated genes in ESCC (18). However, the clinical roles of these gene mutations in the prognosis and clinical response of the CRT in patients with CESCC is unclear. In the present study, we analyzed the mutational status of *EGFR* and its pathway-related gene mutations, *KRAS*, *BRAF*, *NRAS* and *PIK3CA* which are unknown as biomarkers in predicting the prognosis of patients with CESCC. However, we observed *KRAS*, *BRAF* and *PIK3CA* gene mutations in one patient each. Two CESCC patients had *NRAS* mutations. We found a small number and no significant relationship was observed between any gene mutation and the clinical prognostic factors in CESCC patients. We compared our results with those from comprehensive gene analysis of 71 ESCC patients (Table III) (19). The frequency of *KRAS*, *NRAS* and *BRAF* mutations was rare. However, *EGFR* mutation was 15.1 vs. 8%, *PIK3CA* mutation was 3 vs. 24% in CESCC and ESCC, respectively. In addition, in the reports that examined only the *KRAS* and *BRAF* mutations in ESCC, *KRAS* mutation was 0.5% (1/203) and *BRAF* was 0% (0/203), respectively (20). Similarly, in a report examining only *EGFR* gene mutations, L858R missense was found in a minority, 6.3% (8/127) (21). Comparing the results of the reports with our result, we found that our results were similar to those of ESCC.

In patients with non-small cell lung cancer, *EGFR* mutation is an important predictive factor for using EGFR-TKI. The COG trial, a phase III trial conducted in patients with esophageal cancer, could not show the superiority of Gefitinib in overall survival (22). However, the results of biomarker analysis showed that EGFR copy gain was a predictor of efficacy (23). Considering the remarkable progress of EGFR-TKI in non-small cell lung cancer, target treatment for esophageal squamous cell carcinoma may be reconsidered.

Recently, the efficacy of novel molecular-targeted drugs, such as the immune checkpoint inhibitor programmed cell death-1 (PD-1) inhibitors, in ESCC patients has been demonstrated in several studies. Several recent studies described a significant increased density of both effector T lymphocytes and regulatory tumor infiltrating T lymphocytes in HPV-positive compared to HPV-negative oropharyngeal squamous cell carcinoma, and highlighted the predictive value of effector lymphocytes infiltrates (24). We hope that future studies will clarify the association between HPV infection, tumorigenic mutational statuses, and the expression of PD-L1 or the efficacy of novel drugs in CESCC patients.

Several limitations associated with the present study warrant mention. First, CESCC was less than 5% of ESCC, so there was insufficient number of enrolled patients. Second, some data on the patients' background characteristics, such as the staging and treatment modality, were unavailable. However, it is a significant research because it is an area rarely reported.

In conclusion, the present study indicated that HPV infection and *EGFR* and its pathway-related gene mutations were present in low proportions in CESCC patients. Furthermore, these biomarkers might not be associated with the prognosis of CESCC patients. A future study in a larger population including all types of esophageal carcinoma patients will be required to clarify the detailed role of HPV infection, *EGFR* and its pathway-related gene mutations in CESCC patients.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MF and KK analyzed and interpreted the patient data on cervical esophageal squamous cell carcinomas. MF, KK, RO and HT wrote the manuscript. NT, HS, YH and SI made substantial contributions in data analysis and interpretation. HT and RO performed the histological examination of the lesions. AT, TH, YY, YS, YI, JI, NH, HI, YT, KM and

TT helped in acquiring the data for the work. KK and NB conceived the concept and designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The local Ethics Committee of the National Cancer Center Hospital (Tokyo, Japan) approved the present study. The requirement for written informed consent from patients was waived due to the retrospective design of the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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