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.

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 (3). 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 CESC. CESC 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 CESC 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 CESC 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 CESC patients. The dCRT regimen for CESC patients comprised of 70 mg/m² of cisplatin (CDDP) administered intravenously for 120 min on days 1 and 29, 700 mg/m² 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 CESC patients in this study according to the present criteria. The background characteristics of the CESC patients are shown in Table I. Most patients (88%) were males, with only 4 were females (12%).
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 regions.
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 CESC patients who underwent CRT achieved cure and had a good long-term survival. HPV-infected CESC can be a predictive biomarker in sampling and analyzing the survival and efficacy of HPV-positive CESC 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 CESC only. The most common genetic mutations consist of p53, RBL1 (retinoblasoma 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 CESC 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 CESC. However, we observed KRAS, BRAF and PIK3CA gene mutations in one patient each. Two CESC 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 CESC patients. We compared our results with those from comprehensive genetic 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 CESC 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 CESC patients.

Several limitations associated with the present study warrant mention. First, CESC 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 CESC patients. Furthermore, these biomarkers might not be associated with the prognosis of CESC 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 CESC 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, VS, YI, JI, NH, HI, YT, KM and

| Gene   | CESC 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; CESC, cervical esophageal squamous cell carcinoma; ESCC, esophageal carcinoma.
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.

References

1. Grass GD, Cooper SL, Armeson K, Garrett-Mayer E and Sharma A: Cervical esophageal cancer: A population-based study. Head Neck 37: 808-814, 2015.
2. Mendenhall WM, Sombek MD, Parsons JT, Kasper ME, Stringer SP and Vogel SB: Management of cervical esophageal carcinoma. Semin Radiat Oncol 4: 179-191, 1994.
3. Ajanja D’Amico TA, Almanna K, Bentrem DJ, Besh S, Chao J, Das P, Deningter C, Fanta P, Fuchs CS, et al: Esophageal and esophagogastric junction cancers, version 1.2015. J Natl Compr Canc Netw 13: 194-227, 2015.
4. Lordick F, Mariette C, Haustermans K, Obermannova R and Arnold D; ESMO Guidelines Committee: Oesophageal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 27 (Suppl 5): v50-v57, 2016.
5. Li G and Sturgis EM: The role of human papillomavirus in squamous carcinoma of the head and neck. Curr Oncol Rep 8: 130-139, 2006.
6. Allum WH, Stenning SP, Bancewicz J, Clark PI and Langlely RE: Long-term results of a randomized trial of surgery with or without preoperative chemotherapy in esophageal cancer. J Clin Oncol 27: 5062-5067, 2009.
7. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Mendenhall WM, Sombeck MD, Parsons JT, Kasper ME, Stringer SP and Vogel SB: Management of cervical esophageal carcinoma. Semin Radiat Oncol 4: 179-191, 1994.
8. de Roda Husman AM, Walboomers JM, van den Brule AJ, Meijer CJ and Snijders PJ: The use of general primers GP5 and GP6 elongated at their 3'ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J Gen Virol 76: 1057-1062, 1995.
9. Rietdendorf S, Rietdendorf L, Milde-Langosch K, Park TW and Loning T: Differences in HPV 16- and HPV 18 E6/E7 oncoprotein expression between in situ and invasive adenocarcinomas of the cervix uteri. Virchows Arch 437: 491-500, 2000.
10. Nishiwaki M, Yamamoto T, Tone S, Murai T, Ohkawara T, Matsunami T, Koizumi M, Takagi Y, Yamaguchi J, Kondo N, et al: Genotyping of human papillomaviruses by a novel one-step typing method with multiplex PCR and clinical applications. J Clin Microbiol 46: 1161-1168, 2008.
11. Sobin LH and Wittkeind C (eds): TNM classification of malignant tumors, 6th edition. John Wiley & Sons, Hoboken, NJ, 2002.
12. Li X, Gao C, Yang Y, Zhou F, Li M, Jin Q and Gao L: Systematic review with meta-analysis: The association between human papillomavirus infection and oesophageal cancer. Alimint Pharmacol Ther 39: 270-281, 2014.
13. Goto A, Li CP, Ota S, Niki T, Ohtsuki Y, Kitajima S, Yonezawa S, Koriyama C, Akiba S, Uchima M, et al: Human papillomavirus infection in lung and esophageal cancers: Analysis of 485 Asian cases. J Med Virol 83: 1383-1390, 2011.
14. Wu MY, Liang YR, Wu XY and Zhuang CX: Relationship between Egr-1 gene expression and apoptosis in esophageal carcinoma and precancerous lesions. World J Gastroenterol 8: 971-975, 2002.
15. Li QD, Li H, Wang MS, Diao TY, Zhou ZY, Fang QX, Yang FY and Li QH: Multi-sensitivity genes associated with the risk of the development stages of esophageal squamous cell cancer in Feicheng County. BMC Gastroenterol 11: 74, 2011.
16. Yasuda M, Kuwano H, Watanabe M, Toh Y, Ohno S and Sugimachi K: p53 expression in squamous dysplasia associated with carcinoma of the oesophagus: Evidence for field carcinogenesis. Br J Cancer 83: 1033-1038, 2000.
17. Montesano R, Rollstein M and Hainaut P: Genetic alterations in esophageal cancer and their relevance to etiology and pathology: A review. Int J Cancer 69: 225-235, 1996.
18. Zhan C, Yan L, Wang L, Jiang W, Zhang Y, Xi J, Jin Y, Chen L, Shi Y, Lin Z and Wang Q: Landscape of expression profiles in esophageal carcinoma by the cancer genome atlas data. Dis Esophagus 29: 920-928, 2016.
19. Wang K, Johnson A, Ali SM, Klempern SJ, Bekaii-Saab T, Vacirca JL, Khaira D, Yelensky R, Chmielecki J, Elvin JA, et al: Comprehensive genomic profiling of advanced esophageal squamous cell carcinomas and esophageal adenocarcinomas reveals similarities and differences. Oncologist 20: 1132-1139, 2015.
20. Shiogaki H, Baba Y, Watanabe M, Miyake K, Murata A, Iwagami S, Ishimoto T, Iwatsuki M, Yoshida N and Baba H: KRAS and BRAF mutations in 203 esophageal squamous cell carcinomas: Pyrosequencing technology and literature review. Ann Surg Oncol 20 (Suppl 3): S485-S491, 2013.
21. Cui Y, Chang D, Liu M, Lin C, Zhao B, Zhang X and Gong M: Identification of exon 19 and 21 mutations of EGFR gene in Chinese patients with esophageal squamous cell carcinoma. World J Surg Oncol 11: 266, 2013.
22. Dutton SJ, Ferry DR, Blazeby JM, Abbas H, Dahle-Smith A, Mansoor W, Thompson J, Harrison M, Chatterjee A, Falk S, et al: Gefitinib for oesophageal cancer progressing after chemotherapy (COG): A phase 3, multicentre, double-blind, placebo-controlled randomised trial. Lancet Oncol 15: 894-904, 2014.
23. Petry RD, Dahle-Smith A, Stevenson DAJ, Osborne A, Massie D, Clark C, Murray GI, Dutton SJ, Roberts C, Chong IY, et al: Gefitinib and EGFR gene copy number aberrations in esophageal cancer. J Clin Oncol 35: 2279-2287, 2017.
24. Mathur SE, Wilhelmina van Kempen PM, Bovenschen N, van Baarle D and Willems SM: Differences in T-cell infiltrates and survival between HPV+ and HPV−opharyngeal squamous cell carcinoma. Future Sci OA 2: FS088, 2016.