KRAS mutations in tongue squamous cell carcinoma

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ABSTRACT

Background: $p16^{INK4a}$ (p16) expression in tongue cancer (TC) is reportedly not associated with human papillomavirus (HPV). Mutations of *KRAS* in cancer cells are most frequently observed within codon 12. However, few reports have investigated the association between *KRAS* mutations and p16 status in TC.

Objectives: This study aimed to evaluate the influence of *KRAS* mutations on TC.

Methods: Clinical records and surgically resected specimens of 85 TC patients were analyzed. Tumor samples were analyzed for mutations of *KRAS* located within codons 12 and 13. p16 staining was performed and considered positive in cases with moderate to strong nuclear and cytoplasmic staining.

Results: Positive p16 staining was observed in 10 cases (11.8%). A *KRAS* mutation was detected in one case (1.2%). The case with *KRAS* mutation showed negative p16 staining. Despite being at an early stage, the patient died of lung metastasis at 43 months from initial treatment.

Conclusions and Significance: *KRAS* mutations are not associated with p16 expression in TC and may predict poor prognosis in TC patients. Further analysis of mutations in regions other than codons 12 and 13 of *KRAS* will be necessary to determine the relationship between *KRAS* mutations and prognosis of this disease.

Keywords: *KRAS*, p16 ^{INK4a}, tongue cancer

INTRODUCTION

p16 ^{INK4a} (p16) protein, the product of a tumor suppressor gene, is a cyclin-dependent kinase inhibitor that inhibits retinoblastoma protein phosphorylation [1]. Human papilloma virus (HPV) encodes E6 and E7 proteins that create a state competent for DNA replication in squamous epithelial lesions [2]. The high-risk HPV E7 protein is important for upregulating p16 by inactivating phosphorylated retinoblastoma protein [3]. In our previous study, although positive p16 staining was frequently observed in younger adults with tongue cancer (TC), there was no correlation between p16 expression and the detection of HPV DNA [4]. We hypothesized that p16 expression in TC might be caused by activation of cell proliferation signals due to some oncogenes.

RAS is a major oncogene in cancer pathogenesis [5]. In most cases, the somatic missense *RAS* mutations found in cancer cells introduce amino acid substitutions at positions 12, 13, and 61 [6]. In addition, the conversion of *KRAS* to an activated oncogene is usually accomplished by point mutations involving codon 12 and occasionally codons 13 and 61 [7]. Recently, some studies reported the involvement of *KRAS* mutations within codons 12 and 13 in the development of head and neck cancers [7, 8]. A previous study excluded *KRAS* mutations as a cause of p16 expression in head

and neck squamous cell carcinoma (HNSCC) [9]. On the other hand, it was previously reported that there is a significant association between *KRAS* variants and p16 status, and that p16-positive patients with *KRAS* variants exhibit the worst outcomes of all subgroups of patients with HNSCC [10]. Although *KRAS* mutations have been established as a potential biomarker for predicting the efficacy of treatment with an anti-epidermal growth factor receptor (EGFR) monoclonal antibody (i.e., cetuximab) in colorectal cancer, little is known about predictive markers for cetuximab treatment in head and neck cancer [11].

The aim of the present study was to investigate whether *KRAS* mutations, which can cause activation of cell proliferation signals, are associated with TC and to determine the association between p16 expression and *KRAS* mutations in TC.

MATERIALS AND METHODS

Patients

The clinical records of 85 Japanese patients treated for TC at our institution between 2001 and 2015 were analyzed. Tumors originating from the base of the tongue were excluded.

All 85 patients had squamous cell carcinoma (SCC). Disease stage was determined according to the 2009 Union for International Cancer Control Tumor-Node-Metastasis classification. Positive lymph nodes were defined as nodes detected on computed tomography and/or magnetic resonance imaging and ^{18}F-fluorodeoxyglucose positron emission tomography. Data were collected on age, sex, disease stage, p16 staining, and *KRAS* mutations using DNA testing.

Immunohistochemical staining for p16 protein

Paraffin-embedded tissue sections were used for immunostaining with the CDKN2A/p16INK4a antibody (EPR1473, dilution 1:200; Abcam, Cambridge, UK) using an automated Bond Max stainer (Leica Biosystems, Wetzlar, Germany). p16 staining intensity was scored by pathologists as 0, no staining (negative); 1, weak nuclear and cytoplasmic staining (negative); or 2, moderate to strong nuclear and cytoplasmic staining (positive) (**Figure 1a–c**). According to the classification proposed by Orita *et al.* [12], a score of 0–1 was considered negative, and a score of 2 was considered positive.

***KRAS* mutation analysis**

DNA was extracted from formalin-fixed, paraffin-embedded samples using the QIAamp DNA micro kit (Qiagen, Valencia, CA, USA). Template DNA was amplified

by polymerase chain reaction (PCR) using forward (5′-AAGGCCTGCTGAAAATGAC-3′) and reverse (5′-TGGTCCTGCACCAGTAATATG-3′) *KRAS* primers [13]. PCR was performed for 20 cycles of touchdown PCR (starting annealing temperature of 65°C, decremented 0.5°C per cycle) and 15 cycles at a 55°C annealing temperature.

For amplified PCR products, bands were confirmed to be around 170 base pairs using 3% agarose gel electrophoresis. The PCR products were subjected to direct sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and Applied Biosystems 3130xl Genetic Analyzer (Thermo Fisher Scientific). In the present study, tumor samples were analyzed for mutations of *KRAS* located within codons 12 and 13 (**Figure 2**).

The study protocol was approved by the Institutional Review Board of Okayama University (Okayama, Japan). All patients were Japanese and provided written informed consent to participate.

RESULTS

Patient characteristics and overall outcomes

The cohort comprised 57 men and 28 women, with a mean age at diagnosis of

60.4 (range, 23–96) years. Of the 85 patients with TC, 36 (42.4%) had stage I disease, 13 (15.3%) had stage II disease, 13 (15.3%) had stage III disease, and 23 (27.1%) had stage IV disease.

p16 staining intensity scores were 2, 1, and 0 for 10 (11.8%), 25 (29.4%), and 50 (58.8%) patients, respectively. In our previous study, the 10 patients with positive p16 staining were tested for HPV DNA using consensus primer-mediated PCR for high-risk subtypes (16, 18, 31, 33, 35, 52b, and 58) and low-risk subtypes (6 and 11) [4]. As a result, two patients were positive for low-risk HPV subtypes, and the others were negative for HPV DNA. A *KRAS* mutation within codon 12 was positive in one case (1.2%) and negative in 79 cases (92.9%); the other 5 cases (5.9%) could not be analyzed. All 10 cases with positive p16 staining were negative in the DNA sequence examination for *KRAS* mutations.

Characteristics and clinical course of the *KRAS* mutation case

The patient with the *KRAS* mutation detected by DNA sequencing was a 56-year-old man whose clinical stage was I. This case was negative for p16 staining. The patient underwent partial tongue resection as the primary treatment. Two years following the operation, a neck metastasis appeared that was treated with neck dissection and postoperative radiotherapy. However, the patient died because of lung

metastasis 43 months after initial treatment. Histopathologically, the resected specimen was diagnosed as well-differentiated SCC (**Figure 3**).

DISCUSSION

The binding of several specific ligands, such as EGF, promotes EGFR dimerization and the subsequent phosphorylation of several tyrosine residues. In addition, the phosphorylated tyrosines serve as binding sites for several signal transducers that initiate the Ras/Raf/mitogen-activated protein kinase/MEK/extracellular signal-regulated kinase pathway [11]. Ras proteins are activated when GTP is bound, and Ras-GTP binds to various effector proteins to stimulate signaling pathways that control many cellular responses such as proliferation, survival, and differentiation [6].

Mutations activating regulators and effectors of Ras proteins are common in tumor development and cancer [6]. Activating *RAS* mutations occur in ~30% of human cancers and are particularly prevalent in pancreatic, colorectal, endometrial, biliary tract, lung, and cervical cancers [6]. The features of *KRAS* mutations in lung and colon cancer have become increasingly clear [14]. In the present study, a *KRAS* mutation within codons 12 and 13 was detected in one case (1.2%), which is consistent with the

prevalence reported previously in oral SCC (**Table 1**) [7, 9, 15-18]. Specifically, common activating *KRAS* mutations were not present in a large cohort of Asian TC [16].

A *KRAS* mutation is estimated to occur in < 3% of HNSCC, making it of doubtful value to investigate *KRAS* in all candidates for cetuximab treatment, especially from the perspective of cost-versus-benefit [8]. In addition, a *KRAS* mutation analysis is reportedly not useful as a screening test for sensitivity to anti-EGFR therapy in tonsil SCC [19]. On the other hand, because these mutations make at least a minor contribution to oral SCC tumorigenesis, pathway-specific therapies targeting the Ras/Raf/mitogen-activated protein kinase/MEK/extracellular signal-regulated kinase pathway should be considered for oral SCC with *KRAS* mutations [15].

We investigated the association between *KRAS* mutations and p16 expression. *KRAS* mutations were not associated with either p16 expression or the onset of TC. The one case with a *KRAS* mutation presented with early TC that was negative for p16 staining, but the prognosis was extremely poor. In cervical cancer, cases with *KRAS* mutations are reported to have a poorer prognosis than those without such mutations [14]. In addition, *KRAS* variants are reported to be potential predictive biomarkers for poor response to platinum agents in patients with recurrent/metastatic HNSCC [20]. However, another study of HNSCC suggested that advanced stage patients with *KRAS*

variants significantly benefited from the addition of cetuximab to radiotherapy and cisplatin therapy, resulting in an improved prognosis [10]. In the future, studying additional TC cases will be necessary to evaluate whether *KRAS* mutations may also predict poor prognosis in TC patients and to investigate the cetuximab response rate of TC.

There are some limitations to the present study. We analyzed *KRAS* mutations in the codon 12 and 13 regions, but potential mutations at codon 61 were not assessed. A previous study suggested that there were differences in the positions of bases in which *KRAS* mutations tended to occur between SCC and adenocarcinoma [14]. In addition, the *KRAS*/*BRAF* pathway can be activated by mechanisms other than gene mutation [5]. In TC, it remains possible that *KRAS* mutations might occur in regions other than codon 12 or 13, and activation of cell proliferation signals may be caused by factors other than *KRAS*.

CONCLUSIONS

In the present study, although *KRAS* mutations in TC were analyzed, there was no obvious association between such mutations and TC or p16 expression. *KRAS* mutations may predict poor prognosis in TC patients. In the future, analysis of mutations in

regions other than codons 12 and 13 of *KRAS* will be required to elucidate the relationship between *KRAS* mutations and prognosis of this disease. In addition, targets other than *KRAS* may warrant investigation.

Conflict of interest statement

None declared.

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FIGURE LEGEND

Figure 1. Intensity scoring of p16 immunohistochemical staining

(**a**) No staining (classified as score 0; negative), (**b**) weak nuclear and cytoplasmic staining (classified as score 1; negative), and (**c**) moderate to strong nuclear and cytoplasmic staining (classified as score 2; positive) of neoplastic cells.

Figure 2. *KRAS* mutation determined by DNA sequencing

Waveforms show guanine (black), thymine (red), cytosine (blue), and adenine (green). In one case, two base peaks (arrow) in codon 12 were detected, revealing *KRAS* mutation.

Figure 3. Histopathological findings of the case with *KRAS* mutation

Atypical squamous cell proliferation with a cancer pearl (arrow) was observed in resected tongue tissue. Although this patient was diagnosed with well-differentiated squamous cell carcinoma of clinical stage I, the patient died because of lung metastasis 43 months after initial treatment.