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The influence of human papillomavirus on nasopharyngeal carcinoma in Japan

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ABSTRACT

Objective: Although Japan is a non-endemic area with nasopharyngeal carcinoma (NPC), the proportion of WHO type I NPC in Japan are different from that in non-endemic areas such as North America and Europe. Recently, it is said that not only Epstein–Barr virus (EBV) but also human papillomavirus (HPV) has an influence on NPC in non-endemic areas. The aim of this study is to clarify the influence of HPV on NPC in Japan.

Methods: Paraffin-embedded tumor specimens were available for 59 patients with NPC diagnosed between 1996 and 2015. We detected the virus status by p16 immunohistochemistry, HPV PCR, and in situ hybridization for Epstein–Barr virus (EBV)-encoded RNA. Kaplan–Meier curves were used to compare the overall survival by viral status.

Results: Among the 59 patients, 49 (83%) were EBV-positive/HPV-negative, 2 (3%) were EBV-positive/HPV-positive, and 8 (16%) were EBV-negative/HPV-negative. All HPV-positive NPCs were co-infected with EBV. There were no significant differences between the overall survival in the three groups ($p = 0.111$).

Conclusion: In Japan, HPV was detected in a few patients with NPC, and we suggest that HPV has no influence on NPC carcinogenesis in this population.

1. Introduction

Nasopharyngeal carcinoma (NPC) is an endemic disease in Southeast Asia and southern China. In contrast, it is relatively uncommon worldwide [1]. Although NPC is a relatively rare disease in Japan, with an incidence of 0.2–0.3 per 100,000 people, in Southern China, the incidence is 19.5 per 100,000 people [2].

NPC has a variety of contributing etiologies, including environmental factors, genetic susceptibility, and infection with Epstein–Barr virus (EBV) [3]. EBV, a ubiquitous human herpesvirus, is the major etiologic agent in non-keratinizing NPC (WHO type II/III). In contrast, keratinizing carcinomas (WHO type I) lack a consistent association with EBV, suggesting differences in the pathogenesis of NPC [4]. Among non-endemic countries, specifically Japan, the proportion of WHO type I NPC is low, and almost all NPCs are linked to EBV

Abbreviations: ACRT, alternating chemo-radiotherapy; CT, computed tomography; EBV, Epstein–Barr virus; HPV, human papillomavirus; NPC, nasopharyngeal carcinoma; OS, overall survival; pRb, hypophosphorylated retinoblastoma protein; SCC, squamous cell carcinoma.

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[5]. In contrast, there are a certain number of WHO type I NPCs in which no relationship with EBV has been identified in other non-endemic countries.

Many studies have indicated that the high-risk type of human papillomavirus (HPV) is etiologically linked to oropharyngeal squamous cell carcinoma (SCC). Furthermore, patients with HPV-positive oropharyngeal SCC have better prognoses than HPV-negative counterparts [6]. Considering the resemblances between the epithelium and lymphoid tissue of the oropharynx and nasopharynx, the following question arises: what is the role of HPV in NPC carcinogenesis? Some studies have found the presence of HPV in NPC. One study reported a high HPV infection rate in a Caucasian population in non-endemic areas, and another study focusing on patients from endemic areas, reported a smaller HPV infection rate [7–12]. Japan was considered a non-endemic area in these studies, but like endemic areas, it had only a few of WHO type I NPC. Consequently, Japan has characteristics of both endemic and non-endemic areas. To the best of our knowledge, this is the first study on the influence of HPV on NPC in Japan.

2. Materials and methods

2.1. Patients

This study included Japanese patients with primary NPC who were diagnosed at Kanazawa University hospital and Toyama Prefectural Central Hospital between January 1996 and January 2015. Paraffin-embedded tumor specimens were available for 59 of these patients.

All patients underwent clinical examinations, such as nasopharyngoscopy and contrast-enhanced MRI or computed tomography (CT) of the head and neck, to determine the tumor, node, and metastasis (TNM) classification. In this study, TNM classification was re-evaluated according to the 1997 UICC classification.

All tumor specimens were obtained from the nasopharynx by biopsy. On the basis of hematoxylin and eosin staining, these samples were classified according to the WHO classification.

2.2. HPV detection and identification

We detected HPV by PCR and p16 immunohistochemistry.

Isolation of DNA. Genomic DNA was extracted from tumor specimens using the QIAamp DNA Mini Kit (Qiagen, Netherlands).

p16 immunohistochemistry. Primary NPC paraffin-embedded specimens were used for the immunohistochemical analysis of p16 expression. Three-micrometer-thick sections were prepared from each block of tissue embedded in paraffin. Deparaffinized sections were treated with 3% hydrogen peroxide for 10 min to inactivate endogenous peroxidase activity. The sections were incubated with a protein blocker (Dako, Glostrup, Denmark) for 20 min and incubated at 4 °C overnight with a p16 (JC8) antibody (Santa Cruz Biotechnology, U.S.A.) as the primary antibody. The sections were washed three times with PBS (pH 7.2). After washing with PBS, the sections were exposed to EnVision^{+} secondary antibody (Dako) for 30 min. The reaction products were developed by immersing the sections in a 3′,3-diamidobenzidine tetrahydrochloride solution. The sections were counterstained with hematoxylin. We determined that of all carcinoma cells with strong and diffuse nuclear and cytoplasmic staining, >70% were positive [13].

HPV PCR. Samples (genomic DNA was extracted from tumor specimens) were subjected to PCR reactions using general primer GP5+/GP6+ sets and the TaKaRa Ex Taq PCR kit (TaKaRa, Japan). The final 10 μl PCR mixture contained 1 μl sample, 2 μl PCR Master Mix, and 1 μl of each primer. Cycling profile was performed with these amplification settings: incubation for 1 min at 94 °C, 36 cycles of denaturation for 20 s at 94 °C and annealing and elongation for 30 s at 48 °C and 72 °C, respectively, and final extension for 5 min at 72 °C.

HPV thirteen. HPV thirteen is used for the identification and typing of HPVs by Nihon Gene Research Laboratories Inc. This method detects and identifies 13 high-risk HPV subtypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) from genomic DNA.

Of the 59 samples, 10 samples were detected as HPV-positive and false positive using PCR. Then, we used HPV thirteen; HPV thirteen revealed that only two samples were HPV-positive. We adopted HPV thirteen's result because HPV thirteen was more reliable than HPV PCR alone.

2.3. EBV detection

EBV status was assessed by in situ hybridization for EBV-encoded RNA (EBER-ISH) in paraffin embedded tissues, using the PNA ISH Detection kit and Epstein–Barr Virus (EBER) PNA Probe/Fluorescein (Dako, Denmark) according to the manufacturer's protocol. EBER-ISH has been established as the most sensitive and practical method for detecting EBV [14]. We determined that of all carcinoma cells with an intense dark-blue staining limited to the nucleus, >90% showed positive.

2.4. Treatment

Of the 59 patients, 36 patients received radiotherapy with cisplatin-based concurrent chemotherapy. According to the change in treatment protocol from 2005, 18 patients received alternating cisplatin and 5-FU chemo-radiotherapy, as previously described [15]. Four patients received radiotherapy alone due to increased age (>70 years) or renal dysfunction. The accumulated dose of radiation to the nasopharynx was 70–77 Gy in all cases, and the dose to the neck was 40–70 Gy. One patient refused treatment.

2.5. Statistical methods

We excluded the one patient who refused treatment from the statistical analysis. In total, all 58 patients were linked to a mortality registry until December 25, 2015. The primary endpoint was death following NPC diagnosis. Overall survival (OS) was calculated using the duration from diagnosis to the

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