SPANDIDOS PUBLICATIONS

Claudin expression profiles in Epstein-Barr virus-associated nasopharyngeal carcinoma

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Abstract. Claudins are a family of proteins that are structural and functional components of tight junctions and have crucial roles in the maintenance of cellular arrangement, adhesion and paracellular transport. Recent studies have shown that changes and/or loss of claudin expression plays an important role in tumorigenesis and tumor progression, and altered expression of claudins has been reported in various human carcinomas. Non-keratinizing nasopharyngeal carcinoma (NPC) is a common Epstein-Barr virus (EBV)-associated carcinoma with characteristic clinicopathological features. The aim of this study was to investigate claudin expression profiles in EBV-associated non-keratinizing NPC. We analyzed expressions of claudin-1, -2, -3, and -4 in 18 cases of EBV-associated non-keratinizing NPC by immunohistochemical methods. Claudin-1 was expressed in all 18 cases, but claudin-2 was not expressed in any of the 18 cases. Claudin-3 expression was variable, with 8 of the 18 cases (45%) showing no immunoreactivity for claudin-3. Claudin-4 displayed positive immunoreactivity in all cases, even in claudin-3-negative cases. Claudin-3 and -4 are receptors for cytotoxic *Clostridium perfringens* enterotoxin (CPE) and CPE has emerged as a potential therapeutic target for malignant tumors expressing claudin-3 and/or -4, because CPE specifically and rapidly lyses cells expressing these proteins. Clinically, treatment of distant metastases is a serious problem in EBV-associated non-keratinizing NPC, because frequently there is lymph node involvement and distant metastasis before detection of the primary tumor. Therefore, CPE therapy may be a potential therapeutic target for EBV-associated non-keratinizing NPC, since our results clearly showed claudin-3 and/or -4 expression in all cases studied.

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Introduction

Tight junctions (TJs) are specialized regions of cell-cell contact and have crucial roles in maintenance of cell polarity, adhesion, cellular arrangement, and paracellular permeability. Claudins have been recently identified as structural and functional components of the TJs in epithelial and endothelial cells and shown to play an important role in TJ function (1-3). They are a family of 24 proteins and various claudins are expressed in different epithelial cells (1-3). Most tissues express multiple claudin proteins, which is thought to account for the selective variability of different tissue functions (1-3).

Recently, claudins have been of interest in cancer research. It has been hypothesized that changes and/or loss of claudin expression may play an important role in tumorigenesis and tumor progression, and altered expression of claudins has been reported in a variety of human neoplasms, including colorectal, breast, ovarian, pancreatic, prostate, tongue and esophageal carcinomas and in rectal well-differentiated endocrine neoplasms (4-13).

Nasopharyngeal carcinoma (NPC) is a squamous cell carcinoma (14) and is subdivided into non-keratinizing carcinoma and keratinizing squamous cell carcinoma (14). NPC accounts for 3.7% of upper aerodigestive tract carcinomas (15). Epstein-Barr virus (EBV) plays an important role in some human neoplasms and all non-keratinizing NPC cases are thought to be associated with EBV infection (14). Claudin expression profiles were recently studied in EBV-associated gastric carcinoma (16). However, claudin expression profiles have not yet been studied in non-keratinizing NPC. Therefore, this study was designed to investigate claudin expression profiles in EBV-associated non-keratinizing NPC, for comparison with claudin expression profiles in squamous cell carcinomas of other sites and in consideration of therapeutic possibility for EBV-associated non-keratinizing NPC.

Table I. Clinical features and claudin expression profiles in EBV-associated non-keratinizing nasopharyngeal carcinoma.

Case no.	Age (year)/gender	Claudin-1	Claudin-2	Claudin-3	Claudin-4
1	26/M	5	0	5	5
2	41/M	5	0	2	3
3	60/M	5	0	3	4
4	59/M	5	0	0	2
5	53/M	5	0	4	5
6	71/M	4	0	4	5
7	74/M	5	0	0	5
8	43/M	5	0	0	5
9	75/F	4	0	1	2
10	66/F	4	0	3	3
11	74/M	5	0	0	5
12	48/M	4	0	4	3
13	62/F	4	0	0	3
14	56/M	5	0	0	5
15	56/M	4	0	0	3
16	80/F	4	0	2	3
17	55/M	5	0	0	5
18	54/M	5	0	4	5

The percentage of immunoreactive cells for each claudin was scored as follows: 0, 0-5% positive; 1, 6-20% positive; 2, 21-40% positive; 3, 41-60% positive; 4, 61-80% positive; and 5, 81-100% positive.

Materials and methods

Tissue specimens of non-keratinizing NPCs. Eighteen formalin-fixed, paraffin-embedded tissue specimens of non-keratinizing NPCs were obtained from patients at Shiga University of Medical Science Hospital, Kyoto First Red Cross Hospital, and Kyoto Second Red Cross Hospital during 1995-2009. The median age of the patients (14 men and 4 women) was 58.5 years (range, 26-80) (Table I). All specimens were evaluated independently by at least two diagnostic pathologists according to the criteria described in the World Health Organization Classification of the Tumours. Pathology and Genetics of Head and Neck Tumours (14). EBV infection in the non-keratinizing NPCs in this study was confirmed by EBER (EBV encoded early RNA) *in situ* hybridization.

Immunohistochemistry and in situ hybridization. Deparaffinized 3-μm sections of the specimens were used for the immunostains and *in situ* hybridization. Immunostainings and *in situ* hybridization were performed using an autostainer (XT System Benchmark; Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's instructions. Primary antibodies for immunohistochemistry were a rabbit polyclonal antibody against human claudin-1 (JAY.8; Zymed Laboratories Inc., San Francisco, CA, USA), a mouse monoclonal antibody against human claudin-2 (12H12; Zymed), a rabbit polyclonal antibody against human claudin-3 (Z23.JM; Zymed), and a mouse monoclonal antibody against human claudin-4 (3E2C1, Zymed). All sections were pretreated with heat. For *in situ* hybridization, an INFORM EBER probe (Ventana Medical Systems) was used.

Evaluation of immunoreactivity and in situ hybridization. The immunohistochemistry and *in situ* hybridization results were confirmed using both negative and positive controls. For negative controls, the primary antibody was substituted with similar diluted non-immunized mouse or rabbit serum. The specificity of the immunoreactivity with individual antibodies was evaluated using positive controls. Positive controls tissue specimens were as follows: normal human epidermis for claudin-1, pancreatic ductal adenocarcinoma for claudin-2 and -4, and non-neoplastic colorectal mucosae for claudin-3, and -4. Tissue specimens of EBV-associated gastric carcinoma were used as a positive control for EBER *in situ* hybridization.

The immunohistochemistry and *in situ* hybridization results were evaluated independently by at least two diagnostic pathologists.

Scoring for semiquantative analysis for claudins. Immunoreactivity was evaluated as described previously (7,10,13). Ten randomly selected fields in each tissue slide were analyzed using high-power field (x400) with 100 cells counted per field. Immunoreactivity was evaluated semiquantatively as the percentage of positive cells, scored as follows: 0, (0-5% positively); 1, (6-20% positively); 2, (21-40% positively); 3, (41-60% positively); 4, (61-80% positively); and 5, (81-100% positively).

Results

Clinicopathological features of non-keratinizing NPCs. A non-keratinizing NPC is defined as a neoplasm composed of

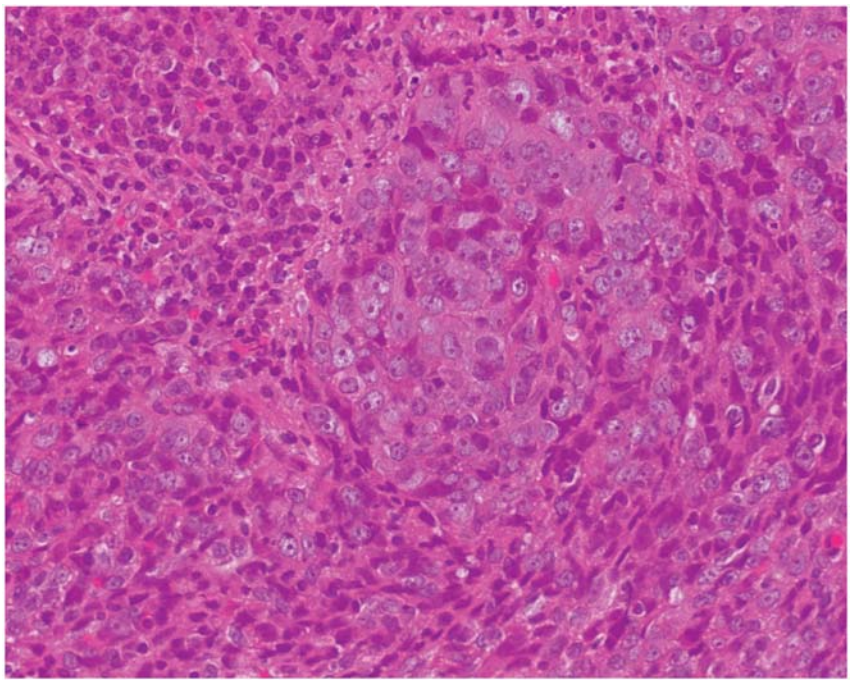

Figure 1. Epstein-Barr virus-associated non-keratinizing nasopharyngeal carcinoma (case 3). H&E staining (original magnification, x200). A typical non-keratinizing nasopharyngeal carcinoma showing solid sheets of tumor cells with enlarged round nuclei, vesicular chromatin, and prominent nucleoli surrounded by rich non-neoplastic lymphoplasmacytic infiltration.

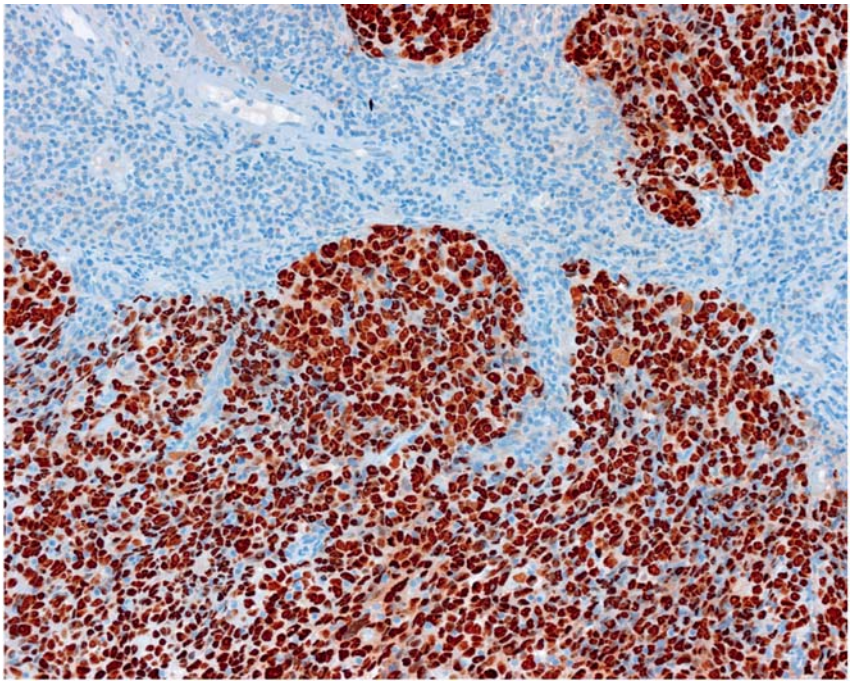

Figure 2. EBER *in situ* hybridization of Epstein-Barr virus-associated non-keratinizing nasopharyngeal carcinoma (case 3). The nuclei of the neoplastic cells are positive (original magnification, x100).

solid sheets or irregular islands of tumor cells separated by a dense infiltration of non-neoplastic lymphocytes and plasma cells (Fig. 1). Lymphocytic infiltration was also observed within the tumor islands. The neoplastic cells are characterized by the presence of enlarged round nuclei, vesicular chromatin, prominent nucleoli, and scant cytoplasm (Fig. 1). There was little or no keratinization. All 18 cases of non-keratinizing NPC in this study were shown to be EBER-positive by *in situ* hybridization (Fig. 2).

Claudin expression in non-keratinizing NPCs. Table I summarizes the claudin expression profile data for 18 cases

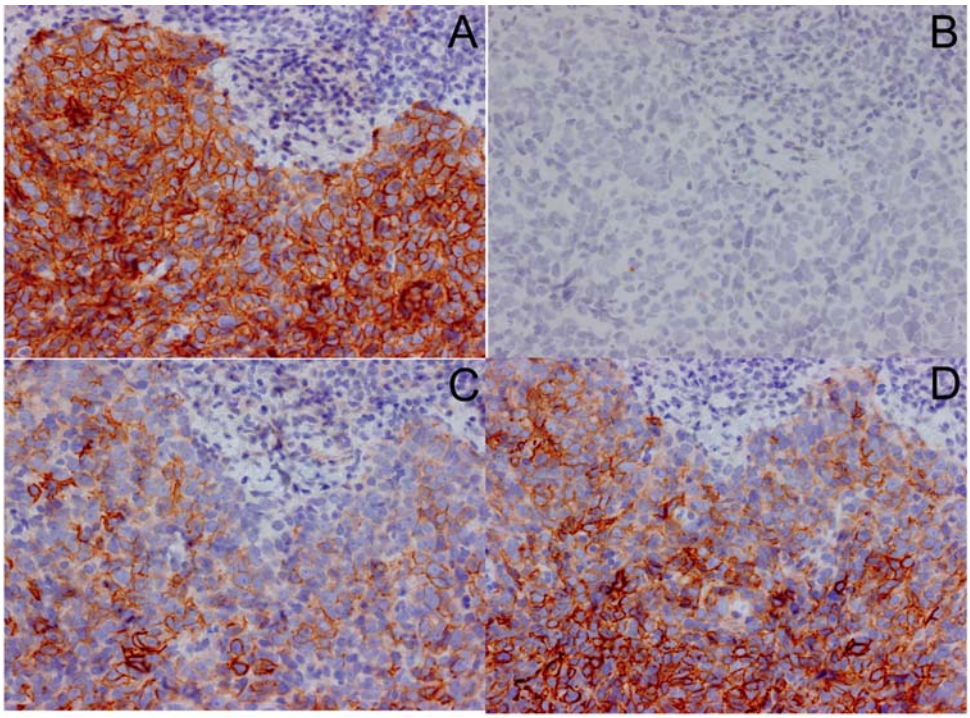

Figure 3. Immunohistochemical results of Epstein-Barr virus-associated non-keratinizing nasopharyngeal carcinoma (case 3). (A) Claudin-1 is expressed diffusely in tumor cells (original magnification, x200). (B) Claudin-2 is not expressed (original magnification, x200). Claudin-3 (C) and -4 (D) expression is observed in approximately 50 and 75% of tumor cells, respectively (original magnification, x200).

of EBV-associated non-keratinizing NPC. Claudin-1 was expressed in the cell membrane of all 18 cases, and 61% (11/18) cases were diffusely expressed (mean score 4.6) (Fig. 3A). None of the 18 cases showed positive immunoreactivity for claudin-2 (all score were 0) (Fig. 3B). Claudin-3 expression was variable (mean score 1.8) (Fig. 3C): 8 cases (45%) showed no expression (score of 0), 9 cases showed membranous immunoreactivity in 6-80% of tumor cells (score of 1-4), and 1 case displayed diffuse membranous expression (score of 5). Claudin-4 had membranous immunoreactivity in all 18 cases (mean score 3.9) (Fig. 3D). All claudin-3-negative cases showed positive immunoreactivity for claudin-4 (score of 2-5).

Discussion

Squamous cell carcinomas develop in various organs, but the molecular events during carcinogenesis can be different in different organs. Recent studies have shown differences in claudin expression in squamous cell carcinomas in different organs. For example, Bello *et al* reported that claudin-1 was strongly expressed and claudin-4 had moderate expression in squamous cell carcinomas of the tongue (11). Takala *et al* also reported that claudin-1 was expressed in almost all esophageal squamous cell carcinomas: 62.3% of these carcinomas showed strong claudin-1 expression with 50% of carcinoma cells being claudin-1 positive (12). In contrast, strong expression of claudin-3 and -4 was observed in only 17.3 and 18.9% of esophageal squamous cell carcinomas, respectively (12). Squamous cell carcinoma of the skin showed strong expression of claudin-1 and -4 in the tumor cells with keratinization, whereas, in non-keratinizing tumor cells of squamous cell carcinoma of the skin, claudin-1 expression was variable and claudin-4 expression was decreased or absent (17). In actinic keratosis, a common intraepidermal squamous cell neoplasm of the skin, claudin-2 was expressed in all cases (mild expression in 3 of 8 cases and strong expression in 5 of 8 cases), but claudin-3 and -4 were not expressed (18).

In the present study, we analyzed the expression profiles of claudin-1, -2, -3, and -4 in 18 cases of EBV-associated non-keratinizing NPC. The results of this study were: i) claudin-1 was expressed in all 18 cases (mean score 4.6); ii) none of the 18 cases showed positive immunoreactivity for claudin-2; iii) claudin-3 expression was variable (mean score 1.8), with 8 cases (45%) showing no claudin-3 immunoreactivity; and iv) claudin-4 was expressed in all 18 cases (mean score 3.9), even in claudin-3-negative cases. These results, together with the previous reports, show that claudin-1 is expressed in squamous cell carcinomas of the tongue, esophagus, and skin, and in EBV-associated non-keratinizing NPC. However, claudin-2, -3 and -4 expression is variable and is dependent on the site of origin of the tumor. For example, claudin-2 is expressed in actinic keratosis, but not in EBV-associated non-keratinizing NPC, and claudin-4 expression is frequently seen in squamous cell carcinomas of the tongue and skin and EBV-associated non-keratinizing NPC, but less frequently in esophageal squamous cell carcinoma (11,12,17,18). These differences in claudin expression seem to be related to differences in the underlying mechanism of carcinogenesis of each squamous cell carcinoma.

Recent progress in therapeutic technology has improved the clinical outcome for patients with EBV-associated non-

ing NPC. In a review of treatment outcome by therapy of NPC, Lee *et al* found that, for 1971-1994, the 5-year overall survival was 71% for stage I, 50% for stage II, 55% for stage III, and 30% for stage IV (19). In contrast, for 1996-2000, the 5-year overall survival for stage I, II, III, and IV was 90, 84, 76%, and 52%, respectively (19). However, one of the major problems of EBV-associated non-keratinizing NPC is frequent lymph node involvement and distant metastasis. At the time of initial presentation, 5% to 11% of patients have distant metastases (20) and painless enlargement of upper cervical lymph node(s) is a common initial presentation of NPC (14). In addition, during the course of the disease, more than 50% of cases develop distant metastases (21), and common metastatic sites are bone, lung and liver (21,22). Accordingly, control of distant metastases is a key issue for improvement in the survival of patients with NPC.

Claudin-3 and -4 are expressed in many normal organs and are overexpressed in several carcinomas, including colorectal, breast, ovary, prostate and pancreatic carcinomas (4-7,9), and these proteins are receptors for cytotoxic *Clostridium perfringens* enterotoxin (CPE) (23,24). CPE is known to injure intestinal epithelial cells expressing claudin-3 and/or -4 by increasing membrane permeability, leading to loss of osmotic equilibrium and subsequent cytolysis and cell death (25). Recently, CPE has emerged as a molecular target in the therapy of malignant tumors expressing claudin-3 and/or claudin-4 (5), because CPE specifically and rapidly lyses cells expressing these proteins. In this study, we have demonstrated that 10 of 18 cases of EBV-associated non-keratinizing NPC expressed both claudin-3 and -4, with claudin-4 being expressed in all 18 cases. Therefore, CPE therapy should be evaluated as a potential therapeutic agent for EBV-associated non-keratinizing NPC, to control lymph node involvement and distant metastasis.

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