Association of Tapasin and HLA Class I Antigen Down-Regulation in Primary Maxillary Sinus Squamous Cell Carcinoma Lesions with Reduced Survival of Patients

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ABSTRACT

Purpose: The purpose of this research was to assess the frequency and clinical significance of antigen processing machinery component and HLA class I antigen down-regulation in primary maxillary sinus squamous cell carcinoma (SCC) lesions.

Experimental Design: Formalin-fixed, paraffin-embedded tumor biopsy specimens at pretreatment status from 70 Japanese patients with maxillary sinus SCC were examined for HLA class I antigen and endoplasmic reticulum chaperone molecule expression using an immunohistochemical method. Furthermore, the results of immunohistochemical staining of the lesions were correlated with their histopathological characteristics and with the clinical course of the disease.

Results: Calnexin, ERP57, calreticulin, tapasin, and HLA class I antigens were down-regulated in 13, 13, 24, 69, and 78% of the 70 lesions tested, respectively. Both tapasin and HLA class I antigen expression were significantly correlated with the number of infiltrating CD3+ T cells into tumor lesions (P < 0.01); furthermore, tapasin expression was significantly correlated with tumor differentiation (P = 0.024). Tapasin expression was correlated with that of HLA class I antigens (P < 0.01). Furthermore, tapasin and HLA class I antigen down-regulation in SCC lesions was significantly associated with reduced survival of patients (P = 0.01 and P = 0.002, respectively). Multivariate Cox proportional hazards model analysis identified HLA class I antigen down-regulation as an independent prognostic marker.

Conclusions: Tapasin expression appears to be associated with HLA class I antigen expression in primary maxillary sinus SCC lesions. Furthermore, defects in tapasin and HLA class I antigen expression in primary maxillary sinus SCC lesions may play a role in the clinical course of the disease, because these defects were associated with poor prognosis.

INTRODUCTION

Malignant transformation of human cells is associated frequently with changes in their HLA antigenic profile (1). The role of HLA antigens in the interaction of tumor cells with a host immune system has stimulated tumor immunologists to investigate the impact of these abnormalities on the clinical course of the disease and on the outcome of T cell-based immunotherapy of malignant diseases. As a result, HLA class I and class II antigen expression has been investigated in a large number of malignant lesions of different histotypes, and correlated with the histopathological characteristics of the lesions and with the clinical course of the disease (2–12). HLA class I antigen down-regulation has been reported to range between 6.0% and 31.6% in head and neck SCC lesions (2, 13–18). Studies of head and neck SCC lesions have lumped together tumors with different anatomical sites within the head and neck, with the exception of laryngeal carcinoma (2). Therefore, it is not known whether the different frequency of HLA class I antigen down-regulation in head and neck SCC lesions reported in the various studies reflects the different representation of various types of head and neck SCC lesions, the different characteristics of the patients included in the various studies, and/or the difference in the sensitivity of the immunohistochemical techniques used. Furthermore, it remains to be determined whether the lack of correlation of HLA class I antigen down-regulation in head and neck SCC lesions with their histopathological characteristics and with the clinical course of the disease reflects the different representation of the various types of head and neck SCC lesions, which have been lumped together in previous studies.

Total or partial HLA class I antigen loss may be caused by different mechanisms. They include HLA class I heavy chain gene mutations (19, 20), β2m gene mutations (21–23), and abnormalities in the antigen processing machinery components.
such as TAP and tapasin (24–26). HLA class I antigens are assembled in ER. The peptides that bind to HLA class I molecules are generated in the cytoplasm from endogenous proteins by proteasome and then are translocated into ER by TAP. In the ER, nascent HLA class I heavy chains interact and are stabilized by ER-retained transmembrane protein calnexin (27). Once β2m associates with HLA class I heavy chain to form the heterodimer, calnexin is replaced by ERp57 and by calreticulin. The former is a thiol oxidoreductase that catalyzes both reduction and oxidation of disulfide bonds (28), whereas the latter is a soluble calnexin homologue (27). Tapasin, a transmembrane protein within ER, bridges the HLA class I heavy chain to TAP and promotes the binding of peptides to the peptide-binding site of HLA class I molecules (25). Finally, the HLA class I heavy chain-β2m-peptide complex dissociates from TAP, ERp57, calreticulin, and tapasin, exits the ER through the Golgi secretory pathway, and travels to the cell surface. Any defect or down-regulation of the ER chaperones may result in HLA class I antigen loss or down-regulation on the cell surface, as indicated by the presence of these defects in calreticulin- and tapasin-deficient cell lines (29–31).

To the best of our knowledge, no information is available about ER chaperone expression in surgically removed carcinoma lesions, probably because of the limited availability of mAb suitable for immunohistochemical staining. As a result it is not known whether ER chaperone expression is defective in malignant cells and has an impact on the clinical course of the disease. Therefore, taking advantage of a panel of mAbs we have developed recently, we have started to analyze ER chaperone expression in different types of head and neck SCC lesions, and to assess the clinical significance of defects in their expression and/or function. In the present study, we have analyzed the expression of ER chaperones of HLA class I antigens, and of HLA class II antigens in primary maxillary sinus SCC lesions, and we have correlated the results of the immunohistochemical assays with the histopathological characteristics of the lesions and with the clinical course of the disease.

MATERIALS AND METHODS

Patients. The study group consisted of 70 Japanese patients (49 males and 21 females) with a median age of 67 years (ranged from 36 to 86 years) who were treated for maxillary sinus SCC in the Department of Otolaryngology, Asahikawa Medical College between 1980 and 2000. According to the 1997 International Union Against Cancer TNM staging systems (identical to the 1997 American Joint Committee on Cancer classification), T1 was not present in any patient; T2 was present in 8 patients (11%), T3 in 33 patients (47%), and T4 in 29 patients (42%). Seven patients (10%) had lymph node metastasis, N1 in all of the cases, at diagnosis. The classification of tumor differentiation was well-differentiated type in 29 patients (42%), moderately differentiated in 24 (34%), and poorly differentiated in 17 (24%). Detailed clinical data of the patients have been reported elsewhere (32).

Of the 70 patients investigated, 58 (83%) were treated with preoperative radiochemotherapy followed by total or partial maxillectomy. The preoperative radiochemotherapy included local irradiation with a total dose of 50 Gy (2.5 Gy × 20 fractions, 5 days/week) along with concomitant intramaxillary arterial infusion of 5-fluorouracil with total dose of 5000 mg (250 mg × 20 times; Ref. 32). The remaining 12 patients (17%) were treated with radiotherapy alone because of intracranial invasion considered to be inoperable, advanced age with poor performance status, refusal of surgery, and concomitant intercurrent illness. Follow-up period ranged from 2 to 189 months with a median of 61 months for all of the patients and of 116 months for surviving patients.

Tissues. Tumor specimens at pretreatment status were obtained by a biopsy of primary maxillary sinus SCC lesions from the 70 patients. Tissue samples were fixed in 20% buffered formalin and embedded in paraffin following standard procedures.

Monoclonal and Polyclonal Antibodies. The mAb HC-10, which recognizes a determinant expressed on all of the β2m-free HLA-B heavy chains and on β2m-free HLA-A10, -A28, -A29, -A30, -A31, -A32, and -A33 heavy chains, the anti-β2m mAb L368, and the anti-HLA class II mAb LGH-612.14, were developed and characterized as described (33–36). The mouse anti-calnexin mAb TO-5, anti-ERp57 mAb TO-2, anti-calreticulin mAb TO-11, and anti-tapasin mAb TO-3 were developed and characterized as described (37). The EPOS anti-CD3/horseradish peroxidase was purchased from DAKO (Glostrup, Denmark).

Immunohistochemical Staining. Immunoperoxidase staining of tissue sections was performed using the EnVision+ system (DAKO, Carpinteria, CA). Briefly, 4 μm-thick, paraffin-embedded tissue sections were deparaffinized with xylene and rehydrated by passage through decreasing concentrations of ethanol. Antigens were retrieved by microwave in citrate buffer (pH 6.0) twice for 5 min before staining with mAb. Tissue sections were incubated with 3% H2O2 in methanol to block endogenous peroxidase activity. Then they were sequentially stained with mAb TO-5, TO-2, TO-11, and TO-3, PBS supplemented with 0.3% Tween 20 was used to incubate in the incubation steps of tissue sections with antibodies and to wash slides (38). The percentage of stained tumor cells in each lesion was evaluated independently by two investigators (10). Normal lymphocytes and vessel endotheliums were used in each specimen as internal controls. Negative controls were performed by omitting primary antibodies. Results were scored as (+), (±) and (−) when the percentage of stained tumor cells in an entire lesion was >75%, 25–75%, and <25%, respectively, according to the criterion established by the HLA and Cancer component of the 12th International Histocompatibility Workshop (39).

Staining with horseradish peroxidase-conjugated anti-CD3 antibody was performed according to the manufacturer’s instructions. Results of staining with anti-CD3 antibody were calculated by counting the number of stained infiltrating cells in 500 μm2 of a SCC lesion.

Statistical Analysis. Correlation of ER chaperone and HLA antigen expression with histopathological and clinical parameters was tested by Spearman rank correlation coefficient.
(40). Disease-free survival time was measured from the date of surgical removal of the tumor to the date of first relapse or to the date of last follow-up. The probability of disease-free survival was calculated using Kaplan-Meier method (41) and compared using log-rank test. A Cox proportional hazards model was used to determine the relationship of variables to disease-free survival (42). $P < 0.05$ was considered to be statistically significant.

RESULTS

HLA Class I and Class II Antigen Expression in Primary Maxillary Sinus SCC Lesions. Seventy primary maxillary sinus SCC lesions were stained with anti-HLA class I heavy chain mAb HC-10, anti-β2m mAb L368, and anti-HLA class II mAb LGGI-612.14 using the immunoperoxidase reaction. Fig. 1 shows representative examples of the staining patterns of the SCC lesions with the mAbs. The majority of tumor cells are stained by anti-HLA class I heavy chain mAb HC-10, anti-calnexin mAb, anti-ERp57 mAb, and anti-HLA class II mAb in the lesion scored as (+; Fig 1, A, C, and E). No staining of tumor cells by the three mAbs is detected in the lesions scored as (−; Fig. 1, B, D, and F). Lymphocytes and vessel endotheliums were used as a quality control of the immunohistochemical staining in each specimen. Table 1 summarizes the results of HLA class I and class II antigen expression in 70 primary maxillary sinus SCC lesions. Anti-HLA class I heavy chain mAb HC-10 and anti-β2m mAb, and anti-HLA class II mAb in the lesion scored as (+; Fig 1, A, C, and E). There were no available for the staining with mAb. Table 2 summarizes the results of tapasin, calnexin, ERp57, and calreticulin expression in 70 primary maxillary sinus SCC lesions. Anti-tapasin mAb TO-3, anti-calnexin mAb TO-5, anti-ERp57 mAb TO-2, and anti-calreticulin mAb TO-11 stained 21 (31%), 59 (87%), 59 (87%), and 50 (76%) of SCC lesions with a score (+), 28 (41%), 9 (13%), 9 (13%), and 16 (24%) of the lesions with a score (±) and 19 (28%), 0 (0%), 0 (0%), and 0 (0%) of the lesions with a score (−), respectively. Only tapasin was found to be down-regulated in a high percentage of maxillary sinus SCC lesions. Furthermore, analysis by the Spearman rank correlation coefficient of the expression of tapasin, calnexin, ERp57, and calreticulin with that of HLA class I antigens showed that only tapasin expression is correlated significantly with that of HLA class I antigens ($P < 0.01$; Fig. 3). This finding suggests that tapasin expression may be associated with HLA class I antigen expression in maxillary sinus SCC lesions.

Correlation of HLA Class I Antigen and ER Chaperone Expression in Primary Maxillary Sinus SCC Lesions with Their Histopathological Characteristics. The level of HLA class I antigen expression tended to be high in well-differentiated tumor lesions. However, the association between level of HLA antigen expression and tumor cell differentiation did not reach the level of statistical significance ($P = 0.097$; Fig. 4A). Furthermore, HLA class I antigen expression was not correlated significantly with T stage ($P = 0.27$; Fig. 4B). In contrast, HLA class I antigen expression was correlated significantly with the degree of CD3+ T-cell infiltration in SCC lesions ($P < 0.01$; Fig. 4C). β2m expression in the lesions was not correlated significantly with tumor differentiation, but was correlated significantly with T stage ($P = 0.029$) and with the degree of CD3+ T-cell infiltration in the lesions ($P = 0.009$; data not shown).

Tapasin expression was correlated significantly with tumor differentiation ($P = 0.024$; Fig. 5A) and with the degree of CD3+ T-cell infiltration in SCC lesions ($P < 0.01$; Fig. 5C), but was not correlated with T stage ($P = 0.13$; Fig. 5B). On the other hand, calnexin, ERp57, and calreticulin expression was not correlated with any of the variables analyzed (data not shown). The same conclusion was reached when the HLA class II antigen expression in the 70 lesions was correlated with the variables analyzed (data not shown).

Association of ER Chaperone and HLA Class I Antigen Expression with Patient Survival. To assess the clinical significance of ER chaperone and HLA class I antigen expression in maxillary sinus SCC lesions, 58 patients treated uniformly with preoperative radiochemotherapy followed by total or partial maxillectomy were selected. Among the ER chaperones analyzed, only tapasin expression was correlated with the disease-free survival in the 58 patients. The disease-free survival of the 14 patients whose lesions were stained by anti-tapasin mAb TO-3 with a score (−) was significantly shorter than that of the 42 patients whose lesions were stained with scores (+) and (±; $P = 0.01$; Fig. 6A). Similar results were obtained when the association between HLA class I antigen expression in the lesions and patient survival was analyzed. The disease-free survival of the 21 patients whose lesions were stained by anti-HLA class I heavy chain mAb HC-10 with a score (−) was significantly shorter than that of the 37 patients whose lesions were stained with score (+) and (±; $P = 0.002$; Fig. 6B). As far
Fig. 1 Representative staining patterns of formalin-fixed, paraffin-embedded primary maxillary sinus SCC lesions with HLA antigens and ER chaperone-specific mAb. The staining with anti-HLA class I heavy chain mAb HC-10 (A and B), with anti-β2m mAb L368 (C and D), and with anti-HLA class II mAb LGII-612.14 (E and F) was scored as (+; A, C, and E) and as (−; B, D, and F). The staining with anti-tapasin mAb TO-3 (G and H), with anti-calnexin mAb TO-5 (I), with anti-ERp57 mAb TO-2 (J), and with anti-calreticulin mAb TO-11 (K) was scored as (+; G, I, J, and K) and as (−; H), respectively. Negative controls were performed by omitting primary antibodies (L; ×200).
as the association of CD3\(^+\) T-cell infiltration with patient survival is concerned, the disease-free survival of the 13 patients whose number of CD3\(^+\) T-cell infiltrations into the lesion were at least 150 tended to be longer than that of the 45 patients whose number of CD3\(^+\) T-cell infiltrations were <150; however, the association did not reach the level of statistical significance (\(P = 0.058\); data not shown).

To determine whether any of the variables analyzed were independent prognostic factors, data were analyzed by multivariate Cox proportional hazards model analysis. The variables analyzed included tumor differentiation (poorly differentiated), CD3\(^+\) T-cell infiltration (<75 cells), HLA class I antigen expression (<25% stained cells), HLA class II antigen expression (<25% stained cells), and tapasin expression (<25% stained cells; Table 3). HLA class I antigen down-regulation was the only one to be found to be an independent prognostic factor (hazards ratio = 7.72; \(P = 0.017\)).

**DISCUSSION**

Changes in the HLA antigenic profile are associated frequently with malignant transformation of cells (1). HLA class I antigen down-regulation has been described in a number of malignant diseases, its frequency ranging from 31% in lung carcinoma to 70% in prostate carcinoma (2–11). Maxillary sinus SCC is no exception to this general finding, because in the present study immunohistochemical staining of 70 formalin-fixed, paraffin-embedded primary maxillary sinus SCC lesions with mAb has shown HLA class I antigen down-regulation in 78% of the lesions tested. This frequency is markedly higher than that reported previously in head and neck SCC lesions, which ranges between 6.0% and 31.6% (2, 13–18). Our study differs from those in the literature in at least three aspects, i.e., the substrate used in the immunohistochemical reactions, the specificity of the mAb used to stain lesions, and the type of patients included in the study. All of the studies in the literature (2, 13–15, 17, 18) with the exception of that by Mattijssen et al. (16) have used frozen tissue sections, whereas we have used formalin-fixed, paraffin-embedded primary maxillary sinus SCC lesions with mAb. This difference is not likely to account for the higher frequency of HLA class I antigen down-regulation we have found, because two previous studies (16, 43) have not found marked differences in the sensitivity of immunohistochemical staining of frozen and formalin-fixed tissue sections with anti-HLA.
mAb. On the other hand, the specificity of the mAb used to detect HLA class I antigens may play a role. Most, if not all of the other investigations have used mAb, which recognize framework determinants expressed on all of the gene products of HLA-A, B, and C loci. In contrast, we have used the mAb HC-10, which recognizes a determinant expressed on all of the HLA-B and C allospecificities but only some HLA-A allospecificities (34). Therefore, we cannot exclude that the restricted reactivity pattern of mAb HC-10 reduces the sensitivity of the immunohistochemical reaction. Lastly, all of the other studies (13–18) with the exception of that by Esteban et al. (2) have lumped together tumors located in different sites in head and neck, whereas we have analyzed only SCC lesions located in maxillary sinus. Tumors located in different sites within head and neck may differ in their biological behavior and prognosis. This possibility is being tested by us in a systematic study, which compares HLA antigen expression in tumors located in different sites within head and neck.

The present study has shown for the first time tapasin down-regulation or loss in 60% of the 70 primary maxillary sinus SCC lesions analyzed. The other components of the antigen processing machinery analyzed, i.e. calnexin, ERp57, and calreticulin, were down-regulated in at most 20% of the lesions tested. To the best of our knowledge, the expression of calnexin, ERp57, calreticulin, and tapasin at the protein level has not been analyzed in any type of carcinoma. The lack of this information in the literature probably reflects the lack or limited availability of antigen processing machinery component-specific antibodies suitable for immunohistochemical staining of malignant lesions. No defects have been detected in the expression of calnexin, ERp57, calreticulin, and tapasin mRNA in human cell lines derived from neuroblastoma, head and neck SCC, and lung, colon, pancreatic, and renal carcinomas (44–46). If not caused by technical reasons, the significant difference between the information in the literature about tapasin expression in malignant cells and our own results suggests that tapasin abnormalities in malignant cells may affect its translation and not its transcription. This finding, which parallels those obtained by analyzing β₂m expression in human melanoma cell lines (47), emphasizes the need to test tapasin expression in malignant lesions by staining with antibodies.

HLA class I antigen down-regulation in primary maxillary sinus SCC lesions appears to have clinical significance, because it

Fig. 4 Correlation of HLA class I antigen expression in primary maxillary sinus SCC lesions with their histopathological characteristics. Tumor cell differentiation in the lesions was scored according to histopathological criteria (A). T stages were based on TNM staging systems (B). Number of infiltrating CD3⁺ T lymphocytes was calculated by counting the number of stained infiltrating cells in 500 μm² of a SCC lesion (C). Statistical analyses were performed by Spearman rank correlation coefficient.

Fig. 5 Correlation of tapasin expression in primary maxillary sinus SCC lesions with their histopathological characteristics. Staining by anti-tapasin mAb TO-3 was evaluated as the percentage of stained tumor cells in each entire lesion. Tumor cell differentiation in the lesions was scored according to histopathological criteria (A). T stages were based on TNM staging systems (B). The number of infiltrating CD3⁺ T lymphocytes was calculated by counting the number of stained infiltrating cells in 500 μm² of a SCC lesion (C). Statistical analyses were performed by Spearman rank correlation coefficient.
is correlated with a statistically significant decrease in patient survival. Association of HLA class I antigen down-regulation with poor prognosis has already been described in esophageal carcinoma (7) and in melanoma (10). However, to the best of our knowledge, maxillary sinus SCC represents the first malignancy in which HLA class I antigen down-regulation in primary lesions has been found to be an independent prognostic factor. As far as the mechanism(s) underlying the described association is concerned, one might argue that the reduced survival of the patient reflects escape of tumor cells from recognition by HLA class I-restricted, tumor-associated antigen-specific cytotoxic T lymphocytes because of HLA class I antigen down-regulation. This possibility is supported by the association between high HLA class I antigen expression level and high T-cell infiltration in primary maxillary sinus SCC lesions. If our interpretation is correct, characterization of the molecular mechanisms underlying HLA class I antigen down-regulation may have a positive impact on the treatment of maxillary sinus SCC because this information may suggest strategies to correct HLA defects in malignant cells. In this regard, two lines of evidence suggest that the association between tapasin and HLA class I antigen expression level found in primary maxillary sinus SCC lesions reflects a cause-effect relationship between these two abnormalities. First, tapasin has been shown to increase the level of TAP, thereby enhancing the efficiency of peptide translocation. Second, tapasin plays a major role in peptide loading and cell surface expression of most HLA class I alleles (25). Furthermore, aberrant tapasin expression may affect the interaction of tumor cells with HLA class I-restricted, tumor-associated antigen-specific cytotoxic T lymphocytes by changing the spectrum of peptides presented by HLA class I molecules. These possibilities are supported by the association of tapasin expression with reduced survival of patients.

HLA class II antigens have been found to be expressed in various types of malignancies (3, 6, 7, 12); the frequency ranges from 12% in laryngeal carcinoma (12) to 80% in cervical carcinoma (3). Conflicting information is available about the clinical significance of HLA class II antigen expression in malignant lesions (3, 6, 7, 12). In the present study, HLA class II antigen expression was found in 18.6% of 70 primary maxillary sinus SCC lesions and was not significantly correlated with their histopathological characteristics and with clinical parameters. This finding, which is at variance with those in laryngeal carcinoma (12), esophageal carcinoma (7), cervical carcinoma (48), and melanoma (49, 50), argues against a major role played by HLA class II antigens in the pathogenesis and clinical course of the disease.

From a methodological viewpoint it is noteworthy that the present study has used successfully formalin-fixed, paraffin-embedded tissue sections as a substrate in immunoperoxidase reactions to evaluate ER chaperone and HLA class I antigen expression in malignant lesions. The use of formalin-fixed, paraffin-embedded tissues, which represent the substrate of choice in immunohistochemical reactions by pathologists, is expected to facilitate the analysis of HLA antigen abnormalities in lesions in the evaluation of patients with malignant diseases, especially those to be enrolled in trials of T cell-based immunotherapy and in trials of gene therapy, which aim at correcting HLA antigen abnormalities.

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