Abstract. HLA class I antigen processing machinery plays a crucial role in the generation of peptides from endogenously synthesized proteins and in their presentation to cytotoxic T lymphocytes. The purpose of this study was to analyze the downregulation of HLA class I antigen, transporter associated with antigen processing (TAP) and tapasin in primary and metastatic lesions of head and neck squamous cell carcinoma (HNSCC) and to compare TAP, tapasin and HLA class I antigen downregulation in metastatic lesions with that of primary lesions. We analyzed expression levels of TAP1, TAP2, tapasin and HLA class I antigen in 25 primary and autologous metastatic lesions by staining formalin-fixed, paraffin-embedded tissue sections in the immunoperoxidase reaction. We identified the expression levels of TAP1, TAP2, tapasin and HLA class I antigen were coordinately downregulated in both primary and metastatic lesions and were significantly lower in metastatic lesions than in autologous primary lesions tested. HLA class I antigen downregulation in metastatic lesion was significantly associated with reduced disease-free survival of patients (P<0.05). Multivariate Cox proportional hazards model analysis identified negativity of HLA class I antigen as an independent prognostic marker. HLA class I antigen and TAP are likely to be downregulated in metastatic lesions compared with primary lesions in HNSCC. The higher frequency of HLA class I antigen and TAP downregulation in metastases play a role in the clinical course of the disease.

Introduction

Malignant transformation of human cells is frequently associated with changes in HLA class I antigen expression and/or function (1-4). These changes may affect the interactions of malignant cells with the host’s immune system and especially with HLA class I antigen restricted, tumor antigen (TA)-specific CTL. As a result they may have a negative impact on the clinical course of malignant disease as well as on the outcome of T cell-based immunotherapy (5). Many molecular mechanisms have been shown to underlie defects in HLA class I antigen expression and processing. Among them are abnormalities in the HLA class I antigen processing machinery (APM). In previous years the role of this machinery in the generation and stable expression of HLA class I antigens on the cell membrane has been well characterized (6). Peptides presented by HLA class I antigens are generated mostly from intracellular antigens by the proteasome which sharpens its proteolytic activity by replacing the delta (Y), MB1 (X) and Z subunits with low molecular weight protein (LMP)2, LMP7 and LMP10 subunits, respectively, when exposed to interferon (IFN)-γ (7). The generated peptides are translocated by the transporter associated with antigen processing (TAP)1 and TAP2 complex into the endoplasmic reticulum (ER) where the chaperones calnexin, calreticulin and ERp57 are involved in the correct folding and assembly of nascent HLA class I heavy chains with β2microglobulin (β2m) (8,9). The resulting complexes are bridged by tapasin to TAP which loads peptides. Following dissociation from TAP and ER chaperones, the trimolecular complex travels through the Golgi secretory pathway to the cell surface for presentation of peptides to CTLs (10). Defects in TAP1, TAP2 and tapasin may result in HLA class I antigen loss or downregulation on the cell surface, as indicated by TAP and tapasin-deficient cell lines (11-13).

Head and neck squamous cell carcinoma (HNSCC) is the most common malignancy of the upper aerodigestive tract. Immunohistochemical staining of frozen and/or formalin-fixed, paraffin-embedded HNSCC primary lesions has detected HLA class I antigen loss and downregulation with a frequency ranging from 6 to 81% (14-21). The mechanisms underlying the HLA class I antigen defects in HNSCC lesions have been analyzed only to a limited extent. In particular scanty
information is available on the role of TAP1, TAP2 and tapasin defects in the generation of the abnormal HLA class I antigen phenotypes identified in HNSCC lesions. The lack of this information has a negative impact on the design of strategies to correct HLA class I antigen defects in HNSCC lesions. Therefore, in the present study we have analyzed TAP1, TAP2 and tapasin expression in primary and autologous metastatic lesions. We have correlated the results of immunohistochemical staining of the lesions with the expression of HLA class I antigen and with the disease progression.

Patients and methods

Patients and tissue samples. Tumor specimens from 25 patients included biopsies for diagnostic purposes and primary lesions and metastatic lymph nodes surgically removed for therapeutic purposes. Tissue samples were fixed with 20% buffered formalin, routinely processed and embedded in paraffin. None of the patients had received preoperative therapy. All the patients signed informed consent for tissue studies, which had received prior approval from the Institutional Review Board.

Monoclonal antibodies. The TAP1-specific mAb NOB-1 and the TAP2-specific mAb NOB-2 are secreted by hybridomas derived from the fusion of murine myeloma cells P3-X63-Ag8.653 with splenocytes from BALB/c mice immunized with partial length TAP1 recombinant fusin protein (amino acids 434-735) and a keyhole limpet hemocianin-conjugated TAP1 peptide (amino acids 717-735) and with partial length TAP2 recombinant protein (amino acids 316-703), respectively. The specificity of the mAbs was assessed by their reactivity with molecules with the size corresponding the immunizing TAP1 and TAP2 when tested in Western blotting with a lysate of the human T2 cells and the mouse myeloma cells P3-X63-AG8.653 (22). The mAb HC-10 which recognizes a determinant expressed on all β2m-free HLA-B and C heavy chains and on β2m-free HLA-A10, -A28, -A29, -A30, -A31, -A32 and -A33 heavy chains were used (23). The tapasin-specific mAb TO-3 was developed as described (24). The anti-idiotypic mAb MK2-23 used as an isotype-negative control was developed as described (25).

Immunoperoxidase staining of formalin-fixed, paraffin-embedded tissue sections. Immunoperoxidase staining of formalin-fixed, paraffin-embedded tissue sections with mAb was performed utilizing the EnVision+ system (Dako Cytomation, Carpinteria, CA, USA) as described (26). The percentage of stained tumor cells in each lesion was evaluated independently by two investigators. Normal lymphocytes and/or vessel endothelia were used in each specimen as internal positive controls. Staining with mAb MK2-23 was used as a negative control. Results were scored as positive (+), heterogeneous (±) and negative (-) when the percentage of stained tumor cells in an entire lesion was (75%, 25-75% and 25%, respectively, according to the criteria established by the HLA and Cancer component of the 12th International Histocompatibility Workshop (27).

Statistical analysis. Differences of TAP1, TAP2, tapasin and HLA class I antigen expression in primary lesions from those in autologous metastatic lesions were tested utilizing Wilcoxon signed rank test. Correlations among the parameters were determined using the Spearman rank correlation coefficient. Disease-free survival (DFS) was calculated using the Kaplan-Meier method. Time was defined as the period starting from the date of diagnosis to the date of disease relapse or that of last follow-up visit. A log-rank test was used for screening the possible prognostic factors in relation to the patients’ survival. A Cox proportional hazards model was used to determine the relationship between DFS and other variables. Statistical tests were based on a level of significance at P<0.05.

Results

Patients. Between 1995 and 2005, 25 Japanese patients (20 males and 5 females) ranged from 51 to 77 with a median age of 65 years old were treated for HNSCC in the Department of Otolaryngology-Head and Neck Surgery, Asahikawa Medical College Hospital. Clinicopathological characteristics of the patients are shown in Table I. The anatomic site of primary lesions was the oral cavity in 10 (40%) patients, the larynx in 6 (24%), the hypopharynx in 6 (24%), and the oropharynx in 3 (12%). Tumors were well differentiated in 23 (37%) patients, moderately differentiated in 33 (52%), and poorly differentiated in 7 (11%). According to the Tumor-Node-Metastasis Classification of Malignant Tumors (6th edition),
the T classification of the disease was T1 in 2 (8%) patients, T2 in 12 (48%), T3 in 8 (32%), and T4a in 3 (13%). the N classification of the disease at diagnosis was N1 in 12 (48%), N2a in 3 (12%), N2b in 8 (32%), and N2c in 2 (8%) patients. All the patients were treated with radical surgery composed of local resection for primary tumor and neck dissection for neck node metastasis. Follow-up period of all patients ranged from 2 to 129 months with a median of 61 months. Ten (40%) of 25 patients died from the disease. The 5-year DFS rate for all patients was 51.2%.

**TAP1, TAP2 and tapasin downregulation in relation to HLA class I antigen downregulation in primary HNSCC lesions.** To determine whether the downregulation was present in primary HNSCC lesions, TAP1, TAP2, tapasin and HLA class I antigen expression was analyzed in 25 primary lesions by staining formalin-fixed, paraffin-embedded tissue sections with TAP1, TAP2, tapasin and HLA class I heavy chain-specific mAbs. Fig. 1 shows representative negative staining patterns for TAP1, TAP2, tapasin and HLA class I antigen expression in primary lesion obtained from a patient. As summarized in Table II, TAP1, TAP2, tapasin and HLA class I antigen expression was downregulated (heterogeneous and negative) in 17 (68%), 16 (64%), 15 (60%) and 15 (60%) of the 25 primary lesions tested, respectively. Analysis by Spearman rank correlation coefficient showed significant correlations of HLA class I antigen with TAP1 (P<0.01), TAP2 (P<0.01), tapasin (P<0.05; Fig. 2).

**Comparison of TAP1, TAP2, tapasin and HLA class I antigen expression in metastatic lesions with that in autologous primary lesions in patients with HNSCC.** TAP1, TAP2 and HLA class I antigen expression was downregulated in 21 (84%), 22 (88%), 17 (68%) and 22 (88%) of the 25 metastatic lesions tested, respectively (Table II). Representative staining pattern of both primary and autologous metastatic lesion is shown in Fig. 1.

**Table II. TAP1, TAP2, tapasin and HLA class I antigen expression in HNSCC lesions.**

<table>
<thead>
<tr>
<th>Stained tumor cells (%)</th>
<th>TAP1 Primary</th>
<th>TAP1 Metastatic</th>
<th>TAP2 Primary</th>
<th>TAP2 Metastatic</th>
<th>Tapasin Primary</th>
<th>Tapasin Metastatic</th>
<th>HLA class I Primary</th>
<th>HLA class I Metastatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;75% (positive)</td>
<td>8 (32%)</td>
<td>4 (16%)</td>
<td>9 (36%)</td>
<td>3 (12%)</td>
<td>10 (40%)</td>
<td>8 (32%)</td>
<td>9 (36%)</td>
<td>3 (12%)</td>
</tr>
<tr>
<td>25-75% (heterogeneous)</td>
<td>15 (60%)</td>
<td>12 (48%)</td>
<td>14 (56%)</td>
<td>16 (64%)</td>
<td>13 (52%)</td>
<td>13 (52%)</td>
<td>13 (52%)</td>
<td>13 (52%)</td>
</tr>
<tr>
<td>&lt;25% (negative)</td>
<td>2 (8%)</td>
<td>9 (36%)</td>
<td>2 (8%)</td>
<td>6 (24%)</td>
<td>2 (8%)</td>
<td>4 (16%)</td>
<td>3 (12%)</td>
<td>9 (36%)</td>
</tr>
</tbody>
</table>

**Figure 1.** Representative staining patterns for TAP1, TAP2, tapasin and HLA class I antigen of formalin-fixed tissue sections from a primary HNSCC lesion. The sections of a primary lesion from patient no. 10 were stained with anti-TAP1 mAb NOB-1 (panel A), anti-TAP2 mAb NOB-2 (panel B), anti-tapasin mAb TO-3 (panel C) and anti-HLA class I heavy chain mAb HC-10 (panel D; x400). Staining of lymphocytes was used as a quality control of the immunohistochemical staining in each tissue section. All the panels were scored as negative.
shown in Fig. 3. Although positive staining of TAP1, TAP2 and HLA class I antigen was seen in the primary lesion, TAP1, TAP2 and HLA class I antigen expression was scored with negative in the autologous metastatic lesion. The expression levels of TAP1, TAP2 and HLA class I antigen were significantly lower in metastatic lesions than in autologous primary lesions tested (P<0.05 each, Fig. 4).

Prognosis according to variables. Kaplan-Meier analysis showed that DFS was significantly worse in patients with positive staining of HLA class I antigen than in patients with negative staining in metastatic lesions (P<0.05; Fig. 5). Significant difference was not identified with regard to TAP1, TAP2 and HLA class I antigen expression. To determine whether any of the variables analyzed was an independent prognostic factor, data were analyzed by multivariate Cox proportional hazards model. The variables analyzed include T classification (T3 and 4), Gender (male), tumor differentiation (moderate + poor differentiation), and HLA class I antigen expression (negative) in metastatic lesions. TAP1, TAP2, and tapasin were excluded from this analysis because each of these variables was correlated with HLA class I antigen expression. Negativity of HLA class I antigen expression in metastatic lesions was the only variable found to be a statistically significant independent prognostic factor for DFS (hazard ratio (HR)=4.32; P=0.048; Table III).

Table III. Multivariate Cox proportional hazards analysis of DFS for independent prognostic factors in patients with HNSCC.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor extension (T3 and 4)</td>
<td>2.63</td>
<td>0.62-9.54</td>
<td>0.19</td>
</tr>
<tr>
<td>Gender (Male)</td>
<td>1.24</td>
<td>0.34-4.31</td>
<td>0.78</td>
</tr>
<tr>
<td>Tumor differentiation (moderate + poor)</td>
<td>2.01</td>
<td>0.55-6.23</td>
<td>0.26</td>
</tr>
<tr>
<td>HLA class I antigen (negative)</td>
<td>4.32</td>
<td>1.02-19.53</td>
<td>0.048</td>
</tr>
</tbody>
</table>

HR, hazard ratio; CI, confidence interval.

Discussion

Immunohistochemical staining of surgically resected tissue sections from 25 HNSCC patients demonstrated that the expression levels of TAP1, TAP2, tapasin and HLA class I antigen was downregulated in both primary and metastatic lesions. In this study, HLA class I antigen downregulation was 60% in 25 primary HNSCC lesions. Ferris *et al* (19) reported that overall defects in HLA class I antigen expression, which range from complete loss to heterogeneous expression, from haplotype loss to selective loss of single allospecificities, has been reported in 60% of the 491 lesions tested. Our results were consistent with the previous studies.

Among the APM components TAP has been the most extensively investigated in HNSCC lesions, but the results have not been concordant. Immunohistochemical staining of HNSCC lesions has yielded conflicting data on TAP expression. Vora *et al* (17) reported that TAP1 and TAP2 were downregulated in only 2 (9%) and 0 (0%) of 34 primary HNSCC lesions tested, respectively. Feenstra *et al* (28) reported that TAP1 and TAP2 were downregulated in 28
and 8 (13%) of 53 primary HNSCC lesions tested, respectively. These results were obtained utilizing frozen tissue sections stained with antisera. Antisera are difficult to standardize because of differences in antibody content in bleeding samples obtained from an immunized animal and also between immunized animals. Meissner et al reported TAP1 and TAP2 were downregulated in 13 (52%) and 22 (88%) of 25 patients with TAP1-specific mAb 148.3 and TAP2-specific mAb 429.4, respectively (20). TAP1-specific mAb NOB-1 and TAP2-specific mAb NOB-2 are also suitable for staining of formalin-fixed tissue sections. Analysis of staining of formalin-fixed tissue section from cervical carcinoma patients with mAb NOB-1 and NOB-2 showed the frequency of TAP1 and TAP2 downregulation was 23 and 37%, respectively (29). Similarly, negativity of TAP1 and TAP2 was 33.3 and 26.7% in patients with ovarian carcinoma with mAb NOB-1 and NOB-2 (30). The association among TAP1, TAP2 and HLA class I antigen expression and their coordinately downregulation in HNSCC in primary lesions suggest that aberrant expression of TAP can be one of mechanisms of tumor escape from immunosurveillance under the circumstances where total loss of HLA class I antigen surface expression is not seen in HNSCC (21).

In this study the expression levels of TAP1, TAP2 and HLA class I antigen were lower in metastatic lesions than in autologous primary lesions in 25 HNSCC lesions tested. The loss of HLA class I antigen expression in primary HNSCC lesions was reported to be associated with regional lymph node metastases (31). It was observed that HLA class I antigen expression was downregulated in 2 (22%) metastatic lesions

![Figure 3. Immunoperoxidase staining for TAP1, TAP2, tapasin and HLA class I antigen of formalin-fixed tissue sections of primary and metastatic HNSCC lesions. The sections of a primary lesion (panels A, C, E and G) and autologous metastatic lesion (panels B, D, F and H) from patient no. 7 were stained with anti-TAP1 mAb NOB-1 (panels A and B), anti-TAP2 mAb NOB-2 (panels C and D), anti-tapasin mAb TO-3 (panels E and F) and anti-HLA class I heavy chain mAb HC-10 (panels G and H; x200). The expression levels of TAP1, TAP2, tapasin and HLA class I antigen in primary lesion were scored as positive. However, the expression levels of TAP1, TAP2, tapasin and HLA class I antigen in autologous metastatic lesion was scored as negative.](image-url)
of 9 autologous primary lesions tested (14). However, scanty information is available on the expression of TAP1 and TAP2 in metastatic HNSCC lesions and about comparison of the expression in primary lesions with that in autologous metastatic lesions. Only one study showed that TAP1 expression was changed to downregulation in 2 (20%) metastatic lesions when compared with 10 autologous primary lesions tested (14). The frequency of TAP1 downregulation was significantly higher in metastases than in autologous primary lesions in cervical carcinoma (32). In addition TAP1 downregulation was observed in metastases compared with primary lesions in breast (33) and renal cell carcinoma (34).

Our results are compatible with the results as reported in these types of carcinomas. Possible explanation for the coordinate downregulation of HLA class I antigen, TAP1 and TAP2 with the development of metastatic lesions includes immune selection of tumors with an abnormal HLA class I antigen phenotype and/or accumulation of mutations by tumor cells. On the other hand HLA class I antigen downregulation in association with TAP downregulation may possess a selective advantage to metastasize in malignant disease, suggesting an association with tumor aggressiveness.

The association between HLA class I antigen downregulation and poor prognosis has already been described in maxillary sinus SCC (18), laryngeal SCC (21) and esophageal carcinoma (35). Similarly, TAP1, TAP2 and HLA class I antigen downregulation in HNSCC lesions from different anatomic sites was found to be associated with reduced survival (20). In the present study, we showed that HLA class I antigen downregulation in metastatic lesions is significantly correlated with an unfavorable clinical course of the disease in patients with HNSCC. Furthermore, using multivariate analysis, we showed that negativity of HLA class I antigen in metastatic lesions is an independent prognostic factor for disease-free survival. These findings may reflect the lack of recognition by HLA class I antigen-restricted, TA-specific CTL of HNSCC metastatic cells which have lost or down-regulated HLA class I antigen expression. This possibility is supported by the positive correlation between HLA class I antigen expression levels and CD8+ T-cell infiltration into the laryngeal SCC lesions (21).

In conclusion, TAP1 and TAP2 downregulation in association with at least in part tapasin downregulation may represent one of the mechanisms of HLA class I antigen downregulation on HNSCC lesions. HLA class I antigen downregulation may provide a selective advantage for HNSCC cells during metastasis. Therefore, analysis for APM
components, especially TAP1 and TAP2, in HNSCC lesions may contribute to the optimization of T-cell-based immunotherapeutic strategies as well as the selection of patients for this treatment. In addition, TAP gene transfer has to be considered for inclusion in therapies of HNSCC patients and might provide a method for increasing immune response against tumors.

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