The Relationship between Secretory Leukocyte Protease Inhibitor Expression and Epstein-Barr Virus Status among Patients with Nasopharyngeal Carcinoma

KA-PO TSE1, CHI-SHENG WU1, CHUEN HSUEN2, KAI-PING CHANG3, SHENG-PO HAO4, YU-SUN CHANG1 and NGAN-MING TSANG5,6

1Molecular Medicine Research Center and 6School of Traditional Chinese Medicine, Chang Gung University, Taoyuan, Taiwan, R.O.C.; Departments of 2Pathology, 3Otolaryngology-Head Neck Surgery and 5Radiation Oncology, Chang Gung Memorial Hospital at Lin-Kou, Taoyuan, Taiwan, R.O.C.; 4Department of Otolaryngology, Head and Neck Surgery, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan, R.O.C.

Abstract. Aim: The aim was to study the expression of Secretory Leukocyte Protease Inhibitor (SLPI) and to explore its correlation with the presence of Epstein-Barr Virus (EBV) among patients with nasopharyngeal carcinoma. Materials and Methods: The expression levels of SLPI mRNA in NPC cell lines and in ten matched-pairs of NPC and adjacent normal tissue were examined by quantitative real-time Polymerase Chain Reaction (PCR). Furthermore, protein expression of SLPI in 71 paraffin-embedded NPC biopsies was assessed by immunohistochemistry. Finally, the serum level of SLPI in 177 NPC patients and 103 healthy controls was evaluated by enzyme-linked immunosorbent assay (ELISA). Results: The expression of SLPI mRNA in NPC cells was significantly lower than in the adjacent normal epithelium (p<0.001). When the expression of SLPI in EBV-positive and -negative NPC cell lines was compared, we found that both mRNA and protein expressions of SLPI were significantly higher in EBV-negative cells. Furthermore, the results of immunohistochemical analysis demonstrated that the frequency of reduced SLPI expression in EBV-positive biopsies was significantly higher than that in EBV-negative biopsies. Conclusion: In this study, we have confirmed that SLPI is significantly down-regulated in NPC tissues. In addition, based on our preliminary results, we propose that the reduction of SLPI in NPC cells is associated with the presence of the EBV genome and/or the expression of EBV-encoded genes. SLPI may play an important role in EBV-mediated NPC tumorigenesis.

Secretory leukocyte protease inhibitor (SLPI MIM 107285), also named antileukoprotease, is a member of the whey acidic protein four-disulfide core family. It is an 11.7 kDa non-glycosylated secretory protein that is produced by neutrophils, macrophages and the epithelial cells lining the respiratory, digestive, and reproductive tracts (1-4). SLPI can be found abundantly in mucosal secretions, including breast milk, seminal fluid, saliva and the secretions of the female genital tract. In addition to the fact that it possesses antiprotease activity which protects tissue from the damaging effects of inflammation (3, 5-6), SLPI has been found to have antibacterial (2, 7), antifungal (8) and antiviral activity (4, 9-10).

Dysregulation of SLPI has been reported in cancer; however, depending on cancer type, both up-regulation and down-regulation have been noted. For example, SLPI has been showed to be overexpressed in ovarian (11-12), gastric (13), papillary thyroid cancer (14) and non-small cell lung carcinoma (15). In these cases, SLPI has been suggested to promote tumorigenesis and invasiveness of cancer cells (16); furthermore, the presence of elevated SLPI expression in these types of cancer often seems to be associated with a poor prognosis among these cancer patients (13, 17). In contrast to this, down-regulation of SLPI has been reported in prostate cancer (18), cervical adenocarcinoma (19) and oral squamous cell carcinoma (20). In addition, down-regulation of SLPI expression has also been reported to be associated with infection-related diseases such as Helicobacter pylori-related gastroduodenal diseases (21-22) and exposure of human cervical epithelial cells to Herpes...
Simplex virus (HSV)-1 or HSV-2 (23). However, the biological significance of SLPI reduction as part of these diseases is still unknown.

Nasopharyngeal carcinoma (NPC) is the most common type of cancer originating in the nasopharynx, and has a remarkable geographical and racial distribution. It is uncommon in Western countries, but is extremely common in south-eastern regions of Asia, such as the southern parts of China, Hong Kong, and Taiwan. Epstein-Barr virus (EBV) has been suggested to be an important co-factor in the etiology of NPC. Elevated levels of EBV-specific IgA can be found in NPC patients compared to normal individuals and patients with other cancer types (24, 25). Furthermore, the detection of EBV nuclear antigen and viral DNA in NPC cells has revealed that EBV is able to infect epithelial cells and that this event is associated with their transformation (26). All these findings strongly suggest a probable oncogenic role for the virus in the genesis of this type of tumor (27).

In our previous microarray data analysis of matched-pair NPC tumor tissue and a pool of adjacent normal tissue, we found that SLPI is one of the genes significantly down-regulated in NPC cells compared to normal nasopharyngeal epithelium (28). A similar observation was also found when the expression profile of NPC was assessed by laser capture microdissection (29). Given the potent anti-inflammatory and antiviral activities of SLPI and the fact that silencing of SLPI has been reported in infection-associated diseases, an evaluation of the expression of SLPI in NPC may help to unravel various tumorigenesis processes. In this study, we compared the mRNA and protein expression levels of SLPI using matched-pair specimens of NPC tumor and adjacent normal nasopharyngeal epithelium. In addition, the correlation between SLPI reduction and EBV status in clinical biopsies was also examined.

Materials and Methods

Patients and samples. Paraffin-embedded tissues and serum samples were collected from consecutively consenting patients suffering from newly diagnosed and untreated NPC who attended oncology clinics at the Department of Radiation Oncology and Otolaryngology-Head Neck Surgery of Chang Gung Memorial Hospital (CGMH), Lin-Kou, Taiwan, ROC, between September 2002 and February 2007. Samples of NPC tissues and adjacent normal nasopharyngeal epithelium were obtained during surgery and were frozen immediately after surgical resection. Tumor-node-metastasis (TNM) stage was defined according to the 2002 revision of the cancer staging systems promulgated by the American Joint Committee on Cancer (AJCC) (30). Histopathological classification of the tumor samples was based on the “Pathology and Genetics of Head and Neck Tumors” published by the World Health Organization in 2005 (31). All stages and histological types were present among the samples obtained from our patients and therefore the patients displayed a full range of NPC symptoms. One hundred and three serum samples were collected from volunteers undergoing routine health examinations as healthy controls; those presenting with hypertension, cardiovascular disease, or diabetes mellitus were excluded from the study.

Based on the typing of lymphomas and the detection of EBV encoded small RNAs (EBER) by ISH given below, 16 cases of characterized nasal Natural Killer/ T cell Lymphoma (NK/TCL) collected from 1999 to 2007 at CGMH, Linkou, Taiwan, ROC, were studied. This project was reviewed and approved by the Institutional Review Board and Ethics Committee of CGMH (authorization no. IRB 98-3215B). Informed consent was obtained from all participants.

Cell culture. NPC cell lines HK1 and HK1/EBV (kindly provided by Dr. Sai-Wah Tsao, University of Hong Kong, Hong Kong SAR, China) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM sodium pyruvate and 2 mM L-glutamate at 37°C under 5% CO2. For HK1/EBV cells, 500 μg/ml of Geneticin (G418) was added to the culture medium for EBV-positive cell selection. For the ELISA assay, cells were cultured in 96-well plates; after 48 h, the culture medium were harvested and centrifuged at 4°C and 1500 × g for 5 min. The resulting pellets were discarded and supernatants were collected and stored at –80°C until use.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA from NPC tissues and cell lines were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instruction. The concentration of RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription of the RNA samples (1 μg) was performed using ImProm-II (Promega, Madison, WI, USA) and oligo (dT) 15 primers (Promega). The reverse transcription products were diluted 20-fold and used as template in a 10 μl PCR reaction containing 1× SYBR Master Mix (Applied Biosystems, Foster City, CA, USA). The primers used to amplify the cDNA corresponding to SLPI and internal control 18S rRNA were as follows: SLPI_F: 5’-TCCTGCCTTACCACATGGAAGTC-3’; and SLPI_R: 5’-AGCCCAAGGTGCCGAGTTT3’; 18S rRNA_F: 5’- CGACGCCCTGGATACC-3’ and 18S rRNA_R: 5’-CCTCAGTTCCGAAAACCAACAA-3’. The quantitative RT-PCR was carried out on an ABI 7500 (Perkin-Elmer Applied Biosystems, Inc., Foster City, CA, USA), running pre-denaturation at 95°C for 10 min, then 40 cycles of the following program: 15 s at 95°C, 1 min at 60°C.

ELISA. Patient blood was obtained at time of diagnosis by peripheral venous puncture and was immediately centrifuged at 3,000 × g for 15 min. The serum was collected and frozen at -80°C until use. The SLPI concentration in the serum samples and culture medium were determined by a commercially available ELISA kit (Quantikine®; R&D Systems, Inc., Minneapolis, U.S.A.). All SLPI analyses were performed in duplicate according to the manufacturer’s instructions.

Immunohistochemistry (IHC). Immunohistochemical staining was performed using an automatic IHC-staining device, Bond-max Automated Immunostainer (Vision Biosystems, Leica, Bannockburn, IL, USA). Paraffin-embedded tissue sections were retrieved using Bond Epitope Retrieval Solution 1 and stained with antibodies against SLPI (NCL-SLPI 1:50 dilution; Novoceastra Laboratories Ltd., Newcastle, UK). Tissue sections were treated with 30-diaminobenzidine tetrahydrochloride (DAB) as the chromogen and hematoxylin as the counterstaining reagent. Images
of the slides were captured using a ScanScope CT automated slide scanning system (Aperio Technologies, Vista, CA, USA) and evaluated by experienced pathologists.

**In situ hybridization (ISH).** ISH to detect EBV-encoded RNA transcripts (EBER) was performed using the EBER probe ISH kit (Novocastra) in accordance with the manufacturer’s instruction.

**Statistical analysis.** Differences in SLPI mRNA between NPC tumor tissue and the corresponding adjacent normal nasopharyngeal epithelium were assessed using paired t-test. Descriptive statistics (mean, SE, SD, median, minimum and maximum) were used to summarize the distribution of serum SLPI levels. The one-sample Kolmogorov-Smirnov test was used to test for the normal distribution of serum SLPI levels within each group, and the results suggested that the data was not normally distributed. Based on this, the statistical difference in SLPI protein concentration between NPC patients and healthy controls was assessed using the Mann-Whitney U-test. The statistical difference in SLPI protein concentration between the culture medium from HK1 versus HK1/EBV cells was assessed using Student’s t-test. All statistical tests were two-sided and significance was set as \( p < 0.05 \) (except where a Bonferroni correction was made to adjust for multiple testing during subgroup analysis, where the significance was set at \( p < 0.05/n \), where \( n \) = number of subgroups). All statistical analysis was performed using SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL, USA).

**Results**

**Down-regulation of SLPI in NPC biopsies.** Firstly, to confirm the expression status of SLPI in the NPC biopsies, qRT-PCR was performed on 10 matched-pairs of NPC tumor and adjacent normal nasopharyngeal epithelium specimens. As shown in Figure 1A, expression of SLPI mRNA was dramatically lower in all NPC tumor tissue samples when compared to the corresponding adjacent normal tissue (\( p < 0.001 \), paired t-test). In addition, we further investigated the protein expression levels of SLPI in paraffin-embedded tissues from the same patients using IHC (Figure 1C). All samples showed abundant SLPI signal in the adjacent normal epithelium (N), but the IHC results were negative for the tumor cells (T). These results demonstrate that the expression of SLPI in NPC cells is significantly reduced compared to the adjacent normal nasopharyngeal epithelium.

**Serum level of SLPI in NPC patients and healthy controls.** In addition to measuring the expression level of SLPI in tumor cells in situ, we also wanted to evaluate the clinical relevance of SLPI. The level (mean±S.D.) of SLPI was quantified by ELISA in serum samples collected from 177 NPC patients and 103 healthy individuals. As shown in Figure 2A, the mean serum level of SLPI in NPC patients was significantly higher than in the healthy control group (46.89±17.22 vs. 41.06±10.22 pg/ml; \( p = 0.003 \), Mann-Whitney U-test). Moreover, to clarify if the serum level of SLPI is associated with disease progression, we compared the serum level of SLPI in patients at different stages (Figure 2B) with that of the healthy control group. The mean serum SLPI level of the patients at stage I and II were found to be 39.66 and 42.75 pg/ml, respectively, which are not significantly different from that of the healthy group (\( p = 0.704 \) and 0.86 for stage I and II vs. control, respectively; Mann-Whitney U-test). However, the mean serum SLPI level of the patients at stage III and IV were found to be 46.22 and 53.39 pg/ml, respectively, which are significantly higher than that in the control group (\( p = 0.006 \) and <0.001 for stage III and IV vs. control, respectively; Mann-Whitney U-test). The results demonstrated that the serum SLPI level is elevated in NPC patients at an advanced stage of the disease, but not in patients at an early stage of the disease.

**Expression of SLPI in EBV-infected nasopharyngeal epithelial cells.** According to previous reports, down-regulation of SLPI has been reported in various types of infectious diseases such as Helicobacter pylori-related gastroduodenal disease (21-22) and when human cervical epithelial cells are exposed to either HSV-1 or HSV-2 (23). Given the fact that NPC is known to be closely associated with EBV infection, we speculated that down-regulation of SLPI in NPC tumor cells might be a phenomenon associated with EBV infection. To verify the association between EBV infection and reduced SLPI gene expression in NPC cells, we examined the SLPI expression status of various NPC cell lines. Specifically, HK1, an NPC cell line that does not carry EBV (32) and HK1/EBV, to which EBV had been introduced by co-culture with infected Akata cells, were used (33). Firstly, the concentration of SLPI protein in culture medium from cell lines was measured by ELISA. As shown in Figure 3A, the level of SLPI in the HK1 culture medium (mean±S.D.=1036.8±197.8 pg/ml) was significant higher than that in the HK1/EBV culture medium (mean±S.D.=134.1±80.6 pg/ml; \( p < 0.001 \), t-test). Similarly, when SLPI mRNA levels for the two cell lines were measured, the mRNA level of SLPI in HK1/EBV cells was ~21% that of the HK1 cells (Figure 3B). These results show that both the mRNA and protein levels of SLPI in HK1/EBV cells were significantly lower than that in parental HK1 cells.

**Expression of SLPI in EBV-positive and -negative NPC biopsies.** In addition to the above cell lines, we also tested the association between EBV status and expression of SLPI using clinical samples. The expression of SLPI protein was evaluated by IHC using EBV-positive and EBV-negative paraffin-embedded NPC tissues (n=71); the presence of EBV in these samples was demonstrated by EBV-encoded small RNA (EBER; Figure 4A and B) staining.

The demographic and pathological data of the studied cases are summarized in Table I. Among the 71 biopsies samples investigated, 64 cases (90.1%) were EBV positive and 7 cases (9.9%) were EBV negative. Overall, SLPI tumor
cell signal was only detected in 4 out of the 71 cases (5.6%), with all the tumor cells from the remaining NPC samples (67 out of 71, 94.4%) being negative for SLPI staining. No association was found between SLPI expression and the various different clinicopathological characteristics, such as gender, age, and disease stage. However, a significant correlation between reduced SLPI expression and the presence of EBER was observed \((p=0.002, \text{Fisher’s exact test})\). Among the 64 EBER-positive biopsies, 63 (94%) exhibited a loss of SLPI expression, which is significantly higher than for the EBER-negative specimens (3 out of 7). The IHC results further support our hypothesis that the presence of EBV or the expression of EBV genes is associated with a reduction in SLPI expression by NPC cells.
Down-regulation of SLPI in EBV-associated nasal type, natural killer (NK)/T-cell lymphoma. In addition to the situation with NPC, a constant association of nasal type NK/T-cell lymphoma (NKTCL) with EBV has also been reported (34-37). To further investigate if down-regulation of SLPI is a general phenomenon among EBV-associated malignancies, the expression of SLPI was evaluated in a pilot study of 16 NKTCL biopsies using IHC staining. All
samples examined were found to be EBV positive. As shown in Figure 5, abundant expression of SLPI protein was detected in normal nasopharyngeal mucosa (Figure 5A and C), but all the NKTCL biopsies examined were negative for SLPI signal by IHC (Figure 5B and D). This result suggests that a reduction in SLPI expression might be a general phenomenon in EBV-associated malignancies.

Discussion

In this study, using real-time qPCR and IHC, we confirmed that expression of SLPI at the mRNA and protein levels is significantly lower in NPC tumor cells than in adjacent normal nasopharyngeal epithelium. Expression of SLPI was dramatically reduced in the NPC tumor mass, with more than 94% of biopsies examined being negative for the presence of SLPI protein. Moreover, when the expression levels of SLPI in EBV-positive and -negative NPC cell lines and paraffin-embedded NPC tissues were compared, we found that a reduction in SLPI expression was also associated with the presence of EBV in tumor cells.

Local inflammation is suspected to play a major role in NPC tumorigenesis, because of the consistent presence of massive lymphoid infiltrate in primary tumors and the intense local production of inflammatory cytokines (38). In addition, it has been shown that SLPI is able to negatively

Figure 4. Representative examples of Secretory Leukocyte Protease Inhibitor (SLPI) expression in EBV-positive (EBER-positive) and EBV-negative (EBER-negative) NPC specimens. A, B: In situ hybridization with EBER probe; C, D: Immunohistochemical staining with SLPI-specific antibody. Bar-100 μm.

Figure 5. Secretory Leukocyte Protease Inhibitor (SLPI) protein expression in EBV-associated Natural Killer T-cell Lymphoma (NKTCL). Immunohistochemical staining of SLPI in normal nasal mucosa (A, C) and NKTCL (B, D). Bar-100 μm.
regulate the inflammatory response, firstly, by inhibiting Nuclear Factor-KappaB (NF-κB) activation (39); secondly, by inhibiting inflammatory infiltrate recruitment and activation (40-41); thirdly, by inhibiting histamine release from mast cells (42); and fourthly, by inhibiting C5a production in the inflamed lung (5). Here, based on our IHC staining results, we confirmed that SLPI down-regulation can be found in more than 94% of NPC biopsies and that this is associated with the presence of the EBV genome and/or the expression of EBV-encoded genes. Given that EBV infection is closely associated with NPC and SLPI is such an important broad-spectrum innate immune molecule with anti-inflammatory properties, which is generally silenced in NPC, we can speculate that the decreased expression of SLPI may help EBV-associated NPC tumorigenesis to occur (43). Further studies are required to clarify the biological significance of SLPI down-regulation in EBV-positive NPC tumor cells.

Although our results show that expression of SLPI mRNA and protein in tumor cells is significantly reduced when compared to adjacent normal nasopharyngeal epithelium, the serum level of SLPI in the NPC patients at advanced stages of the disease was significantly higher than among the healthy control group. Since the source of SLPI found in serum remains unclear, it is suggested that this contradiction may be explained by the reduction in SLPI being a local event associated with the NPC tumor mass, while the elevated level of serum SLPI may reflect an activation of the systematic antitumor immune response when a larger tumor mass at advanced stage is present.

In addition to previous published expression profiling information, a recent report in 2012 also demonstrated that SLPI is one of the genes that is consistently down-regulated in NPC tissues (44), which supports our findings. However, although the biological significance and underlying mechanism remain unknown, this report is the first showing that SLPI down-regulation is associated with the presence of EBV in NPC cells. It is just the beginning for understanding the biological significance of SLPI in EBV-mediated tumorigenesis of NPC. Functional assays of SLPI protein in NPC cells should help us to further clarify its roles and its interaction with EBV.

Acknowledgements

We thank our colleagues in the Department of Radiation Oncology for their kind assistance, and we gratefully acknowledge the skill and dedication of our laboratory technicians, Shu-Tsen Kao, Yu-Ling Hsieh and Hsiao-Yun Cheng. We are grateful to Lai-Chu See (Department of Public Health, Chang Gung University, Taoyuan, Taiwan) for helpful discussions about our results. This study was supported by a Chang Gung Memorial Hospital Research Grant, no. CMRPG360221.

Table I. Clinical characteristics and Secretory Leukocyte Protease Inhibitor (SLPI) expression in the patients with Nasopharyngeal Carcinoma (NPC).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All patients</th>
<th>SLPI-positive</th>
<th>SLPI-negative</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>46 (64.8%)</td>
<td>3 (75%)</td>
<td>43 (64.2%)</td>
<td>0.66a</td>
</tr>
<tr>
<td>Female</td>
<td>25 (35.2%)</td>
<td>1 (25%)</td>
<td>24 (35.8%)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>47.85</td>
<td>47.21</td>
<td>47.9</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>46.78</td>
<td>45.21</td>
<td>47.07</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>13-71</td>
<td>33-66</td>
<td>13-71</td>
<td></td>
</tr>
<tr>
<td>T-Stage (2002 AJCC criteria)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>41 (57.7%)</td>
<td>3 (75%)</td>
<td>38 (56.7%)</td>
<td>0.472a</td>
</tr>
<tr>
<td>T3-T4</td>
<td>30 (42.3%)</td>
<td>1 (25%)</td>
<td>29 (43.3%)</td>
<td></td>
</tr>
<tr>
<td>N-Stage (2002 AJCC criteria)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>14 (19.7%)</td>
<td>2 (50%)</td>
<td>12 (17.9%)</td>
<td>0.117a</td>
</tr>
<tr>
<td>N1-N3</td>
<td>57 (80.3%)</td>
<td>2 (50%)</td>
<td>55 (82.1%)</td>
<td></td>
</tr>
<tr>
<td>Overall stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>24 (33.8%)</td>
<td>2 (50%)</td>
<td>22 (32.8%)</td>
<td>0.481a</td>
</tr>
<tr>
<td>III-IV</td>
<td>47 (66.2%)</td>
<td>2 (50%)</td>
<td>45 (67.2%)</td>
<td></td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCC</td>
<td>3 (4.2%)</td>
<td>1 (25%)</td>
<td>2 (3%)</td>
<td>0.102a</td>
</tr>
<tr>
<td>NKC</td>
<td>19 (26.8%)</td>
<td>1 (25%)</td>
<td>18 (26.9%)</td>
<td></td>
</tr>
<tr>
<td>UC</td>
<td>49 (69%)</td>
<td>2 (50%)</td>
<td>47 (70.1%)</td>
<td></td>
</tr>
<tr>
<td>EBER Positive</td>
<td>64 (90.1%)</td>
<td>1 (25%)</td>
<td>63 (94%)</td>
<td>0.002b</td>
</tr>
<tr>
<td>Negative</td>
<td>7 (9.9%)</td>
<td>3 (75%)</td>
<td>4 (6%)</td>
<td></td>
</tr>
</tbody>
</table>

AJCC, American Joint Committee on Cancer; NKC, non-keratinizing differentiated carcinoma; SCC, squamous cell carcinoma; UC, undifferentiated carcinoma. Calculated using the $\chi^2$ test, $^a$Fisher’s exact test.

References


