Expression of cathepsin S and its inhibitor stefin A in sinonasal inverted papilloma*


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INTRODUCTION

Sinonasal inverted papilloma (IP) is a locally aggressive benign tumour characterized by a tendency of recurrence and the potential for malignant transformation. Its surface epithelium is derived from the Schneiderian membrane and is multilayered and formed of squamous or ciliated columnar cells (1). Under microscope examination, the epithelium invaginates and proliferates into the underlying stroma, thus earning the name “inverted papilloma” (2). The etiology of sinonasal IP remains uncertain, but chemical exposure, chronic inflammation, and viral infection, notably with human papillomavirus (HPV) have been reported (3-5). After HPV infection, HPV might evade the immune system recognition through interference with MHC assembly and integrate into the host genomic DNA, which is associated with progression from polyclonal to monoclonal expansion in IP (6,7). These events may play a fundamental role in tumourigenesis, but the actual trigger that ultimately leads to tumour formation and recurrence is still unknown.

In the microenvironment of a developing tumour, the extracellular matrix (ECM) and cells surrounding the tumour all contribute to tumourigenesis (8). The primary families of proteases released into the extracellular space following cell activation include members of the matrix metalloprotease (MMP), serine protease, and the cysteinyl cathepsin groups of proteases (9-11). Cathepsins have been found in the lysosomes and various specific physiological roles have been ascribed to these enzymes.

SUMMARY

Background: Dysregulation of cysteinyl cathepsins and their inhibitors, cystatins (stefins), were implicated in progression of tumorgenesis; nevertheless, their role in sinonasal inverted papilloma (IP) is still unrecognized.

Methods: The differential expression of cathepsins and stefins in IP and normal tissues were revealed by data of human Affymetrix U133A gene chips, real-time polymerase chain reaction (PCR) and immunohistochemistry.

Results: Among the cathepsins and stefins family, expression of cathepsin S and stefin A were most differentially expressed (down- and up-regulated, respectively) in IP tissue as compared with normal tissues. Their expression levels were validated by real-time PCR, which showed the expression level of cathepsin S was significantly down-regulated, whereas the expression of stefin A was significantly up-regulated in IP tissue compared to normal sinus mucosa. Using immunohistochemistry, expression of cathepsin S was observed in stromal and epithelial area macrophages of normal sinus mucosa, but no obvious expression of cathepsin S was found in IP tissue. In contrast, over-expression of stefin A was present in nearly all layers of the proliferative squamous cells of IP, but expression of stefin A was only detected in a scattered area of normal sinus mucosa.

Conclusion: Down-regulation of cathepsin S and up-regulation of its endogenous inhibitor, stefin A, were found in IP tissues as compared with their expression level in normal sinus mucosa tissues. The biological significance of inverse expression of both stefin A and cathepsin S in sinonasal IP need further investigation in the future.

Key words: inverted papilloma, sinonasal tract, cathepsin S, stefin A
including taking part in the dissolution and remodeling of connective tissue, major histocompatibility complex (MHC) class II mediated antigen presentation, keratinocyte differentiation, bone remodeling, reproduction and apoptosis (11,12). In addition, cystatins are natural cysteine protease inhibitors, which belong to a superfamily of proteins with wide distribution in tissues. They are classified into three distinct subfamilies. The type 1 cystatins are represented by cystatin A (stefin A) and cystatin B (stefin B), and function intracellularly. The type 2 cystatins (C, D, S, SA, SN, M and F) are mainly secreted proteins, and the type 3 cystatins (L- and H-kininogens) are intravascular proteins (13). The enzymatic function of cathepsins is regulated by stefins either in patients with malignant or nonmalignant tumours and both are suggested as target proteins for prognosis, diagnosis and therapy in several cancers (15). However, till now, there is no literature to discuss their roles in IP formation. To address that, the differential expression of cathepsins and stefins in IP and normal tissues were revealed by data of human Affymetrix U133A gene chips. In addition, we chose the most differentially expressed genes among cathepsins and their endogenous inhibitors (cystatins) for further validation by real-time PCR and immunohistochemistry. Meanwhile, we analyzed the correlation between expression of these genes with clinical stage and eventual recurrence rate of these IP patients. Finally, we will discuss the possible mechanism.

MATERIALS AND METHODS

Patients

This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital (CGMH) (IRB number 96-1511B). All patients recruited for this study provided written informed consent for sample collection and study participation. Subsequently, the sinonasal IP tissues investigated were surgically resected (i.e. not merely intranasal biopsied) from a total of 15 patients (10 male and 5 female patients; mean age 48.2 years, range 18-78). All cases were staged according to the Krouse staging system (14). The enzymatic function of cathepsins is regulated by stefins either in patients with malignant or nonmalignant tumours and both are suggested as target proteins for prognosis, diagnosis and therapy in several cancers (15). However, till now, there is no literature to discuss their roles in IP formation. To address that, the differential expression of cathepsins and stefins in IP and normal tissues were revealed by data of human Affymetrix U133A gene chips. In addition, we chose the most differentially expressed genes among cathepsins and their endogenous inhibitors (cystatins) for further validation by real-time PCR and immunohistochemistry. Meanwhile, we analyzed the correlation between expression of these genes with clinical stage and eventual recurrence rate of these IP patients. Finally, we will discuss the possible mechanism.

RNA extraction, microarray data processing, and statistical analysis

Total RNA from 6 normal and 6 IP tissues were isolated with TRIzol® (Invitrogen, Life Technologies Corporation) and further purified and concentrated with the RNeasy® MinElute clean-up kit (Qiagen, Valencia, CA, USA), and the quality and quantity of RNA were analyzed with Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Total RNA (1 μg) isolated from six patients with IP and 6 patients with normal mucosal tissue, was converted using SuperScript® II reverse transcriptase (Invitrogen, Life Technologies Corporation) to labeled cDNA, and 25 μg of labeled probe was hybridized to the GeneChip® Human Genome U133A 2.0 Array (Affymetrix, Santa Clara, CA, USA). The gene expression data were generated and normalized by the robust multi-chips average method using RMAEXPRESS (http://stat-www.berkeley.edu/~bolstad/RMAExpress/RMAExpress.html)(12). The cathepsins and cystatins genes that were differentially expressed between IP tissues versus the normal control tissues were selected.

Real-time reverse-transcriptase polymerase chain reaction

Total RNA from the normal and IP tissues was isolated with TRIzol® (Invitrogen) and further purified and concentrated with the RNeasy® MinElute kit (Qiagen). The quality and quantity of RNA were analyzed with the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). One μg of amplified anti-sense RNA (aRNA) pre-treated with DNase was mixed with the Reverse Transcriptor kit (Roche, Basel, Switzerland) and oligo-dT primers to generate 25 μl of first strand cDNA. Real-time quantitative (RTQ)-PCR was performed in a total volume of 10 μl in 384 - well plates using 0.5 μl of the 1st strand cDNA synthesis mixture as template with TaqMan® (Assay-on-DemandTM, Applied Biosystems, Carlsbad, CA, USA) and amplification was according to the manufacturer’s recommendation. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as internal control for gene expression. The catalogue numbers for these assay genes were Hs00175403 (Cathepsin S), Hs00193257 (Stefin A), and Hs99999905 (GADPH). For each sample, the relative quantitation of gene expression was calculated using the differences in threshold cycles (ΔCt = Ct internal control - Ct sample) and a calibrated ΔCt value (ΔCt = Ct internal control - Ct sample) was analyzed and shown in Figure 1.

Immunohistochemistry

Archived, surgically resected specimens (formalin-fixed, paraffin-embedded) were sectioned in the Department of Pathology at CGMH. Sequential tissue sections were cut using a microtome set at 4 μm. All formalin-embedded sections were deparaffinized by xylene and rehydrated through serial alcohol rinses before antigen retrieval. After antigen retrieval by boiling or proteinase K digestion, the slides were incubated with an appropriate dilution of antibodies at room temperature for 1 hour. Cathepsin S protein was detected with an anti-human cathepsin S polyclonal antibody (Abcam, Cambridge, United Kingdom). Stefin A protein was detected with a monoclonal antibody (Abcam). After incubation, these slides were washed...
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with phosphate-buffered saline (PBS) three times, and then incubated with horseradish peroxidase (HRP) polymer antibody (Zymed, San Francisco, CA, USA) at room temperature for 10 minutes. The slides were then developed by the addition of 3,3’-Diaminobenzidine tetrahydrochloride (DAB) at room temperature for 10 minutes.

Semiquantification of immunostaining

Stained tissue sections were examined at 400 x magnification using an Olympus microscope and photographed using the Arc digital software package. To avoid any sampling errors due to possible non-homogeneous distribution of the markers investigated within the tissues, semiquantification was performed by randomly selecting a total of 5 high-powered fields from each specimen. Staining results of cathepsin S and stefin A were expressed as the number of positive cells per square millimeter of lamina propria and epithelium.

Statistics

Group differences were analyzed using the Mann-Whitney U test for skewed data distribution and Student’s t-test for normally distributed data. The group data was summarized as mean values ± 95% confident intervals in Figures. The analyses were performed using the SPSS (version 11.0) software system. P-values < 0.05 were considered statistically significant.

RESULTS

Expression of cathepsins and cystatins between inverted papilloma and normal mucosa as revealed by Affymetrix U133A genechip

Among 25,000 genes in the Affymetrix U133A chip, expression of fifteen genes of cathepsins family and 10 genes of cystatins family were selected for further analysis (Table 1). Among stefins, we found a significant up-regulation of stefin A (10.14 fold of up-regulation, p < 0.029) in IP tissues as comparing with normal tissues (Table 1). Although expression of cathepsins showed no significant difference between normal and IP,

<table>
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cathepsin S (0.42 fold of reduction, p < 0.102) and K (0.45 fold of reduction, p < 0.135) showed the most decrease in IP tissues among the cathepsin family (Table 1). Since stefin A is an inhibitor for cathepsin S and they both were most differentially expressed (down- and up-regulated, respectively) between IP and healthy sinus mucosa, they were chosen for further validation.

Expression of cathepsin S and stefin A in sinonasal IP tissue and normal sinus mucosa

To confirm microarray data, expression of both cathepsin S, and its endogenous inhibitor, stefin A were examined in other IPs (n = 15) and normal sinus mucosa (n = 15) tissues by real-time PCR. As shown in Figure 1, the expression level of stefin A was significantly up-regulated in IP tissues than in the normal sinus mucosa (p < 0.001; Figure 1, right panel). In contrast, the expression level of cathepsin S was down-regulated in the IP tissues as compared to the control mucosa (p < 0.001; Figure 1, left panel). This result was consistent with microarray data showing up-regulation of stefin A and down-regulation of cathepsin S in IP tissues as comparing with normal tissues.

Detection of cathepsin S and stefin A in IP and normal tissues by immunohistochemistry

To distinguish the specific cell types in sinonasal IP tissue and normal sinus mucosa that expressed cathepsin S and its endogenous inhibitor, stefin A, tissue sections from IP patients and normal tissues were subjected to immunohistochemical staining. As shown in Figure 2, expression of cathepsin S was found in macrophages of the superficial epithelium and the stroma areas (Figures 2B, 2D, Figure 4). In contrast, expression of cathepsin S in sinonasal IP was weakly detected only in macrophages of the stroma areas (Figures 2A, 2C, Figure 4). Nevertheless, expression of stefin A was detected in cytoplasm of either epithelium or submucosal glands in both sinonasal IP and in normal mucosa, but the staining intensity of stefin A appeared stronger in sinonasal IP (Figures 3A, 3C, Figure 4) than in normal mucosa (Figures 3B, 3D, Figure 4) (p < 0.001). Taken together, dysregulation of cathepsin S and its endogenous inhibitor, stefin A, were found in sinonasal IP tissues either by using real-time PCR or immunohistochemistry.

The correlation of stefin A or cathepsin S with tumour recurrence and tumour stage

Among fifteen patients with IP, all cases were staged by Krouse T stages: T1 (n = 1), T2 (n = 5) and T3 (n = 9). Because limited numbers of T1 and T4 stage cases were available, we divided these cases into two groups: group 1 and 2; and group 3 and 4. There was no obvious correlation between the mRNA expression of these two genes with both groups (p = 0.40 for stefin A and p = 0.11 for cathepsin S). In addition, in immunohistochemistry analysis, the differences between these two groups were also insignificant (p = 0.76 for stefin A and p = 0.48 for cathepsin S).
All patients received endoscopic surgery to remove the tumour with an average follow-up period of 10.8 months (range from 6 months to 26 months), and only one case with recurrence was found. There was also no obvious significant correlation found between the expression of these two genes with tumour recurrence.

**DISCUSSION**

In this study, we examined the expression of cathepsin S and its inhibitor stefin A in sinonasal IP and normal mucosa tissues. We found a significant inverse expression of cathepsin S (down-regulation) and stefin A (up-regulation) in IP as compared with healthy sinus mucosa. To our knowledge, this is the first study to examine the expressions of cysteine cathepsins and cystatins in IP tissues.

Sinonasal IP is a disease of viral origin caused by HPV and a benign tumour with potential for malignant transformation. A previous report revealed that HPV may evade immune recognition by down-regulating MHC cell surface expression via decreased TAP-1 levels. This could facilitate the entrapment of viral peptides in the rough endoplasmic reticulum, making these peptides available to be complexed with MHC-I molecules and further destruction. Moreover, cells in the invasive malignant stage were found to have a very low level of MHC class I and II, which could hamper the presentation of the antigen and lead to a decreased immune response. Connecting these two together, HPV infection with an impaired host immune system might play important roles in IP formation.

Cathepsin S is expressed in the lysosome of antigen-presenting cells and is best known for its critical function in the proteolytic digestion of invariant chain chaperone molecules, thus controlling antigen presentation to CD4+ T-cells by MHC class II molecules. Although the information concerning cathepsin S in tumourigenesis has been presented in several recent papers, its possible role in tumourigenesis remained controversial. Originally, the study of cathepsin S in cancer, like that of other proteases, was focused on its potential role in the degradation of ECM during invasive processes. For example, over-expression of cathepsin S has been linked to prostate cancer invasion and metastasis. However, in our study, the expression level of cathepsin S was decreased in IP compared to normal sinus mucosa. This might suggest that, unlike the role of other cathepsins in degradation of ECM and cancer progression, the inactivation of cathepsin S in sinonasal IP could result in impaired immune response and abnormal levels of MHC. Furthermore, it would contribute to the latent HPV infection in IP tissue. This phenomenon may be similar to previous results. In lung cancer, the patients with high levels of cathepsin S in tumours and lung parenchyma experienced significantly better survival probability than those with low levels of this protease. In this case, high cathepsin S expression in tumours may reflect an immune response to the tumour and inhibit the aggressive ability of these tumour cells. In our study, cathepsin S was expressed in antigen-presenting cells - macrophages - of normal sinus mucosa, but it was difficult to detect them in the areas of IP. For these immune cells to exit the mucosa into the draining lymphatics for presentation, it is necessary that matrix degradation occurs. This is performed by enzymes such as cathepsin S, which is secreted by the migrating cells. In IP, our studies found a decreased production of cathepsin S and impaired migration ability of these antigen-presenting cells following exposure to HPV antigen. However, the relation of HPV infection with cathepsin S expression and the underlying mechanisms of immune response should further be examined by using an in vitro cell culture system in detail.

The roles of cystatins in tumourigenesis are still obscure. Although some reports revealed that cystatins had a putative tumour-suppressing function, some recent findings appeared to contradict this view. Stefin A was first reported to be associated with malignant tumours. For example, in breast cancer patients, an inverse correlation of stefin A expression with metastatic potential and a relapse-free period was suggested. However, some reports demonstrated that in head and neck tumours, stefin A and stefin B levels were moderately increased compared to the control matched pair tissues.

In an animal study, serial analysis of gene expression (SAGE) was used to identify differential gene expression in ultraviolet induced skin papilloma and squamous cell carcinoma. In another DNA microarray study, epidermal abrasion was used to induce skin papilloma genesis in transgenic Tg.AC mice. Both of the results showed increased expression of stefin A in papilloma and squamous cell carcinoma. In our results, formalin-fixed whole cross-sections labeled with anti-stefin A demonstrated that this protein was localized within the cytoplasm of proliferating squamous cells in IP. It suggested that stefin A might be cross-linked to squamous metaplasia, epithelial acanthosis, and hyperplasia, as well as being important in tumourigenesis.

In addition, stefin A was implied to have a potential role of inhibiting the migration of immune cells from neonatal skin. Enhanced expression of stefin A found in neonatal skin will inhibit the activity of the cathepsins, thus preventing cell migration beyond the cellular matrix. When the skin develops, stefin A decreases and the antigen-presenting cell density increases. It means that the microenvironment of IP is similar to that of neonatal skin with abnormal stefin A expression.

All true cystatins inhibit cysteine cathepsins. The cysteine cathepsins family includes cathepsins B, L, H, S, K, F, V, X, W, O and C. At the protein level, cathepsins B, L, H, F, O, X and C are expressed ubiquitously, whereas the expressions of cathepsins S, K, W, and V are limited (e.g., antigen-presenting cells, cornea, testes and spleen). It was also suggested that stefin A can inhibit the activity of cathepsin S. Therefore, it may be reasonable to speculate that over-expression of stefin...
A may inhibit cathepsin S and break down MHC assembly, which results in latent HPV infection and tumour formation in sinonasal IP patients. However, we still await proof from additional studies.

In addition, it has been observed that interferon-γ (IFN-γ), a factor stimulating MHC class II antigen presentation, can also induce cathepsin S expression (10). IFN-γ is produced by many cell types, including T cells and B cells, and is an important component of the antiviral response. The upregulation of cathepsin S and enhancement of antigen presentation at the cell surface by IFN-γ potentially allows the infected cell to become a target for the immune system again. Though there were no significant correlations of these two genes with clinical stage and recurrence rate found in this study, the roles of cathepsin S and stefin A are worth investigating further to gain a more complete understanding of the development of sinonasal IP and to provide some promise of nonsurgical treatment for sinonasal IP in the future.

CONCLUSION
In conclusion, we have identified two proteins, which are differentially expressed in sinonasal IP and normal sinus mucosa. A decrease of cathepsin S and an increase of stefin A were observed in IP compared to healthy sinus mucosa. The dysregulation of stefin A and cathepsin S may break down the MHC assembly, resulting in latent HPV infection and sinonasal IP formation.

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