Identification of Galectin-1 as a novel biomarker in nasopharyngeal carcinoma by proteomic analysis

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Abstract. Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors in southern China and Southeast Asia. It is characterized as a multistep process involved in multiple genetic and epigenetic events. The mechanism of carcinogenesis still needs to be further clarified. In this study, two-dimensional gel electrophoresis, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS), RT-PCR, Western blot and immunohistochemical (IHC) analyses were used to detect Galectin-1 expression in NPC compared with normal nasopharyngeal epithelial tissues (NNET). We found that Galectin-1 was expressed at a significantly higher level in NPC compared with NNET. Our results indicated that high expression level of Galectin-1 might correlate with the development of NPC and Galectin-1 may serve as a potential diagnostic marker or therapeutic target for NPC.

Introduction

NPC is a disease with remarkable racial and geographic distribution, which is one of the most common malignant tumors in southern China and Southeast Asia. The incidence rate is about 25-50 per 100,000 person year and 100-fold higher than that in the Western world (1,2). With the advances in NPC study, many NPC-related molecules have been reported, such as serum antibodies against various EBV proteins (3), cathepsin D (4), stathmin 14-3-3, Annexin I (5), serum amyloid A (6), Bmi-1 (7) and Met protein (8). However, the molecular basis of NPC is still unclear. A better understanding of the molecular basis of NPC is fundamental for developing more effective diagnostic, prognostic, treatment and prevention approaches.

High-throughput technologies such as microarrays and proteomics offer the potential ability to find alterations previously unidentified in NPC. Analyses for gene expression profiles of NPC with a cDNA array found that genes with aberrant expressions possibly contributed to the pathogenesis of NPC (9). Proteomics has provided new opportunities to uncover biomarkers and therapeutic targets for NPC as well as reveal the molecular mechanism underlying this disease. Using tissue samples from patients may be the most direct and persuasive way to find biomarkers and therapeutic targets for cancers by a proteomic approach.

In the present study, 2-DE and mass spectrometry (MS) were used to identify differential expression proteins between NPC and NNET samples. The differential expression of Galectin-1 was confirmed significantly up-regulated in NPC compared with NNET by RT-PCR and Western blot analysis. Our data will facilitate the better understanding of NPC carcinogenesis and reveal the function of Galectin-1 in NPC. Investigation of protein that occurs during carcinogenesis can provide new insights into the pathogenesis of cancer and is useful in developing new tumor biomarkers for the diagnosis and treatment of the disease.

Materials and methods

Materials and chemicals. Immobilene pH-gradient (IPG) DryStrips (pH 3-10, length 24 cm), IPG buffer (pH 3-10), DryStrip cover fluids, thiourea, urea, CHAPS, DTT, Pharmalyte (pH 3-10), bromophenol blue, Bis, TEMED, Coomassie Brilliant Blue G-250, molecular weight marker, Tris-base, SDS, glycine, horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG, and the enhanced chemiluminescence (ECL) system were purchased from Amersham Biosciences (Stockholm, Sweden). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI, USA). PVDF membrane was obtained from Millipore (Boston, MA, USA). Polyclonal rabbit anti-human Galectin-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).
Tissue samples. Thirty-four cases of fresh undifferentiated NPC tissues and 20 cases of fresh NNET from healthy individuals were obtained from the First and Third XiangYa Hospital of Central South University, China. Among them, 10 cases of tissues selected randomly from each group were performed to separate proteins for 2D analysis, the remaining were used for the following validation. Informed consent was obtained from all patients before collection of the specimens, which were frozen in liquid nitrogen immediately after surgical removal. The study was approved by the hospital ethics committee, and all patients in the study gave their consent before tissue donation.

Two-dimensional electrophoresis. 2-DE was performed to separate proteins as previously described by us (10). Briefly, 600 μg of protein samples were diluted to 450 μl with a rehydration solution [7 mol/l urea, 2 mol/l thiourea, 0.2% DTT, 0.5% (v/v) pH 3-10 IPG buffer, and trace bromophenol blue], and applied to IPG strips (pH 3-10 L, 24 cm) by 14 h rehydration at 30 V. The proteins were focused successively for 1 h at 500 V, 1 h at 1000 V and 8.5 h at 8000 V to give a total of 68 kVh on an IPGphor (Amersham Biosciences). Focused IPG strips were equilibrated for 15 min in a solution (6 mol/l urea, 2% SDS, 30% glycerol, 50 mmol/l Tris-HCl, pH 8.8 and 1% DTT), and then for another 15 min in the same solution except that DTT was replaced by 2.5% iodoacetamide. After equilibration, SDS-PAGE was performed on Ettan DALT II system (Amersham Biosciences). After SDS-PAGE, the Blue Silver staining method, a modified Neuhoff's colloidal Coomassie Brilliant Blue G-250 stain, was used to visualize the protein spots in the 2-DE gels. Triplicate gels were made for each conditioned medium.

Image analysis. The stained 2-DE gels were scanned with Magic-Scan software on Image-scanner (Amersham Biosciences), and analyzed using PDQuest system (Bio-Rad Laboratories, Hercules, CA, USA) according to the protocols provided by the manufacturer. To minimize the contribution of experimental variations, three separate gels were prepared for each conditioned medium. The gel spot pattern of each conditioned medium was separated on a 12% SDS-PAGE gel prior to transfer onto a PVDF membrane. After blocking with 5% milk in TBS/0.2% Tween-20 (TBS-T) for 1 h at room temperature, the membrane was incubated with rabbit anti-mouse Galectin-1 antibody (1:500 dilution) (Cell Signaling Technology, Inc., Beverly, MA) for 1 h at room temperature, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1:10000 dilution, Amersham Biosciences) for 1 h at room temperature. Detection of β-actin (Sigma, 1:4000 dilution) was used as loading control. The reactions were visualized using an enhanced chemiluminescence detection system (ECL, Amersham Biosciences). Signals on the blots were visualized by autorgraphy.

Immunohistochemical (IHC) analysis. Thirty-two cases of NPC and 10 cases of NNET (none of them were analyzed using 2-DE) were used to measure the distribution and abundance of the protein Galectin-1 by a standard immunohistochemical technique. Paraffin blocks were cut into 4-μm-thick sections. The sections were then deparaffinized in xylene and rehydrated in graded alcohol concentrations. Non-specific binding was blocked by pre-incubation with blocking solution for 5 min followed by incubation overnight at 4˚C with rabbit anti-mouse Galectin-1 (1:200 dilution). The sections were then washed 3 times with phosphate-buffered solution (PBS) for 5 min each and then incubated with HRP-conjugated goat anti-rabbit IgG (1:200 dilution) for 30 min at room temperature. The sections were then
washed with PBS and incubated with substrate-chromogen 3,3’-diaminobenzidine (DAB) reagent. Hematoxylin solution was then used to stain the nuclei. Negative controls without primary antibody were processed along with all the samples.

Statistical analysis. Statistical analysis was done by using SPSS (version 10.0). The significant difference of Galectin-1 expression between the tumor and normal tissues was determined by using the t-test or Mann-Whitney’s U test. A difference of P<0.05 was considered statistically significant. Results are expressed as means ± standard deviation.

Results

2-DE maps of NPC and NNET. Proteins from 2 sets of pooled (10 samples/group) NPC and NNET were resolved by 2-DE,
respectively, and stained with Coomassie Brilliant Blue. These experiments were repeated three times under identical experimental conditions and parameters to confirm reproducibility. Well-resolved and reproducible Coomassie Brilliant Blue-stained 2-DE maps from NPC and NNET were obtained. The volume of the Galectin-1 protein spot derived from the NPC was obviously larger than that of the NNET tissues (Fig. 1).

Identification of Galectin-1 protein expression by MALDI-TOF MS. The differentially expressed protein spots were excised from Coomassie Brilliant Blue-stained gels and subjected to in-gel digestion with trypsin and analyzed by MALDI TOF-MS. We focused on an interesting differential protein spot, as shown in Fig. 1. It was analyzed by mass spectroscopy and indentified as Galectin-1. Galectin-1 received a protein score of 196 and has a MW of 15048 Da, a PI of 5.34 and accession no. IPI00219219 (Fig. 2).

Verification of Galectin-1 expression by RT-PCR and Western blot analysis. The Galectin-1 mRNA expression was evaluated by RT-PCR in the remaining 24 cases of fresh-frozen NPC tissues samples and 10 cases of NNET tissues samples. There was a statistically significant difference in Galectin-1 mRNA expression between NPC and NNET samples (P<0.05). Galectin-1 mRNA was up-regulated in NPC compared with NNET tissues (Fig. 3A). Western blotting was also performed to detect the Galectin-1 protein expression and the results

Table I. The difference of Galectin-1 expression among NNET and NPC.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>0-2</th>
<th>3-4</th>
<th>5-6</th>
<th>Total</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNET</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>10</td>
<td>0.006*</td>
</tr>
<tr>
<td>NPC</td>
<td>2</td>
<td>11</td>
<td>19</td>
<td>32</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.01 by Mann-Whitney U test.
were consistent with the RT-PCR and 2-DE results (P<0.05, Fig. 3B). Both the RT-PCR and Western blot results indicated that overexpression of Galectin-1 might be related to development and progression of NPC, and the variables of Galectin-1 occurred at the transcription level.

**IHC pattern of Galectin-1 expression.** To examine the relevance of this finding to clinical NPC cancer, we used IHC to detect Galectin-1 in 32 cases of NPC and 10 cases of NNET. As shown in Fig. 4 and Table I, intense staining was detected in the tumor cells. In contrast, the overall density of Galectin-1+ cells in the sections from the NNET was significantly lower than that in NPC. The result also indicates the association of Galectin-1 gain with NPC occurrence.

**Discussion**

NPC is one of the most common malignant tumors in Southern China and Southeast Asia (1). Although NPC is classified as a subtype of head and neck squamous cell carcinoma, the epidemiology, clinical characteristics, etiology and histopathology are unique. Therefore, early diagnosis, exact histologic grading and accurate prognostication of NPC are critical for guiding the treatment and improving the prognosis of NPC. Identification of new NPC biomarkers may be an important way to achieve these objectives.

A number of studies have recently identified multiple genetic and epigenetic alterations that occur in NPC. Differential proteomic analysis of tumor and normal tissues allows the identification of aberrantly expressed proteins in cancer which might provide key information for finding biomarkers for the diagnosis and treatment of cancer as well as an understanding of carcinogenesis (14). In this study, we focused on up-regulated protein Galectin-1 in cancer tissues.

Galectin-1 is a member of the carbohydrate binding protein family that shows affinity for β-galactosides. It has been reported to play an important role in interaction with p27, Ras, Raf and PI-3K (15-18). Galectin-1 is extra-cellularly expressed in many tissues including normal and pathological conditions, and its expression has been shown to be higher in colorectal, pancreatic and non-small cell lung cancer (19-22). However, there is no previous report on association between Galectin-1 and NPC.

Our current study showed that Galectin-1 expression level was significantly higher in NPC tissues. This observation was supported by 2-DE, MS, RT-PCR, Western blot and immunohistochemical analysis. The 2-DE and MS analysis showed that Galectin-1 protein expression was significantly higher in NPC than the control group. RT-PCR and Western blot analysis confirmed these results. The percentage of Galectin-1+ cells in NPC cell population was significantly higher than the control. These results provide new mechanistic insight into the occurrence of NPC.

In summary, Galectin-1 overexpression was observed in NPC tissues using a proteomic approach and may serve as a novel biomarker for NPC. These findings suggest that Galectin-1 might have clinical value in predicting the prognosis of NPC, and identifying NPC patients who are at high risk of progression.

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**References**


