**S100A8 mediates the activation of P65/HLA-B/S100A8/BCL-2/Caspase-9 (-3) pathway in laryngeal carcinogenesis**

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S100 calcium binding protein A8 (S100A8), a possible novel member of NF-kappa B signal pathway in laryngeal squamous cell carcinoma (LSCC), interacts with human leukocyte antigen B (HLA-B) which carries an NF-kappa B binding site within the enhancer A. The objective of this study was to explore the molecular mechanism of S100A8 in laryngeal carcinogenesis. RT-PCR, Western blotting and immunohistochemistry staining were applied to evaluate the expression levels of IKKa, P65, REL-B, S100A8, APAF-1 and BCL-2 genes. The signal transduction passway in which S100A8 might participate was explored by RNA interference. Flow cytometry, TUNEL assay and cell invasion in vitro were used to detect the biological behavior of Hep2 cells induced by S100A8 gene. Our results showed that high expression of S100A8 was related to tumorigenesis in LSCC and negatively correlated with the degree of differentiation, indicating that S100A8 gene could inhibit apoptosis and promote metastasis in LSCC. Additionally, the suppression of S100A8 by RNA interference down-regulated BCL-2 but not APAF-1, P65 and IKKa, while, the suppression of P65 could significantly down-regulate the expression of S100A8 gene. In conclusion, S100A8 plays an important role in P65/HLA-B/S100A8/BCL-2/Caspase-9 (-3) pathway in laryngeal carcinoma.

Laryngeal carcinoma is the second most common respiratory system cancer among Chinese people, especially in the northeast China and 95% of the cases are laryngeal squamous cell carcinoma (LSCC). Now, the morbidity of LSCC has shown an increasing tendency\(^{[1,2]}\). Infection and inflammation are two most important potential predisposing risk factors for LSCC. The main treatment paradigm for LSCC has been surgical, usually total laryngectomy in advanced cases, followed by radiotherapy. This strategy may seriously impair patient’s laryngeal function and affect their life quality. Some researchers have been trying to develop a new targeted intervention approach for LSCC\(^{[3]}\).

S100A8, as an important inflammation factor, localized in the cytoplasm and/or nucleus of a wide range of cells, are involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. Accumulating evidence suggests that S100A8 of high concentration is present in the body fluid at local inflammatory sites, which might cause a delay in tissue repair and exert a deleterious effect on the infectious tissue\(^{[4]}\). S100A8 and S100A9 are co-expressed as a complex, suggesting a common mechanism of transcriptional regulation in inflammatory diseases and cancers\(^{[5]}\). It is reported that infection and inflam-

Received December 20, 2007; accepted March 31, 2008
doi: 10.1007/s11434-008-0238-0
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Supported by National Natural Science Foundation of China (Grant No. 30171008) and National 863 Project of China (Grant No. 2002BA711A08-18)
mation contribute to 15%—20% of all malignancies, and are predisposing risk factors to many kinds of cancers, such as gastric cancer, colorectum cancer, lung cancer, etc.\[6–8\]. Our clinical analysis also revealed that LSCC patients often have a background of chronic irritation and inflammation. At present, it has been thought that the nuclear factor-kappa B (NF-kappa B) that plays a key role in the inflammatory process, immune response, cell growth control and apoptosis, provides a mechanistic link between inflammation and cancer\[9,10\].

Our previous studies revealed that S100A8, hypothetical protein L0C80154, MHC class I HLA-B, similar to T-box 1 isform C and sarcrolemmal associated protein 1, are interactive proteins in LSCC, which suggest that S100A8 might be a novel member of NF-Kappa B signal pathway\[11\]. At present, the molecular mechanism of S100A8 in the signaling pathway in the tumorigenesis is still unclear. In this study, using RT-PCR, immunohistochemistry staining, Western blotting, RNA interference, apoptosis detection and in vitro invasion, we tried to solve the above problem, aiming to establish a novel LSCC therapeutic strategy.

1 Materials and methods

1.1 Samples

56 Primary LSCC, 6 lymphocyte node metastases and 7 precancerous lesion were obtained from patients treated at the E.N.T. Department of the First Affiliated Hospital of China Medical University, the 463 Hospital of PLA and the General Hospital of Shenyang Military Region with their informed consent and the approval of the hospital authorities. All subjects were unrelated ethnic Chinese from Northeastern China. None of them received radiotherapy or chemotherapy prior to the genetic analysis. The clinical pathological characteristics of the patients were confirmed according to the International Union Against Cancer. All the specimens, including cancerous tissues and paired adjacent normal laryngeal tissues (PANLs) typically 4—15 mm in diameter, were frozen immediately after surgery and stored at ~80°C.

1.2 Cell culture

The cell lines Hep-2 (human laryngeal carcinoma) and NIH3T3 (mouse) obtained from Cell Biology Institute of Shanghai, Chinese Academy of Science, were grown in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C in a 5% CO2 humidified atmosphere. Only cells in a log phase were used for the following experiments.

1.3 Semi-quantitative RT-PCR

Total RNA was extracted by using TRIzol™ reagent following the protocols of the manufacturer (Invitrogen, California, USA). The quality of RNA was confirmed on a formaldehyde agarose gel, and concentration was determined by reading the absorbance at 260/280 nm. 1 μg of total RNA was used to synthesize the first strand cDNA with Reverse Transcription system (Promega, Madison, WI, USA). Then 1 μL of RT product was used as the template to amplify specific fragments. PCR conditions were optimized individually for individual gene, and the cycle number for PCR was adjusted so that the reactions fell within the linear range of product amplification. The expression of housekeeping gene β-actin was used as an internal control. The mRNA levels of the related genes were also evaluated following RNA interference (Table 1). The RT-PCR product was analyzed by

<table>
<thead>
<tr>
<th>Gene</th>
<th>Dir</th>
<th>Primer sequences (5′—3′)</th>
<th>Product size</th>
<th>Anne (℃)</th>
<th>PCR cycles</th>
</tr>
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<tbody>
<tr>
<td>S100A8</td>
<td>F</td>
<td>CAGCTGGAAACGCAACATAGA</td>
<td>238</td>
<td>58.0</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TCAGCATGATGAACTCCTCG</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>P65</td>
<td>F</td>
<td>AATGGCTAAGAGCAGAGGAGG</td>
<td>416</td>
<td>61.0</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>ATCTTGAAGCTGGAAGTGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IKK-α</td>
<td>F</td>
<td>CTTACCCAGGCCTGCGAGT</td>
<td>600</td>
<td>59.0</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CAGGTAAATGGCTGCAGAT</td>
<td></td>
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<tr>
<td>BCL-2</td>
<td>F</td>
<td>GTCAAGTGCCTTCCATCAGTCT</td>
<td>759</td>
<td>58.0</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>ACAAAACCCCCACGAAAAAG</td>
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<tr>
<td>APAF-1</td>
<td>F</td>
<td>TTGGCGCTCTTCCATGAT</td>
<td>334</td>
<td>58.0</td>
<td>35</td>
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<tr>
<td></td>
<td>R</td>
<td>TCCCAACTGGAAAAGGTGGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>F</td>
<td>GTGGGGGGCAGACGACGACCA</td>
<td>498</td>
<td>According to target genes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CTCCCTAATGTCAGCAGATTCTC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) F, R, Dir and Anne represent the forward primer, reverse primer, direction and annealing temperature, respectively.
Tissues

1.5 Western blotting analysis

Tissues were lysed in 1× SDS sample buffer (62.5 mmol/L Tris-HCl, pH 6.8 at 25°C, 2% (w/v) SDS, 10% glycerol, 50 mmol/L DTT, and 0.01% bromophenol blue) and the lysate was heated for 5 min at 95°C before loading. 30 μg of cell protein per lane was separated by electrophoresis on a 1%-agarose gel. The images were scanned by Fluor-S MultImager (Bio-Rad, California, USA) and the original intensity of each specific band was quantified with the software Multi-Analyist (Bio-Rad, California, USA). Data were compared after being normalized by the intensity of β-actin.

1.4 Immunohistochemistry

41 cases of representative Paraffin blocked laryngeal carcinoma tissues were selected for immunohistochemistry staining. A paraffin block of normal part was used for positive control and primary antibody-added normal slices for negative control. The experiment was performed by StreptAvidin peroxidase conjugated method for S100A8. Antibodies and SP immunohistochemistry kit were commercially available from Santa Cruz (CA, USA) and Zhongshan (Beijing, China) Co., respectively. The deparaffinized and rehydrated slides were incubated with the primary antibodies in a dilution of 1:200 at 4°C overnight and secondary antibody in 1 :500 at 37°C for 30 min, after thorough washing with 1× PBS for 10 min, with streptavidin for 30 min, and liquid Diaminobenzidine (DAB) for 5 min. The cells with obvious brown stained granules were defined as S100A8 protein expression positive, and others were defined as negative expression. 500—1000 cells were selected randomly in five different scopes to count the positive cells.

1.6 Small interference RNA synthesis and transfection

The siRNA oligos of S100A8, REL-B and P65 were designed according to Ambion pSilence software and their siRNAs were synthesized by Silencer® siRNA Construction Kit (Ambion). Plasmid transfection was performed when the cell reached a confluency of about 80% using the TransMessenger™ Transfection Reagent (Qiagen) according to the manufacturer’s instructions and confirmed by detecting the mRNA level of the target gene. Cells were plated in 6-well plates with RPMI 1640 medium and incubated for 24—48 h prior to transfection. On the day of transfection, medium was replaced with 900 μL serum-free and antibiotic-free RPMI 1640. Transient transfections were performed using 90 μL of complex with suitable amounts of S100A8 or P65 dsRNA for 4 h at 37°C. The medium was then replaced with serum-containing maintenance media and the cells were incubated for a specified time. Groups of cells transfected with 1× PBS and control dsRNA served as vehicle controls for specificity. Each experiment was repeated at least three times. The cells showing obvious interference effect were harvested and used for the following detection. The sequences of the siRNA templates were listed in Table 2.

<table>
<thead>
<tr>
<th>Name</th>
<th>Template sequence</th>
</tr>
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<tbody>
<tr>
<td>S100A8</td>
<td>Anti: 5′-AAGTACTCCCTGATCGAACGGGCTCTGTC-3′ Sen: 5′-AACCCTTTATATGACCGGTACCCCTGTC-3′</td>
</tr>
<tr>
<td>Rel-A (p65)</td>
<td>Anti: 5′-AATGCTCTGTCAGTATGTCACCCTGTC-3′ Sen: 5′-AAGTGCACTACACAGACAGCACCTGTC-3′</td>
</tr>
<tr>
<td>Rel-A21 (p65)</td>
<td>Anti: 5′-AAGATCAATGGCTACACAGACAGCACCTGTC-3′ Sen: 5′-AATCCCTCTGATCCATATGCTTC-3′</td>
</tr>
<tr>
<td>Rel: A31 (p65)</td>
<td>Anti: 5′-AATCCAGTGCTGAAAGAGAGGCGCCTGTC-3′ Sen: 5′-AACGCTTCTTCACACAGTACCCCTGTC-3′</td>
</tr>
<tr>
<td>Rel: A41 (p65)</td>
<td>Anti: 5′-AAGTCTCTTGATACAGAGAGGAGGAGCCCTGTC-3′ Sen: 5′-AAGTCCTCTGCTCTTCTGTAAGAACCTGTC-3′</td>
</tr>
<tr>
<td>Rel: B11</td>
<td>Anti: 5′-AATGAGATACATCATCGAGACGTGACCTGTC-3′ Sen: 5′-AACACTGCTGCATATGCTACCCCTGTC-3′</td>
</tr>
<tr>
<td>Rel: B21</td>
<td>Anti: 5′-AAGGAGACCGGCTCCGCTGACTGACCTGTC-3′ Sen: 5′-AACGCGGAGAAGCCGCTCCCTGTC-3′</td>
</tr>
</tbody>
</table>

a) Anti and Sen represent the anti-sense and sense strands, respectively.

1.7 Apoptosis detection

Apoptotic cells were measured by using Annexin V-FITC apoptosis detection kit (Biosea, Beijing, China). Bands were then quantified by scanning densitometry (THERMAL IMAGING SYSTEM FTI-500, Pharmacia Biotech). β-actin was used as a housekeeping protein.
following the procedure of the manufacturer. Cells \((5\times 10^6 \sim 10\times 10^6)\) were collected with trypsinization, resuspended with binding buffer, followed by incubation with both Annexin V-FITC and propidium iodide (PI) for 15 min at room temperature. Cells were subjected to flow cytometry (FACScan, Becton Dickinson) (Ex = 488 nm, Em=635 nm) in an hour and analyzed using CELLQuest software. Annexin V-positive cells were regarded as apoptosis. For the detection of apoptotic cells with nuclear fragmentation, TUNEL (terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP-biotin nick end-labeling) assay was performed using in situ cell death detection kit (Roche). Treated or untreated cells were collected via trypsinization and fixed with paraformaldehyde solution in PBS (4%, pH 7.4) at room temperature for 30 min. After incubation with permeabilisation solution (0.1% TritonX-100, 0.1% sodium citrate), cells were incubated with labeled nucleotide mixture in the presence of Tdt at 37°C for 1 h. After three times rinsing with 1× PBS, apoptotic cells with fragmented nuclei were visualized and counted under a fluorescence microscope. A general caspase inhibitor, Z-VAD-fmk (benzoyloxycarbonyl-Val-Asp-fluoromethylketone) was purchased from R&D Systems (Minneapolis, MN).

1.8 \textit{In vitro} invasion assay

After a pretreatment with siRNA in different days, cells were removed by trypsinizing and then seeded to Transwell chamber (Coster, USA) at the upper part at a density of \(2\times10^5\) cells/well in 25 \(\mu\)L of serum free medium and then incubated for 12 h at 37°C. The bottom chamber contained the supernatant of mouse NIH3T3. The cells invading to the lower surface of the membrane were fixed with methanol and stained with haematoxylin and eosine. Cell numbers in randomly selected fields were counted under a light microscope.

1.9 Statistical analysis

Each experiment was performed in triplicate. Data were analyzed with software SPSS for Window 12.0 and expressed as mean ± standard deviation (SD). Statistical significance was analyzed with one-way analysis of variance (ANOVA). Difference was assumed to be statistically significant when \(P\) values were less than 0.05.

2 Results

2.1 \textit{S100A8} expression in LSCC

(i) RT-PCR. The results of \textit{S100A8} gene expression were rectified by the control gene \((\beta\text{-actin}).\) Semi-quantitative RT-PCR analysis revealed that \textit{S100A8} mRNA was up-regulated in 17 cases of 36 LSCC (47.2%) compared to PANL (Figure 1(a)). Only 1 in 7 (14.3%) benign laryngeal tumors showed \textit{S100A8} mRNA up-regulation (data not shown). ANOVA analysis showed that \textit{S100A8} mRNA expression was more significantly up-regulated in LSCC than in PANL (\(P<0.05\)).

(ii) Immunohistochemistry. The result of immunohistochemistry showed that \textit{S100A8} protein was mainly located in cytoplasm. Positive \textit{S100A8} protein in LSCC (19/35, 53.4%) significantly increased compared to that in PANL (1/6, 16.6%). ANOVA analysis also indicated that \textit{S100A8} expression was related to the pathological classification of tumors, the positive rate and expression level increased as the pathological level of LSCC decreased (\(P<0.05\), Figure 1(b)).

(iii) Western blotting. Using PANL as a normal control, the results of \textit{S100A8} protein expression were revised by the control gene \((\beta\text{-actin}),\) and it was found that \textit{S100A8} protein were up-regulated significantly in LSCC samples (7/13, 53.85%), especially in metastatic
lymph nodes \( (P<0.05, \text{Figure 1(c)}) \). All the RT-PCR, Western blotting and immunohistochemistry results were corresponding with each other.

### 2.2 RNA interference of target genes

The results of target gene expression were rectified by the control gene (\( \beta\text{-actin} \)). RT-PCR analysis showed that the down-regulation of \( P65 \), \( REL-B \) and \( S100A8 \) mRNA levels in Hep2 cell lines emerged at approximately 3rd d, and reached the peak at about 4th\( -10\)th d. After 10th d of interference, the expression of target gene was restored gradually and almost reached the same level as that in vehicle groups at 12th d (Figure 2). Statistically analysis revealed that transfection successful rate was nearly 85\% \((17/20)\), which ensured the subsequent detection.

**Figure 2** Representative gel picture of RNA interference target gene. \( S100A8 \) mRNA levels of Hep2 cells in different time points after the interference of \( S100A8 \) using a semi-quantitative PCR analysis. A DNA ladder on right indicates the size of the fragments.

### 2.3 Biological behavior of \( S100A8 \) gene on LSCC Hep2 cell line

After RNA interference (RNAi), flow cytometry and TUNEL assay were used to identify the apoptosis in Hep2 cells. Both the results of FACS and TUNEL assay showed that the apoptosis rate increased with the down-regulation of \( S100A8 \) mRNA. Compared to blank and negative controls, the apoptotic rate of Hep2 cells undergoing RNA interference went up from 1.98\% and 2.04\% to 17.1\% at 6th d, nearly 9-fold higher than that of vehicle groups (Figure 3). Meanwhile, the result of the cell invasion test showed that the invasion ability of the cells undergoing \( S100A8 \) interference was much lower than that in vehicle groups, especially at 5th\( -9\)th d, and almost lost at 9th d (Figure 4). ANOVA analysis indicated that there was significant difference between the RNAi groups and the vehicle groups \((P<0.05)\).

### 2.4 Putative localization of \( S100A8 \) in NF-Kappa B signal pathway

In LSCC, the expression of P65 and S100A8 at protein level were clearly in accordance with that at mRNA level (data not shown). RNAi results showed that the down-regulation of \( S100A8 \) gene affected the expression of \( BCL-2 \) but not those of \( APAF-1 \), \( P65 \) and \( IKK\alpha \), especially at 5th\( -10\)th d (Figure 5(a)\( -(c) \)). Meanwhile, the apoptosis rate increased significantly during this period (Figure 3), indicating that \( S100A8 \) may be located upstream of \( BCL-2 \) gene and inhibit Hep2 cell apoptosis partly via down-regulated \( BCL-2 \) expression. On the other hand, down-regulation of \( P65 \) gene affected \( S100A8 \) gene expression, while low level of \( REL-B \) gene expression had no effect on \( P65 \) gene (Figure 5(d)).
following RNA interference of gene; (d) Representative gel image indicates the gene expression profiles in LSCC Hep2 cell line. (a) stained with haematoxylin and eosine are counted under light microscopy. (a) Vehicle control; (b) 7 and 9, respectively.

is associated with human bladder cancer stage S100A8 Convincing evidences suggest that over-expression of S100A8 may be a key step during cancer development[12].

Discussion

S100A8, an important inflammatory factor, has been functionally invoked to inhibit casein kinases I and II that are essential for phosphorylation of various molecules participating in the gene transcription and translation. Recent clinical and experimental data have suggested that changes in the expression and/or function of S100A8 may be a key step during cancer development[12]. Convincing evidences suggest that over-expression of S100A8 is associated with human bladder cancer stage progression, invasion, metastasis and poor survival[13]. Additionally, significantly up-regulation of S100A8 was found in breast, lung, gastric, colorectal, pancreatic, and prostate cancers, while down-regulation of the gene was detected in squamous oesophageal carcinoma[14–22].

However, the association between S100A8 and LSCC is still obscure. Our present RT-PCR, Western blotting and immunohistochemical staining suggest that over-expression of S100A8 is related to tumorigenesis in LSCC and negatively correlated with the degree of differentiation, indicating that the over-expression of S100A8 might be an early event in LSCC carcinogenesis. Meanwhile, the apoptosis rate of Hep2 cell line was increased with down-regulation of S100A8 gene expression using RNA interference technology. At 6th d, the proportion of apoptosis reaches a peak (17.1%), which is nearly 9-fold higher than those in vehicle groups (1.98% and 2.04%). Invasion cells also decrease significantly with the down-regulation of S100A8 gene. The results are like the case of chronic inflammation that promotes carcinogenesis by inhibiting apoptosis, and stimulating cell metastasis[23].

Calprotectin (S100A8/S100A9), with a growth-inhibiting and cell death-inducing activities, plays a regulatory role in inflammatory processes through its effect on the survival and/or growth state of fibroblasts and other cells involved in inflammation. Calprotectin, an inflammatory protein complex, exerts a broad apoptosis-inducing activity mainly by S100A9, which is also a member of S100 protein family, while S100A8 itself had weak effects on apoptosis even if in high concentrations[23–26]. In contrast, our study shows that the hypoxia-induction of S100A8 promoted Hep-2 cell apoptosis, and that S100A8 gene antagonizes the apoptosis-inducing activity of S100A9 gene and participates in laryngeal carcinogenesis partly via inhibiting Hep2 cell apoptosis. APAF-1 and BCL-2 are two mediators in the caspase-dependent mitochondrial apoptosis pathway. Inhibition of apoptosis resulting from BCL-2 mutations can promote lymphomagenesis and influence the sensitivity of tumor cells to chemo- or radiotherapy[27]. In our study, BCL-2 expression was regulated by S100A8 while APAF-1 not, especially at 5th—8th d following RNA interference of S100A8 gene. The apoptosis rate of Hep2 cells is positively coherent with the expression of BCL-2. Therefore, we propose that S100A8, located upstream of BCL-2, triggers mitochondrial apoptosis pathway partly by down-regulating BCL-2 gene expression (Figure 6). Viemann et al[28] showed that S100A8 associates with both caspase-dependent and caspase-independent cell death program and initiates the caspase-dependent pathway by activating caspase-9 and -3 expression. Our results localize BCL-2 between the down-stream S100A8 and the up-stream caspase-9 (-3), which might shed some light on the inflammation-induced carcinogenesis process.
NF-kappa B, a key gene in the inflammatory process, provides a mechanistic link between inflammation and cancer[3,7]. Ott et al.[29] indicated that NF-Kappa B might regulate the expression of S100A8 gene, but experimental data is lacking to support this viewpoint. In the present study, we predict an NF-Kappa B core motif (CGGGGT) at 933—938 bp within the first exon of S100A8 with software Patch. Our RNA interference results suggest that S100A8 mediates NF-Kappa B and apoptosis signaling pathway. The pathway is enriched according to the related references and our present study.

Previous studies in this field mainly focus on numerous pro-inflammatory cytokines (TNF-a, IL-1, etc.) and upstream transcript factors (AP-1, NF-Kappa B, C/EBP)[32–34]. But little is known of how S100A8 induces carcinogenesis. Our study is an attempt to reveal the mechanism of S100A8 in LSCC through analyzing the relationship of S100A8 to the transcription and protein regulation network, immunological molecule (HLA-B), apoptosis related gene (BCL-2) and NF-Kappa B. An in-depth investigation into the way that inflammation leads to carcinogenesis may help to develop drugs targeting at the specific genes, and provide a powerful tool for preventing cancer development.
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