SPANDIDOS PUBLICATIONS

HLA-B gene participates in the *NF-κB* signal pathway partly by regulating *S100A8* in the laryngeal carcinoma cell line Hep2

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Abstract. Human leukocyte antigen B (HLA-B), a novel member of the *NF-κB* signal pathway in laryngeal squamous cell carcinoma (LSCC), mediates immunological surveillance of tumor cells by presenting peptides to cytotoxic T-lymphocytes (CTLs) together with S100 calcium binding protein A8 *(S100A8)*. The objective of this study was to investigate the molecular mechanism of *HLA-B* and *S100A8* in laryngeal carcinogenesis. Flow cytometry, 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay and cell invasion *in vitro* were used to detect the biological effect of the Hep2 cell line induced by *HLA-B* RNA interference. RT-PCR and Western blotting were applied to evaluate the expression level of the *S100A8* gene after *HLA-B* RNA interference. Our results showed that *HLA-B* had negative effects on Hep2 cells by inhibiting apoptosis and cell invasion while decreasing cell proliferation. Additionally, the expression level of *HLA-B* and *S100A8* in LSCC were down-regulated after *HLA-B* RNA interference. The abnormal expression of *HLA-B* is thus relevant to the biological effect of laryngeal carcinoma and participates in the *NF-κB* signal pathway partly by regulating the expression of the *S100A8* gene.

Introduction

Laryngeal carcinoma is the second most common respiratory system cancer among Chinese people, especially in the northeast, and 95% of the cases are laryngeal squamous cell carcinoma (LSCC) (1,2). The morbidity of LSCC is showing an increasing trend. Numerous studies support that immuno-escape plays an important role in the resistance of laryngeal carcinoma to chemo- and radiotherapy. Immunological surveillance of tumor cells is performed by cytotoxic T-lymphocytes (CTLs), which recognize aberrant peptides presented by human leukocyte antigen *(HLA)* class I molecules. Activated T cells directed against tumor-associated antigens are fully capable of recognizing and eradicating neoplastic cells (3). Therefore, a decrease in the expression of HLA antigens is considered a characteristic of tumor progression and an important tumor-escape mechanism.

The crucial role played by HLA class I antigens in the interactions of malignant cells with HLA class I antigen-restricted, tumor antigen-specific cytotoxic T lymphocytes (CTL) and NK cells has stimulated interest in the characterization of their expression by malignant cells. Evidence generated by the immunohistochemical staining of surgically removed malignant lesions with monoclonal antibodies recognizing framework, locus specific and polymorphic determinants of HLA class I antigens indicates that the surface expression of these molecules is frequently defective on malignant cells (4). This abnormality appears to have clinical significance, since in addition to its association with the histopathological characteristics of the lesions, it may be significantly associated with disease-free interval and patient survival (4,5). These associations have been suggested to reflect the effect of HLA class I antigen abnormalities on the interactions of tumor cells with tumor antigen-specific CTL and NK cells. However, there are many examples in which disease progresses in the face of appropriate HLA class I antigen and tumor antigen expression by malignant cells and of functional tumor antigen-specific CTL in the investigated patients (6).

Our previous studies showed that *S100A8* interacting with *HLA-B*, participates in the development and progression of LSCC through the *NF-κB* signal pathway (7). However, some researchers believe that *HLA-B* has no relationship with the biological effects of tumors, and no description of *HLA-B* in triggering tumor-escape mechanisms in signal transduction pathways has been reported. In this study, RNA interference, apoptosis detection, cell cycle analysis, cell proliferation ability, *in vitro* invasion, RT-PCR and Western blotting were used to investigate the biological effect and detect the expression of mRNA and protein, in order to explain the potential mechanism of the *HLA-B* gene in the development and progression of LSCC, which may pave the way for developing LSCC therapeutic vaccine.

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Materials and methods

Cell culture. The cell lines in this study were Hep-2 (human laryngeal carcinoma) and NIH3T3 (mouse) obtained from Cell Biology Institute of Shanghai, Chinese Academy of Science, grown in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37˚C in a 5% CO_2 humidified atmosphere. All the cells in log phase were used for the following experiments.

Small inference RNA synthesis and transfection. For RNA interference experiments, the *HLA-B* siRNA oligonucleotides were designed using free online website (http://www.ambiom.com/techlib/misc/siRNA_finder.html.ben) and synthesized by Silencer® siRNA construction kit (Ambion). Plasmid transfection was performed when the cells reached a confluence of ~80% using the TransMessenger™ transfection reagent (Qiagen) according to the manufacturer's instructions and confirmed by detecting the mRNA level of the target gene. Briefly, 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, media were replaced with 900 μl serum-free and antibiotic-free RPMI-1640. Transient transfections were performed using 90 μl of complex with 3 ng HLA-B dsRNA for 4 h at 37˚C. The media were then replaced with serum-containing maintenance media and the cells were incubated for the specified time. Groups of cells transfected with PBS and control dsRNA served as vehicle controls for specificity. Each experiment was repeated at least 3 times. The cells showing an obvious interference effect were harvested and used for the following detections. The sequences of the *HLA-B* siRNA templates are listed in Table I.

Table I. *HLA-B* siRNA templates.

Name	Template sequence
siRNA 1	
Antisense	5'-AAGAGCAGAGATACACATGCCCCTGTCTC-3'
Sense	5'-AAGGCATGTGTATCTCTGCTCCCTGTCTC-3'
siRNA 2	
Antisense	5'-AACACACAGATCTACAAGGCCCCTGTCTC-3'
Sense	5'-AAGGCCTTGTAGATCTGTGTGCCTGTCTC-3'

Biological detect of the Hep2 cell line after HLA-B RNA interference

Detection of apoptosis. Apoptotic cells were measured by using Annexin V-FITC apoptosis detection kit (Biosea, Beijing, P.R. China) according to the manufacturer's instructions. Cells (5-10x10^5) 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) within 1 h and analyzed using CELLQuest software. Annexin V-positive cells were regarded as apoptotic cells.

Cell cycle analysis. Cells (1x10^6) were harvested and fixed in 75% ethanol for 20 min at 4˚C. After thorough rinsing, the cells were digested with RNase (20 μg/ml) and stained with propidium iodide (50 μg/ml) for 40 min at 37˚C. The rate of cell apoptosis and DNA content at each cell cycle stage were determined by flow cytometry.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. To assess the proliferation ability of the Hep2 cell line after the interference of the *HLA-B* gene, 2-3x10^3 cells were cultured for 48 h in 96-well plates. Each group contained five wells. Then cells were stained with 100 μl sterile MTT dye (0.5 mg/ml, Sigma) for 4 h at 37˚C, culture medium was removed and 150 μl of DMSO was added and thoroughly mixed for 15 min. Spectrometric absorbance at a wavelength of 570 nm was measured on a microplate reader (Bio-Rad) and the cell growth curve was constructed by using OD_{570} as ordinate axis.

Cell invasion assays. After pretreatment with transfected HLA-B siRNA, cells were removed by trypsinization and then seeded to Transwell chamber (Coster, USA) at the upper compartment at a density of 2x10^5 cells/well in 25 μl of serum-free medium. Supernatant (0.5 ml) of human NIH3T3 was added to the bottom chamber. Following incubation for 12 h at 37˚C, cells that invaded to the lower surface of the membrane were fixed with methanol and stained with haematoxylin and eosin. The mean of cell numbers in 5 fields randomly selected represented the cell invasion ability. Four duplicated wells in each group were designed to evaluate reproducibility.

Expression of the S100A8 gene after RNA interference of HLA-B

Semi-quantitative RT-PCR. Total RNA was extracted by using TRIzol™ reagent following the manufacturer's protocol (Invitrogen, CA, USA). The quality of RNA was confirmed on a formaldehyde agarose gel, and the concentration was determined by reading the absorbance at 260/280 nm. Total RNA (1 μg) was used to synthesize the first-strand cDNA with reverse transcription (RT) system (Promega, Madison, WI, USA). Then RT product (1 μl) was used as the template to amplify specific fragments. PCR reaction conditions were optimized for each gene, and the cycle number for PCR was adjusted so that the reactions fell within the linear range of product amplification. The expression of the housekeeping gene *ß-actin* was used as an internal control. The mRNA levels of the RNA inference genes were also detected. The RT-PCR product was analyzed by electrophoresis on a 1% agarose gel. The images were scanned by Fluor-S MultiImager (Bio-Rad, CA, USA) and the original intensity of each specific band was quantified with the software Multi-Analyst (Bio-Rad). Data were compared after being normalized by the intensity of ß-actin (Table II).

Western blotting. Cells were lysed in RIPA buffer [20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na_3VO_4, 0.5 μg/ml leupeptin, 1 mM phenylmethanesulfonylfluoride (PMSF)] and proteins were isolated by centrifugation at 10,000 x g at

Gene primers and semi-quantitative RT-PCR conditions.

Gene	Dir	Primer sequences (5'-3')	Product size	Annealing (˚C)	PCR cycles
S100A8	F	TTGCTAGAGACCGAGTGTCC	169	58.0	35
	R	CTTTGTGGCTTTCTTCATGG			
HLA-B	F	CCAGCAGGAGATAGAACCTT	349	58.0	35
	R	TCAGTCCCTCACAAGACAG			
ß-actin	F	CCAGATCATGTTTGAGACCT	480	According to target gene	
	R	TTGAAGGTAGTTTCGTGGAT			

F, forward primer; R, reverse primer; Dir, direction.

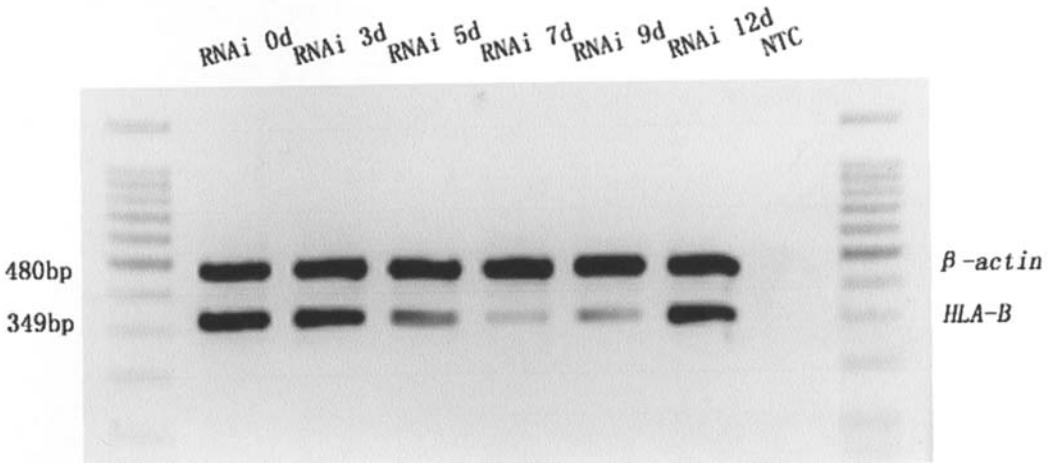

Figure 1. *HLA-B* mRNA levels of Hep2 cells in different time points after the interference of *HLA-B* using semi-quantitative PCR analysis. A DNA ladder on each side indicates the size of the fragments. NTC, non-template control.

4˚C for 5 min. Protein concentrations were determined using Pierce Micro BCA protein assay system (Pierce, Rockford, IL, USA). Western blotting was performed according to the procedure of Towbin *et al* (8). Briefly, proteins (30 μg) were loaded on 12% of SDS-polyacrylamide gel for electrophoresis, then transferred onto nitrocellulose transfer membranes (Osmonics, USA) at 0.8 mA/cm^2 for 2 h. Membranes were blocked at room temperature for 1 h with blocking solution [5% skimmed milk in Tris-buffered solution plus Tween-20 (TBST): 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.1% v/v Tween-20]. Membranes were then incubated overnight at 4˚C with anti-S100A8 goat polyclonal antibody at 1:1000 (Santa Cruz Biotechnology, USA) in blocking solution. After two 10-min washings in TBST, membranes were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-goat secondary antibody at 1:5000 in blocking solution. Detection was performed by enhanced chemiluminescence (ECL) using a Western blot luminol reagent (Santa Cruz Biotechnology) according to the manufacturer's instructions. Bands were then quantified by scanning densitometry (Thermal Imaging System FTI-500, Pharmacia Biotech). Mouse ß-actin was used as a housekeeping protein, and was determined following the same procedure as given above, using a specific anti-actin mouse monoclonal antibody (Sigma-Aldrich, Madrid, Spain) at 1:1000 and a horseradish peroxidase-conjugated anti-mouse secondary antibody at 1:5000 in blocking solution.

Statistical analysis. All data were obtained from three independent experiments and were analyzed with software SPSS for Window 12.0, expressed as mean ± standard deviation (SD). Statistical comparisons were made using One-way analysis of variance (ANOVA). Differences in data were assumed to be statistically significant at $P<0.05$.

Results

RNA interference of HLA-B. The results of *HLA-B* gene expression were rectified by the control gene (*ß-actin*). RT-PCR analysis showed that the down-regulation of *HLA-B* mRNA level in Hep2 cell lines emerged at day 3, and reached the peak at day 7. After the 9th day of interference, the expression of *HLA-B* mRNA was restored gradually to almost the same level as the control groups at day 12 (Fig. 1). ANOVA analysis revealed that the inference effect at 7th day was most obvious and there was no significant difference

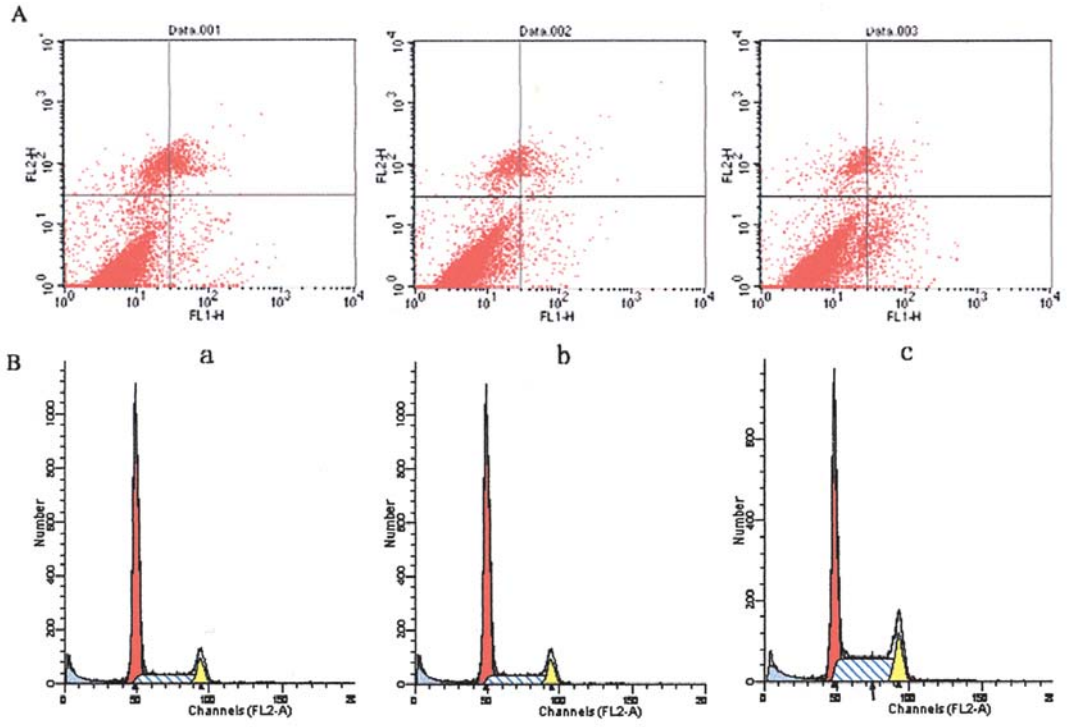

Figure 2. Cellular analysis of Hep2 by a flow cytometry assay. (A) Hep2 cell apoptosis identified with Annexin V-FITC/PI double staining. The cell percentage located in the lower right quadrant represents the content of the apoptotic cells. (B) Cell cycle analysis of Hep2 by PI staining. Significant S phase arrest are shown compared with other groups. a, blank control; b, negative control; c, transfected group.

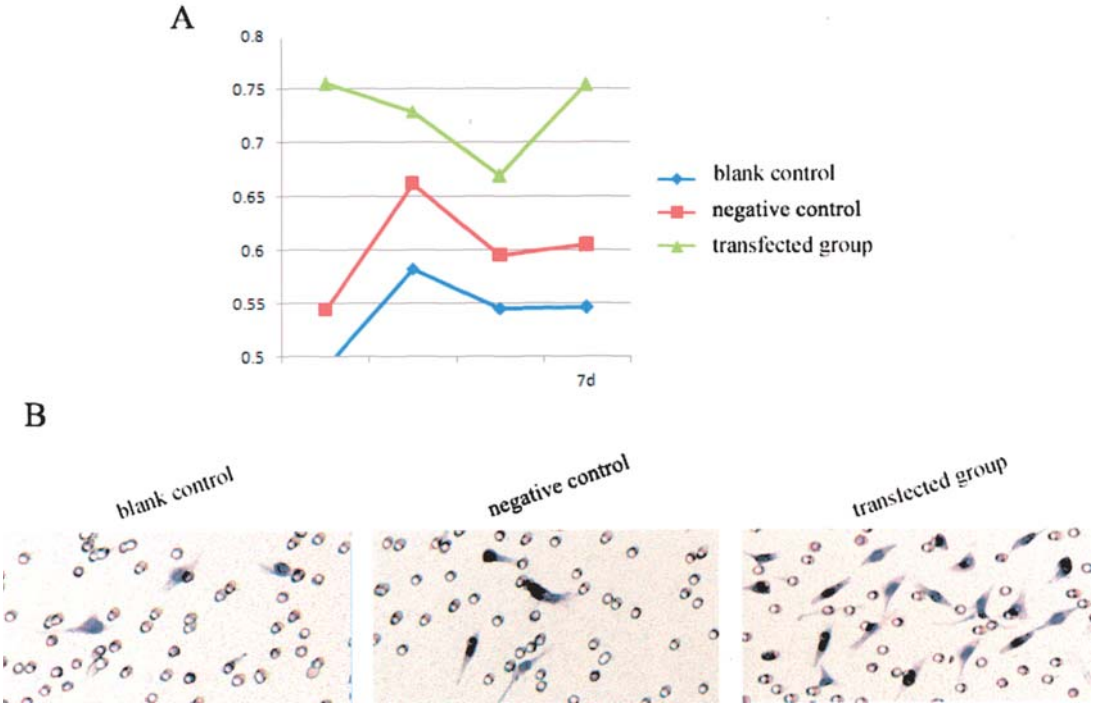

Figure 3. Influence of *HLA-B* on Hep2 cell proliferation and invasion ability. (A) Cell growth curve of four repeated detections. A high OD_{570} value indicates strong cell proliferation ability. (B) Cell invasion assay *in vitro*. Transmembrane cells stained with haematoxylin and eosin were counted under light microscopy.

between negative and blank control groups. Therefore, the cells at day 7 of interference were collected and harvested for the following assays.

Biological effect of the Hep2 cell line after RNA interference of HLA-B. After RNA interference (RNAi), flow cytometry was used to identify any changes in apoptosis and cell cycle profiles of the Hep2 cell line. Comparison of non-transfected and

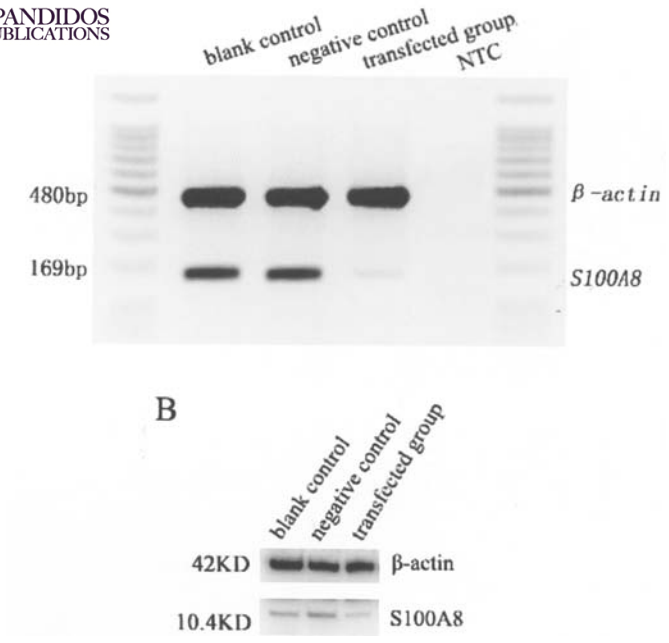

Figure 4. Expression level of the *S100A8* gene in Hep2 cell line undergoing the RNA interference of *HLA-B*. (A) Representative gel image shows the RNA level of *S100A8*. A DNA ladder on each side indicates the size of the fragments. NTC, non-template control. (B) Representative image of the protein level of *S100A8*. *ß-actin* is normal control.

transfected control groups to transfected siHLA-B groups, the apoptotic rate of Hep2 cells increased with S phase cell cycle block. The proportion of apoptotic cells increased from 2.04% and 2.13% to 4.79%, the proportion of S stage cells increased from 23.67% and 24.75% to 43.45%, whereas the proportion of G1 stage cells was reduced from 68.33% and 67.25% to 48.65% in the Hep-2 cells, respectively (Fig. 2). Meanwhile, cell proliferation ability also increased by monitoring OD_{570} nm. The invasion effect of RNAi *HLA-B* on Hep2 cells by Transwell showed the increase of transmembrane Hep2 cells undergoing *HLA-B* RNAi were much greater than those in control groups at day 7. The transmembrane Hep2 cell number ranged from 16.87±3.51 and 18.05±2.94 to 29.48±4.82 after RNAi (Fig. 3). ANOVA analysis showed a significant statistical difference between the RNAi and control groups ($P<0.05$).

S100A8 gene expression after RNA interference of HLA-B. Using non-transfected and transfected control siRNA groups as normal controls, the results of *S100A8* gene expression levels of RNA and protein were revised by the housekeeper gene *(ß-actin)*. It was found that the *S100A8* gene was expressed in all of the three groups, but only showed weak bands in the *HLA-B* siRNA transfection group in which *HLA-B* was down-regulated significantly. The RT-PCR and Western blotting results corresponded with each other. The results showed that the inhibition of *HLA-B* affected the expression of *S100A8* (Fig. 4).

Discussion

The human *HLA-B* gene together with *HLA-A, and HLA-C* belongs to the HLA class I heavy chain paralogues and is characterized by broad tissue expression and high polymorphism (9-11). *HLA-B*, regulating organism immunoresponses and determining individual sensitivity, plays an important role in human tumors and infectious diseases. Several studies have shown that down-regulation or complete loss of *HLA* class I gene expression in a variety of cancers was followed by enhanced tumor cell invasion and poor prognosis. Rockett *et al* found that *HLA-ABC* is abnormal in a high proportion of oesophageal carcinomas. In 20 of 37 (54%) carcinomas *HLA-ABC* was expressed weakly, with heterogeneous expression in nine (24%). Two tumors showed strong expression of *HLA-ABC*, but 15 of 37 (41%) were negative (12). This discrepancy may partly explain the aggressive nature of oesophageal carcinomas. By use of immunohistochemistry, several studies have shown defects in class I expression in gastric and other cancers, such as the colorectum, head and neck, breast, lung, kidney, uterine cervix, and prostate (5,13-16). However, Esteban *et al* reported that *HLA* class I losses were not associated with an increased metastatic potential in LSCC (17). Our results show that the down-regulation of *HLA-B* leads to S phase arrest, high proliferation and enhanced cell invasion potential of LSCC, which implies that *HLA-B* is associated with the biological effect of LSCC and retards the development of LSCC.

The increase of the apoptotic rate in the Hep2 cell line after RNAi *HLA-B* was not as obvious as that of the RNAi *S100A8* gene, which was enhanced nearly 9-fold compared to the control (unpublished data). *S100A8*, a novel member of the *NF-κB* signal pathway, is involved in the regulation of a number of cellular processes such as cell cycle progression, differ-entiation, chemotaxis and the inflammation signal transduction pathway in a variety of cancers, including LSCC (7,18-21). In our previous study, S100A8 localized upstream of *BCL-2* and downstream of *P65* (unpublished data). By using immunoprecipitation assay with anti-S100A8 antibody followed by MALDI-TOF identification, we found that S100A8 participates in the *NF-κB* signal pathway together with its new partner HLA-B (7). While the transcriptional regulation of *HLA-B* is controlled by *NF-κB* through the NF-κB binding site on enhancer A of *HLA-B* (22), it indicates that *HLA-B* participates in *NF-κB* signal pathway. However, the regulation mechanism between *S100A8* and *HLA-B* is still unclear. RT-PCR and Western blotting showed that the *S100A8* gene was significantly down-regulated after RNAi *HLA-B* which confirmed our hypothesis: *HLA-B* participates in the *NF-κB* signal pathway partly by regulating the expression of *S100A8* gene.

Our previous studies showed that *S100A8* might regulate Hep2 cell apoptosis via BCL-2, which is an anti-apoptosis integral outer mitochondrial membrane protein (unpublished data). Co-immunoprecipitation and immunofluorescence assay revealed that HLA-B and S100A8 are interactive proteins (7). Also, HLA-B can partly regulate S100A8 gene expression. Inhibition of apoptosis resulting from *BCL-2* mutations can promote lymphomagenesis and influence the sensitivity of tumor cells to chemo- or radiotherapy (23). These associations suggest that *HLA-B* triggers apoptosis of LSCC partly by *S100A8/BCL-2*. Evidence has shown that ß2m-associated and ß2m-free soluble HLA class I heavy

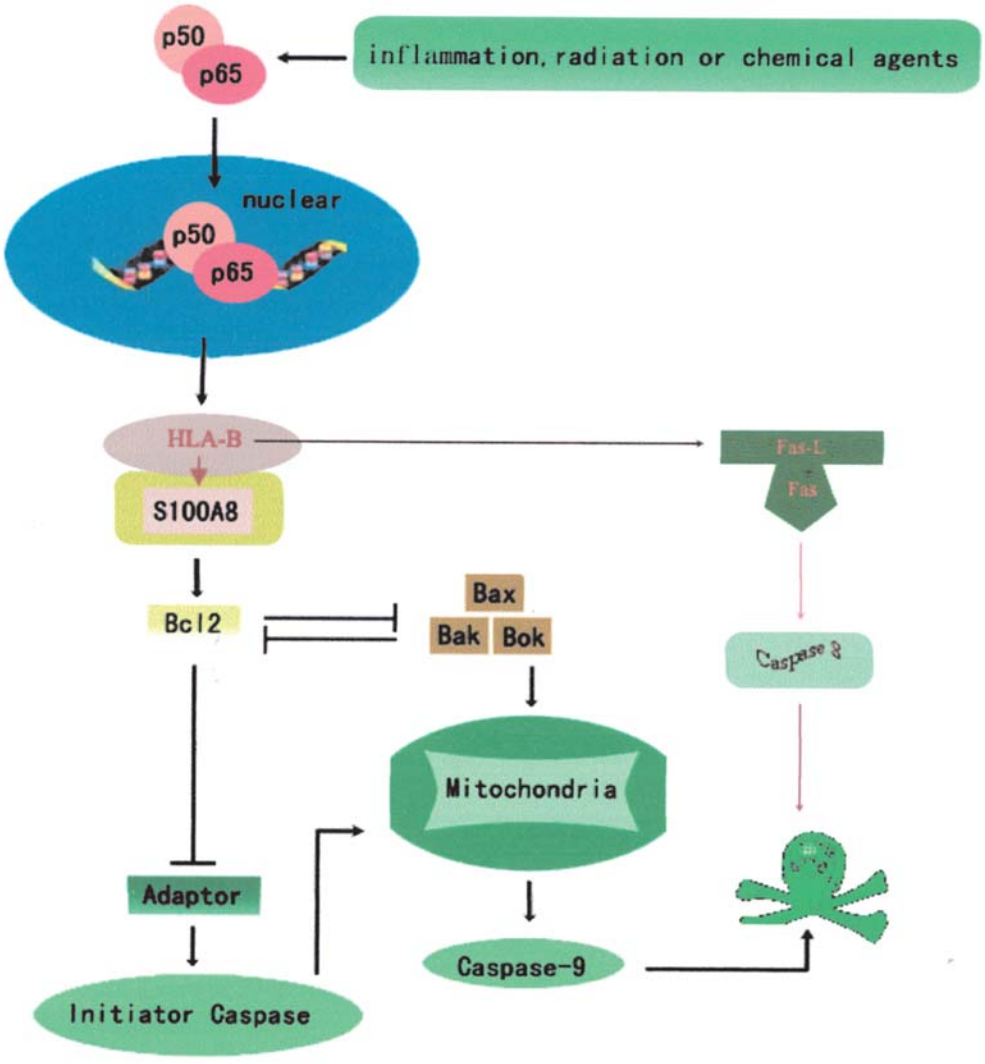

Figure 5. Possible localization of *HLA-B* in the *NF-κB* signal pathway. The pathway is shown according to related references, our previous unpublised results and the present study.

chains can induce apoptosis of activated CD8 T cells *in vitro* (24). Conflicting mechanisms have been shown to trigger the induction of apoptosis in activated T cells. Zavazava *et al* have shown that apoptosis results from interaction of soluble HLA class I antigens with the T cell receptor (25). In contrast, Puppo and colleagues showed that apoptosis mediated by soluble HLA class I antigens is dependent on CD8 in both T and NK cells suggesting that interaction with the TCR is not required for this effect (24,26). However, there is agreement on the requirement of fas and fasL in the induction of apoptosis by soluble HLA class I antigens (24-27). The present study not only enriches the knowledge of NF-κB signal pathway but also contributes to the understanding of the apoptosis pathway, providing a theoretical basis for tumor therapy, especially immunological therapy of LSCC (Fig. 5).

Knowledge of the exact mechanism by which cancer cells can elude the immune system can be a key factor in the choice of a particular vaccination strategy or therapeutic approach. Such mechanisms include reduced or absent antigenic expression due to defects in the antigenic presentation machinery, lack of expression of tumor antigens or of molecules that participate in the triggering of T cell-induced apoptosis, secretion of suppressor cytokines, appearance of surface molecules that modulate the activity of T and NK cells, and induction of abnormalities in the signal transduction of T cells (28). Among these different mechanisms, alterations in the expression of *HLA* class I molecules on tumor cells play a leading role in the tumor-host scenario because of the crucial interaction of *HLA* molecules with T- and NK-specific receptors present in both immune effector cells (29).

Acknowledgements

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