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Regulation of neutrophil gelatinase-associated lipocalin expression by C/EBPβ in lung carcinoma cells

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Abstract. Neutrophil gelatinase-associated lipocalin (NGAL), a member of the lipocalin family, has been found to be overexpressed in a variety of tumors, including lung adenocarcinomas. However, the mechanism by which NGAL expression is regulated in lung carcinoma needs further evaluation. In this study, immunohistochemistry was employed to analyze the expression of NGAL in lung carcinoma tissue samples, including lung squamous carcinomas, adenocarcinomas, adenosquamous carcinomas and bronchial alveolar cell carcinomas. The results showed that NGAL was expressed in 82.61% (19/23) of the samples. RT-PCR and immunofluorescent staining showed that NGAL was localized to the cytoplasm in lung carcinoma cell lines. To explore the transcriptional regulation mechanism of NGAL basal expression in lung carcinoma, a 1515-bp fragment (-1431 to +84) of the *NGAL* promoter region was cloned and a series of deletion and mutation constructs were generated. These constructs were analyzed using the luciferase reporter assay. The results indicated that the *cis*-acting elements important for the basal activity of *NGAL* transcription were likely located between -152 and -141. Further analysis using site-directed mutagenesis and the luciferase reporter assay suggested that the C/EBP binding sites were responsible for the activity of the *NGAL* promoter. Finally, the binding ability and specificity of the transcription factors were determined by electrophoretic mobility-shift assay (EMSA). The results showed that C/EBPβ was able to bind to the -152 and -141 segments. Taken together, these findings suggest that NGAL is expressed in lung carcinomas and that NGAL expression is mediated by the binding of C/EBPβ to the -152 and -141 segment of the *NGAL* promoter.

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Introduction

Neutrophil gelatinase-associated lipocalin (NGAL), a member of the lipocalin family, was originally identified as a protein stored in specific granules of the human neutrophil (1). Besides the neutrophil, NGAL is expressed in most tissues normally exposed to micro-organisms and is induced in epithelial cells during inflammation (2). NGAL binds bacterial catecholate-type ferric siderophores and acts as a potent bacteriostatic agent by sequestering iron, indicating that NGAL participates in the iron-depletion strategy of the innate immune system (2). In addition, increased NGAL expression has been observed in a variety of pathological conditions, including inflammation, acute ischemic renal injury and various types of human cancer (3). The upregulation of NGAL has been found in human tumors of various organs, including the ovary, colon, pancreas, lung, esophagus and thyroid (2). Previous studies have also demonstrated that the association of NGAL with cancer progression may be due to the ability of NGAL to interact and protect MMP-9 from degradation, resulting in increased MMP-9 activity (4).

Some findings have been reported concerning the mechanisms of *NGAL* transcriptional regulation. The induction of *NGAL* expression by the co-stimulation of IL-17 and TNFα was controlled by IκB-ζ, but not by C/EBPβ or C/EBPδ in lung cancer A549 cells (5). *NGAL* was consistently upregulated in lung cancer A549 cells following IL-1β stimulation, and in thyroid cancer FRO cells following IκB-ζ stimulation (6,7). Moreover, the region of the *NGAL* promoter from -153 to -90 contained *cis*-acting elements that may be significant for the basal expression of *NGAL* in A549 cells (8). C/EBPε also enhanced the transcription of *NGAL* in neutrophilic granulocytes, as demonstrated by C/EBPε-/- mice experiments (9). We previously studied the responsiveness of *NGAL* to TPA stim-

ulus in EC109 cells, an esophageal squamous cell carcinoma cell line, and found that the region between -152 and -60 of the *NGAL* promoter contained TPA response elements, which were identified to the region required for basal expression (10). Our more recent study showed that NGAL was overexpressed in gastric cancer; the binding of C/EBPβ to the TRE of the *NGAL* promoter mediated its TPA-induced overexpression in gastric carcinoma cells (11). However, to date the core promoter elements for *NGAL* basal expression are uncertain.

To further explore the transcriptional regulation of NGAL, in the current study, we cloned a 1515-bp fragment (-1431 to +84) of the *NGAL* promoter region in lung cancer cells and generated a series of deletion and mutation constructs. Further studies using these constructs revealed that the region from -152 to -141 was the core promoter of *NGAL* and that *NGAL* expression was mediated by the binding of C/EBPβ to the -152 and -141 segments of the *NGAL* promoter.

Materials and methods

Cell culture. Two cell lines, 95D (a lung carcinoma cell line of high metastatic propensity) and A549 (type II pneumocyte-derived cell line), were purchased from the Chinese Academy of Sciences. They were all epithelial-like lung carcinoma cells and were cultured in DMEM medium (Invitrogen, Carlsbad, CA, USA) and maintained in a humidified atmosphere with 5% CO_2 at 37˚C. For transfection, the cells were seeded in 96-well plates at $1.5x10^5$ cells/ml.

Immunohistochemical staining. Specimens of human lung carcinomas were obtained from the Pathology Department of the Medical College of Shantou University (Shantou, China). Immunohistochemical staining was performed as previously described (12). The slides were incubated overnight at 4˚C with rat anti-human NGAL monoclonal antibody (R&D Systems, Minneapolis, MN, USA). Following rinsing with PBS, the slides were incubated for 20 min at room temperature with peroxidase-conjugated goat anti-rat antibody (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Subsequently, the slides were stained with 0.003% 3, 3'-diaminobenzidine tetrahydrochloride and 0.005% hydrogen peroxide in 0.05 M Tris HCl (pH 7.2), counterstained, dehydrated and mounted. Blank controls were prepared by substituting PBS for the primary antibody. NGAL-positive samples were defined as those showing brown staining in the cytoplasm.

RT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with 1 μg of total RNA using Reverse Transcription system (Promega, Madison, WI, USA) according to the manufacturer's recommendations. PCR analyses were then performed and the primer sequences were as follows: 5'-GGATCCGTCAGGACTCCACCTCAGA-3' (forward primer) and 5'-GGTACCTCAGCCGTCGATACA CTG-3' (reverse primer) for *NGAL* and 5'-GAAGGT GAAGGTCGGAGTC-3' (forward primer) and 5'-GAAGAT GGTGATGGGATTTC-3' (reverse primer) for GAPDH.

Immunofluorescent staining. The cells were seeded on coverslips and incubated for 24 h. After washing with PBS, the cells were fixed with 100% methanol at -20˚C for 15 min and then treated with 0.2% Triton X-100 in PBS for 10 min. The cells were subsequently incubated with a blocking solution (10% normal goat serum in PBS) for 20 min and incubated with primary antibody overnight at 4˚C. The cells were washed and incubated with fluorescein-conjugated goat anti-rat IgG (Zymed, Carlsbad, CA, USA) for 30 min at 37˚C. Finally, the cells were washed and mounted in glycerol and examined using a fluorescence microscope.

Construction of plasmids. The construction of the original luciferase expression vector pGLB (Promega), containing the human *NGAL* promoter region, has been described previously (10). To further study the putative *cis*-acting elements located at -152 to -141, we constructed mutational plasmids by PCR amplification with mutated primers. The -152 deletion plasmid was generated by PCR amplification with the following primers: 5'-TACTCGAGCTGTCTTGCC CAATCCTGAC-3', containing a C/EBPs binding site (underlined) and 5'-ATAGATCTGAGACCTAGGGGCATGA TTT-3'. Then a series of mutants with a -152 deletion mutation were generated by PCR amplification. The mutations of the C/EBPs binding site were generated using the following primers: i) 147m, 5'-TACTCGAGCTGTCAACCCGTT TCCTGAC-3'; ii) 144m, 5'-TACTCGAGCTGTCTTGGA TGGTCCTGAC-3'; iii) 141m, 5'-TACTCGAGCTGTCTT GCCCGATCCT GAC-3'; iv) CEBP (conc), 5'-TACTCGAGCTGTCTTGCG CAATCCTGAC-3'. The DNA fragments containing the mutated region were inserted into the *Xho*I and *Bgl*II sites of the pGLB vector. Relevant regions of the final constructs were confirmed by sequencing.

Transient transfection and luciferase reporter gene assay. The cells in 96-well plates were transfected using Superfect transfection reagent (Qiagen, Hilden, Germany) with pGLB promoter constructs. To compensate for differences in transfection efficiency, the cells were co-transfected with the internal control plasmid pRL-TK containing Renilla luciferase (Promega). Following transfection, the cells were cultured at 37˚C in a humidified atmosphere with 5% CO_2 for 48 h. Subsequently, the cells were harvested and the lysates were assayed for luciferase activity using the Dual Luciferase Reporter Assay system (Promega). Luciferase activity data are expressed as the mean ± SE of at least three independent experiments.

Western blot analysis. Nuclear extracts from 95D and A549 cells were prepared using the method described by Sambrook and Russell (13). The protein concentration was estimated using the Bradford method. Equal amounts of nuclear extracts (100 μg) were separated by SDS-PAGE and transferred to the PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked in 5% skimmed milk in PBST (phosphate-buffered saline, containing 0.1% Tween 20) for 1 h at room temperature followed by the addition of the primary antibody for 2 h at room temperature. The membrane was subsequently incubated with secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 2 h at room temperature. The protein-antibody complexes were then identified by western blot luminol reagent (Santa Cruz Biotechnology, Inc.).

Table I. Oligonucleotides used in EMSA analysis.

Name	Sequence (5'-3')
-152/-114	CTGTCTTGCCCAATCCTGACCAGGTGCAGAAATCTTGCCGGCAAGATTTCTGCACCTGGTCAGGATTGGGCAAGACAG
-147/-140m	CTGTC*GACTAGTC*TCCTGACCAGGTGCAGAAATCTTGCCGGCAAGATTTCTGCACCTGGTCAGGA*GACTAGTC*GACAG
-145/-143m	CTGTCTT*TAA*CAATCCTGACCAGGTGCAGAAATCTTGCCGGCAAGATTTCTGCACCTGGTCAGGATTG*TTA*AAGACAG

Mutated bases indicated in italics. EMSA, electrophoretic mobility shift assay.

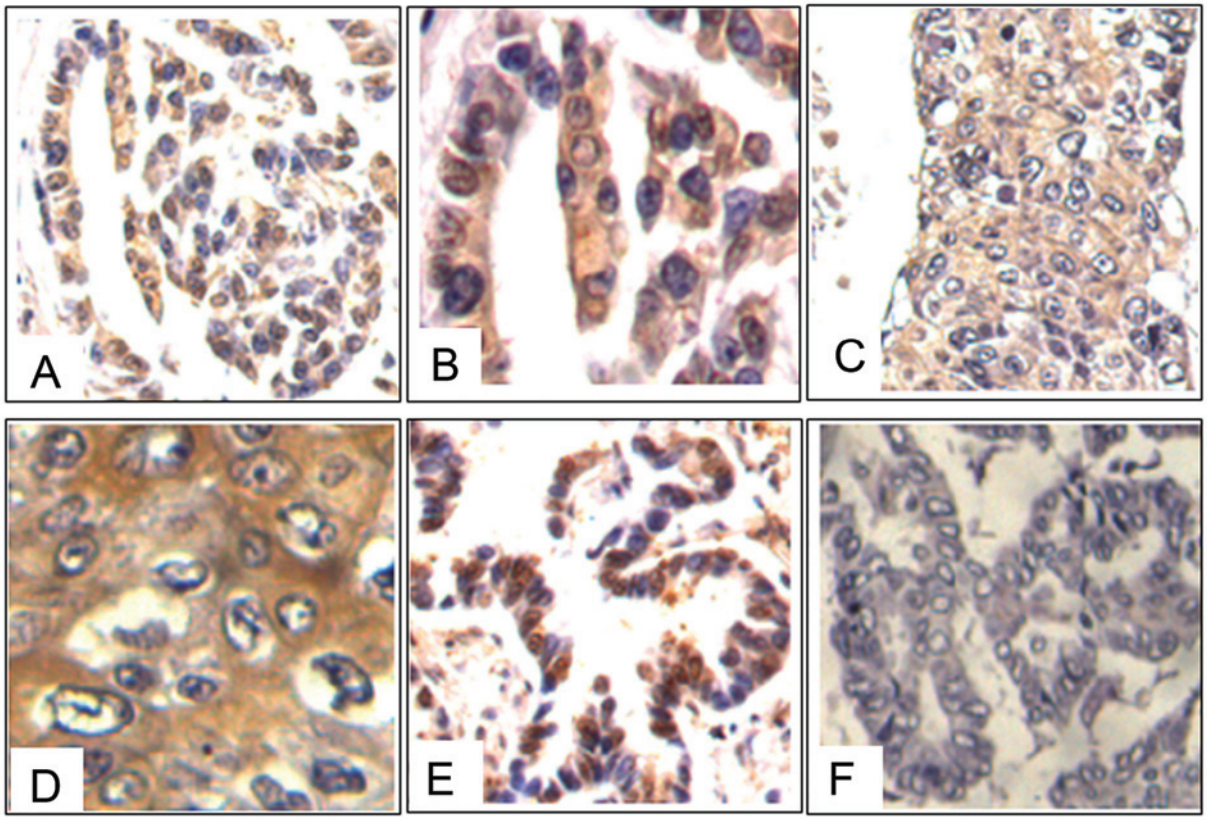

Figure 1. Immunohistochemistry staining for NGAL expression in lung carcinoma tissues. (A) (x100) and (B) (x200), lung adenocarcinomas; (C) (x100) and (D) (x200), lung squamous carcinomas; (E) (x100), lung adenosquamous carcinomas; (F) (x100), blank control. NGAL, neutrophil gelatinase-associated lipocalin.

Electrophoretic mobility shift assay (EMSA) and supershift analysis. The sequences of the oligonucleotides used in the EMSA are listed in Table I and the mutated bases are presented in italics. Complementary oligonucleotides were annealed and labeled with DIG-ddUTP by terminal transferase using DIG Gel Shift kit (Roche Diagnostics, Mannheim, Germany). Nuclear extracts were incubated with labeled probes for 30 min at room temperature in a 20 μl reaction mixture containing 20 mM Hepes (pH 7.9), 1 mM EDTA (pH 8.0), 1 mM dithiotreitol, 10 mM $(NH_4)_2SO_4$, 0.2% Tween-20 (w/v), 30 mM KCl, 1 μg poly-[d(I-C)] and 0.1 μg poly L-lysine. Then, complexes were resolved on 6% nondenatured polyacrylamide gel (acrylamide/bis-acrylamide ratio of 29:1) in 0.5X TBE at 80 V for 100 min at 4˚C. The gels were transferred to a positively-charged nylon membrane. Alkaline phosphatase-conjugated anti-digoxigenin antibody and the chemiluminescent substrate CSPD were used to detect digoxigenin according to the manufacturer's instructions (Dig Gel Shift kit, Roche Diagnostics) and immunoreactive bands were photographed and analyzed by FluorChemTMIS-8900 (Alpha Innotech, Santa Clara, CA, USA). For supershift analysis, 2 μl anti-C/EBPβ antibodies (Santa Cruz Biotechnology, Inc.) were incubated with nuclear extracts for 20 min prior to the addition of labeled probes. In the competition experiments, different folds molar excess of unlabeled oligonucleotide was added to the binding reactions.

Results

Expression of NGAL in human lung carcinoma tissues. Previous studies have reported that in lung cancer tissues, NGAL showed moderate to strong positive expression in adenocarcinomas; whilst staining for the protein was negative or weakly positive in squamous cell or large cell carcinomas (14). To further evaluate the expression of NGAL

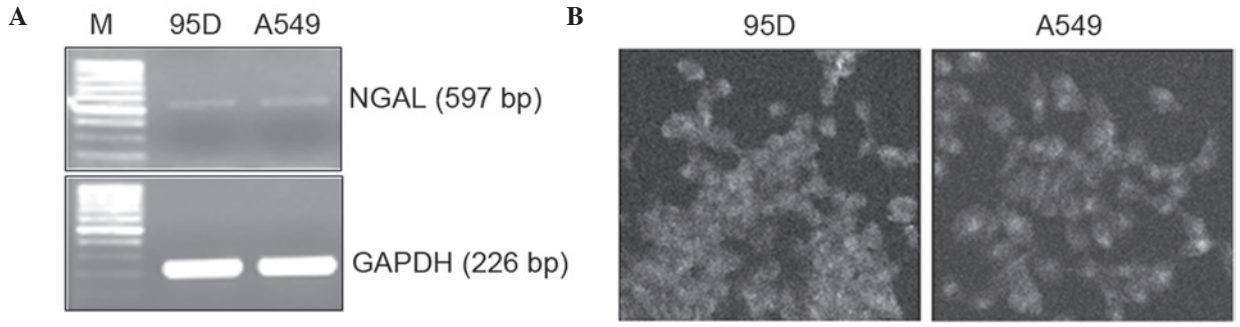

Figure 2. Expression of NGAL in 95D and A549 cells. (A) RT-PCR analysis for NGAL expression. Detection of GAPDH was used as a control. (B) Distribution of NGAL expression was determined by immunofluorescent staining. NGAL was observed in the cytoplasm of the 95D cells (x200) and A549 cells (x200).

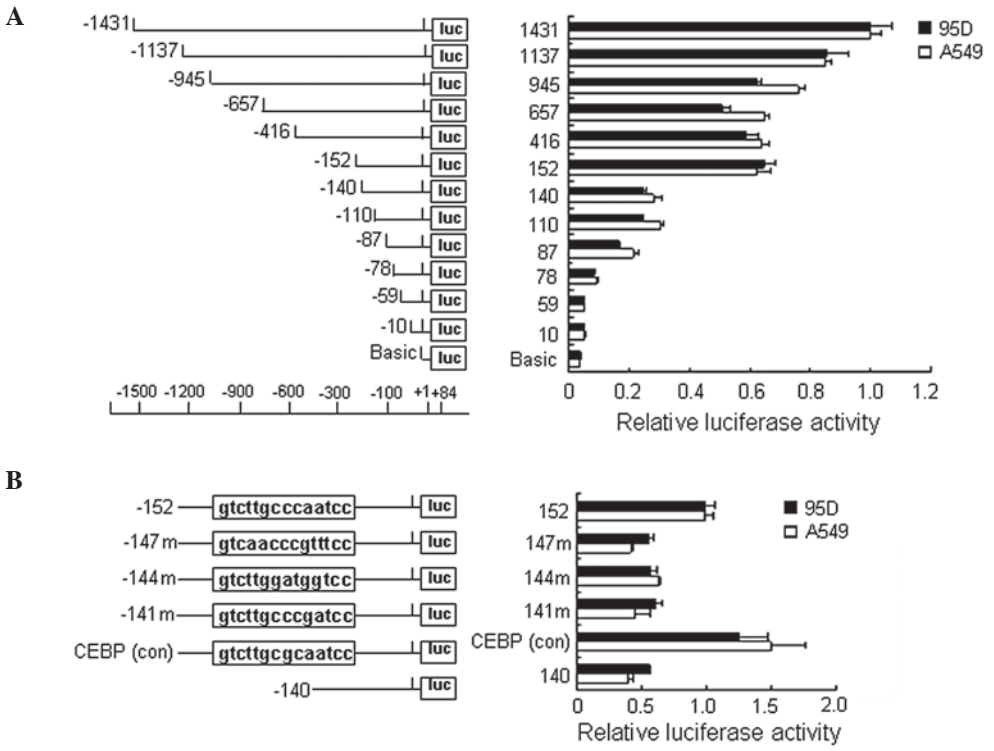

Figure 3. The basal activity of the *NGAL* promoter in 95D and A549 cells. (A) Relative luciferase activities of various 5' deletion constructs of the NGAL promoter ranging from -1431 to -10. Luciferase activity was normalized to the *Renilla* luciferase activity and then shown relative to that of -1431 deletion construct. Background activity was determined by including the reporter plasmid pGL3-Basic with no promoter insert (Basic). (B) Relative luciferase activities of the 152 mutants. Luciferase activity was normalized to the *Renilla* luciferase activity and then shown relative to that of the -152 deletion construct. The data were obtained from a single experiment repeated two more times with similar results. Each value is presented as mean ± SE of at least three independent experiments. (A and B) Schematic representation of the NGAL promoter constructs used for transient transfection is shown in the left panel, with the relative luciferase activity from each construct in the right panel.

in lung carcinomas, 23 tissue samples were examined by immunohistochemical staining. The samples consisted of a spectrum of tissues ranging from low- to high-grade human lung squamous carcinomas, adenocarcinomas, adenosquamous carcinomas and bronchial alveolar cell carcinomas. The results showed that 82.61% (19/23) of the cases were positive for NGAL staining. All the positive cases revealed a diffuse cytoplasmic distribution of NGAL in the cancer cells (Fig. 1).

Expression of NGAL in 95D and A549 cells. The mRNA level of NGAL was analyzed by RT-PCR. A band of ~537 bp was detected in 95D and A549 cells, suggesting that NGAL was expressed in these cells (Fig. 2A). The distribution of NGAL in lung carcinoma cells was determined by means of immunofluorescent staining. NGAL was localized to the cytoplasm of these cells, showing a diffuse to granular pattern (Fig. 2B).

Region -152 to -141 was the core promoter of NGAL. To determine the core promoter of human *NGAL* and to identify regulatory elements within the promoter, a 1515-bp fragment (-1431 to +84) of the *NGAL* promoter region was cloned and a series of deletion and mutation constructs were obtained. These constructs were then co-transfected with the pRL-TK plasmid into 95D and A549 cells and subjected to the luciferase reporter assay. It was shown that, when truncated from -152 to -140, the promoter activity of *NGAL* was decreased by ~70%

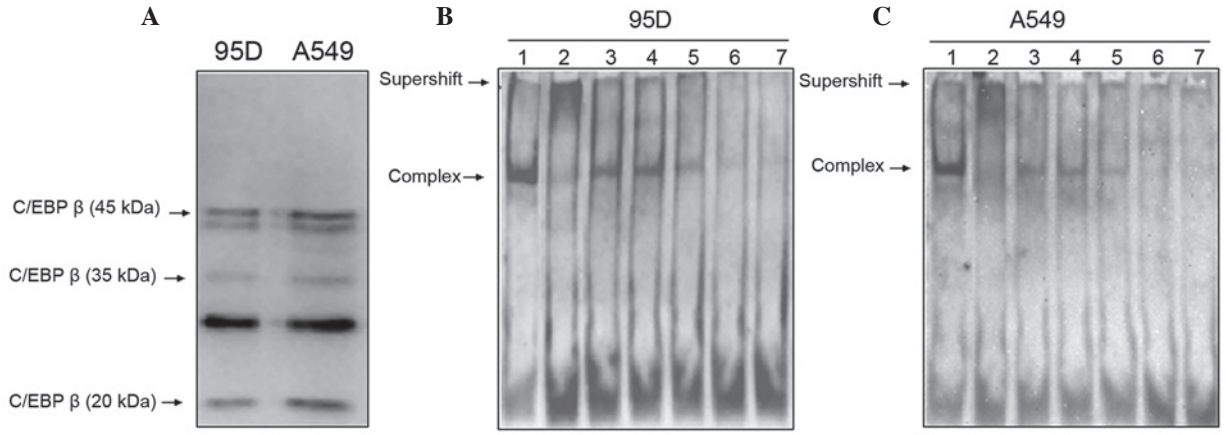

Figure 4. C/EBPβ was present in 95D and A549 cells and bound to the *NGAL* promoter. (A) Nuclear extracts were collected from 95D and A549 cells and tested for C/EBPs by western blot analysis; (B and C) EMSA analysis for the binding of C/EBPβ to the core promoter of *NGAL* in (B) 95D and (C) A549 cells. Lane 1, EMSA was performed using Dig-labeled C/EBP sequence of the *NGAL* promoter (-152/-114) and nuclear extracts; lane 2, supershift experiment; lanes 5-7, a competition EMSA was carried out to determine the specificity of nuclear extracts binding to the C/EBP binding sites. The binding reaction contained a 125-, 375- and 725-fold molar excess of specific competitors (unlabeled -152/-114), respectively. A 125-fold molar excess of two non-specific competitors with two different mutated C/EBP sites (-147/-140m and -145/-143m) were also used (lanes 3-4). EMSA, electrophoretic mobility shift assay; NGAL, neutrophil gelatinase-associated lipocalin.

(Fig. 3A), indicating that the -152 to -141 region was essential for the basal activity of the *NGAL* promoter and this region was identified as the core promoter of *NGAL*. We analyzed the sequence using TESS software (http://www.cbil.upenn.edu/cgi-bin/tess) and found several putative binding sites of transcription factors, including C/EBPs, in this region. We then generated a series of mutants with a -152 deletion plasmid by PCR amplification with the mutated primers (see Materials and methods). The relative activity of the -152 mutations, including 147m, 144m and 141m, were reduced by ~50% compared with the -152 deletion plasmid, and the activity of the CEBP(conc) was slightly increased, suggesting that C/EBPs binding sites were responsible for promoter activity (Fig. 3B).

C/EBPβ was probably the transcription factor regulating NGAL basal expression. According to the results of the luciferase reporter assay, we presumed that C/EBPs regulated the basal expression of *NGAL*. Nuclear extracts from 95D and A549 cells were collected and the levels of expression of C/EBPs were analyzed using western blotting. The results showed that C/EBPα and γ were undetected (data not shown), but C/EBPβ could be detected in different isoforms (42, 35 and 20 kDa; Fig. 4A). In order to further confirm that C/EBPβ played a critical role in regulating the activity of the *NGAL* promoter, we synthesized oligonucleotide probes corresponding to C/EBPs binding sites, and simultaneously, oligonucleotide probes with a mutated C/EBPs binding site served as non-specific competitors (Table I). We then examined nuclear extracts from 95D and A549 cells by EMSA, using the C/EBPs site of the *NGAL* promoter (-152/-114) as a probe. Similar DNA-protein complexes were found in the two types of cells (Fig. 4B and C, lane 1). To determine the specificity of these binding complexes, we added 125- to 725-fold molar excess of unlabeled specific competitor to the binding reaction. With the increasing molarity of unlabeled oligonucleotide, the complex disappeared gradually (Fig. 4B and C, lanes 5-7). However, when the unlabeled mutated oligonucleotide was added to the reaction, the complex remained prominent [Fig. 4B and C, lane 3 (-147/-140m): TTGCCCAA→GACTAGTC; lane 4 (-145/-143m): GCC→TAA]. In supershift analysis, anti-C/EBPβ antidody led to the disappearance of the DNA-protein complex and later a migrating band appeared (Fig. 4B and C, lane 2). These results suggest that C/EBPβ binds to the core promoter of *NGAL* in lung carcinoma cells.

Discussion

NGAL has been identified in a variety of normal and pathological human tissues (14). The expression of NGAL has been demonstrated in several types of cancer, including carcinoma of the colon, lung, pancreas, breast and esophagus (2). NGAL also promotes breast tumor growth by enhancing MMP-9 activity and facilitating tumor progression. The detection of urinary MMP-9 and NGAL complexes in breast cancer patients may serve as new independent predictors of disease status (4). Also, our previous studies have indicated that NGAL was overexpressed in esophageal squamous cell carcinoma and gastric carcinoma, and that altered NGAL expression may play a significant role in the transformation and progression of esophageal squamous cell carcinoma (10,11,15). In the current study, we further showed that NGAL was expressed in lung squamous carcinoma and adenocarcinoma tissues.

The significant correlation between NGAL and tumor progression led to studies concerning the expression regulation mechanisms of *NGAL*. Increasing evidence suggests that certain transcription factors, including IκB-ζ, NF-κB, C/EBPα, C/EBPβ and C/EBPε, play dominant roles in the induction of *NGAL* expression within tumor cells (5). Our previous study in esophageal squamous cell carcinoma cells indicated that there was a TPA response element located at the -152 and -60 regions of the *NGAL* promoter which mediated TPA stimulation (10). More recently, in gastric cancer, we found that the binding of C/EBPβ to the TRE of the *NGAL* promoter mediates its TPA-induced overexpression (11). In lung cancer,

it has been reported that *NGAL* was induced by IL-1β through the NF-κB and IκB-ζ pathway in A549 cells (6,8). However, the mechanisms for *NGAL* basal expression have not been thoroughly investigated. In the current study, we aimed to find the *cis*-acting elements which regulate *NGAL* basal expression. We identified the core promoter region of *NGAL*, which contained C/EBPs binding sites located at -152 to -141 and showed that the C/EBPs binding sites were crucial for the basal expression of *NGAL*.

C/EBPβ belongs to the C/EBP family of basic region leucine zipper (bZIP) transcription factors that consists of six members: C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ. With the exception of C/EBPζ, which lacks a canonical basic region, each protein contains a similar basic region and leucine zipper sequences at its C-terminus, which mediate DNA binding and dimerization, respectively (16). C/EBPs form both homo- and heterodimers and may interact with other non-bZIP transcription factors (17). C/EBPs play significant roles in a number of cellular processes, including metabolism and inflammatory response, and are specifically involved in the differentiation of adipocytes, myeloid cells, hepatocytes, mammary epithelial cells, intestinal epithelial cells, keratinocytes and ovarian luteal cells (18). Therefore, C/EBPs lead to not only granulocytic differentiation, but also the expression of granulocyte-specific genes, including the expression of *NGAL*, which is stored in specific granules of the human neutrophil (1,19). In the present study, we demonstrated that C/EBPβ exists as different isoforms in 95D and A549 cells. C/EBPβ binds to the C/EBPs binding site of the *NGAL* promoter and regulates the basal expression of *NGAL*. C/EBPβ has been shown to function as a pro-oncogenic transcription factor that promotes the proliferation and/or survival of certain tumor cells, and its levels were increased in a number of tumors, including lung cancer (20). This may be the reason for NGAL overexpression in these tumors. Moreover, NGAL has been implicated in epithelial cell differentiation (21). We presume that C/EBPβ promotes NGAL expression in the cell differentiation process.

In summary, we identified the core promoter of *NGAL* in lung carcinoma cells and revealed the regulation mechanism of *NGAL* basal expression by the binding of transcriptional factor C/EBPβ to this core promoter.

Acknowledgements

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