NGALR Is Overexpressed and Regulated by Hypomethylation in Esophageal Squamous Cell Carcinoma

Lei Cui, Li-Yan Xu, Zhong-Ying Shen, Qian Tao, Shu-Ying Gao, Zhuo Lv, Ze-Peng Du, Wang-Kai Fang, and En-Min Li

Abstract

Purpose: Neutrophil gelatinase-associated lipocalin receptor (NGALR) mRNA level is reduced in isolated chronic myelogenous leukemia blasts but up-regulated in esophageal squamous cell carcinoma (ESCC). The mechanism of NGALR regulation is unknown. Here, we show the expression pattern of NGALR and examine the aberrant methylation of its gene in ESCC and esophageal carcinoma cell lines.

Experimental Design: The expression pattern of NGALR was analyzed by immunohistochemistry in 59 ESCCs and compared with noncancerous tissues. The DNA methylation status was investigated by methylation-specific PCR and by bisulfite genomic sequencing in esophageal carcinoma cell lines and surgically resected samples. Methylated cell lines were treated with a methylation inhibitor to restore NGALR expression.

Results: The expression of NGALR in ESCC was significantly higher in tumor cell membrane and cytoplasm than in normal esophageal epithelium (P < 0.01). Methylated alleles were detected in three NGALR-nonexpressing cell lines but were not detected in three NGALR-expressing cell lines. Treatment of methylated cell lines with 5-aza-2'-deoxycytidine, a methylation inhibitor, restored NGALR expression. In surgically resected samples, 31 of 77 (40.3%) primary esophageal carcinomas and 46 of 77 (59.7%) paired normal tissues contained methylated NGALR alleles (P < 0.05).

Conclusions: Our results suggest that NGALR hypomethylation contributes to its expression in esophageal carcinomas and that this overexpression may play a role in the pathogenesis of esophageal carcinomas.

Neutrophil gelatinase-associated lipocalin (NGAL), a member of the lipocalin family, shows a high degree of similarity to the mouse protein 24p3 (1). Several reports indicate that NGAL expression is induced in various diseases such as renal tubular injury, vascular injury, and respiratory distress syndrome (2–4). In particular, elevated NGAL was observed in several types of cancer, such as colorectal neoplasm, pancreatic cancer, primary breast cancer, ovarian cancer, and esophageal squamous cell carcinoma (ESCC; refs. 5–9). Additionally, it was reported that NGAL expression was highly suggestive of a poor prognosis in primary human breast cancer (10). Our previous studies showed that NGAL overexpression plays an important role in the process of malignant transformation of human immortalized esophageal epithelial cells and is involved in the invasion of esophageal carcinoma cells (11–13). We found that the expression of NGAL in esophageal carcinoma cells could be induced by TPA (14). We also showed that NGAL overexpression enhanced the differentiation and invasiveness of ESCC (9).

Recently, a specific cell-surface receptor for 24p3/NGAL (24p3R/NGALR) was isolated from murine FL5.12 cells (15). In light of the correlation between NGAL overexpression and ESCC, a series of experiments was carried out to investigate the relationship between NGAL receptor (NGALR) and ESCC. In addition to the known alternatively spliced NGALR variants NGALR1 and NGALR2, we identified a new alternatively spliced variant, NGALR3, in esophageal carcinoma cells, and all three variants were up-regulated in ESCC (16). Thus, both NGAL and NGALR were up-regulated in ESCC, a situation that differed from their expression pattern in chronic myelogenous leukemia blast crisis patients, who expressed high levels of...
NGAL is implicated in progression of various tumors including ESCC. Recently, a specific cell-surface receptor of 24p3/NGAL (24p3R/NGALR) was isolated from murine FL5.12 cells by Devireddy et al. Research suggested that NGALR mRNA level is reduced in isolated chronic myelogenous leukemia blasts but up-regulated in ESCC. However, nothing is yet known of the regulation mechanism of NGALR. Here, we show that not only NGALR mRNA but also the protein were overexpressed in tumors compared with nontumors. Additional experiments showed that hypomethylation of the CpG island located within the NGALR promoter region plays a crucial role in overexpression of NGALR in ESCC, suggesting that hypomethylation of the NGALR promoter may contribute to the progression of ESCC.

Materials and Methods

Cell culture, 5-aza-2′-deoxycytidine treatment, and tumor samples. The ESCC cell lines EC109 (17), EC1.71 (also named EC/CUHK1; ref. 18), EC18 (also named EC/CUHK2; provided by Prof. Z.Y. Shen, Medical College of Shantou University), EC8712 (19), and SHEEC (20) were used. All cells were cultured in 199 medium (Invitrogen) or DMEM (Alpha Innotech). With ethidium bromide, and photographed with AlphaEaseFC Software

Reverse primer GAAGATGGTGATGGGATTTC

NGALR bisulfite genomic sequencing Forward primer GCCATTGCGCCACTGTCAC

Reverse primer AGGAGAAGGACCCAAGGAACAGA

NGALR MSP methylated Forward primer ATCGTAGAGATTAAAGGAGCGTT

Reverse primer CACCTAAACCTCRAAACACTTACTC

NGALR MSP unmethylated Forward primer ATGTGAGATTAAGGAGCGTTT

Reverse primer CACTTACCTCATACTCAGATCG

Glyceraldehyde 3-phosphate dehydrogenase Forward primer GAAAGTGAAGGCTGCGGACTC

Reverse primer GAAAGTGGATGGCAGGATTTC

Table 1. PCR primer sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotide</th>
<th>Sequence (5′-3′)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGALR</td>
<td>Forward primer</td>
<td>GCCATTGCGCCACTGTCAC</td>
<td>308-254</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>AGGAGAAGGACCCAAGGAACAGA</td>
<td></td>
</tr>
<tr>
<td>NGALR bisulfite genomic</td>
<td>Forward primer</td>
<td>AGAAGTTTTGATGGAAGAGTTT</td>
<td>435</td>
</tr>
<tr>
<td>sequencing</td>
<td>Reverse primer</td>
<td>CACCTAAACCTCRAAACACTTACTC</td>
<td></td>
</tr>
<tr>
<td>NGALR MSP methylated</td>
<td>Forward primer</td>
<td>ATCGTAGAGATTAAAGGAGCGTT</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>CACCTACCTCTAATCTCAATGCCG</td>
<td></td>
</tr>
<tr>
<td>NGALR MSP unmethylated</td>
<td>Forward primer</td>
<td>ATGTGAGATTAAGGAGCGTTT</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>CACTTACCTCATACTCAGATCG</td>
<td></td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate</td>
<td>Forward primer</td>
<td>GAAAGTGAAGGCTGCGGACTC</td>
<td>226</td>
</tr>
<tr>
<td>dehydrogenase</td>
<td>Reverse primer</td>
<td>GAAAGTGGATGGCAGGATTTC</td>
<td></td>
</tr>
</tbody>
</table>
(5’-CAACTGGGAGCAGACAGGAAA-3’ and 5’-GATGACACCTGA-
CATGCGCTGG-3’), and TaqMan probe (NGALR1, 5’-FAM-
ACAGTGGCTGGTCAACAGAAAGGCGC-3’; NGALR2, 5’-
FAM-ACCCCCACCTCCACCGACCAGG-TCLP3E-3’; and β-actin, 5’-
FAM-TCTGGCACCACACCTCTACACTAGC-TAMRA-3’). Each primer
was placed in a different exon to avoid amplification of contaminating
genomic DNA.

Real-time quantitative PCR was done using the Rotor-Gene 6000
(Corbett Research). Briefly, each PCR mixture contained 1 µL cDNA,
Premix Ex Taq (Perfect Real-time; TaKaRa), primer pair, and TaqMan
probe in a final volume of 10 µL. After activation of Taq polymerase at
95 °C for 10 s, PCR was done for 40 to 50 cycles, with each cycle
consisting of denaturing at 95 °C for 5 s, annealing, and extending at
60 °C for 30 s. Preliminary experiments were done with each primer
pair. Specificity of the reverse transcription-PCR (RT-PCR) amplifica-
tion products was documented with 2% agarose gel electrophoresis and
resulted in a single product with the desired length. Then, PCR products
were ligated into the pMD-19 Simple T vector, and serial 1:10 dilutions
of pMD-19 Simple T/PCR product plasmids were analyzed for each
target cDNA. These served as standard curves from which we
determined the rate of change of threshold cycle values. The amount
of target gene expression was calculated as the standard change,
and quantitative normalization of NGALR1 and NGALR2 cDNA in each
sample was done using β-actin as an internal control. Real-time PCR
assays were done in duplicate, and the mean values were used for
calculations of mRNA expression. Finally, the NGALR mRNA expres-
sion ratios for tumorous (T) and nonmalignant (N) tissues were
calculated as follows: R = [NGALR1 or NGALR2 (T) / β-actin (T)] /
[NGALR1 or NGALR2 (N) / β-actin (N)].

Construction of luciferase reporter gene constructs.
PCR using nested deletation was applied to obtain genomic DNA clones containing the
5’-flanking region of NGALR. For the PCR-based method, the primers 5’-
GGGATCCGCTGATCCGATGCTAATC-3’ (sense) and 5’-CTAGCAGCGGATCAGGAGGACAGG-GAGG-3’ (antisense) were designed to obtain the fragment from -2,985 to -1 (GenBank accession no. EU1374714). The nucleotide immediately upstream of the translation initiation codon (ATG) was denoted as -1. The use of TaKaRa LA Taq
with GC Buffer (TaKaRa), designed for PCR of highly GC-rich regions,
was critical to successful amplification. The Kpn1/NheI-digested final
PCR product (−2.9 kb) was cloned into the Kpn1/NheI-digested pGL3-
Basic vector (Promega) and sequenced on both strands.

The resultant plasmid (pB-2985) was digested with KpnI and EcoRI and
then directly directionally with the exonuclease III/I S1 nuclease system
(Erase-a-Base System; Promega) to make a series of constructs. All plas-
mid DNAs were purified with a QIAprep Spin Miniprep kit (Qiagen). All constructs were sequenced to confirm the expected deletions, and
their quantity and quality were routinely checked by agarose gel
electrophoresis.

Transfection and luciferase assay.
EC109 or EC8712 cells (−1 × 10^5 per well) were plated in 96-well plates for 24 h and cotransfected with
NGALR promoter/luciferase reporter plasmid (500 ng) or pGL3-Basic vector (as a negative control) together with pRL-TK (10 ng; Promega; as
an internal control for transfection efficiency) using SuperFect Transfection Reagent (Qiagen) according to the supplier’s protocol. Transfected cells were treated with or without 5-aza-dC (10 µmol/L) for
48 h. Cell lysates were prepared 48 h post-transfection, and firefly and
Renilla luminescence activities were quantified by a TD-20/20 luminometer according to the manufacturer’s instructions (Dual-Luciferase Reporter Assay System; Promega).

Bisulfite treatment, bisulfite genomic sequencing, and methylation-
specific PCR. Genomic DNA was extracted from fresh cell pellets and
tissues with DNA Extraction Buffer (10 mmol/L Tris-HCl (pH 8.0),
100 mmol/L EDTA (pH 8.0), and 0.5% (w/v) SDS).Briefly, the frozen
issues or cells were homogenized in 600 µL DNA Extraction Buffer;
then, 6 µL of 10 mg/ml RNase and 10 µL of 10 mmol/L proteinase K
were added and mixed well. The samples were incubated at 55 °C for
at least 3 h or overnight, after which extractions were extracted one by one
with Tris-saturated phenol, phenol-chloroform, and chloroform.
Subsequently, DNA was precipitated from the aqueous phase by adding 2× volume of cold anhydrous ethanol. The pellet was washed with 70% (v/v) ethanol, air-dried, and redissolved in 100 µL buffer
containing 10 mmol/L Tris-HCl and 1 mmol/L EDTA (pH 8.0).

Bisulfite modification of DNA was carried out as described previously
(24). In brief, 5 µg genomic DNA was denatured by adding 3 µL of
3 mol/L NaOH at 37 °C for 15 min. Denatured DNA was mixed with
333 µL bisulfite solution and incubated in the dark for 4 h at 55 °C.
The bisulfite solution was prepared as 2.4 mol/L sodium metabisulfite
(pH 5.0.5)/0.5 mmol/L hydroquinone. Treated DNA was desalted and
purified using the Wizard DNA Clean-up System (Promega). DNA
was then treated with 0.3 mol/L NaOH at 37 °C for 15 min and precipitated with 3 mol/L ammonium acetate and 3× volume of anhydrous ethanol. Recovered DNA was dissolved in 100 µL buffer
containing 10 mmol/L Tris-HCl and 1 mmol/L EDTA (pH 8.0).

In the bisulfite sequencing technique, the fragment of interest is
amplified from bisulfite-modified DNA, cloned, and sequenced to
obtain an accurate map of the distribution of CpG methylation. A
new primer set for NGALR bisulfite genomic sequencing (Table 1)
was designed to amplify a 435-bp region surrounding TSS2 that
contained 29 CpG dinucleotides. The PCR mixture contained 1×
PCR Gold Buffer, 2 mmol/L MgCl2, 0.2 mmol/L deoxynucleotide
triphosphates, 0.6 mmol/L of each primer, 0.625 units AmpliTaq
Gold DNA Polymerase (Applied Biosystems), and 25 ng bisulfite-
modified DNA in a final volume of 12.5 µL. After activation of Taq
polymerase at 95°C for 10 min, PCR was done in a 2720 thermal
cycler (Applied Biosystems) for 40 cycles, with each cycle consisting of
denaturing at 95°C for 30 s, annealing at 56°C for 30 s, and
extending at 72°C for 60 s followed by a final 7-min extension at
72°C. PCR products were visualized on 2.0% agarose gels stained
with ethidium bromide, and DNA was extracted from excised gel
slices (MiniElute Gel Extraction Kit; Qiagen) and cloned into the
pGEM-T Vector (Promega) or pMD-19 Simple T (TaKaRa). Clones
containing the desired insert were sequenced in an ABI 3700 Genetic
Analyzer (Applied Biosystems).

 Primer sequences for the NGALR methylation-specific PCR (MSP)
are listed in Table 1. These primer sets were designed using Methyl
Primer Express Software v1.0 (Applied Biosystems) and contained TSS2,
coincident with the maximum density of CpG sites. The PCR reactions
were done in a total volume of 15 µL. Reactions contained 2× GoTaq
Green MasterMix (7.5 µL; Promega), primer (0.6 µL each at 10 µmol/L),
and bisulfite-modified DNA (1.5 µL). PCR conditions were as follows:
5 min at 95°C, 40 cycles of amplification (30 s at 95°C and 30 s at
58°C for methylation-specific primers or at 54°C for non-methylation-
specific primers, 30 s at 72°C), and 7 min at 72°C. All reactions were
done with negative controls (without DNA) for both unmethylated
and methylated alleles. The PCR products were loaded onto a 2.0% agarose
gel, stained with ethidium bromide, and visualized under UV
illumination.

Statistical analysis. The correlation between methylation status and
antigen expression was evaluated using either the Pearson χ² test or,
when appropriate, the Fisher’s exact test. Differences in expression level
of NGALR1 and NGALR2 mRNA between normal and malignant
tissues were assessed with the Wilcoxon signed rank test. Statistical
significance was defined as P<0.05. All P values are two-sided.
Statistical Package for the Social Sciences for Windows 13.0 software
(SPSS) was used for all analyses.

Results
Increased expression of NGALR protein in esophageal carcinoma.
We examined NGALR (NGALR1 and NGALR2) expression by immunohistochemical staining in 59 ESCCs
and paired adjacent normal epithelial tissues. In cancerous
tissue, intense immunostaining for NGALR was observed in a

Human Cancer Biology

Clin Cancer Res 2008;14(23) December 1, 2008 7676 www.aacrjournals.org
Downloaded from clincancerres.aacrjournals.org on May 4, 2017. © 2008 American Association for Cancer Research.
cytoplasmic and membranous distribution (Fig. 1A), whereas absent/weak immunostaining was detected in the membrane of normal epithelium of the esophagus, however. Cells of the basal layer were always negative for NGALR (Fig. 1B). We found a similar difference between cancerous and normal tissues in the expression pattern of NGAL (Fig. 1C and D). Interestingly, NGAL was found within the NGALR-abundant area in cancerous tissue. When assessed for staining intensity, the 59 paired ESCC versus normal samples were graded as negative ($n=0$ versus 10, respectively), weakly positive ($n=12$ versus 29), moderately positive ($n=29$ versus 13), or strongly positive ($n=18$ versus 7). Quantitation of the immunohistochemical result revealed that the expression of NGALR in ESCC was significantly higher than in normal esophageal epithelium ($P<0.01$).

**NGALR mRNA expression in human tissue samples of ESCC.** To determine the status of gene expression in ESCC, 33 matched pairs of primary tumor tissues and histologically normal adjacent mucosa were examined for mRNA levels of NGALR1 and NGALR2 using real-time quantitative RT-PCR. NGALR1 mRNA levels ranged from 14.03 to 6503.69 in cancerous tissue and from 6.50 to 1091.34 noncancerous tissue, respectively. The $R$ of NGALR1 ranged from 0.33 to 63.48. The mRNA expression of NGALR1 was higher in malignant tissues than in histologically normal mucosa ($P<0.05$). NGALR2 mRNA levels were between 32.75 and 14,266.91 in cancerous tissue and between 16.88 and 4,092.23 in noncancerous tissue, respectively. The $R$ of NGALR2 ranged from 0.05 to 47.43. The mRNA expression level of NGALR2 was also significantly higher in tumor tissues than in normal tissues ($P<0.05$). We defined the cutoff value for differentiation between normal and overexpression of NGALR1 and NGALR2 mRNA as a ≥2-fold increase in tumor mRNA expression relative to matched normal esophageal controls. Using this criterion, 54.55% (18 of 33) of patients expressed high levels of NGALR1, 45.45% (15 of 33) expressed high levels of NGALR2, and 33.33% (11 of 33) expressed high

![Fig. 1. NGALR and NGAL expression assessed by immunohistochemistry staining in ESCC and adjacent normal epithelium.](https://www.aacrjournals.org/doi/10.1158/1078-0432.CCR-08-1811-S/figure?figure=1)
levels of both NGALR1 and NGALR2. The results of the real-time quantitative RT-PCR agreed well with those obtained by immunohistochemistry staining.

Characterization of NGALR mRNA expression in various ESCC cell lines. Using RT-PCR with glyceraldehyde 3-phosphate dehydrogenase as a control, the expression of NGALR mRNA was evaluated in human ESCC cell lines and in an immortalized esophageal cell line (Fig. 2). NGALR mRNA level was abundant in three tumor cell lines (EC1.71, EC18, and EC8712) but was undetectable in two other tumor cell lines (EC109 and SHEEC) and in the normal immortalized esophageal cell line SHEE. These cell lines provided a panel of NGALR-expressing and NGALR-nonexpressing cells for additional analyses to define the mechanism of NGALR expression.

Analysis of NGALR promoter-luciferase activity in NGALR-expressing and NGALR-nonexpressing cells. To study transcriptional activity of the putative promoter region within the NGALR 5'-flanking region in NGALR-expressing and NGALR-nonexpressing cells, we generated deletion constructs, cloned them upstream of the firefly luciferase gene into the promoterless pGL3-Basic plasmid (Fig. 3A), and analyzed them by transfection in NGALR-expressing ESCC EC8712 cells and NGALR-nonexpressing EC109 cells. We used pRL-TK encoding Renilla luciferase under the control of a constitutive TK promoter as an internal control for transfection efficiency. Using a transient transfection assay system in EC8712 cells, all of the NGALR promoter-luciferase constructs showed activity above the pGL3-Basic background (Fig. 3B). As shown in Fig. 3B, the NGALR promoter had high reporter gene activity in EC8712, in contrast to residual reporter gene activity in EC109.

Analysis of NGALR promoter sequences for CpG islands. According to the results of RT-PCR and NGALR promoter transcriptional activity described above, the expression of NGALR may be altered in esophageal carcinomatous tissues and cell lines. We next analyzed sequence characteristics of the NGALR 5'-flanking region; CG dinucleotides were abundant in this region, indicating the possibility of CpG islands. CpG-rich regions were defined as stretches of DNA with a high G + C content (>50%) and an observed CpC/expected CpG ratio of ≥0.6. Methyl Primer Express Software v1.0 predicted one CpG island from -1,028 to +117 (+1 is A of initiating ATG), containing 76 CpG sites (Fig. 4A). A simplified schematic overview of the region showing the location of the CpG sites within the NGALR promoter (Fig. 4A) indicated that epigenetic alterations may underlie the observed alteration of NGALR expression in ESECs and tumor cells.

DNA methylation status of the NGALR promoter in ESCC cell lines. To examine whether the altered expression of NGALR in tumor cell lines was associated with methylation of the CpG island in the promoter, we determined the frequency of NGALR methylation in ESCC cell lines by MSP (Fig. 2D). Methylated alleles were detected in cell lines (EC109, SHEEC, and SHEE) lacking NGALR expression (Fig. 4B). Methylation was not detected in the NGALR-expressing cell lines EC1.71, EC18, and EC8712 (Fig. 4B). The cell line MSP result confirmed that activation of expression was associated with lack of methylation in the 5’ CpG island.

To examine NGALR methylation in more detail, we analyzed the five ESCC cell lines and one immortalized esophageal cell line described above using high-resolution bisulfite genomic sequencing analysis (Fig. 4C). For bisulfite genomic sequencing, a region of 345 bp (from -754 to -410) surrounding TSS2 (the transcription start site of NGALR, transcript variant 2, mRNA) and containing 29 CpG sites (Fig. 4A) was amplified with bisulfite-modified DNA from ESCC cell lines, and the entire desired fragments were sequenced (Fig. 4C). The bisulfite genomic sequencing result strongly correlated with the MSP analysis. Methylated CpG sites were absent or sparsely distributed in cell lines expressing NGALR. In contrast, a high density of methylated CpG sites was detected in all silenced cell lines; the major methylated CpG sites were located in the region from the 3rd to the 15th CpG in EC109 cells and from the 9th to the 20th CpG in SHEEC and SHEE cells (Fig. 4C). Thus, the 9th to the 15th CpG sites constituted the consensus methylated sequence.

NGALR promoter hypomethylation in ESCC. To assess whether the NGALR promoter hypermethylation observed in
cell lines was reflected in ESCC, we further examined NGALR methylation in a large collection of primary ESCC tumors with paired normal tissues via MSP (Fig. 4D). Methylation was observed in 31 of 77 (40.3%) ESCC tumor samples and in 46 of 77 (59.7%) paired normal tissues, showing statistically different methylation of NGALR between normal and tumor tissues (P < 0.05). The results of MSP agreed well with the results of immunohistochemistry staining.

5-aza-dC activated NGALR promoter in hypermethylated cells. To test whether methylation was directly responsible for silencing NGALR, we administered the methylation inhibitor 5-aza-dC to three cell lines (EC109, SHEEC, and SHEE) having a hypermethylated and silenced NGALR promoter. The 5-aza-dC restored NGALR expression in all three lines, albeit with varying efficiency (Fig. 5A). Concomitantly, after 5-aza-dC treatment, unmethylated NGALR alleles were increased as determined by MSP (Fig. 5B). By quantitating the intensity of each MSP product, we evaluated the level of NGALR methylation \( \frac{U}{(M + U)} \times 100\% \) in methylated cell lines and in those treated with 5-aza-dC. After treatment with

---

**Fig. 4.** NGALR methylation in ESCC cell lines and carcinoma samples. A, schematic structure of the NGALR promoter CpG island (dark rectangle). CpG sites within the CpG island (short vertical lines), the two transcription start sites (TSS), and the initiation codon (ATG + t three right-angled arrows). The MSP and bisulfite genomic sequencing regions analyzed are also indicated. B, MSP analysis of ESCC cell lines. Representative high-resolution analyses of the methylation status of individual CpG sites in the NGALR promoter by bisulfite genomic sequencing in ESCC cell lines. Each CpG site is shown in the top row as an individual number. Each row indicates an analyzed promoter allele, and each circle corresponds to a single CpG site. Also shown are methylated sites (filled circles), unmethylated sites (empty circles), and a deletion (filled triangle). Four to six clones from each cell line were bisulfite sequenced to obtain a representative sampling of methylation patterns. D, representative MSP results of ESCC primary tumors (T) and paired normal tissues (N). Numbers on top, sample number. M, methylation; U, unmethylation.
5-aza-dC, the level of NGALR unmethylation increased from 0.07% to 73.31% in EC109, from 5.62% to 46.26% in SHEEC, and from 0.02% to 25.44% in SHEE, respectively, confirming the role of the 5′ CpG region methylation in regulating NGALR expression.

For further confirmation of the critical role of methylation in NGALR expression, we have compared the NGALR promoter activity in control and 5-aza-dC-treated EC109 cells using a serial NGALR promoter-luciferase construct. 5-aza-dC-treated EC109 cells (AZA-EC109) showed higher promoter activity than control cells (Fig. 5C).

**Discussion**

This study showed that the expression of NGALR protein and mRNA was significantly higher in ESCC compared with normal esophageal epithelium and showed that NGALR activation was associated with hypomethylation. Using RT-PCR, we reported previously that NGALR mRNA was up-regulated in ESCC (16). Here, we found that NGALR was overexpressed with high frequency in the membrane and cytoplasm of esophageal carcinoma cells, whereas it was largely absent from normal esophageal epithelium. These data were confirmed by real-time RT-PCR in clinical samples, clearly showing that NGALR mRNA expression was significantly higher in ESCC than in adjacent normal tissue.

A series of studies has suggested that NGAL is a novel iron transporter that, along with NGALR, mediates a new iron delivery pathway that functions distinctly from the transferrin pathway (15, 25, 26). The function of NGALR seems to be determined by the status of NGAL. Expression of a recombinant apo-NGAL (no iron bound) could remove iron from cells expressing NGALR, thereby decreasing intracellular iron concentration and inducing apoptosis; a recombinant holo-NGAL (loaded with iron), however, failed to induce apoptosis (15). Immunohistochemistry showed that ferritin, the concentration of which reflects the intracellular iron level, was overexpressed in ESCC tissues (Supplementary Data). Based on the above results, combined with the colocalization of NGAL and NGALR in tissues and heterologous systems (16), we propose that NGAL-bound iron may be delivered into ESCC cells by NGALR, which increases the intracellular iron level and adds to the growth potential of the tumor. Combined with iron-related free radical reactions and DNA mutations (27), this could promote malignancy in esophageal carcinoma. Future studies will be required to directly address these issues.

Thus, NGALR may have a crucial role in tumor development and progression. However, the mechanisms controlling the tissue-specific and cancer-specific expression of NGALR are not known. In the present study, sequence analysis revealed a CpG island located in the 5′-flanking region of NGALR. DNA methylation of the CpG islands is a developmentally regulated process that acts in concert with other regulatory mechanisms to control gene expression (28, 29), including tissue-specific expression (30, 31). Therefore, we hypothesized that NGALR in human ESCC tissue might be regulated by aberrant CpG methylation of the NGALR promoter. Using MSP, we found that all ESCC cancer cell lines with a hypomethylated NGALR allele overexpressed NGALR and that treatment with a methylation inhibitor induced NGALR expression in cell lines not expressing NGALR. Bisulfite sequencing was also done and confirmed the completeness of the bisulfite modification and the accuracy of the MSP results. As cultured cells may have altered methylation patterns compared with their tissue counterparts (32), we also examined ESCC cancer tissue samples and found a high frequency of hypomethylated NGALR alleles in those samples. In addition, transient transfections showed that the NGALR promoter had higher reporter gene activity in the hypomethylated cell line EC8712 compared with the promoter in the hypermethylated cell line EC109 and the 5-aza-dC-activated NGALR promoter in EC109. This method has also been used to identify inhibition of transcriptional activity by methylation of the ABCG2 promoter (33).

Several studies that have examined hypomethylation of promoter regions in various cancers have reported similar results. Paredes et al. (34) found that P-cadherin mRNA expression in invasive breast carcinomas was associated with hypomethylation of the CDH3 promoter. Sato et al. (35) showed frequent hypomethylation of multiple genes that were overexpressed in pancreatic ductal adenocarcinoma. A recent report showed that the aberrant overexpression of claudin-4 in epithelial ovarian cancer may be regulated by hypomethylation of its gene promoter region (36). Similarly, we found that expression levels of NGALR mRNA were significantly higher in ESCC and that NGALR promoter hypomethylation was more common than in adjacent noncancerous tissues. This finding suggests that NGALR promoter hypomethylation may be closely related to up-regulation of NGALR transcription and that hypomethylation-associated activation of NGALR mRNA
expression may play an important role in human esophageal carcinogenesis.

The reason for the different methylation patterns in the NGALR promoter region from the NGALR-methylated cells is unclear. However, when the consensus sequences for common DNA transcription factors are compared with the NGALR promoter region, the significance of methylation at these sites is evident (Fig. 4C). TFSEARCH revealed that CpG sites 11 to 13 are located within an E2F sequence (CCGCGCGGCAACTGTA) and that CpG sites 15 to 17 are located within another E2F sequence (TTGCCGCCGG). Studies have shown that CpG methylation within the E2F consensus sequence blocks protein binding (37), suggesting that methylation of these six sites may affect the binding affinity of transcriptional regulatory elements that activate transcription from NGALR. Mancini et al. (38) also suggested that the presence of specific methylated sites may effectively alter gene expression; however, further investigation of the E2F motif should be done to establish whether the binding of this putative transcription factor to the target promoter sequence might be affected by methylated CpG sites. In summary, our results strongly suggest that NGALR is overexpressed in cells lacking NGALR promoter methylation in the majority of ESCC and that this overexpression may play a role in the pathogenesis of esophageal carcinomas.

References

NGALR Is Overexpressed and Regulated by Hypomethylation in Esophageal Squamous Cell Carcinoma

Lei Cui, Li-Yan Xu, Zhong-Ying Shen, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/14/23/7674

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2008/12/04/14.23.7674.DC1

Cited articles
This article cites 37 articles, 10 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/14/23/7674.full.html#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
/content/14/23/7674.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.