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 NGAL 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 and NGALR.
NGAL but virtually no NGALR (15). These results suggest that NGALR expression may be transcriptionally regulated. Here, we report the identification of a CpG island within the NGALR promoter sequence and show that methylation of this promoter plays a crucial role in the expression of NGALR.

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). The ESCC cell lines EC109, EC1.71, EC18, and SHEEC were treated with medium containing 10% heat-inactivated fetal bovine serum in a humidified atmosphere containing 5% CO2. The EC109, SHEEC, and SHEE cells were treated with medium containing 10 μM 5-aza-2'-deoxycytidine (5-aza-dC) for 9 days. The culture medium was changed every 24 h for long periods. The immunogen and affinity purified on a peptide-coupled Sepharose antibody against NGALR was raised using a COOH-terminal NGALR peptide. Specimens from esophageal carcinoma tissues and incisural margin tissues used for this study were acquired from the collection of the Pathology Department of the Medical College of Shantou University.

Antibodies and immunohistochemical staining. A polyclonal rabbit antibody against NGAL was raised using a COOH-terminal NGAL (both NGALR1 and NGALR2) peptide (CDHVPLATPNAL) as the immunogen and affinity purified on a peptide-coupled Sepharose column (Beijing Biosynthesis Biotechnology). Rat anti-human NGAL monoclonal antibody was obtained from R&D Systems. An assay for NGAL expression was done as described previously (9). SuperPicTure Polymer Detection kit and Liquid DAB Substrate kit (Zymed/Invitrogen) were used to carry out NGAL immunohistochemistry. Slides were submerged in peroxidase quenching solution containing 1 part of 30% hydrogen peroxide to 9 parts of absolute methanol for 10 min followed by a wash with PBS. Serum blocking solution (100 μL) was added to each section for a 10 min incubation followed by application of rabbit anti-human NGAL polyclonal antibody [100 μL; 1:10 dilution in PBS containing 0.01% (w/v) Triton X-100] to each section for 30 min. After rinsing, horseradish peroxidase polymer conjugate (100 μL) was added to each section for a 10 min incubation followed by application of DAB chromogen (100 μL) and incubated for 3 to 10 min. After rinsing, slides were counterstained with Mayer’s hematoxylin, dehydrated, and mounted. Negative controls were prepared by substituting PBS for primary antibody.

The immunohistochemical expression of NGALR was examined via light microscopy. The percentage of positive tumor cells was determined semiquantitatively by assessing the whole tumor section, and each sample was assigned to one of the following categories: 0 (0-4%), 1 (5-24%), 2 (25-49%), 3 (50-74%), or 4 (75-100%; ref. 22). The intensity of immunostaining was determined as 0 (no staining), 1+ (weak staining), 2+ (medium staining), and 3+ (strong staining). The intensity of immunostaining was judged relative to smooth muscle cells within the sample, which were arbitrarily designated as 1+. Additionally, an immunoreactive score was calculated by multiplying the percentage of positive cells and the staining intensity. In the case of heterogeneous staining intensities within one sample, each component was scored independently, and the results were summed. Using this system, the maximum score was 12. For statistical analysis, the 13-tier scoring was simplified by combining the criteria as follows: 0, negative; 1 to 4, weakly positive; 5 to 8, moderately positive; and 9 to 12, strongly positive (23).

Total RNA isolation and reverse transcription-PCR. Total RNA was extracted from cells and tissues with TRIzol (Invitrogen). First-strand cDNA was reverse transcribed from 2 μg total RNA in a total volume of 20 μL using the Reverse Transcription System (Promega) according to the manufacturer’s protocol. The nucleotide sequences of the forward and reverse primers for each gene are listed in Table 1. Each primer was placed in a different exon to avoid amplification of contaminating genomic DNA. The amplification protocol was as follows: 5-min preincubation at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C and ending with a 7-min incubation at 72°C. The amplified products were separated on 1.8% agarose gels, visualized with ethidium bromide, and photographed with AlphaEaseFC Software (Alpha Innotech).

Real-time quantitative reverse transcription-PCR. The oligonucleotide primers and TaqMan probes designed for NGALR1, NGALR2, and β-actin were as follows: NGALR1 (5’-TGGGATTGTGAGCATCCTATCTT-3’ and 5’-GGGACGCTCTGCAGATCTC-3’), NGALR2 (5’-GCAGAACGCTGAG-3’ and 5’-CTGGGCCACCCGATCT-3’), β-actin forward primer GCACTTGCACACTGCTAC 308 or 254
Reverse primer GAGAAAGAGCCCAAGGCAG 435
Reverse primer AGACCTTTGATGAGGTATT 105
Reverse primer ATGCCGAGGATTTAGGACTT 106
Reverse primer GATTGAGGATTTAGGACTT 226
Reverse primer GAAAAGGAAATTGCCGAG 226
Reverse primer GGAAGTGGATGGCAGTAC

Table 1. PCR primer sequences

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<th>Oligonucleotide</th>
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<td></td>
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<td></td>
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<td>Reverse primer</td>
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NGALR Gene Is Regulated by Hypomethylation in ESCC
with GC Buffer (TaKaRa), designed for PCR of highly GC-rich regions, initiation codon (ATG) was denoted as -1. The use of TaKaRa LA Taq (EU374714). The nucleotide immediately upstream of the translation obtain the fragment from -2,985 to -1 (GenBank accession no. Basic vector (Promega) and sequenced on both strands. PCR product (2.9 kb) was cloned into the Kpn I/ Nhe I-digested final PCR productswas 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 by threshold cycle value, 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 NGAL mRNA expression ratios for tumorous (T) and nontumorous (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 deletion was applied to obtain genomic DNA clones containing the 5'-flanking region of NGALR. For the PCR-based method, the primers 5'-GGGTTAGCTGGACGACACTCTGAG-3' (sense) and 5'-CTAGC-TAGGCCCAGAACGAAAGAG-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/Nhe1-digested final PCR product (~ 2.9 kb) was cloned into the Kpn1/Nhe1-digested pGL3- Basic vector (Promega) and sequenced on both strands.

The resultant plasmid (pB-2985) was digested with Kpn1 and EcoRI and then directly directioned by the exonuclease III/S1 nuclease system (Erase-a-Base System; Promega) to make a series of constructs. All plasmid 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 pG53-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 luciferase 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% (v/v) SDS). Briefly, the frozen tissues 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 5.0-5.2)/0.5 mmol/L hydroquinine. 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 cycle (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 (MinElute 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 3700x Genetic Analyzer (Applied Biosystems).

NGALR1 and NGALR2 mRNA expression between normal and malignant tissue, intense immunostaining for NGALR was observed in a

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
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 \((n = 0 \text{ versus } 10, \text{ respectively})\), weakly positive \((n = 12 \text{ versus } 29, \text{ respectively})\), moderately positive \((n = 29 \text{ versus } 13, \text{ respectively})\), or strongly positive \((n = 18 \text{ versus } 7, \text{ respectively})\). Quantitation of the immunohistochemical result revealed that the expression of NGAL 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

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Fig. 1. NGALR and NGAL expression assessed by immunohistochemistry staining in ESCC and adjacent normal epithelium. **A**, significant expression of NGALR was observed in ESCC. **B**, weak expression of NGALR was detected in adjacent normal mucosa. **C**, ESCC showed abundant expression of NGAL. **D**, negative or weak immunostaining for NGAL was observed in epithelium.
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 ESCCs 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 ($U / (M + U) \times 100\%$) in methylated cell lines and in those treated with 5-aza-dC. After treatment with...
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 NGAL 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 NGAL, 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...
NGALR Gene Is Regulated by Hypomethylation in ESCC

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 (CCGGCCGGCAACTGTA) and that Cpg sites 15 to 17 are located within another E2F sequence (TCCGGCCGGG). 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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

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