

Analysis of the methylation status of genes up-regulated by the demethylating agent, 5-aza-2'-deoxycytidine, in esophageal squamous cell carcinoma

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Abstract. To determine the clinical significance of gene promoter methylation in esophageal squamous cell carcinoma (ESCC), we examined the promoter methylation status of genes showing elevated expression, as determined by DNA microarray-based transcriptomic analysis, in three ESCC cell lines (TE-1, TE-2, TE-10) after 5-aza-2'-deoxycytidine (DAC) treatment. We observed a high degree of DNA methylation within the promoter regions of three genes, namely cathepsin L2 (*CTSL2*), normal mucosa of esophagus specific 1 (*NMES1*), and fatty acid binding protein 5 (*FABP5*). Overexpression of *NMES1* in ESCC cell lines increased cell motility. Down-regulation of *NMES1* might play an important role in the cell motility of ESCC or be a potent marker of malignancy.

Introduction

CG-rich DNA sequences are found within the promoter region and first exon of ~50% of all genes in the human genome (1). These sequences can be targets of epigenetic phenomena that are associated with altered chromatin structure, transcriptional repression and cancer development

(2,3). With respect to esophageal squamous cell carcinoma (ESCC), hypermethylation events within the promoter regions of p14ARF, p16INK4a, HLA class I genes, E-cadherin, and Ras association (RalGDS/AF-6) domain family 1, have been reported to be related to the development of ESCC (4-7). However, a global analysis of the genes regulated by epigenetic factors in ESCC has rarely been performed.

The nucleotide analogue 5-aza-2'-deoxycytidine (DAC) inhibits DNA methyltransferase, resulting in the reactivation of methylation-silenced genes (8). DAC has been used as an anti-cancer agent for patients with chronic myelogenous leukemia in the expectation of gene reactivation via its demethylation properties (9). In a previous study, we clarified the expression profile of up-regulated genes in hepatoma cell lines by DAC using a DNA microarray-based transcriptomic approach (10).

Herein, we assessed, on a global level, the genes induced by DAC in ESCC cell lines using DNA microarrays, with a goal to find candidate genes regulated by aberrant DNA methylation. From the cohort of genes up-regulated by DAC treatment, we then assessed the methylation status of the promoter CpG islands of four genes in ESCC cell lines. In addition, we focused on the normal mucosa of esophagus specific 1 (*NMES1*), which was up-regulated by DAC treatment and displayed abnormal methylation in its promoter region in ESCC cell lines. We report on its function in cancer cell lines.

Materials and methods

Cell lines. ESCC cell lines (TE-1, TE-2, TE-10) were provided by the Cell Resource Center for Biomedical Research, Tohoku University (11). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sanko Junyaku Co., Ltd., Tokyo, Japan). Cell lines were treated with DAC (Sigma-Aldrich Co.), dissolved in DMEM just before use. Cells were grown in medium containing 1 μ M DAC for 96 h, with the medium and the drug being replaced every 24 h. As a control, cell lines were cultured in normal medium, with the medium also being replaced every 24 h.

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Abbreviations: ESCC, esophageal squamous cell carcinoma; DAC, 5-aza-2'-deoxycytidine; *NMES1*, normal mucosa of esophagus specific 1; S.D., standard deviation; HIST1H2BF, histone 1, H2bf; *FABP5*, fatty acid binding protein 5; *CTSL2*, cathepsin L2; *GADD45A*, growth arrest; DNA-damage-inducible, α ; HIST1H1C, histone 1, H1c

Key words: methylation, esophageal squamous cell carcinoma, microarray, normal mucosa of esophagus specific 1

Table I. Primer sets for quantitative RT-PCR analysis.

Gene name	Symbol	RefSeq	Sense	Antisense	Size
Cathepsin L2	CTSL2	NM_001333	acagtgtggttcttctgtggg	ccgttctccttgacatactgg	183
Normal mucosa of esophagus specific 1	NMES1	NM_032413	aggaactcattcccttggtg	tccacagtttccaaggttc	133
Growth arrest and DNA-damage-inducible, α	GADD45A	NM_001924	ggaagtgctcagcaaagccc	cgttctcgcagcaaaaagccc	185
Histone 1, H1c	HIST1H1C	NM_005319	aacagccgatcaaaacttgg	aactggcttcttaggttgg	168
Actin, β	ACTB	NM_001101	gacaggatgcagaaggagattact	tgatccacatctgatggaaggt	142

Gene name, gene symbol, RefSeq, sense and antisense primers used for PCR amplification and the size of respective PCR products are listed.

DNA and RNA extraction of cell lines. DNA and RNA were extracted from treated and untreated cell lines after the 96 h time point. Total RNA was prepared using TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) and DNA was extracted through the use of a QIA amp DNA mini kit (Qiagen Co., Hilden, Germany) according to the manufacturer's instructions.

Microarray analysis. From 5 μ g of total RNA, RNA probes were generated using a MessageAmpTM aRNA kit (Ambion Inc., Austin, TX, USA) (<http://www.dna-chip.co.jp/thesis/AceGeneProtocol.pdf>). For transcriptomic profiling, we used an oligonucleotide-based DNA microarray, AceGene[®] (HumanOligoChip30K, DNA Chip Research Inc., Yokohama, Japan). Fluorescence detection was performed essentially as previously described (12). Images were analyzed with Quant Array[®] (GSI Lumonics, Nepean, Canada) and DNASIS[®] Array (Hitachi Software Engineering, Tokyo, Japan) according to the manufacturer's instructions. The mean and standard deviation (S.D.) of background levels were calculated and those genes whose intensities were less than the mean plus two S.D. of background levels were excluded from further analysis. The Cy5/Cy3 ratios of all spots on the DNA microarray were normalized by the method of global normalization. Microarray analyses were performed twice with the swapping of Cy3 or Cy5 dyes. We evaluated whether there were CpG islands in the promoter region of genes [from -500 to -1 base pairs (bp)] via CpG Island Searcher (<http://ccnt.hsc.usc.edu/cpgislands2/cpg.aspx>) (13,14).

Analysis of mRNA expression by quantitative RT-PCR in cancer cell lines. First-strand synthesis of cDNA was performed with the Superscript First Strand synthesis system for real-time polymerase chain reaction (RT-PCR; Invitrogen) according to the manufacturer's instructions. The cDNA templates were synthesized from 1 μ g total RNA. Quantitative PCR amplification was performed by Applied Biosystems 7300 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions using the primer pairs shown in Table I, which shows the predicted sizes of the resulting PCR products. PCR amplification conditions were as follows: pre-incubation 95°C for 10 min, with 40 cycles at

95°C for 30 sec and 63°C for 30 sec; β -actin expression was used as a control (15). TaqMan real-time primers and probes were used for validation when the adequate primers for quantitative RT-PCR could not be designed due to technical difficulties. A set of primers and a probe for each gene tested was obtained from Applied Biosystems. TaqMan[®] Gene expression assay IDs for the genes were as follows: histone 1, H2bf (HIST1H2BF), Hs00371409_g1; fatty acid binding protein 5 (FABP5), Hs02339439_g1.

Methylation status of the up-regulated genes in cancer cell lines. Using extracted DNA, bisulfite modification of DNA (1 μ g) was performed through the use of a Cp GenomeTM DNA modification kit (Chemicon International Inc., Temecula, CA, USA) according to the manufacturer's instructions. To clarify the methylation status of promoter regions, bisulfite genomic sequencing analysis was used. We amplified the bisulfite-modified gene promoters from genomic DNA (0.04 μ g) via PCR (details of primer sets are given in Table II). PCR conditions were 95°C for 15 min and 40 cycles at 95°C for 30 sec, annealing temperature for 30 sec, and 72°C for 90 sec, ending with an extension at 72°C for 15 min. The amplified DNAs were subcloned into the TA-cloning vector (Invitrogen). We determined the sequence of the promoter in eight independent colonies in each case and examined the extent of methylation by determining the number of methylated cytosine residues.

Selection of NMES1 stable-transfected cells. TE-1 and TE-2 cells were plated in 100-mm culture dishes, incubated overnight, and then transfected with 2 μ g of plasmid DNA (OmicsLinkTM Expression clone purchased from GeneCopoeia, Inc., Germantown, MD, USA) using lipofectin reagent (Invitrogen), according to the manufacturer's protocol. After incubation in the growth medium for 2 days, cells were fed with DMEM/10% FBS containing G418 (0.6 mg/ml). After two weeks, the G418-resistant clones were individually picked and expanded. The expression of NMES1 in each individual clone was checked by the RT-PCR method, and their cell viability was examined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium

Table II. Primer sets of PCR amplification of promoter regions of candidate methylated genes.

Gene symbol	Sense	Antisense	Position	Size	°C
CTSL2	aatgtaatagattggaag	acaccctcaacaaacaaacc	From -523 to +163	686	60
NMES1	gttggaatatagtggtgtaa	ccccctacaaaacattctac	From -562 to -101	462	50
FABP5	gggtaggttttttagtgat	ttccaactactaaactataa	From -313 to +80	393	51
GADD45A (part 1)	gggatttttatatgtggttattagttttt	acaattacattaaaaaatatctccaacc	From -768 to -449	320	54
GADD45A (part 2)	ggtagttgttttagtttaagttgagg	cctaccaaccactaaaaacaaaaaac	From -323 to +14	337	51

Indicated gene promoter elements were PCR amplified from bisulfite-modified genomic DNA. Sense and antisense primers used for PCR amplification and the size of respective PCR products are shown. Positions were the distance from the transcription site defined by using the Entrez Gene home page at (www.ncbi.nlm.nih.gov/sites/entrez?db=gene). The primer sets for *GADD45A* were designed by Wang *et al* (27).

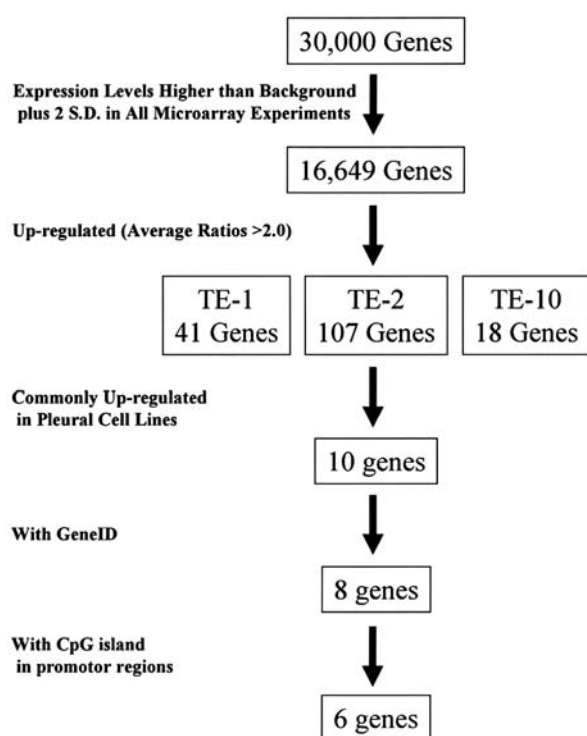


Figure 1. Flowchart for selection of candidate genes regulated by epigenetic factors in esophageal squamous cell carcinoma (ESCC) cell lines. We used three ESCC cell lines to find candidate genes regulated by epigenetic factors. We obtained 16,649 genes that demonstrated expression levels greater than the mean plus two S.D. of background levels in all experiments. Among them, we selected the genes up-regulated (average of ratios >2.0) by DAC treatment. The numbers of the genes in TE-1, TE-2, and TE-10 cells were 41, 107, and 18, respectively. We excluded the genes without GeneID or CpG islands in their promoter regions. As a result, we selected six genes for the examination of the restoration of their expression levels after DAC treatment.

(MTS) assay (CellTiter 96 Aqueous One Solution cell proliferation assay; Promega Corp., Madison, WI, USA). The results from six assays per sample were quantified by reading the absorbance at 490 nm with a microplate colorimeter.

Analysis of the role of NMES1 in cell motility. Cell motility was assessed using the Boyden chamber assay according to the manufacturer's protocol (BD, Franklin Lakes, NJ, USA). To

exclude clonal variation, the cell motility was confirmed using two different *NMES1* overexpressed clones. Briefly, 200,000 parental (TE-1) and *NMES1* overexpressing cell lines (TE1-1 and TE1-4) in optiMEM (Invitrogen) without FBS were each placed on an insert with 8.0- μ m pore size membrane in 24-well plate, respectively. Six hundred thousand parental (TE-2) and *NMES1* overexpressing cells (TE2-3 and TE2-5) were placed into 24-well plates using the same procedure. DMEM plus 10% FBS was placed in the bottom wells as a chemoattractant. After 24 h, the cells that did not migrate were removed from the top side of the inserts with a cotton swab. Cells that had migrated to the underside of the inserts were stained with Diff-Quik (Sysmex International Reagents Co., Kobe, Japan). After staining, the number of cells on each insert in a field of view was counted at x100 magnification; this count was performed 5 times. Results were expressed as mean \pm S.D.

Statistical analysis. To determine the statistical significance of the data relating to measurement of cell viability and induction of apoptosis, the Mann-Whitney U test was performed using StatView (SAS Institute Inc., Cary, NC, USA). $p < 0.05$ was considered significant.

Results

Genes up-regulated by DAC treatment revealed by DNA microarray analysis. An oligonucleotide microarray representing 30,000 genes was used. Microarray analyses were performed twice in each cell line with the swapping of Cy3 or Cy5 dyes. In total, six analyses were performed. The number of genes with expression levels greater than the mean plus two S.D. of background levels in all experiments was 16,649 (Fig. 1); these genes were analyzed further. The number of genes that were up-regulated (average of ratios >2.0) by DAC treatment in TE-1, TE-2, and TE-10 cells were 41, 107, and 18, respectively. Among them, ten genes were selected due to up-regulation in plural cell lines. Two genes without GeneID, and other two genes without a CpG island in their promoter regions, were excluded from further analysis. As a result, we studied the expression levels of six genes as follows using the quantitative RT-PCR method: cathepsin L2 (*CTSL2*), *NMES1*, *FABP5*, growth arrest and DNA-damage-inducible, α

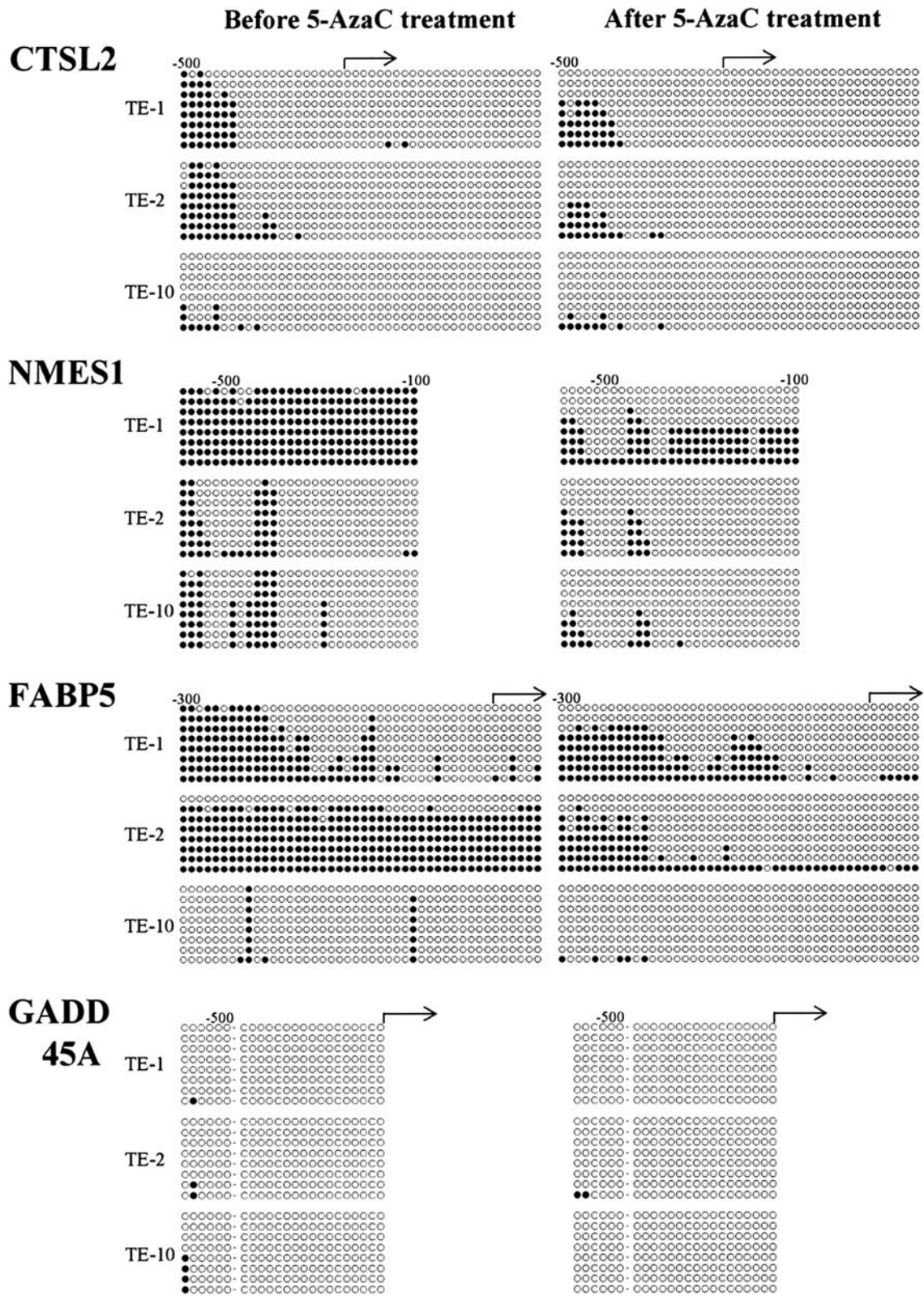


Figure 2. The methylation status of promoter regions of *CTSL2*, *NMES1*, *GADD45A*, and *FABP5* in esophageal squamous cell carcinoma (ESCC) cell lines before and after DAC treatment. The methylation status of CpG islands in each promoter region and first exon were analyzed by a bisulfite genomic sequencing method. After DAC treatment, demethylation effects were obvious in all genes and cell lines. Blank circle, non-methylated; solid circle, methylated. Transcription start sites are as indicated above each methylation mapping. *CTSL2*, cathepsin L2; *NMES1*, normal mucosa of esophagus specific 1; *FABP5*, fatty acid binding protein 5; *GADD45A*, growth arrest and DNA-damage-inducible, α .

(*GADD45A*), Histone 1, H1c (*HIST1H1C*), and *HIST1H2BF*. The results of the microarray and quantitative RT-PCR analyses are shown for the six genes in Table III; restoration

of the expression level after DAC treatment was confirmed for four of the six as follows: *CTSL2*, *NMES1*, *FABP5*, and *GADD45A*.

Table III. DNA microarray and quantitative RT-PCR data for the genes up-regulated by DAC treatment.

Gene symbol	GeneID	Ratios of DAC treatment/control by quantitative RT-PCR			Ratios of DAC treatment/control by microarray analysis		
		TE-1	TE-2	TE-10	TE-1	TE-2	TE-10
CTSL2	1515	5.79	2.33	2.15	3.99	1.55	2.10
NMES1	84419	4.62	8.24	1.65	2.25	3.67	1.33
FABP5	2171	2.47	5.47	1.01	2.30	2.89	1.60
GADD45A	1647	1.58	4.04	2.38	1.78	2.67	3.56
HIST1H1C	3006	0.81	0.36	0.45	2.23	3.13	1.48
HIST1H2BF	8343	40.36	0.28	0.55	52.11	2.01	1.71

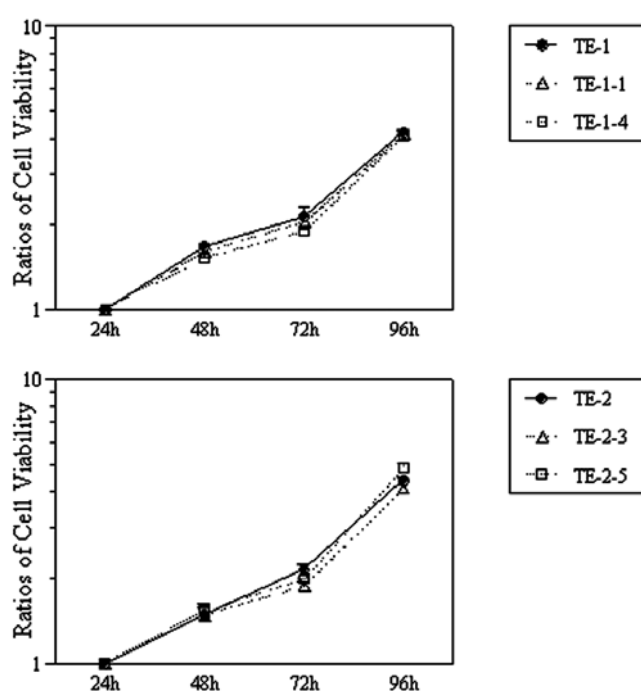


Figure 3. Cell viability in ESCC lines with overexpression of *NMES1*. The numbers of viable cells were measured by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium assay at 24, 48, 72, and 96 h after incubation and the ratio at 48, 72, and 96 compared to 24 h after incubation were calculated. Results are the mean of six independent experiments. There was no significant difference between the cell lines with regard to the overexpression of *NMES1* and their parental cells (Mann-Whitney U test). Error bars indicate S.D.

Methylation status of the up-regulated genes in ESCC cell lines. We determined the methylation status of the CpG islands in the promoter regions of *CTSL2*, *NMES1*, *FABP5*, and *GADD45A* (Fig. 2). Using bisulfite genomic sequencing analysis, aberrant methylation was observed in the promoter regions of *CTSL2*, *NMES1*, and *FABP5*, with only slight methylation observed in the *GADD45A* promoter.

Cell motility in ESCC lines with overexpression of *NMES1*. After selection for 2 weeks, G418-resistant colonies began to appear. Among them, we selected clones which expressed *NMES1* stably, and had the same cell viability as the parental

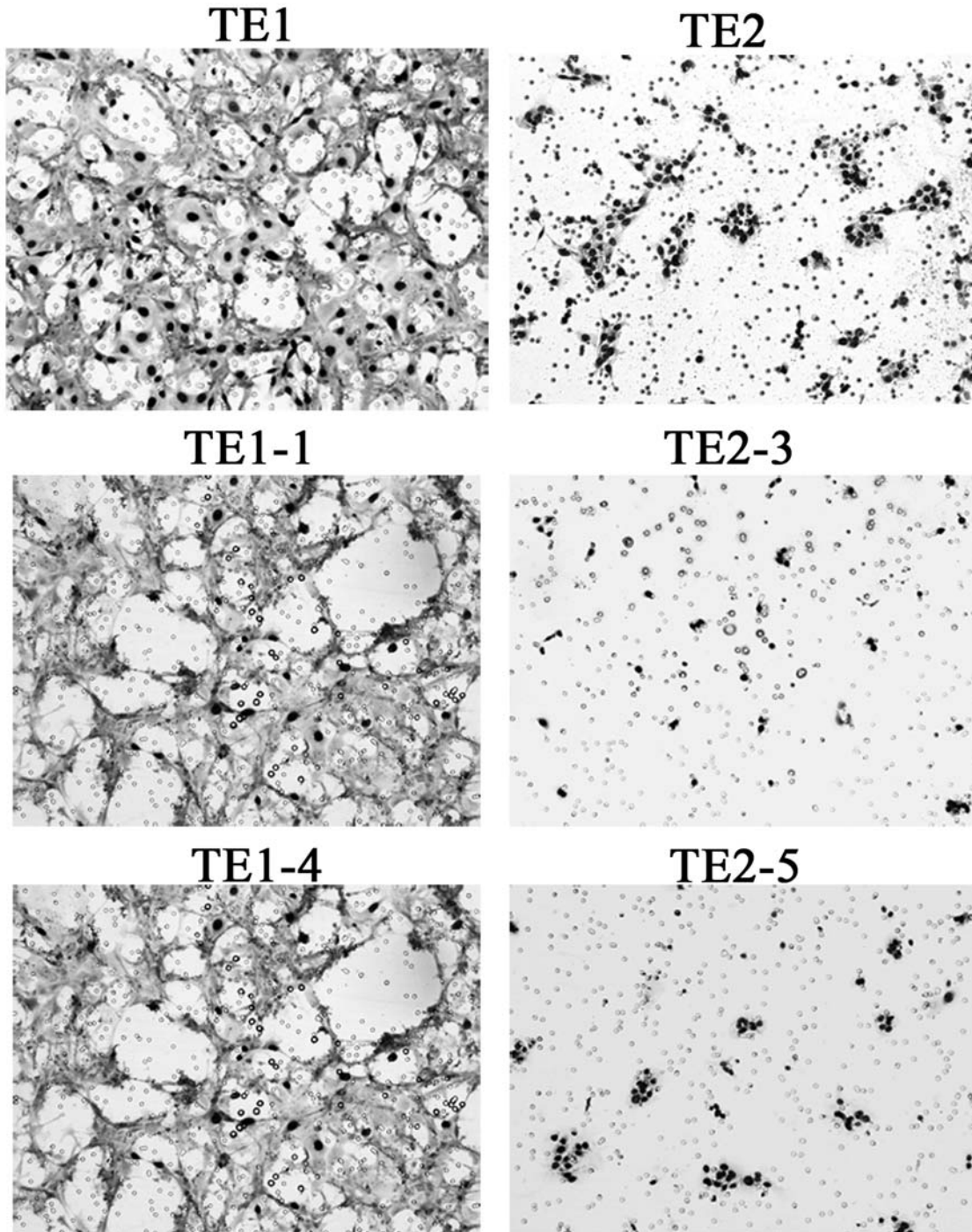
cell lines. These were: two sublines (clone TE1-1 and TE1-4) from TE-1 and two (clone TE2-3 and TE2-5) from TE-2 (Fig. 3). Overexpression of *NMES1* was confirmed by the quantitative RT-PCR method. The ratios of expression levels of *NMES1* in TE1-1 and TE1-4 compared to TE-1 and in TE2-3 and TE2-5 compared to TE-2 were 2.1 ± 0.1 , 1.6 ± 0.1 , 3.6 ± 0.4 , and 2.3 ± 0.2 , respectively. Boyden chamber assays were performed to compare the migratory capability of *NMES1* overexpressed cells with the parental ones (Fig. 4A). At 24 h, the number of cells migrating were 111.3 ± 5.5 , 20.3 ± 1.5 , and 39.0 ± 6.2 for TE-1 (parental cells), TE1-1, and TE1-4 (*NMES1* overexpressed cells), respectively. The corresponding numbers for TE-2 (parental cells), TE2-3, and TE2-5 (*NMES1* overexpressed cells) were 137.0 ± 13.7 , 29.0 ± 4.0 , and 54.7 ± 22.7 , respectively. There was a significant difference between *NMES1* overexpressed cells and parental cells ($p < 0.01$) (Fig. 4B).

Discussion

Finding genes that are down-regulated in cancer tissue due to epigenetic factors may lead to the discovery of new tumor suppressor genes. In lung cancer, the frequency of aberrant methylation of the p16INK4a gene increases during disease progression from basal cell hyperplasia to squamous metaplasia and carcinoma *in situ* lesions (16). ESCC is also thought to develop through a multi-stage process, from normal cells to hyperplasia, dysplasia, carcinoma *in situ*, and finally, carcinoma. In fact, hypermethylation of multiple genes has been detected in association with the development of ESCC (17). Discovery of genes with aberrant methylation in accordance with the multi-stage process of ESCC might be useful for the accurate diagnosis and prediction for the risk of disease progression.

Herein, we investigated the promoter methylation status of four genes and revealed aberrant methylation of the following three genes in ESCC cell lines: *CTSL2*, *NMES1*, and *FABP5*. *NMES1* was identified due to its strikingly low or completely absent expression in clinical samples of ESCCs (18). Furthermore, the aberrant methylation of the *NMES1* promoter region was present in a high proportion of cervical cancers, but not in normal samples (19). Therefore, we decided to investigate its function further. As for tissue distribution, the expression of *NMES1* was observed in normal esophagus,

A



B

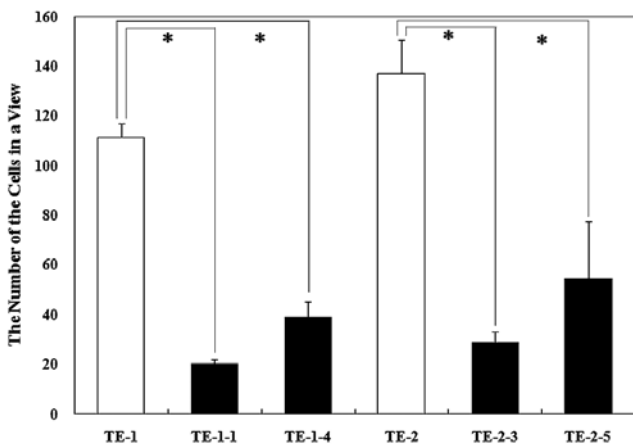


Figure 4. Effect of overexpression of normal mucosa of esophagus specific 1 (*NMES1*) on cell migration. We used the clones which expressed *NMES1* stably, namely two sublines (clone TE1-1 and TE1-4) from TE-1, and two (clone TE2-3 and TE2-5) from TE-2. Boyden chamber assay was done to compare the migratory capabilities of *NMES1* overexpressed cells with the parental ones. (A) The cells with overexpression of *NMES1* (TE-1-1, TE1-4, TE2-3, and TE2-5) showed a decrease in the number of cells migrating compared with parental cells (TE-1 and TE-2). (B) The number of cells in a field of view were counted. There was a significant difference between *NMES1* overexpressed cells and parental cells ($p < 0.01$, Mann-Whitney U test). * $p < 0.01$. Error bars indicate S.D.

stomach, small intestine, colon and placenta, with the *NMES1* protein being located mainly in the nucleus, as determined by immunohistochemical analysis. In addition, *NMES1* has been identified as a potential candidate for modifiers of susceptibility to skin tumor promotion by phorbol ester (20). In this study, using the Boyden chamber assay, we clarified that over-expression of *NMES1* in ESCC cell lines could prohibit cell motility. We also performed the wound healing assay in TE-1, TE1-1, and TE1-4 according to a previous report (21). The speed of wound healing in TE-1 was faster than seen in TE1-1 and TE 1-4 (data not shown). Taken together, we speculated that *NMES1* was related to cell motility of ESCC, and that maintenance of the expression of *NMES1* in normal esophagus was important for the inhibition of the development of ESCC. To clarify this, further investigation using clinical samples is needed.

CTSL2 is a member of the papain family of cysteine proteinases and is highly expressed in colorectal and breast carcinomas, but not in normal colon, mammary gland, or peritumoral tissues. In addition, high expression levels of *CTSL2* have been reported in ESCC; however, only a small number of samples were used in this study (22). *FABP5* is a member of the fatty acid binding protein family that binds long-chain fatty acids (23). Overexpression of *FABP5* has been observed in prostate cancer (24). *FABP5* is a major target of c-Myc and has been shown to be rapidly up-regulated upon induction of EpCAM, which was itself up-regulated in squamous cell carcinoma of the head and neck (25,26). Additionally, *FABP5* levels have been shown to correlate with the amount of EpCAM *in vivo* in primary head and neck squamous cell carcinomas (25). Thus, it follows that *FABP5* levels may be up-regulated in squamous cell carcinomas as well. In our study, the expressions of *CTSL2* and *FABP5* in cancer cell lines were down-regulated and restored by DAC treatment, which conflicted with the reports using clinical samples. Thus, the roles of *CTSL2* and *FABP5* in ESCC will need to be investigated further.

Transcript levels of *GADD45A* are increased following stressful growth arrest conditions and treatment with DNA-damaging agents. Wang *et al* reported that four CpGs, located ~700 bp upstream of the transcriptional start site, were methylated in the majority of breast cancer cell lines and clinical samples. Furthermore, this study showed that DAC induced the expression of *GADD45A* in breast cancer cell lines (27). In our study, the promoter region of *GADD45A* was slightly methylated, but almost identical to the methylated regions revealed by Wang *et al*. The methylation of this region might be important for the carcinogenesis of ESCC as well as breast cancer.

By DNA microarray-based transcriptomic analysis after DAC treatment, Yamashita *et al* have clarified previously the epigenetically silenced tumor suppressor genes in ESCC cell lines (28). However, the candidate genes silenced epigenetically in their study did not coincide with the ones silenced in ours. We speculate that the difference might be caused by using different microarrays or by the difference in the way candidate genes were selected from the microarray data.

In conclusion, we determined the methylation status, within the respective promoter regions, of *CTSL2*, *NMES1*, and

FABP5 in ESCC cell lines. Our results revealed that over-expression of *NMES1* inhibits cell motility in ESCC cell lines. *NMES1* might play an important role in metastasis of cancer cells and serve as a potential biomarker for ESCC.

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