Downregulation of *ST6GALNAC1* is associated with esophageal squamous cell carcinoma development

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Abstract. Tylosis is an inherited disorder characterized by abnormal palmoplantar skin thickening and a highly elevated risk of esophageal squamous cell carcinoma (ESCC). Analyses of tylosis in families have localized the responsible gene locus to a region of chromosome 17q25.1. Frequent loss of heterozygosity (LOH) in 17q25.1 was also observed in the sporadic form of ESCC. A putative tumor suppressor gene for ESCC may exist at this locus. We investigated the expression patterns of genes on 17q25.1 in tumor and corresponding normal tissues from patients with sporadic ESCC using RNA sequence analysis. For candidate genes, quantitative real-time reverse transcription-PCR (qRT-PCR), direct sequence, LOH and methylation analyses were performed using 93 clinical ESCC samples and 10 cell lines. A significant downregulation of *ST6GALNAC1* was demonstrated in ESCC tissues compared to its expression in normal tissues by qRT-PCR (n=93, p<0.0001). Frequent LOH (17/27, 62.9%) and hyper-methylation in *ST6GALNAC1* were also observed in all cell lines. Our results indicated that *ST6GALNAC1* was downregulated in sporadic ESCC via hyper-methylation and LOH, and it may be a candidate responsible gene for ESCC. Furthermore, recent studies suggest that multiple genes on chromosome 17q25 are involved in ESCC development.

Introduction

Tylosis is an extremely rare autosomal, dominantly inherited disorder characterized by abnormal thickening of the palmoplantar skin and a highly elevated risk of esophageal squamous cell carcinoma (ESCC). Five families with high frequencies of tylosis have been reported from the UK, USA, Germany, Spain and Finland (1-7). Linkage and haplotype analyses in these families have localized the tylosis esophageal cancer (TOC) gene locus to a region of chromosome 17q25 (3-5,8,9). It has also been reported that frequent loss of heterozygosity (LOH) in 17q25.1 was observed in the sporadic form of ESCC (10,11). These reports on inherited and sporadic ESCC indicated the presence of a putative tumor suppressor gene for ESCC at this locus. Although abnormalities of genes on 17q25.1, such as *CYGB* and *RHBDF2*, have been demonstrated in families with tylosis, abnormalities of these genes have not been clearly demonstrated in sporadic forms of ESCC (12,13). Therefore, additional genes on 17q25.1 other than *CYGB* and *RHBDF2* may also be involved in ESCC development.

Additionally, we recently reported the result of whole-exome sequencing of paired DNA samples from 144 Japanese patients with ESCC (14). The most frequently mutated gene was *TP53* (mutated in 93.1% of patients), followed by *NOTCH1*, *MLL2*, *NFE2L2*, *ZNF750*, *FAT1* and *PIK3CA* (mutated in 10-20% of patients), in line with the results of next-generation sequencing analysis in other studies of sporadic ESCC (14-17). Although 17,189 non-synonymous mutations in 10,552 genes were identified in 144 ESCC samples in our previous study, recurrent mutations were not observed in genes on chromosome 17q25.1, including *RHBDF2* (14). Other studies did not uncover *RHBDF2* mutations either in sporadic ESCC (16,17).

In this study, we investigated the expression patterns of genes in an ~1500 kb region on 17q25.1, including the TOC locus, in tumor and corresponding normal tissues using RNA sequence (RNA-seq) analysis data from patients with sporadic ESCC. We demonstrated frequent downregulation of ST6 N-acetylgalactosaminide α-2,6-sialyltransferase 1 (*ST6GALNAC1*) on 17q25.1 in ESCC tissues compared to its expression in corresponding normal tissues.

Patients and methods

Patients and sample collection. A total of 93 ESCC samples obtained by surgery were used after obtaining written

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Table I. Primer sequences and amplified regions of *ST6GALNAC1* gene in direct sequencing analysis.

Gene name	Forward primer	Reverse primer	Probe no.
ST6GALNAC1	CGAAATAGGAGGCCTTCAGA	AGAGAGTGAGGTTGGGCAGA	#50
EVPL	TACCGTGCCCTGTACGAGA	GCGCAGACCTGCTTCTGT	#67
CYGB	CCGCTGCCTACAAGGAAGT	GGGTGGAGTTAGGGGTCCT	#62
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC	#60

Table II. Primer sequences and amplified regions of *ST6GALNAC1* gene in direct sequencing analysis.

	Forward primer	Reverse primer	Amplified region
Segment 1	CTTCCTTTAAGCCACGCCAGCTTAT	TGAAATATGAGGAGTGGAAAGGACA	3'UTR and Exon 1
Segment 2	GCCTTCATCAAAGGTTATCTCTGTC	AAAGTCTAGAAGCAGAGCCCAGGAG	Exon 2
Segment 3	CCTTCCTGACTTCGTCCTCCTGTAT	GAGCTTGGGTGGGGACAGCTTAC	Exons 3 and 4
Segment 4	CCGTCTGATTGTGTCTTTCTGCAC	CAACCCTTTAGAGCCACTCATGACA	Exons 5 and 6
Segment 5	CCAGGTAAGGGAGCTGAGTCTGAAT	CCAGGAGCTGTTTCTCCAGGTATTT	Exons 7 and 8
Segment 6	CGTGATGTAGGTGAGTGCTTATGGC	TTCACTGTAGAAAATTTATTTGCCT	Exon 9 and 5'UTR

informed consent. All patients underwent resection of the primary tumor at Iwate Medical University, Kyushu University Beppu Hospital and affiliated hospitals between 1992 and 2007. Resected cancer and corresponding normal tissues were immediately cut and stored at -80˚C until DNA/RNA extraction. Total RNA and DNA were obtained using an RNeasy mini kit and QIAamp DNA mini kit (Qiagen Inc., Valencia, CA, USA), respectively.

Gene expression profiling on chromosome 17q25.1 using RNA-seq data of samples from three patients with ESCC. The expression patterns of genes on chromosome 17q25.1 were analyzed using RNA-seq data for tumor and corresponding normal tissues from three patients with ESCC. The characteristics of the three patients were as follows: well-differentiated SCC in the cervical esophagus (female, 70 years old, T1N0M0, stage I), poorly differentiated SCC in the middle thoracic esophagus (male, 73 years old, T3N0M0, stage II) and moderately differentiated SCC in the lower thoracic esophagus (male, 68 years old, T3N1M0, stage III). One microgram of extracted RNA was used as a template to construct RNA-seq libraries. Detailed protocols of RNA-seq analysis were described previously (18). Fold enrichment of the RNA-seq tags in the samples was calculated for each mRNA using the assigned tag counts and normalized to reads per kilobase (kb) mRNA.

Evaluation of ST6GALNAC1 and EVPL expression in clinical samples. Quantitative real-time reverse transcription-PCR (qRT-PCR) was performed to measure *ST6GALNAC1*, *EVPL*, *CYGB* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA expression using a LightCycler 480 Probes Master kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol with specific primers and universal probes that were designed at the Universal Probe Library's assay design center (http://lifescience.roche.com/shop/CategoryDisplay?catalogId=10001&t ab=Assay+Design+Center&identifier=Universal+Probe+Libr ary&langId=-1). Gene expression levels were normalized with respect to those of *GAPDH*. Primer sequences and universal probe number for each gene are listed in Table I.

Direct sequencing analysis of ST6GALNAC1. In 46 cases of ESCC, coding exons of *ST6GALNAC1* were amplified using KOD FX (Toyobo, Tokyo, Japan) according to the manufacturer's protocol and sequenced using BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA, USA) as previously described (19). Primer sequences are listed in Table II and Fig. 1.

Microsatellite LOH analysis of the ST6GALNAC1 locus. In samples from 34 patients with ESCC, PCR was performed for three dinucleotide repeat microsatellite markers (D17S2238, D17S2243 and D17S2245) within the *ST6GALNAC1* locus using fluorescent primer pairs (Applied Biosystems). LOH was analyzed using an ABI PRISM 3100 Genetic Analyzer and GeneScan Analysis and Genotyper software version 3.7.1 (Applied Biosystems).

Cell lines and cell culture. Ten human ESCC cell lines (KY150, KY270, KYSE410, KYSE450, KYSE510, TE1, TE6, TE8, TE9 and TE10) were purchased from the Japanese Collection of Research Bioresources Cell Bank and the Riken Bioresource Center. Cells were maintained in RPMI-1640 containing 10% fetal bovine serum and cultured in a humidified 5% CO_2 incubator at 37˚C.

Methylation levels and response to 5-aza-2'-deoxycytidine (5-Aza-dC) treatment in ESCC cell lines. ESCC cells were seeded at a density of $1x10^6$ cells/10 cm dish and cultured for 24 h with an inhibitor of DNA methyltransferase, 5-Aza-dC (Sigma-Aldrich, St. Louis, MO, USA), at a final concentration of 2 μM. Control cells were treated with the diluent

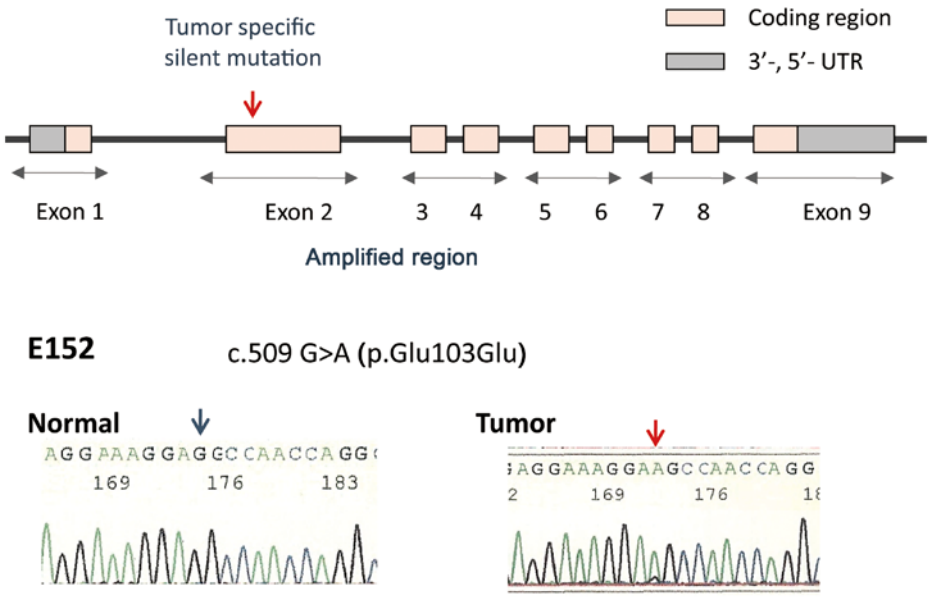


Figure 1. Sequence analysis of *ST6GALNAC1*. Sequence of *ST6GALNAC1* is illustrated in the upper panel. All protein-coding regions were amplified by PCR. A tumor-specific silent mutation was noted in case E152.

phosphate-buffered saline (PBS) alone. After 48 h of incubation, total RNA was extracted from collected cells in each dish.

Statistical analysis. Data from RNA-seq analyses, qRT-PCR and methylation assays were analyzed using JMP 12 software (SAS Institute, Inc., Cary, NC, USA) and GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Differences between the gene expression levels of samples were estimated using the Wilcoxon rank test or paired t-test. All differences were considered statistically significant at the level of $p<0.05$.

Results

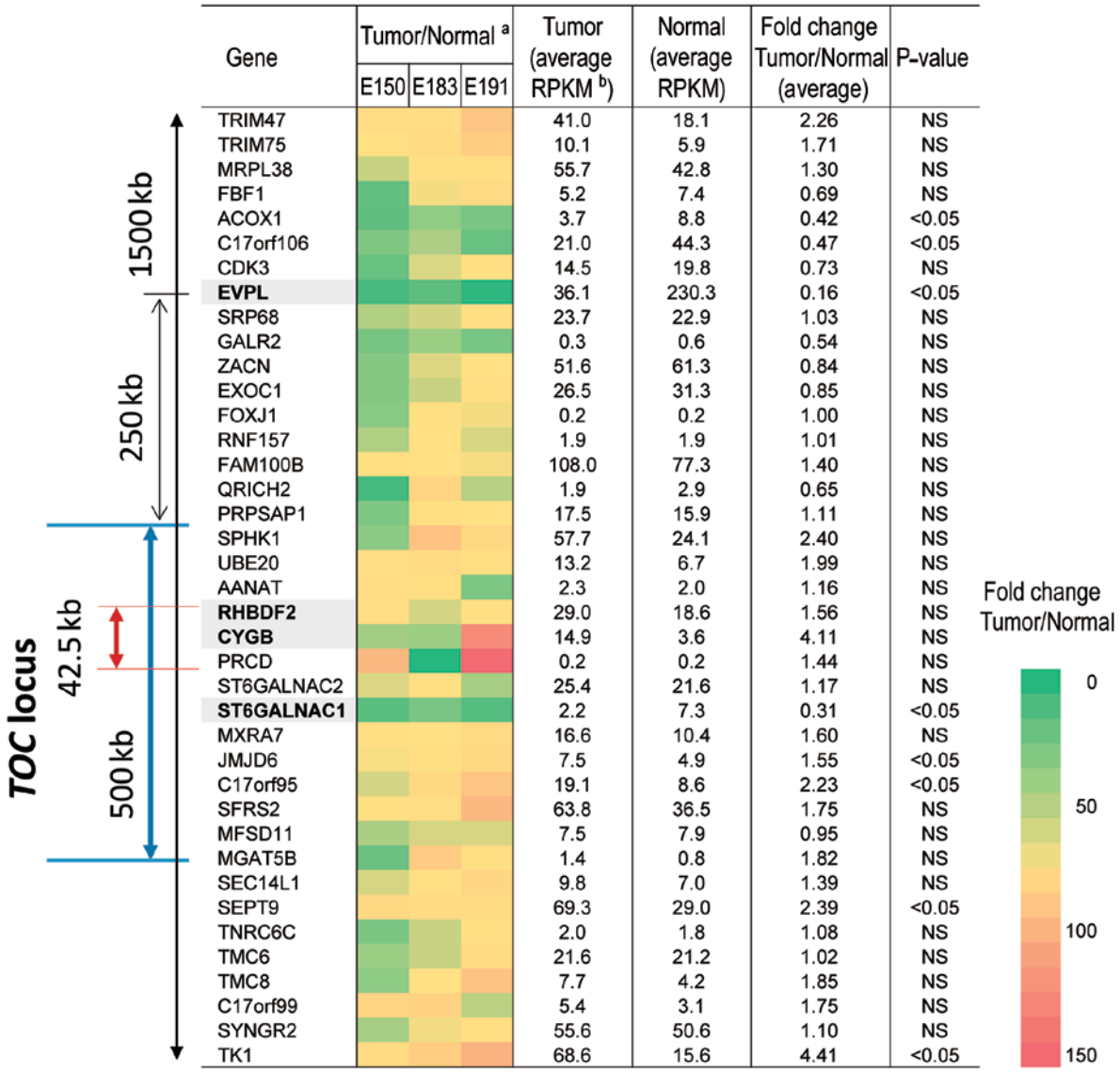
Differential gene expression profiling on chromosome 17q25.1 in ESCC samples by RNA-seq analysis. In our RNA-seq analysis from three patients with ESCC with differences in tumor stage, histological differentiation and tumor location, 17,673 genes were detected with an RPKM value of at least 2.0 in either normal or tumor tissues. The first 10 genes showed significant increase or decrease in their expression in tumor tissues compared with their expression in normal tissues and are listed in Table III. Among the expression data obtained by RNA-seq analysis, we focused on the expression profile of genes in a 1500 kb region on 17q25.1 including the TOC locus, which has been mapped to the 500 kb region in UK, USA and German pedigrees (8) and narrowed to a 42.5 kb region in the UK pedigree (9). The differences in expression levels between normal and tumor samples for 39 genes in the region are shown in Fig. 2. Among these genes, the expression levels of *EVPL* and *ST6GALNAC1* in tumor tissues were significantly decreased to less than one-third of the levels in normal tissues (Fig. 2).

Downregulation of ST6GALNAC1 and EVPL in ESCC tissues. We validated the expression levels of these two genes using qRT-PCR in samples from 93 patients with ESCC. Both *EVPL* and *ST6GALNAC1* displayed significant downregulation in tumor samples compared to their corresponding normal tissue levels ($p<0.0001$) (Fig. 3). Although downregulation of *CYGB* has been demonstrated in esophageal tissues in tylotic patients

Table III. Upregulated and downregulated genes in ESCC tumor by RNA-seq analysis.

Genes	Tumor (average RPKM)	Normal (average RPKM)	Fold-change Tumor/ normal
Upregulated			
CST1	160.41	0.21	763.84
GRP	6.88	0.01	516.25
OBP2A	7.77	0.02	466.4
IL-17C	3.23	0.01	322.67
MMP11	160.47	0.64	250.74
RNASE10	5.27	0.03	175.56
MMP13	2.44	0.02	146.6
HIST1H3G	3.61	0.03	135.25
HOXD11	7.5	0.06	132.35
MMP3	2.17	0.02	130
Downregulated			
TMPRSS11B	0.7	156.48	222.48
SFTA2	0.22	42.3	192.26
MUC21	3.24	494.75	152.54
CRNN	20.17	3015.85	149.5
KRT4	75.48	11106.92	147.15
MAL	21.72	2735.73	125.97
CWH43	0.21	15	70.3
KRT78	9.69	617.84	63.76
KRT13	610.37	37852.87	62.02
CD207	0.06	3.79	59.89

RPKM, read per kilobase per million mapped reads.



Gene	Tumor/Normal [a]			Tumor (average RPKM [b])	Normal (average RPKM)	Fold change Tumor/Normal (average)	P-value
	E150	E183	E191				
TRIM47				41.0	18.1	2.26	NS
TRIM75				10.1	5.9	1.71	NS
MRPL38				55.7	42.8	1.30	NS
FBF1				5.2	7.4	0.69	NS
ACOX1				3.7	8.8	0.42	<0.05
C17orf106				21.0	44.3	0.47	<0.05
CDK3				14.5	19.8	0.73	NS
EVPL				36.1	230.3	0.16	<0.05
SRP68				23.7	22.9	1.03	NS
GALR2				0.3	0.6	0.54	NS
ZACN				51.6	61.3	0.84	NS
EXOC1				26.5	31.3	0.85	NS
FOXJ1				0.2	0.2	1.00	NS
RNF157				1.9	1.9	1.01	NS
FAM100B				108.0	77.3	1.40	NS
QRICH2				1.9	2.9	0.65	NS
PRPSAP1				17.5	15.9	1.11	NS
SPHK1				57.7	24.1	2.40	NS
UBE20				13.2	6.7	1.99	NS
AANAT				2.3	2.0	1.16	NS
RHBDF2				29.0	18.6	1.56	NS
CYGB				14.9	3.6	4.11	NS
PRCD				0.2	0.2	1.44	NS
ST6GALNAC2				25.4	21.6	1.17	NS
ST6GALNAC1				2.2	7.3	0.31	<0.05
MXRA7				16.6	10.4	1.60	NS
JMJD6				7.5	4.9	1.55	<0.05
C17orf95				19.1	8.6	2.23	<0.05
SFRS2				63.8	36.5	1.75	NS
MFSD11				7.5	7.9	0.95	NS
MGAT5B				1.4	0.8	1.82	NS
SEC14L1				9.8	7.0	1.39	NS
SEPT9				69.3	29.0	2.39	<0.05
TNRC6C				2.0	1.8	1.08	NS
TMC6				21.6	21.2	1.02	NS
TMC8				7.7	4.2	1.85	NS
C17orf99				5.4	3.1	1.75	NS
SYNGR2				55.6	50.6	1.10	NS
TK1				68.6	15.6	4.41	<0.05

Figure 2. Gene expression patterns on chromosome 17q25.1 in esophageal squamous cell carcinoma (ESCC) samples. RPKMs of genes on 17q25.1 in tumor and corresponding normal tissues from three patients with ESCC are shown. a, Fold difference of RPKMs between tumor and normal tissues is schematized. The minimal region (42.5 kb) and 500 kb region of the TOC locus are shown on the left of the table. b, RPKMs, reads per kilobase per million mapped reads.

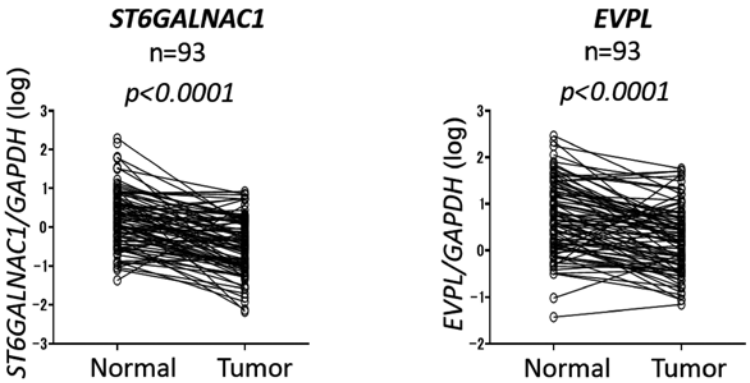


Figure 3. *ST6GALNAC1* and *EVPL* expression in patients with esophageal squamous cell carcinoma (ESCC). *ST6GALNAC1* and *EVPL* expression levels in tumor and corresponding normal tissues were validated by quantitative real-time reverse transcription-PCR (analysis in 93 patients with ESCC). The expression levels of both genes were normalized to those of *GAPDH*, and the results are shown as $\log_{10}$.

compared with that in the normal esophagus (12), a significant difference in its expression was not observed between sporadic ESCC and normal samples by qRT-PCR analyses in our series (data not shown).

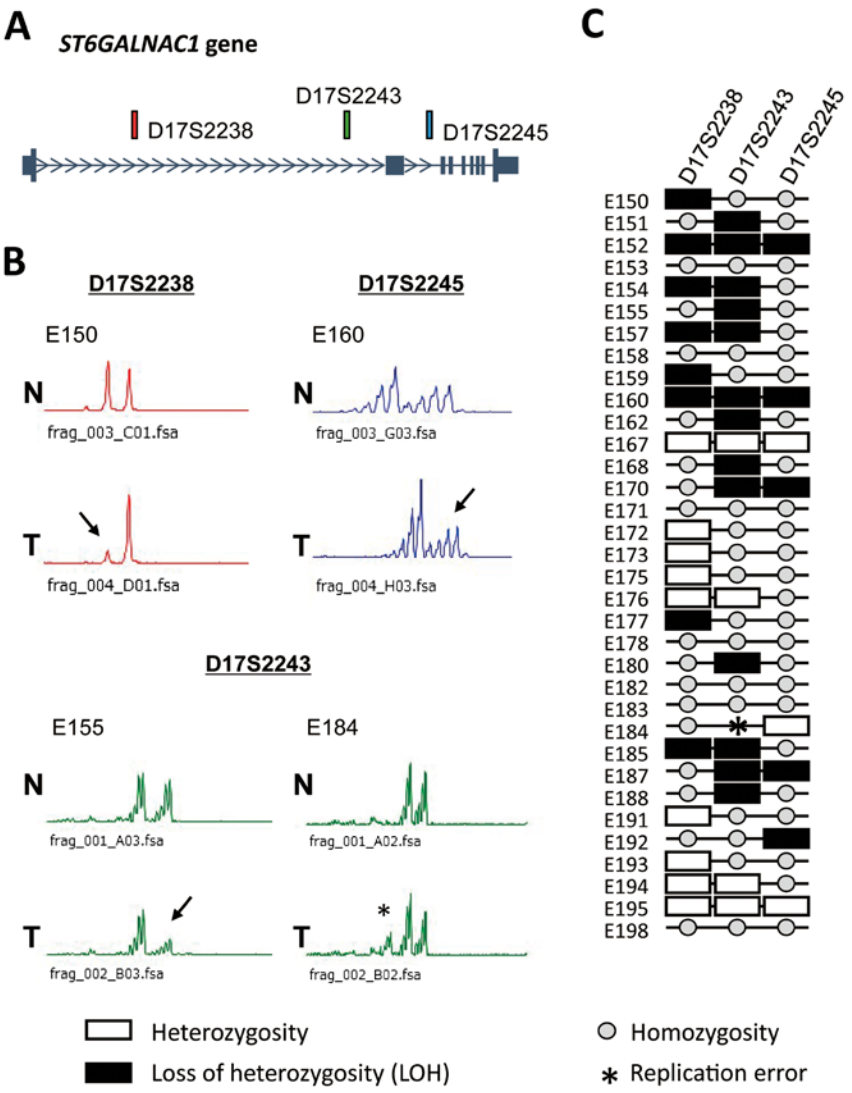


Figure 4. PCR microsatellite analysis at the *ST6GALNAC1* locus in esophageal squamous cell carcinoma (ESCC) samples. (A) The locations of three microsatellite markers in the *ST6GALNAC1* locus. D17S2238 and D17S2243 are located in intron 1 of *ST6GALNAC1*, and D17S2245 is located in intron 2. (B) Histograms of each marker in representative cases. Arrows indicate loss of heterozygosity (LOH), whereas the asterisk indicates a replication error. N, normal; T, tumor. (C) LOH mapping on the *ST6GALNAC1* locus. The microsatellite status, namely heterozygosity, homozygosity, LOH or replication error, at each marker in examined cases is represented schematically.

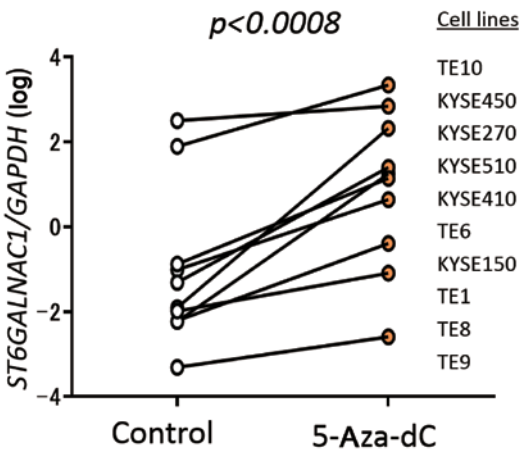


Figure 5. Methylation analysis of *ST6GALNAC1* in esophageal squamous cell carcinoma (ESCC) cell lines. ESCC cell lines were treated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-Aza-dC). *ST6GALNAC1* expression levels were normalised to those of GAPDH, and the results are shown as $\log_{10}$.

Nucleotide variants in ST6GALNAC1. Direct sequence analyses of samples from 46 patients with ESCC revealed several nucleotide variants in ST6GALNAC1. Two missense variants [c.400 C>T (p.Pro67Leu) and c.724 C>T (p.Thr175Met)] were observed in two patients. In four patients, 3 bp in-frame deletions [c.752_754delTGG (p.His184del)] were observed. These variants were detected in both tumor and corresponding normal tissues, and they have been registered in dbSNP as rs143927446, rs138569950 and rs565363235. A one-base G insertion in intron 3 registered as rs146144287 in dbSNP was also detected in both tumor and normal tissues from six patients with ESCC. Only one patient (1/46, 2.1%) displayed a tumor-specific mutation in exon 2 of *ST6GANAC1*, although this mutation was silent [c.509 G>A (p.Glu103Glu)] (Fig. 1). Therefore, no tumor-specific non-synonymous mutations were observed in the coding region of *ST6GALNAC1*.

Frequent LOH was observed in the ST6GALNAC1 locus in ESCC samples. Three microsatellite markers, D17S2238,

Table IV. Summary of altered genes on chromosome 17q25 in sporadic or hereditary forms of esophageal squamous cell carcinoma (ESCC).

Genes	Locus	Position (start-end)	Known function	Gene alterations in sporadic ESCC or tylosis families
EVPL	17q25.1	76004502 -76027452	Link the cornified envelop to desmosomes and intermediated filaments	LOH and infrequent mutation Sporadic ESCC
RHBDF2	17q25.1	76471069 -76501790	Activation of the altered protein leading to constant EGF receptor signalling and hyper-proliferation	Germ-line missense mutations Tylosis families
CYGB	17q25.1	76527356 -76537905	Collagen synthesis, O2 sensing and transport or detoxification of reactive oxygen species	LOH and hyper-methylation Tylosis families
ST6GALNAC1	17q25.1	76624763 -76643838	Synthesis of the sialyl-Tn antigen in cancer cells	LOH and hyper-methylation Sporadic ESCC
ZNF750	17q25.3	82828435 -82840578	Control terminal epidermal differentiation via interactions with KDM1A, RCOR1 and CTBP1/2	LOH and frequent mutation Sporadic ESCC

D17S2243 and D17S2245, were located in the introns of *ST6GALNAC1* (Fig. 4A). PCR microsatellite analysis of the *ST6GALNAC1* locus in 34 patients using these three markers demonstrated LOH in 17/27 (62.9%) informative cases at one or more sites and a replication error in 1/27 (3.7%) cases at D17S2243 (Fig. 4B and C).

ST6GALNAC1 downregulation by methylation in ESCC cell lines. Treatment with 5-Aza-dC significantly elevated *ST6GALNAC1* expression compared to the control level in all 10 ESCC cell lines assessed (Fig. 5).

Discussion

Because the region in chromosome 17q25.1 was affected in both hereditary and sporadic forms of ESCC, it is believed that a gene responsible for the oncogenesis or development of ESCC existed in this region. Several genes in the TOC locus have been studied in inherited and sporadic forms of ESCC. In our present study, *ST6GALNAC1* expression was significantly decreased in ESCC tumor tissue compared with that in the corresponding normal tissue, and the gene was located in the vicinity of the minimal region of the TOC locus (Figs. 2 and 3). It was reported that *ST6GALNAC1* was associated with biosynthesis of the sialyl-Tn (sTn) antigen in cancer cells and that it was overexpressed in gastric, breast and prostate cancer cell lines, inducing sTn expression (20). In contrast to these findings, our results suggest that *ST6GALNAC1* may have a tumor-suppressor function in ESCC. In terms of the downregulation mechanisms of *ST6GALNAC1* expression, frequent LOH (62.9%) of the gene locus was demonstrated in ESCC, although no tumor-specific mutation was observed in the coding region (Figs. 1 and 4). Furthermore, demethylation using 5-Aza-dC recovered *ST6GALNAC1* expression in all 10 ESCC cell lines examined (Fig. 5). There are no CpG islands in the promoter region of *ST6GALNAC1*. However, it has been reported that *ST6GALNAC1* was downregulated by hyper-methylation of GC 2 bp upstream of the transcription start site in estrogen receptor- and progesterone receptor-positive breast cancers (21). These results suggest that *ST6GALNAC1* was inactivated by LOH and hyper-methylation of the transcription start site.

Several candidate genes for sporadic and inherited ESCC have been demonstrated. *EVPL* is a member of the desmosomal plaque protein family that attaches to desmosomal cadherin and keratin filaments. We previously reported infrequent mutations and frequent LOH of this gene in sporadic ESCC (22). In this study, we demonstrated that *EVPL* expression was significantly decreased in ESCC tissues (Fig. 3). Downregulation of *EVPL* may be involved in ESCC development, although the gene is located 250 kb to the telomeric side of the minimal region of the TOC locus (Fig. 2). It has been demonstrated that *CYGB* was a tumor suppressor gene inactivated by DNA hyper-methylation of its promoter in several types of cancer, including ESCC (12,23). However, downregulation of *CYGB* was not observed in our series of tumor tissues from Japanese patients with ESCC compared to its levels in the corresponding normal tissues (data not shown). *CYGB* methylation has also been demonstrated in multiple malignancies other than ESCC, such as leukaemia as well as breast, bladder, lung and colon cancers (23). These findings indicated that alterations of *CYGB* were limited to a subset of ESCCs and that the tumor-suppressor role of *CYGB* may not be specific in esophageal tissues but instead may be common among many types of malignancies.

Recently, missense mutations of *RHBDF2* in the minimal region of the TOC locus were identified in patients with tylosis from US/UK, German and Finnish families (7,13). It may be clear that *RHBDF2* is a responsible gene for tylosis. Blaydon *et al* demonstrated that the altered *RHBDF2* represents a gain-of-function allele that results in sustained EGFR signaling within the cells, and the signaling leads to a hyperproliferative phenotype. Furthermore, it was suggested that *RHBDF2* may also be dysregulated in a similar manner in sporadic ESCC according to immunohistochemical data (13). In our previous study, however, *RHBDF2* mutation was not observed by whole-exome sequence analysis using next-generation sequencing in 144 patients with sporadic ESCC (14). This sequencing analysis also demonstrated that recurrent mutations were observed only in *ZNF750*, with the mutation

rate of 16.7%, on chromosome 17q. Frequent *ZNF750* mutations were also demonstrated in Chinese patients with sporadic ESCC by whole-exome sequence analysis (15). Furthermore, we found that the mutational APOBEC signature was predominantly observed in the ESCC genome, and *ZNF750* mutations were positively associated with the APOBEC signature (14). *ZNF750* was located on 17q25.3 telomeric to the TOC locus, and its mutations were null mutations accompanied by LOH (14). Therefore, *ZNF750* may be a strong candidate tumor suppressor gene for ESCC. It remains unclear as to which gene dysregulation of in the chromosomal region is essential for the development of hereditary and sporadic forms of ESCC. Mutations in one allele of *RHBDF2* gene induced sustained EGFR signaling in the cells and led to a hyperproliferative phenotype during wound repair in patients with tylosis (13). Although *RHBDF2* mutation was not observed in sporadic ESCC, the EGFR signaling were frequently dysregulated in sporadic ESCC cells (14-17). Therefore, an abnormality in the RHBDF2-EGFR pathway may lead to precancerous lesions in the esophagus. In addition to the oncogenic change in RHBDF2-EGFR, further inactivation of several tumor suppressor genes on 17q25, such as *EVPL*, *CYGB* and *ZNF750*, by a two-hit mechanism may induce ESCC.

In conclusion, *ST6GALNAC1* was downregulated in sporadic ESCC by hyper-methylation and LOH, and it may be a candidate responsible gene for ESCC. Furthermore, our results on sporadic ESCC and recent studies on tylotic families suggest that multiple genes on chromosome 17q25 are involved in ESCC development (Table IV).

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