SPANDIDOS PUBLICATIONS

Decreased expression of *RUNX3* is correlated with tumor progression and poor prognosis in patients with esophageal squamous cell carcinoma

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Abstract. Runt-related transcription factor 3 (*RUNX3*) has been reported to be a candidate tumor suppressor gene in gastric cancer. However, in esophageal cancer, the role of *RUNX3* has not been studied. The expression of *RUNX3* mRNA was quantified by real-time reverse transcription polymerase chain reaction using Taq Man PCR in 15 esophageal cancer cell lines (TE1-15) and 70 esophageal squamous cell carcinoma (ESCC) specimens and their paired normal esophageal mucosa. The data were analyzed with reference to clinicopathological factors. Using specific primers, methylation of the promoter region of *RUNX3* was examined. *RUNX3* mRNA expression in ESCC tissue was significantly lower than that in the corresponding normal esophageal mucosa (3.913±4.617 vs. 7.795±15.361, P=0.0345). *RUNX3* mRNA expression levels in locally invasive T4 tumors were significantly lower than those in less invasive T1-3 tumors (P=0.0454). Patients who had low *RUNX3* mRNA expression levels had a significantly shorter survival after surgery compared with patients who had high *RUNX3* mRNA expression (P=0.0299). Among the 15 esophageal cancer cell lines studied, one had methylation of the promoter region of *RUNX3*. Only 4 in 70 ESCC tumors had methylation in this region. In conclusion, *RUNX3* expression may be involved in the tumor invasion and poor prognosis of patients with ESCC. The methylation of the *RUNX3* promoter region in esophageal cancer is rare. A study on the mechanisms that underlie the reduced expression of *RUNX3* in ESCC is warranted.

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Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common causes of cancer death in Japan. The prognosis for patients with ESCC is poor, prompting a search for new treatment strategies. Although preoperative chemotherapy and chemoradiation therapy are currently used for patients with advanced ESCC, their results are not satisfactory. Even with early-stage disease, many patients will develop locally recurrent tumors or distant metastases within a short period after surgery.

Recent molecular biological studies have revealed that several genes are involved in the carcinogenesis and/or progression of esophageal carcinoma: e.g. TP53 (1), CDKN2A (2), DEC1 (3), DCC (4) and DLC1 (5). It has been reported that mRNA expression of these genes was correlated with survival. Our previous studies have identified candidate genes that may affect the survival of patients with ESCC; *survivin* (6), *DFF45* (7), *PTTG1* (8), *PPAR γ* (9), *ERCC3* (10), *PABPC1* (11), *NDRG1* (12), *ACP6* (13) and *RNASEN* (14).

The *RUNX3* gene, a candidate tumor suppressor gene, was cloned and identified as a human runt-domain containing gene in 1994 (15). Its precise function is unknown and its role in ESSC has not been studied. In this study, we investigated the *RUNX3* mRNA expression in ESCC and its paired normal esophageal mucosa by real-time RT-PCR. We analyzed the results in reference to the patients' clinicopathological characteristics and the prognosis of the ESCC patients. We searched for methylation of the promoter region of *RUNX3* in ESCC tissue and cell lines.

Materials and methods

Cell lines and cell culture. Esophageal cancer cell lines (TE1-15) were obtained from the Japanese Collection of Research Bioresources (JCRB). Cultures were maintained in RPMI-1640 (Sigma) medium supplemented with 10% fetal bovine serum (FBS) (Gibco) at 37˚C in a humidified 5% CO_2 incubator.

Tissue samples. Samples were obtained from 70 patients with primary ESCC who had undergone radical esophagectomy at

Table I. Correlation of *RUNX3* mRNA expression in esophageal cancer with clinicopathological factors, including patient and tumor characteristics.

Characteristics	No. of patients (n=70)	*RUNX3* expression relative to GAPDH[a]	P-value
Normal	70	7.795±15.361	
Tumor	70	3.913±4.617	0.0345
Age at surgery			
≤65 years	41	3.643±4.327	
>65 years	29	4.165±5.086	0.835
Gender			
Male	59	3.644±4.167	
Female	11	5.234±6.465	0.7341
Tumor status			
T1	9	5.007±4.282	
T2	8	3.1±3.383	
T3	34	4.604±5.435	
T4	19	2.303±3.29	0.1903
T1-3 vs. T4			0.0454
Lymph node status			
NO	14	5.152±4.707	
N1	11	1.898±1.729	
N2	23	4.525±5.41	
N3	9	4.986±5.103	
N4	11	2.357±4.245	0.5341
Unknown	2	1.141 ±0.689	
Pathological stage			
0	4	3.42±2.18	
I	2	6.698±9.194	
II	12	4.199±3.161	
III	23	4.799±6.016	
IV	29	2.838±3.798	0.4835
Histological differentiation			
Well	26	4.78±5.232	
Moderate	34	2.952±3.486	
Poor	5	2.752±2.701	0.1114
Unknown	5		
Lymphatic invasion			
Negative	10	5.239±4.37	
Positive	45	3.713±4.545	0.4768
Unknown	15		
Blood vessel invasion			
Negative	22	5.695±5.263	
Positive	33	2.854±3.587	0.0164
Unknown	15		

[a]Mean ± standard deviation (SD). *RUNX3*, esophageal cancer related gene 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

the Department of Surgery II, Nagoya City University Medical School between 1996 and 2002. The study design was approved by the IRB (Institutional Review Board) of our University hospital, and a written consent was obtained from all the patients. Tumors were classified according to the Guidelines for the Clinical and Pathological Studies on Carcinoma of the Esophagus. Normal esophageal mucosa was taken from the apparently noncancerous mucosa as far apart as possible from the tumor (at least 5 cm). All samples were frozen immediately in liquid nitrogen and stored at -80˚C until use. The characteristics of the 70 patients with ESCC are shown in Table I.

Nucleic acid isolation and cDNA synthesis. Total RNA was extracted from ESCC tissue and its corresponding normal esophageal mucosa using the Absolutely RNA™ RT-PCR Miniprep kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The concentration of total RNA

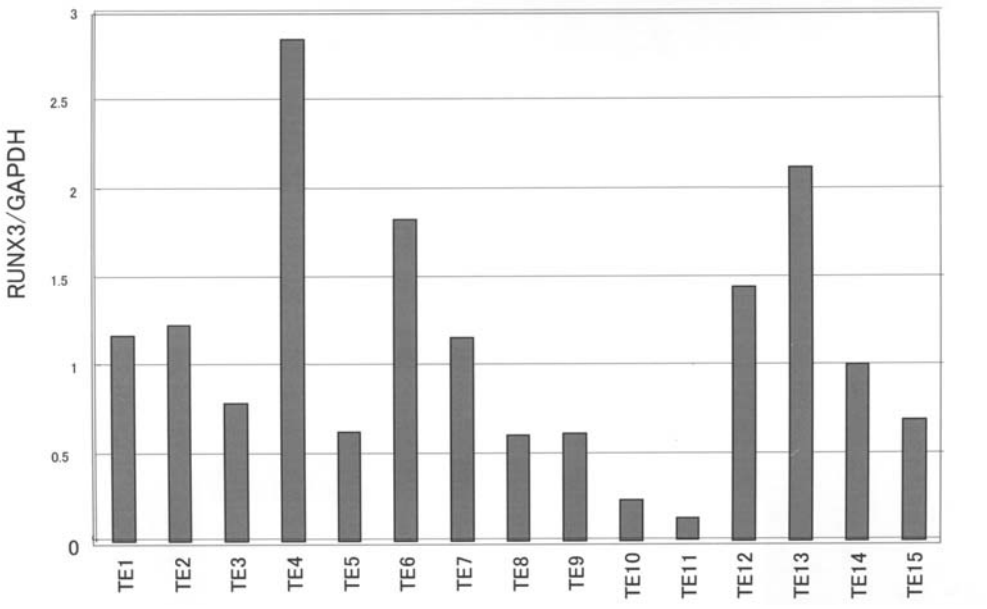

Figure 1. *RUNX3/GAPDH* mRNA expression levels in esophageal cancer cell lines. TE11 had significantly lower expression than other esophageal cancer cell lines.

was adjusted to 200 ng/ml using a spectrophotometer. Reverse transcriptase reaction was carried out at 42˚C for 90 min and at 95˚C for 5 min followed by incubation at 72˚C for 15 min using 1 μg of total RNA, 0.5 μg oligo(dT) primer, and Superscript II enzyme (Gibco BRL, Gaithersburg, MD).

All samples were quantified after PCR amplification using a Lightcycler-Faststart DNA Master SYBR-Green I kit (Roche Molecular Biochemicals, Mannheim, Germany).

Real-time RT-PCR using Taq Man Probes. Real-time quantitative PCR amplification of the cDNA template corresponding to 20 ng total RNA was performed using Taq Man® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM 7500 (Applied Biosystems). PCR conditions were at 50˚C for 2 min and at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. *RUNX3*-specific Taq Man probes were designed from sequences in exons 4 and 5 (Assays-on-Demand Gene Expression system, *RUNX3*-assay ID:Hs00231709_m1, Applied Biosystems). Expression levels were normalized against *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* (Assays-on-Demand Gene Expression system, assay ID Hs99999905_m1, Applied Biosystems).

Genomic DNA and methylation-specific PCR. Genomic DNA and total RNA were isolated from each sample of ESCC and its corresponding normal esophageal mucosa. Genomic DNA was extracted using a standard SDS-proteinase K procedure.

Sodium bisulfite modification of genomic DNA was performed by the CpGenome™ DNA modification kit (Chemicon International, CA, USA). Methylation-specific PCR was performed using PCR primers which were designed to be specific to either methylated or unmethylated DNA after sodium bisulfite modification described previously. Methylated DNA-specific primers were 5'-TTACGAGG GGCGGTCGTACGCGGG-3' (sense) and 5'-AAAACG ACCGACGCGAACGCCTCC-3' (antisense). Unmethylated DNA-specific primers were 5'-TTATGAGGGGTGGTTG TATGTGGG-3' (sense) and 5'-AAAACAACCAACACA AACACCTCC-3' (antisense).

Statistical analysis. Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using the Stat-View software package (Abacus Concepts, Berkeley, CA). The Wilcoxon signed-ranks test, Mann-Whitney U test, and Kruskal-Wallis test were used to evaluate the significance of differences in expression levels of *RUNX3/GAPDH* mRNA. Statistical analyses were carried out using Stat-View software (Abacus Concepts). The survival of ESCC patients after surgery was examined using the Kaplan-Meier method and survival times were compared using the log-rank test. Multivariate analysis was performed using Cox's regression model and the logistic multivariate regression model. P-values were considered significant at the $P<0.05$ level.

Results

Expression of RUNX3 in esophageal cancer cell lines. We examined the expression of *RUNX3* mRNA in 15 esophageal cancer cell lines (TE1 to TE15) using quantative RT-PCR. Expression of *RUNX3* and *GAPDH* mRNA was detectable in all esophageal cancer cell lines. TE11 had the lowest expression and TE4 had the highest expression of *RUNX3* among the cell lines studied (Fig. 1).

Expression of RUNX3 in esophageal cancer tissues. *RUNX3* mRNA expression was detectable in all ESCC tissue and noncancerous esophageal mucosa. It was standardized using the expression of *GAPDH*. The levels of expression of *RUNX3* mRNA in ESCC tissue were significantly lower than those in the corresponding normal esophageal mucosa (3.913±4.617 vs. 7.795±15.361, P=0.0345; Wilcoxon signed-rank test) (Table I). We examined the relationship between *RUNX3/GAPDH* mRNA expression in 70 ESCC samples and the

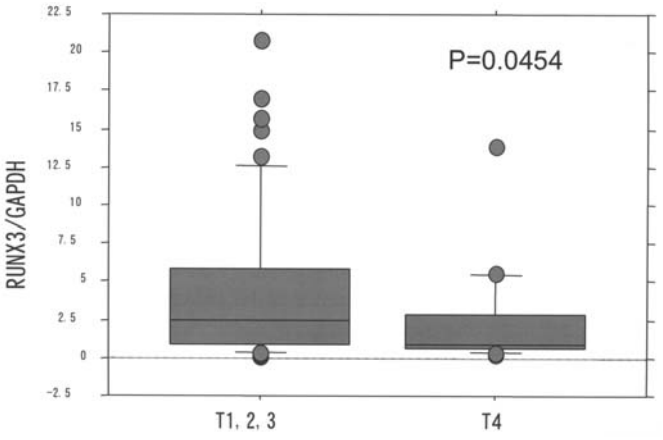

Figure 2. *RUNX3/GAPDH* mRNA expression levels in patients with locally invasive T4 tumors were significantly lower than those in less invasive T1-3 tumors (P=0.0454; Mann-Whitney U test).

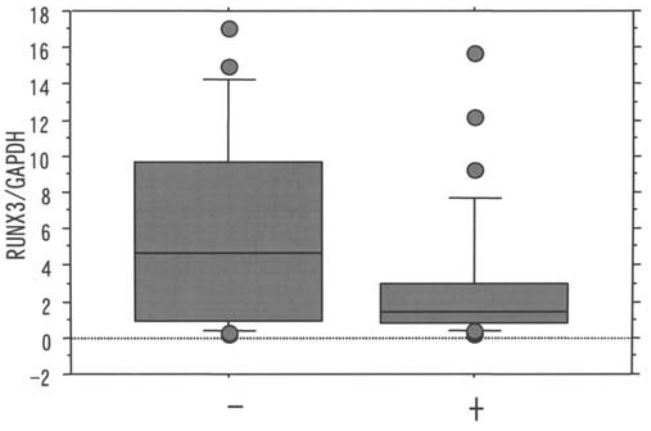

Figure 3. *RUNX3/GAPDH* mRNA expression levels in blood vessel invasion positive ESCC tissue were significantly lower than those of negative ESCC tissues (P=0.0164; Mann-Whitney U test).

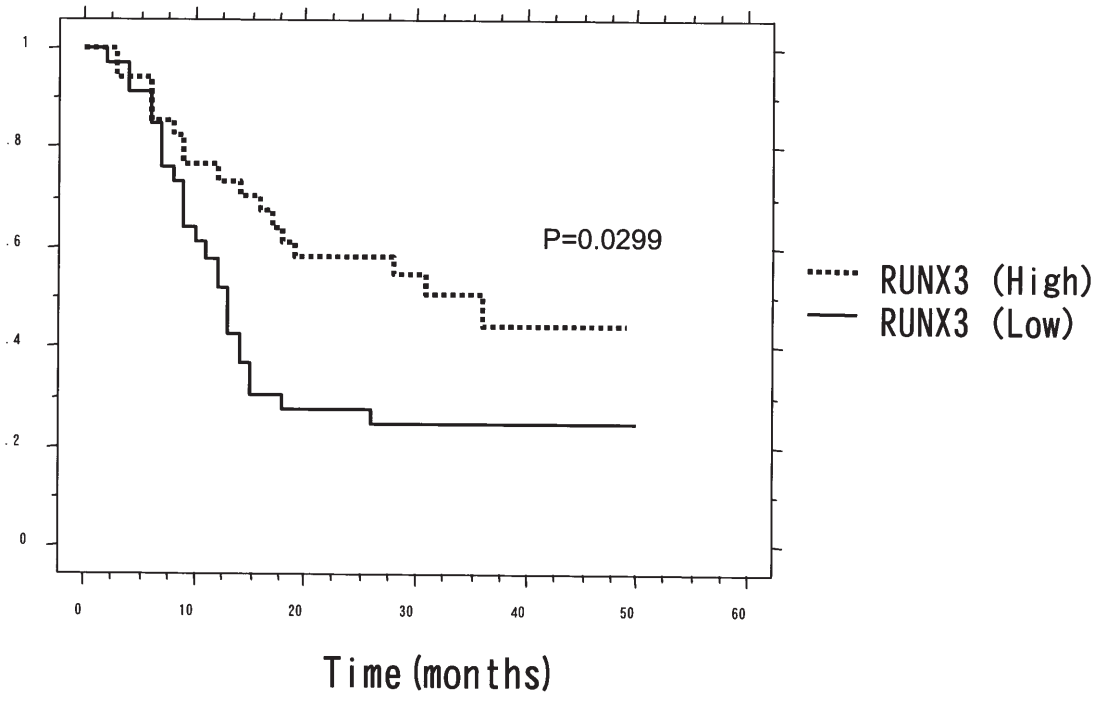

Figure 4. Patients who had low *RUNX3/GAPDH* mRNA expression levels (*RUNX3/GAPDH* mRNA expression in the tumor <2.0; n=36) had a significantly shorter survival (14.3±1.4 months) after surgery compared with patients who had high *RUNX3* mRNA expression levels (*RUNX3/GAPDH* >2.0, n=34; 28.3±2.6 months) (P=0.0299, log-rank test).

patients' clinicopathological factors (Table I). Of the 70 ESCC samples studied, there were no significant differences in *RUNX3/GAPDH* mRNA according to age, gender, lymph node status, histological differentiation and lymphatic invasion. *RUNX3/GAPDH* mRNA expression levels in patients with locally invasive T4 tumors were significantly lower than those in less invasive T1-3 tumors (P=0.0454; Mann-Whitney U test) (Table I, Fig. 2). *RUNX3/GAPDH* mRNA expression levels in tumors with blood vessel invasion were significantly lower than those in vessel invasion negative tumors (P=0.0164; Mann-Whitney U test) (Table I, Fig. 3).

We investigated the correlation between the *RUNX3/GAPDH* mRNA expression levels and the survival of ESCC patients after surgery (median follow-up, 24.8 months). Patients who had low *RUNX3/GAPDH* mRNA expression levels (indicated as the ratio of *RUNX3/GAPDH* <2.0, n=34) had a significantly shorter survival (14.3±1.4 months) after surgery compared with patients who had high *RUNX3/GAPDH* mRNA expression levels (*RUNX3/GAPDH* mRNA >2.0, n=36; 28.3±2.6 months) (P=0.0299, log-rank test) (Fig. 4). Univariate analysis showed that among the clinicopathological factors, the local invasiness (tumor status) (risk ratio 4.716; P<0.0001), lymph node metastasis (node status) (risk ratio 32.849; P=0.0019), lymphatic invasion (risk ratio 10.526; P=0.0012), blood vessel invasion (risk ratio 3.27; P=0.0005) and *RUNX3* expression (<2.0) (risk ratio 1.908; P=0.0355) were statistically significant prognostic factors (Table II). However, multivariate analysis revealed that *RUNX3*

Univariate analysis.

Parameter	Risk ratio	95% Cl	P-value
Age at surgery			
>65 years	1		
≤65 years	1.083	0.660-1.776	0.7523
Gender			
Female	1		
Male	1.220	0.65-2.30	0.5225
Tumor status			
T1-3	1		
T4	4.716	2.710-8.196	0.0001
Lymph node status			
N0-3	1		
N4	2.849	1.472-5.494	0.0019
Lymphatic invasion			
Negative	1		
Positive	10.526	2.551-43.472	0.0012
Blood vessel invasion			
Negative	1		
Positive	3.270	1.672-6.410	0.0005
RUNX3 expression			
High	1		
Low	1.908	1 .044-3.484	0.0355

Cl, confidence interval.

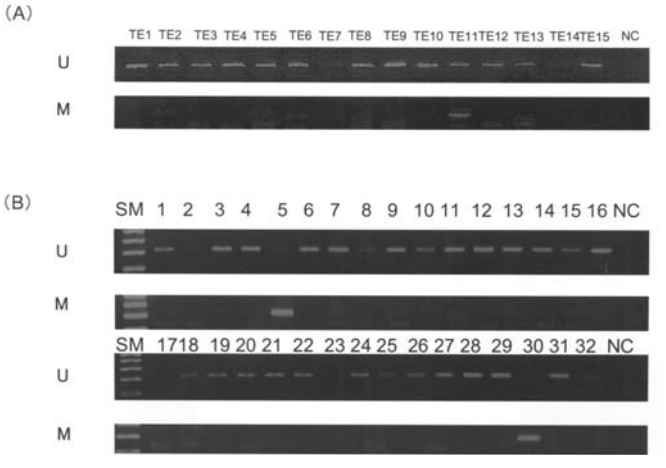

Figure 5. (A) Analysis of 15 esophageal cancer cell lines using MSP. M, methylated sequence-specific PCR; U, unmethylated sequence-specific PCR; SM, size marker. (B) Analysis of esophageal cancer tissues after surgery using MSP. Representative 32 cases are shown. Patients #5 (upper panel) and #30 have methylation in the promoter region of *RUNX3*. M, methylated sequence-specific PCR; U, unmethylated sequence-specific PCR; SM, size marker; NC, negative control.

expression (*RUNX3/GAPDH* <2.0) was not an independent prognostic factor (data not shown).

Methylation PCR of RUNX3 CpG islands. Methylation-specific PCR was performed in order to analyze the methylation status of *RUNX3* CpG islands in the esophageal cancer cell lines. Genomic DNA extracted from 15 esophageal cancer cell lines, TE1-15, were treated with sodium bisulfite. Methylation-specific PCR was performed using PCR primers which were designed to be specific to either methylated or unmethylated DNA after sodium bisulfite modification. Among the 15 cell lines, methylation was detected only in TE11.

We then analyzed the methylation status of *RUNX3* CpG islands in 70 primary ESCC samples and their paired normal esophageal tissues using the MSP technique. Among the 70 primary ESCC samples, only four samples were found to have *RUNX3* promoter methylation (Fig. 5). In any of the normal esophageal mucosa, methylation of *RUNX3* was not observed (data not shown).

Discussion

In this study, we examined *RUNX3* expression in 70 cases of ESCC at the mRNA level by quantitative RT-PCR. The *RUNX3* gene was expressed abundantly in noncancerous esophageal epithelium, though it was down-regulated in ESCC tissue. Among the clinicopathological factors examined, we observed a significant correlation between *RUNX3* expression and the depth of invasion (T factor), vascular invasion and survival of patients with ESCC. These results indicate a close association between the *RUNX3* expression and the growth and invasion of ESCC.

In search of possible mechanism(s) of the down-regulation of *RUNX3*, we examined DNA methylation of CpG islands of *RUNX3* promotor region. We found that the hypermethylation

in *RUNX3* promotor region is rare (only 4/70, 5.7% of all the patients). Other possible mechanisms that downregulate *RUNX3*, including mutation and loss of heterozygosity (LOH), was not examined in this study. This gene is located at chromosome 1p36, where frequent LOH has been reported in human malignancies, such as neuroblastoma (16), oral and laryngeal squamous carcinoma (17), lung carcinoma (18) and prostate carcinoma (19). LOH in 1p36 has been detected in esophageal cancer patients and within this region, it was suggested to harbor a putative tumor suppressor gene (20).

Many tumor suppressor genes are downregulated by promoter methylation during the development and progression of cancer, and hypermethylation of gene-promoter regions is being revealed as one of the most frequent mechanisms in the loss of gene function, thus detection of CpG methylation is important in understanding the gene regulation of cancer. It has been reported that the expression of some tumor suppressor genes, such as *p15INK4b*, *p16INK4a*, *FHIT* and *E-cadherin* are commonly down-regulated by CpG island hypermethylation in ESCC (10,21,22). Aberrant methylation of CpG islands in the core promoter of the *RUNX3* gene was reported to be a frequent molecular event in gastric cancer (23-25), lung cancer (26) and bladder tumor (27). These results indicate that the inactivation of the *RUNX3* gene by hypermethylation in gastric cancer may be involved in the carcinogenesis of the cancer. However, in our study, the hypermethylation of the *RUNX3* promoter region was a rare event. In addition to epigenetic change including methylation, silencing due to LOH or mutation may be responsible for the downregulation of *RUNX3*. This possibility remains to be examined.

RUNX3 is a downstream target of the TGF-ß signaling pathway. Hanai *et al* reported that *RUNX3* interacts with Smads which transduce TGF-ß signals (28). The down-regulation of SMAD4 in esophageal cancers is correlated with poor prognosis (29). Furthermore, *RUNX3* is a transcription factor that regulates numerous downstream genes, for example, the cell-cycle regulator p21 (30). Thus, *RUNX3* is a candidate tumor suppressor.

Although the precise molecular mechanism of the down-regulation of *RUNX3* expression needs to be clarified, our data indicate that *RUNX3* may be involved in prognosis and tumor invasion in patients with ESCC. The locally invasive T4 ESCCs are not candidates for surgery. In view of the reduced expression of *RUNX3* in T4 ESCC and in ESCC with poor prognosis, *RUNX* may be the target of future therapy in this intractable disease.

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References

1. Robert V, Michel P, Flaman JM, *et al*: High frequency in esophageal cancers of p53 alterations inactivating the regulation of genes involved in cell cycle and apoptosis. Carcinogenesis 21: 563-565, 2000.
2. Suzuki H, Zhou X, Yin J, *et al*: Intragenic mutations of CDKN2B and CDKN2A in primary human esophageal cancers. Hum Mol Genet 4: 1883-1887, 1995.
3. Nishiwaki T, Daigo Y, Kawasoe T and Nakamura Y: Isolation and mutational analysis of a novel human cDNA, DEC1 (deleted in esophageal cancer 1), derived from the tumor suppressor locus in 9q32. Genes Chromosomes Cancer 27: 169-176, 2000.
4. Miyake S, Nagai K, Yoshino K, Oto M, Endo M and Yuasa Y: Point mutations and allelic deletion of tumor suppressor gene DCC in human esophageal squamous cell carcinomas and their relation to metastasis. Cancer Res 54: 3007-3010, 1994.
5. Daigo Y, Nishiwaki T, Kawasoe T, Tamari M, Tsuchiya E and Nakamura Y: Molecular cloning of a candidate tumor suppressor gene, DLC1, from chromosome 3p21.3. Cancer Res 59: 1966-1972, 1999.
6. Kato J, Kuwabara Y, Mitani M, *et al*: Expression of survivin in esophageal cancer: correlation with the prognosis and response to chemotherapy. Int J Cancer 95: 92-95, 2001.
7. Konishi S, Ishiguro H, Shibata Y, *et al*: Decreased expression of DFF45/ICAD is correlated with a poor prognosis in patients with esophageal carcinoma. Cancer 95: 2473-2478, 2002.
8. Shibata Y, Haruki N, Kuwabara Y, *et al*: Expression of PTTG (pituitary tumor transforming gene) in esophageal cancer. Jpn J Clin Oncol 32: 233-237, 2002.
9. Terashita Y, Sasaki H, Haruki N, *et al*: Decreased peroxisome proliferator-activated receptor gamma gene expression is correlated with poor prognosis in patients with esophageal cancer. Jpn J Clin Oncol 32: 238-243, 2002.
10. Terashita Y, Ishiguro H, Haruki N, *et al*: Excision repair cross complementing 3 expression is involved in patient prognosis and tumor progression in esophageal cancer. Oncol Rep 12: 827-831, 2004.
11. Takashima N, Ishiguro H, Kuwabara Y, *et al*: Expression and prognostic roles of PABPC1 in esophageal cancer: correlation with tumor progression and postoperative survival. Oncol Rep 15: 667-671, 2006.
12. Ando T, Ishiguro H, Kimura M, *et al*: Decreased expression of NDRG1 is correlated with tumor progression and poor prognosis in patients with esophageal squamous cell carcinoma. Dis Esophagus 19: 454-458, 2006.
13. Ando T, Ishiguro H, Kuwabara Y, *et al*: Expression of ACP6 is an independent prognostic factor for poor survival in patients with esophageal squamous cell carcinoma. Oncol Rep 15: 1551-1555, 2006.
14. Sugito N, Ishiguro H, Kuwabara Y, *et al*: RNASEN regulates cell proliferation and affects survival in esophageal cancer patients. Clin Cancer Res 12: 7322-7328, 2006.
15. Levanon D, Negreanu V, Bernstein Y, Bar-Am I, Avivi L and Groner Y: AML1, AML2, and AML3, the human members of the runt domain gene-family: cDNA structure, expression, and chromosomal localization. Genomics 23: 425-432, 1994.
16. Abel F, Sjoberg RM, Ejeskar K, Krona C and Martinsson T: Analyses of apoptotic regulators CASP9 and DFFA at 1P36.2, reveal rare allele variants in human neuroblastoma tumours. Br J Cancer 86: 596-604, 2002.
17. El-Naggar AK, Lai S, Clayman GL, *et al*: p73 gene alterations and expression in primary oral and laryngeal squamous carcinomas. Carcinogenesis 22: 729-735, 2001.
18. Nomoto S, Haruki N, Tatematsu Y, Konishi H, Mitsudomi T, and Takahashi T: Frequent allelic imbalance suggests involvement of a tumor suppressor gene at 1p36 in the pathogenesis of human lung cancers. Genes Chromosomes Cancer 28: 342-346, 2000.
19. Badzioch M, Eeles R, Leblanc G, *et al*: Suggestive evidence for a site specific prostate cancer gene on chromosome 1p36. The CRC/BPG UK Familial Prostate Cancer Study Coordinators and Collaborators. The EU Biomed Collaborators. J Med Genet 37: 947-949, 2000.
20. Nimura Y, Mihara M, Ichimiya S, *et al*: p73, a gene related to p53, is not mutated in esophageal carcinomas. Int J Cancer 78: 437-440, 1998.
21. Xing EP, Nie Y, Song Y, Yang GY, Cai YC, Wang LD and Yang CS: Mechanisms of inactivation of p14ARF, p15INK4b, and p16INK4a genes in human esophageal squamous cell carcinoma. Clin Cancer Res 5: 2704-2713, 1999.
22. Si HX, Tsao SW, Lam KY, *et al*: E-cadherin expression is commonly downregulated by CpG island hypermethylation in esophageal carcinoma cells. Cancer Lett 173: 71-78, 2001.
23. Homma N, Tamura G, Honda T, Matsumoto Y, Nishizuka S, Kawata S and Motoyama T: Spreading of methylation within RUNX3 CpG island in gastric cancer. Cancer Sci 97: 51-56, 2006.
24. So K, Tamura G, Honda T, *et al*: Quantitative assessment of RUNX3 methylation in neoplastic and non-neoplastic gastric epithelia using a DNA microarray. Pathol Int 56: 571-575, 2006.

Γ, Aoyagi K, Saeki N, *et al*: Expression status of 1/AML1 in normal gastric epithelium and its mutational analysis in microdissected gastric cancer cells. Int J Oncol 29: 779-784, 2006.
26. Yanada M, Yaoi T, Shimada J, *et al*: Frequent hemizygous deletion at 1p36 and hypermethylation downregulate RUNX3 expression in human lung cancer cell lines. Oncol Rep 14: 817-822, 2005.
27. Kim WJ, Kim EJ, Jeong P, *et al*: RUNX3 inactivation by point mutations and aberrant DNA methylation in bladder tumors. Cancer Res 65: 9347-9354, 2005.
28. Hanai J, Chen LF, Kanno T, *et al*: Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Calpha promoter. J Biol Chem 274: 31577-31582, 1999.
29. Natsugoe S, Xiangming C, Matsumoto M, *et al*: Smad4 and transforming growth factor beta1 expression in patients with squamous cell carcinoma of the esophagus. Clin Cancer Res 8: 1838-1842, 2002.
30. Chi XZ, Yang JO, Lee KY, *et al*: RUNX3 suppresses gastric epithelial cell growth by inducing p21(WAF1/Cip1) expression in cooperation with transforming growth factor {beta}-activated SMAD. Mol Cell Biol 25: 8097-8107, 2005.