

Clinical significance of *ZNF750* gene expression, a novel tumor suppressor gene, in esophageal squamous cell carcinoma

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Abstract. The present authors previously identified a novel candidate tumor suppressor gene, zinc finger protein 750 (*ZNF750*), in esophageal squamous cell carcinoma (ESCC) (1). The present study aimed to clarify the clinical significance of *ZNF750* expression in ESCC. The association between *ZNF750* DNA mutation status and the mRNA expression was examined by whole exome sequence analysis and quantitative reverse transcription polymerase chain reaction (RT-qPCR). The expression of *ZNF750* in 76 patients with ESCC (Kyushu University Beppu Hospital) was measured using immunohistochemistry and RT-qPCR. Using this dataset, the association between *ZNF750* mRNA expression and clinicopathological factors was examined. Additionally, survival analysis was performed using datasets from the Kyushu University Beppu Hospital and The Cancer Genome Atlas (TCGA). The biological effects of *ZNF750* expression were explored using gene set enrichment analysis (GSEA) and were validated using datasets from the Cancer Cell Line Encyclopedia (CCLE) and the Kyushu University Beppu Hospital. *ZNF750* expression analyses demonstrated that *ZNF750* mRNA expression was lower in patients with the DNA mutations compared with those without the mutations ($P < 0.05$), and *ZNF750* expression was downregulated in tumor tissues compared with normal tissues ($P < 0.00005$). In the clinicopathological analysis, the low *ZNF750* expression group exhibited a higher incidence of undifferentiated histology ($P < 0.05$) compared with the high expression group. The low *ZNF750* expression group exhibited a poorer prognosis in the Kyushu and TCGA datasets ($P < 0.0005$ and $P < 0.05$, respectively). GSEA indicated that

ZNF750 expression was significantly correlated with epithelial differentiation in ESCC. This was confirmed using the datasets from CCLE and the Kyushu University Beppu Hospital by analyzing the levels of small proline rich protein 1A mRNA, an epithelial differentiation-associated gene. In conclusion, the results of the present study suggested that *ZNF750* serves a role as a tumor suppressor; potentially via regulating epithelial differentiation and that it may be a promising biomarker of poor outcomes in ESCC.

Introduction

Esophageal squamous cell carcinoma (ESCC) is the most common histological type of esophageal cancer worldwide (2). ESCC tends to have a poor prognosis due to high rates of lymph node metastasis and invasion into surrounding organs (2). ESCC has been identified to harbor frequent somatic mutations in tumor protein 53 (3), Notch homolog 1, tumor protein P53 (3) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (4). However, previous cohorts used to investigate the genomic abnormalities of ESCC were too small to encompass the full range of genomic abnormalities (3,4). The present authors have recently performed whole-exome sequence (WES) analysis on 144 Japanese patients with ESCC, and identified a previously uncharacterized mutant gene, zinc finger protein 750 (*ZNF750*) (1), which is an essential regulator of epithelial proliferation and differentiation (5-7).

In general, in order to identify a sporadic tumor suppressor gene, studies should focus on the high frequency aberrant genomic loci in familial cancer syndromes. As such, it was identified that *ZNF750* is located at chromosome 17q25, the same location for the gene responsible for hereditary tylosis esophageal cancer, a familial esophageal cancer (8,9).

In a previous study by the present authors, mutations in *ZNF750* were identified in 16.7% of ESCC tumors and *ZNF750* was identified to be the fifth most frequent mutated genes. Additionally, 87.5% of mutations in *ZNF750* led to decreased mRNA expression (1). Similarly, Lin *et al* (10) and Zhang *et al* (11) also revealed inactivating mutations (70.0 and 64.0% of mutations in each study, respectively) in *ZNF750* by WES analysis of ESCC and demonstrated that the depletion of *ZNF750* in ESCC cell lines promoted proliferation and

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invasiveness. These results suggested that *ZNF750* serves a role as a tumor suppressor in ESCC. However to the best of our knowledge, no study has investigated whether aberrant *ZNF750* expression affected clinical outcomes in ESCC. Elucidation of the clinical significance of *ZNF750* expression may clarify whether *ZNF750* may be targeted in clinical therapy. Therefore, the present study aimed to clarify the clinicopathological and prognostic significance of *ZNF750* expression in ESCC using two independent ESCC datasets.

Materials and methods

ESCC patients and sample collection. A total of 76 primary ESCC samples and 76 paired normal tissues were obtained from 76 patients who underwent surgery at Kyushu University Beppu Hospital (Beppu, Japan) and affiliated hospitals [Iwate Medical University (Iwate, Japan), Kurume University School of Medicine (Kurume, Japan), and Kagoshima University Graduate School of Medical and Dental Sciences (Kagoshima, Japan)] from January, 1998 to December, 2012 (Kyushu dataset). The samples were taken as a part of routine examination. The patient group comprised 67 males and 8 females (the gender of one patient was not available). The mean age of the patients was 64.7 years (range, 40.0-79.0). All patients had a histological diagnosis of ESCC and were closely followed at 3-month intervals. The median follow-up period was 3.9 years. All patients were treated in accordance with the Japan Esophageal Society Guidelines for the treatment of esophageal cancer (12). Written informed consent was obtained from all patients, and the Institutional Review Board of Kyushu University (Fukuoka, Japan) approved the present study. Sample collection was performed as previously described (13). Data on patient age, sex, histology, tumor depth of invasion, lymph node metastasis, lymphatic invasion, venous invasion and distant metastasis were obtained from the clinical and pathological records. A total of two patients who exhibited distant metastasis were included in the present study. Of these patients, one exhibited distant lymph node metastasis and the other liver metastasis.

ZNF750 mutation. *ZNF750* mutation status data were obtained from WES analysis that the authors of the present study previously performed (1). RNA from 28 patients of the 144 used in the previous study was available for quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis.

Total RNA extraction and RT. Total RNA from tissues was extracted by a modified AGPC method using ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan), in which the aqueous phase extraction step by chloroform is unrequired (14). RT was performed using 8 μ g of total RNA with Moloney Murine leukemia virus reverse transcriptase according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Quantitative (q)PCR. qPCR reactions were performed using a LightCycler 480 probes master kit (Roche Diagnostics, Indianapolis, IN, USA) and repeated three times as previously described (15). The following primers were used: *ZNF750*; sense primer, 5'-GAACAGGTACTGCTTCCTGAGC-3' and

antisense primer, 5'-GAGAGCCTCCGTCATCTGG-3'; small proline rich protein 1A (*SPRR1A*); sense primer, 5'-TCCACCCAGGAACCATGC-3' and antisense primer, 5'-CTTGGTCTTCTGCTGGGCTG-3'; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*); sense primer, 5'-TTGGTATCGTGGAAGGACTCTA-3' and antisense primer, 5'-TGTCATATTTGGCAGGTT-3. The expression levels of *ZNF750* and *SPRR1A* were normalized by *GAPDH* as an internal control. The expression levels were calculated as the values relative to the expression level of the cDNA using Human Universal Reference Total RNA with the calibration curve method (Clontech Laboratories, Inc., Mountainview, CA, USA) (16).

Immunohistochemical analysis. Immunohistochemistry of *ZNF750* in 5 randomly selected representative ESCC cases was performed on formalin-fixed, paraffin-embedded tissues as previously described (15). A polyclonal rabbit anti-*ZNF750* antibody (cat. no., HPA023012, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany; dilution, 1:100) was used as the primary antibody. Tumor histology was independently reviewed by an experienced pathologist.

The Cancer Genome Atlas (TCGA) data analysis. Paired RNA sequencing and survival data of 86 available patients with ESCC were obtained from TCGA (<http://cancergenome.nih.gov/>). *ZNF750* mRNA expression and survival data were extracted from this reference.

Gene set enrichment analysis (GSEA). The correlations between *ZNF750* expression and previously annotated gene expression signatures were analyzed by applying GSEA (17). ESCC expression profiles from the National Center for Biotechnology Information (NCBI) gene expression omnibus database (NCBI accession no. GSE25333) were acquired, and analyzed using GSEA, as previously described (18). Gene sets of *ZNF750* targets were extracted from C2 curated gene sets in the Broad Institute database (<http://www.broadinstitute.org/gsea/msigdb/collections.jsp>).

Cancer Cell Line Encyclopedia (CCLE) data analysis. Normalized mRNA expression data of 22 available ESCC cell lines were obtained from the CCLE dataset (<http://www.broadinstitute.org/ccle/home>). *ZNF750* and *SPRR1A* mRNA expression data were extracted from this reference.

Statistical analysis. For continuous variables, statistical analyses were performed using Student's t-test for comparisons of *ZNF750* mRNA expression according to the DNA mutation status (un-paired t-test) and between corresponding tumor and normal tissues (paired t-test). The degree of linearity was estimated by Pearson's correlation coefficient. The association between *ZNF750* mRNA expression and clinicopathological factors was estimated by the χ^2 test with Yates' correction. Overall survival was estimated using the Kaplan-Meier method, and survival curves were compared using log-rank tests. Univariate and multivariate analyses were used to determine patient prognosis and were performed by Cox regression analysis with a backward stepwise model. Based on the levels of *ZNF750* mRNA expression in the Kyushu and the TCGA datasets, patients were divided into two groups using

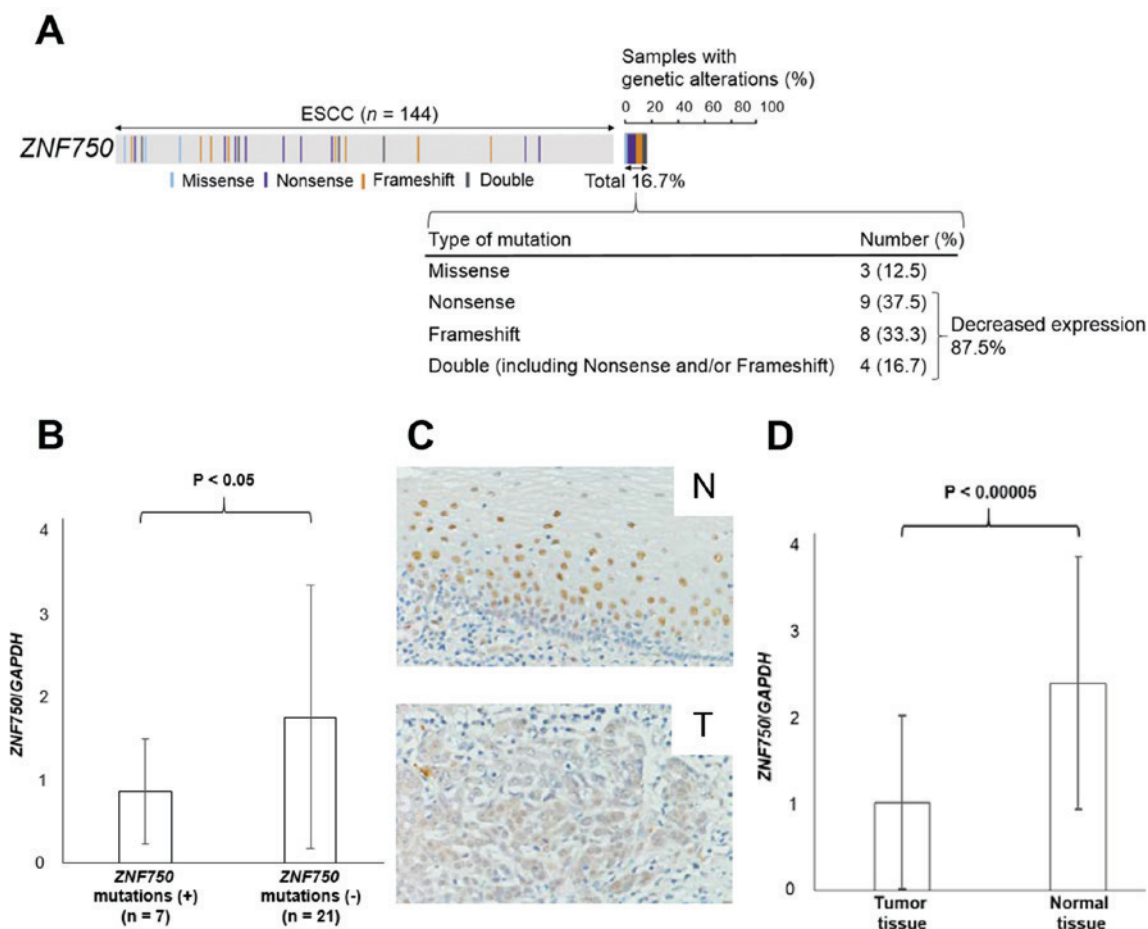


Figure 1. *ZNF750* expression in ESCC. (A) Overview of genetic alterations of *ZNF750* across 144 patients with ESCC from a previous report by the present authors (4). (B) *ZNF750* mRNA expression in ESCC according to DNA mutation status. ($P < 0.05$; Student's t-test). (C) Immunohistochemical staining of *ZNF750* expression in representative samples of ESCC. Original magnification, x400. (D) *ZNF750* mRNA expression in ESCC assessed by quantitative reverse transcription polymerase chain reaction. ($P < 0.00005$; Student's t-test). N, normal tissue; T, Tumor tissue. ESCC, esophageal squamous cell carcinoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *ZNF750*, zinc finger protein 750.

the minimum P-value approach, a comprehensive method that determines the optimal risk separation cutoff point in continuous gene expression measurement (19). The cutoff values for high and low *ZNF750* expression groups were 8.34 (*ZNF750*/*GAPDH* expression) in the Kyushu dataset and 2.75 (log₂ expression) in the TCGA dataset. All tests were analyzed by using the JMP v.11 software (JMP, Cary, NC, USA). Depth of tumor invasion was classified according to the 7th edition of the International Union against Cancer TNM classification system (20). Histological grade of the tumor was classified according to the 11th edition of the Japanese Classification of Esophageal Cancer (21).

Results

Downregulation of *ZNF750* expression in ESCC. It was revealed that 16.7% of patients with ESCC exhibited mutations in the *ZNF750* gene (4) (Fig. 1A). The majority of the mutations (87.5%) were nonsense and/or frameshift mutations that caused reduced expression of *ZNF750* (Fig. 1A). As expected, *ZNF750* mRNA expression was significantly lower in patients with *ZNF750* nonsense and/or frameshift mutations (n=7) compared with those without mutations (n=21) ($P < 0.05$; Fig. 1B). Consistent with these data, the immunohistochemical

analysis revealed that *ZNF750* was stained less intensely in the nuclei of ESCC tumor cells compared with those of normal esophageal epithelial cells in 4 of 5 (80.0%) patients (Fig. 1C). Additionally, RT-qPCR analysis also demonstrated that *ZNF750* mRNA expression in tumor tissues was significantly lower compared with paired normal tissues ($P < 0.00005$) (Fig. 1D). In fact, *ZNF750* mRNA expression levels in tumor tissues were lower compared with normal tissues in 78.9% of 76 patients with ESCC.

Clinicopathological significance of *ZNF750* mRNA expression in ESCC. The association between *ZNF750* mRNA expression levels in tumor tissues and clinicopathological factors in ESCC is summarized in Table I. The low *ZNF750* expression group (n=37) exhibited a higher frequency of tissues showing poorly differentiated histology ($P < 0.05$) compared with the high expression group (n=39). No significant associations between *ZNF750* mRNA expression and age, sex, depth of invasion, lymph node metastasis, lymphatic invasion, venous invasion or distant metastasis were observed.

Prognostic significance of *ZNF750* mRNA expression in ESCC. The overall survival rate of patients in the low *ZNF750* expression group was significantly lower compared with

Table I. Association between *ZNF750* mRNA expression of tumor tissues and clinicopathological factors in ESCC.

Factors	High <i>ZNF750</i> expression (n=39) n, (%)	Low <i>ZNF750</i> expression (n=37) n, (%)	P-value
<65	20 (51.3)	18 (48.6)	NS
≥65	19 (48.7)	19 (51.4)	
Male	35 (89.7)	32 (86.5)	NS
Female	4 (10.3)	4 (10.8)	
NA	0 (0)	1 (2.7)	
Well	17 (43.6)	7 (18.9)	<0.05
Moderate/poor	17 (43.6)	22 (59.5)	
NA	5 (12.8)	8 (21.6)	
≤submucosa (T1)	8 (20.5)	2 (5.4)	NS
≥muscularis propria (T2-T4)	31 (79.5)	35 (94.6)	
Absent	12 (30.8)	10 (27.0)	NS
Present	27 (69.2)	27 (73.0)	
Absent	8 (20.5)	3 (8.1)	NS
Present	31 (79.5)	33 (89.2)	
NA	0 (0)	1 (2.7)	
Absent	8 (20.5)	2 (5.4)	NS
Present	31 (79.5)	34 (91.9)	
NA	0 (0)	1 (2.7)	
Absent	38 (97.4)	36 (97.3)	NS
Present	1 (2.6)	1 (2.7)	

NA, not available; NS, not significant.

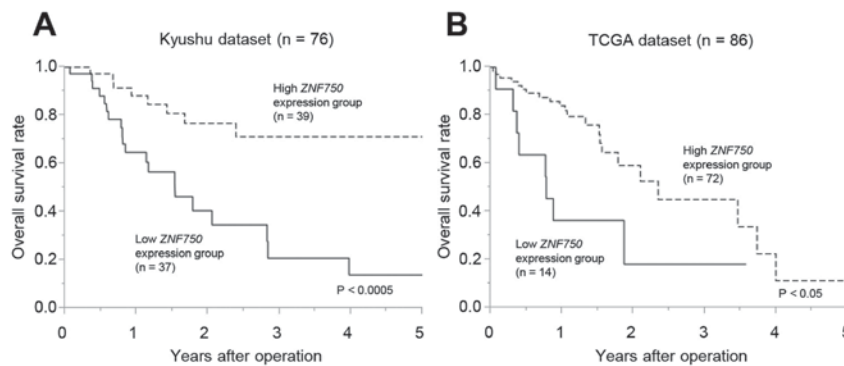


Figure 2. Prognostic significance of *ZNF750* mRNA expression in ESCC. Overall survival rate of patients with ESCC according to *ZNF750* mRNA expression in tumor tissues. (A) Kyushu dataset ($P < 0.0005$). (B) TCGA dataset ($P < 0.05$). TCGA, The Cancer Genome Atlas; ESCC, Esophageal squamous cell carcinoma; *ZNF750*, zinc finger protein 750.

the high expression group in the Kyushu dataset ($P < 0.0005$; Fig. 2A) and the TCGA dataset ($P < 0.05$) (Fig. 2B). In the univariate analysis, higher T factor (T2-4), lymph node metastasis, lymphovascular invasion and low *ZNF750* mRNA expression were significantly associated with a lower overall survival rate (Table II). The multivariate analysis demonstrated that lymphatic invasion ($P < 0.05$) and low *ZNF750* mRNA expression ($P < 0.05$) were independent poor prognostic factors in ESCC (Table II).

Correlation between ZNF750 mRNA expression levels and epithelial differentiation genes in ESCC. In order to validate the

clinicopathological analysis of *ZNF750* in ESCC, GSEA was applied to patients with ESCC (NCBI accession no. GSE2533). GSEA demonstrated that *ZNF750* expression was significantly correlated with epithelial differentiation genes (Fig. 3A), which suggests that *ZNF750* serves a role in epithelial differentiation in the development of ESCC. Among those genes, *SPRR1A*, an early epithelial differentiation marker (22), was ranked at the upper level of epithelial differentiation gene signature. The analysis confirmed that *ZNF750* mRNA expression was positively correlated with *SPRR1A* mRNA expression using the CCLE dataset ($R = 0.71$; Fig. 3B) and the Kyushu dataset ($R = 0.56$; Fig. 3C).

Table II. Univariate and multivariate analysis of clinicopathological factors affecting overall survival rate.

Factors	Univariate analysis			Multivariate analysis		
	RR	95% CI	P-value	RR	95% CI	P-value
Age (≥ 65 / <65)	0.93	0.46-1.91	NS	1.68	0.58-5.15	NS
Sex (male/female)	1.19	0.45-4.13	NS	1.21	0.26-7.54	NS
Histology grade (well/moderate and poor)	0.62	0.24-1.51	NS	1.89	0.59-5.66	NS
T factor (T1/T2-4)	0.31	0.07-0.94	<0.05	0.22	0.03-1.11	NS
Lymph node metastasis (negative/positive)	0.43	0.17-0.98	<0.05	0.53	0.11-1.92	NS
Lymphatic invasion (negative/positive)	5.42×10^{-10}	0.34-2.96	<0.005	3.37×10^{-10}	0.47-2.11	<0.05
Venous invasion (negative/positive)	6.02×10^{-10}	0.48-2.08	<0.05	1.67×10^{-9}	0.37-2.68	NS
Distant metastasis (negative/positive)	5.84	0.92-20.79	NS	11.01	0.51-23.34	NS
<i>ZNF750</i> mRNA expression (high/low)	3.83	1.82-8.59	<0.0005	3.83	1.22-13.44	<0.05

RR, relative risk; CI, confidence interval; NS, not significant; T, Depth of tumor invasion.

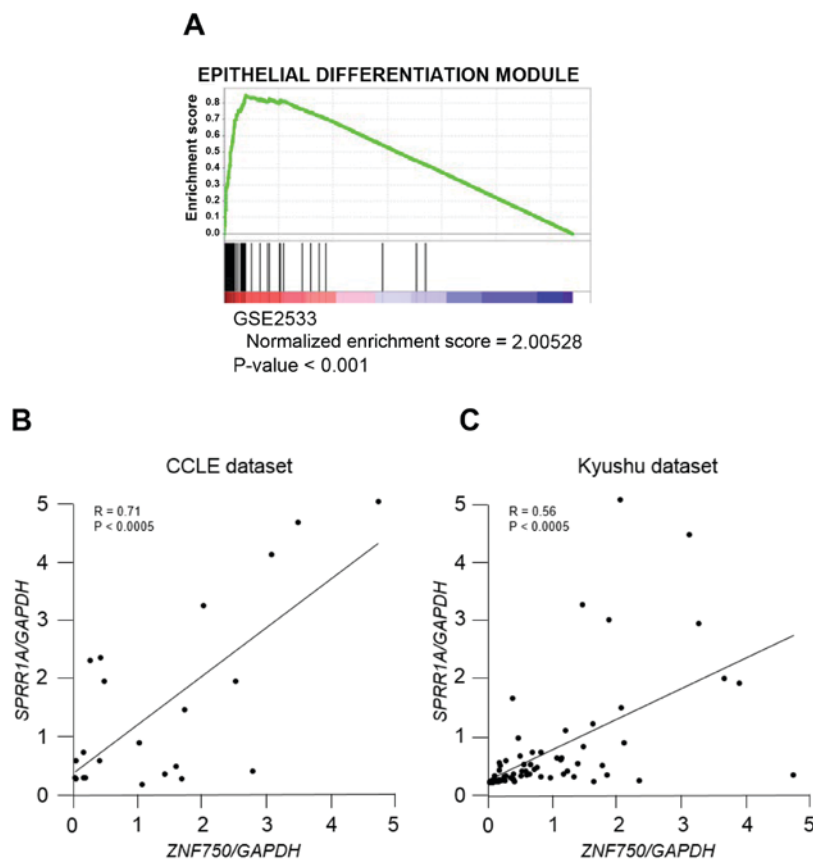


Figure 3. Correlation between *ZNF750* mRNA expression levels and epithelial differentiation genes in ESCC. (A) Gene set enrichment analysis of interrelated *ZNF750* expression and epithelial differentiation module using reference gene sets in the ESCC dataset. (B) Correlation between *ZNF750* and *SPRR1A* expression in the CCLE dataset. (C) Correlation between *ZNF750* and *SPRR1A* expression in the Kyushu dataset. ESCC, esophageal squamous cell carcinoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *SPRR1A*, small proline rich protein 1A; *ZNF750*, zinc finger protein 750; CCLE, Cancer Cell Line Encyclopedia.

Discussion

In the present study, the clinicopathological and prognostic significance of *ZNF750* expression, a novel candidate tumor suppressor gene previously described by the present authors (4), was assessed. In order to assess the prognostic significance of *ZNF750* expression, two independent ESCC

datasets were used. In addition, an association between *ZNF750* expression and epithelial differentiation in ESCC was identified. The data of the present study suggested that the downregulation of *ZNF750* expression due to inactivating mutations (nonsense/frameshift mutations) induced the development or aggressiveness of ESCC, potentially via dysregulation of esophageal epithelial differentiation.

Although other factors such as hyper-methylation or regulation by microRNA may also downregulate the expression of *ZNF750*, to the best of our knowledge, this is the first study to explore the clinical significance of *ZNF750* expression in ESCC.

The present clinicopathological study revealed that the tissues from the low *ZNF750* expression group exhibited a higher frequency of poorly differentiated histology compared with the high expression group. Previously, it was suggested that *ZNF750* function was associated with epithelial differentiation (5-7,10,11). These data suggested that a low expression of *ZNF750* is associated with tumor aggressiveness in ESCC.

Additionally, it was demonstrated that *ZNF750* expression was reduced in tumor tissues, and that low expression of *ZNF750* was an independent poor prognostic factor in ESCC. In a previous study by the present authors (4), it was also demonstrated that the majority of the *ZNF750* mutations were accompanied by loss of heterozygosity. These data strongly support the hypothesis that *ZNF750* is a tumor suppressor gene, as previously identified by WES analysis. In addition, low *ZNF750* expression levels in tumor tissues suggest that it is a promising biomarker capable of predicting poor outcomes for patients with ESCC.

It has been reported that *ZNF750* controls epithelial homeostasis by repressing proliferation genes and inducing differentiation genes (5-7). Previous studies indicated that mutations in *ZNF750* were pathogenic and disrupt epithelial homeostasis, as observed in psoriasis (23). The present study suggested that *ZNF750* expression was significantly correlated with epithelial differentiation genes such as *SPRR1A*, an early epithelial differentiation marker, in ESCC. The downregulation of *ZNF750* expression in ESCC may also disrupt epithelial homeostasis, followed by the development of ESCC, according to Tetreault *et al* (24), who demonstrated that the downregulation of *Kruppel like factor 4*, an important epithelial differentiation gene, induced malignant transformation of esophageal epithelium. However, the function of the *ZNF750* gene in ESCC has not been fully elucidated. Additional large clinical studies and biological experiments for *ZNF750* in ESCC are required to clarify this.

In conclusion, *ZNF750* expression was low in ESCC tumor tissues, and its reduction was associated with poor differentiation. In addition, low *ZNF750* expression was an independent poor prognostic factor in ESCC. These data provide evidence that *ZNF750* is a novel tumor suppressor gene, and may be a therapeutic target and useful biomarker of poor clinical outcome in patients with ESCC.

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