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PROGNOSTIC VALUE OF HIGH DDX39 EXPRESSION IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA

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Abstract **: Background:** This study was aimed to validate a prognostic biomarker, adenosine triphosphate-dependent RNA helicase DDX39, in esophageal squamous cell carcinoma (ESCC). Novel prognostic biomarkers may help design more effective treatments for ESCC in the clinic.
Methods: The expression level of DDX39 was bioinformatically detected and immunohistochemically examined in 95 ESCC cases. Correlation analyses were performed by χ^2 test between DDX39 expression and clinicopathological parameters, and univariate analyses were performed to assess the prognostic value of DDX39 expression in ESCC.
Results: Bioinformatics analysis showed DDX39 upregulated in esophageal cancer , while immunohistochemical study revealed that 61.1% of the 95 patients showed high DDX39 expression in ESCC tissues, and univariate analyses demonstrated that DDX39 expression was a prognostic factor in ESCC.
Conclusions: These results established the clinical utility of DDX39 expression as a novel prognostic biomarker in ESCC, suggesting that DDX39 may be a potential therapeutic target for treating ESCC patients with elevated DDX39 expression.

Keywords **–** esophageal squamous cell carcinoma, DDX39, survival analysis

I. INTRODUCTION

Esophageal cancer is the ninth most common cancer in the world and causes 9.8 million disability-adjusted life years in 2013 [1]. Esophageal squamous cell carcinoma (ESCC), one of the major histological types of esophageal cancer, has a poor diagnosis rate at advanced stages and accounts for more than 90% of all esophageal cancer cases in China [2]. Current treatments for ESCC include surgery, chemotherapy and radiation. In the clinic, biomarkers have been used to diagnose and treat cancer, thereby improving the long-term survival of cancer patients [3,4]. Although diagnostic biomarkers for ESCC are emerging, such as TP53, it is critical to discover more biomarkers to improve ESCC diagnosis and treatment

The present study was aimed to assess the potential of the DEAD-box helicase 39 (DDX39), a member of the Asp-Glu-Ala-Asp box RNA helicase family [5], as a prognostic marker in ESCC. DDX39 regulates mRNA export, RNA secondary structure unwinding, and gene expression [6], and DDX39 expression is de-regulated in cancer. For example, DDX39 expression is up-regulated in lung squamous cell carcinoma [7]. In gastrointestinal stromal cancer, both proteomics study and immunochemistry staining showed that high DDX39 expression correlated with poor prognosis [8,9]. Moreover, DDX39 suppresses tumor invasion in urinary bladder cancer [10].

Previous reports have shown that DDX39 participates in biological processes such as cancer cell survival and growth, and overexpression of DDX39 promotes tumor cell growth [7,8]. The oncogenic potential of DDX39 may be related to its role in maintaining genome and telomere integrity. Yoo and Chung found that DDX39 overexpression in telomerase-positive human cancer cells led to progressive telomere elongation, and depletion of endogenous DDX39 by small hairpin RNA resulted in telomere shortening [11]. Telomere maintenance requires the interaction between telomeric DNA repeats and specific binding proteins [12], in which TRF2 signaling pathway plays an important role, and DDX39 modulates TRF2 signaling by binding to the TRFH domain of TRF2 [11]. Here we found that DDX39 expression is up-regulated in ESCC, and high DDX39 expression is an indicator of poor ESCC prognosis.

II. MATERIALS AND METHODS

A. Bioinformatics analysis

Data on 11 normal esophagi and 162 ESCC samples from the Cancer Genome Atlas database (TCGA) were collected and analyzed with Ballgown to search for genes showing ≥2-fold differential tumor vs. normal tissue expression. The search revealed that the average expression level of DDX39 was increased in ESCC by 2.0-fold. We then chose DDX39 as our candidate to compare its expression in different patient

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groups, performed survival analysis using the log-rank test, and generated survival curves.

B. Patients and tissue samples

Ninety-five patients with diagnosed and pathologically confirmed ESCC were evaluated in the Meizhou People's Hospital of Guangdong Province. Demographic and clinicopathological data, including age, sex, histological grade, T classification, lymphatic invasion, metastasis and vital states (at follow-ups), were collected from patient's medical records. All patients underwent curative surgery between June 2003 and June 2007, and none of them received radiotherapy or chemotherapy prior to curative surgery. Patients' health information was collected at follow-up examinations from 1 to 39 months after surgery. Written informed consent was obtained from all patients, and this study was approved by the Research Ethics Committee of Meizhou People's Hospital. Tissues were sectioned and histologically examined by hematoxylin and eosin (H&E) staining for the presence of ≥80% tumor cells (cancer samples) or of only normal cells without inflammation and tumor invasion (matched normal samples).

C. Immunohistochemical staining

Formalin-fixed, paraffin-embedded tissues of 95 ESCC patients were analyzed by immunohistochemical staining. Sections (4 μm-thick) were cut, baked at 65°C for 60 min, deparaffinized in xylene, and rehydrated in alcohol and distilled water. Epitope retrieval was performed by incubating sections in boiling citrate buffer (pH 6.0) for 10 min. Sections were washed in phosphate buffered saline three times, and incubated in 3% hydrogen peroxide for 10 min to eliminate endogenous peroxidase. Sections were then incubated with rabbit monoclonal DDX39 antibody (ab176348, 1:100; Abcam, Cambridge, MA, USA) at room temperature for 3 h, followed by incubation with goat anti-rabbit IgG horseradish peroxidase polymer (Envision PO System; DAKO, Glostrup, Denmark) for 30 min at room temperature. Antigen was detected with 3,3-diaminobenzidine, and sections were counterstained with hematoxylin.

D. Scoring

Immunohistochemical analysis was carried out by two pathologists who were blind to the clinical data (Y.J.C and H.J.X.). DDX39 signal was observed in the nuclei of ESCC tissue cells but not in normal epithelia. Tissues with ≥50% cancer cells showing DDX39 expression in ten random fields under a microscope were deemed high-expression, and those with <50% cells showing DDX39 expression were deemed low-expression [9].

E. Statistical analysis

Data were analyzed with SPSS software (version 13.0). The correlation between DDX39 protein levels and clinicopathological features was analyzed by the Chi-square test. Kaplan-Meier and log-rank tests were used to compare overall survival rates. The univariate analysis model includes gender, age at surgery, tumor size (<5 cm vs. >5 cm), histological grade (grade I vs. grade II and grade III), TNM stage (stage I+II vs. III+IV), T stage (T1+T2 vs. T3+T4), and DDX39 expression (high vs. low). In all statistical analyses, a P value of <0.05 was considered statistically significant, and all P values were calculated by two-tailed tests.

III. RESULTS

A. DDX39 expression is upregulated in human esophageal cancer

Based on our bioinformatics analysis (Table S1), DDX39 expression is upregulated in esophageal carcinoma patients at RNA level. We collected gene expression data on 162 esophageal cancer and 11 normal cases, and examined the relationship between DDX39 expression and patients' overall survival. Based on the survival curves in Fig. 1, high DDX39 expression correlates with shorter survival time (P < 0.05).

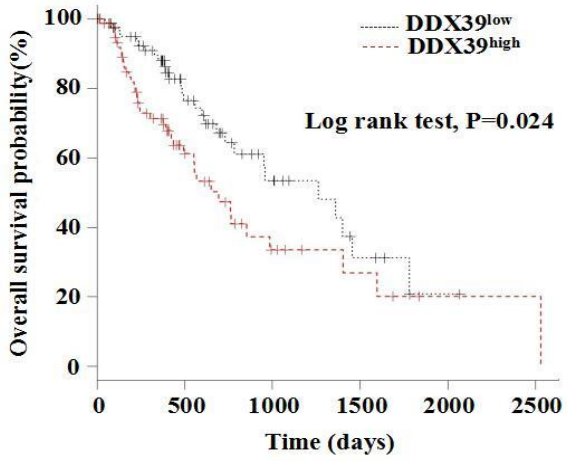

Fig. 1 Survival curves of esophageal carcinoma patients divided into two groups based on DDX39 expression levels. Data were obtained from the Cancer Genome Atlas database, and high expression level of DDX39 correlates with poor prognosis.

B. Clinical characteristics of ESCC patients

The characteristics of the 95 ESCC patients enrolled in this study were listed in Table 1. Their median age was 55 years (ranging from 26 to 75 years), and the ratio of male to female patients was 3.75:1. The number of cases in histological grade I, II and III was 14 (14.7%), 73 (76.8%) and 8 (8.4%), respectively. The number of cases with and without lymphatic invasion was 55 (57.9%) and 40 (42.1%), respectively. The distribution of tumor stages was: 27 T1+T2 cases (28.9%), and 68 T3+T4 cases (68.8%).

C. Expression and cellular localization of DDX39 in ESCC tissues

We examined DDX39 expression in primary tumors of the 95 ESCC patients by immunohistochemical staining. Immunohistochemistry showed DDX39 is localized in the tumor cell nuclei and is absent in normal tissues (Figure 2). Low DDX39 expression was observed in sample B from a patient with good treatment outcome, and high DDX39

expression was observed in sample C from a patient with poor treatment outcome.

TABLE I. CLINICOPATHOLOGICAL CHARACTERISTICS OF PATIENTS, AND DDX39 EXPRESSION IN ESCC TISSUES

Characteristics	Number of cases
Age (years)	
≥ 60	35 (36.8%)
< 60	60 (63.2%)
Gender	
Male	75 (78.9%)
Female	20 (21.1%)
Histological grade	
I	14 (14.7%)
II + III	81 (85.3%)
T classification	
T1	2 (2.1%)
T2	25 (26.8%)
T3	54 (54.7%)
T4	14 (14.1%)
Lymphatic invasion	
No	55 (57.9%)
Yes	40 (42.1%)
Metastasis	
No	75 (78.9%)
Yes	20 (21.1%)
Vital states (at follow-ups)	
Alive	32 (33.7%)
Dead	63 (66.3%)
DDX39 Expression	
Low expression	37 (38.9%)
High expression	58 (61.1%)

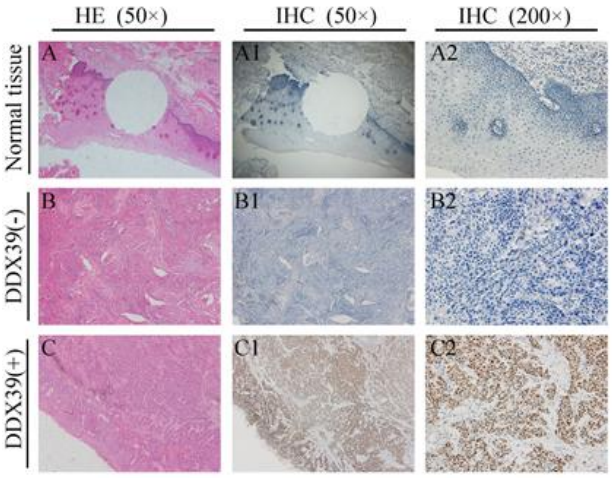

Fig. 2 Detection of DDX39 by immunohistochemical staining, shown here are examples of normal tissue, low and high DDX39 expression in patient samples. A, B, and C: H&E staining, 50×; A1, B1, and C1: DDX39 immunohistochemistry, 50×; A2, B2, and C2: DDX39 immunohistochemistry, 200×.

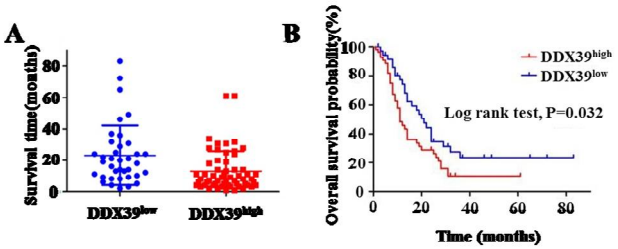

Fig. 3 Evaluation of the association between DDX39 expression and clinical outcomes. (A) Comparison of survival time between patients with low and high DDX39 expression. (B) High DDX39 expression significantly correlated with poor prognosis (P = 0.032).

D. Survival analysis of ESCC patients based on DDX39 expression

After scoring the expression level of DDX39 and grouping 95 ESCC patients based on DDX39 expression, as shown in Table 2, we found that 58 patients showed high DDX39 expression in ESCC tissues and the other 37 had low DDX39 expression. Chi-Square test showed that DDX39 expression correlated with the histological grade of ESCC (P < 0.05), but not with tumor size, T stage or N stage.

TABLE III. CORRELATION BETWEEN DDX39 EXPRESSION AND CLINICOPATHOLOGIC CHARACTERISTICS OF ESCC PATIENTS

Parameters	***DDX39* expression**		***P* value**
	Low	**High**	
Age (years)			
≤ 60	22 (59.5%)	15 (40.5%)	0.551
> 60	38 (65.5%)	20 (34.5%)	
Gender			
Male	29 (38.7%)	46 (61.3%)	0.913
Female	8 (40.0%)	12 (60.0%)	
Histological grade			
I	9 (64.3%)	5 (35.7%)	0.035
II + III	28 (34.6%)	53 (65.4%)	
T grade			
T1 + T2	10 (37.0%)	17 (63.0%)	0.810
T3 + T4	27 (39.7%)	41 (60.3%)	
Lymphatic invasion			
No	22 (40.0%)	33 (60.0%)	0.805
Yes	15 (37.5%)	25 (62.5%)	
Metastasis			
No	29 (38.7%)	46 (61.3%)	0.913
Yes	8 (40.0%)	12 (60.0%)	
Vital states (at			
Alive	12 (37.5%)	20 (62.5%)	0.837
Dead	61 (39.7%)	38 (60.3%)	

TABLE IIIII
UNIVARIATE SURVIVAL ANALYSIS OF 95 ESCC PATIENTS BY LOG-RANK TEST

Patients	N	Mean ±	Median ±	*P*
Gender				0.257
Male	75	24.43 ±	13.00 ± 1.42	
Female	20	27.34 ±	24.00 ± 4.	
Age at surgery				0.411
≤ 55	52	24.49 ±	13.00 ± 1.52	
> 55	43	25.57 ±	20.00 ± 3.44	
Tumor size				0.478
≤ 5	53	23.10 ±	18.00 ± 3.29	
> 5	42	25.19 ±	12.00 ± 1.36	
Histological				0.775
I	14	22.11 ±	24.00 ± 2.91	
II + III	81	26.56 ±	14.00 ± 0.97	
TNM stage				0.352
I + II	60	27.29 ±	14.00 ± 2.52	
III + IV	35	21.36 ±	12.00 ±2.68	
T stage				0.960
T1 + T2	27	25.35 ±	14.00 ± 5.22	
T3 + T4	68	24.34± 3.12	14.00 ± 3.03	
DDX39				0.032
Low	37	32.57 ±	21.00 ± 2.84	
High	58	18.38 ±	11.00 ± 1.58	

*: Low DDX39 expression versus high *DDX39* expression: $P < 0.05$

E. UPREGULATION OF DDX39 IS ASSOCIATED WITH POOR PROGNOSIS OF ESCC

We analyzed the association between DDX39 expression and the clinicopathological features of ESCC patients. As shown in Table 3, in the 58 patients with high DDX39 expression, the mean overall survival was 18.38±2.59 years, which was significantly shorter than that of 37 patients with low DDX39 expression (32.57±5.17 years). The median overall survival was 11.00±1.58 years for DDX39high patients, significantly shorter than that of DDX39low patients (21.00±2.84 years). The survival curves in Fig. 2B showed that high DDX39 expression correlated with poor prognosis of ESCC ($P < 0.05$).

IV. DISCUSSION

ESCC is an aggressive disease with high mortality, and patients with advanced ESCC usually show poor prognosis. Novel biomarkers are valuable for developing effective personalized and targeted therapies [13]. Biomarkers involved in the mRNA processing pathway were reported to play a vital role in ESCC, such as TWIST1 and OCT4 [14].

DEAD-box RNA helicases, which contain a conserved Asp-Glu-Ala-Asp motif, are vital players of RNA metabolism. They unwind double-stranded RNA molecules, and play important roles in transcription, splicing, RNA transport, ribosome biogenesis, RNA editing, RNA decay, and translation [15-17]. The role of DEAD-box RNA helicases in cancer is complex, for many of them are deregulated in cancer and have been reported to possess either oncogenic potential or tumor suppressive properties in a context-dependent manner [18]. For example, one member of this family, DDX46, promotes ESCC occurrence and development [19].

DDX39, another member of the DEAD-box RNA helicase family, has been reported to be involved in the carcinogenesis and be associated with poor prognosis of several digestive cancers [8-9, 20]. Proteomic analysis revealed that DDX39 is a potential biomarker in gastrointestinal stromal tumor, hepatocellular carcinoma and neuroblastoma [9,21-22]. In the present study, we performed immunohistochemical staining to detect DDX39 expression in ESCC patients, and found that DDX39 is overexpressed in ESCC, and higher DDX39 expression is associated with shorter overall survival of ESCC patients, suggesting that DDX39 is potentially a novel prognostic biomarker in ESCC.

Although there was no correlation between DDX39 expression and clinicopathological features in this study, univariate survival analyses suggest that poor prognosis of ESCC patients could be related to high DDX39 expression. Based on previous cancer research, DDX39 plays important roles in RNA splicing, metastasis and invasion, and drug resistance [19,20]. We plan to investigate the role of DDX39 in ESCC at molecular and cellular levels in the future.

In summary, we have demonstrated that DDX39 expression is associated with the prognosis of ESCC patients. Our findings warrant further investigation of the molecular mechanism underlying DDX39 overexpression in ESCC, and large-scale studies to validate the prognostic value of DDX39 expression in ESCC.

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