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High expression of HLA-DQA1 predicts poor outcome in patients with esophageal squamous cell carcinoma in Northern China

Fang-Fang Shen, MS[a],[*], Ying Pan, MD[a], Jing-Zhong Li, BS[a], Fang Zhao, MS[b], Hai-Jun Yang, MS[b], Jun-Kuo Li, MS[b], Zhao-Wei Gao, MS[b], Jing-Fen Su, MS[b], Li-Juan Duan, MS[b], Shu-Min Lun, MS[b], Peng Zhang, MD[c], Lin-Qiang Tian, MD[a], Gang Sun, MS[a], Da Huang, MS[a], Yan-Tian Cao, MS[a], Fu-You Zhou, MD, PhD[a],[b],[*]

Abstract

Background: Our previous studies demonstrate that the major histocompatibility complex (MHC) is associated with the progression of esophageal squamous cell carcinoma (ESCC). HLA-DQA1, which belongs to the MHC Class II family, may be a potential biomarker in ESCC progression. However, the association between HLA-DQA1 and ESCC in high-incidence area of northern China has not been well characterized. The purpose of this study is to investigate the relationship of HLA-DQA1 expression with the progression and prognosis of ESCC.

Methods: We analyzed the expression profiles of HLA-DQA1 in esophageal cancer (EC) samples in the TCGA database and validated HLA-DQA1 expression by immunohistochemistry, western blotting, and quantitative reverse-transcription polymerase chain reaction in matched EC and normal tissues, respectively. The correlation between HLA-DQA1 expression and clinicopathologic characteristics of ESCC was further analyzed.

Result: Immunohistochemical analysis indicated that the expression level of HLA-DQA1 in ESCC tissues was significantly higher than the matched normal tissues ($P < .001$). HLA-DQA1 mRNA and protein expression were significantly higher in ESCC tissues compared to the matched normal tissues. Patients with family history negative or with tumor sizes >4 cm were associated with higher HLA-DQA1 expression levels. A prognostic significance of HLA-DQA1 was also found by the Log-rank method, in which high expression of HLA-DQA1 was correlated with a shorter overall survival time. The receiver operating characteristic (ROC) curve analysis yielded the area under the ROC curve value of 0.693. Univariate and multivariate analyses also suggest that high expression of HLA-DQA1 is a potential indicator for poor prognosis of ESCC.

Conclusions: Our results demonstrate that HLA-DQA1 plays an important role in ESCC progression and may be a biomarker for ESCC diagnosis and prognosis, as well as a potential target for the treatment of patients with ESCC.

Abbreviations: AUC = area under the ROC curve, EC = esophageal cancer, ESCC = esophageal squamous cell carcinoma, HR = hazard ratio, MHC = major histocompatibility complex, ROC = receiver operating characteristic.

Keywords: esophageal squamous cell carcinoma, HLA-DQA1, prognosis, progression

[a] The Key Laboratory for Tumor Translational Medicine, The Third Affiliated Hospital of Xinxiang Medical University, Xinxiang, [b] Anyang Key Laboratory for Esophageal Cancer Research, Anyang Tumor Hospital, Anyang, [c] Henan Key Laboratory for Esophageal Cancer Research, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China.

[*] Correspondence: Fang-Fang Shen, The Third Affiliated Hospital of Xinxiang Medical University, Xinxiang, Henan 453003, China (e-mail: ffshen2010@163.com); Fu-You Zhou, Anyang Key Laboratory for Esophageal Cancer Research, Anyang Tumor Hospital, Anyang, Henan 455000, China (e-mail: ayzhoufuyou@163.com).

1. Introduction

Despite the current advances in the treatment strategies for esophageal cancer (EC), still, it ranks the 6th most common cause of cancer-related deaths worldwide.[1,2] The 5-year overall survival of patients with EC ranges from 15% to 25%.[3] Around 80% (328,800/400,200) of EC-related deaths occur in less developed regions, especially the rural regions around the Taihang Mountain in the junction of Henan, Hebei, and Shanxi provinces in northern China.[2,4–6] In those places, esophageal squamous cell carcinoma (ESCC) is the predominant histologic type of EC. Due to the fact that ESCC is tightly associated with extensive lymphatic spread and vascular invasion, ESCC is often diagnosed at advanced stage and the survival rate of ESCC is very poor.[7] Therefore, a better understanding of the molecular mechanisms underlying ESCC progression could improve the early diagnosis, treatment strategy, and overall prognosis of this deadly disease.

Our previous studies[8–10] have demonstrated that the major histocompatibility complex (MHC) region of human chromosome is associated with the progression of ESCC. HLA-DQA1, which is one of the MHC Class II family members and locates on

chromosome 6p21, may be a potential prognostic biomarker for ESCC.[8] A major function of MHC Class II molecules in the immune system is to present antigens derived from extracellular proteins for recognition by CD4+ T-cells.[11] Aberrant expression of HLA-II may result in insufficient immune response or autoimmunity reaction, which may result in lots of diseases including cancers.[12,13] More importantly, numerous studies have revealed that HLA-II members are involved in cancers like ESCC.[14–17] However, the association between HLA-DQA1 and ESCC in high-incidence area of northern China has not been well characterized.

In this study, we analyzed the expression profiles of HLA-DQA1 in EC samples in the database (from TCGA: The Cancer Genome Atlas), and validated the expression of HLA-DQA1 in matched EC and normal tissues by techniques including immunohistochemistry, western blotting, and reverse-transcription polymerase chain reaction (RT-PCR). The relationship between HLA-DQA1 expression level and clinicopathologic characteristics was investigated. Moreover, we assessed the link between HLA-DQA1 expression and the prognosis of ESCC. To our knowledge, no previous studies have reported HLA-DQA1 expression associated with the prognosis of ESCC.

2. Materials and methods

2.1. Ethics statement

The study protocol was approved by the ethical review committee of the Third Affiliated Hospital of Xinxiang Medical University. All the assays were conducted according to Declaration of Helsinki principles. Written informed consent was obtained from all the participants.

2.2. Recruitment of patients and controls

A total of 97 cases of matched ESCC and normal tissue samples (3–7 cm away from the tumor margin) were obtained from Anyang Tumor Hospital (Henan, China) ranging from January, 2011 to February, 2012. All specimens for total RNA and protein extraction were stored at −80°C after immediate snap-frozen in liquid nitrogen. All the cases were histopathologically confirmed and there was no prior treatment to those patients. Patient demographic, clinical, and pathologic data were recorded. All the tumors were confirmed to contain >80% tumor cells by histologic examination of sequential sections. Patients were classified according to TNM staging (American Joint Committee on Cancer, 7th edition). All the personal information was concealed to ensure the privacy. The clinicopathologic characteristics of the patients with ESCC are summarized in Table 1.

2.3. Real time PCR

Total RNA of the tissues was extracted by using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, California), according to the manufacturers protocol. The complementary DNA was synthesized using reverse transcription kit (Tiangen, Beijing, China) and stored at −20°C. The levels of messenger RNA (mRNA) of HLA-DQA1 expression and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined using the ABI 7500 FAST sequence detection system (Applied Biosystems, Thermo Fisher Scientific, Inc., Carlsbad, California). GAPDH was used as a control gene for the quantitative RT-PCRs (qRT-PCRs). The primer sequences were sense/anti-sense:

Table 1

Clinicopathologic characteristics of 97 esophageal squamous cell carcinoma cases.

Variables	N	% of cases
Age, yr		
≤65	58	59.8
>65	39	40.2
Gender		
Female	43	44.3
Male	54	55.7
Family history		
Positive	35	36.1
Negative	62	63.9
Drinking history, yr		
≤10	66	68.0
>10	31	32.0
Smoking history, yr		
≤10	63	64.9
>10	34	35.1
Differentiation		
Well	13	13.4
Moderate	52	53.6
Poor	32	33.0
T classification		
T1–T2	25	25.8
T3	72	74.2
N classification		
N0	50	51.5
N1 + N2 + N3	37	38.1
Tumor size, cm		
≤4	59	60.8
>4	38	39.2
Location		
Upper	17	17.5
Middle	65	67.0
Low	15	15.5

HLA-DQA1:

5′-GAGGTGAAGACATTGTGGCT-3′
5′-CGTAGAACTGCTCATCTCCAT-3′

GAPDH:

5′-GACTCATGACCACGTCCATGC-3′
5′-AGAGGCAGGGATGATGTTCTG-3′

All the reactions were done in triplicate using 20 μL samples containing 50 ng of complementary DNA. The cycling condition is listed as follows: 10 minutes at 95°C, 1 cycle; (95°C, 15 seconds; 60°C, 60 seconds) for 40 cycles. The data were analyzed using the ABI 7500 FAST Sequence Detection software. Each reaction was performed in triplicate and the $2^{-\Delta\Delta Ct}$ method was used to calculate the expression of HLA-DQA1.[18]

2.4. Western blotting analysis

The tumor tissues were collected and lysed on ice according to the manufacture protocol. After spinning at 12,000 g for 20 minutes, the concentration of protein was measured, and then the samples were denatured by boiling for 10 minutes at 100°C and loaded into sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%) gel for electrophoresis. The proteins were transferred onto polyvinylidene fluoride membrane (Millipore, Bedford, Massachusetts), which was then incubated in the blocking

solution at room temperature for 1 hour. Anti-HLA-DQA1 (1:500; Bioss, Woburn, Massachusetts) and anti-GAPDH (1:1000; Cell Signaling Technology, Massachusetts) were incubated at 4°C overnight. The membranes were subsequently incubated with horse-radish peroxidase-labeled goat anti-rabbit IgG and protein expression was normalized against GAPDH expression. Digital imaging and signal quantification were performed on a Beyo ECL Plus Detection System (Beyotime, Shanghai, China) using Bio-Rad Image Lab Software (Hercules, California).

2.5. Immunohistochemistry staining

Paraffin-embedded sections (5 μm) of human esophageal histologic ESCC (n=97) and matched normal tissues (n=97) were collected on gelatin-coated slides for HLA-DQA1 immunostaining (Abcam Group, Inc; Abcam, Cambridge, UK). The avidin–biotin–peroxidase complex method was applied as previously described.[19] In brief, after dewaxing, inactivating, endogenous peroxidase activity, and blocking cross-reactivity with preimmune serum, the sections were incubated over night at 4°C with the primary antibodies (antibody for HLA-DQA1 was diluted at 1:100). Localization of the primary antibodies was achieved by subsequent incubation of a biotinylated anti-primary antibody, an avidin–biotin complex conjugated to horseradish peroxidase, and diaminobenzidine (Vectastain elite kit, Vector Laboratories Inc., Burlingame, CA). The slides were washed 3 times with phosphate-buffered saline after incubation. As negative controls, some slides were subjected to normal serum blocking and omission of the primary antibody. Two experienced pathologists (H-JY and F-FS) were specified to read the immunostaining slides.

2.6. Statistical analysis

The SPSS 22.0 (IBM SPSS; SPSS Inc, Chicago, IL) was used for statistical analysis. In all stratification analysis, comparisons of categorical variables were made using the Pearson test. Continuous variables were compared using independent 2-sample t test. Receiver operating characteristic (ROC) curve was plotted to determine how well the HLA-DQA1 expression level could differentiate survival time from patients with ESCC. The area under the ROC curve (AUC) >0.5 was taken to indicate reasonable biomarker performance. ROC curves optimal cutoff values were defined as the point that maximized the Youden index, defined as sensitivity −- (1-specificity). Survival analysis was conducted by Kaplan–Meier method and log-rank test. Univariate and multivariate Cox regression analyses were used to assess the prognostic value of biomarkers alone and when adjusted for clinical parameters. $P < .05$ was considered to be statistically significant.

3. Results

3.1. Immunohistochemical analysis for HLA-DQA1 protein

Immunohistochemical analysis was used to detect HLA-DQA1 expression in 97 paired ESCC cases and matched normal tissues. Intense nuclear or cytoplasm staining was the criterion for a "positive" reaction. We applied the criteria established by our laboratory previously to describe the patterns of positive result as follows: "scattered," in which only some isolated positive cells were identified; "papillary," where immunostain-positive cells were identified only in the esophageal epithelial papillary area; "focal," where wide clusters of positive cells were seen in some areas of the epithelia; and "diffuse," in which the sheets of

positive cells were found throughout most areas of the lesions. We found that HLA-DQA1 protein was mainly expressed in ESCC tissues (81.44%, 79/97), while there was weak or no positive staining in most matched normal tissues (16.49%, 16/97) (Fig. 1, Table 1). Various expression pattern of HLA-DQA1 protein in ESCC tissues was observed (Fig. 1C, D). Most ESCC cases in this study showed a significantly high HLA-DQA1 protein expression when compared to the matched normal tissues ($P < .001$). The positive reactants of HLA-DQA1 were yellow or brown substances present primarily in the cytoplasm.

3.2. HLA-DQA1 expression in ESCC

We 1st analyzed the HLA-DQA1 data from TCGA in EC by using the bioinformatics tool UALCAN (http://ualcan.path.uab.edu). The results revealed that HLA-DQA1 expression levels were upregulated approximately 6-fold in EC ($P < .001$, Fig. 2A). Then, we analyzed the expression of HLA-DQA1 in ESCC cases and normal tissue samples from TCGA. As shown in Figure 2B, HLA-DQA1 expression levels were significantly upregulated in ESCC ($P < .001$). We also evaluated the expression levels of HLA-DQA1 in 97 paired ESCC cases and matched normal tissues by qRT-PCR. The expression of HLA-DQA1 mRNA was 8-fold higher in ESCC tissue samples, compared to which in matched normal esophageal tissue samples ($P < .001$, Fig. 2C). These results were confirmed by western blot (Fig. 1D). These results demonstrate that HLA-DQA1 is highly expressed in human ESCC.

3.3. Clinicopathologic characteristics and HLA-DQA1 mRNA expression level in ESCC

The clinicopathologic characteristics of the 97 ESCC cases are summarized in Table 1. To further demonstrate the clinical significance of HLA-DQA1 expression in ESCC, we used t test to examine the correlation of HLA-DQA1 expression levels and clinicopathologic factors in patients. The results demonstrated that there was an obvious positive correlation between increased HLA-DQA1 levels and family history (21.332 ± 8.753 vs 5.520 ± 2.564, $P = .021$) and larger tumor size (27.057 ± 13.886 vs 8.265 ± 2.645, $P = .004$). Patients with family history negative or with tumors sizes >4 cm were associated with higher HLA-DQA1 expression levels, whereas patients with family history positive or with tumors sizes <4 cm were associated with lower HLA-DQA1 expression levels (Fig. 2E, F). A Chi-squared test was performed to assess the clinical pathologic characteristics between the 2 groups. The results in Table 2 show that HLA-DQA1 expression was significantly associated with family history ($P = .004$) and tumor size ($P = .029$). However, there was no correlation detected in the expression of HLA-DQA1 with age ($P = .586$), gender ($P = .836$), and smoking history ($P = .805$) in our study (Table 2).

3.4. HLA-DQA1 expression and the prognosis of ESCC

To assess the potential role of HLA-DQA1 expression on the prognosis of ESCC, the Kaplan–Meier survival analysis and log-rank tests for overall survival were conducted on 97 patients who had intact follow-up information. The overall survival rate over 3 years for the high HLA-DQA1 group was 30.2%, while it was 43.2% for the low HLA-DQA1 group. The median survival time for the low HLA-DQA1 group was 25.045 ± 3.016 months, but it was only 24.604 ± 2.270 months for the high HLA-DQA1 group. Remarkably, high HLA-DQA1 expression was demonstrated to be associated with a shorter overall survival time ($P = .029$). The 5-year overall survival rate for the high HLA-DQA1 group was
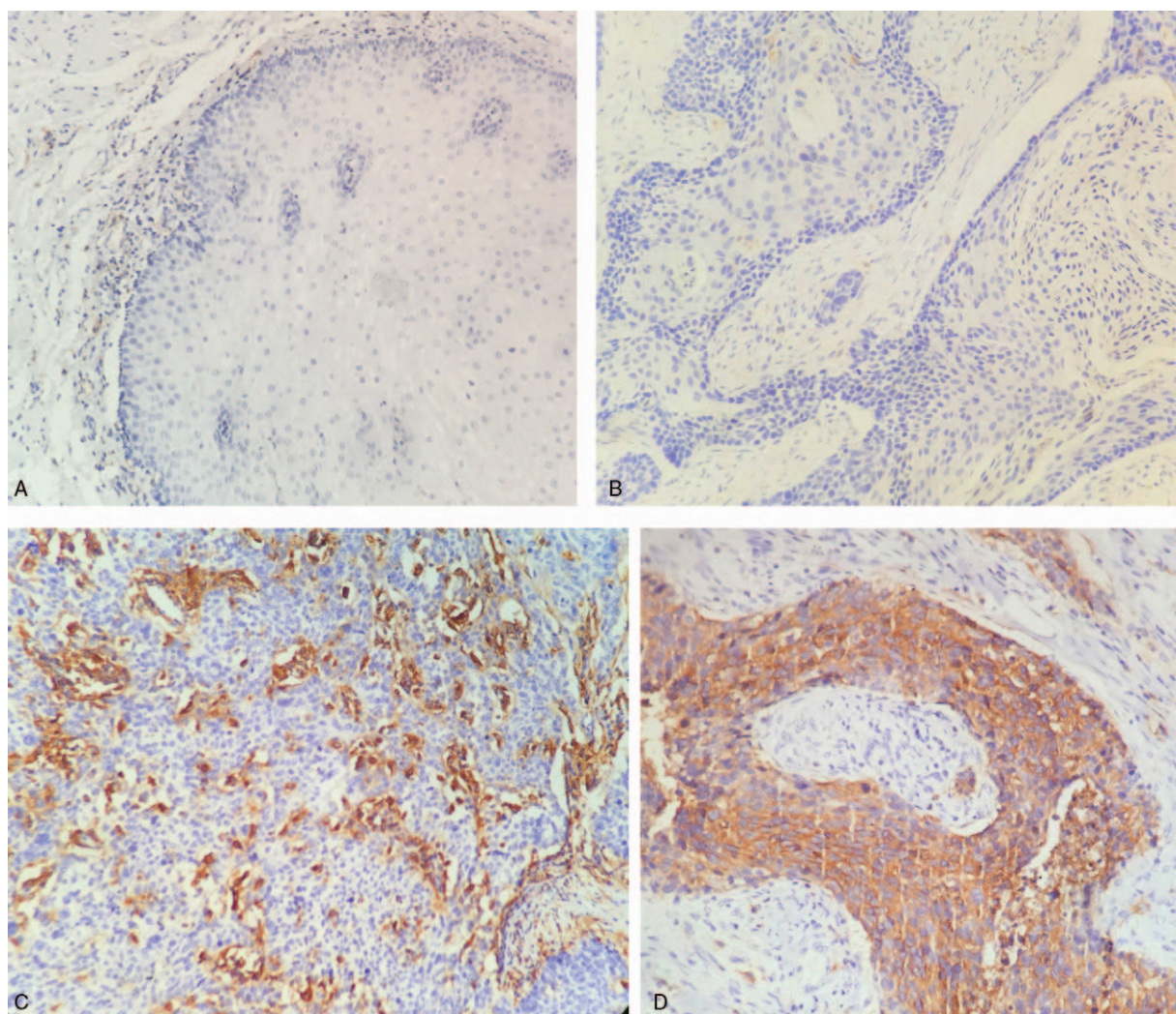
Figure 1. Immunohistochemical staining of HLA-DQA1 in normal tissues and esophageal squamous cell carcinoma (ESCC) tissues. Representative images of negative HLA-DQA1 immunohistochemical staining of normal tissue (A, 20×) and ESCC tissue (B, 20×). (C, 20×; D, 20×): HLA-DQA1 staining in cancer tissue with +1 (weak) and +3 (strong) score.

9.43%, while it was 27.27% in the compared groups. As it is shown in Figure 3, the overall survival is shorter in HLA-DQA1 highly expressed patients than those of low levels of HLA-DQA1 (P = .0024).

3.5. ROC curve of HLA-DQA1 expression in the prognosis of ESCC

We further analyzed ROC curve of HLA-DQA1 expression to assess the prognosis of ESCC (Fig. 4). We found that HLA-DQA1 expression level could differentiate survival time from patients with ESCC, with an AUC of 0.693 (95% confidence interval: 0.577–0.808, P = .013). And the optimal cutoff values were 1.323 (sensitivity 0.539, specificity 0.824).

3.6. HLA-DQA1 expression and the indicator for survival

The association between HLA-DQA1 expression and prognosis of patients with ESCC was evaluated by univariate analysis and multivariate analysis. As shown in Table 3, the overall survival of those patients was significantly dependent on gender (hazard ratio [HR] = 0.404, P = .016), tumor location (HR = 0.034,

P = .034), and HLA-DQA1 expression levels (HR = 2.096, P = .008). Thus, these factors and smoking history were enrolled in the multivariate Cox regression analysis. Highly expressed HLA-DQA1 was demonstrated to be an independent indicator for the poor overall survival (HR = 1.958, P = .009) in ESCC, along with the gender (HR = 0.446, P = .013) and tumor location (HR = 2.886, P = .012) (Table 3). These results indicate that HLA-DQA1 could be a useful marker for the prognosis of ESCC.

4. Discussion

The ESCC has long been recognized as one of the most malignant tumors worldwide, with the current median survival time <1 year.[20] With the improvement of early diagnosis and the application of new techniques, and the 5-year survival rate has increased significantly.[3] Of note, the application of endoscopic techniques, including endoscopic mucosal resection, endoscopic submucosal dissection, and radiofrequency ablation, has improved the prevention and curative treatment of early esophageal lesions.[21] Both neoadjuvant chemotherapy and chemoradio-
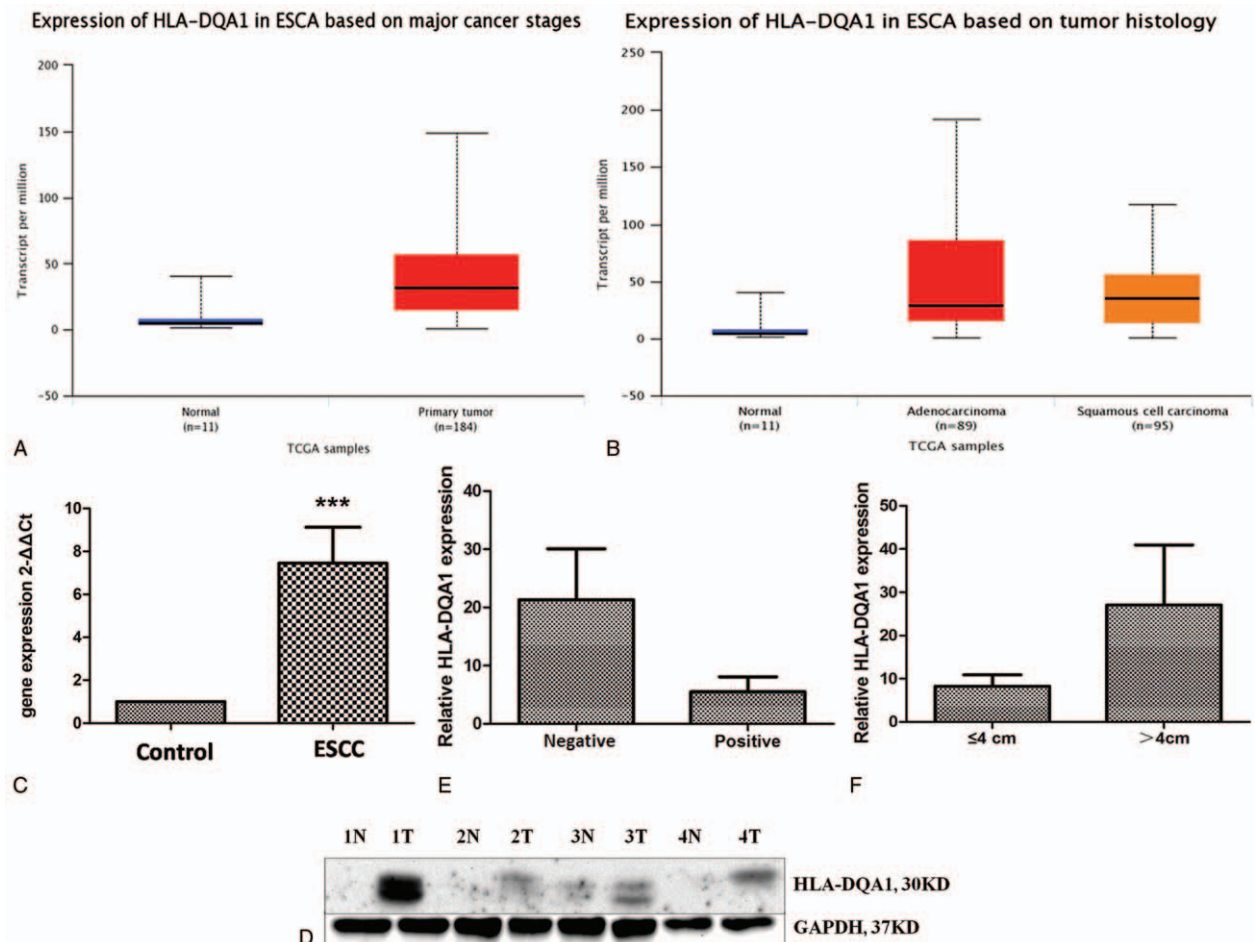
Figure 2. Relative HLA-DQA1 expression in esophageal squamous cell carcinoma (ESCC) tumor tissues and control samples. (A) HLA-DQA1 expression in esophageal cancer tissues (n=184) compared with noncancerous tissues (n=11) analyzed using TCGA database. (B) Relative expression levels of HLA-DQA1 in ESCC tissues (n=95) compared with nontumor tissues (n=11). (C) HLA-DQA1 expression was examined using quatitative polymerase chain reaction and was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. (D) Representative western blotting analysis of HLA-DQA1 protein in ESCC tissues, and GAPDH was used as the control. N: normal tissues; T: cancer tissues. (E) HLA-DQA1 expression was significantly higher in patients with family history negative than in those with family history positive (P=.021). (F) HLA-DQA1 expression was significantly higher in patients with tumors sizes >4 cm than that in those with smaller tumor sizes (≤4 cm) (P=.004).

therapy improve overall survival for patients with operable ESCC. Even through many factors were reported to be related to the progression of ESCC, no reliable prognostic markers were available in clinic.[22–27] Discovery of new prognostic marker is of urgent need for the treatment of those patients.

Over the past few decades, the roles of MHC in human disease, including cancer, have attracted much attention. Many studies have revealed that MHC has crucial roles in various cancer types by functioning as potential oncogenes or tumor suppressors.[28–30] In our previous studies,[8–10] we found that multiple genetic factors within the MHC region confer risk to ESCC from high-risk area in northern China, but the mechanism of MHC has not been studied. Further functional studies were needed to elucidate the molecular mechanisms about the MHC genes.

Based on our previous study, we have investigated HLA-DQA1 expression in ESCC samples and matched normal tissue samples. Firstly, we analyzed the expression profiles of HLA-DQA1 in EC samples in the TCGA database and validated the results in a cohort of 97 pairs of ESCC samples and normal tissue samples. The results showed that HLA-DQA1 was upregulated in ESCC tissues and correlated with poor prognosis and shorter

survival rate. Specifically, patients with higher HLA-DQA1 expression levels seemed to harbor larger tumor sizes and were characterized by family history. We further determined that HLA-DQA1 expression was an independent predictor for overall survival. These results indicated that overexpressed HLA-DQA1 played an important role in the occurrence of ESCC.

Previous studies have indicated that family history, smoking, alcohol consumption, aging, and gender are risk factors for ESCC.[10,31,32] Our results demonstrated that family history (P=.004) and tumor size (P=.029) were preferentially associated with HLA-DQA1 expression. The percentage of patients expressing HLA-DQA1 is higher for negative family history than that of positive family history. The mRNA expression in this study was consistent with the protein expression in our previous study. The other factors (age, gender, drinking history, smoking history, differentiation, T classification, N classification, and location) did not significantly associated with HLA-DQA1 expression. The results further supported the potential role of HLA-DQA1 in esophageal carcinogenesis.

However, certain limitations in our study should be addressed as follow. The population of enrolled cases was relatively small,

Table 2

Clinicopathologic characteristics and HLA-DQA1 expression level of 97 esophageal squamous cell carcinoma cases.

Variables	N	HLA-DQA1 expression		P-value
		High (n=53)	Low (n=44)	
Age, yr				
≤65	58	33	25	.586
>65	39	20	19	
Gender				
Female	43	24	19	.836
Male	54	29	25	
Family history				
Positive	35	13	22	.009
Negative	62	40	22	
Drinking history, yr				
≤10	66	35	31	.642
>10	31	18	13	
Smoking history, yr				
≤10	63	35	28	.805
>10	34	18	16	
Differentiation				
Well	13	6	7	.514
Moderate	52	27	25	
Poor	32	20	12	
T classification				
T1–T2	25	14	11	.847
T3	72	39	33	
N classification				
N0	50	24	26	.176
N1 + N2 + N3	37	29	18	
Tumor size, cm				
≤4	59	27	32	.029
>4	38	26	12	
Location				
Upper	17	10	7	.929
Middle	65	35	30	
Low	15	8	7	

which might result in bias in the final results. Therefore, we will increase the number of samples for further investigation. Besides, the concise molecular mechanisms underlying the altered expression of HLA-DQA1 in ESCC cell lines and mouse model need to be further addressed.
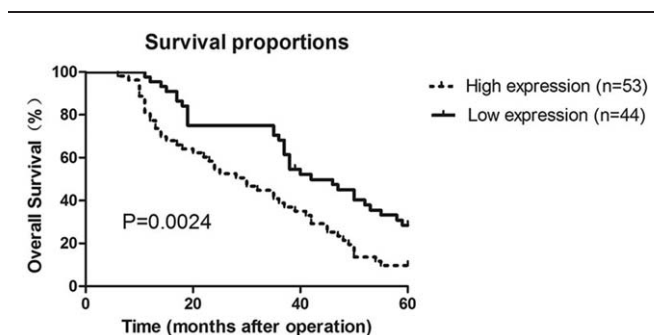

Figure 3. Kaplan–Meier survival curves for 97 esophageal squamous cell carcinoma based on HLA-DQA1 mRNA expression level. Patients with high HLA-DQA1 mRNA expression (imaginary line) had a significantly poorer prognosis (shorter 5-year survival rate) than those with low HLA-DQA1 mRNA expression ($P=.0024$). The x axis represents survival time after surgery, and the y axis represents cumulative overall survive.
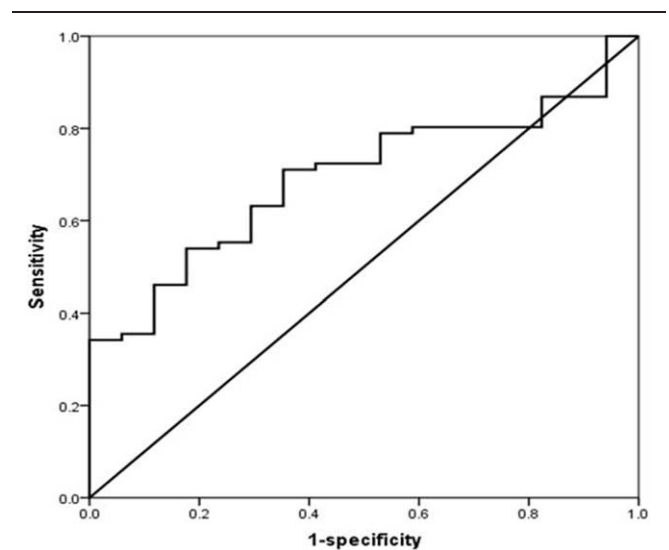

Figure 4. Receiver operating characteristic curve analysis of HLA-DQA1 expression in the prognosis of esophageal squamous cell carcinoma (ESCC). HLA-DQA1 expression level could differentiate survival time from patients with ESCC, with an AUC of 0.693 ($P=.013$).

Table 3

Univariate and multivariate Cox regression analysis of overall survival in 97 esophageal squamous cell carcinoma cases.

Variables	N	Univariate analysis HR (95% CI)	P-value	Multivariate analysis HR (95% CI)	P-value
Age, yr			.423		
≤65	58	1			
>65	39	1.231 (0.740–2.049)		–	
Gender			.016		.013
Female	43	1		1	
Male	54	0.404 (0.193–0.847)		0.446 (0.236–0.843)	
Family history			.780		
Positive	35	1			
Negative	62	1.086 (0.610–1.933)		–	
Drinking history, yr			.713		
≤10	66	1			
>10	31	1.141 (0.564–2.309)		–	
Smoking history, yr			.123		.241
≤10	63	1		1	
>10	34	0.565 (0.274–1.167)		0.684 (0.362–1.291)	
Differentiation			.401		
Well	13	1			
Moderate	52	0.578 (0.256–1.308)	.188	–	
Poor	32	0.959 (0.570–1.613)	.874	–	
T classification			.579		
T1–T2	25	1			
T3	72	0.839 (0.452–1.559)		–	
N classification			.817		
N0	50	1			
N1 + N2 + N3	37	0.938 (0.546–1.611)		–	
Tumor size, cm			.880		
≤4	59	1			
>4	38	1.041 (0.616–1.761)		–	
Location			.034		.012
Upper	17	1		1	
Middle	65	2.674 (1.116–6.405)	.027	2.886 (1.304–6.391)	.009
Low	15	1.132 (0.577–2.220)	.719	1.203 (0.645–2.243)	.561
HLA-DQA1 expression			.008		.009
High	53	1		1	
Low	44	2.096 (1.217–3.610)		1.958 (1.184–3.238)	

CI = confidence interval, HR = hazard ratio.

5. Conclusion

Collectively, in this study, we showed that HLA-DQA1 was upregulated in ESCC tissues and correlated with poor prognosis in patients with ESCC. Our finding implies that HLA-DQA1 could be a potential prognostic marker in patients with ESCC.

Author contributions

Conceptualization: Fang-Fang Shen, Fu-You Zhou.

Data curation: Fang-Fang Shen, Ying Pan, Jing-Zhong Li, Fang Zhao, Hai-Jun Yang, Jun-Kuo Li, Zhao-Wei Gao, Jing-Fen Su, Li-Juan Duan, Shu-Min Lun, Lin-Qiang Tian, Gang Sun, Da Huang, Yan-Tian Cao.

Funding acquisition: Fang-Fang Shen.

Investigation: Fang-Fang Shen.

Methodology: Fang-Fang Shen, Fu-You Zhou.

Project administration: Fu-You Zhou.

Resources: Fang-Fang Shen, Fu-You Zhou.

Software: Fang-Fang Shen, Peng Zhang.

Supervision: Fu-You Zhou.

Writing – original draft: Fang-Fang Shen, Fu-You Zhou.

Writing – review & editing: Fang-Fang Shen, Fu-You Zhou.

Fangfang Shen orcid: 0000-0002-1130-7847.

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