Biomarkers, 2012; 17(4): 372–378

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.3109/1354750X.2012.680609

informa
healthcare

RESEARCH ARTICLE

Serological antibodies against LY6K as a diagnostic biomarker in esophageal squamous cell carcinoma

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Abstract

Objectives: To evaluate the diagnostic values of autoantibodies against lymphocyte antigen 6 complex locus K (LY6K) in esophageal squamous cell carcinoma (ESCC).

Methods: After cloning, expressing, and purifying LY6K as fusion proteins, LY6K autoantibodies were measured in 62 patient and 58 control serum samples using enzyme-linked immunosorbent assay (ELISA). Reverse transcription polymerase chain reaction (RT-PCR) was used to measure the LY6K mRNA levels in ESCC and adjacent tissues.

Results: LY6K autoantibodies were found significantly higher in patients than controls. The area under the receiver-operating characteristic (ROC) curve (AUC) was 0.85, and the optimal sensitivity and specificity for ESCC detection were 80.6 and 78.7%, respectively. LY6K mRNA expressions in patients were upregulated.

Conclusions: Autoantibodies against LY6K may be a good diagnostic biomarker for ESCC.

Keywords: Gastric cancer, gene expression, proteomics

Background

Esophageal squamous cell carcinoma (ESCC), the major histological form of esophageal cancer in East Asian countries, is one of the leading causes of cancer death worldwide (Jemal et al., 2010; Muto et al., 2010). Further reduction in the mortality requires successful strategies for early detection and screening of the disease. Serological biomarkers that may accurately determine onset tumors have been considered as a promising approach for early cancer detection. Much of the efforts in the past have been centered on the discovery and characterization of circulating tumor-associated antigens (TAAs) as diagnostic markers. Three serum antigen biomarkers, carcinoembryonic antigen (CEA), squamous cell carcinoma antigen (SCC), and cytokeratin 19-fragment (CYFRA21-1), have been found useful for advanced ESCC patients (Kawaguchi et al., 2000). However, their sensitivity remains from 20 to 50%, thus none of them is clinically helpful for early detection of ESCC (Mealy et al., 1996; Yamabuki et al., 2007).

In contrast to the use of serum antigens, the detection of serum antibodies against TAAs may provide more reliable information for early cancer diagnosis (Hanash, 2003; Desmetz et al., 2009; Tan et al., 2009; Belousov et al., 2010). The immune system is sensitive enough in detecting very low levels of TAAs that may originate in only a few neoplastic cells by generating very high affinity T cells and antibodies (Finn, 2005). Autoantibodies to p53 have been reported in patients with early stage ovarian or colorectal cancers (Finn, 2005; Sahin et al., 1995), and a

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(Received 29 January 2012; revised 21 March 2012; accepted 26 March 2012)

panel of serum antibodies can detect non-small cell lung cancer (NSCLC) 5 years prior to autoradiograph detection (Zhong et al., 2006). Thirty percentage of patients with ductal carcinoma *in situ* in which the proto-oncogene HER-2/*neu* is overexpressed have serum antibodies specific to this protein (Old & Chen, 1998; Minenkova et al., 2003). Therefore, it is logical and practical to employ the body's endogenous immune system as a natural "amplification strategy" to detect cancer at early stage.

Lymphocyte antigen 6 complex locus K (LY6K) was initially described as an unannotated transcript by several groups (Maruyama et al., 2010) but was recently identified as a member of cancer-testis antigens (CTAs) (Boon & Old, 1997; Chen et al., 2005). CTAs are defined as proteins that are highly expressed in cancer cells but not in normal cells, except for cells in reproductive tissues, such as testis, ovary, and placenta (Old, 2001; Scanlan et al., 2004). As these tissues do not express major histocompatibility complex (MHC) Class I molecules, CTAs are considered to be promising targets for immunotherapy and cancer vaccine development. Meanwhile, CTAs are also good candidates of biomarkers for diagnosis of cancer and monitoring of relapse (Caballero and Chen, 2009). LY6K has been reported to be highly expressed in human head and neck SCC (de Nooij-van Dalen et al., 2003), breast cancers (Lee et al., 2006), and ESCC (Ishikawa et al., 2007). Serological protein levels of LY6K have been reported as biomarkers for ESCC and NSCLC diagnosis and prognosis (Ishikawa et al., 2007). As one of the early immune responses to cancer development, autoantibody levels against LY6K protein have not been studied. This humoral immune response to LY6K might be a good early indicator for ESCC.

In this study, we evaluate the diagnostic values of serum autoantibodies against LY6K in ESCC patients. We further discuss the correlation between LY6K mRNA expressions and the humoral immune response, and the potential utility of these autoantibodies as tumor markers in clinical diagnosis.

Methods

Clinical samples

A total of 62 ESCC patient and 58 risk-matched control serum samples were collected from Baoding Cancer Hospital, Hebei, China. In addition, 20 specimens of ESCC tumor and 15 adjacent nontumor tissues were collected from the same hospital. Detailed information of serum and tissue samples is listed in Table 1. Patient serum samples were collected before surgical removal, chemotherapy, or radiation therapy. Informed consents were obtained from patients before all the samples were collected.

Cloning LY6K cDNA from ESCC tissues

About 12 g of ESCC tissue samples were snap-frozen in liquid nitrogen. RNA extraction was performed according to procedures described in the TRIzol Reagent manual (Invitrogen, Carlsbad, CA) and was reversely transcribed to single-stranded cDNA using Olig (dT) 15 and M-MLV reverse transcriptase (Promega, Madison, WI). The LY6K cDNA was amplified using the following set of synthesized primers specific to LY6K mRNA (GenBank, NM_017527) by PCR. The sequence of the forward primer was 5′-CTA*GCTAGC*GACGCCAACCTGACTGC-3′, and the sequence of the reverse primer was 5′-CCC*AAGCTT*CTAACCACAGCTCTCACCCA-3′. *Nde I* and *Hind III* (underlined) restriction sites were introduced into the two primers.

Table 1. Characteristics of serum and tissue specimens.

	ESCC sera ($n = 62$)				Control sera ($n = 58$)	ESCC tissues ($n = 20$)				Nontumor tissues ($n = 15$)
		Stage					Stage			
	Number	I/II	III	IV	Number	Number	I/II	III	IV	Number
Age										
≤45	3	1	1	1	3	0				0
45–54	8	2	3	3	11	6	1	4	1	2
55–64	25	5	12	8	20	11	1	7	3	11
65–75	18	4	7	7	20	3	1	1	1	2
≥75	8	1	4	3	4	0				0
Sex										
Female	16				15	5				3
Male	46				43	15				12

ESCC, esophageal squamous cell carcinoma.

The ProtoScript® M-MuLV Taq RT-PCR Kit (New England BioLabs, Ipswhich, MA) was used for setting up the PCR reactions. The reactions were carried out under the following conditions: after denaturing at 94°C for 4 min, the reactions were exposed to 30 cycles of 94°C for 45 s, 62°C for 60 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The final products were then subjected to electrophoresis on 1% agarose. The amplified inserts were purified using a DNA purification kit (QIAGEN, Valencia, CA), digested with *Nde I* and *Hind III*, and then ligated to a prokaryotic expression vector pET28b(+) (Novagen, Gibbstown, NJ) that was also digested with the same restriction enzymes. The constructed plasmid was transformed into competent *E. coli* DH5 α cells and grown in Luria–Bertani (LB) broth supplemented with kanamycin (30 μg/mL). The recombinant plasmid was confirmed by double endonuclease digestion and DNA sequencing.

LY6K protein expression and purification

The recombinant plasmid pET-28b/LY6K was transformed into expression strain *E. coli Rosseta (DE3)* cells by heat-shock. One colony was picked and grown in 20 mL LB medium containing 30 μg/mL kanamycin at 37°C until an optical density (OD) at 600 nm of 0.6 was reached. Isopropyl-b-D-thiogalactopyranoside (IPTG) was then added to induce protein expression at 28 and 37°C. To determine the optimal condition for LY6K expression, *DE3* cells were induced at different concentrations (0.4,

0.8, and 1.0 mM) of IPTG for different lengths of time (1, 2, 4, 6, and 7 h). At the end of each condition, cells were harvested by centrifugation, resuspended in 1 mL PBS, and sonicated on ice until the suspension became transparent (5 min). The lysate was centrifuged for 30 min at 12,000 g, and then both the supernatant and the pellets were tested for the LY6K protein expression by 15% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).

To purify this His-tagged LY6K recombinant protein, a QIAexpressionist (QIAGEN) kit was used. Briefly, 3 mL of cell culture at the most optimal expression condition was pelleted and resuspended in 600 µl lysis buffer (8 M urea, 10 mM NaH_2PO4, 10 mM Tris.Cl, pH 8.0) at room temperature for 4 h. Cell debris was cleared by centrifugation, and the supernatants were transferred to a fresh tube and incubated with 60 µl of a 50% slurry of Ni-NTA resin (10 µl resin has a capacity for 50–100 µg His-tagged protein) for 60 min at 4°C with agitation. The resin was then pelleted by centrifugation and washed twice with 300 µl wash buffer (8 M urea, 100 mM NaH_2PO4, 10 mM Tris.Cl, pH 6.3). The protein was then eluted three times with 30 µl elution buffer (8 M urea, 100 mM NaH_2PO4, 10 mM Tris.Cl, pH 4.5). The purification process was tested by 15% SDS-PAGE followed by Coomassie Brilliant Blue staining.

To further confirm the expression of the target protein, the purified LY6K protein was separated by 15% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. Immunoblotting was carried out with diluted (1:1000) polyclonal LY6K antibody (Abnova, Taiwan). After being washed, the membrane was incubated with goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad, Hercules, CA). Specific complexes were visualized with an DAB (diaminobenzidine) colorimetric detection system (Thermo Scientific, Barrington, IL).

Measurement of serum autoantibodies against LY6K

Ninety-six-well Costar ELISA plates (Jet Biofil, Beijing, China) were separately coated (overnight at 4°C) with 2 µg/mL of the purified LY6K protein or nontransformed *DC3* cell lysate (negative control) to test specific affinity of antibodies in individual serum samples to LY6K proteins. The plates were washed four times with PBST (PBS buffer containing 0.05% Tween 20) and then, blocked with PBS containing 1% BSA at 37°C for 1 h, followed by four washes in PBST. Patient serum samples were assayed in limiting dilution from 1:20 to 1:5,120 and incubated in the both type of coated enzyme-linked immunosorbent assay (ELISA) plates at 37°C for 1 h. After washing four times with PBST, 100 µl of goat antihuman IgG-HRP (1:1000 dilutions) was added to each well for 1h at 37°C. After washing four times with PBST, the color was developed with 3,4,5-trimethoxy benzaldehyde (TMB) for exactly 15 min and then stopped with 0.5 M H_2SO_4. The absorbance of each well was read at OD = 450 nm by a plate microplate reader (New Air Electrical Technology, Beijing, China). In separated experiments serum samples from 62 ESCC patients or 58 controls were diluted 1:160 in 1 × PBS/1% BSA and incubated with the LY6K-coated ELISA plates. The absorbance was used as a measure of antibody reactivity in each independent assay. Each serum sample was tested in triplicate.

Analysis of LY6K mRNA expression by RT-PCR

Total RNA from 20 ESCC tissues and 15 adjacent nontumor tissues were extracted by the method of TRIZOL reagent. RNA of each sample was reversely transcribed to single-stranded cDNAs using Olig (dT) 15 and M-MLV reverse transcriptase (Promega). Following primer sets for LY6K (forward: 5′-GCATGGCGCTGCTCGCCTT-3′; reverse: 5′-CCCTCAAGACAGGCTGAGGC-3′) or β-actin (forward: 5′-GGAAGGCTGGAAGAGTGCC-3′; reverse 5′-GTGATGGTGGGCATGGGTC3′) were used to detect their molecular expressions using semiquantitative reverse transcription-PCR. The conditions for PCR were denaturing at 94°C for 5 min, followed by 30 cycles of 45 s at 94°C, annealing at 62°C for 45 s, and extension at 72°C for 1 min. After the reaction, the samples were continuously incubated at 72°C for 10 min. The PCR products were subjected on 2% agarose gels and stained with ethidium bromide and then visualized with ultraviolet light. Band intensity was analyzed using Quantity one software (Bio-Rad, Hercules, CA). All experiments were repeated three times.

Statistical analysis

To analyze the difference of autoantibodies reaction to LY6K proteins between ESCC and risk-matched control samples, the absorbance of each serum sample in the ELISA plate was averaged from triplicate experiments. Unequal variance *t*-test was run between ESCC and normal control samples. Nonparametric ROC in which the value for sensitivity was plotted against false-positive rate (1-specificity) was generated. In addition, an AUC with 95% confidence intervals was calculated. In addition, the absorbance of the early stage (Stages I & II) patient samples were also analyzed separately for the diagnostic sensitivity. Relative LY6K mRNA expression values were normalized with β-actin expression. ROC analysis was used to determine cutoff scores for the positive protein expression. In all testes, $p < 0.05$ was considered to be statistically significant. All statistical analysis was done using the SPSS software package version 16.0 (SPSS, Chicago, IL).

Results

LY6K expression and purification

The cDNA of LY6K was cloned from ESCC tissues and inserted into a prokaryotic expression plasmid, pET-28b. After double endonuclease digestion and PCR confirmation, a single band of 495 bp was obtained (Figure 1A) at the expected location. The inserted DNA

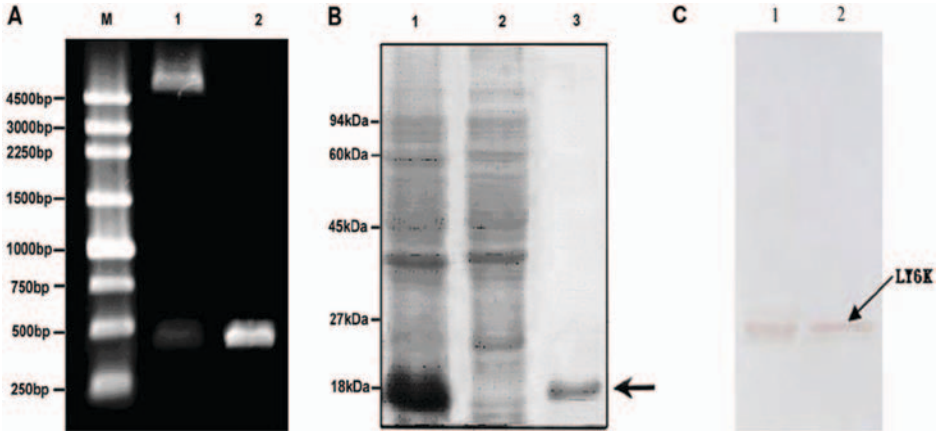

Figure 1. Expression of lymphocyte antigen 6 complex locus K (LY6K) recombinant protein. (A) Confirmation of the LY6K cDNA construct. Lane M, DNA ladder; lane 1, double endonuclease digestion with *Nhe I* and *Hind III* from the recombinant vector pET-28b/LY6K; lane 2, polymerase chain reaction product of LY6K. (B) Sodium dodecyl sulfate polyacrylamide gel electrophoresis of expressed LY6K protein. Lane 1, induced *DE3* cells with isopropyl-b-D-thiogalactopyranoside (1 mM) for 3 h at 37°C; lane 2, uninduced *DE3* cells; lane 3, purified LY6K recombinant protein. (C) Western blot verification of the expressed LY6K protein. Lanes 1 and 2, purified LY6K protein.

was further purified and sequenced. The sequence was found to be a perfect match to the sequence of LY6K mRNA deposited in NCBI GenBank.

This pET-28b/LY6K plasmid was transformed into *DE3* cells for LY6K expression. Optimal IPTG concentration and time course determinations were performed for the kinetics of protein expression in the bacterial culture. The results showed that *DE3* cells had the highest LY6K protein expression level after 3 h of 1 mM IPTG induction at 37°C. The pET-28b/LY6K recombinant proteins were mainly observed in the precipitate of the *DE3* lysate, which indicated that the expressed protein was mainly in sequestered to inclusion bodies. The expressed proteins were further purified to approximately 95% purity by Ni-NTA resin and subjected to 15% SDS-PAGE followed by Coomassie Brilliant Blue staining. The LY6K recombinant protein showed a clean band with a molecular weight of 18 kD (Figure 1B) and was confirmed by anti-LY6K antibody in Western blotting assay (Figure 1C).

Comparison of LY6K autoantibodies between cancer and control samples

To confirm antibody affinity to LY6K proteins in individual serum samples, serum was assayed in limiting dilution from 1:20 to 1:5120 by ELISA constructed with LY6K proteins or with nontransformed *DC3* cell lysates as a negative control. Absorbance values from antibodies in sera of three patients decreased over serial dilutions in the LY6K coated-plates (Figure 2A), whereas exhibited little change and small values in the negative control plates (Figure 2B), suggesting that the affinity of serum antibodies against LY6K is specific.

To test the levels of autoantibodies against LY6K proteins, 62 ESCC along with 58 risk-matched control serum samples were tested using ELISA, plates which were coated with the purified LY6K proteins. The means OD_{450} (±SD) of autoantibodies against LY6K were 0.630 ± 0.052 in ESCC patients and 0.571 ± 0.041 in control individuals. The levels of autoantibodies against LY6K were significantly higher in ESCC than in the risk-matched controls ($p < 0.001$, Figure 2C).

ROC curves were plotted to identify an optimal value that would distinguish case from control samples. According to the ROC curve, the AUC for LY6K was 0.85, whereas the optimal sensitivity was 80.6% and specificity was 78.7% (Figure 2D) in detecting ESCC patients. In addition, the analysis of 13 early stage serum samples generated a sensitivity of 73.2% for detection of ESCC.

Expression of LY6K mRNA in ESCC and adjacent nontumor tissues

Expression of LY6K mRNA in 20 tumor tissues and 15 adjacent nontumor tissues was measured using semi-quantitative RT-PCR and analyzed with Quantity one software (Bio-Rad). The agarose gel electrophoresis of the PCR products for LY6K and β-actin from individual samples showed different fragments (Figure 3A). The mean (±SD) of band intensity ratios (LY6K/β-actin) between ESCC tissues and adjacent nontumor tissues was 0.509 ± 0.035 and 0.436 ± 0.072, respectively (Figure 3B). Statistical analysis indicated that LY6K mRNA was upregulated in 14 out of 20 (70%) of the ESCC tumor tissues, whereas all the adjacent nontumor tissues showed low LY6K expressions except one upregulation was observed. It was considered that this exception was caused by tissue contamination with ESCC.

Discussion

Changes in the level of gene expression and aberrant expression of tissue-restricted gene products are factors in the development of humoral immune response in cancer patients (Tan, 2001). So far, promising results have been reported using autoantibodies as biomarkers for detection of breast cancer (Jäger et al., 2001), head and neck cancer (Lin et al., 2007), prostate cancer (Wang

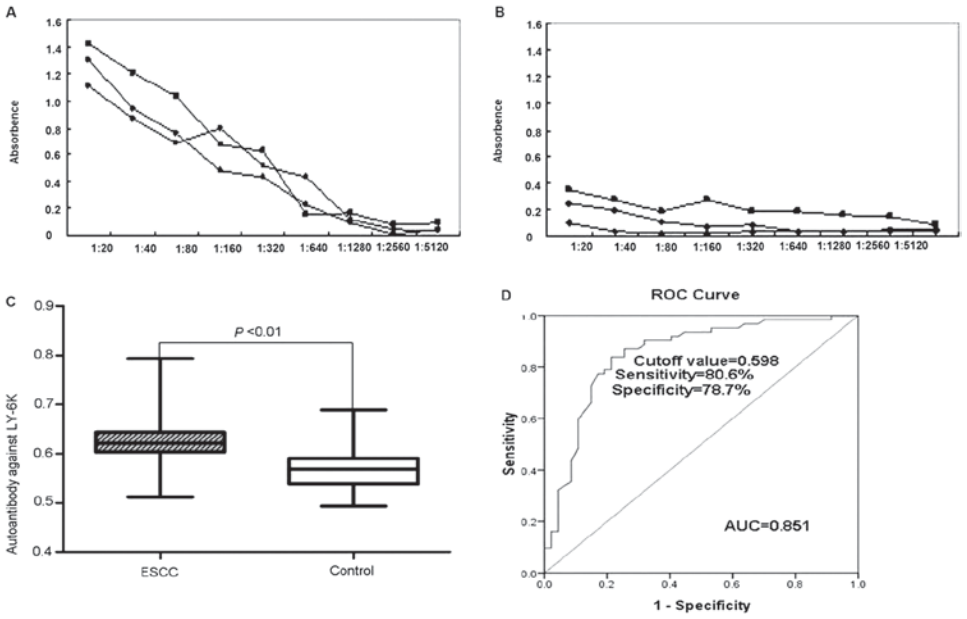

Figure 2. Comparisons of the serum autoantibodies against lymphocyte antigen 6 complex locus K (LY6K) between patients and control serum samples. (A) Absorbance values from antibodies in sera of three patients showed a decrease over serial dilutions (1:20 to 1: 5120) in the LY6K coated-plates. (B) Absorbance values from antibodies in sera of three patients exhibited little change over serial dilutions (1:20 to 1: 5120) in the negative control plates. (C) Serum samples from 62 esophageal squamous cell carcinoma (ESCC) patients and 58 risk-matched controls were tested using indirect enzyme-linked immunosorbent assay (ELISA). The mean (optical density) OD_{450} (±SD) of serum autoantibodies against LY6K was 0.630 ± 0.052 in ESCC patients and 0.571 ± 0.041 in control individuals. (D) Nonparametric receiver-operating characteristic curve was generated based on the ELISA data in A. The area under curve (AUC) was 0.85 with an optimal sensitivity of 80.6% and specificity of 78.7% when a cutoff value was 0.598.

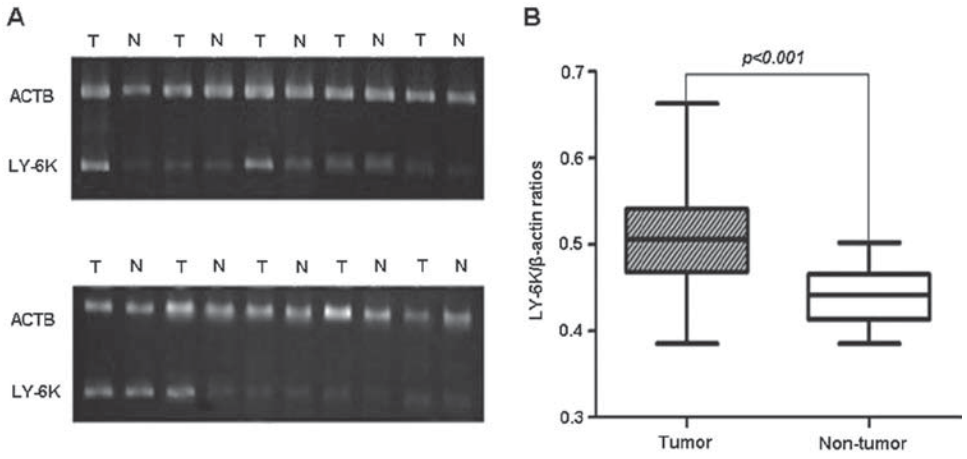

Figure 3. Analysis of lymphocyte antigen 6 complex locus K (LY6K) mRNA expression using semiquantitative reverse transcription polymerase chain reaction. (A) Expression of LY6K mRNA in 10 esophageal squamous cell carcinoma tumor (T) and 10 adjacent nontumor (N) tissues. The total number of tissues tested was T = 20 and N = 15. (B) Each LY6K mRNA expression value was normalized with β-actin expression and compared between T and N (0.509 ± 0.035 vs. 0.436 ± 0.072).

et al., 2005), and lung cancer (Zhong et al., 2005, 2006). There are several advantages of using serum antibodies as markers for tumor detection: (i) it is a blood test, which is minimally invasive and therefore acceptable to most asymptomatic people, (ii) antibodies are stable and resistant to degradation, (iii) antibodies are highly specific and can be produced by the immune system against unique peptides found in a very small number of cells, and (iv) many efficient and low cost methods exist to detect antibodies, allowing possible widespread implementation of such a screening method in resource-poor populations.

In this study, we evaluated the diagnostic values of serum autoantibodies against LY6K in ESCC patients. Our results showed significant elevation of autoantibody levels against LY6K in patients with ESCC than in the risk-matched control samples. The AUC was 0.85, and the optimal sensitivity and specificity for detection of ESCC

were 80.6 and 78.7%, respectively. This diagnostic value is more sensitive and specific than the current traditional serological antigen markers. More importantly, our data showed a promising diagnostic sensitivity (73.2%) for the early stage ESCC. In addition, we also tested the LY6K mRNA expression levels between ESCC and adjacent nontumor tissues. The results showed that 70% ESCC tumors had LY6K mRNA upregulation on comparing with the adjacent nontumor tissues. Although the number of tissue samples were not enough to make a powerful conclusion, the similar ratios between mRNA expression and autoantibody distribution in ESCC patient imply that overexpression of LY6K may be the cause to trigger the humoral immune response. Therefore, not only autoantibody against LY6K is a promising type of biomarker for ESCC early detection but LY6K might also be a good target for immunotherapy.

Although the diagnostic values in this study is promising for ESCC, the sample size in this study was relatively small. In addition, most of the patient serum samples were collected from patients with advanced disease. Therefore, a large cohort of well-characterized patient samples, especially with early stage patient samples, may be needed for further validation. Furthermore, in order to maintain or increase the diagnostic value for detection of ESCC, additional autoantibody biomarkers may be needed to use combinedly when testing a large cohort of patient samples. Due to the genetic heterogeneity of cancer, it would be impossible to obtain a high degree of diagnostic accuracy using a single marker. A panel of autoantibody biomarkers would be useful to profile the difference between patient and normal samples and would ultimately be used in clinical tests for early cancer detection.

Conclusion

We have shown that autoantibody against LY6K is a novel diagnostic biomarker for ESCC. Overexpression of LY6K in ESCC may induce the humoral immune response, which implies that LY6K may be a promising target for immunotherapy.

Declaration of interest

This study is supported by NIH grant CA137570 (L Zhong) and NSFC Grant #81071795.

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