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Original Article

Expression and role of MIC-AB and ULBP-1,2,3 in primary resected esophageal squamous cell carcinoma

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Purpose: Esophageal squamous cell carcinoma (ESCC) is a highly malignant tumor with a poor prognosis. The study aims to detect the potential significance of NKG2D ligands in predicting the prognosis and guiding treatment decisions for patients with esophageal squamous cell carcinoma.

Methods: A total of 39 surgically resected esophageal squamous cell carcinoma tumor samples and adjacent non-tumor counterparts to detect the presence of NKG2D ligands (MIC-A/B and ULBPs) using reverse transcription and PCR analysis. Immunohistochemical methods are also employed to identify these ligands in the tumor specimens. The research aims to understand the expression and role of NKG2D-specific ligands in esophageal squamous cell carcinoma patients.

Results: The difference of expression between normal esophagus and ESCC was significantly increased in ULBP1 and ULBP3. The correlation between expression of ULBP1, ULBP2, and ULBP3 in ESCC was significant. But, there was no significant correlation between expression of MIC-A/B and ULBP1,2,3 and the disease status of the patients (stage, tumor differentiation, and lymph node metastasis).

Conclusion: MIC-A/B and ULBP1,2,3 molecules are expressed in both normal esophagus and esophageal squamous cell carcinoma. There was no significant correlation with the disease status of esophageal squamous cell carcinoma patients.

Keywords: Esophageal Squamous Cell Carcinoma, NKG2D ligands, MIC-AB, ULBP-1,2,3

1. Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most malignant tumors with a dismal prognosis. Despite dramatic advances in diagnostic methods and meticulous execution of radical surgery with or without adjuvant treatment, one-half of the patients die within 3 years, and the overall 5-year survival rate is only about 20%(1). In Taiwan, squamous cell carcinoma accounts for almost all esophageal malignancies. Even in the resectable stage, the results of surgical excision have been unsatisfactory. This situation makes the availability of prognostic factors for outcome highly desirable to guide the use of (neo)adjuvant treatment modalities and to aid the decision about the extent of the operative procedure.

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Natural killer (NK) cells are important effector cells for the first line of defense again tumors. NK cells employ various modes of immune recognition. Recent studies have demonstrated that NK-mediated effector functions are regulated by a balance between inhibitory and stimulatory signals. In general, tumor cells expressing little or no MHC Class I molecules are highly susceptible to NK-mediated cytolysis. The molecular basis of this "missing self" hypothesis is that NK cells recognize MHC Class I on target cells via surface receptors that deliver signals to suppress NK cell function(2, 3). On the other hand, there are several receptors on NK cells engaged in activating the signal transduction that leads to augmented NK-mediated cytolysis(4, 5). NKD2 differs from most other receptors as it exists only in an activating form. The crucial role of NKG2D in the activation of NK cells depends on its unique signal pathway. In contrast to other activating receptors, which are associated with KARAP/DAP12 containing immune receptor tyrosine-based activating motifs (ITAM), NKG2D pairs with the adapter protein DAP10 that carries a tyrosine motif for binding of the p85 subunit of the phospho-inositide-3 kinase(3, 6, 7). Activation through NKG2D, therefore, occurs through a pathway, raising the possibility that NKG2D is not subject to inhibition by ITAM-containing receptors. NKG2D could potentially play a central role in tumor immunity, as killing via the NKG2D pathway is largely resistant to inhibitory signals delivered by the interaction of inhibitory NK receptors with their MHC class I ligands in vitro(8, 9).

The molecular cloning of ligands for NKG2D provided further insight into the function of the NKG2D receptor. In humans, NKG2D recognizes two families of unusual MHC class I molecules on chromosome 6: the highly polymorphic family of the MHC class I-related chain(MIC) genes including MICA and MICB, and the polymorphic ULBP(UL-16 binding protein) family(10, 11). The MIC proteins consist of the α1, α2, and α3 domains with MHC class I folds. They are expressed on the cell surface without β2-microglobulin and ~~without~~ peptides. The ULBP proteins have an even more minimalist MHC class I structure, consisting of onlyα1 and α2 domains and they use glycosylphosphatidylinositol (GPI) anchors for cell surface expression(12). The ligands of NKG2D are induced by cellular stress and are especially expressed by some tumor cells. Recent studies show that MIC-A/B and ULBPs are detected in several carcinoma cells such as from lung, breast, ovary, prostate, and colon cancer(13). This raises the possibility that MIC-A/B and ULBPs may be specific targets on tumor cells for NK-mediated cytolytic activity. Recent studies also reveal that the expression of MIC and ULBP on human tumor cells is sufficient to overcome the inhibitory effects of MHC class I expression on NK cell killing and indicate that NKG2D provided first-line surveillance against stressed or abnormal cells that have been induced to express one of its ligands(13).

Our previous studies have demonstrated surgically resected esophageal squamous cell carcinoma patients with a high level of NK infiltration are found to have a better prognosis than those with a low level of NK infiltration(14). In the present study, we try to demonstrate the presence of MIC-A/B and ULBP1,2,3 mRNA by reverse transcription and PCR (RT-PCR) analysis in 39 consecutive surgically resected esophageal squamous cell carcinoma tumor samples and adjacent non-tumor counterparts (normal esophagus). Immunohistochemical detection of MIC-A/B and ULBP will be done with cryosections of the tumor specimens. We try to find the expression and role of NKG2D-specific ligands, MIC-A/B, and ULBPs, in the primary resected esophageal squamous cell carcinoma patients.

2. Material and Methods

2.1. Patients and tissue samples

Thirty-nine pairs of primary resected esophageal squamous cell carcinomas and adjacent non-tumor counterparts (normal esophagus) were obtained at the time of surgical resection at the division of Thoracic Surgery of Taichung Veterans General Hospital between 2001 and 2003. No pre-operative radio- or chemotherapy had been performed. The tissues were frozen in liquid nitrogen and stored at –80C until use.

2.2. Immunohistochemistry

These tissue blocks were cut into thin sections (4

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μm). Immunohistochemical staining was performed by the streptavidin-biotin-complex method (DAKO LSAB® + Kit, DAKO, CA, USA). Before staining, antigens were retrieved by heating in 0.1M citrate buffer (pH 6.0) for 10 min. Section was treated with 3% hydrogen peroxide in absolute methanol for 10 min at room temperature to inactivate the endogenous peroxidase, and then washed in PBS (pH 7.2). Following incubation with protein block serum-free (DAKO, CA, USA) for 10 min, the sections were incubated with the primary antibody for 1.5 hr in a moist chamber at room temperature (anti-MIC-A/B - No.558032, BD Biosciences, 1:100 dilution, anti-ULBP – No.AF1298, R&D systems, 1:40 dilution). The primary antibodies were anti-MIC-A/B and anti-ULBP. After incubation, the sections were washed twice in PBS and treated with biotinylated anti-rabbit, mouse, and goat Ig and peroxidase-conjugated streptavidin for 20 min. Then the section was reacted with DAKO® Liquid DAB+ large-volume substrate-chromogen solution (DAKO, CA, USA), and counterstained with hematoxylin.

2.3. Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

For semi-quantification utilizing the reverse transcriptase-polymerase chain reaction (RT-PCR), total RNA extracted from the tissue by TRI-Reagent (Invitrogen, TECH-LINE^{SM}, U.S.A.), and cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen, TECH-LINE^{SM}, U.S.A.). Using first strand cDNA as the template, the RT-PCR method was performed with 39 cancerous tissues and non-cancerous tissues. For both MICA and MICB, the oligonucleotide 5'-ACA CCC AGC AGT GGG GGG AT-3' was prepared as a sense primer. The MICA antisense primer was 5'-GCA GGG AAT TGA ATC CCA GCT-3', and the MICB antisense primer was 5'-AGC AGT CGT GAG TTT GCC CAC-3'. For ULBP-1, the oligonucleotide 5'-AGCTCCAGGTCTACAATGGCA-3' was prepared as a sense primer. The ULBP-1 antisense primer was 5'-CATGCTCACAAGACATCCTGG-3'. For ULBP-2, the oligonucleotide 5'-TGATGGGCATGGACAGCA-3' was prepared as sense primer. The ULBP-2 antisense primer was 5'-AATTCCATCAGGTAGCACCAAGAG-3'. For ULBP-3, the oligonucleotide 5'-CCTAGAAGAGCAGCTGTATGCCA -3' was prepared as sense primer. The ULBP-3 antisense primer was 5'-GTGCTGTGGGTTCCAGCC -3'. For GADPH, an internal control, two primers (5'-GTC AAC GGA TTT GGT CGT ATT-3' and 5'-AGT CTT CTG GGT GGC AGT GAT-3') were used. PCR was performed using a DNA Thermal Cycler under the following conditions: template DNA 50-100ng, dNTP 200μM, primer 2 pM, ProTaq plus DNA polymerase 2U (Protech, Taiwan,R.O.C.); denaturation, 94℃, 30 sec; annealing, 55℃, 30 sec; extension, 72℃, 1 min; 28 cycles. Electrophoresis of the PCR products on a 2%agarose gel revealed an hsp90 band of about 406bp, and a GADPH band of about 540bp. Each band was analyzed using the Bio-1D image analysis software version, and the ratio of hsp90 to GADPH was calculated and semi-quantitatively analyzed.

2.4. Statistical analysis

The difference in NKG2D ligands expression between normal esophagus and ESCC was examined by Wilcoxon signed-rank test. The difference in expression rate in NKG2D ligands between normal esophagus and ESCC was analyzed by Yate's correction of contingency. The correlation between NKG2D ligands expression in ESCC was examined by Spearman's rho correlation. The association of NKG2D ligands expression and disease status was analyzed by the Kruskal-Wallis test. For all statistical tests, the level of significance was set at 0.05.

3. Results

A total of 39 pairs of ESCCs and adjacent non-tumor counterparts were processed for MIC-A/B and ULBP1,2,3 expressions by immunohistochemistry and RT-PCR. All 39 patients were male. The age ranged from 37 to 86 years old (mean age 61). There were 2 stage I, 8 stage IIa, 5 stage IIb, 18 stage III, and 6 stage IV patients. Table 1 lists the clinicopathological features of the 39 patients.

3.1. Expression of MIC-A/B and ULBP1,2,3 in the normal esophagus and ESCC

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Table 1. Clinicopathological features of 39 ESCC patients

Variable	
Gender	
Male	39
Female	0
Age	
≤60	20
>60	19
Stage	
I+II	15
III+IV	24
Differentiation	
Moderate	22
Moderate to poor	11
Poor	6
Lymph node metastasis	
Negative	11
Headache	28

The expression of MICA, MICB, ULBP1, ULBP2, and ULBP3 in normal esophagus and ESCC was shown in Table 2. The difference in expression between normal esophagus and ESCC was significantly increased in ULBP1 and ULBP3.

3.2. The Expression rate of MIC-A/B and ULBP1,2,3 in the normal esophagus and ESCC

The expression rate of MICA, MICB, ULBP1, ULBP2, and ULBP3 in normal esophagus and ESCC were 25%,100%, 38.5%, 69.2%, 71.8% and 30.8%,100%, 64.1%, 82.1%, 89.7%. The difference in expression rate between normal esophagus and ESCC was significantly increased in ULBP1. (Table 3)

3.3. The correlation between expression of MIC-A/B, ULBP1,2,3 in ESCC.

The correlation between the expression of ULBP1, ULBP2, and ULBP3 in ESCC was significant. (Table 4)

Table 2. Expression of MIC-A/B and ULBP1,2,3 in normal esophagus and ESCC

	Normal(%)	Tumor(%)	*p*-value
MICA	6.78+2.01	6.83+2.05	0.826
MICB	63.83+11.6	48.36+5.82	0.706
ULBP-1	10.25+2.91	37.01+6.15	<0.001*
ULBP-2	60.73+11.3	65.20+8.25	0.478
ULBP-3	39.91+8.46	59.55+7.85	0.003*

Wilcoxon signed-rank test. *p<0.05

Table 3. Expression rate of MIC-A/B and ULBP1,2,3 in normal esophagus and ESCC

	Normal(%)	Tumor(%)	*p value*
MICA	25.6	30.8	0.801
MICB	100.0	100.0	
ULBP-1	38.5	64.1	0.041*
ULBP-2	69.2	82.1	0.291
ULBP-3	71.8	89.7	0.085

Yate's correction of contigency. *p<0.05

3.4. The correlation between expression of MIC-A/B, ULBP1,2,3 in ESCC and disease status of the patients

There was no significant correlation between the expression of MIC-A/B and ULBP1,2,3 and the disease status of the patients (stage, tumor differentiation, and lymph node metastasis). (Table 5)

4. Discussion

There are at least 7 subgroups of human NKG2, such as NKG2A, B, C, D, E, F, and H. The function of NKH2A and B are inhibitory receptors. The NKG2C, D ,and E are activating receptors. However, NKG2D participates in immunities including innate and adaptive(15).

To prevent autoimmune tissue injured, the expression of NKG2D ligands is strictly controlled. Expressing of NKG2D ligands is not found in normal tissue. Compared with different NKG2D ligands, the

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Table 4. The correlation between MIC-A/B, ULBP1,2,3 in ESCC.

	MICA	MICB	ULBP-1	ULBP-2	ULBP-3
MICA	1				
MICB	0.812	1			
ULBP-1	0.338	0.078	1		
ULBP-2	0.753	0.868	<0.001*	1	
ULBP-3	0.323	0.210	<0.001*	<0.001*	1

Spearman's rho correlation. *p<0.05

Table 5. The correlation between MIC-A/B, ULBP1,2,3 in ESCC and disease status

	Stage	Tumor Differentiation	Lymph node metastases
MICA	0.844	0.825	0.611
MICB	0.880	0.286	0.845
ULBP-1	0.619	0.460	0.680
ULBP-2	0.749	0.539	0.425
ULBP-3	0.448	0.504	0.508

Kruskal-Wallis Test

MIC family play an important role in certain cancer cell(16). Further, in this category, MICA expresses much more on MICB on the tumor cell surface. Besides, the ULBP families are also regarded as unfavorable prognosis in some cancers.

For example, ~~to~~ against hepatocellular carcinoma, the NKG2D pathway also plays a decisive role as an inhibitor. By recruiting interferon-gamma, tumor necrosis factor, and releasing certain enzymes, protein promotes the process of eliminating cancer cells(15). Besides, in esophageal squamous cell carcinoma, infiltrated ~~of~~ NK cells are related to better prognosis via the cytotoxic pathway of NKG2D expression.

In this study, we analyzed the expression and role of different NKG2D-specific ligands; MICA, MICB, ULBP-1, ULBP-2, and ULBP-3, in human esophagus and ESCC. We have demonstrated for the first time that MIC-A/B and ULBP1,2,3 molecules express in both normal esophagus and esophageal squamous cell carcinoma. Moreover, we show the upregulation of these NKG2D ligands in esophageal carcinoma, especially in ULBP-1 and ULBP-3. A detailed analysis of NKG2D ligand expression on tumor cells and their regulation by exogenous factors could be very useful in designing strategies to exploit immune defenses against cancer. The ligands of NKG2D are MIC-A/B and ULBPs, which are encoded within the human MHC. MIC-A/B are stress-induced class I molecules with three extracellular domains that neither associate with β2-microglobulin nor bind peptides. ULBP class I-like molecules have the class I related α1 and α2 domains but lack aα3 domain, and have a GPI linkage to the plasma membrane(17, 18). In this study, we have demonstrated the significant correlation of expression between ULBP-1, ULBP-2, and ULBP-3, but not with MIC-A/B in ESCC. The relationship between these NKG2D ligands in ESCC needs further investigation.

NK cells are important effector cells in the defense of tumors. Our previous study has also demonstrated that high-level NK cells infiltration in primary resected ESCC was found to have a better prognosis(14). Because NK cell function is the result of a fine-tuning between opposite signals by inhibitory and activating receptors, it is conceivable that the final signal delivered to a given NK cell may depend on the relative number of the various receptor/ligand interactions and on the avidity of each receptor engagement. Indeed, in humans, it has been proposed that even HLA class I+ target cells may be killed by NK cells via NKG2D(11, 19). So the NKG2D receptor-ligand system represents a new theme in immune recognition in which cells in distress, such as tumor cells, up-regulate ligands that are specifically designed to enlist an attack from immune effector cells.

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Recent studies demonstrated that MIC/NKG2D interactions are present in the initial phases of an immune response to uveal melanoma, but this interaction seemed to be less involved in response to metastatic disease suggesting an immune selection of MIC-negative tumor cells(18). In this study, the lack of correlation between NKG2D ligands expression and the clinical course of the disease has provided the impetus to investigate the mechanisms used by malignant cells to escape immune recognition and interaction with NK cells in ESCC.

In recent years, immunotherapy has demonstrated encouraging outcomes when used as a component of the treatment approach for esophageal cancer. The initial utilization for esophageal squamous cell carcinoma treatment was immune checkpoint inhibitors (PD-1/PD-L1 blockade). Following the recent CheckMate-577 trial(20), which suggested a substantial advantage of adjuvant nivolumab after the standard CROSS regimen(21), several current research projects are evaluating the effectiveness of checkpoint of esophageal cancer as immunotherapy studies are now being conducted in a curative approach.

Recent research has revealed the potential of NK cells in cancer immunotherapy due to their exceptional capability to recognize and eliminate transformed cells without requiring prior sensitization. As a result, investigations centered on the antitumor immune response of NK cells have become a focal point in the field of tumor immunology. The NKG2D-NKG2D ligand (NKG2DL) pathway is regarded as a promising candidate for immunotherapy due to its selective expression on tumor cells of "stress-induced ligands" and the potent activation of NK cells through NKG2D (23). Various approaches to target the NKG2D pathway for cancer therapy are developed based on a comprehensive understanding of this mechanism, including NKG2D-mediated cancer immunity.

In conclusion, we have demonstrated for the first time that MIC-A/B and ULBP1,2,3 molecules express in both normal esophagus and esophageal squamous cell carcinoma. Even upregulation of these NKG2D ligands expression is noted, there is no significant correlation between the expression of these ligands and the disease status of the patients in esophageal squamous cell carcinoma. The mechanism of work by natural killer cells and the NKG2D receptor-ligand system and the immunotherapy by NK cells in esophageal squamous cell carcinoma need further investigation.

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