Expression of immunoglobulin-like transcript (ILT)2 and ILT3 in human gastric cancer and its clinical significance

YI ZHANG[1], NAN LU[2], YUWEN XUE[3], MIN ZHANG[4], YINGJIE LI[1], YUANQUAN SI[1], XIAOKUN BIAN[1], YANFEI JIA[5] and YUNSHAN WANG[5]

[1]Department of Clinical Laboratory Medicine, Qilu Hospital of Shandong University; [2]Institute of Diagnostics, School of Medicine, Shandong University; [3]Department of Medicine, Qilu Hospital of Shandong University; [4]Department of Medicine, Shandong Chest Hospital; [5]Shandong Province Key Laboratory for Tumor Target Molecules, Medical Research and Laboratory Diagnostic Center, Jinan Central Hospital Affiliated to Shandong University, Jinan, P.R. China

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Abstract. Immune inhibitory receptors play an important role in organ transplantation, autoimmune diseases and cancers. Immunoglobulin-like transcript (ILT)2 and ILT3 belong to the inhibitory receptors of the ILT family, which have been reported to regulate a broad range of cellular functions involved in the immune response. They contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which are related to immune regulation. Although ILT receptors have been studied in dendritic cells (DCs), T cells, NK cells and other cell types, the expression and clinical significance of ILT2 and ILT3 in gastric cancer have yet to be elucidated. Here, the expression of ILT2 and ILT3 in gastric cancer cell lines and pathologic tissues, as well as their effects on the cytotoxicity of NK92MI against the gastric cancer cell lines MKNI with $ILT2^{low}ILT3^{low}$ and HGC-27 with $ILT2^{high}ILT3^{high}$ were detected. The results suggest that ILT2 and ILT3 are expressed with diverse degrees in gastric cancer cells and tissues, and the expression of ILT2 is related with differentiation and size of tumors. Furthermore, the cytotoxic activity of NK92MI against the MKNI cell line was stronger than that against HGC-27. This study indicates that ILT2 and ILT3 play a key role in gastric cancer immune escape, and ILT2 may be a new target in the clinical diagnosis and treatment of gastric cancer.

Correspondence to: Professor Yi Zhang, Department of Clinical Laboratory Medicine, Qilu Hospital of Shandong University, 107 Wen Hua Road, Jinan 250012, P.R. China
E-mail: yizhang@sdu.edu.cn

Professor Yunshan Wang, Medical Research and Laboratory Diagnostic Center, Jinan Central Hospital Affiliated to Shandong University, 115 Jie Fang Road, Jinan 250013, P.R. China
E-mail: sdjnwys@163.com

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Introduction

Gastric cancer is one of the most serious diseases threatening human health. It is the fourth most common cancer and the second leading cause of cancer-related death in the world (1,2). According to the Chinese Ministry of Health in 2006, over 400,000 new cases of gastric cancer are diagnosed and approximately 300,000 deaths in China are reported each year.

Interaction between the host immune response and gastric cancer cells results in the occurrence and development of gastric cancer. This involves multilateral factors, such as host effector cells, stromal cells and tumor cells (3-5). Shortage of co-stimulation molecules on the surface of tumor cells is the main reason for immune evasion among all of the factors. There are two types of co-stimulation molecules discovered at present, which include positive and negative co-stimulation molecules. The former influences the activation and proliferation of T lymphocytes and secretion of cytokines by combining with lymphocyte receptors, while the latter induces T cells anergy through two ways as follows. One is to induce inhibitory factors to express on the effector cells and the other is to elicit them to express on tumor cells (6).

Immunoglobulin-like transcripts (ILTs), also called leukocyte immunoglobulin-like receptors (LIRs/LILRs) or CD85 (7), are a group of membrane receptors coded by more than 10 genes located on the 19q13.4 chromosome (8,9). Nakajima *et al* (10) identified a new class of immunoglobulin superfamily, whose members were named ILT1 and ILT2, and other ILT family members were later found and identified (11-14). Characterized by different transmembrane and cytoplasmic domains, the members of the ILT family are divided into activating receptors, inhibitory receptors and soluble receptors (15). ILT2 and ILT3 as the two primary types of inhibitory receptors, which regulate the functions of cells involved in the immune system (16). ILT2 is detected on the surface of a proportion of NK cells (~75%) and T lymphocytes (4-20%), on all B lymphocytes, monocytes, DCs and macrophages (17). However, ILT3 is expressed only on monocytes, DCs and endothelial cells, but not on T and B lymphocytes (18,19). They play an extensive role by transmitting inhibitory signals, for instance, they restrain the killer activity of

killer cells, and regulate signal transmission between DCs and T lymphocytes (20-23). ILT3 also induces the generation of $CD8^+$ T suppressor cells and renders antigen-presenting cells tolerogenic (22,24). In addition, it has been reported that ILT2 and ILT3 integrate with MHC-I molecules, especially HLA-G, and deliver negative signals by immunoreceptor tyrosine-based inhibitory motifs (ITIM) to repress the activation of $CD4^+$ and $CD8^+$ T lymphocytes and down-regulate recognition of antigens by $CD8^+$ T lymphocytes, which leads to the immune escape of tumor cells (25-27).

The mechanisms through which tumor cells escape from host immunity are not yet clear. However, one important reasons is that ILTs induce immunological tolerance. Lefebvre *et al* (28) discovered that partial tumor infiltrating lymphocytes in human breast cancer express ILT2. Yet, the role of ILT receptors in tumors is not clear and many issues require further study. Our research aimed to investigate the expression and role of ILT2 and ILT3 in gastric cancer via *in vivo* and *in vitro* experiments.

Materials and methods

Cell lines and culture. Six human gastric cancer cell lines were used, which included the well-differentiated gastric cancer cell line MKNI, moderately differentiated cell lines SGC7901 and AGS, the poorly differentiated cell line MGC803 and undifferentiated cell lines HGC27 and BCG823, respectively. Human NK92MI cells were used as effector cells. All gastric cancer cell lines and human NK92MI were from Shandong Province Key Laboratory for Tumor Target Molecules in Jinan Central Hospital which is affiliated to Shandong University. All cell lines were maintained in RPMI-1640 or F-12 medium (Gibco Co., USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin and 100 IU/ml streptomycin at 37˚C in a humidified atmosphere with 5% CO_2.

Patients and information. Tumor specimens were obtained from 50 primary gastric cancer patients (37 men and 13 women; aged 38-76 years; mean age, 53.3 years) without having received any preoperative therapy at Qilu Hospital of Shandong University, China, between February, 2008 and August, 2009. The main clinicopathological variables of the patients are recorded in Tables I and II. According to pathological Tumor-Node-Metastasis International System and Lauren classification, 31 cases were characterized as gastric adenocarcinomas, 10 were mucinous adenocarcinomas and 9 were signet ring cell cancers. Considering pathological grading, 20 were staged as well differentiated and 30 as poorly differentiated. Two-thirds of the patients with lymph node metastasis were validated by conventional postoperative pathological examination. There were 19 cases with tumor size >5 cm, and 31 cases with tumor size <5 cm. We obtained informed consent from each patient, and the study protocol was approved by the Ethics Committee of Qilu Hospital of Shandong University and was performed in accordance with the ethical standards of the 2000 Declaration of Helsinki as well as the Declaration of Istanbul 2008.

Total-RNA isolation and RT-PCR. The gastric cancer cells ($3\text{-}5x10^5$) with various degrees of differentiation were collected respectively, and the total-RNA was extracted using the TRIzol RNA isolation kit (Invitrogen, USA). Total-RNA (1 μg) was reverse transcribed using High-Capacity cDNA reverse transcription kits (Applied Biosystems, USA) according to the manufacturer's instructions and then was detected by SYBR-Green I (Invitrogen) real-time PCR. The ILT primers were synthesized by Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). The primers for ILT2, ILT3 and ACTB (internal control) were designed as follows: ILT2 primer, forward, 5'-GGGGTTGTGATCGGCATCTT and reverse sequence, 5'-CTGGCCTGGACTCGATGTC; ILT3 primer, forward, 5'-CATCCATGACAGAGGACTATGC and reverse sequence, 5'-GGGCTGAAAGGGTGGGTTTA; ACTB forward, 5'-TTGCCGACAGGATGCAGAA and reverse sequence, 5'-GCCGATCCACACGGAGTACT. The lengths of the amplified fragments were 189, 311 and 101 bp, respectively. The FQ-PCR system contained SYBR-Green I 10 μl, PCR forward and reverse primers (5 μM) 1 μl, respectively, cDNA 2 μl, ddH_2O 6 μl. There were 2 μl DEPC in the negative control instead of cDNA. The reaction was incubated for 40 cycles at 95˚C for 10 sec, 60˚C for 10 sec and 72˚C for 10 sec.

Flow cytometry. We collected and counted the gastric cancer cells in the media which were in the logarithmic phase, and adjusted the consistency of the cells to $1\text{-}2.5x10^5$ cells/ml. The cells were washed twice with phosphate-buffered saline (PBS, pH 7.4), and incubated with mouse anti-human ILT2 (R&D, USA; clone 292305) and ILT3 antibody (R&D; clone 293623) for 30 min. After washing thoroughly with PBS, the cells were stained with PE-labeled goat anti-mouse antibodies (R&D) for 30 min at 4˚C. Cells were washed twice with PBS and analyzed by FACSCalibur flow cytometry with CellQuest software and associated software (BD Company, USA). Meanwhile, non-specific staining was determined using control goat immunoglobin.

Cytotoxicity assay. Two types of target cells ($1x10^5$/ml) in a logarithmic growth phase, $ILT2^{low}ILT3^{low}$ human well differentiated gastric cancer MKNI cells and $ILT2^{high}ILT3^{high}$ undifferentiated gastric cancer HGC27 cells, were seeded in 96 well culture plates at 37˚C, in a 5% CO_2 incubator for 4 h, respectively. Each well contained 100 μl RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). Whereafter, NK92MI cells were added in a volume of 100 μl at effector and target cell ratios of 10:1, 5:1, 2.5:1 and 1.25:1. Blank controls, target cell controls (T) and effector cell controls (E) were set synchronously. The cell mixtures were then incubated at 37˚C, in 5% CO_2 for 24 h, after which 20 μl MTT (5 mg/ml) was added and cultured for a further 4 h. The absorbance was determined at 570 nm by an ELISA reader. The percentage of kill (% kill) was calculated using the following equation: $\%\ \text{kill} = [1 - (OD_{E+T} - OD_E)/OD_T] \times 100\%$.

Immunohistochemistry. Immunohistochemical staining was performed using the biotin-streptavidin-peroxidase method with a Vectastain ABC kit (Invitrogen). Resected tissue specimens were collected and fixed in formalin, embedded in paraffin, and cut into 4-μm serial sections. Sections were incubated with mouse anti-ILT2 and ILT3 antibodies (diluted

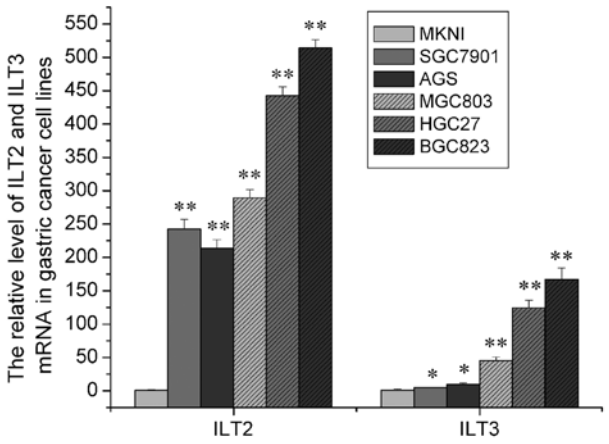

Figure 1. Expression of ILT2 and ILT3 mRNA in the gastric cancer cell lines. Expression of ILT2 and ILT3 at the level of mRNA increased with the decreased degree of gastric cancer cell differentiation. *P<0.05, **P<0.01 by comparision with the well-differentiated gastric cancer cell line (MKN1).

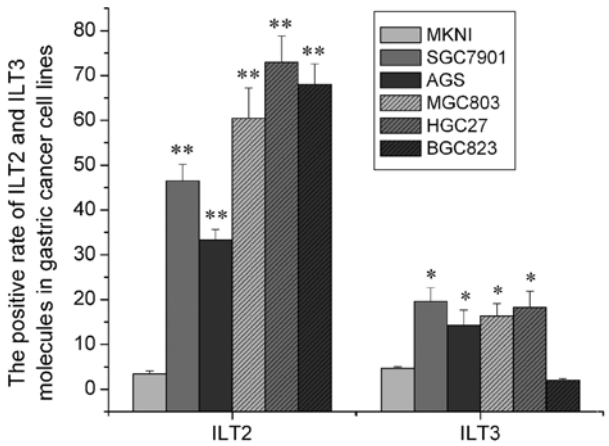

Figure 2. Expression of ILT2 and ILT3 protein in gastric cancer cell lines. The positive rates of ILT2 protein were related to the differentiation of the gastric cancer cells. However, the expression of ILT3 protein did not obviously vary in the gastric cancer cells with different degrees of differentiation. *P<0.05, **P<0.01 by comparision with the well-differentiated gastric cancer cell line (MKN1).

1:300) in a humidty chamber at 4˚C overnight. After washing with PBS, the sections were incubated with biotinylated goat anti-mouse antibodies for 30 min and then washed three times with PBS, and then incubated with streptavidin-conjugated peroxidase for 30 min. The sections were then visualized by incubation with 3,3'-diaminobenzidine solution (0.3% hydrogen peroxide and 0.05% 3,3'-diaminobenzidine) and stained with hematoxylin. Negative controls were carried out by substituting a normal mouse IgG for the primary antibody. Histological analysis was performed by two investigators simultaneously by microscopy without knowledge of the patient clinical records, and the number of ILT2- and ILT3-stained tumor cells among 1,000 tumor cells in each section was determined. Cell counts were performed at a magnification x400 in at least five fields in randomly selected tumor areas.

Statistical analysis. Statistical analysis was performed using SPSS 10.0. Comparison of the means between the two groups

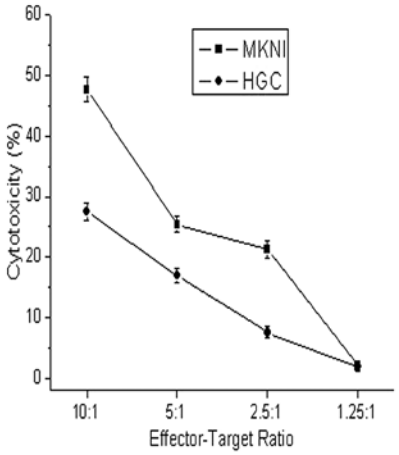

Figure 3. Killing activity of NK92MI to MKNI and HGC-27 with diverse effector-target ratios. The cytotoxicity of NK92MI to ILT2^{low}ILT3^{low} MKNI was stronger than that to ILT2^{high}ILT3^{high} HGC-27 at different effector-target ratios (except at 1.25:1).

was different analyzed using the t-test, and comparison of the means among the groups was assayed by variance analysis and SNK test. The result of the immunohistochemistry was analyzed using the χ^2 test, and the relationship between the expression of ILT2 and ILT3 and clinical parameters was assessed by the Fisher's test. Data were represented as the means ± standard deviation. Values of P<0.05 were considered to denote statistical signification.

Results

Expression of ILT2 and ILT3 mRNA in gastric cancer cells. We detected ILT2 and ILT3 mRNA expression in the 6 gastric cancer cell lines using FQ-PCR SYBR-Green I. The relative expression of ILT2 and ILT3 mRNA is shown in Fig. 1. Due to the absence of a negative standard control, we used the expression of ILT2 and ILT3 mRNA in the MKNI cell line as cardinal number. Relative expression of ILT2 and ILT3 mRNA in the other groups was derived from values compared with the cardinal number. As shown in Fig. 1, it was demonstrated that ILT2 and ILT3 mRNA expression increased along with increased differentiation, and the difference among them was significant (P<0.05).

Expression of ILT2 and ILT3 protein in gastric cancer cells (Fig. 2). The positive rate of ILT2 and ILT3 protein expression in the wel- differentiated gastric cancer cell line MKN1 was low, which was significantly different from the expression levels in the moderately or poorly differentiated gastric cancer cell lines and the undifferentiated tumor cell lines as well (P<0.05). Among the 6 gastric cancer cell lines, BCG823 hardly expressed the ILT3 protein while it expressed a high level of the ILT2 protein. The positive rates of ILT3 protein expression were lower than those of the ILT2 protein in the five cell lines (MKN1, SGC7901, AGC, MGC803 and HGC27), and expression of the ILT3 protein did not vary obviously between the moderately or the poorly differentiated and undifferentiated cell line (P>0.05). However, the positive rate of ILT2 protein expression in poorly differentiated and undifferentiated gastric

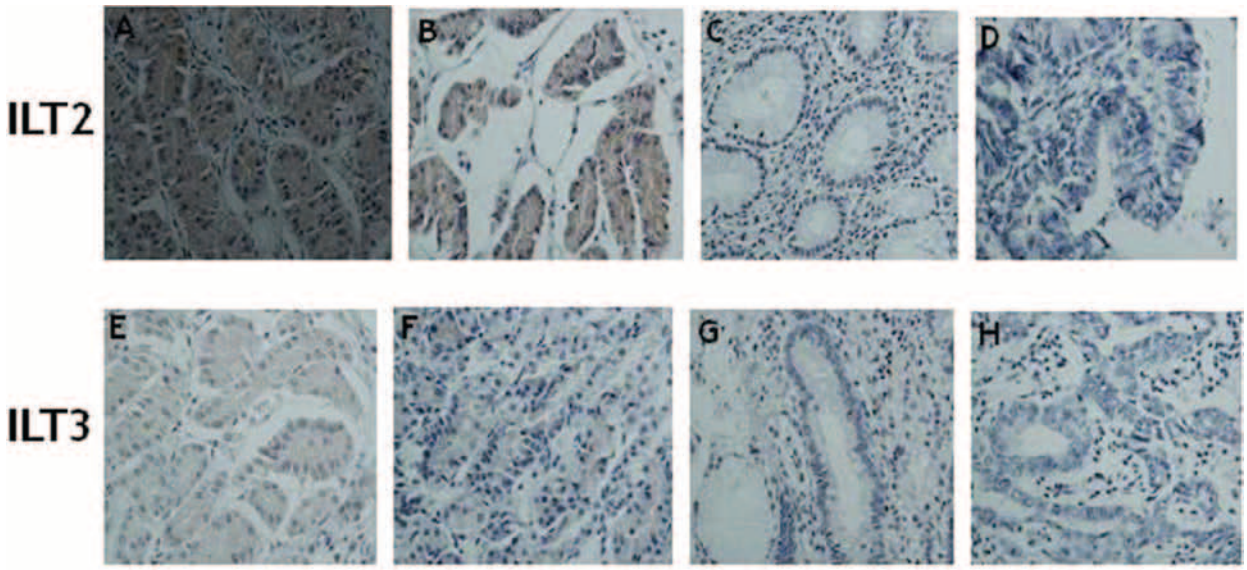

Figure 4. ILT2 and ILT3 immunohistochemical staining of (A and E) poorly differentiated gastric cancer, (B and F) moderately differentiated gastric cancer, (C and G) well-differentiated gastric cancer and (D and H) normal gastric tissue. (Original magnification, x400) As shown above, ILT2 was expressed in gastric cancer tissues with diverse differentiation degrees and pathologic types, and the expression was correlated with differentiation degrees. Furthermore, there was barely any expression of ILT2 noted in the normal stomach tissue. However, no significantly statistical difference was achieved between ILT3 expression and the differentiation degree and size of the cancer tumors.

Table I. Relationship between ILT2 expression in the tumor cells and clinicopathological variables.

		ILT2 expression			
Clinicopathological variables	No. of patients	Negative	Positive	χ^2	P-value
Gender				0.002	0.968
Male	37	14	23		
Female	13	5	8		
Age (years)				0.085	0.771
>60	25	9	16		
≤60	25	10	15		
Tumor location				0.656	0.720
Ridge 1/3	30	11	19		
Medi 1/3	13	6	7		
Bottom 1/3	7	2	5		
Differentiation				4.089	0.043[a]
Well/moderately	20	11	9		
Poorly	30	8	22		
Muscular infiltration				0.195	0.659
No	14	6	8		
Yes	36	13	23		
Metastasis				0.110	0.740
Negative	17	7	10		
Positive	33	12	21		
Tumor size				6.417	0.011[a]
≤5 cm	31	16	15		
>5 cm	19	3	16		

Well-differentiated gastric cancer included mastoid adenocarcinoma, well-differentiated tubular adenocarcinoma, and moderately differentiated adenocarcinoma; poorly differentiated cancer included poorly differentiated adenocarcinoma, signet ring cell cancer and Mucus cell carcinoma. [a]P<0.05. There was a significant relationship between ILT2 expression and the differentiation and size of tumors.

Table II. Relationship between ILT3 expression on tumor cells and clinicopathological variables.

Clinicopathological variables	No. of patients	ILT3 expression		χ^2	P-value
		Negative	Positive		
Gender				0.081	0.755
Male	37	30	7		
Female	13	11	2		
Age (years)				0.136	0.712
>60	25	20	5		
≤60	25	21	4		
Tumor location				2.414	0.087
Ridge 1/3	30	24	6		
Medi 1/3	13	11	2		
Bottom 1/3	7	6	1		
Differentiation				0.203	0.652
Well/moderately	20	17	3		
Poorly	30	24	6		
Infiltration muscular				0.182	0.670
No	14	12	2		
Yes	36	29	7		
Metastasis				0.002	0.963
Negative	17	14	3		
Positive	33	27	6		
Tumor size				0.101	0.750
≤5 cm	31	25	6		
>5 cm	19	16	3		

Well-differentiated gastric cancer included mastoid adenocarcinoma, well-differentiated tubular adenocarcinoma, moderately differentiated adenocarcinoma; poorly differentiated cancer included poorly differentiated adenocarcinoma, signet ring cell cancer and mucus cell carcinoma. No significant relationship was noted between ILT3 expression and clinicopathological variables.

cancer cells was much higher than that in the moderately and well-differentiated cancer cells (P<0.05).

Differential expression of ILT2 and ILT3 in gastric cancer cells affected NK92MI cytotoxicity. Here, we chose the $ILT2^{low}ILT3^{low}$ well-differentiated gastric cancer cell line MKNI and the $ILT2^{high}ILT3^{high}$ undifferentiated gastric cancer cell line HGC27 as target cells, and the NK92MI as effector cells. The cytotoxicity of NK92MI to MKNI and HGC27 was detected, respectively, using different effector-target ratios. The cytotoxicity of NK92MI to difference MKNI at effector-target ratios from 10:1 to 2.5:1 was stronger than that to HGC27 after 24 h of co-culture, and a significant difference was noted (P<0.05) (Fig. 3). We conclude that the different expression of ILT2 and ILT3 in gastric cancer cells influenced the killer activity of NK92MI cells.

Relationship between the expression of ILT2 and ILT3 in patients with gastric cancer and clinicopathologic variables. Expression of ILT2 and ILT3 in gastric cancer cells from 50 patient pathological tissues with diverse degrees of differentiation and pathological types were found to be dissimilar by immunohistochemistry, and the positive rate of ILT2 and ILT3 expression was 62.0% (31/50) and 18.0% (9/50), respectively, which indicates that there was a significant difference between ILT2 and ILT3 in the gastric cancer cells (P<0.01). ILT2 and ILT3 protein were expressed either in the cytoplasm or the cell membrane of the gastric cancer cells, but no ILT2 and ILT3 expression was noted in the normal stomach tissue (Fig. 4).

Further information concerning the pathological characteristics and the clinical data of these patients were analyzed (Tables I and II). It was demonstrated that there was no significant different between ILT2 and ILT3 expression and factors such as cancer origin, invasive depth and lymphatic metastasis (P>0.05). Yet, a distinct relationship was noted between ILT2 and the differentiation and size of the tumor. The positive rate of ILT2 expression was 73.3% (22/30) and 45% (9/20), respectively, in the poorly differentiated cancers including adenocarcinoma, signet ring cell cancer and well differentiated cancer including mastoid adenocarcinoma, well- and moderately differentiated tubular adenocarcinoma (χ^2=4.089, P<0.05). The positive rate of ILT2 expression was 84.2% when the tumor size was >5 cm, which was higher than that of 48.4% when the tumor size was <5 cm (χ^2=6.417, P=0.011). Nevertheless, no significantly statistical relation was noted between ILT3 expression and the differentiation degree and the size of the tumors.

Discussion

ILT2 and ILT3 belong to a family of inhibitory receptors, which are characterized by long cytoplasm tails that contain three immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and either two or four homologous extracellular C-2 type Ig-like domains (8,29,30). It has been suggested that the interaction of ILT2 or ILT3 with HLA-G inhibits the activation of T cells, natural killer cells and myelomonocytic cells (9,31,32). They also regulate $CD8^+$ T cell activation by blocking CD8 binding as well as by recruiting inhibitory molecules through their ITIM motifs (33). Recent studies have demonstrated that monocytes and dendritic cells (DCs) exposed to T suppressor or regulatory cells acquire a tolerogenic phenotype characterized by upregulation of ILT2 and ILT3 expression (34-36). Antigen-presenting cells (APCs), which express high levels of these receptors, can become tolerogenic and induce anergy in $CD4^+$ T helper cells (32,37). Then the antigen-presenting function of monocytes and DCs is modulated by the level of expression of ILT2 and ILT3. Studies have also shown that ILT3 on the surface of B lymphocytes is related to tumor-specific T lymphocyte tolerance in B-cell chronic lymphatic leukemia, and regardless of whether ILT3 was membrane (mILT3) or soluble (sILT3), they all had strong immunosuppressive activity (38). In addition, high expression of ILT2 and ILT3 was found to highly induce Th cells and CTLs into Treg and Ts cells, respectively, *in vitro* while ILT was expressed in DCs. $CD8^+CD28^-$ Ts cells upregulated the expression of HLA-G in tumor cells, which result in immune escape (25,39). Yet, no report exists to date concerning ILT2 and ILT3 expression in human tumor tissues and to our knowledge this is the first description of the expression of these molecules in primary gastric cancer.

Our studies demonstrate that ILT2 and ILT3 mRNA and protein were expressed to a different extent in the gastric cancer cells with different degrees of differentiation. Moreover, there was a significant relationship between the expression of ILT2 and ILT3 and the pathological grade of the gastric cancer. We also found that the cytotoxicity of NK cells against gastric cancer cells with different expression of ILT2 and ILT3 was distinct *in vitro*. It was previously reported that inhibitory receptors influence the function and state of T lymphocytes, NK cells and DCs, and regulate immunological tolerance (40,41). Thus, we speculated that tumor cells express inhibitory receptors via certain pathways and affect the killer activity of immunocytes.

We found that ILT2 and ILT3 were expressed to some extent in the gastric cancer pathological tissues, and their expression in cancer tissues was characterized by a scattered pattern and was located in both the plasma membrane and the cytoplasm of the cancer cells. The intensity and positive rates of ILT2 were higher than that of ILT3, and the expression of ILT2 was obviously related to the differentiation and size of the tumors, which demonstrated that expression of this inhibitory receptor by the tumor cells restrained the immune response. Sun *et al* (42) discovered that ILT4 was expressed in non-small cell lung cancer cells *in vitro* as well. By assessing clinical pathological tissues, they also found that there was a significant relation between the expression of ILT4 and surrounding tumor infiltrating lymphocytes (TILs), which suggested that the tumor cells induced infiltrating lymphocyte tolerance by contacting them indirectly after upregulating inhibitory ILT4. In conclusion, our research indicates that ILT2 and ILT3 expression in gastric cancer cells and tissues may play an important role in tumor development, and ILT2 and ILT3 may be applied as targets for the diagnosis and therapy of gastric cancer. However, further research is needed to understand the specific mechanism.

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