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Expression of the nonclassical HLA class I and MICA/B molecules in human hepatocellular carcinoma

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HLA-G and HLA-E are nonclassical human MHC class I molecules, which promote tolerance to NK cytotoxicity. MICA and MICB are known to enhance the functions of NK and T cells. However, the expression of these molecules has never been investigated in liver cancer.

Using RT-PCR and western blot, we aimed to identify the expression of HLA-G, HLA-E, MICA and MICB in a panel of 41 tissues dissecting from liver cancer patients in China.

HLA-G mRNA was expressed in 8 of 41 Human Hepatocellular Carcinoma (HCC) specimens and in 1 adjacent normal hepatocellular tissue. The expression of HLA-G protein was found in 7 of the 8 HLA-G mRNA-positive HCC tissues. HLA-E mRNA was up-regulated in 56% HCC specimens but the expression of HLA-E protein was only upregulated in 29% HCC tissues in comparison with their adjacent normal counterpart. MICA and MICB mRNA was decreased in 5% and 8% HCC specimens, while the expression of their proteins decreased in 21% and 24% HCC tissues.

These results suggested that the expressions of HLA-G, HLA-E, MICA and MICB were differently up-regulated in HCC tissues. Furthermore, HLA-E and MICA/B genes showed obviously distinctive expression pattern at transcription and translation level.

Key words: Hepatocellular carcinoma; HLA-G; HLA-E; MICA; MICB

Non-classical HLA class I molecules HLA-E, HLA-F and HLA-G have a limited polymorphism and display a restricted expression pattern [1]. Among them, expressions of HLA-E and HLA-G have been reported to be able to support tumor escaping from host immunosurveillance. HLA-G with the immunosuppressive properties was first evidenced at the fetal–maternal interface, where its expression by classical HLA class I-negative cytotrophoblast cells protects the fetus from destruction by maternal NK cells [2]. Ectopic expression of HLA-G was observed in tumor biopsies and their expression confers tumor cells resistance to NK cell cytolysis [3]. This has positioned HLA-G as a molecule capable of contributing to immune escape by tumors [4]. Indeed, HLA-G expressed by tumors may inhibit NK cytotoxicity by interacting with ILT-2 and/or KIR2DL4 receptors on NK cells [5]. Similarly, HLA-E might also contribute to tumor cell escaping from NK cytotoxicity. HLA-E binds specifically to the conserved leader sequence peptides of the classical HLA class I molecules and interacts with the inhibitory receptor CD94/NKG2 on the cell surface of NK cells [6,7]. The MHC class I-chain related (MIC) gene family has been identified within the HLA class I region [8]. The family consists of seven members, among which, only MICA and MICB encode transcripts that give rise to functional glycoproteins [9]. The product of MICA and MICB are the ligands for the activating receptor NKG2D on NK cells. By engaging the receptor, MICA/ MICB provide powerful stimulation for NK cells and can therefore determine the magnitude and outcome of certain effector functions [10].

Hepatocellular carcinoma (HCC) is one of the most common malignant and highly lethal tumors over the world. Intervention of HCC progression as well as its recurrence is one of the most challenging aspects for reducing the mortality of HCC. The liver is rich of natural immune cells, including NK cells, natural killer T cells (NKT), γδT cells and Kuffer cells. It has been shown that NK cells account for 31 percent of the total number of lymphocytes in the liver. Therefore, the liver might most likely the largest full-time natural immune organ [11]. However, NK cell activity has been demonstrated to be decreased in HCC patients [12,13]. Since NK cells are known to play a distinct role in first-line immunologic defense

against tumor cells [14,15], it was thought that augmenting the NK-mediated innate immune response might be an attractive strategy for preventing HCC. For this purpose, it is important to get the basic knowledge of the expression of multiple ligands on HCC cells that determine the magnitude and outcome of the killing effect of NK cells.

So far, the expression of HLA-E in HCC has not been reported and the expression of MICA and MICB in HCC has only been reported in 10 fresh tissues by RT-PCR assay [16]. The expression of HLA-G in HCC has been reported in 173 samples by immunohistochemical staining of tissue microarray blocks and membrane-bound HLA-G1 protein was detected by Western blot in five human HCC cell lines [17]. In this study, we aimed to identify the expression of HLA-G, HLA-E, MICA and MICB in a panel of 41 fresh tissues of liver cancer patients in China who had already been investigated on classical HLA class I expression. These results suggested that the expressions of HLA-G, HLA-E, MICA and MICB were up-regulated in HCC tissues to different extents. Furthermore, HLA-E and MICA/B gene showed obviously different expression pattern at transcription and translation level. The expression of HLA-G was correlated with the expression of HLA-A, while the expressions of HLA-A and HLA-B/C and the expression of MICA and MICB molecules were positively correlated with each other.

Materials and methods

Tissue samples. Forty one pairs of samples of HCC with its adjacent normal part were obtained from liver cancer patients who had undergone surgical resection without any prior radio- or chemotherapy in Jiangsu Provincial People's Hospital from 2004 to 2008. Surgically removed biopsies were immediately snap-frozen in liquid nitrogen and stored at -70°C for RNA or protein extraction. All these samples were selected according to the pathological diagnosis and reviewed by a pathologist to confirm the diagnosis of HCC. The adjacent normal tissue was used to evaluate the basal level of various gene expressions.

Semi-quantitive reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted with Trizol (Invitrogen, San Diego, CA) and reverse transcribed (Promega, Madison, WI) according to the manufacturer's protocol. Polymerase chain reaction (PCR) products were amplified in a 25μL mixture containing 1μL first-strand complementary DNA, 0.5μL dNTP mix (10 mM each), 1μL of 25mM 5'and 3' primers, 2.5μL 10×PCR buffer (TaKaRa Bio Inc., Shiga, Japan), $MgCl_2$ at a final concentration of 2 mM, and 0.5μL TaKaRa Ex Taq DNA polymerase (5 units/μL). The programs and primers used for amplification of HLA-G, HLA-E, MICA and MICB were previously confirmed for the detection of each gene (Table 1). The sizes of the PCR products were verified to the predicted sizes by 2% agarose gel electrophoresis and each of the PCR procedure was repeated at least three times. We estimated the expression levels of the mRNA after normalization with GAPDH.

Table 1 Primers used for reverse transcription polymerase chain reaction amplification

Gene	Sequence 5'–3'	Length of products (bp)
HLA-G	5'-GGAAGAGGAGACACGGAACA-3'	726
	5'-CCTTTTCAATCTGAGCTCTTCTTTT-3'	
HLA-E	5'-CCACCATGGTAGATGGAACCCTC-3'	1085
	5'-GCTTTACAAGCTGTG-3'	
MICA	5'-GCGGTACCAACCTATGTAGGGTCCCTTC-3'	423
	5'-GCAAGCTTCCTCGGCGTCTGGGGAG-3'	
MICB	5'-GCGGTACCTGTCGTCGCGGTCGCTG-3'	680
	5'-GCAAGCTTCCTCGGCGTCTGGGGAG-3'	
GAPDH	5'-GCCACCCAGAAGACTGTGGATGGC-3'	442
	5'-CATGTAGGCCATGAGGTCCACCAC-3'	

Western blot assays. The cell lysate (10-15 μg protein) were separated by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis and electrophoretically transferred onto PVDF membrane in a trans blot cell (Bio-Rad, Hercules, CA, USA). After blocking with 5% non-fat milk, the membrane was incubated separately with anti-HLA-E mAb (MEM-E/02) (Santa Cruz technology), anti-HLA-G mAb (4H84) (Santa Cruz technology), anti-MICA mAb and anti-MICB mAb (R&D Systems, Minneapolis, MN, USA) overnight at 4°C. After washed, the blot was incubated with HRP-conjugated goat anti-mouse IgG (Pierce Chemical Co., Rockford, IL, USA) for one hour at room temperature, and immunoreactive bands were visualized with the ECL reagent (Pierce). After the blot was stripped with stripping solution, the blot was reprobed with anti-GAPDH (Sigma) mAb for comparison of the protein load in each lane. The X-ray film was scanned (Kodak, Fuji, Agfa, Konica Sony) following chemiluminescence was done and the expression levels of the proteins were estimated after normalization with GAPDH.

Statistical method. Chi-squared test and Spearman correlation coefficient were used. A P-value < 0.05 was considered statistically significant. Statistical analysis were performed using SPSS software (Version 11.0 Chicago IL, SPSS Inc.).

Results

The expression of HLA-G, HLA-E, MICA and MICB mRNA in HCC tissues. The expression of these non classical HLA class I molecules at mRNA levels in HCC specimens and their adjacent hepatocellular tissues were determined by semi-quantitative RT-PCR. Gel-pro32 image analysis software was used to quantify the bands density in scanned agarose gels and GAPDH was used as internal control (Representative data showed in Fig.1). We found that HLA-G mRNA was only expressed in 8 HCC specimens and in 1 normal liver tissue

(p=0.009, Table 2). Different to the sparsely expression of HLA-G molecules, the expression of HLA-E was commonly seen in adjacent normal tissues. When compared to their normal counterparts, 56% of the lesions showed increased HLA-E antigen expression, 22% demonstrated consensus HLA-E expression, while 22% had lower HLA-E expression in their cancerous parts (Table 3). Regarding the expression of MICA and MICB, 43% and 38% of HCC samples had increased MICA and MICB mRNA expression, with only 5% and 8% samples showing decreased expression in HCC samples with comparison to their normal counterparts (Table 4).

The expression of HLA-G, HLA-E, MICA and MICB protein in HCC tissues.

In order to analysis whether the transcriptional changes of HLA-G, HLA-E, MICA and MICB ultimately lead to translational changes, we applied Western Blot assay to analyze HLA-G, HLA-E, MICA and MICB protein expression in the same group of samples (Representative data showed in Fig.. 2). The expression of HLA-G protein was shown in 7 of the 8 HCC samples which had HLA-G mRNA. No HLA-G protein was detected in the adjacent normal hepatocelular tissues even in the sample showing HLA-G mRNA (Table 2). According to the expression of HLA-E protein, the percentage of upregulation was much lower than that detected by RT-PCR. The expression of HLA-E protein was increased in only 12 of 41 (29%) HCC samples, and was decreased in 22 of 41 (54%) samples compare to the normal part (Table 3). The percentage of the increased expressions of MICA and MICB at protein level were consensus with those detected by semi-quantitative RT-PCR, while there were more decreased expressions of MICA (21%)

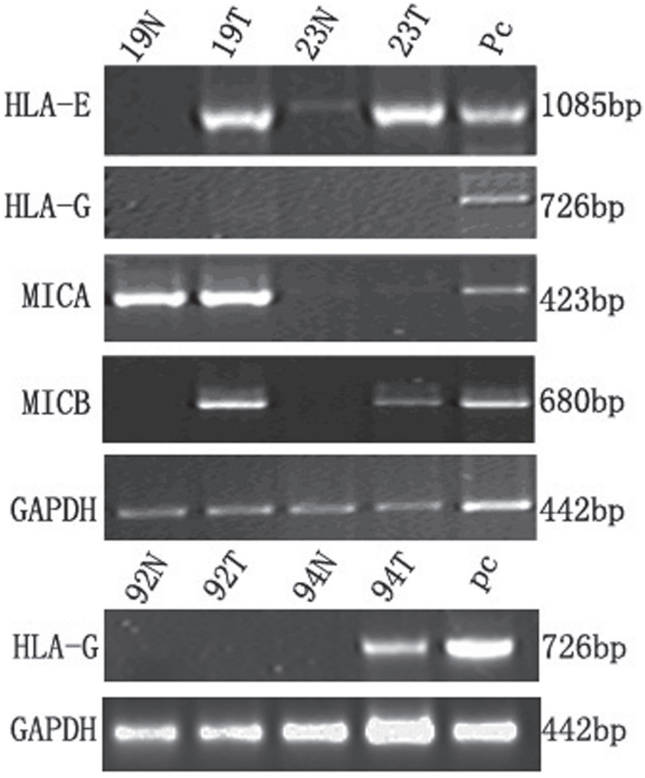

Figure 1. Reverse transcription polymerase chain reaction (RT-PCR) analysis of the mRNA expression of HLA-E, HLA-G, MICA and MICB in hepatocellular carcinoma (HCC) tissues and their normal counterpart (patient ID 19, 23, 92, 94). Total RNAs of fresh tissues were extracted and subjected to RT-PCR analysis. The detailed information of the primers is described in Materials and Methods. The PCR products were run on 2% agarose gels and stained with ethidium bromide with GAPDH as internal control. T, hepatocellular carcinoma; N, adjacent normal liver cell; Pc, positive control (The RNA extracted from extravillous trophoblast cells were used as positive control for amplification of HLA-E and HLA-G gene. The RNA extracted from HepG2 cell line was used as positive control for amplification of MICA and MICB gene).

Table 2 The mRNA and protein expression of HLA-G in hepatocellular carcinoma tissues (HCC) and adjacent normal hepatocellular tissues detected by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot (WB)

	HLA-G			
	RT-PCR		Westernblot	
	+	-	+	-
Adjacent normal liver cell	1	40	0	41
Hepatocellular Carcinoma	8	33	7	34
P Value		0.009		0.001

(+) means gel band was present; (-) means gel band was absent.

Table 3 Analysis of Transcript and translation profiles of HLA-E molecules in hepatocellular carcinoma tissues (HCC) and adjacent normal hepatocelular tissues by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot (WB)

	HLA-E	
	RT-PCR	WB
Up-regulation	23 (56%)	12 (29%)
Down-regulation	9 (22%)	22 (54%)
Consensus expression	9 (22%)	7 (17%)
Total	41	41

and MICB (24%) proteins in HCC tissue with comparison to the results shown at mRNA level (Table 4).

The correlation of the expression of HLA-A, HLA-B/C, HLA-G, HLA-E, MICA and MICB proteins. Classical HLA molecules have been investigated in this group of samples and the expression of HLA-A proteins were positively correlated

Table 4 Analysis of Transcript and translation profiles of MICA and MICB molecules in hepatocellular carcinoma tissues (HCC) and adjacent normal hepatocelular tissues by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot (WB)

	MICA		MICB	
	RT-PCR	WB	RT-PCR	WB
Up-regulation	17 (43%)	15 (39%)	15 (38%)	16 (42%)
Down-regulation	2 (5%)	8 (21%)	3 (8%)	9 (24%)
Consensus expression	21 (53%)	15 (39%)	22 (55%)	13 (34%)
Total	40	38	40	38

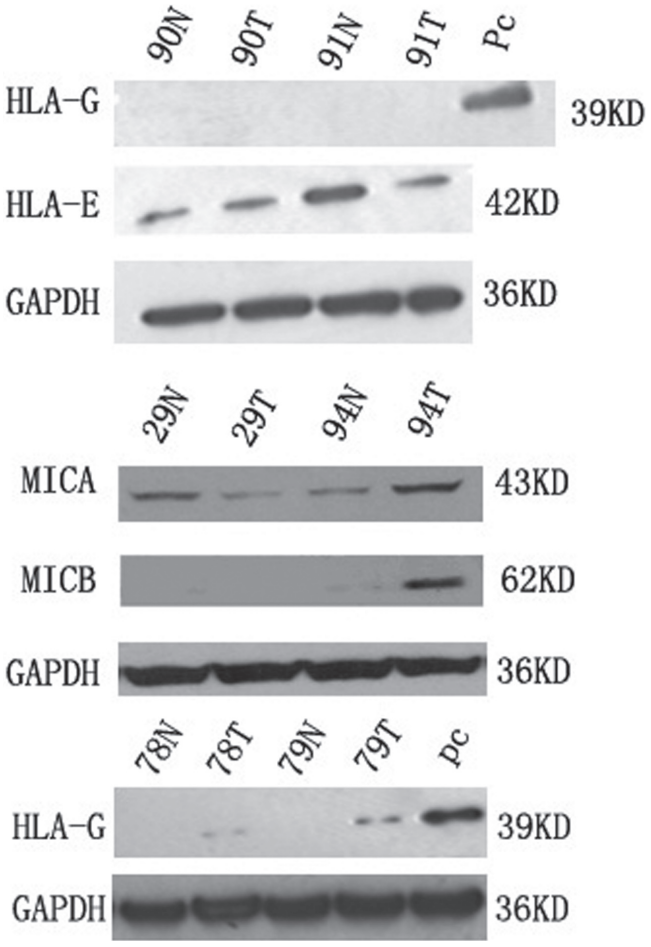

Figure 2. Western blot (WB) analysis of the protein expression of HLA-E, HLA-G, MICA and MICB in hepatocellular carcinoma (HCC) tissues and their normal counterpart (Patient ID 29, 78, 79, 90, 91, and 94). Total proteins of fresh tissues were extracted and subjected to WB analysis (10μg proteins per lane). The membranes were stained with HLA-E, HLA-G, MICA and MICB monoclonal antibody as described in Materials and Methods. GAPDH was used as the internal control. T, hepatocellular carcinoma; N, adjacent normal liver cell; Pc, positive control (The proteins extracted from extravillous trophoblast cells were used as positive control for the detection of HLA-G expression).

with the expression of HLA-B/C ($p<0.01$), also did the expression of MICA and MICB ($p<0.05$). The expression of HLA-G were also correlated with the expression of HLA-A ($p<0.05$).

The correlation of the expression of HLA-G, HLA-E, MICA and MICB proteins with clinic-pathology parameters. The increased expression of MICA, MICB and HLA-E were obviously in female patient. The upregulation of HLA-E was correlated with the older age (>=50 years old, Table 5, $p<0.05$). The expression of other molecules did not significantly correlate with age, gender, tumor size and the expression of HBsAg.

Discussion

Abnormal expression of HLA I molecules appears to be an important mechanism in cancer development and immune escaping of tumor cells [18]. Several studies have found that there were varying degrees of reduction or loss of classical HLA I molecules in head and neck cancer, breast cancer, lung cancer, colon cancer, prostate cancer and melanoma [19]. All these cancers also showed different amount of HLA-G expression or highly expression of HLA-E molecules [20]. Researchers speculate that these tumor cells might avoid immune surveillance through down-regulating the classical HLA I molecules while up-regulating the non-classical HLA I molecules at the same time to escape immune surveillance. HCC is one of the most common forms of malignancy and is the major cause of mortality in East Asian countries. China has the highest incidence of HCC in the world that about 300,000 of Chinese died of HCC annually, which accounts approximately half of the total death caused by HCC worldwide [21]. Our previous study showed strong classical HLA class I molecules expression in HCC patients, which should facilitate the cytotoxic T lymphocytes to kill tumor cells [22]. Despite these advantages, the liver cancer tends to progress quickly and give just months to live without treatment. These results promote us to find out the expression of non classical HLA class I molecules, which might provide a way to explain the immune escape mechanisms using by liver cancer cell.

Table 5. Correlation analyze of the expression of HLA-G, HLA-E, MICA and MICB proteins with clinical parameters in HCC patients

Parameters	MICA			*P* value	MICB			*P* value	HLA-G		*P* value	HLA-E			*P* value
	+	±	−		+	±	−		+	−		+	±	−	
Age (years)				0.524				0.265			0.069				0.022(*)
>=50	8	11	5		9	9	6		3	23		9	5	12	
<50	7	4	3		7	4	3		4	11		3	2	10	
Tumor size (cm^2)				0.755				0.705			0.731				0.554
>=7.5	9	7	4		8	8	4		4	17		5	3	13	
<7.5	6	8	4		8	5	5		3	17		7	4	9	
HBsAG				0.079				0.117			0.055				0.335
Positive	10	15	7		11	13	8		4	30		9	7	18	
Negative	4	0	1		4	0	1		3	3		3	0	3	
GENDER				0.044(*)				0.027(*)			0.069				0.037(*)
Female	6	1	3		6	0	4		4	7		7	0	4	
Male	9	14	5		10	13	5		3	27		5	7	18	

* Correlation is significant at the 0.05 level

This study reported the expression of non-classical HLA class I molecules HLA-E, HLA-G, and also investigated the expression of MICA and MICB molecules in HCC tissues of Chinese patients. Notably, HLA-G mRNA and protein expressions in HCC were similarly to the previous report in other cancers that showed a trend of upregulation [23-27] and this overexpression of HLA-G protein in HCC was reported as an independent indicator for poor outcome. In addition, we found that the expression of HLA-G proteins were correlated with the expression of HLA-A proteins ($p<0.05$). It has been reported that the classical HLA class I molecules, as well as HLA-G can inhibit NK cytotoxicity by interacting with the inhibitory receptor on the surface of NK cells. Because of the number, the way and the speed to start the cytotoxic effect, NK cells are absolutely advantaged to cytotoxic T lymphocytes (CTLs) in the liver [28], we speculated that HCC cells may escape immune surveillance through increased expression of HLA-G and the classical HLA class I molecules at the same time.

Recognition of HLA-E by CD94/NKG2 receptors on NK cells could allow indirect inhibition of NK cytotoxicity. In our studies, HLA-E mRNA was up-regulated in 56% HCC tissues in comparison with their adjacent normal hepatocellular tissues. However, there were significant differences between the transcription and translation level of HLA-E expression in some HCC specimens. Posttranscriptional regulation mechanisms could be used to explain the increased or normal HLA-E expression at mRNA level with decreased expression at protein level (for example, Case No.4 and No.14). We also found that the expression of HLA-E mRNA was up-regulated, but the expression of HLA-E protein cannot be detected when we co-cultured HCC cell line BEL-7405 cell with a NK cell line NK92 cell (Fig. 3). However, the detailed mechanism still needs to be clarified. The same phenomena were also detected in MICA and MICB expression of HCC patients. MICA and MICB were found to be decreased in some patients at protein level while they had normal expressions at mRNA level (for example, Case No. 2). It is reported that microRNA encoded by cytomegalovirus downregulate MICB expression by targeting MICB mRNA [29]. The same regulatory mechanism might be underlining the different expression pattern of MICA and MICB mRNAs and proteins in HCC patient, which need to be investigated further. In addition, we suggested that detection the expression of these molecules at both mRNA and protein level should be necessary since there were multiple mechanisms of posttranscriptional regulation.

Since the expression of HLA-E and MICA/B molecules were not consensus at mRNA and protein level, we only analysis the correlation of their expression at protein level. The expression of HLA-A were correlated with HLA-B/C while the expression of MICA were correlated with MICB. This could be explained by some same transcriptional factors that bind to their promoter region that regulate their expression [30-31]. Besides that, although it did not reach statistically significance, the increased HLA-G or HLA-E expression was negatively correlated with the increased expression of MICA or MICB. As we all know, MICA and MICB can activate the function of NK cells while the expression of HLA-G and HLA-E can inhibit the cytotoxicity of NK cells. There might be dynamic balance between the expression of HLA-G, HLA-E, MICA and MICB in the progress of liver cancer formation which may control the outcome of HCC. Functions of immunocyte in vivo regulated by a balance of inhibitory and activating signals should be studied further.

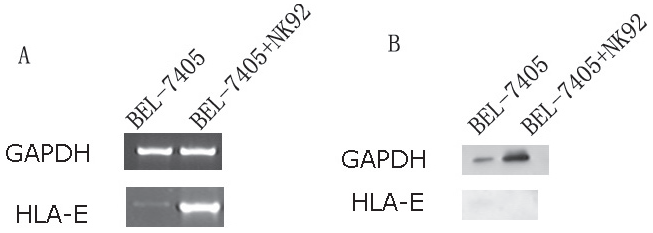

Figure 3. Compared with the control, HLA-E mRNA was obviously up-regulated (A), but HLA-E protein remained unchanged (B) when we co-cultured HCC cell line BEL-7405 with NK92 cell line. NK92 cells were cultured in α -MEM supplemented with 12.5% fetal calf serum, 12.5% equine serum, 0.2 mM inositol, 0.1 mM β-mercaptoethanol, 0.02 mM folic acid and 100 U/ml recombinant IL-2 at 37°C in a 5% CO2 humidified incubator. 3×10^5 7405 cells in normal medium (RPMI1640 medium, supplemented with 10% fetal bovine serum) were added to a 20 ml flask and cultured for 6 hours to let them adhere to the flask. 3×10^5 NK92 cells were then added to the flask to co-culture with 7405 cells for 6 hours. Remove the NK cell suspension to stop co-culture and added normal culture medium. Repeated this process when 7405 cells covered two third of the area of the flask. Before the examination of HLA-E expression, ten times of this co-culture process were carried out. Total RNA of the BEL7405 cells (with or without incubation with NK92 cells) were extracted and subjected to RT-PCR analysis. Total protein of the BEL7405 cells (with or without incubation with NK92 cells) were extracted and subjected to Western blot analysis. The experimental conditions were the same as that described in Material and Methods.

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