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A high-throughput assay of NK cell activity in whole blood and its clinical application

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ABSTRACT

Natural killer (NK) cells are lymphocytes of the innate immune system and have the ability to kill tumor cells and virus-infected cells without prior sensitization. Malignant tumors and viruses have developed, however, strategies to suppress NK cells to escape from their responses. Thus, the evaluation of NK cell activity (NKA) could be invaluable to estimate the status and the outcome of cancers, viral infections, and immune-mediated diseases. Established methods that measure NKA, such as ^{51}Cr release assay and CD107a degranulation assay, may be used to determine NK cell function, but they are complicated and time-consuming because they require isolation of peripheral blood mononuclear cells (PBMC) or NK cells. In some cases these assays require hazardous material such as radioactive isotopes. To overcome these difficulties, we developed a simple assay that uses whole blood instead of PBMC or isolated NK cells. This novel assay is suitable for high-throughput screening and the monitoring of diseases, because it employs serum of ex vivo stimulated whole blood to detect interferon (IFN)-γ secreted from NK cells as an indicator of NKA. After the stimulation of NK cells, the determination of IFNγ concentration in serum samples by enzyme-linked immunosorbent assay (ELISA) provided a swift, uncomplicated, and high-throughput assay of NKA ex vivo. The NKA results microsatellite stable (MSS) colorectal cancer patients was showed significantly lower NKA, 263.6 ± 54.5 pg/mL compared with healthy subjects, 867.5 ± 50.2 pg/mL (p value <0.0001). Therefore, the NKA could be utilized as a supportive diagnostic marker for microsatellite stable (MSS) colorectal cancer.

1. Introduction

Natural killer (NK) cells are large granular lymphocytes that play a key role in innate immune responses. NK cells are classified as group 1 innate lymphoid cells (ILCs) [1] and are involved in the early defense against infections and tumors [2]. Recruitment of NK cells to the site of inflammation, infection or transformation is the first step in the response to infection and tumors and occurs prior to the initiation of an adaptive immune response against infections or tumors [3]. Without prior sensitization, NK cells are able to eliminate target cells with intracellular microbe infection or with malignant transformation [4]. The mechanisms by which NK cells defend the body are through targeted cell death and release of chemokines and cytokines (innate immune system), as well as by helping other immune cells in their targeted cell elimination (adaptive immune system) [2], thus linking innate and cellular immunities. NK cells are activated by the binding of NK cell activating receptors with activating ligands on target cells. Proinflammatory cytokines such as interleukin (IL)-2, IL-12, IL-15 and IL-18 are also able to activate NK cells [5]. Activated NK cells induce cell death of target cells through secreting perforins and granzymes, engaging death receptors on target cells, and secreting interferon (IFN)-γ [6]. The cytokines induce proliferation and maturation of NK cells, promote cytokine production, and enhance NK cell cytotoxicity [7]. IFN-γ, as well as TNF-α, is a major cytokine secreted

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from activated NK cells and exerts immune responses against cancer cells and virus-infected cells [8].

NKA is a stable trait in individuals, and healthy individuals' low, medium or high NKA remains unchanged over the years unless disease occurs [9]. The activity of NK cells can differ in many different populations and can be influenced by lifestyle such as sleep, exercise, stress, diet, and by infection or disease. NKA decreases in many cancers and virus infections. It is well known that NKA negatively correlates with the tumor stage [10,11]. In addition, reduced NKA is related to low 5-year survival rates after operation, meaning high survival rates are associated with increased NKA [12]. Besides virus infections and tumors, recent studies identified relationships between NKA and inflammatory diseases [13,14], autoimmune disorders [15], Alzheimmer's disease [16], chronic fatigue [17], pregnancy/miscarriages [18] by employing various methods that measure NK cell functions.

A number of different assays are available for measuring NK cell cytotoxicity or NKA. Since 1968, the 'gold standard' assay for NKA has been the Chromium 51 release assay used to determine the cytolytic activity of effector cell populations from whole blood taken from subjects and incubated with radiolabelled sodium chromate ($Na_2{}^{51}CrO_4$) [19]. The percentage of lysis is measured by determining the proportion of free ^{51}Cr over the amount of cell-bound ^{51}Cr. However, the chromium release assay has many limitations, such as, hazardous radioactivity, high cost, short half-life, increased staff requirements for radiation safety training and licensing, and disposal of radioactive waste. Established other methods to assess NKA, such as CD107a degranulation assay [20], ELISA detecting IFN-γ secreted from NK cells, and intracellular staining of IFN-γ produced in NK cells [21], need isolation of peripheral blood mononuclear cells (PBMC) or NK cells from whole blood. Moreover, they require large amount of whole blood to obtain enough PBMC or NK cells. It is, however, difficult to get large volumes of blood from patients.

Alternative methods to the ^{51}Cr release assay have been proposed. These assays require NK cells to be isolated from PBMC using flow cytometry. This method does not distinguish between the NK cell subsets (dim, bright) as the flow cytometer isolates $CD45^+CD3^-CD16^+CD56^+$ cells [19].

NK Vue™ is an innovative in vitro diagnostic test kit that uses a quantitative sandwich ELISA to measure NKA from whole blood. NKA in vitro cannot be measured without stimulating NK cells since NK cells do not secrete effector cytokines ex vivo in the absence of appropriate activating stimuli. Therefore, the principle of NK Vue™ is to stimulate whole blood with PROMOCA™, an engineered recombinant cytokine that specifically activates NK cells in whole blood, and measure the release of IFN-γ from NK cells using sandwich ELISA. In other words, NK Vue™ measures the cytotoxic potential of the whole NK cell population, including both $CD56^{bright}$ and $CD56^{dim}$ subsets of NK cells as well as the ability of these cells to amplify the immune response to tumors [22]. Therefore, the advantage of NK Vue™ is that it measures the immune potential of both NK cell subsets of $CD56^{dim}$ and $CD56^{bright}$, as opposed to the ^{51}Cr release assay, which only measures the cytotoxic potential of NK cells isolated from whole blood ($CD56^{dim}$ subset). The amount of IFN-γ released is indicative of both the cytotoxic ability of the $CD56^{dim}$ subset and the immunomodulation by this cytokine, released from both subsets, to stimulate dendritic cells, and aid in the T-cell mediated antitumor response. Therefore, NK Vue™ is a true measurement of NKA that includes both innate immune cytotoxicity and immunomodulation of the adaptive immune response.

This paper describes a feasibility study of the novel assay for evaluation of NKA in whole blood samples using NK Vue™ conducted at Yonsei University, Gangnam Severance Hospital. To investigate the clinical value, the NKA of 41 patients with newly diagnosed microsatellite stable (MSS) colorectal cancer was compared with 41 healthy volunteers.

2. Materials and methods

2.1. Materials

BD Vacutainer® heparinN1 tube, BD Vacutainer® K_2EDTA tube, BD Vacutainer® acid citrate dextrose (ACD) tube, BD vacutainer® CPTTM heparin tube, BD vacutainer® needle and holder were purchased from BD (Franklin Lakes, NJ). RosettSep enrichment cocktail was purchased from STEMCELL Technologies (Vancouver, Canada) and recombinant TGF-β was purchased from Cell Signaling Technology (Danvers, MA). RBC lysis buffer was purchased from BioLegend (San Diego, CA) and 10% normal human serum was purchased from Sigma (St. Louis, MO). All dye labeled monoclonal antibodies for flow cytometry, Brefeldin A, Fix/Perm solution, and Perm/Wash buffer were purchased from BD Biosciences (San Jose, CA).

2.2. Preparation of PROMOCA™

PROMOCA™ expression vectors were transformed into the *Escherichia coli* strain BL21 (DE3). For expression of PROMOCA™, transformed bacteria were grown in medium containing lactose. The produced PROMOCA™ was concentrated and was purified on an FPLC gel-filtration column equilibrated in PBS, pH 7.5 PROMOCA™ solution was prepared with RPMI 1640, 0.05% BSA, 0.05% Proclin 300. Molecular weight of PROMOCA™ was characterized by FPLC against standard calibration samples (Supplemental Fig. 1).

2.3. Isolation of PBMC, NK cells, T cells, B cells, and monocytes

Peripheral blood mononuclear cells (PBMC) were isolated from 1 mL whole blood by density gradient centrifugation using BD Vacutainer CPT heparin tubes. NK cells, T cells, B cells, and monocytes were directly isolated from 4 mL whole blood by negative selection using RosettSep enrichment cocktail.

2.4. NK Vue™ assay

One mL of whole blood was incubated with 1 mL PROMOCA™ solution for 24 h in a 37 °C CO_2 chamber. After incubation, the supernatant of the mixture was collected and the release of IFN-γ from NK cells was measured using NK Vue™. Culture supernatants of cytokine-treated whole blood as well as isolated leukocytes, such as PBMC, NK cells, T cell, B cells, monocytes were harvested by centrifugation, and IFN-γ levels in the supernatants were determined using NK Vue™ according to the standard operating protocols. In short, standards and samples were incubated in an anti-human IFN-γ-coated plate for 2 h at room temperature and washed 4 times with washing buffer (0.05% Tween 20 in PBS, pH 7.4). IFN-γ conjugate was added to wells and incubated for 1 h, washed seven times and then 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added, incubated for 30 min. Stop solution was added to wells and absorbance was measured at 450 nm.

2.5. Flow cytometry

One mL of whole blood was mixed with 1 mL of PROMOCA™ solution, and the mixture was incubated for 24 h. BD GolgiPlug containing Brefeldin A was added to the culture after 20 h incubation, and the cells were cultured for additional 4 h. The cells were then harvested and the red blood cells (RBC) were lysed with 1X RBC lysis buffer. The samples were washed and resuspended in

staining buffer containing 10% normal human serum to pre-block Fc receptors. The cells were stained with fluorochrome-conjugated monoclonal antibodies including FITC mouse anti-human CD56, APC mouse anti-human CD3, PE mouse anti-human CD19, and PerCP-Cy5.5 mouse anti-human CD14. The cells were washed twice with staining buffer and incubated with Fix/Perm solution for 20 min at 4 °C. After wash with Perm/Wash buffer, the cells were stained with PE-Cy7 mouse anti-human IFN-γ or PE-Cy7 isotype control. Samples were analyzed with LSRII flow cytometer (BD Biosciences).

2.6. Clinical study design

This prospective cross-sectional analysis involved 41 patients with newly diagnosed microsatellite stable (MSS) colorectal cancer. Blood samples of self-volunteered 41 healthy individuals were investigated as controls. None of the patients had received prior treatment for colon cancer, were known to have immunological or other malignant conditions, and were all free of active infection or inflammation. Moreover, the patients who had high microsatellite instability tumors were excluded to eliminate potential bias [23]. Independent approval was obtained from Yonsei University Ethics Committee (3-2012-0029), with all blood samples collected after obtaining informed consent prior to surgery. All participants provided written consent to participate in the

Table 1
Demographic data of clinicopathological characteristics of colorectal patients as well as controls.

Category		Normal	Patients
Total		41	41
Gender	Male	20	19
	Female	21	22
Age	Age range (yr)	27–74	33–86
	40>	12	1
	40⩽	29	40
Stage	I	–	7
	II	–	12
	III	–	19
	IV	–	3
NK activity mean (pg/mL)	867.5 ± 50.2	263.6 ± 54.5	

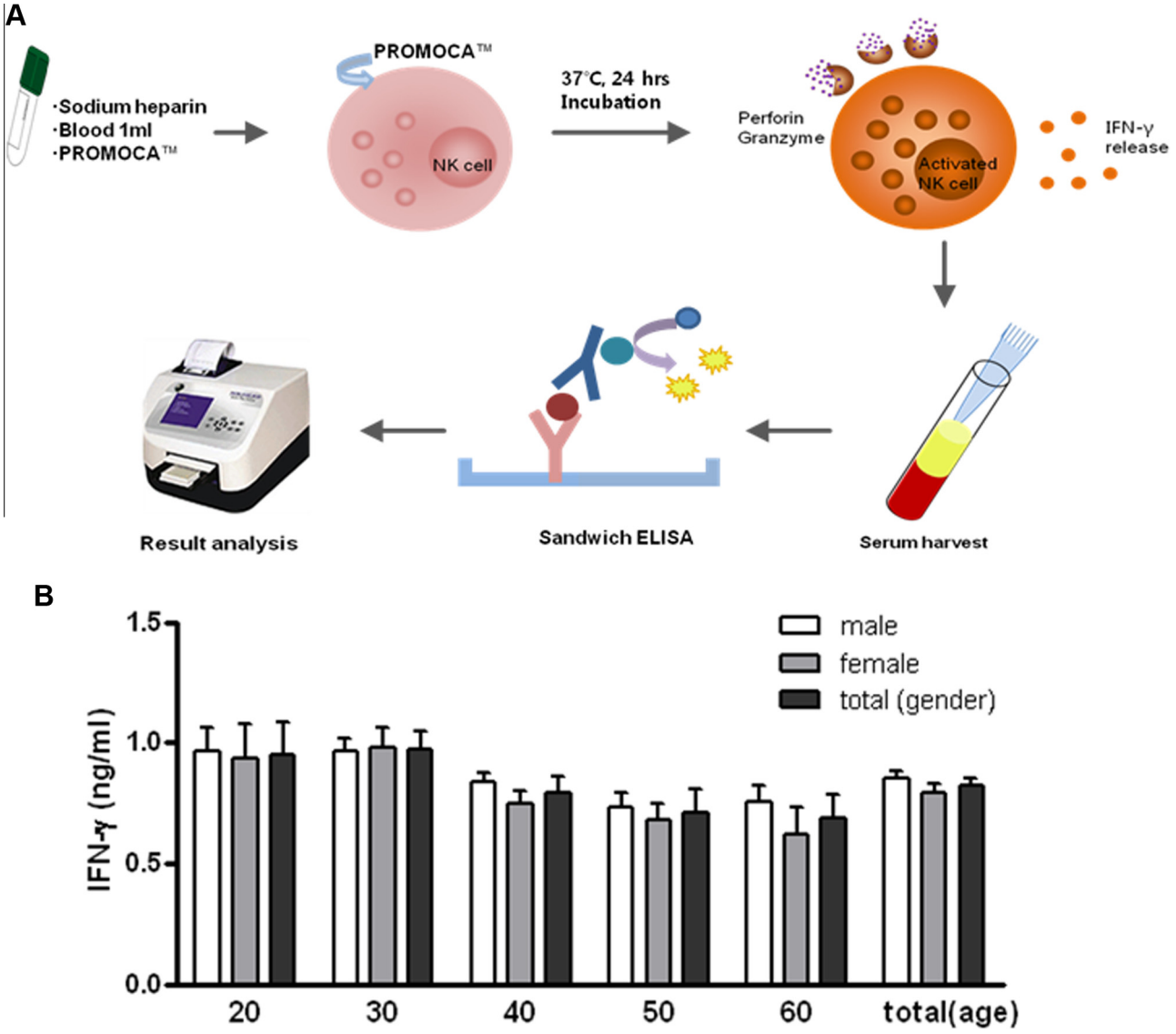

Fig. 1. (A) Schematics of NKA measurement using NK Vue™. (B) Geometric mean and standard deviation of NKA as a function of gender and age group of volunteers.

current study and the study cohort was summarized in Table 1. The NK Vue™ assay results from apparently healthy subjects and cancer patients were analyzed by ANOVA and two-tail *t*-test to assess the statistical significance of difference [24].

3. Results

The secretion of IFN-γ ex vivo from PROMOCA™-stimulated primary human NK cells in whole blood and the quantitation of secreted IFN-γ is depicted in the principle of NK Vue™ (Fig. 1A). After collecting whole blood, the blood was immediately mixed with media containing PROMOCA™ and incubated for 24 h. The supernatant of the mixture was collected and the concentration of IFN-γ was measured by ELISA. NKA was measured through detecting NK cell-secreted IFN-γ because IFN-γ secretion typically represents NK cell activation and effector function. PROMOCA™ was added to whole blood since NK cells does not secrete effector cytokines ex vivo in the absence of appropriate activating stimuli. Freshly isolated NK cells from 4 mL whole blood were incubated with several doses of PROMOCA™ for 48 h, and the secreted IFN-γ of the supernatant was measured by ELISA (Supplemental Fig. 2). Under these conditions, PROMOCA™ induced IFN-γ secretion from NK cells in a dose-dependent manner. Although PROMOCA™ induced IFN-γ secretion could be detected in both PBMC and whole blood, the IFN-γ secretion from PBMC was relatively inconsistent with that from whole blood. It could be attributable to the damage caused by Ficoll–Plaque during the isolation of PBMC from whole blood, which is skill dependent.[25]. In PBMC, the delay caused by the lengthy isolation protocols affect the stability of NK cells. In contrast, NK cells can effectively an immediately be activated by mixing whole blood and PROMOCA™. NK Vue™ was used to measure the NKA of 578 subjects donated by healthy volunteers was depicted with respect to their gender, and age group (Fig. 1B). Data analysis demonstrated no significant differences (at the $p < 0.05$) in NKA between gender and age groups which is consistent with the result trend of ^{51}Cr release assay [26].

To identify the cells responsible for IFN-γ secretion after adding PROMOCA™ to whole blood, IFN-γ production by individual cell types after PROMOCA™ treatment was measured. T cells, B cells, NK cells, and monocytes were isolated from whole blood and individually incubated with 8 ng/mL PROMOCA™ for 48 h. Only NK

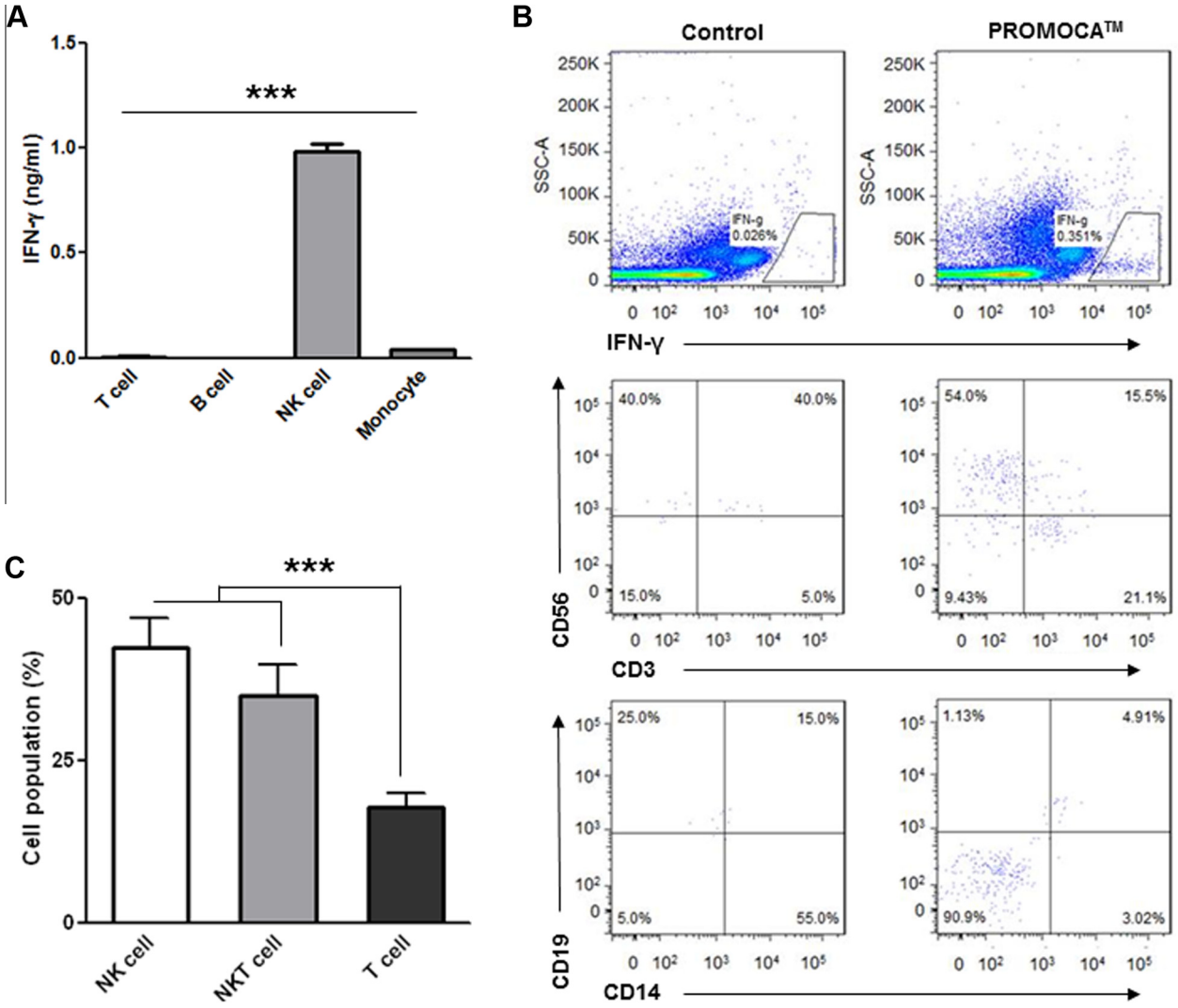

Fig. 2. Determination of major cell type secreting IFN-γ by PROMOCA™-stimulated whole blood. (A) Isolated T cells, B cells, NK cells, or monocytes from 4 mL of whole blood were incubated with PROMOCA™ for 48 h, and the supernatant was harvested. Secreted IFN-γ was measured by ELISA. (B) Whole blood was incubated with PROMOCA™ for 24 h. Cells were stained to detect markers for T cells (CD3), B cells (CD19), NK cells (CD56), and monocytes (CD14). Intracellular IFN-γ staining was done after fixation and permeabilization of cells. (C) The population ratio of NK cells ($CD3^-CD56^+$, 42.3%), NKT cells ($CD3^+CD56^+$, 35.1%) and T cells ($CD3^+CD56^-$, 17.9%) was average over seven healthy individuals' FACS results. $^{***}p < 0.001$ in relation to controls.

cells secreted detectable amount of IFN-γ (Fig. 2A). This result was consistent with a finding reported in a previous paper describing that the major IFN-γ secreting cells after in vivo PROMOCA™ administration are NK cells [27]. Although isolated T cells did not secrete IFN-γ after PROMOCA™ stimulation, interactions among activated immune cells in whole blood might induce T cells to secrete IFN-γ after PROMOCA™ treatment. Thus, we incubated whole blood with 8 ng/mL PROMOCA™ and performed intracellular cytokine staining to identify IFN-γ-producing cells in the stimulated whole blood. More than 50% of IFN-γ^+ cells were $CD3^-CD56^+$ NK cells and around 30% of IFN-γ^+ cells were $CD3^+$ T cells and NKT cells. $CD19^+$ B cells and $CD14^+$ monocytes were negative for IFN-γ staining (Fig. 2B). Although the interplay of T cells with other immune cells in whole blood could induce T cells to produce IFN-γ, NK cells were still responsible for the production of the majority of IFN-γ after PROMOCA™ stimulation. In the light of these two results, the majority of IFN-γ in whole blood after PROMOCA™ stimulation was secreted by NK cells. The population ratio of NK cells ($CD3^-CD56^+$, 42.3%), NKT cells ($CD3^+CD56^+$, 35.1%) and T cells ($CD3^+CD56^-$, 17.9%) was average over seven healthy individuals' FACS results (Fig. 2C as well as Supplemental Fig. 3 for raw FACS data). The results also suggested that the majority cell type containing IFN-γ in the cytosol activated by PROMOCA™ was the sum of NK and NKT cells. Therefore, the NKA from NK Vue™ truly reflected innate immunity.

To confirm whether IFN-γ produced by PROMOCA™-stimulated whole blood was mainly from NK cells, whole blood or NK cells samples were incubated in the presence of both PROMOCA™ and TGF-β, because TGF-β inhibits NK cell function [28]. IFN-γ production decreased in whole blood as well as in NK cells under the influence of TGF-β (Fig. 3A). The results suggested that reduced IFN-γ secretion observed in whole blood samples was associated with NK cell inhibition by TGF-β and thereby verified that NK cells were the major cell type that secreted IFN-γ upon addition of PROMOCA™ to whole blood.

We also identified factors that affect the results of NKA assay. There are various types of blood collection tubes that may contain heparin, EDTA, or ACD as an anticoagulant. The selection of blood collection tubes depends on the assays to be conducted with the blood. We tested, therefore, three types of tubes including heparin, EDTA and ACD tubes. Blood was collected in each tube from a donor, and the blood samples were incubated with PROMOCA™ for 24 h. Interestingly whole blood collected using heparin tubes secreted IFN-γ, but blood collected with other tubes did not produce detectable amount of IFN-γ (Fig. 3B).

Next, we evaluated the effect of the storage time of the blood samples. Whole blood collected in heparin tube was stored at room temperature for 0, 1, 2, 3, or 4 h after collection, then treated with PROMOCA™. As shown in Fig. 3C, NKA was substantially reduced if whole blood was left at room temperature for more than 1 h. It appeared that PROMOCA™ treatment should be started within 1 h after blood collection (Fig. 3C).

Finally, we compared IFN-γ secretion before and after food intake. Blood was collected before and after a meal and the collected blood was treated with PROMOCA™ and incubated for 24 h. IFN-γ secretion was considerably reduced in two donors whose blood was collected after food intake. This suggested that bleeding before eating would be better to analyze NK cell function correctly (Fig. 3D).

All patients and controls of study cohort were clinically and pathologically investigated with respect to certain factors. These factors along with mean NKA and standard deviation were summarized in Table 1. This prospective cross-sectional analysis involved 41 patients with newly diagnosed MSS colorectal cancer. The NKA results from apparently 41 healthy subjects and cancer patients were analyzed by two-tail *t*-test and plotted in Fig. 4. As indicated,

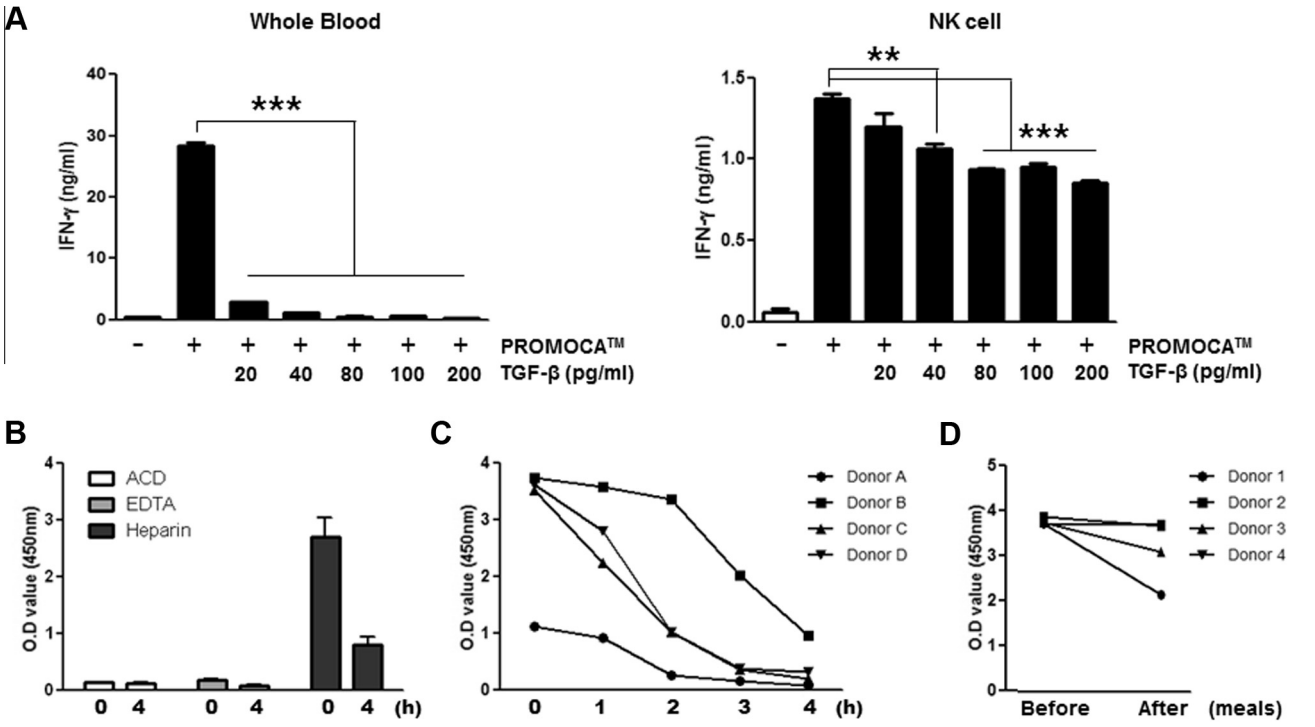

Fig. 3. Factors affecting the NKA assay. (A) Whole blood or NK cells were incubated with PROMOCA™ and the indicated doses of TGF-β, and the supernatant was collected. All secreted IFN-γ was measured by ELISA. The results are representative of three independent experiments. (B) Whole blood was collected and PROMOCA™ was added immediately or 4 h after bleeding, and the blood was incubated for 24 h. The results are representative of three independent experiments. (C) PROMOCA™ was added to whole blood at the indicated time after blood collection, and the blood was incubated for 24 h. (D) Whole blood was collected before and after food intake. The blood was incubated with PROMOCA™ for 24 h, and the serum was collected. $^{**}p < 0.01$ and $^{***}p < 0.001$ in relation to controls.

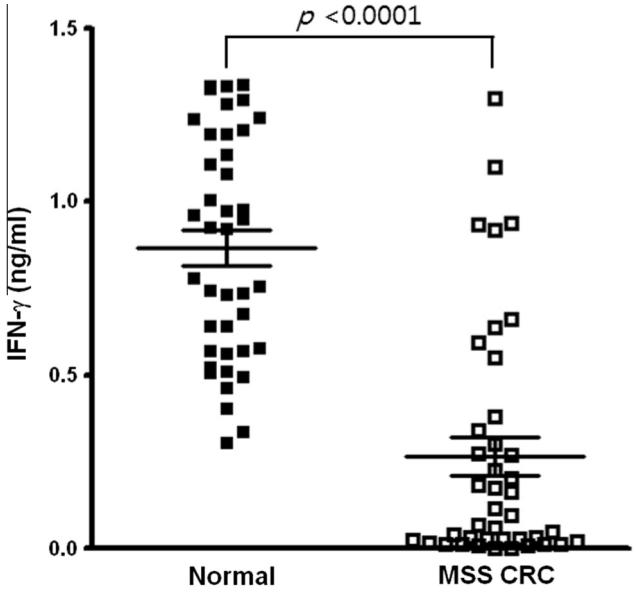

Fig. 4. Scatter plot comparing NKA between the MSS colorectal cancer patients compared with healthy subjects. $^{***}p < 0.001$ in relation to controls.

the patients showed significantly lower NKA, 263.6 ± 54.5 pg/mL compared with healthy subjects, 867.5 ± 50.2 pg/mL. (p value <0.0001). In the light of clinical results, the NKA could be utilized as a supportive diagnostic marker for MSS colorectal cancer.

4. Discussion

It appears that the measurement of NKA is important to predict prognosis of diseases associated with decreased NK cell function, such as tumors, virus infections, and immune-mediated diseases. Low NKA correlates with increased tumor volume, higher metastasis, and lower 5-year survival rate after the operation [12]. Furthermore, Imai et al. demonstrated that low NKA is associated with the increased risk of cancer [29]. This suggests that determination of NKA would be helpful for early diagnosis of cancer.

Conventional methods to measure NKA [20,30] require large amount of whole blood for PBMC or NK cell isolation, and their experimental processes are complicated. In this study, we have developed and verified a simple assay to measure NKA in a small volume (1 mL) of whole blood samples. Heparinized whole blood was incubated with PROMOCA™ for 24 h, and the serum was harvested to determine secreted IFN-γ by ELISA.

Claus and colleagues recently described assays that measure NK cell function in whole blood samples. They conducted ^{51}Cr release assay, CD107a degranulation assay, and intracellular IFN-γ staining with whole blood. They did not, however, try IFN-γ ELISA with cytokine-stimulated whole blood to assess NK cell function, because they believed that ELISA could not distinguish IFN-γ secreted by NK cells from that secreted by T cells [21]. Our feasibility experiments showed, however, that NK cells were the major cell type that secreted IFN-γ after treatment of whole blood with PROMOCA™. Because it requires serum with PROMOCA™-stimulated whole blood rather than blood cells. The serum can be frozen to be analyzed later. As a result, the new method described here is more adequate to perform high-throughput screening and follow-up for diseases associated with reduced NKA.

In order to obtain accurate results with this whole blood NK cell assay, heparin tubes are required to collect blood samples, and PROMOCA™ treatment should be completed within 1 h after bleeding. It also appeared that blood collection before eating produced better results.

The NKA was validated with MSS colorectal cancer patients by showing lower NKA comparing with healthy subjects so that the NKA measurement by NK Vue™ could be utilized for diagnosis of MSS colorectal cancer.

In summary, we developed a simple and high-throughput assay to assess NKA in whole blood samples. It is capable of measuring NKA with small amount of whole blood and is simpler than other established methods. This ELISA-based assay could be useful to help in diagnosing and in monitoring diseases associated with functional inhibition of NK cells, such as malignant tumors, viral diseases, and immune-mediated disorders. The diagnostic utility of NK Vue™ will be further validated by clinical studies of various types of cancers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc. 2014.02.040.

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