การประเมินความสัมพันธ์ทางคลินิกของ HLA CLASS I, KIR GENOTYPES และ MISSING KIR LIGAND ในคนไข้มะเร็งเม็ดเลือดขาวที่ได้รับการปลูกถ่ายเซลล์ต้นกำเนิดเม็ดโลหิตจากผู้บริจาคพี่น้องที่มี HLA-IDENTICAL MATCHED

นางสาวศรีประไพ ขนุนทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ASSOCIATION BETWEEN HLA CLASS I, KIR GENOTYPES AND MISSING KIR LIGAND AND CLINICAL OUTCOME IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES RECEIVING HLA-IDENTICAL HSCT

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ศรีประไพ ขนุนทอง : การประเมินความสัมพันธ์ทางคลินิกของ HLA CLASS I, KIR GENOTYPES และ MISSING KIR LIGAND ในคนไข้มะเร็งเม็ดเลือดขาวที่ได้รับการปลูกถ่ายเซลล์ต้นกำเนิดเม็ดโลหิตจากผู้บริจาคพี่น้องที่มี HLA-IDENTICAL MATCHED (ASSOCIATION BETWEEN HLA CLASS I, KIR GENOTYPES AND MISSING KIR LIGAND AND CLINICAL OUTCOME IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES RECEIVING HLA-IDENTICAL HSCT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. พญ. ดร. ณัฏฐิยา หิรัญกาญจน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: น.ส. ภาวิณี คุปตวินทุ, 82 หน้า.

โมเลกุลรับสัญญาณชนิดเกียร์มีบทบาทสำคัญในการควบคุมการทำงานของเอนเคเซลล์ ซึ่งโมเลกุลที่จับจำเพาะกับโมเลกุลรับสัญญาณยับยั้งชนิดเกียร์ส่วนใหญ่คือโมเลกุลชนิดเอชแอลเอคลาสวัน โดยมีบทบาทสำคัญในการกำจัดเซลล์มะเร็งให้กับคนไข้มะเร็งเม็ดเลือดขาวที่ขาดโมเลกุลเอชแอลเอไปจับกับโมเลกุลรับสัญญาณยับยั้งชนิดเกียร์บนเอนเคเซลล์ของผู้บริจาค ส่งผลให้ผลรวมของสัญญาณในเอนเคเซลล์เป็นสัญญาณกระตุ้นไปทำลายเซลล์มะเร็งได้ จากการตรวจหาโมเลกุลชนิดเอชแอลเอบนเม็ดเลือดขาวของคนไข้และโมเลกุลรับสัญญาณยับยั้งชนิดเกียร์บนเม็ดเลือดขาวของผู้บริจาค ด้วยเทคนิคพีซีอาร์เอสเอสโอพีในคนไข้มะเร็งเม็ดเลือดขาวจำนวน 66 ราย ที่ได้รับบริจาคเซลล์ต้นกำเนิดเม็ดโลหิตจากผู้บริจาคพี่น้องที่มีโมเลกุลชนิดเอชแอลเอตรงกัน ประกอบด้วยคนไข้มะเร็งเม็ดเลือดขาวชนิดเอเอ็มแอล 40 ราย, ชนิดเอแอลแอล 12 ราย และชนิดซีเอ็มแอล 14 ราย เพื่อศึกษาย้อนหลังในคนไข้มะเร็งเม็ดเลือดขาวหลังการปลูกถ่ายเซลล์ต้นกำเนิดเม็ดโลหิตที่ขาดโมเลกุลเอชแอลเอไปจับกับโมเลกุลรับสัญญาณยับยั้งชนิดเกียร์บนเอนเคเซลล์ของผู้บริจาคที่ส่งผลต่อผลลัพธ์ทางคลินิก จากการศึกษาพบว่าคนไข้ส่วนใหญ่ขาดโมเลกุลเอชแอลเออย่างน้อยหนึ่งชนิดที่ไม่สามารถไปจับจำเพาะกับโมเลกุลรับสัญญาณยับยั้งชนิดเกียร์ของผู้บริจาคพี่น้องจำนวน 58 ราย (87.9%) และมีคนไข้ 8 ราย (12.1%) ที่มีโมเลกุลเอชแอลเอจับจำเพาะกับโมเลกุลรับสัญญาณยับยั้งชนิดเกียร์ของผู้บริจาคพี่น้อง หากมาพิจารณาร่วมกับผลทางคลินิก พบว่า คนไข้มะเร็งเม็ดเลือดขาวที่ขาดโมเลกุลเอชแอลเอตั้งแต่หนึ่งชนิดขึ้นไปจะไม่ส่งผลลัพธ์ทางคลินิก แต่ถ้าคนไข้ขาดโมเลกุลเอชแอลเอตั้งแต่สองชนิดขึ้นไปจะช่วยลดการกลับมาเป็นมะเร็งซ้ำ (p-value=0.035) โดยเฉพาะคนไข้มะเร็งเม็ดเลือดขาวชนิดเอเอ็มแอล (p-value=0.033) นอกจากนี้จะช่วยลดการการเกิดกราฟท์เวอร์สัสโฮสท์ดีซีสชนิดฉับพลันในคนไข้มะเร็งเม็ดเลือดขาวด้วย (p-value=0.005) หากพิจารณาตามชนิดของมะเร็งเม็ดเลือดขาวแล้ว คนไข้มะเร็งเม็ดเลือดขาวชนิดเอเอ็มแอลจะมีชีวิตรอดมากขึ้นด้วย (p-value=0.018) ดังนั้นการขาดโมเลกุลเอชแอลเอตั้งแต่สองชนิดขึ้นไปจะส่งผลดีต่อคนไข้มะเร็งเม็ดเลือดขาว โดยลดการเกิดมะเร็งซ้ำ ลดการเกิดกราฟท์เวอร์สัสโฮสท์ดีซีสชนิดฉับพลัน และมีชีวิตรอดมากขึ้น จึงเป็นข้อพิจารณาหนึ่งในการเลือกผู้บริจาคที่เหมาะสมเพื่อช่วยเหลือคนไข้มะเร็งเม็ดเลือดขาว

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5587175420 : MAJOR MEDICAL MICROBIOLOGY
KEYWORDS: KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTORS (KIRS) / HLA LIGAND / MISSING KIR LIGAND / GRAFT-VERSUS LEUKEMIA (GVL)

SRIPRAPAI KHANUNTONG: ASSOCIATION BETWEEN HLA CLASS I, KIR GENOTYPES AND MISSING KIR LIGAND AND CLINICAL OUTCOME IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES RECEIVING HLA-IDENTICAL HSCT. ADVISOR: PROF. NATTIYA HIRANKARN, M.D.,Ph.D., CO-ADVISOR: MS. PAWINEE KUPATAWINTU, 82 pp.

Killer cell immunoglobulin-like receptors (KIRs) are subpopulation of receptors on NK cells. The ligands for most KIRs are HLA class I. The lack of HLA ligand for KIR receptor known as missing KIR ligand plays a role in the elimination of malignant cells by a graft-versus leukemia (GVL) effect. The aim of this study was to analyze the impact of missing KIR ligand on clinical outcome. This study was a retrospective analysis in patients undergoing T-replete hematopoietic stem cell transplant from HLA-identical sibling donors. We investigated 66 patients, including 40 patients with AML, 12 patients with ALL and 14 patients with CML. The KIR genes and HLA ligands were typed by polymerase chain reaction-sequence specific oligonucleotide probe (PCR-SSOP). We found that as high as 58 patients (87.9%) had at least one missing KIR ligand, while only 8 patients (12.1%) had no missing KIR ligand. There was no significant association of 1 or more than 1 missing KIR ligand on the clinical outcome regarding relapse, GVHD, and survival. However, the 2 or more than 2 missing KIR ligands could improve patient outcome e.g., reducing relapse (*p*-value=0.035), particularly in the AML patients (*p*-value=0.033). Moreover, this model could influent clinical outcome by reducing acute GVHD (*p*-value=0.005). In AML patients, significant association could also be found with increased survival rate (*p*-value=0.018). In addition, a dose effect of missing KIR ligand could impact patients clinical outcome on relapse, aGVHD and survival. This result is important for the considering of KIR missing ligand in the donor selection along with HLA matching and the development of NK adoptive therapy for AML treatment.

Field of Study: Medical Microbiology Student's Signature .....

Academic Year: 2013 Advisor's Signature .....

Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

HSCT	Hematopoietic Stem Cell Transplant
BM	Bone Marrow
PBSC	Peripheral Blood Stem Cell
CB	Cord Blood
HLA	Human Leukocyte Antigen
NK	Natural Killer cell
GVHD	Graft-Versus-Host Disease
KIR	Killer cell Immunoglobulin-like Receptor
GVL	Graft-Versus Leukemia
AML	Acute Myeloid Leukemia
MDS	Myelodysplastic Syndrome
ALL	Acute Lymphoblastic Leukemia
CML	Chronic Myeloid Leukemia
G-CSF	Granulocyte-Colony Stimulating Factor
aGVHD	Acute Graft-Versus-Host Disease
cGVHD	Chronic Graft-Versus-Host Disease
TRM	Transplant-Related Mortality
CTLs	Cytotoxic T Lymphocytes
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
ITAM	Immunoreceptor Tyrosine-based Activating Motif
PCR-SSP	Polymerase Chain Reaction-Sequence Specific Primer
PCR-SSOP	Polymerase Chain Reaction-Sequence Specific Oligonucleotide Probe
TBI	Total Body Irradiation
CsA	Cyclosporine-A
MTX	Methotrexate

CMV	Cytomegalovirus
SAPE	Streptavidin, R-Phycoerythrin-conjugated
MFI	Mean Fluorescence Intensity
FI	Fluorescence Intensity

CHAPTER I
INTRODUCTION

Background information and rationale

Hematopoietic stem cell transplant (HSCT) has been a standard treatment for hematological malignancies. Three different sources include bone marrow (BM), peripheral blood stem cells (PBSC) and cord blood (CB). The human leukocyte antigen (HLA) identical sibling is the important source of allogeneic hematopoietic stem cell transplantation (HSCT). Although HLA antigens are fully matched, cancer relapse and graft-versus-host disease (GVHD) remain problems for overall survival of the patients. NK cells play an important role in immunity against viral infection and allogeneic cancer, particularly cancer cells of hematologic malignancy. NK cells express several receptors including activating, inhibiting, adhesion and cytokine receptors. Killer cell immunoglobulin-like receptors (KIR) are a family of transmembrane glycoproteins expressed on NK cells. The KIR gene is located on human chromosome 19q13.4 and is inherited independently of HLA on chromosome 6; therefore, NK cells may express KIR in which they have no HLA ligand. Therefore, there are possibilities of persons who lack KIR receptors for their HLA ligands. There are several evidences that NK cell alloreactivity can contribute to the clinical outcome of hematopoietic stem cell transplantation through KIR and HLA molecules. The situation in HLA nonidentical transplants in which the donor NK cells encountered recipient target cells lacking HLA class I allele present in the donor HLA genotype can be called “KIR-ligand mismatch”. Interestingly, this KIR ligand mismatch can improve overall survival by mediate antileukemic effects and even decrease acute GVHD, particularly in myeloid leukemia. Moreover, the NK graft versus leukemia (GVL) effect is not only occur in HLA nonidentical transplantation, as is required for the KIR ligand incompatibility studies, but instead can be observed in the “missing KIR-ligand” situation that can be found even in HLA-identical transplants. In the latter case, the NK cells from the donor who has missing KIR-ligand can mediate the GVL effect in the HLA identical recipient. Therefore, the KIR-HLA class I relationship between recipient and donor might become the additional part of the donor evaluation and selection process. A better

understanding of the biologic mechanisms involved in the observed effects should be further investigated.

The missing ligand effect in HLA identical transplantation has been mostly shown to decrease leukemia relapse and improve survival in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). However, some studies also report no association or even contradict result. There are several reasons for the differences including the variation in treatment protocols, the type of disease treated, disease stage, patient age, conditioning regimen, GVHD prophylaxis and stem cell source. Only one previous study has been reported in Thai population about the effect of KIR genotype in HLA-identical sibling HSCT with leukemia. Their result showed no difference in clinical outcome correlated with missing KIR ligand but reported an association with activating KIR genotype [1].

In this study, we aimed to analyze KIR genes in HLA-identical sibling and the effect of missing KIR ligand and activating KIR on the outcome of HSCT in another independent set of Thai patients with leukemia.

Research Question/Objective

To study the association between HLA class I, KIR genotypes and missing KIR ligand and clinical outcome in Thai patients with hematological malignancies receiving HLA-identical HSCT?

Benefits

We can use this result as a reference data in Thai population to select the best donor KIR for optimal outcome following transplantation and to predict the outcome after donor KIR transplantation for hematologic malignancy. Moreover, it is a basic knowledge to use NK cells in donor KIR transplantation as adoptive immunotherapy against hematological malignancies.

CHAPTER II
LITERATURE REVIEW

Haematopoietic Stem Cell Transplantation (HSCT) in Cancer

From the 1950s, E. Donnall Thomos who was later rewarded with a Nobel Prize was a pioneer using haematopoietic stem cells from bone marrow. That work showed that the bone marrow can produce new blood cells and also lower of development of a threatening graft-versus-host disease [2]. In 1958, Georges Mathe who is oncologist performed the bone marrow transplant on five nuclear workers whose marrow had been damaged by irradiation. Later in 1963, he used the bone marrow transplants in the treatment of leukemia [3]. In 1968, Robert A. Good is the physician first performed the successful human bone marrow transplant on immunodeficiency disease other than cancer [4]. Next in 1975, John Kersey also performed the first successful bone marrow transplant to cure lymphoma which resulted in the longest-living lymphoma transplant survivor [5]. Many leukemia patients who were already resistant to chemotherapy would have benefit from HSCT. Most transplantation to cure hematological malignancies disease used allogeneic HSCT that appear to improve chances for cure cancer or long-term survival [6, 7]. The perfect allogeneic HSC donors must have human leukocyte antigen (HLA) type that matches the recipient. The preferable allogeneic transplant donors are HLA matched between patient and sibling donor as HLA identical matched sibling. However, in case that an identical sibling donor is not available, the patients can be offered source of HLA matched stem cells from unrelated donors who have very close degree of HLA matching. Unrelated donors may be found through a registry of bone marrow donors. In general, matching is performed on the basis of variability at three (HLA-A, HLA-B and HLA-DR) or more HLA gene loci. Currently, the National Marrow Donor Program recommends to test for all six of these HLA genes (HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DQ and HLA-DP) before transplant process. Currently, there are 3 sources of HSCT. First, stem cells from the bone marrow, which are removed from a large bone of the donor by a large needle performed under anesthesia. Second, the most common source of stem cells for allogeneic HSCT nowadays is peripheral blood stem cells. These are collected from

the blood through a process known as apheresis. The peripheral stem cell is boosted by daily subcutaneous injections of granulocyte-colony stimulating factor (G-CSF) to mobilize stem cells from the donor's bone marrow into the peripheral circulation. Third source of HSCT is the cord blood, which contains less and rather naïve T cells. However, the amount of the HSCT might be suitable only to pediatric patients. The drug or total body irradiation that is given prior to a transplant is called the conditioning regimen. This process aim to help reducing the cancer cells in the patient before the infusion of the newly donor cells and also suppress immune reactions to allow the HSCT to engraft.

In HSCT, the major complication is the graft-versus-host disease (GVHD). Since the conditioning regimen clears all the immune cells in the patients, graft rejection from host immune response is minimal. Instead, the T cells from the donor respond against recipient host. When transplanted into the patient, the donor's T cells identify the host cell as non-self and attack patient's tissues and organ mainly against the mismatched HLA. It can be classified into acute and chronic forms on the basis of histological pattern. Acute GVHD usually occurs during the first 100 days after transplanted. The effector T cells cause epithelial cell injury including skin, liver, gastrointestinal tract [8]. Chronic GVHD usually develops after 100 days after transplanted. It is believed to mediate by the new T cells that are produced after donor cells are engrafted. Patients are characterized by fibrosis and atrophy of one or more of the organs. Chronic GVHD may involve product obliteration of small airways. When it is severe, chronic GVHD leads to complete dysfunction of the organ and may be fatal [9]. After the transplantation, immunosuppressive drugs are necessary to prevent the acute and chronic graft-versus-host disease (GVHD) by donor's immune system. These drugs can cause other side effect e.g., opportunistic infections and cancer relapse [10]. If the patients and their donors have very similar HLA, the chance of GVHD will be reduced [11]. Another technique sometimes used to reduce incidence of GVHD is called "T cell depletion" by removing mature T cells from donor's grafts [10]. Moreover, although HLA antigens are fully matched, GVHD can still occur against minor histocompatibility antigens [12].

Cancer relapse remains the most frequent cause of treatment failure and mortality in HSCT. Relapse is the return of cancer after treatment[13]. The possible explanation is that the cancer may be resistant to treatment, chemotherapy or radiation. The cancer cells that are left behind can then grow and show up again. Another reason is from the immune system weakens, and the disease undergoes immune escape as a result leukemia will recur [14]. The term graft-versus-leukemia (GVL) effect of the allograft, mediated through donor-derived T cells and NK cells is a powerful effect that can control the allogeneic recipient's leukemic cells from relapsing [15-17]. HLA mismatching is associated with increased GVL resulting in a lower risk of relapse [18, 19]. After this observation, an alternative approach has been used to enhance GVL by selecting haploidentical donors or mismatch HLA donors [20, 21]. These grafts contain donor T lymphocytes and Natural killers (NK cells) that are beneficial to eliminate malignant residual leukemic cells [15, 16]. It is clear that donor T lymphocytes are established immunity against malignancy cells by destroying residual leukemia cells, whereas the manipulated T cell depletion has been associated with a loss of graft-versus-leukemia (GVL) activity, particularly in patients undergoing BMT for CML [22, 23]. The donor cytotoxic T lymphocytes (CTLs) can largely mediate this respond but this protocol has the disadvantage of causing severe GVHD [24]. The donor NK cells can also be employed, particularly in T-cell-depleted HSCT [15]. The alloreactive NK cells represent an attractive source of GVL-reacting cells, which believed to have the advantage of minimized GVHD [25].

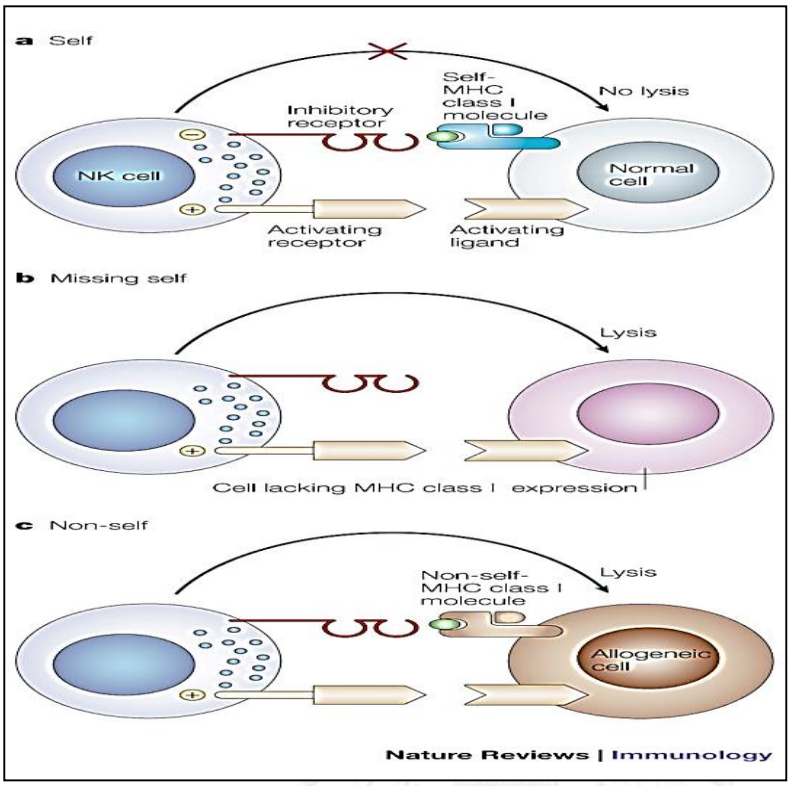

Figure 1. NK cell activation in the absence of MHC class I (missing self) or non-self [26].

Role of Human NK cells in Cancer

Natural killer (NK) cells are a subset of innate lymphoid cells [27]. They are established as the first member to defend against viral infections and cancer cells [28]. Many cancer cells are capable of immune escape to HLA-restricted cytotoxic T lymphocytes (CTLs) by the loss of class I molecules. However, NK cells have been described as alternate effectors cells populations to destroy cancer cells because the NK cells sense a loss of MHC class I on the target cells. This mechanism is called the missing-self hypothesis. Normally, the NK cells posses the NK inhibitory receptors, which can bind to MHC class I: therefore, preventing NK cell from activation to normal healthy cells (Figure 1a). In the tumor cells that loss the MHC class I expression (missing self) to interact with the inhibitory receptor on the NK cells, the NK activating receptors will be activated and result in tumor lysis (Figure1b) [29, 30]. In addition, each inhibitory receptor has specificity to certain MHC allele. In the case of non-self in which the MHC ligand on the target cell cannot bind and inhibit NK cells, it will result in cell lysis as

well (Figure 1c). The main effector functions of NK cells are the cytotoxic granules, the death-receptor-ligand, and the nitric oxide to lyse the target cells. The main cytokine produced by NK cell is interferon gamma (Figure 2) [31].

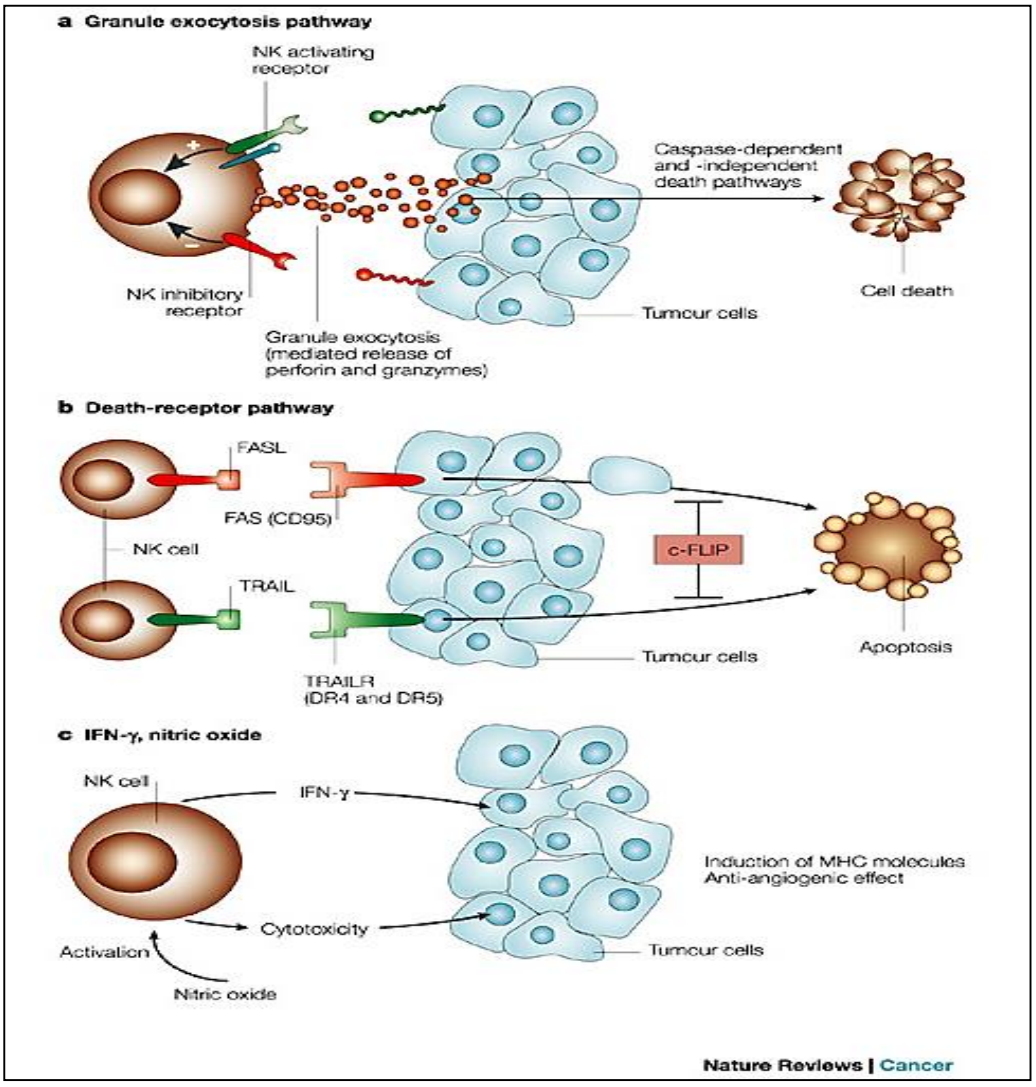

Figure 2. Functional NK cells in eliminated tumor cells [31].

NK cells possess various receptors on their surface, which composed of both activating signals and inhibitory signals (Figure 3) [32]. Natural killer cell function is determined by the net effect of signaling by activating and inhibitory receptors (Figure 4) [33]. The negative signals are provided by the killer cells inhibitory receptors (KIR)

recognizing HLA class I molecule, and positive signals are produced by stress-induced ligands for viral infection or cancer.

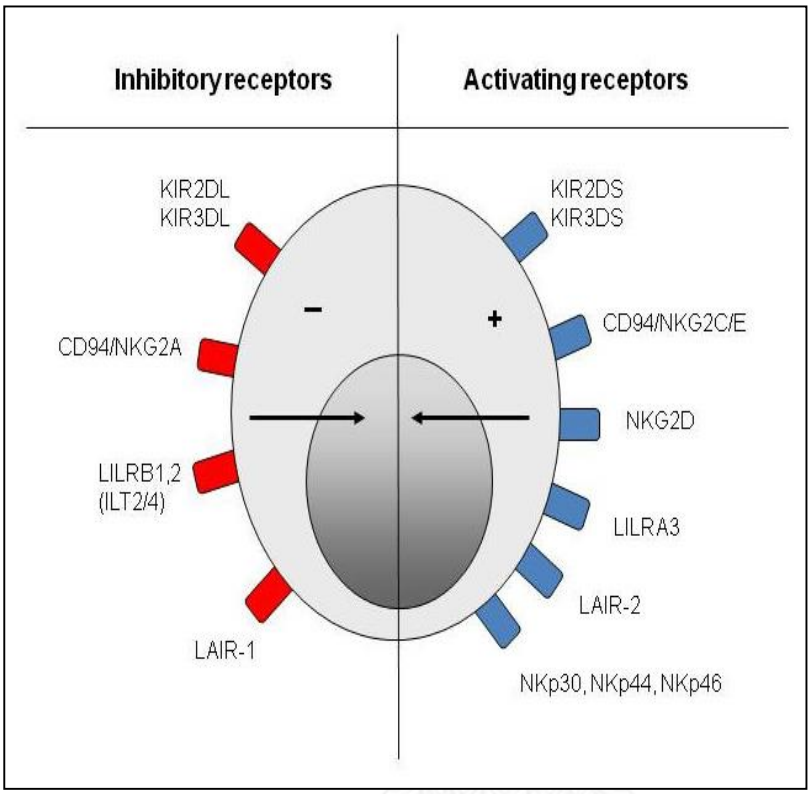

Figure 3. Expression of activating and inhibitory receptors on NK cell [32].

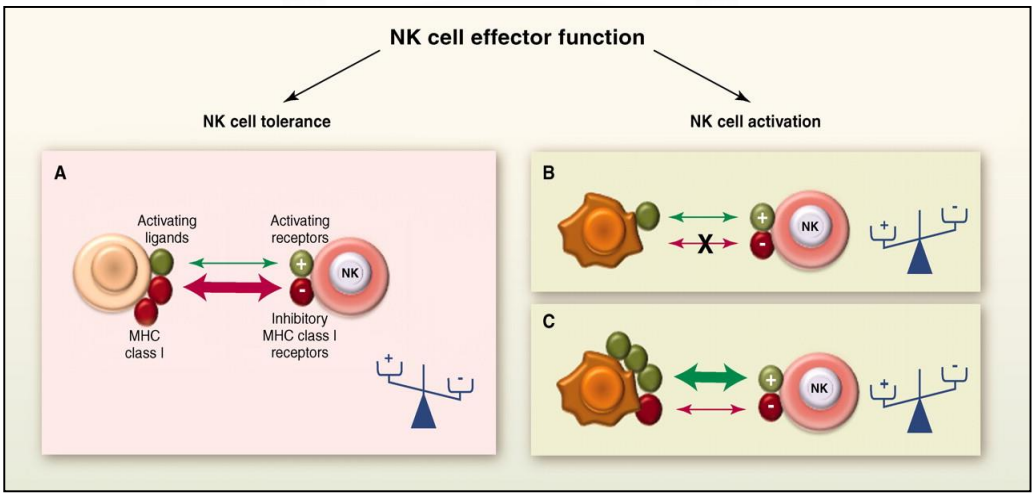

Figure 4. NK cell activation on balancing of activating and inhibitory receptors [33]

Education of NK cells

NK cells mature in the bone marrow. They undergo an education process similar to other lymphocytes [34]. They protect the host cells from pathogen invasion while avoiding mechanisms of autoimmune responses [35]. During NK cells development in the bone marrow, they are educated to distinguish healthy from abnormal tissues. First in education process, inhibitor receptor will engage cognate autologous MHC class I results in the generation of functional effector NK cells in peripheral. This process has been called licensing of NK cells [36, 37]. The failure of the inhibitory receptors to engage to MHC class I will generate a subset of anergic or hyporesponsive peripheral NK cells, since their receptors are absence or lack of interaction with self MHC class I [38-40]. All these process are thought to ensure that NK cells do not response to healthy self-tissue.

Killer immunoglobulin-like receptor (KIR)

Killer cell immunoglobulin-like receptor (KIR) is a family of transmembrane glycoproteins expressed on NK cells. They are key regulators of the tolerance and activation of NK cells [33]. The KIR receptors are encoded by the leukocyte receptor complex (LRC) gene on human chromosome 19q13.4 (Figure5).

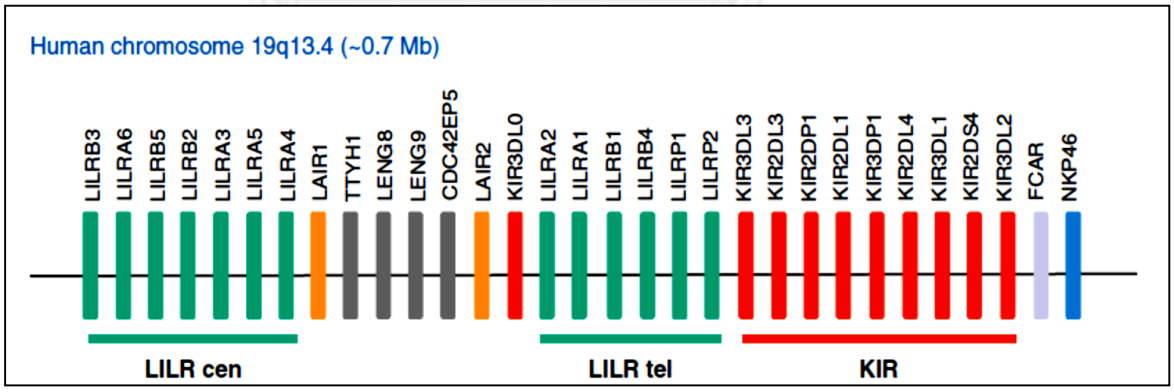

Figure 5. The leukocyte receptor complex (LRC) with KIR genes [41]

The major ligands for KIRs are HLA (MHC) class I (HLA-A,-B,-C) molecules. Interestingly, KIR and HLA genes are inherited independently [42]. The structural features of KIRs are based on their extracellular domains including 2D or 3D standing for the number of Ig-like domains and based on the length of cytoplasmic tails

including L or S for long or short tails, respectively. The transmembrane portions of the KIR molecules can reflect their function activities. The long tailed KIR are inhibitory while short tailed KIR are activating receptors (Figure 6) [43]. The long cytoplasmic tail contains immunoreceptor tyrosine-based inhibitory motif (ITIM), which can recruit and activate SHP-1 and SHP-2 phosphatases for inhibitory signal transduction. In contrast, the short-tailed KIRs that lack ITIM to drive inhibitory signal, have DAP-12, which is an adaptor molecule for delivering activating signals [44]. The Inhibitory KIR recognizes amino acids in the COOH-terminal portion of the MHC class I at alpha1 helix. The KIR2DL1 specifically recognizes the HLA-C allele characterized by amino acids of lysine in position 80 (HLA-C related group 2 allele), whereas KIR2DL2 and KIR2DL3 have specificity for HLA-C characterized by amino acids of asparagine in position 80 (HLA-C related group 1). The KIR3DL1 exhibits specificity for HLA-B allotypes with Bw4 motifs at position 77-83. Finally KIR3DL2 has ligand specificity for HLA-A3/11[45, 46]. In activating signals such as KIR2DS1-5, KIR3DS1, these signals were transferred to immunoreceptor tyrosine based activating (ITAM) motif for immune activation (Figure 7)[44].

KIR haplotype

In terms of KIR gene content, there are framework genes that are present in everyone namely KIR3DL3, KIR3DP1, KIR2DL4 and KIR3DL2 [41]. Two KIR haplotypes (group A and B) have been identified based on difference in KIR genes content [47]. Group A haplotypes are generally non-variable with all four framework genes present (KIR2DL1, KIR2DL3, KIR3DL1, and KIR2DP1) plus only one stimulatory KIR2DS4. In contrast, group B haplotypes are characterized by having one or more of the following genes: inhibitory KIR2DL2, KIR2DL5 and stimulatory KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5, KIR3DS1. Therefore, it should be noted that group B haplotypes have variable gene contents due to gene duplication and deletion [48]. KIR haplotypes have been associated with clinical outcome of HSCT [49]. Each individual can be categorized as 1) A/A, which is homozygous for group A KIR haplotypes, or 2) B/x, which contains either 1 or 2 group B haplotypes (A/B heterozygous or B/B homozygotes). Previous

HSCT studies have reported inconsistent associations between KIR haplotype and clinical outcome [48, 50, 51].

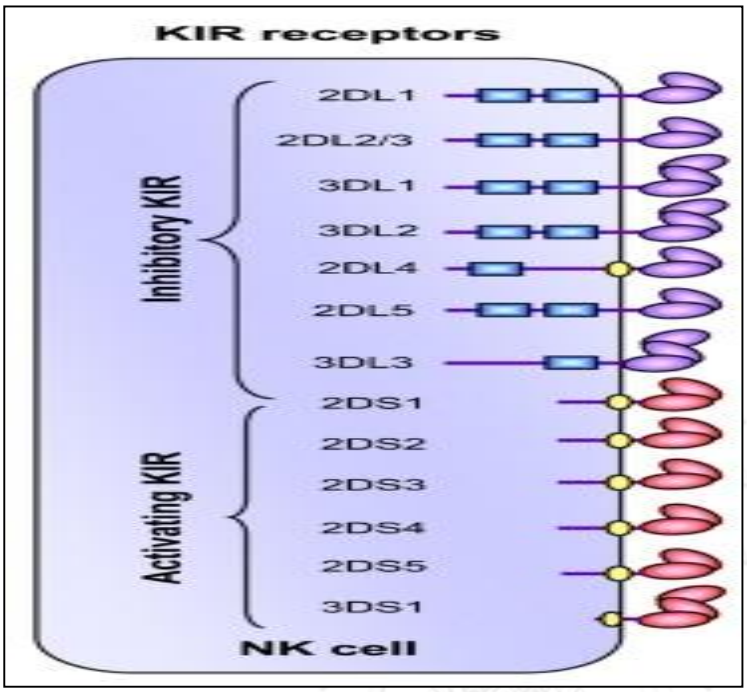

Figure 6. Inhibitory and activating KIR[43]

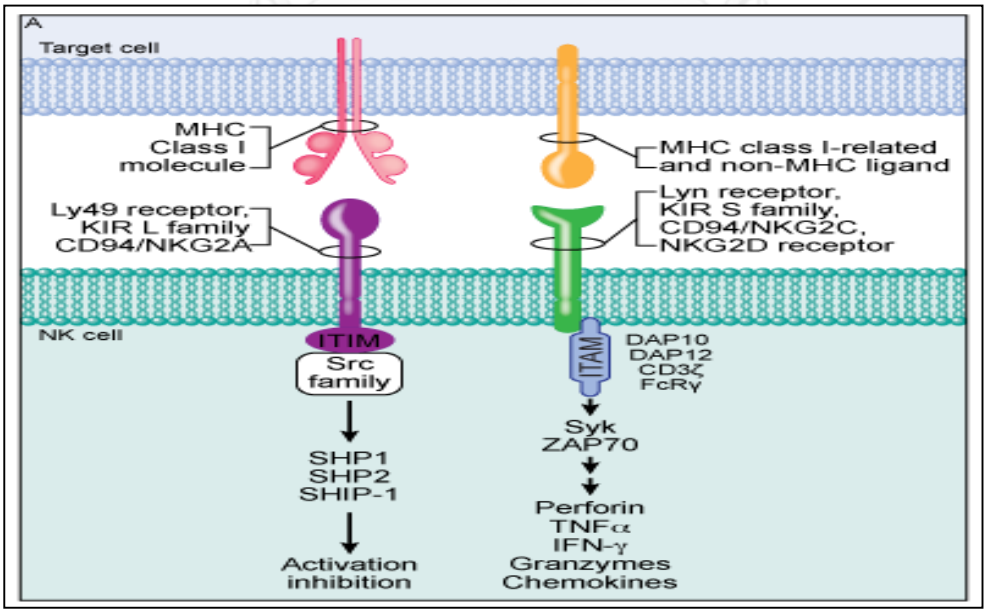

Figure 7. NK cell inhibitory and activating receptors with signaling mediated molecules [44]

Ligand of KIR

Natural killer cell-mediated cytotoxicity can be inhibited by the interaction between killer cell inhibitory receptors (KIR) with specific HLA class I molecule on target cells [52]. It was predicted based on the observation that NK cells lysed the deficient HLA class I lymphoblastoid cell lines but NK cell did not kill target cell which contained HLA class I molecule. Individual KIR involved in the recognition HLA class I were identified by using monoclonal antibodies blocking experiment, which disrupted interactions between the inhibitory receptors on the NK cells and their class I ligand on target cell [53]. Several studies used direct binding assay with soluble KIR production. The KIR and HLA class I binding assay was determined by flow cytometry [46, 54-57]. In the functional assay, the HLA class I was recognized by KIR that was determined by NK cell activation. The human NK cell line was infected with recombinant viruses encoding KIR of interest and then tested for its ability to kill a panel of HLA class I transfectants [57].

The HLA class I molecules are the ligands for several of the KIR genes (table 1). In humans, the inhibitory KIR receptors for HLA class I molecules, KIR2DL/3DL are specific for determinants shared by groups of HLA-A, -B, or –C. Two groups of HLA-C alleles differ by the amino acid at position 80. HLA-C group 1 has asparagine at position 80 provides the ligand for KIR2DL2 and KIR2DL3, whereas HLA-C group 2 has lysine at position 80 provides the ligand for KIR2DL1. The KIR3DL1 has specificity for the HLA-B molecules with an HLA-Bw4 epitope at residues 77–83. The KIR3DL2 has specificity for the HLA–A3 and –A11 allele. Finally, HLA-G is the ligand for KIR2DL4. Among the activating KIR, they were KIR2DS1, KIR2DS2 and KIR2DS4 have specificity for HLA class I molecules, while the other activating KIR has been unequivocally documented [58, 59].

Table 1. KIR and KIR ligands

KIR	KIR-ligand	Function
2DL1	HLA-C^{lys80} (C group 2)	Inhibitory

KIR	KIR-ligand	Function
2DL2/2DL3	HLA-C^{Asn80} (C group 1), HLA-B*46:01, HLA-B*73:01 Low affinity : HLA-C^{lys80} (C group 2)	Inhibitory
2DL4	HLA-G	Inhibitory and activating
2DL5	Unknown	Inhibitory
3DL1	HLA-Bw4	Inhibitory
3DL2	HLA-A*03 and HLA-A*11	Inhibitory
2DS1	HLA-C^{lys80} (C group 2)	Activating
2DS2	HLA-C^{Asn80} (C group 1)	Activating
2DS3	Unknown	Activating
2DS4	HLA-A*11 and some HLA-C alleles	Activating
2DS5	Unknown	Activating
3DS1	Unknown	Activating

HLA-C allele group:
HLA-C^{Asn80} - C*01, 03, 07, 08, 12, 13, 14:02, 15:07, 16:01
HLA-C^{lys80}- C*02, 04, 05, 06, 07:07, 12:04, 14:01, 15 (without 15:07), 16:02, 17

Missing KIR Ligand

KIR and HLA genotypes segregate independently of each other; therefore, NK cells may express KIR in which they have no HLA ligand and there is the possibility of persons who lack KIR receptors for their HLA ligands. Population KIR genotyping studies have revealed that there is significant KIR diversity between persons [52, 60]. One or more inhibitory KIR may be lacking in a number of population. It is common for a person to lack HLA ligand for his or her own KIR. As shown by some study, more than 60% of the white population is missing one or more ligands for inhibitory KIR [61]. In Thai population, there are as high as 80-90% of the normal population that have at least one missing KIR ligand [1]. This observation raises the possibility of the NK autoreactivity. However, a recent study by Grau et al demonstrated that the frequency of the autoreactive NK clones was very low [62]. They believed that it was due to the NK education process that leads to NK anergy to prevent autoreactivity.

However, studies investigating the KIR-HLA relationship and susceptibility to viral disease progression and autoimmunity have recognized that the lack of HLA ligand for inhibitory KIR receptors within a patient can have significant effects on clinical outcome in several diseases. For example, the homozygosity for KIR2DL3 with its group 1 HLA-C ligand was associated HCV protection [63]. For KIR susceptibility in combination with specific HLA class I ligands, the present of activating KIR2DS2 was susceptibility to systemic sclerosis. It results from a significant decrease in the amount of the inhibitory KIR for recognition to HLA-C ligand [64]. Type 1 diabetes mellitus was associated with KIR2DS2 and HLA-C1 genotype and had decreasing amount of inhibitory KIR for HLA ligand [65]. The HLA-C homozygous with KIR2DS1 and 2DS2 genotype was associated with psoriatic arthritis [66]. Furthermore, the 2DS1 was increased in SLE patients [67]. Most of these situations seem to be associated with either lack of NK cell inhibition or with an excessive NK cell activation through HLA class I receptor signaling. Furthermore, a study in the mother and the fetus has demonstrated that certain KIR-HLA combinations predispose to the development of preeclampsia [68].

KIR-Ligand Mismatch or Missing KIR-Ligand in HSCT

There is also evidence that NK cell alloreactivity contribute to the clinical outcome of hematopoietic stem cell transplantation through KIR and HLA molecule. The situation in HLA nonidentical transplants in which the donor NK cells recountering recipient target cells lacking HLA class I allele present in the donor HLA genotype can be called “KIR-ligand mismatch”. Interestingly, this KIR ligand mismatch can improve overall survival by mediate antileukemic effects and even decrease acute GVHD, particularly in myeloid leukemia [69]. Moreover, the NK GVL effect is not only occur in HLA nonidentical transplantation, as is required for the KIR ligand incompatibility studies, but instead can be observed in the “missing KIR-ligand” situation that can be found even in HLA-identical transplants. In the latter case, the NK cells from the donor who has missing KIR-ligand can mediate the GVL effect in the HLA identical recipient. Despite the fact that these NK cells are anergized against self through NK education process, when the NK cells are transferred into the new environment, it is hypothesized that 1) the anergy state of the NK cell is decreasing, 2) there is an upregulation of

activating KIR ligand on the cancer cells (especially the myeloid cells) [15, 70]. Therefore, the KIR-HLA class I relationship between recipient and donor might become the additional part of the donor evaluation and selection. A better understanding of the biologic mechanisms involved in the observed effects must be further investigated.

NK mediated GVL effects and HSCT

There are growing evidences that KIR ligand mismatch has beneficial effects in HSCT as the treatment for leukemia [71-73]. In haploidentical transplantation, the survival advantage was observed only in patients with AML but not in patients with acute lymphoblastic leukemia (ALL) [69]. Most of the studies interested in the role of NK cells in acute myeloid leukemia (AML) [69, 71, 74]. Adult ALL patients are not as susceptible to missing KIR ligand in allogeneic HSCT [26, 69]. These results suggest a less efficient interaction between NK cells and ALL blasts compared to the interaction between NK cells and AML blasts. It may be possible that the surface density of HLA class I molecules seem to be higher on ALL compared to AML blasts [75]. Likewise, one of the immune escape in ALL may involve the absence or down-regulation of ligands for activating NK cell receptors. However, in children with ALL, transplantations from NK-alloreactive donors were also reported to decrease the risk of relapse [76, 77]. However, the outcomes reports following allogeneic HSCT greatly varied. Some reports described beneficial effects of KIR genotype outcome, whereas other reports have described either no effect or worse outcome [61, 74, 78-80] (See summary in Table 2).

The clinical contribution of NK cells to alloreactivity for AML patients who received mismatched KIR ligand transplantation promote a strong graft versus leukemia (GVL) and reduce GVHD [69, 81]. The effects of missing KIR ligand had a lower hazard of relapse compared with patients who were not missing ligand [61, 82]. Interestingly, transplant of KIR ligand mismatch also had better outcome to decrease risk of GVHD [69]. Indeed, none of the patients in the KIR ligand-mismatched group developed grade III-IV acute GVHD [74]. There are evidences that NK cells can kill dendritic cells that are essential in the induction of GVHD both *in vitro* and *in vivo* [83]. Therefore, the most likely hypothesis is that alloreactive NK cells are induced to kill recipient antigen

presenting cells, thus further reducing the risk of GVHD. In summary, the HLA-KIR mismatched patients have higher survival rate than HLA-KIR matched group [84]. In addition, the number of the missing KIR-ligand is important too [61]. Some transplant studies have described upon the impact of numerous missing KIR ligands. In the AML and MDS patients with missing ligand, the overall survival of missing 2 ligands were higher compared with no missing or missing only 1 ligand [70]. When donor-recipient pairs were separated into specific missing ligand group, one study showed that the patients with lack 2 ligand for HLA-C and HLA-Bw4 for donor KIR have lower relapse and higher survival compared with other groups [61].

For contribution of donor-activating KIR receptors to transplantation outcome, patients with AML who had received allografts from donor with KIR2DS1 positive had a lower rate of relapses than patients received negative KIR2DS1 donor [85]. In addition, the activating KIR3DS1 was associated with improved outcome in patients who received an unrelated donor and was associated with lower-grade II-IV acute graft-versus-host disease [86].

As for the KIR haplotype analysis, the donor B haplotype KIR genes included KIR2DL5, KIR2DS1 and KIR3DS1 have been shown to have an impact on outcome for treatment of malignant relapse who were AML with undergoing HLA-identical sibling HSCT [87]. Similarly, KIR B haplotype donors in AML also showed beneficial effect in unrelated HLA-matched. They showed that the use of donor B haplotype compared with group A haplotype was associated with significant improvement in relapse and survival [88].

Table 2. The summary of KIR genotypes on clinical HSCT in leukemia patients

HSCT in patient with HLA identical sibling

Investigation	Sample size	KIR	Observation	Disease	treatment	Reference
Inhibitory KIR	n=178 (AML=114)	Missing KIR ligand	-decrease relapse (HR=0.41, p=0.04) -Increase survival (HR=0.53, p=0.03)	AML,MDS	T cell deplete	Hsu et al.,2005
Inhibitory KIR	n=43 (AML=30)	Missing KIR ligand	-decrease relapse (p=0.028) -Increase survival (p=0.046)	AML,MDS	T cell deplete	Clausen et al.,2007
Inhibitory KIR	n=151 (AML=62)	Missing KIR ligand	-Increase survival (HR=0.79, p<0.05)	AML,MDS	T cell deplete	Linn et al.,2009
Inhibitory KIR	n=100 (AML=43)	Missing KIR ligand (Homozygous HLA-C1 or HLA-C2)	-Increase relapse (RR=0.28, p=0.003) -decrease survival (RR=0.56, p=0.046)	Hematological malignancies	T cell replete	Clausen et al.,2010
Inhibitory KIR	n=105 (AML=81)	KIR ligand mismatch	Unaffected on relapse, survival and GVHD	AML,MDS	T cell replete	Bjorklund et al.,2010
Inhibitory KIR & activating KIR	n=51 (AML=25)	Missing KIR ligand, KIR ligand mismatch	-Unaffected on relapse, survival and GVHD	AML,CML,ALL	T cell replete	Wongwuttisaroj et al.,2012
Inhibitory KIR	n=52 (AML=20)	Missing KIR ligand (Homozygous HLA-C1 or HLA-C2) KIR ligand mismatch	-decrease relapse (p=0.024) -Increase survival (p=0.034) -decrease relapse (p=0.002) -Increase survival (p=0.003)	AML,MDS,CML	T cell replete	Wang et al.,2013

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia

HSCT in patients with Haploidentical Transplant

Investigation	Sample size	KIR	Observation	Disease	treatment	Reference
Inhibitory KIR	n=92 (AML=92)	Missing KIR ligand	-Decrease relapse (p=0.0008) -Increase survival (p=0.0005) -Decrease aGVHD (p≤0.01)	AML	Related donor, T cell deplete	Ruggeri et al.,2002
Inhibitory KIR	n=62 (AML=15)	KIR ligand mismatch	-A trend toward less relapse (p=0.09) -Increase aGVHD (p<0.02)	AML,CML,ALL	Related donor, T cell deplete	Bishara et al.,2004
Inhibitory KIR	n=112 (AML=112)	Missing KIR ligand	-Decrease relapse (p=0.003) -Increase survival (p=0.04)	AML	Related donor, T cell deplete	Ruggeri et al.,2007
Inhibitory KIR	n=86 (AML/MD S=33)	KIR ligand mismatch	-Decrease relapse (p=0.025) -Increase survival (p=.0003)	Hematological malignancies	Related donor, T cell replete	Symons et al.,2010

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia

HSCT in patients with unrelated donor

Investigation	Sample size	KIR	Observation	Disease	Treatment	Reference
Inhibitory KIR	n=175 (AML=14)	Missing KIR ligand	-Worse survival -No effect in relapse and aGVHD	AML	HLA mismatch, T cell replete	Davies et al, 2002
Inhibitory KIR	n=130 (AML/MDS =45)	KIR ligand mismatch	-Decrease relapse (p=0.07) -Increase survival (p=0.006)	AML	HLA mismatch , T-cell replete	Giebel et al.,2003
Inhibitory KIR	n=1770 (AML=286)	Missing KIR ligand	-Decrease relapse (HR=0.47, p=0.004)	AML,CML,MDS	HLA mismatch, T cell replete	Hsu et al.,2006

Investigation	Sample size	KIR	Observation	Disease	Treatment	Reference
Inhibitory KIR	n=2062 (AML=556)	KIR ligand mismatch	-Decrease relapse (RR=0.54, p=0.03) -Increase GVHD (RR=1.58, p=0.008)	AML,CML,MDS	HLA match, T cell deplete	Miller et al.,2007
Inhibitory KIR	n=24 (AML=17)	KIR ligand mismatch	Unaffected on relapse, survival and GVHD	AML,CML,MDS	HLA mismatch, T cell deplete	Weisdorf et al.,2012

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia

CHAPTER III
MATERIALS AND METHODS

Patient and donor samples

The retrospective study was conducted from sixty-six hematologic malignancy patients receiving stem cell transplantation. The DNAs from donor-recipient pairs were collected from Tissue Typing Laboratory, King Chulalongkorn Memorial Hospital during April 2006 – December 2013. These samples were stored in -30°C. The study was approved by an institutional review board, Faculty of Medicine, Chulalongkorn University, Thailand. All grafts were HLA-identical sibling. The hematopoietic stem cells were either from peripheral blood stem cells (PBSC) or bone marrow (BM) without T cells depletion.

The baseline characteristic of patient are confounding factors that may affect the transplant outcome including, the patients' age, the donors' age, a preparative condition regimen which comprised of total body irradiation (TBI) base or Busulphan base, the using of GVHD prophylaxis (combination of cyclosporine-A (CsA) and methotrexate (MTX) were mainly used condition in the study), the ABO blood group (it was classified as matching or mismatching), the CMV status of donor-recipient pairs, stem cell dose (it was assorted into 3 groups, including $5x10^6$/kg, $5x10^6$ to $10x10^6$/kg, above $10x10^6$/kg), type of disease (AML, ALL and CML patients), the difference in gender between donor and recipient. The clinical outcome of the patients were determined by the hematologist including relapse, survival, acute graft versus host disease (aGVHD), chronic graft versus host disease (cGVHD) and transplant-related mortality (TRM). Relapsed disease for AML and ALL was defined by morphologic or cytogenetic evidence, either in peripheral blood or in bone marrow. Relapsed disease for CML was defined by hematological, cytogenetic or molecular evidence of recurrence. The time to event of each clinical outcomes (relapse, acute GVHD and chronic GVHD) are defined as time from stem cell infusion (day 0) to event (at date of relapse or acute GVHD or chronic GVHD) or last follow up in without each event. Acute GVHD was determined within 100 days after transplantation, and chronic GVHD was an event occurred more than 100 days after transplantation. Overall survival was determined from the time

between date of transplantation to date of last follow up or death from any reasons. Transplant-related mortality (TRM) was defined by death due to any reason without evidence of relapse.

HLA and KIR genotyping

All DNA samples have been completely identified for donor-recipient HLA-A,-B,-DR,-DQ typing. HLA-A,-B were typed by microlymphocytotoxicity test and HLA-DR, -DQ were typed at low resolution by sequence specific primer (PCR-SSP) (MicroSSP, One Lambda Inc., California, USA). The data was obtained from pre-existing record done at Tissue Typing Laboratory, King Chulalongkorn Memorial Hospital before transplant. In the present study, we additionally typed both HLA-C loci in patients and KIR genotype in donors by the same reactive condition of polymerase chain reaction-sequence specific oligonucleotide probes (PCR-SSOP) commercial kit (LABType® SSO Typing Tests, One Lambda Inc., California, USA) as described by the manufacturer protocol. The KIR genotyping test in the kit can identify the presence or absence of the following KIR genes and variants: 2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1, 3DL2, 3DL3, 3DS1, 2DP1 and 3DP1

Amplification steps

In brief, the reactions of 10 ul PCR solution contained 0.1 ug test DNA, master mix buffer, 0.5 U Taq DNA polymerase (GoTaq® Flexi DNA Polymerase, Promega ,USA) and primer mixture. The amplification was performed in thermalcycler (Gene Amp PCR System 9600, Applied Biosystems, California, USA) under thermal cycling condition as the following, 3 min denaturing step at 96^0C, 5 cycles of 96^0C for 20 seconds, 60^0C for 20 seconds, 72^0C for 20 seconds, then 30 cycles of 96^0C for 10 seconds, 60^0C for 15 seconds, 72^0C for 20 seconds and final extension step at 72^0C for 10 min. The PCR products were kept in 4^0C until the analysis by gel electrophoresis. Confirmation of the amplified product band prior to hybridization assay was done to ensure the generation of optimal signals.

Hybridization steps

The PCR products were hybridized with oligonucleotide coated on micropartical bead. Before hybridization steps, the hybridization mixture was prepared

using Hybridization Buffer and Bead Mixture. First, amplified DNA samples were allowed to denature by Denaturation Buffer. Then, the reaction was stopped by Neutralization Buffer. The color was changed to clear or pale yellow. The Hybridization Mixture was allowed to hybridize to complementary DNA conjugated on fluorescent microspheres bead at 60^0C. Then, Wash Buffer was added to reaction mixture. During the last wash step, the R-Phycoerythrin-conjugated Streptavidin (1XSAPE) was prepared by the combination of SAPE buffer and 100XSAPE. After washing, R-Phycoerythrin-conjugated Streptavidin (1XSAPE) was tagged to amplified DNA at 60^0C. After the latest washing and adding buffer, the hybridization was visualized by flow analyzer, the LABScan™ 100 (One Lambda Inc., California, USA) to identify the fluorescent intensity of phycoerythrin on each microsphere. The results were reported in pattern of .csv file and then the file was imported into HLA fusion™ software for data analysis.

Data Calculation

The mean fluorescence intensity (MFI) is generated by Luminex® Data Collection software. Each bead contains the fluorescence intensity (FI) in each different sample. The percent positive value was calculated as below.

$$\text{Percent positive value} = 100 \times \left[\frac{\text{MFI(Probe n)} - \text{MFI(Probe Negative Control)}}{\text{MFI(Probe Positive Control)} - \text{MFI(Probe Negative Control)}}\right]$$

The positive reaction is defined by the percent of positive values for each probe higher than cut off value and the negative reaction is defined as the percent of positive values lower than the cut off value.

The Determination of Missing KIR ligand

There are four categories for missing KIR ligand, which can be identified depending on their KIR recognition as following.

1. The patient is lack of HLA-C group 2 ligand for donor inhibitory KIR2DL1
2. The patient is lack of HLA-C group 1 ligand for donor inhibitory KIR2DL2 or 2DL3
3. The patient indicates lack of HLA-Bw4 ligand for donor inhibitory KIR3DL1
4. The patient is lack of HLA-A11 ligand for donor inhibitory KIR3DL2

The Determination of KIR haplotype

1. KIR haplotype group AA. There are 5 inhibitory KIR and 1 activating KIR including KIR2DL3, KIR2DL1, KIR2DL4, KIR3DL1, KIR3DL2 and KIR2DS4.
2. KIR haplotype group Bx. There are at least 1 of the KIR B loci including KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5 and KIR3DS1.

Statistical analysis

The association between baseline characteristics as well as KIR genes with transplant outcome variables were determined by Chi-square or Fisher's exact test (two-tailed). For Fisher's exact test, it can be used in small sample sizes with expected counting less than 5 events. The difference between time-to-event distributions was compared by Kaplan-Meier method. Significance was established at p-value less than 0.05. Probability of relapse, overall survival, acute GVHD and chronic GVHD were estimated using the Kaplan-Meier method (SPSS software version 19).

CHAPTER IV
RESULTS

Baseline Characteristics on Clinical Outcome

This study retrospectively analyzed a group of 66 leukemia patients. The patients and transplant characteristics included in this study were as follows. The median age of patients and donors were 35 and 37 years, respectively. Most of them had previous CMV infection (45 out of 47 pairs with known data, 95.7%). The majority of the patients have AML (60%). Peripheral blood stem cell sources were the most common source of stem cells (98.5%). Patients received stem cell with blood group matching more than mismatching (60.6% VS 39.4%). Before transplantation, 44 patients (66.7%) were treated by total body irradiation based and 22 patients (33.3%) used Busulfan based. Cyclosporine with methotrexate combination were mostly given before and after transplantation in 62 patients (93.9%) (Table 3).

Table 3. Baseline characteristics

Patient and treatment characteristics	Data value
Median recipient age (n=66) , median (range)	35 (15-62)
Median donor age (n =66) , median (range)	37 (8-66)
Sex match,(donor/recipient) (n=66)	
Male/male, n (%)	17 (25.8%)
Male/female, n (%)	14 (21.2%)
Female/male, n (%)	14 (21.2%)
Female/female, n (%)	21 (31.8%)
CMV status, donor/recipient (n=47)	
Negative/positive, n (%)	1 (2.1%)
Positive/positive, n (%)	45 (95.7%)
Positive/negative, n (%)	1 (2.1%)
Diagnosis (n=66)	
AML, n (%)	40 (60.6%)
ALL, n (%)	12 (18.2%)
CML, n (%)	14 (21.2%)

Patient and treatment characteristics	Data value
Source of stem cells (n=66)	
PBSC, n (%)	65 (98.5%)
BM+PBSC, n (%)	1 (1.5%)
CD34+ cells infused (n=66), median (range)	$5.95X10^{6}$/kg (2.3 - $17.3X10^{6}$/kg)
ABO (n=66)	
Match, n (%)	40 (60.6%)
Mismatch, n (%)	26 (39.4%)
Condition regimen (n=66)	
TBI-based, n (%)	44 (66.7%)
Busulfan-based, n (%)	22 (33.3%)
GVHD prophylaxis (n=66)	
CsA/MTX, n (%)	62 (93.9%)
Other, n (%)	4 (6.1%)

Clinical Outcome

We investigated 66 patients after hematopoietic stem cell transplant and donor pairs. The patients have been transplanted during April 2006 and December 2013. These patients have different clinical outcome. Twenty patients (30.3%) had relapse. Acute GVHD and chronic GVHD were observed in 14 (21.2%) and 20 (30.3%) patients, respectively. There was only one patient who died from a transplant-related event (1.5%). There were 50 patients (75.7%) who were considered to be survival while 16 patients (24.2%) were death after transplantation (Table 4).

Table 4. Clinical outcomes

Incidence	n (%)
Relapse	20 (30.3%)
Acute GVHD	14 (21.2%)
Chronic GVHD	20 (30.3%)
TRM	1 (1.5%)

Incidence	n (%)
Alive	50 (75.7%)
Dead	16 (24.2%)

HLA and KIR Genotyping Frequencies

The frequencies of the HLA antigens, which are the main KIR ligands in the 66 patients are shown in Figure 8. There were 62.1% of HLA-C group 1 (n=41), 56.1% of HLA-Bw4 (n=37), 48.5% of HLA-A11 (n=32), 33.3% of the heterozygous HLA-C group1/2 (n=22) and only 4.5% of HLA-C group 2 (n=3).

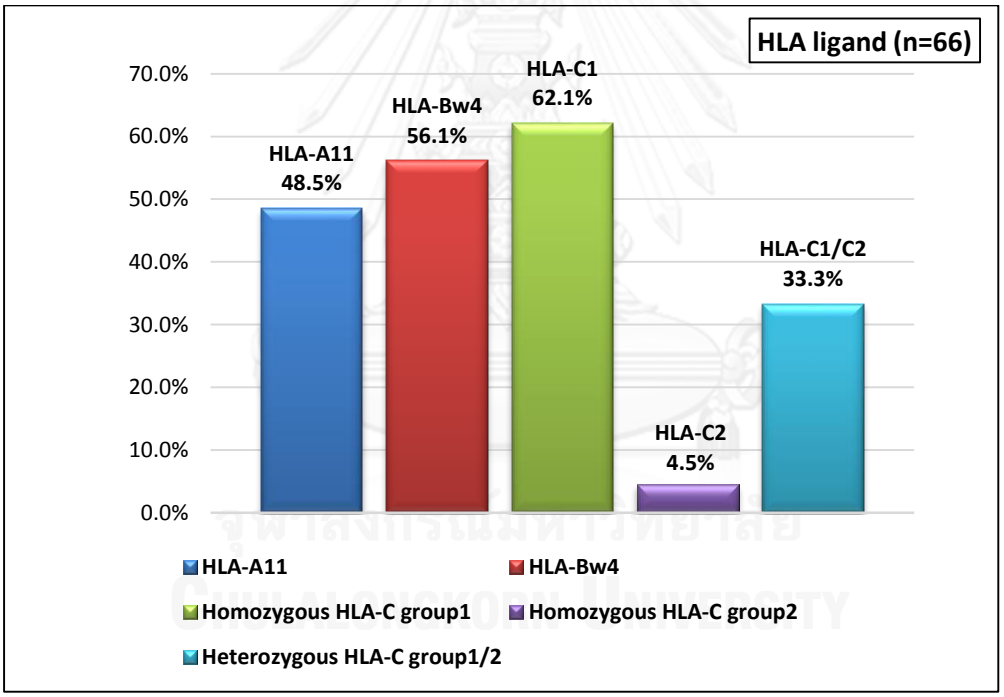

Figure 8. Frequencies of HLA antigens, which are the main KIR ligands

The distribution of inhibitory KIR genotypes in the sibling donors was shown in in Figure 9. The inhibitory KIR gene found in all donors was KIR3DL2. Sixty-three donors had KIR2DL1 (95.5%), and 62 donors had KIR2DL3 (93.9%). Inhibitory KIR3DL1 were seen in 63 donors (95.5%). KIR2DL2 receptor was seen in 26 donors (39.4%).

The most common activating KIR gene was KIR2DS4. It was seen in 60 donors (90.9%). The other activating KIR genes have the frequency less than 50%, there were KIR2DS1, KIR2DS2, KIR3DS1, KIR2DS5 and KIR2DS3. There were 31 donors with KIR2DS1 (46.9%). Both KIR2DS2 and KIR3DS1 were found in 25 donors (37.9%), KIR2DS5 was seen in 24 donors (36.4%), while KIR2DS3 receptor was found in 13 donors (19.7%) which was the lowest frequency compared to other types of activating KIR genotype (Figure 10).

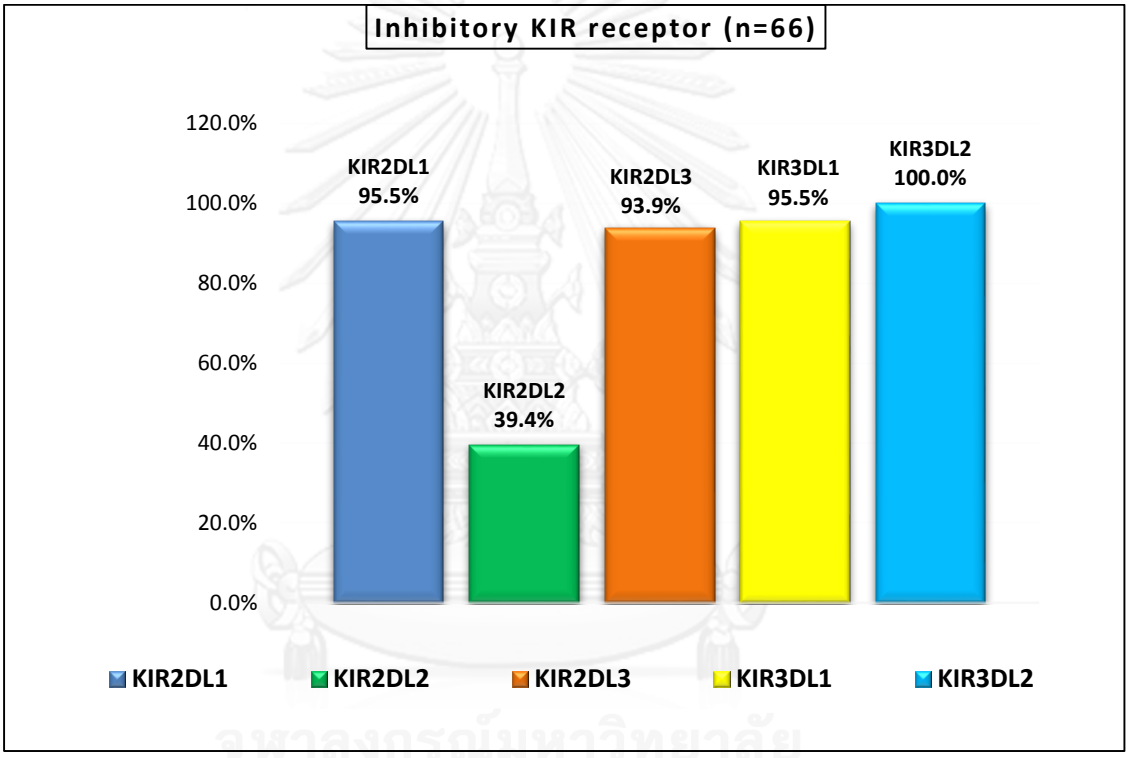

Figure 9. Inhibitory KIR genotype frequencies

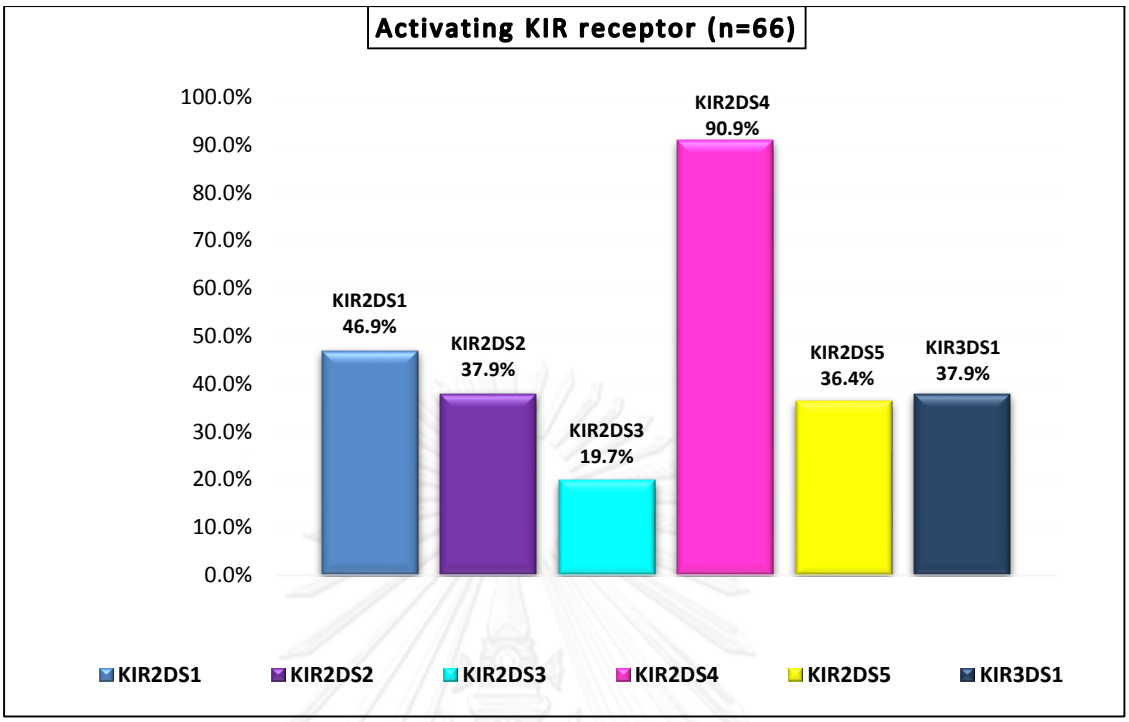

Figure 10. Activating KIR genotype frequencies

KIR Haplotype Frequencies

In this study, the distribution of KIR haplotypes in the 66 donors was shown in Figure 11. The donor with haplotype AA group was found in 26 patients (39.4%) and haplotype Bx group was found in 40 patients (60.6%).

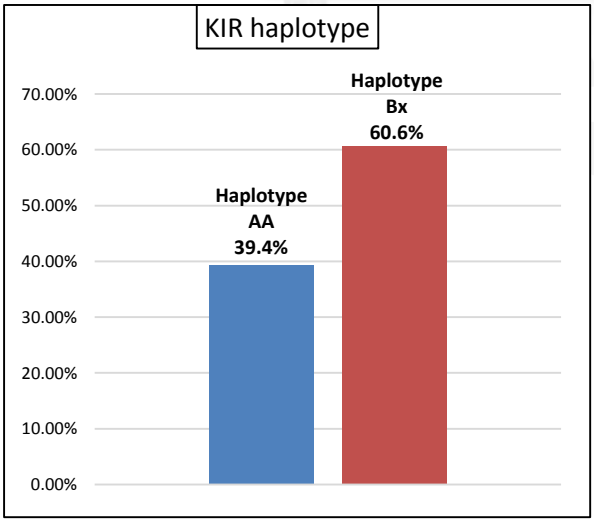

Figure 11. KIR haplotype frequencies

Missing ligand frequencies for inhibitory KIR

Among the 66 donor-recipient pairs, there were a high number of pairs with missing HLA ligand for inhibitory KIR receptor KIR2DL1, KIR2DL2/3, KIR3DL1 and KIR3DL2. The missing ligand for KIR2DL1 and KIR3DL2 were the most frequent group, seen in 40 patients (60.6%) and 34 patients (51.5%), respectively. The other missing ligand for KIR3DL1 and KIR2DL2/3 were seen in 27 patients (40.9%) and 3 patients (4.5%), respectively (Figure 12).

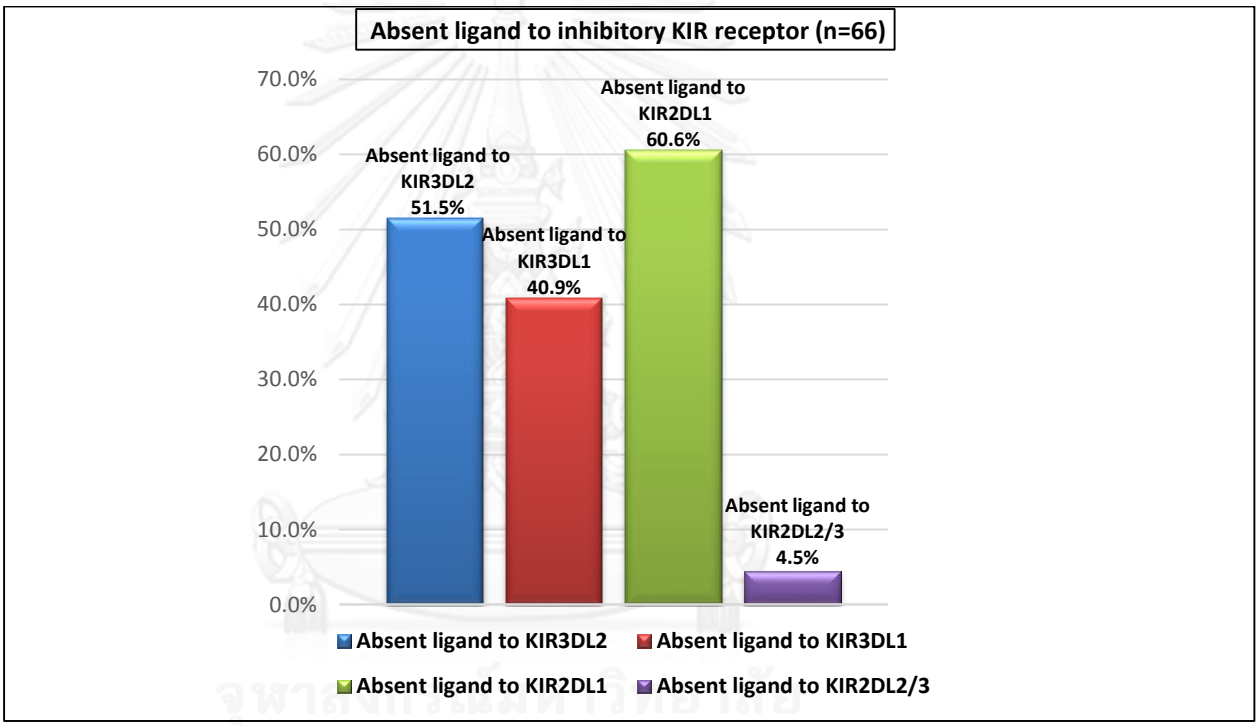

Figure 12. The lack of HLA ligand for inhibitory KIR receptor

We investigated the influence of different baseline characteristics variables on relapse, acute GVHD, chronic GVHD and survival. None of these variable factors regarding patient age, donor age, sex donated matching, CMV status, diagnosis, source of stem cell, CD34+ cells infusion, ABO blood group, condition regiment and GVHD prophylaxis were found to have a significant influence on relapse, acute GVHD, chronic GVHD and survival. (Table 5-8).

Table 5. Baseline characteristics on relapse

Characteristics	Relapse	No relapse	p-value
Recipient age (n=66)	20 (30.3%) (mean=36.4)	46 (69.7%) (mean=35.6)	0.795
Donor age (n=66)	20 (30.3%) (mean=36.7)	46 (69.7%) (mean=36.9)	0.959
Sex match, donor/recipient (n=66)			
Male/male	1 (1.5%)	16 (24.2%)	
Male/female	4 (6.1%)	10 (15.2%)	0.059
Female/male	6 (9.1%)	8 (12.1%)	
Female/female	9 (13.6%)	12 (18.2%)	
CMV status, donor/recipient (n=42)			
Negative/positive	1 (2.4%)	0 (0%)	
Positive/positive	8 (19.0%)	32 (76.2%)	0.387
Positive/negative	0 (0%)	1 (2.4%)	
Diagnosis (n=66)			
AML	13 (19.7%)	27 (40.9%)	
ALL	6 (9.1%)	6 (9.1%)	0.054
CML	1 (1.5%)	13 (19.7%)	
Source of stem cells (n=56)			
PBSC	19 (28.8%)	46 (69.7%)	0.126
BM+PBSC	1 (1.5%)	0 (0%)	
CD34+ cells infused (n=55)			
< 5x106 / kg	3 (4.5%)	15 (22.7%)	
5-10X106/kg	16 (24.2%)	26 (39.4%)	0.190
> 10x106 / kg	1 (1.5%)	5 (7.6%)	
ABO (n=60)			
Match	14 (21.2%)	26 (39.4%)	0.303
Mismatch	6 (9.1%)	20 (30.3%)	
Condition regimen (n=57)			

Characteristics	Relapse	No relapse	p-value
TBI-based	15 (22.7%)	29 (43.9%)	0.344
Busulfan-based	5 (7.6%)	17 (25.8%)	
GVHD prophylaxis (n=59)			
CsA/MTX	19 (28.8%)	43 (65.2%)	0.812
Other	1 (1.5%)	3 (4.5%)	

Table 6. Baseline characteristics on aGVHD

Characteristics	Acute GVHD	No acute GVHD	p-value
Recipient age (n=66)	14 (21.2%) (mean=33.6)	52 (78.8%) (mean=36.5)	0.396
Donor age (n=66)	14 (21.2%) (mean=35.1)	52 (78.8%) (mean=37.3)	0.547
Sex match, donor/recipient (n=66)			
Male/male	2 (3.0%)	15 (22.7%)	
Male/female	2 (3.0%)	12 (18.2%)	0.485
Female/male	4 (6.1%)	10 (15.2%)	
Female/female	6 (9.1%)	15 (22.7%)	
CMV status, donor/recipient (n=42)			
Negative/positive	0 (0%)	1 (2.4%)	
Positive/positive	11 (26.6%)	29 (69.0%)	0.689
Positive/negative	0 (0%)	1 (2.4%)	
Diagnosis (n=66)			
AML	8 (12.1%)	32 (48.5%)	
ALL	3 (4.5%)	9 (13.6%)	0.933
CML	3 (4.5%)	11 (16.7%)	
Source of stem cells (n=66)			
PBSC	13 (19.7%)	52 (78.8%)	0.212
BM+PBSC	1 (1.5%)	0 (0%)	
CD34+ cells infused (n=66)			

Characteristics	Acute GVHD	No acute GVHD	p-value
< $5x10^6$ / kg	4 (6.1%)	14 (21.2%)	
5-$10X10^6$/kg	10 (15.2%)	32 (48.5%)	0.407
> $10x10^6$ / kg	0 (0%)	6 (9.1%)	
ABO (n=66)			
Match	6 (9.1%)	34 (51.5%)	0.126
Mismatch	8 (12.1%)	18 (27.3%)	
Condition regimen (n=66)			
TBI-based	11 (16.7%)	33 (50.0%)	0.287
Busulfan-based	3 (4.5%)	19 (28.8%)	
GVHD prophylaxis (n=66)			
CsA/MTX	14 (21.2%)	48 (72.2%)	0.284
Other	0 (0%)	4 (6.1%)	

Table 7. Baseline characteristics on cGVHD

Characteristics	Chronic GVHD	No chronic GVHD	p-value
Recipient age (n=66)	20 (30.0%) (mean=34.8)	46 (69.7%) (mean=36.4)	0.590
Donor age (n=66)	20 (30.3%) (mean=34.9)	46 (69.7%) (mean=37.6)	0.412
Sex match, donor/recipient (n=66)			
Male/male	6 (9.1%)	11 (16.7%)	
Male/female	3 (4.5%)	11 (16.7%)	0.841
Female/male	4 (6.1%)	10 (15.2%)	
Female/female	7 (10.6%)	14 (21.2%)	
CMV status, donor/recipient (n=42)			
Negative/positive	0 (0%)	1 (2.4%)	
Positive/positive	13 (31.0%)	27 (64.3%)	0.561
Positive/negative	1 (2.4%)	0 (0%)	

Characteristics	Chronic GVHD	No chronic GVHD	p-value
Diagnosis (n=66)			
AML	9 (13.6%)	31 (47.0%)	
ALL	3 (4.5%)	9 (13.6%)	0.054
CML	8 (12.1%)	6 (9.1%)	
Source of stem cells (n=66)			
PBSC	19 (28.8%)	46 (69.7%)	0.126
BM+PBSC	1 (1.5%)	0 (0%)	
CD34+ cells infused (n=66)			
< 5x106 / kg	5 (7.6%)	13 (19.7%)	
5-10X106/kg	14 (21.2%)	28 (42.4%)	0.682
> 10x106 / kg	1 (1.5%)	5 (7.6%)	
ABO (n=66)			
Match	12 (18.2%)	28 (42.4%)	0.947
Mismatch	8 (12.1%)	18 (27.3%)	
Condition regimen (n=66)			
TBI-based	12 (18.2%)	32 (48.5%)	0.449
Busulfan-based	8 (12.1)%	14 (21.2%)	
GVHD prophylaxis (n=66)			
CsA/MTX	18 (27.3%)	44 (66.7%)	0.376
Other	2 (3.0%)	2 (3.0%)	

Table 8. Baseline characteristics on survival

Characteristics	Alive	Dead	p-value
Recipient age (n=66)	50 (75.8%) (mean=35.5)	16 (24.2%) (mean=37.1)	0.618
Donor age (n=66)	50 (75.8%) (mean=37.3)	16 (24.2%) (mean=35.4)	0.604

Characteristics	Alive	Dead	p-value
Sex match, donor/recipient (n=66)			
Male/male	15 (22.7%)	2 (3.0%)	
Male/female	10 (15.2%)	4 (6.1%)	0.585
Female/male	10 (15.2%)	4 (6.1%)	
Female/female	15 (22.7%)	6 (9.1%)	
CMV status, donor/recipient			
(n=42)	0 (0%)	1 (2.4%)	
Negative/positive	30 (71.4%)	10 (23.8%)	0.460
Positive/positive	1 (2.4%)	0 (0%)	
Positive/negative			
Diagnosis (n=66)			
AML	28 (42.4%)	12 (18.2%)	
ALL	9 (13.6%)	3 (4.5%)	0.228
CML	13 (19.7%)	1 (1.5%)	
Source of stem cells (n=66)			
PBSC	49 (74.2%)	16 (24.2%)	0.569
BM+PBSC	1 (1.5%)	0 (0%)	
CD34+ cells infused (n=66)			
< 5x106 / kg	15 (22.7%)	3 (4.5%)	
5-10X106/kg	30 (45.5%)	12 (18.2%)	0.555
> 10x106 / kg	5 (7.6%)	1 (1.5%)	
ABO (n=66)			
Match	30 (45.5%)	10 (15.2%)	0.859
Mismatch	20 (30.3%)	6 (9.1%)	
Condition regimen (n=66)			
TBI-based	31 (47.0%)	13 (19.7%)	0.155
Busulfan-based	19 (28.8%)	3 (4.5%)	
GVHD prophylaxis (n=66)			
CsA/MTX	47 (71.2%)	15 (22.7%)	0.971
Other	3 (4.5%)	1 (1.5%)	

Missing KIR Ligand

We counted the patients who have at least one missing ligand as missing KIR ligand. Missing KIR ligand was found in the majority of the patients as high as 58 patients (87.9%), while only 8 patients had no missing KIR ligand (Figure 13).

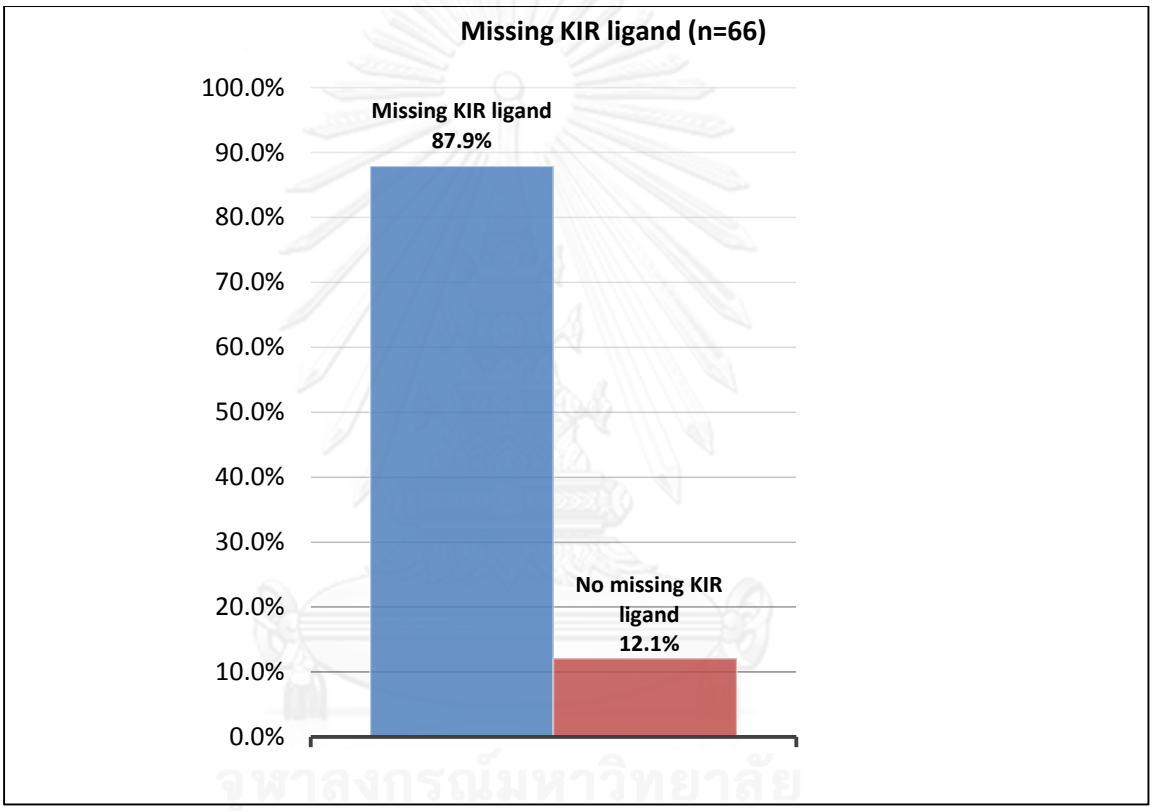

Figure 13. Frequencies of missing KIR ligand

The donor-recipient pairs were segregated into specific missing ligand group. Figure 14 showed the frequencies of each specific missing KIR ligand and their combinations. The most common of one missing KIR ligand is the missing of HLA-C2 (12 patients, 18.2%). Missing HLA-A11 and HLA-Bw4 ligand were seen in 4 (6.1%) and 5 patients (7.6%), respectively. The most common of more than one missing KIR ligand included the missing of HLA-A11 combined with HLA-C2 (15 patients, 22.7%), missing of HLA-Bw4 combined with HLA-C2 (7 patients, 10.6%) and HLA-A11 combined with

HLA-Bw4 (6 patients, 9.1%). Three missing KIR ligand was seen in 9 patients (13.6%), while no missing KIR ligand was seen in 8 patients (12.1%) (Figure 14).

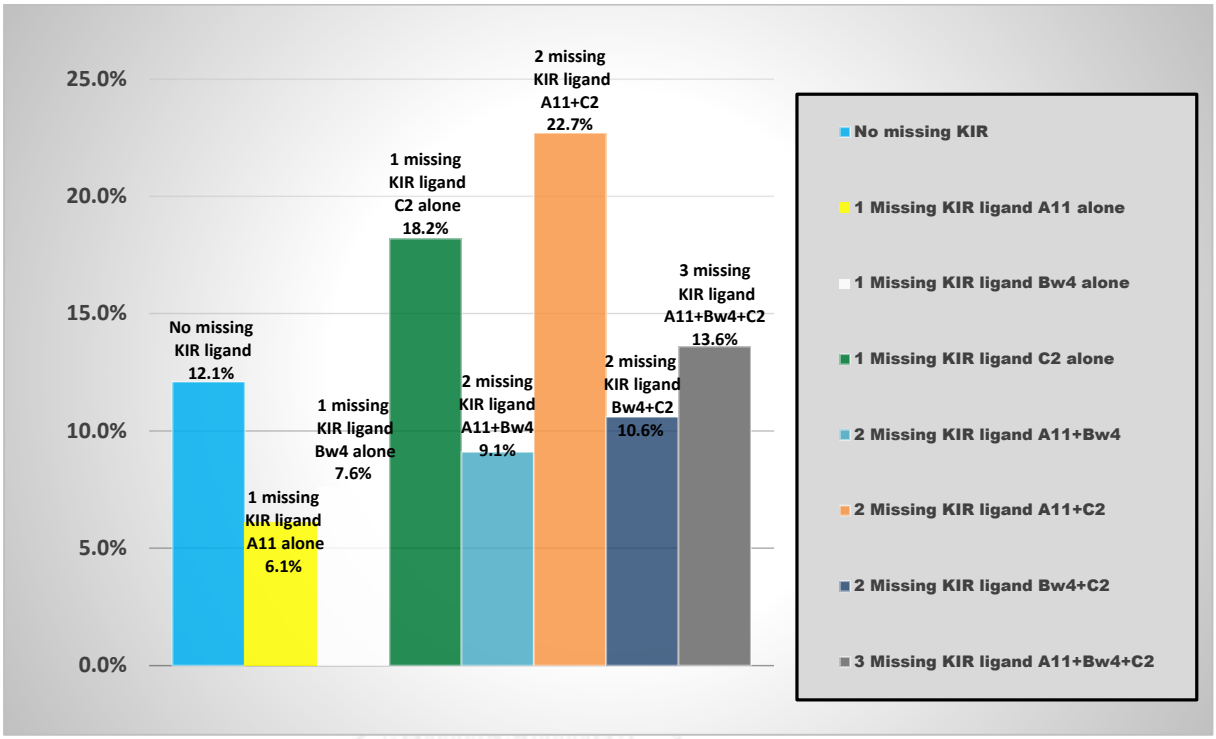

Figure 14. The frequencies of specific missing KIR ligand

The Number of Missing KIR ligand Effect on Clinical Outcome

We analyzed the effect of the number of missing KIR ligand on clinical outcome (Table 9-12). The beneficial effect of one or more than one missing KIR ligand was not seen. The patients with 2 or more than 2 missing KIR ligands had statistically significant better clinical outcome by reducing relapse and acute GVHD (p-value = 0.035, 0.005 respectively) (Figure 15-16). Moreover, we found that there were no relapse and no death in the 9 patients who had 3 missing KIR ligands (Table 9, 12). When the data were divided into individual dose of missing KIR ligand, statistical significance for decreasing acute GVHD could be detected (p-value=0.041) and there was a trend for statistically significance for decrease relapse (p-value = 0.092) (Figure 17, Table 9-10).

Table 9. The number of missing KIR ligand effect on relapse

Missing KIR ligand	Relapse	No relapse	p-value
1 more missing ligand (n=66)			
1 or more than 1 missing ligand	16 (24.2%)	42 (63.6%)	0.232
No missing ligand	4 (6.1%)	4 (6.1%)	
2 more missing ligand (n=66)			
2 or more than 2 missing ligand	7 (10.6%)	29 (43.9%)	0.035
Less than 2 missing ligand	13 (19.7%)	17 (25.8%)	
Number of missing ligand (n=66)			
0 missing ligand	4 (6.1%)	4 (6.1%)	
1 missing ligand	8 (12.1%)	13 (19.7%)	0.092
2 missing ligand	8 (12.1%)	20 (30.3%)	
3 missing ligand	0 (0%)	9 (13.6%)	

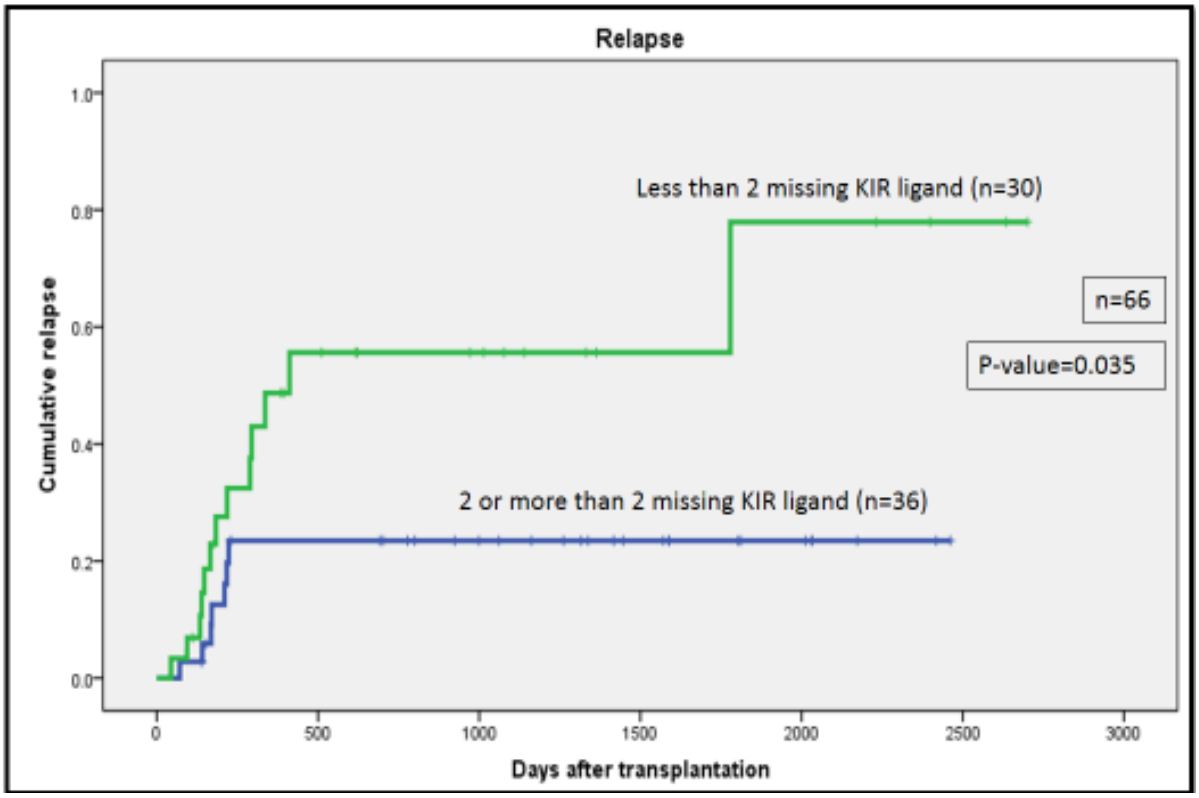

Figure 15. The number of 2 or more than 2 missing KIR ligand decrease relapse

Table 10. The number of missing KIR ligand effect on aGVHD

Missing KIR ligand	Acute GVHD	No acute GVHD	p-value
1 more missing ligand (n=66)			
1 or more than 1 missing ligand	13 (19.7%)	45 (68.2%)	0.520
No missing ligand	1 (1.5%)	7 (10.6%)	
2 more missing ligand (n=66)			
2 or more than 2 missing ligand	3 (4.5%)	33 (50.0%)	0.005
Less than 2 missing ligand	11 (16.7%)	19 (28.8%)	
Number of missing ligand (n=66)			
0 missing ligand	1 (1.5%)	7 (10.6%)	
1 missing ligand	9 (13.6%)	12 (18.2%)	0.041
2 missing ligand	3 (4.5%)	25 (37.9%)	
3 missing ligand	1 (1.5%)	8 (12.1%)	

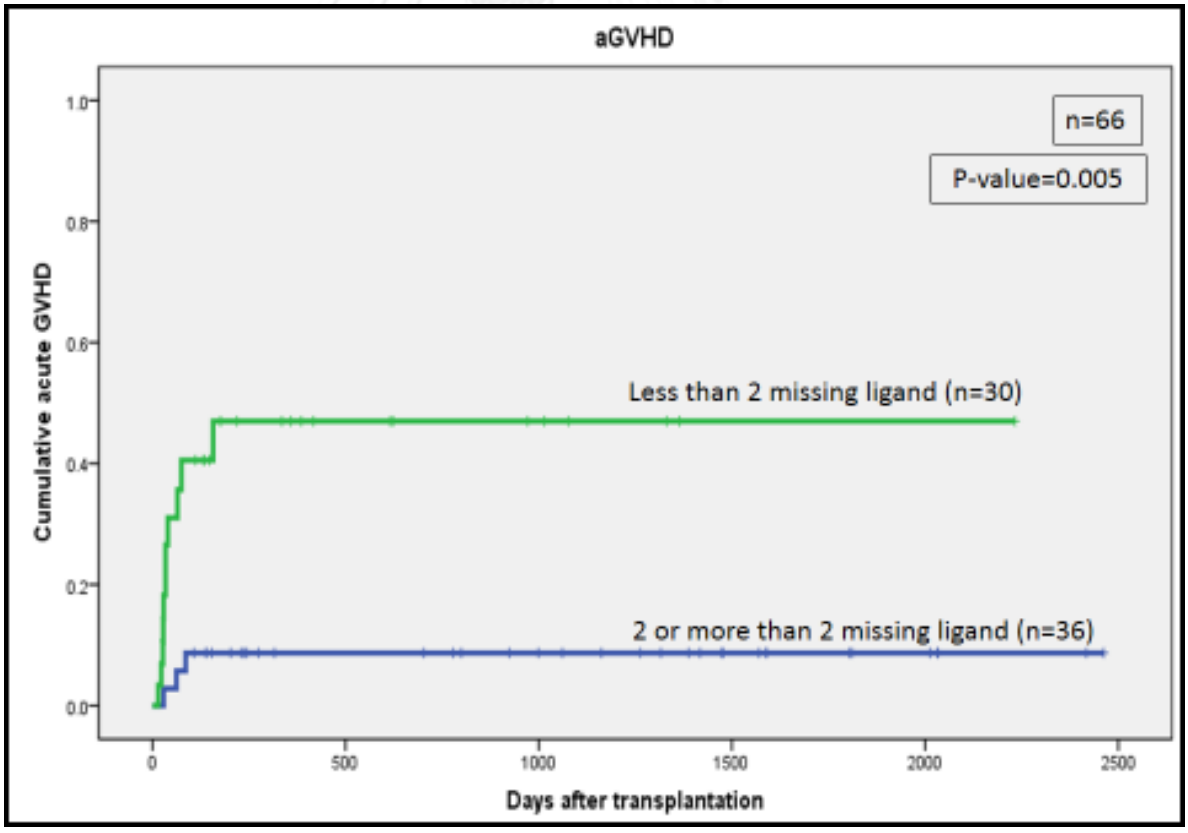

Figure 16. The number of 2 or more than 2 missing KIR ligand decrease aGVHD

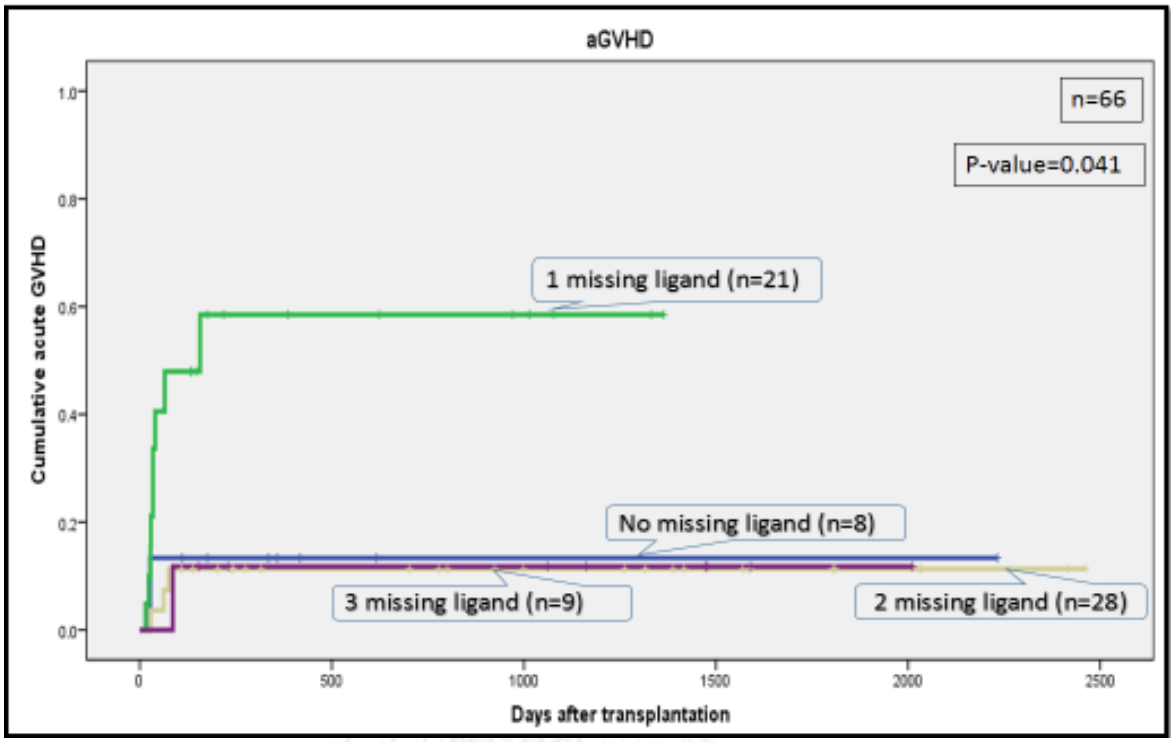

Figure 17. The number of missing KIR ligand effect on aGVHD

Table 11. The number of missing KIR ligand effect on cGVHD

Missing KIR ligand	Chronic GVHD	No chronic GVHD	p-value
1 more missing ligand (n=66)			
1 or more than 1 missing ligand	19 (28.8%)	39 (59.1%)	0.418
No missing ligand	1 (1.5%)	7 (10.6%)	
2 more missing ligand (n=66)			
2 or more than 2 missing ligand	8 (12.1%)	28 (42.4%)	0.118
Less than 2 missing ligand	12 (18.2%)	18 (27.3%)	
Number of missing ligand (n=66)			
0 missing ligand	1 (1.5%)	7 (10.6%)	
1 missing ligand	10 (15.2%)	11 (16.7%)	0.228
2 missing ligand	7 (10.6%)	21 (31.8%)	
3 missing ligand	2 (3.0%)	7 (10.6%)	

Table 12. The number of missing KIR ligand effect on survival

Missing KIR ligand	Alive	Dead	p-value
1 more missing ligand (n=66)			
1 or more than 1 missing ligand	45 (68.2%)	13 (19.7%)	0.390
No missing ligand	5 (7.6%)	3 (4.5%)	
2 more missing ligand (n=66)			
2 or more than 2 missing ligand	30 (45.5%)	6 (9.1%)	0.116
Less than 2 missing ligand	20 (30.3%)	10 (15.2%)	
Number of missing ligand (n=66)			
0 missing ligand	5 (7.6%)	3 (4.5%)	
1 missing ligand	14 (21.2%)	7 (10.6%)	0.175
2 missing ligand	22 (33.3)	6 (9.1%)	
3 missing ligand	9 (13.6%)	0 (0%)	

The Effect of Specific Missing KIR Ligand on Clinical Outcome

The analysis of specific missing KIR ligand on clinical outcome which were relapse, aGVHD, cGVHD, and survival were shown in table 13-16. Interestingly, the data have shown that the patients who had one absent ligand (HLA-C group 2) for KIR2DL1 was significantly associated with increase a risk of acute GVHD (p-value=0.014) (Figure 18). The combination of three missing KIR ligand of HLA-A11, HLA-Bw4 and HLA-C2 was significantly associated with the reducing of relapse when compared with other group (p-value=0.048) (Table 13).

Table 13. The effect of specific missing KIR ligand on relapse

Specific missing KIR ligand n=(66)	Relapse	No relapse	p-value
No missing KIR ligand			
Yes	4 (6.1%)	4 (6.1%)	0.232
No	16 (24.2%)	42 (63.6%)	
Missing KIR ligand A11 alone			
Yes	1 (1.5%)	3 (4.5%)	0.812
No	19 (28.8%)	43 (65.2%)	

Specific missing KIR ligand n=(66)	Relapse	No relapse	p-value
Missing KIR ligand Bw4 alone			
Yes	2 (3.0%)	3 (4.5%)	0.624
No	18 (27.3%)	43 (65.2%)	
Missing KIR ligand C2 alone			
Yes	5 (7.6%)	7 (10.6%)	0.488
No	15 (22.7%)	39 (59.1%)	
Missing ligand A11+Bw4			
Yes	1 (1.5%)	5 (7.6%)	0.659
No	19 (28.8%)	41 (62.1%)	
Missing ligand A11+C2			
Yes	5 (7.6%)	10 (15.2%)	0.771
No	15 (22.7%)	36 (54.5%)	
Missing ligand Bw4+C2			
Yes	2 (3.0%)	5 (7.6%)	0.916
No	18 (27.3%)	41 (62.1%)	
Missing ligand A11+Bw4+C2			
Yes	0 (0%)	9 (13.6%)	0.048
No	20 (30.3%)	37 (56.1%)	

Table 14. The effect of specific missing KIR ligand on aGVHD

Specific missing KIR ligand n=(66)	acute GVHD	No acute GVHD	p-value
No missing KIR ligand			
Yes	1 (1.5%)	7 (10.6%)	0.520
No	13 (19.7%)	45 (68.2%)	
Missing KIR ligand A11 alone			
Yes	2 (3.0%)	2 (3.0%)	0.195
No	12 (18.2%)	50 (75.8%)	

Specific missing KIR ligand n=(66)	acute GVHD	No acute GVHD	p-value
Missing KIR ligand Bw4 alone			
Yes	1 (1.5%)	4 (6.1%)	0.945
No	13 (19.7%)	48 (72.7%)	
Missing KIR ligand C2 alone			
Yes	6 (9.1%)	6 (9.1%)	0.014
No	8 (12.1%)	46 (69.7%)	
Missing ligand A11+Bw4			
Yes	1 (1.5%)	5 (7.6%)	0.775
No	13 (19.7%)	47 (71.2%)	
Missing ligand A11+C2			
Yes	1 (1.5%)	14 (21.2%)	0.161
No	13 (19.7%)	38 (57.6%)	
Missing ligand Bw4+C2			
Yes	1(1.5%)	6 (9.1%)	0.635
No	13 (19.7%)	46 (69.7%)	
Missing ligand A11+Bw4+C2			
Yes	1 (1.5%)	8 (12.1%)	0.671
No	13 (19.7%)	44 (66.7%)	

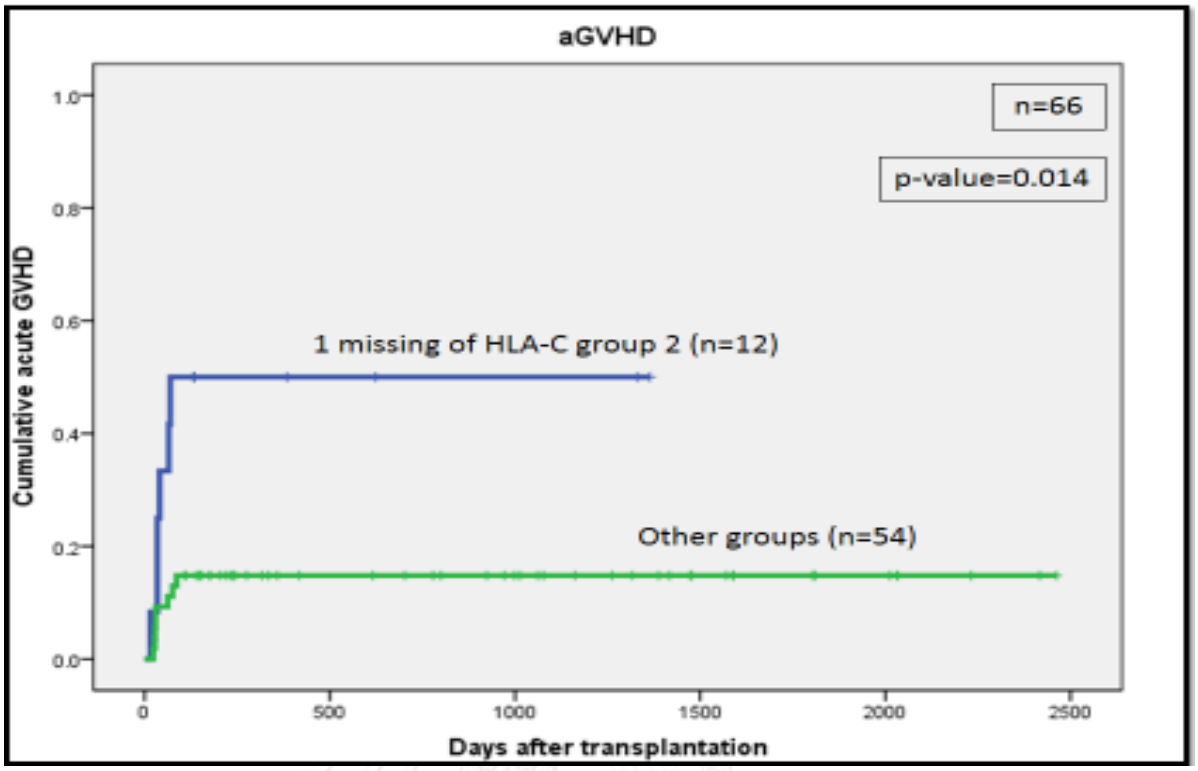

Figure 18. The effect of missing HLA-C group 2 alone on aGVHD

Table 15. The effect of specific missing KIR ligand on cGVHD

Specific missing KIR ligand n=(66)	Chronic GVHD	No Chronic GVHD	p-value
No missing KIR ligand			
Yes	1 (1.5%)	7 (10.6%)	0.418
No	19 (28.8%)	39 (59.1%)	
Missing KIR ligand A11 alone			
Yes	1 (1.5%)	3 (4.5%)	0.812
No	19 (28.8%)	43 (65.2%)	
Missing KIR ligand Bw4 alone			
Yes	2 (3.0%)	3 (4.5%)	0.624
No	18 (27.3%)	43 (65.2%)	
Missing KIR ligand C alone			
Yes	7 (10.6%)	5 (7.6%)	0.054
No	13 (19.7%)	41 (62.1%)	

Specific missing KIR ligand n=(66)	Chronic GVHD	No Chronic GVHD	p-value
Missing ligand A11+Bw4			
Yes	1 (1.5%)	5 (7.6%)	0.659
No	19 (28.8%)	41 (62.1%)	
Missing ligand A11+C2			
Yes	3 (4.5%)	12 (18.2%)	0.524
No	17 (25.8%)	34 (51.5%)	
Missing ligand Bw4+C2			
Yes	3 (4.5%)	4 (6.1%)	0.425
No	17 (25.8%)	42 (63.6%)	
Missing ligand A11+Bw4+C2			
Yes	2 (3.0%)	7 (10.6%)	0.712
No	18 (27.3%)	39 (59.1%)	

Table 16. The effect of specific missing KIR ligand on survival

Specific missing KIR ligand (n=66)	Alive	Death	p-value
No missing KIR ligand			
Yes	5 (7.6%)	3 (4.5%)	0.390
No	45 (68.2%)	13 (19.7%)	
Missing KIR ligand A11 alone			
Yes	3 (4.5%)	1 (1.5%)	0.971
No	47 (71.2%)	15 (22.7%)	
Missing KIR ligand Bw4 alone			
Yes	3 (4.5%)	2 (3.0%)	0.588
No	47 (71.2%)	14 (21.2%)	
Missing KIR ligand C alone			
Yes	8 (12.1%)	4 (6.1%)	0.465
No	42 (63.6%)	12 (18.2%)	
Missing ligand A11+Bw4			
Yes	4 (6.1%)	2 (3.0%)	0.627

Specific missing KIR ligand (n=66)	Alive	Death	p-value
No	46 (69.7%)	14 (21.2%)	
Missing ligand A11+C2			
Yes	12 (18.2%)	3 (4.5%)	0.663
No	38 (57.6%)	13 (19.7%)	
Missing ligand Bw4+C2			
Yes	6 (9.1%)	1 (1.5%)	0.516
No	44 (66.7%)	15 (22.7%)	
Missing ligand A11+Bw4+C2			
Yes	9 (13.6%)	0 (0%)	0.100
No	41 (62.1%)	16 (24.2%)	

Missing ligand effect for inhibitory KIR on clinical outcome

The contributions of specific absence of HLA ligand for KIR receptor effect on clinical outcome were shown in table 17-20. This study investigated the patients with lacking of ligands for inhibitory KIR3DL2, KIR3DL1 and KIR2DL1. We compared the effect on clinical outcome between absence and presence of HLA ligand for individual inhibitory KIR receptor. Both of absence of ligand for KIR3DL1 and KIR3DL2 had trend to reach the statistical significance for reducing relapse incidence (Table 17). The trend to reach statistically significance for reducing chronic GVHD was also found in patients with absent ligand for KIR3DL2.

Table 17. Missing ligand effect for inhibitory KIR on relapse

Inhibitory KIR	Relapse	No relapse	p-value
Inhibitory KIR3DL2 (n=66)			
Absence of A11 ligand to KIR3DL2	7 (10.6%)	27 (40.9%)	0.077
Present of A11 ligand to KIR3DL2	13 (19.7%)	19 (28.8%)	
Inhibitory KIR3DL1 (n=66)			
Absence of Bw4 ligand to KIR3DL1	5 (7.6%)	22 (33.3%)	0.083
Present of Bw4 ligand to KIR3DL1	15 (22.7%)	24 (36.4%)	

Inhibitory KIR	Relapse	No relapse	p-value
Inhibitory KIR2DL1 (n=66)			
Absence of C2 ligand to KIR2DL1	11 (16.7%)	29 (43.9%)	0.539
Present of C2 ligand to KIR2DL1	9 (13.6%)	17 (25.8%)	
Inhibitory KIR2DL2/3 (n=66)			
Absence of C1 ligand to KIR2DL2/3	1 (1.5%)	2 (3.0%)	0.907
Present of C1 ligand to KIR2DL2/3	19 (28.8%)	44 (66.7%)	

Table 18. Missing ligand effect for inhibitory KIR on aGVHD

Inhibitory KIR	Acute GVHD	No acute GVHD	p-value
Inhibitory KIR3DL2 (n=66)			
Absence of A11 ligand to KIR3DL2	5 (7.6%)	29 (43.9%)	0.183
Present of A11 ligand to KIR3DL2	9 (13.6%)	23 (34.8%)	
Inhibitory KIR3DL1 (n=66)			
Absence of Bw4 ligand to KIR3DL1	4 (6.1%)	23 (34.8%)	0.290
Present of Bw4 ligand to KIR3DL1	10 (15.2%)	29 (43.8%)	
Inhibitory KIR2DL1 (n=66)			
Absence of C2 ligand to KIR2DL1	8 (12.1%)	32 (48.5%)	0.765
Present of C2 ligand to KIR2DL1	6 (9.1%)	20 (30.3%)	
Inhibitory KIR2DL2/3 (n=66)			
Absence of C1 ligand to KIR2DL2/3	1 (1.5%)	2 (3.0%)	0.517
Present of C1 ligand to KIR2DL2/3	13 (19.7%)	50 (75.8%)	

Table 19. Missing ligand effect for inhibitory KIR on cGVHD

Inhibitory KIR	Chronic GVHD	No chronic GVHD	p-value
Inhibitory KIR3DL2 (n=66)			
Absence of A11 ligand to KIR3DL2	7 (10.6%)	27 (40.9%)	0.077
Present of A11 ligand to KIR3DL2	13 (19.7%)	19 (28.8%)	

Inhibitory KIR	Chronic GVHD	No chronic GVHD	p-value
Inhibitory KIR3DL1 (n=66)			
Absence of Bw4 ligand to KIR3DL1	8 (12.1%)	19 (28.8%)	0.921
Present of Bw4 ligand to KIR3DL1	12 (18.2%)	27 (40.9%)	
Inhibitory KIR2DL1 (n=66)			
Absence of C2 ligand to KIR2DL1	14 (21.2%)	26 (39.4%)	0.303
Present of C2 ligand to KIR2DL1	6 (9.1%)	20 (30.3%)	
Inhibitory KIR2DL2/3 (n=66)			
Absence of C1 ligand to KIR2DL2/3	1 (1.5%)	2 (3.0%)	0.907
Present of C1 ligand to KIR2DL2/3	19 (28.8%)	44 (66.7%)	

Table 20. Missing ligand effect for inhibitory KIR on survival

Inhibitory KIR	Alive	Dead	p-value
Inhibitory KIR3DL2 (n=66)			
Absence of A11 ligand to KIR3DL2	28 (42.4%)	6 (9.1%)	0.197
Present of A11 ligand to KIR3DL2	22 (33.3%)	10 (15.2%)	
Inhibitory KIR3DL1 (n=66)			
Absence of Bw4 ligand to KIR3DL1	22 (33.3%)	5 (7.6%)	0.367
Present of Bw4 ligand to KIR3DL1	28 (42.4%)	11 (16.7%)	
Inhibitory KIR2DL1 (n=66)			
Absence of C2 ligand to KIR2DL1	32 (48.5%)	8 (12.1%)	0.319
Present of C2 ligand to KIR2DL1	18 (27.3%)	8 (12.1%)	
Inhibitory KIR2DL2/3 (n=66)			
Absence of C1 ligand to KIR2DL2/3	3 (4.5%)	0 (0%)	0.316
Present of C1 ligand to KIR2DL2/3	47 (71.2%)	16 (24.2%)	

Missing Ligand Effect on Clinical Outcome in Specific Cancer Type

We analyzed the missing KIR ligand effect on clinical outcome in different types of hematological malignancy diseases. There were AML, ALL and CML patients. First, we compared the effect of one or more than one missing KIR ligand with no missing

KIR ligand on the patient outcome. In each type of disease, one or more missing KIR ligand was not significantly associated with any clinical outcome. However, the AML patients with 2 or more than 2 missing KIR ligand had significantly lower relapse (p-value=0.033) (Figure 19) and higher survival (p-value=0.018) (Figure 20), but it did not affect the relapse and overall survival in ALL and CML patients (Figure 21-24).

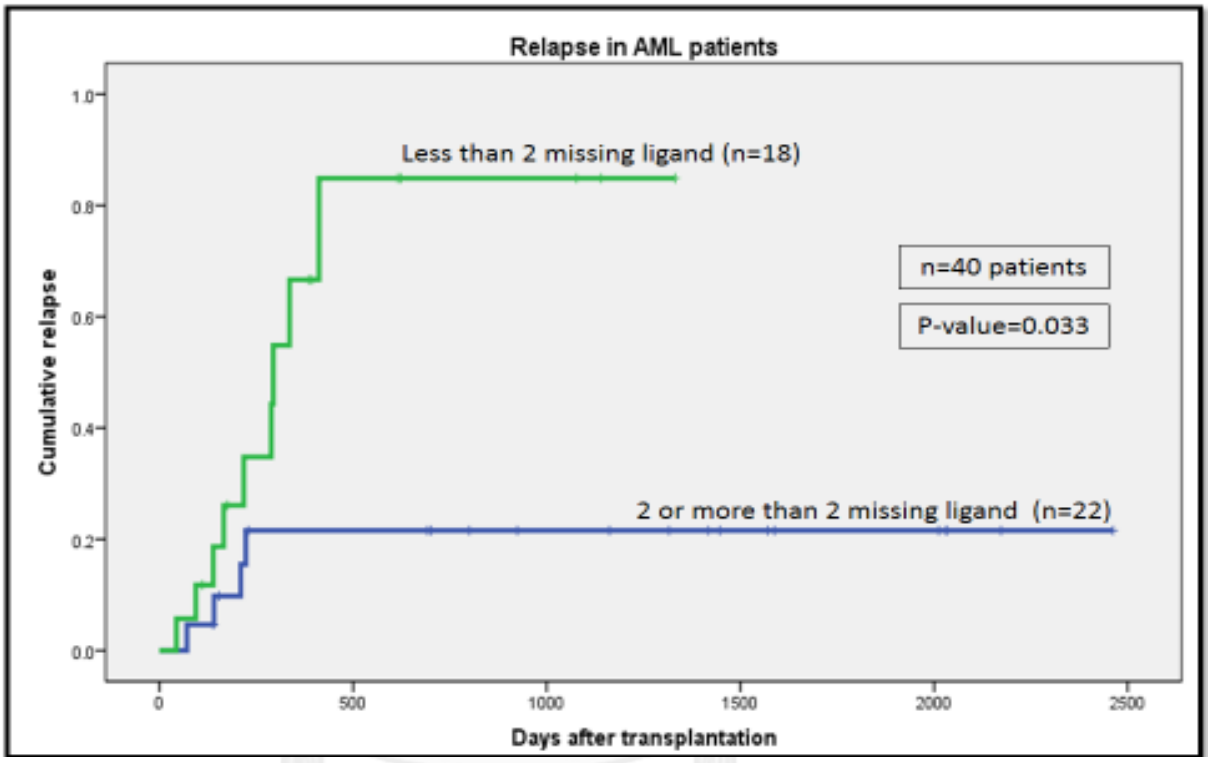

Figure 19. Relapse in AML patients with 2 or more than 2 missing KIR ligands compared to the patients with less than 2 missing KIR ligands.

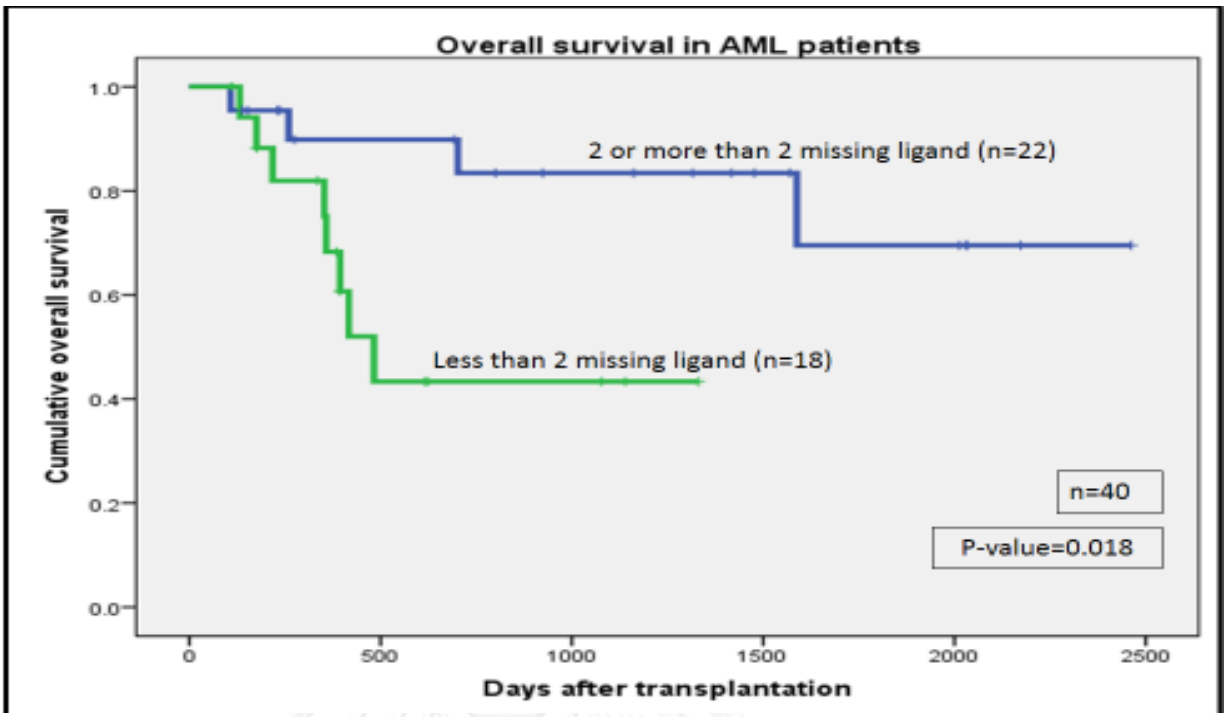

Figure 20. Overall survival in AML patients with 2 or more than 2 missing KIR ligands compared to the patients with less than 2 missing KIR ligands.

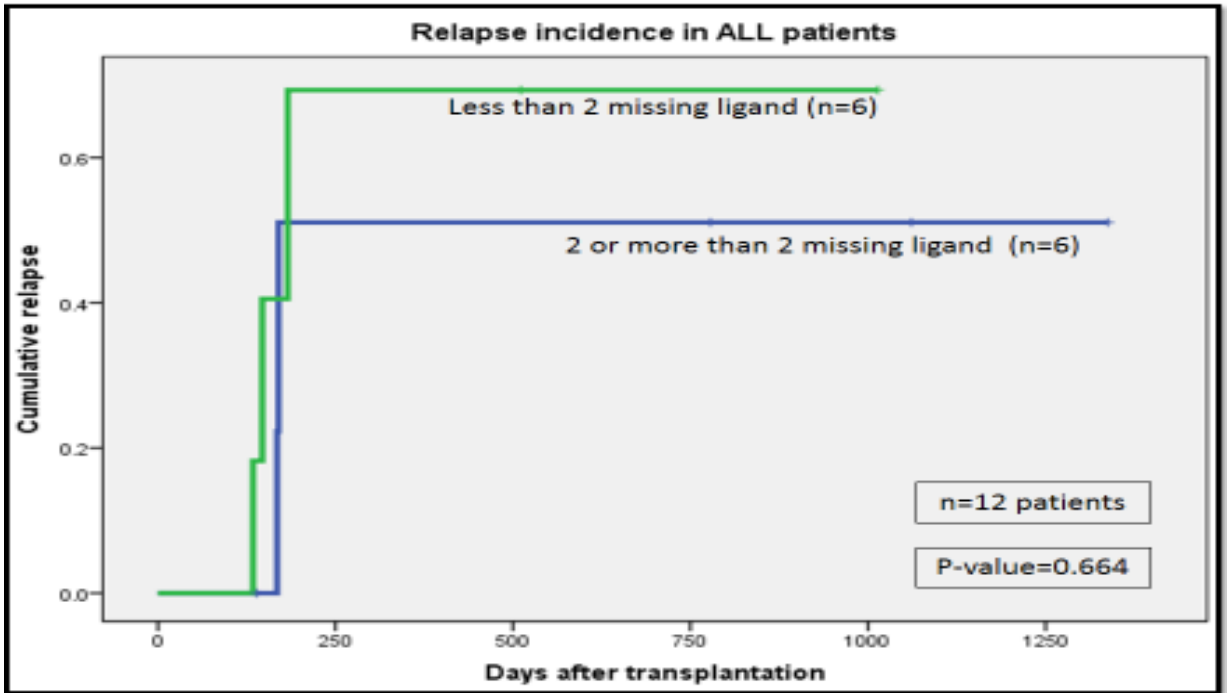

Figure 21. Relapse in ALL patients with 2 or more than 2 missing KIR ligands compared to the patients with less than 2 missing KIR ligands.

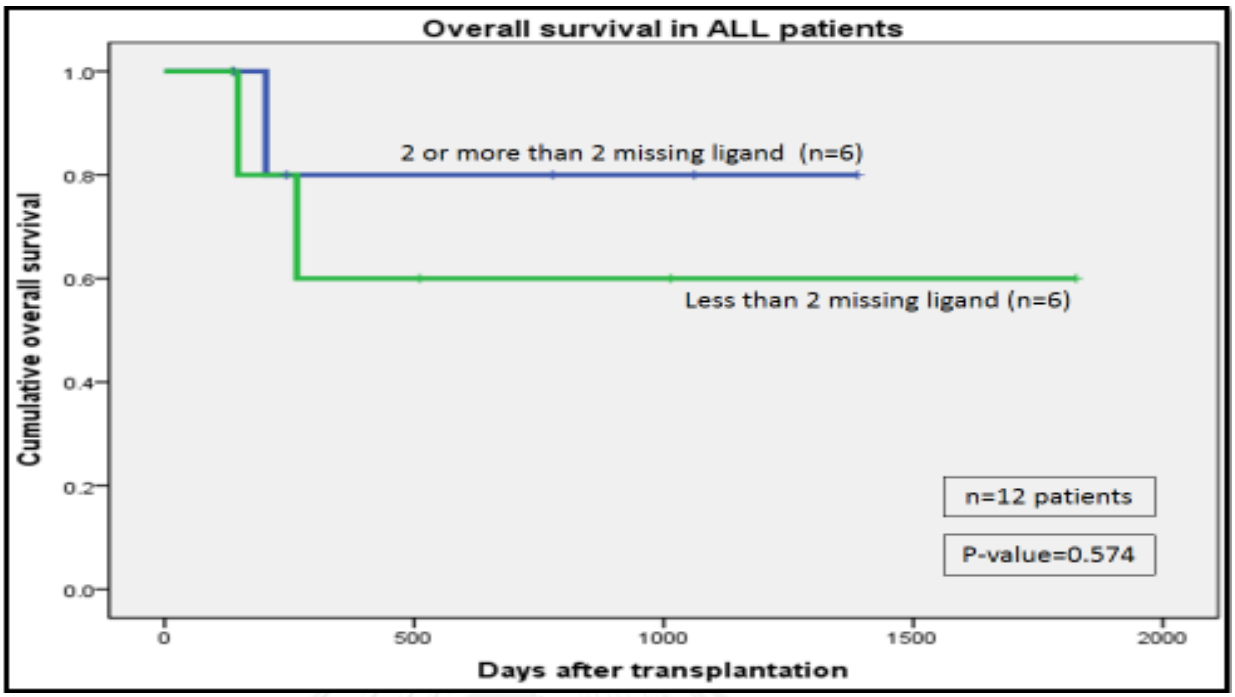

Figure 22. Overall survival in ALL patients with 2 or more than 2 missing KIR ligands compared to the patients with less than 2 missing KIR ligands.

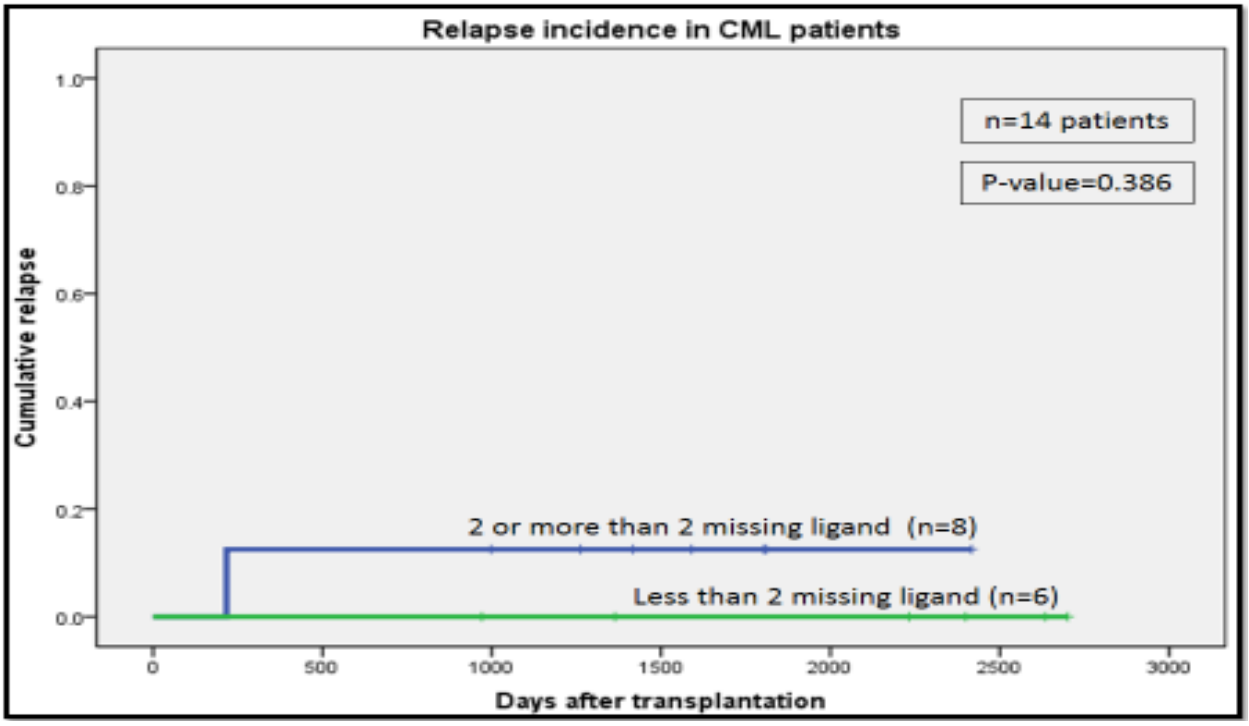

Figure 23. Relapse in CML patients with 2 or more than 2 missing KIR ligands compared to the patients with less than 2 missing KIR ligands.

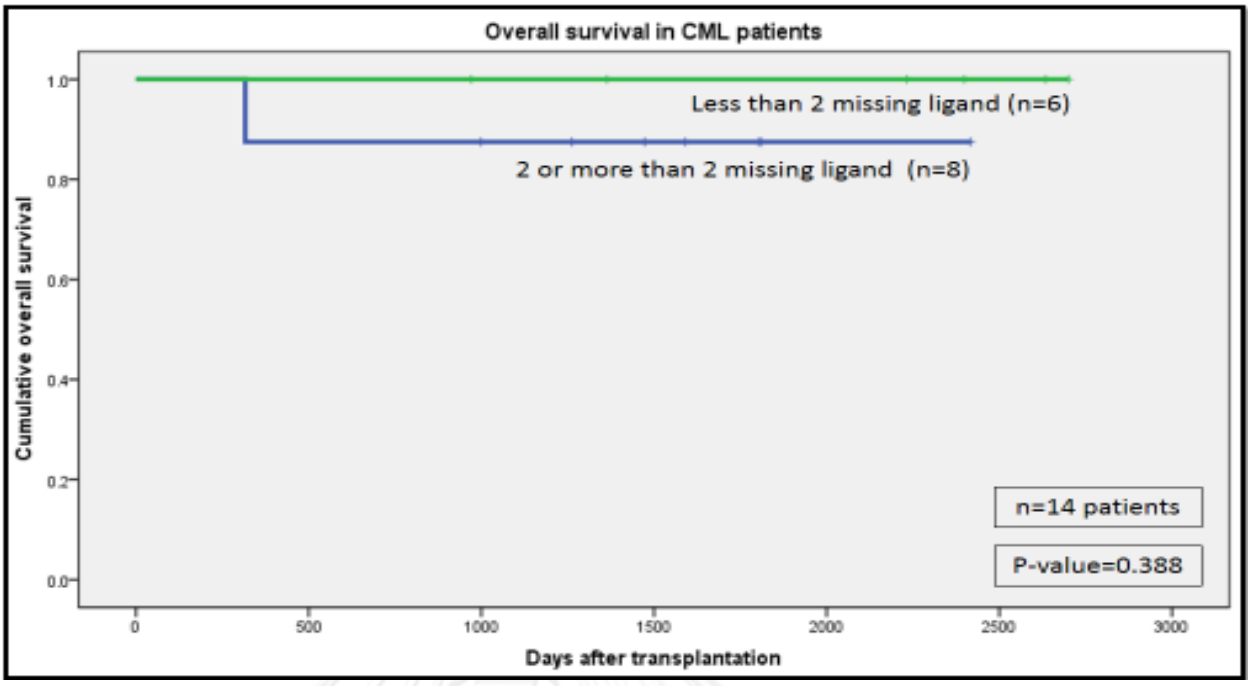

Figure 24. Overall survival in CML patients with 2 or more than 2 missing KIR ligands compared to the patients with less than 2 missing KIR ligands.

We compared the effect of 2 or more than 2 missing KIR ligand on acute GVHD in patients with different disease groups. Interestingly, the CML Patients with 2 or more missing KIR ligands had lower acute GVHD than other group (p=0.028) (Figure 25). On the contrary, the ALL and AML patients with 2 or more missing KIR ligands had trend of decreasing acute GVHD (Figure 26-27).

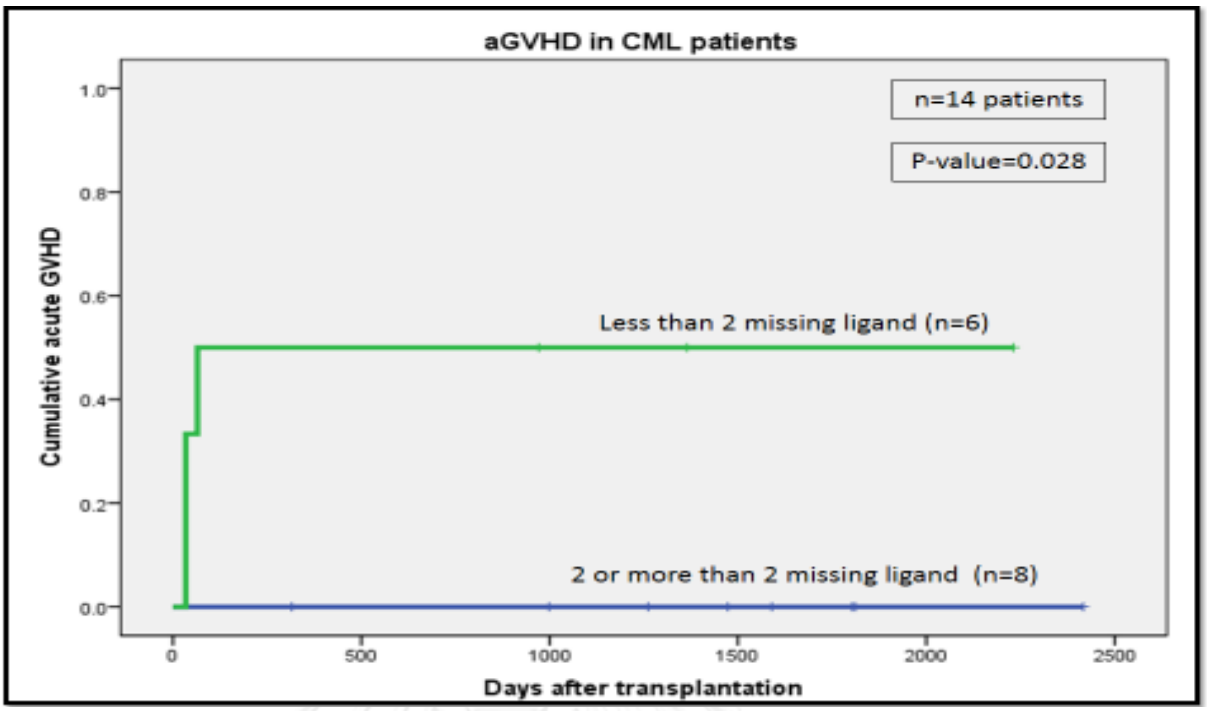

Figure 25. Acute GVHD in CML patients with 2 or more than 2 missing KIR ligands compared to the patients with less than 2 missing KIR ligands.

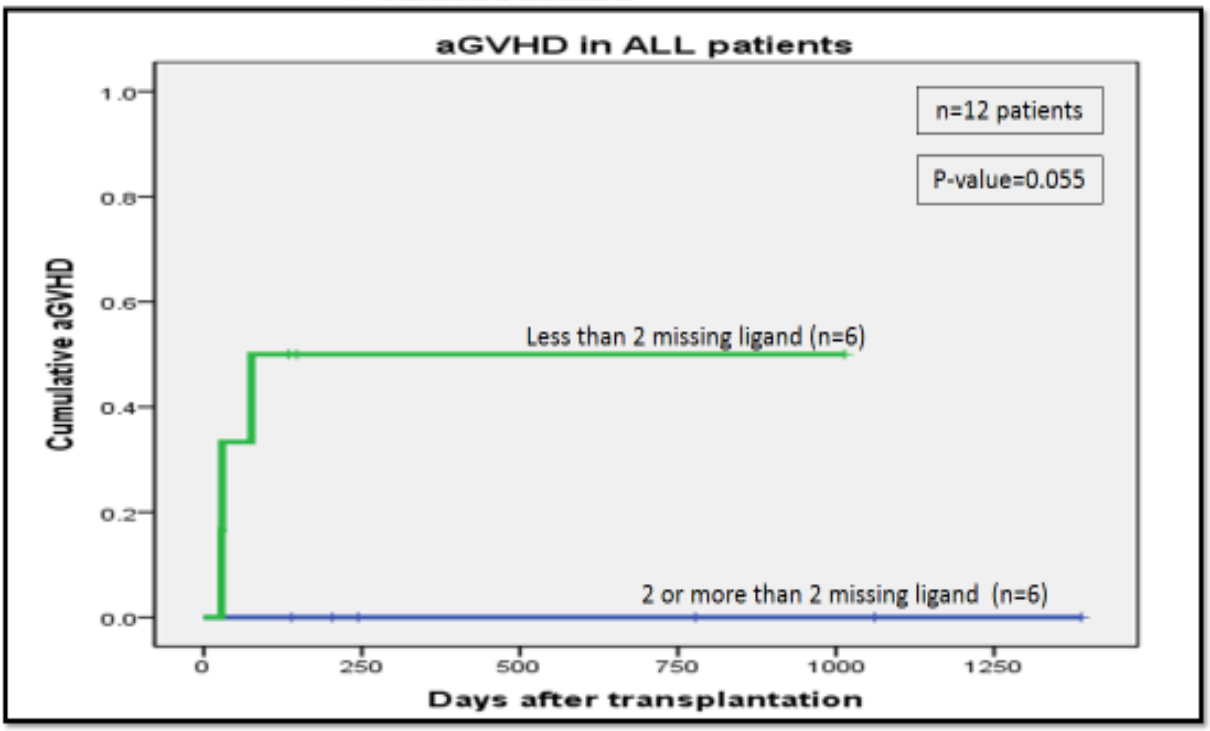

Figure 26. Acute GVHD in ALL patients with 2 or more than 2 missing KIR ligand compared to the patients with less than 2 missing KIR ligands.

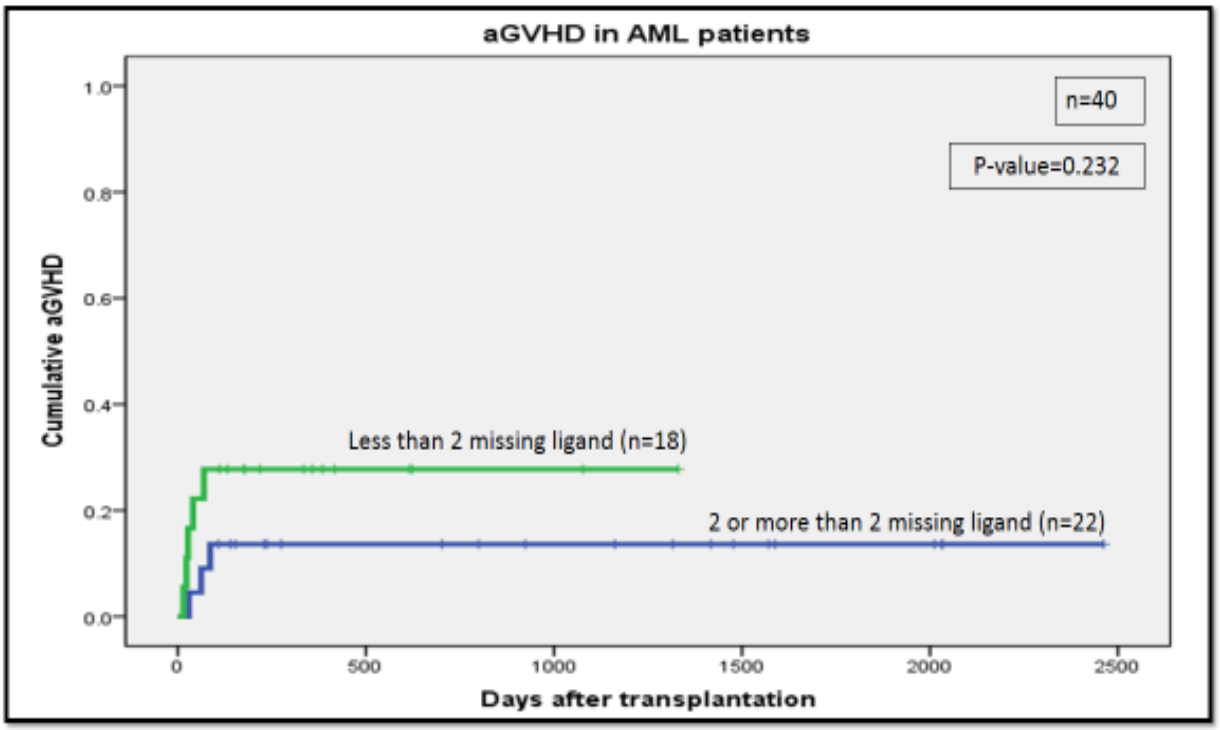

Figure 27. Acute GVHD in AML patients with 2 or more than 2 missing KIR ligand compared to the patients with less than 2 missing KIR ligands.

KIR Haplotype Effect on Clinical Outcome

In this study, donors with haplotype AA group were found in 26 patients (39.4%) and haplotype Bx group were found in 40 patients (60.6%). We analyzed the effect of donor KIR haplotype by comparing haplotype AA and haplotype Bx with various clinical outcomes. Our analysis showed no effect on relapse, aGVHD, cGVHD, and survival (Table 21).

Table 21. The effect of donor KIR haplotype on transplant outcomes

Donor KIR haplotype (n=66)	Relapse	No relapse	p-value
KIR haplotype AA (n=26)	9 (13.6%)	17 (25.8%)	0.539
KIR haplotype Bx (n=40)	11 (16.7%)	29 (43.9%)	
Donor KIR haplotype (n=66)	**Acute GVHD**	**No acute GVHD**	**p-value**
KIR haplotype AA (n=26)	6 (9.1%)	20 (30.3%)	0.765
KIR haplotype Bx (n=40)	8 (12.1%)	32 (48.5%)	

Donor KIR haplotype (n=66)	Chronic GVHD	No chronic GVHD	p-value
KIR haplotype AA (n=26)	9 (13.6%)	17 (25.8%)	0.539
KIR haplotype Bx (n=40)	11 (16.7%)	29 (43.9%)	
Donor KIR haplotype (n=66)	**Alive**	**Death**	**p-value**
KIR haplotype AA (n=26)	19 (28.8%)	7 (10.6%)	0.682
KIR haplotype Bx (n=40)	31 (47.0%)	9 (13.6%)	

Next, we analyzed the effect of KIR haplotype on clinical outcome in AML patients. The data also showed no significant association with any outcomes (Table 22).

Table 22. The donor KIR haplotype in AML patients on transplant outcomes

Donor KIR haplotype (n=40)	Relapse	No relapse	p-value
KIR haplotype AA (n=16)	5 (12.5%)	11 (27.5%)	0.890
KIR haplotype Bx (n=24)	8 (20.0%)	16 (40.0%)	
Donor KIR haplotype (n=40)	**Acute GVHD**	**No acute GVHD**	**p-value**
KIR haplotype AA (n=16)	4 (10.0%)	12 (30.0%)	0.519
KIR haplotype Bx (n=24)	4 (10.0%)	20 (50.0%)	
Donor KIR haplotype (n=40)	**Chronic GVHD**	**No chronic GVHD**	**p-value**
KIR haplotype AA (n=16)	4 (10.0%)	12 (30.0%)	0.757
KIR haplotype Bx (n=24)	5 (12.5%)	19 (47.5%)	
Donor KIR haplotype (n=40)	**Alive**	**Death**	**p-value**
KIR haplotype AA (n=16)	11 (27.5%)	5 (12.5%)	0.888
KIR haplotype Bx (n=24)	17 (42.5%)	7 (17.5%)	

The Activating KIR Effect on Clinical Outcome

The following activating KIRs were analyzed including KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5 and KIR3DS1. There was no difference between positive and negative presence of donor activating KIR genes on transplant outcomes (Table 23-26).

Table 23. Activating KIR Effect on relapse

Activating KIR	Relapse	No relapse	p-value
KIR2DS1 (n=66)			
Positive	8 (12.1%)	23 (34.8%)	0.454
Negative	12 (18.2%)	23 (34.8%)	
KIR2DS2 (n=66)			
Positive	7 (10.6%)	18 (27.3%)	0.751
Negative	13 (19.7%)	28 (42.4%)	
KIR2DS3 (n=66)			
Positive	4 (6.1%)	9 (13.6%)	0.967
Negative	16 (24.2%)	37 (56.1%)	
KIR2DS4 (n=66)			
Positive	19 (28.8%)	41 (62.1%)	0.446
Negative	1 (1.5%)	5 (7.6%)	
KIR2DS5 (n=66)			
Positive	7 (10.6%)	17 (25.8%)	0.879
Negative	13 (19.7%)	29 (43.9%)	
KIR3DS1 (n=66)			
Positive	7 (10.6%)	18 (27.3%)	0.751
Negative	13 (19.7%)	28 (42.4%)	

Table 24. Activating KIR Effect on on aGVHD

Activating KIR	Acute GVHD	No acute GVHD	p-value
KIR2DS1 (n=66)			
Positive	7 (10.6%)	24 (36.4%)	0.798
Negative	7 (10.6%)	28 (42.4%)	
KIR2DS2 (n=66)			
Positive	4 (6.1%)	21 (31.8%)	0.419
Negative	10 (15.2%)	31 (47.0%)	
KIR2DS3 (n=66)			
Positive	1 (1.5%)	12 (18.2%)	0.183
Negative	13 (19.7%)	40 (60.6%)	
KIR2DS4 (n=66)			
Positive	14 (21.2%)	46 (69.7%)	0.183
Negative	0 (0%)	6 (9.1%)	
KIR2DS5 (n=66)			
Positive	6 (9.1%)	18 (27.3%)	0.569
Negative	8 (12.1%)	34 (51.5%)	
KIR3DS1 (n=66)			
Positive	7 (10.6%)	18 (27.3%)	0.292
Negative	7 (10.6%)	34 (51.5%)	

Table 25. Activating KIR Effect on cGVHD

Activating KIR	Chronic GVHD	No chronic GVHD	p-value
KIR2DS1 (n=66)			
Positive	10 (15.2%)	21 (31.8%)	0.745
Negative	10 (15.2%)	25 (37.9%)	
KIR2DS2 (n=66)			
Positive	6 (9.1%)	19 (28.8%)	0.384
Negative	14 (21.2%)	27 (40.9%)	
KIR2DS3 (n=66)			
Positive	3 (4.5%)	10 (15.2%)	0.527
Negative	17 (25.8%)	36 (54.5%)	

Activating KIR	Chronic GVHD	No chronic GVHD	p-value
KIR2DS4 (n=66)			
Positive	20 (30.3%)	40 (60.6%)	0.167
Negative	0 (0%)	6 (9.1%)	
KIR2DS5 (n=66)			
Positive	7 (10.6%)	17 (25.8%)	0.879
Negative	13 (19.7%)	29 (43.8%)	
KIR3DS1 (n=66)			
Positive	7 (10.6%)	18 (27.3%)	0.751
Negative	13 (19.7%)	28 (42.4%)	

Table 26. Activating KIR Effect on survival

Activating KIR	Alive	Dead	p-value
KIR2DS1 (n=66)			
Positive	24 (36.4%)	7 (10.6%)	0.767
Negative	26 (39.4%)	9 (13.6%)	
KIR2DS2 (n=66)			
Positive	20 (30.3%)	5 (7.6%)	0.530
Negative	30 (45.5%)	11 (16.7%)	
KIR2DS3 (n=66)			
Positive	9 (13.6%)	4 (6.1%)	0.540
Negative	41 (62.1%)	12 (18.2%)	
KIR2DS4 (n=66)			
Positive	44 (66.7%)	16 (24.2%)	0.146
Negative	6 (9.1%)	0 (0%)	
KIR2DS5 (n=66)			
Positive	19 (28.8%)	5 (7.6%)	0.625
Negative	31 (47.0%)	11 (16.7%)	
KIR3DS1 (n=66)			
Positive	19 (28.8%)	6 (9.1%)	0.971
Negative	31 (47.0%)	10 (15.2%)	

This study also analyzed 31 donor-patient pairs with KIR2DS1 and HLA-C genotypes for their association with clinical outcomes. HLA-C2 is the ligand for activating KIR2DS1 and controlled NK cell to activation. From previous investigation, patients who were homozygous or heterozygous HLA-C1 antigens and received donor KIR2DS1 gene had better outcome in AML [89]. However, their interaction was not associated with any outcome in our study. When analyzed only in patients with AML, we could not observe any advantages either (Table 24-25)

Table 27. The KIR2DS1 and HLA-C1 ligand on transplant outcomes

KIR2DS1 and HLA-C ligand (n=31)	Relapse	No relapse	p-value
KIR2DS1 with HLA-C1 or C1/C2 (n=29)	7 (22.6%)	22 (71.0%)	0.419
KIR2DS1 with HLA-C2 (n=2)	1 (3.2%)	1 (3.2%)	
KIR2DS1 and HLA-C ligand (n=31)	**Acute GVHD**	**No acute GVHD**	**p-value**
KIR2DS1 with HLA-C1 or C1/C2 (n=29)	7(22.6%)	22(71.0%)	0.430
KIR2DS1 with HLA-C2 (n=2)	0 (0%)	2(6.5%)	
KIR2DS1 and HLA-C ligand (n=31)	**Chronic GVHD**	**No chronic GVHD**	**p-value**
KIR2DS1 with HLA-C1 or C1/C2 (n=29)	10 (32.3%)	19 (61.3%)	0.313
KIR2DS1 with HLA-C2 (n=2)	0 (0%)	2 (6.5%)	
KIR2DS1 and HLA-C ligand (n=31)	**Alive**	**Death**	**p-value**
KIR2DS1 with HLA-C1 or C1/C2 (n=29)	23 (74.2%)	6 (19.4%)	0.406
KIR2DS1 with HLA-C2 (n=2)	1 (3.2%)	1 (3.2%)	

Table 28. AML Patients with KIR2DS1 and HLA-C1 ligand on transplant outcomes

KIR2DS1 and HLA-C ligand (n=19)	Relapse	No relapse	p-value
KIR2DS1 with HLA-C1 or C1/C2 (n=17)	5 (26.3%)	12 (63.2%)	0.554
KIR2DS1 with HLA-C2 (n=2)	1 (5.3%)	1 (5.3%)	
KIR2DS1 and HLA-C ligand (n=19)	**Acute GVHD**	**No acute GVHD**	**p-value**
KIR2DS1 with HLA-C1 or C1/C2 (n=17)	3 (15.8%)	14 (73.7%)	0.517

KIR2DS1 with HLA-C2 (n=2)	0 (0%)	2 (10.5%)	
KIR2DS1 and HLA-C ligand (n=19)	**Chronic GVHD**	**No chronic GVHD**	**p-value**
KIR2DS1 with HLA-C1 or C1/C2 (n=17)KIR2DS1 with HLA-C2 (n=2)	5 (26.3%) 0 (0%)	12 (63.2%) 2 (10.5%)	0.591
KIR2DS1 and HLA-C ligand (n=19)	**Alive**	**Death**	**p-value**
KIR2DS1 with HLA-C1 or C1/C2 (n=17) KIR2DS1 with HLA-C2 (n=2)	12 (63.2%) 1 (5.3%)	5 (26.3%) 1 (5.3%)	0.554

In addition, 19 donor-patient pairs with KIR2DS2 and HLA-C genotypes were analyzed for their association with clinical outcomes. KIR2DS2 is an activating receptor for NK activation, which its interactive ligand is HLA-C group 1. Similar to previous report [48], activating KIR and HLA-C gene were determined for interaction between KIR2DS2 with HLA-C group 1. Patients who had HLA-C1 group did not have beneficial effect from donor KIR2DS2-positive allogragts (Table 29-30). HSCT performed for acute myeloid leukemia patients who were homozygous HLA-C1 or heterozygous HLA-C1 had no advantage in clinical outcome.

Table 29. The KIR2DS2 and HLA-C ligand on transplant outcomes

KIR2DS2 and HLA-C ligand (n=25)	**Relapse**	**No relapse**	**p-value**
KIR2DS2 with HLA-C1 or C1/C2 (n=24) KIR2DS2 with HLA-C2 (n=1)	7 (28.0%) 0 (0%)	17 (68.0%) 1 (4.0%)	0.524
KIR2DS2 and HLA-C ligand (n=25)	**Acute GVHD**	**No acute GVHD**	**p-value**
KIR2DS2 with HLA-C1 or C1/C2 (n=24) KIR2DS2 with HLA-C2 (n=1)	4 (16.0%) 0 (0%)	20 (80.0%) 1 (4.0%)	0.656
KIR2DS2 and HLA-C ligand (n=25)	**Chronic GVHD**	**No chronic GVHD**	**p-value**
KIR2DS2 with HLA-C1 or C1/C2 (n=24) KIR2DS2 with HLA-C2 (n=1)	6 (24.0%) 0 (0%)	18 (72.0%) 1 (4.0%)	0.566
KIR2DS2 and HLA-C ligand (n=25)	**Alive**	**Death**	**p-value**
KIR2DS2 with HLA-C1 or C1/C2 (n=24) KIR2DS2 with HLA-C2 (n=1)	19 (76.0%) 1 (4.0%)	5 (20%) 0 (0%)	0.610

Table 30. AML Patients with KIR2DS2 and HLA-C ligand on transplant outcomes

KIR2DS2 and HLA-C ligand (n=15)	**Relapse**	**No relapse**	**p-value**
KIR2DS2 with HLA-C1 or C1/C2 (n=14)	5 (33.3%)	9 (60.0%)	0.464
KIR2DS2 with HLA-C2 (n=1)	0 (0%)	1 (6.7%)	
KIR2DS2 and HLA-C ligand (n=15)	**Acute GVHD**	**No acute GVHD**	**p-value**
KIR2DS2 with HLA-C1 or C1/C2 (n=14)	3 (20.0%)	11 (73.3%)	0.605
KIR2DS2 with HLA-C2 (n=1)	0 (0%)	1 (6.7%)	
KIR2DS2 and HLA-C ligand (n=15)	**Chronic GVHD**	**No chronic GVHD**	**p-value**
KIR2DS2 with HLA-C1 or C1/C2 (n=14)	2 (13.3%)	12 (80.0%)	0.685
KIR2DS2 with HLA-C2 (n=1)	0 (0%)	1 (6.7%)	
KIR2DS2 and HLA-C ligand (n=15)	**Alive**	**Death**	**p-value**
KIR2DS2 with HLA-C1 or C1/C2 (n=14)	10 (66.7%)	4 (26.7%)	0.533
KIR2DS2 with HLA-C2 (n=1)	1 (6.7%)	0 (0%)	

CHAPTER V
DISCUSSION

The KIR genes have been proposed to help predict inhibitory KIR-driven donor NK alloreactivity in the clinical situation besides HLA compatibility between donors and patients. Since KIR and HLA genotypes segregate independently, the possibility of NK cells with KIR receptor have no HLA ligand exist. This study was designed to see the impact of the missing KIR ligand on various clinical outcomes in the Thai HLA-identical sibling transplantation.

First, our study determined the frequencies of HLA ligand, killer cell Immunoglobulin-like receptors (KIRs) and missing KIR ligands, we compared our result to previous reports. The distribution of KIR gene frequencies in donors was mostly similar to previous results in Thai populations (as shown in Table 31) [90]. If compared with other ethnic groups, the frequencies of inhibitory KIR2DL1, KIR2DL2 and KIR2DL3 gene and activating KIR2DS2, KIR2DS3 and KIR3DS1 were significantly different among other population from the present Thai population (Table 31-32) [91-94].

Table 31. The distribution of inhibitory KIR genes of Thai population and other populations

Inhibitory KIR genotype	Thai		Chinese	Vietnamese	Caucasian	African
	Present study (n=66)	Tammakorn et al.,2011 (n=500)	Jiang et al.,2005 (n=104)	Toneva et al.,2001 (n=59)	Clausen et al.,2010 (n=100)	Norman et al.,2002 (n=62)
KIR2DL1, n (%)	63 (95.5%)	492 (98.4%)	103 (99%)	57 (98%)	97 (97%)	49 (79%)[a]
KIR2DL2, n (%)	26 (39.4%)	187 (37.4%)	18 (17.3%)[b]	26 (45%)	55 (55%)[c]	32 (52%)
KIR2DL3, n (%)	62 (93.9%)	483 (96.6%)	103 (99%)	39 (66%)[d]	91 (91%)	52 (85%)

Inhibitory KIR genotype	Thai		Chinese	Vietnamese	Caucasian	African
	Present study (n=66)	Tammakorn et al.,2011 (n=500)	Jiang et al.,2005 (n=104)	Toneva et al.,2001 (n=59)	Clausen et al.,2010 (n=100)	Norman et al.,2002 (n=62)
KIR3DL1, n (%)	63 (95.5%)	463 (92.6%)	98 (94.2%)	52 (88%)	91 (91%)	60 (98%)
KIR3DL2, n (%)	66 (100%)	500 (100%)	104 (100%)	59 (100%)	100 (100%)	62 (100%)

The inhibitory KIR were compared to the present study by Chi-square test; [a]p=0.0006, [b]p=0.0009, [c]p=0.034, [d]p=0.0001

Table 32. The distribution of activating KIR genes of Thai population and other populations

Activating KIR genotype	Thai		Chinese	Vietnamese	Caucasian	African
	Present study (n=66)	Tammakorn et al (n=500)	Jiang et al.,2005 (n=104)	Toneva et al.,2001 (n=59)	Clausen et al.,2009 (n=100)	Norman et al.,2002 (n=62)
KIR2DS1, n (%)	31 (46.9%)	213 (42.6%)	35 (33.7%)	22 (37%)	35 (35%)	14 (23%)
KIR2DS2, n (%)	25 (37.9%)	188 (37.6%)	18 (17.3%)[a]	24 (41%)	56 (56%)[b]	28 (45%)
KIR2DS3, n (%)	13 (19.7%)	152 (30.4%)	13 (12.5%)	20 (34%)[c]	30 (30%)	11 (19%)
KIR2DS4, n (%)	60 (90.9%)	463 (92.6%)	84 (80.7%)	52 (88%)	84 (84%)	60 (97%)
KIR2DS5, n (%)	24 (36.4%)	155 (31%)	24 (23%)	ND	30 (30%)	14 (24%)
KIR3DS1, n (%)	25 (37.9%)	208 (41.6%)	34 (32.8%)	24 (41%)	38 (38%)	8 (13%)[d]

The activating KIR were compared to the present study by Chi-square test; [a]p=0.0015, [b]p=0.016, [c]p=0.038, [d]p=0.0001 ; ND = no data

When we determined the missing KIR ligand, It is important to note that nearly 90% of the Thai patients possessed missing KIR ligands similar to previous report [1]. There seem to be less missing KIR ligands in the American and the Caucasian studies

(Table 30) [1, 61, 70, 95]. However, it should be noted that the reports in American and the Caucasian did not include the analysis of HLA-A11 ligand.

Table 33. The frequencies of missing KIR ligands in Thai population and other populations

KIR genotype	Thai		American[a]	Caucasian[b]	Asian
	Present study (n=66)	Wongwuttisaroj et al (n=74)	Hu et al.,2005 (n=178)	Clausen et al.,2007 (n=35)	Linn et al.,2010 (n=151)
Missing KIR ligand, n (%)	58 (87.9%)	60 (81.1%)	112 (62.9%)	21 (60%)	122 (81%)
No missing ligand, n (%)	8 (12.1%)	14 (18.9%)	66 (37.1%)	14 (40%)	29 (19%)

The missing KIR ligand were compared to the present study by Chi-square test; [a]p=0.0001, [b]p=0.0001

As expected, the specific KIR ligand and missing KIR ligand frequencies from our study were mostly similar to previous results in Thai populations, but there were more different from Caucasian and Chinese populations (Table 34, 35) [1, 84, 93] [61, 82, 96].

Table 34. The frequencies of KIR-ligands in Thai population and other populations

KIR-ligand	Thai		Caucasian	Chinese
	Present study (n=66)	Wongwuttisaroj et al.,2012 (n=74)	Clausen et al.,2010 (n=100)	Wang et al.,2013 (n=52)
HLA-Bw4, n (%)	37 (48.5%)	36 (49%)	74 (74%)[a]	ND
Homozygote HLA-C1, n (%)	41 (62.1%)	45 (60.8%)	40 (40%)[b]	25 (48%)[c]
Homozygote HLA-C2, n (%)	3 (4.5%)	0	13 (13%)	3 (5.7%)
Heterozygote HLA-C1/C2, n (%)	22 (33.3%)	29 (39.2%)	47 (47%)	24 (46.1%)

The KIR-ligand were compared to the present study by Chi-square test; [a]p=0.0005, [b]p=0.003, [c]p=0.0001 ; ND = no data

Table 35. The frequencies of absent KIR ligand in Thai population and other populations

KIR ligand absent	Thai	American	Japanese	Caucasian
	Recent study (n=66)	Hu et al.,2005 (n=112)	Hu et al.,2006 (n=568)	Bjorklund et al.,2010 (n=67)
HLA-C group 1 absent for donor KIR2DL2/3, n (%)	3 (4.5%)	27 (24.1%)[a]	2 (0.37%)	14 (20.9%)[b]
HLA-C group 2 absent for donor KIR2DL1, n (%)	40 (60.6%)	31 (27.7%)[c]	319 (56%)	17 (25.4%)[d]
HLA-Bw4 absent for donor KIR3DL1, n (%)	27 (40.9%)	19 (17.0%)[e]	33 (6%)[f]	9 (13.4%)[g]
HLA-Bw4 and HLA-C absent for donor KIR, n (%)	7 (10.6%)	35 (31.3%)[h]	173 (30%)[i]	27 (40.3%)[j]

The absent KIR ligand were compared to the present study by Chi-square test; [a]p=0.0003, [b]p=0.0016, [c]p=0.0001, [d]p=0.0001, [e]p=0.0003, [f]p=0.0001, [g]p=0.0001, [h]p=0.001, [i]p=0.0016, [j]p=0.0001

Next, we analyzed missing KIR ligand, activating KIR genotypes, and KIR haplotypes to each clinical outcome. Although, previous study in Thai population reported the positive association between the activating KIR2DS5 with decreased acute GVHD in HLA identical transplantation [1], there was no association between any particular activating KIR genotypes or haplotypes with any clinical outcomes in our study. However, it should be noted that both Thai studies had limited sample sizes, and there also had different disease distributions and transplant protocols (Table 36).

Table 36. The characteristics in Both Thai Studies

characteristics	Wongwuttisaroj et al.,2012	Present study
Sample size	51 patients	66 patients
Condition regimen		
TBI-based[1]	5.4%	66.7%
Busulfan-based[2]	89.2%	33.3%
Unknown	5.4%	ND
Patient age ,median(range)	37.85(17-57)	35(15-62)

characteristics	Wongwuttisaroj et al.,2012	Present study
Patient/donor sex		
M/M	27.0%	25.8%
M/F	32.4%	21.2%
F/F[3]	16.2%	31.8%
F/M	24.3%	21.2%
Diagnosis		
AML[4]	33.8%	60.6%
ALL	9.5%	18.2%
CML[5]	56.8%	21.2%
Clinical outcome		
Relapse	19.6%	30.8%
aGVHD	27.45%	21.5%
cGVHD[6]	49.01%	31.3%

The characteristic factors were compared between both Thais study by Chi-square test; [1]p=0.0001, [2]p=0.0001, [3]p=0.013, [4]p=0.0002, [5]p=0.0001, [6]p=0.014 ; ND = no data

We also could not detect any protective role of KIR2DS1 in association with HLA-C1, which had been reported previously in AML [89]. One of a reason might be due to a small comparative sample size of homozygous group 2 HLA-C in our study.

Interestingly, our study can clearly demonstrated the significant association between the 2 or more than 2 missing KIR ligands with decrease in relapse and acute GVHD. If sub-analysis into each disease, we even see the significant association between the 2 or more than 2 missing KIR ligands with increase survival in AML group too. Our result can demonstrate the dose effect of missing KIR ligands as well. The patients with lack of 3 ligands for donor-inhibitory KIR resulted in no relapse and death. I hypothesized that the number of KIR ligand mismatch has impact on NK functions. It seems that the GVL effect correlates with the numbers of KIR ligand mismatch. Previous studies in HLA-identical sibling transplantation have not produced consistent findings. The beneficial effect of NK cells was mostly seen clearly in the T cell depleted rather than T cell repleted protocol. Interestingly, most of the studies did not analyze dose of missing KIR ligands. It is possible that in the identical sibling transplantation

particularly with the T cell repleted protocol, the role of KIR alloreactivity is not so strong. The protective role of NK alloreactivity might be recognized only in more than 2 missing ligands, which is true in our study. This might be the reason for the inconsistent result.

The previous study demonstrated that NK cell amount was a major factor in reduce risk of relapse when considered with missing KIR ligand [95]. Clausen et al. suggested that the benefit of missing KIR ligand was not different between T cell repleted and T cell depleted protocol. NK cell function following unmanipulated protocol is similar to T cell depletion [93]. However, we did not have information on NK cell amount in this study. The degree of post-transplantation immunosuppression is another possible variation. This information was lacking in most studies. The increased use of immunosuppression post-transplantation may promote NK alloreactivity through T cell suppression [82].

Disease type is another major factor. The strong NK effects against AML have previous been demonstrated, but they were less clear in CML and ALL [31, 61, 69]. The component of AML in each study was varies and might result in inconsistent association [1, 78]. Previous study in Thai patients reported no association between the missing KIR ligands ($\geq$ 1) with any clinical outcome [1]. We suggest that this mainly might be the result from the difference in patient cohorts because most of our patients were AML (60%) while previous study were mostly CML (56.8%) (Table 33). Previous data showed that the patients with AML/MDS showed a significantly reducing relapse and increasing survival for patients with 2 missing KIR ligand compared with 1 or 0 missing KIR ligand group [70]. When myeloid leukemia patients were selected for analysis, these effects became prominent, suggesting that patients with myeloid malignancies were more responsive to treatment [69, 71, 74]. Several studies have suggested that ALL is not as susceptible to missing KIR ligand [69, 74].

When specific type of missing KIR ligand was analyzed, our study showed that patients who lack HLA-C group 2 had increased risk of acute GVHD. Interestingly, previous report e.g., Cook et al. showed that in HLA-matched (T-replete) sibling transplantation for myeloid leukemia, patients homozygous for C2 alleles receiving a graft from a donor carrying KIR gene 2DS2 had a significantly reduced survival [48]. In

addition, Bjorhlund et al., 2010 reported that missing HLA-C2 was associated with increased hazard ration of acute GVHD and TRM [96]. However, in the latter study, the author discussed that this specific association could not be explained by NK-cell alloreactivity, because recipients lacking 2 ligands (HLA-Bw4 and HLA-C1 or HLA-C2) had a similar risk to develop GVHD as those with all ligands present. However, there is no clear explanation for this observation. Another possible hypothesis is the role of HLA-C2 in association with the stimulatory KIR. Recently, KIR2DS1 that also recognizes the HLA-C group 2 epitope, was demonstrated to play a role in mediating alloreactivity [76, 97]. Therefore, in the absence of HLA-C 2 group, the activating KIR2DS1 might function less efficiently in killing APC and increased the risk of GVHD.

In summary, our result helps emphasize the important role of NK alloreactivity as a protective factor in patients with myeloid leukemia including the HLA-identical sibling using our treatment protocol. The development of adoptive transfer of NK cells should be useful and in our HLA-identical patients.

CHAPTER VI
CONCLUSION

Although the impact of donor inhibitory KIR and the HLA ligand remains an unresolved issue, the role of HLA class I with specific KIR in clinical implication for allogeneic HSCT in myeloid leukemia is quite strong. Our present study demonstrated the beneficial effects of missing KIR ligand based on the absence of HLA ligand for particular inhibitory KIR genes in leukemia patients particularly AML. Moreover, the model of 2 or more than 2 missing KIR ligand in patients could influence the clinical outcome by reducing acute GVHD. Their capability of killing leukemic blast as well as antigen presenting cell by a dose effect of missing KIR ligand become a criteria of growing importance as clinicians are offered another important factor concerning donor selection in sibling transplant settings along with HLA matching. In addition, the development of adoptive transfer of NK cells should be useful both in HLA-mismatch and HLA-identical patients. Towards this goal, our understanding of HLA-KIR interaction with leukemic target cells needs to be further improved.

REFERENCES

1. Wongwuttisaroj N, V.S., Chongkolwatana V, Issaragrisil S, *Analysis of KIR Genes in HLA-identical Sibling Hematopoietic Stem cell Transplantation in Thai Patients with Leukemia.* J Med Assoc Thai, 2012. 95(10): p. 1261-5.
2. Thomas, E.D., H.L. Lochte, Jr., W.C. Lu, and J.W. Ferrebee, *Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy.* The New England journal of medicine, 1957. 257(11): p. 491-6.
3. Martin, D., *"Dr. Georges Mathe, Transplant Pioneer, Dies at 88".* New York Times, 20 October 2010.
4. Saxon, W., *"Robert A. Good, 81, Founder Of Modern Immunology, Dies".* New York Times., 18 June 2003.
5. Foundation, T.B.M., *"Cancer Research Pioneer Dies"* Retrieved 6 October 2013.
6. Baron, F., M.B. Maris, B.M. Sandmaier, B.E. Storer, M. Sorror, R. Diaconescu, A.E. Woolfrey, T.R. Chauncey, M.E. Flowers, M. Mielcarek, D.G. Maloney, and R. Storb, *Graft-versus-tumor effects after allogeneic hematopoietic cell transplantation with nonmyeloablative conditioning.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2005. 23(9): p. 1993-2003.
7. Toze, C.L., A. Galal, M.J. Barnett, J.D. Shepherd, E.A. Conneally, D.E. Hogge, S.H. Nantel, T.J. Nevill, H.J. Sutherland, J.M. Connors, N.J. Voss, T.L. Kiss, H.A. Messner, J.C. Lavoie, D.L. Forrest, K.W. Song, C.A. Smith, and J. Lipton, *Myeloablative allografting for chronic lymphocytic leukemia: evidence for a potent graft-versus-leukemia effect associated with graft-versus-host disease.* Bone marrow transplantation, 2005. 36(9): p. 825-30.
8. Goker, H., I.C. Haznedaroglu, and N.J. Chao, *Acute graft-vs-host disease: pathobiology and management.* Experimental hematology, 2001. 29(3): p. 259-77.
9. Lee, S.J., G. Vogelsang, and M.E. Flowers, *Chronic graft-versus-host disease.* Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation, 2003. 9(4): p. 215-33.
10. Wagner, J.E., J.S. Thompson, S.L. Carter, and N.A. Kernan, *Effect of graft-versus-host disease prophylaxis on 3-year disease-free survival in recipients*

of unrelated donor bone marrow (T-cell Depletion Trial): a multi-centre, randomised phase II-III trial. Lancet, 2005. 366(9487): p. 733-41.

11. Morishima, Y., T. Sasazuki, H. Inoko, T. Juji, T. Akaza, K. Yamamoto, Y. Ishikawa, S. Kato, H. Sao, H. Sakamaki, K. Kawa, N. Hamajima, S. Asano, and Y. Kodera, *The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors.* Blood, 2002. 99(11): p. 4200-6.
12. Hale, G. and H. Waldmann, *Control of graft-versus-host disease and graft rejection by T cell depletion of donor and recipient with Campath-1 antibodies. Results of matched sibling transplants for malignant diseases.* Bone marrow transplantation, 1994. 13(5): p. 597-611.
13. Barrett, A.J. and M. Battiwalla, *Relapse after allogeneic stem cell transplantation.* Expert review of hematology, 2010. 3(4): p. 429-41.
14. Khong, H.T. and N.P. Restifo, *Natural selection of tumor variants in the generation of "tumor escape" phenotypes.* Nature immunology, 2002. 3(11): p. 999-1005.
15. Savani, B.N., S. Mielke, S. Adams, M. Uribe, K. Rezvani, A.S. Yong, J. Zeilah, R. Kurlander, R. Srinivasan, R. Childs, N. Hensel, and A.J. Barrett, *Rapid natural killer cell recovery determines outcome after T-cell-depleted HLA-identical stem cell transplantation in patients with myeloid leukemias but not with acute lymphoblastic leukemia.* Leukemia, 2007. 21(10): p. 2145-52.
16. Truitt, R.L. and A.A. Atasoylu, *Contribution of CD4+ and CD8+ T cells to graft-versus-host disease and graft-versus-leukemia reactivity after transplantation of MHC-compatible bone marrow.* Bone marrow transplantation, 1991. 8(1): p. 51-8.
17. Bleakley, M. and S.R. Riddell, *Molecules and mechanisms of the graft-versus-leukaemia effect.* Nature reviews. Cancer, 2004. 4(5): p. 371-80.
18. Huang, X.J. and Y.J. Chang, *Unmanipulated HLA-mismatched/haploidentical blood and marrow hematopoietic stem cell transplantation.* Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation, 2011. 17(2): p. 197-204.
19. Cooper, D.L., *HLA matching for hematopoietic stem-cell transplants.* The New England journal of medicine, 2002. 346(16): p. 1251-2; author reply 1251-2.
20. Kawase, T., K. Matsuo, K. Kashiwase, H. Inoko, H. Saji, S. Ogawa, S. Kato, T. Sasazuki, Y. Kodera, and Y. Morishima, *HLA mismatch combinations*

associated with decreased risk of relapse: implications for the molecular mechanism. Blood, 2009. 113(12): p. 2851-8.

21. Fuchs, E.J., *Haploidentical transplantation for hematologic malignancies: where do we stand?* Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program, 2012. 2012: p. 230-6.
22. Sehn, L.H., E.P. Alyea, E. Weller, C. Canning, S. Lee, J. Ritz, J.H. Antin, and R.J. Soiffer, *Comparative outcomes of T-cell-depleted and non-T-cell-depleted allogeneic bone marrow transplantation for chronic myelogenous leukemia: impact of donor lymphocyte infusion.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 1999. 17(2): p. 561-8.
23. Marmont, A.M., M.M. Horowitz, R.P. Gale, K. Sobocinski, R.C. Ash, D.W. van Bekkum, R.E. Champlin, K.A. Dicke, J.M. Goldman, R.A. Good, and et al., *T-cell depletion of HLA-identical transplants in leukemia.* Blood, 1991. 78(8): p. 2120-30.
24. Schroeder, M.A. and J.F. DiPersio, *Mouse models of graft-versus-host disease: advances and limitations.* Disease models & mechanisms, 2011. 4(3): p. 318-33.
25. Olson, J.A., D.B. Leveson-Gower, S. Gill, J. Baker, A. Beilhack, and R.S. Negrin, *NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects.* Blood, 2010. 115(21): p. 4293-301.
26. Kumar, V. and M.E. McNerney, *A new self: MHC-class-I-independent natural-killer-cell self-tolerance.* Nature reviews. Immunology, 2005. 5(5): p. 363-74.
27. Lanier, L.L., *NK cell recognition.* Annu Rev Immunol, 2005. 23: p. 225-74.
28. Parham, P., *MHC class I molecules and KIRs in human history, health and survival.* Nat Rev Immunol, 2005. 5(3): p. 201-14.
29. Ljunggren, H.-G. and K. Kärre, *In search of the 'missing self': MHC molecules and NK cell recognition.* Immunology Today, 1990. 11(0): p. 237-244.
30. Hinson, J.W., G.S. Huang, J. Lee, D.H. Miller, V. Pavlunin, R. Rangarajan, B. Sanghi, E.I. Shibata, I.P. Shipsey, D. Cronin-Hennessy, C.S. Park, W. Park, J.B. Thayer, E.H. Thorndike, T.E. Coan, Y.S. Gao, F. Liu, R. Stroynowski, M. Artuso, C. Boulahouache, S. Blusk, E. Dambasuren, O. Dorjkhaidav, R. Mountain, H. Muramatsu, R. Nandakumar, T. Skwarnicki, S. Stone, J.C. Wang, S.E. Csorna, I. Danko, G. Bonvicini, D. Cinabro, M. Dubrovin, S. McGee, A. Bornheim, E. Lipeles, S.P. Pappas, A. Shapiro, W.M. Sun, A.J. Weinstein, R.A. Briere, G.P. Chen, T. Ferguson, G. Tatishvili, H. Vogel, M.E. Watkins, N.E. Adam, J.P.

Alexander, K. Berkelman, V. Boisvert, D.G. Cassel, J.E. Duboscq, K.M. Ecklund, R. Ehrlich, R.S. Galik, L. Gibbons, B. Gittelman, S.W. Gray, D.L. Hartill, B.K. Heltsley, L. Hsu, C.D. Jones, J. Kandaswamy, D.L. Kreinick, A. Magerkurth, H. Mahlke-Kruger, T.O. Meyer, N.B. Mistry, J.R. Patterson, D. Peterson, J. Pivarski, S.J. Richichi, D. Riley, A.J. Sadoff, H. Schwarthoff, M.R. Shepherd, J.G. Thayer, D. Urner, T. Wilksen, A. Warburton, M. Weinberger, S.B. Athar, P. Avery, L. Breva-Newell, V. Potlia, H. Stoeck, J. Yelton, K. Benslama, C. Cawlfield, B.I. Eisenstein, G.D. Gollin, I. Karliner, N. Lowrey, C. Plager, C. Sedlack, M. Selen, J.J. Thaler, J. Williams, K.W. Edwards, D. Besson, S. Anderson, V.V. Frolov, D.T. Gong, Y. Kubota, S.Z. Li, R. Poling, A. Smith, C.J. Stepaniak, J. Urheim, Z. Metreveli, K.K. Seth, A. Tomaradze, P. Zweber, S. Ahmed, M.S. Alam, J. Ernst, L. Jian, M. Saleem, F. Wappler, K. Arms, E. Eckhart, K.K. Gan, C. Gwon, K. Honscheid, H. Kagan, R. Kass, T.K. Pedlar, E. von Toerne, H. Severini, P. Skubic, S.A. Dytman, J.A. Mueller, S. Nam and V. Savinov, *Improved measurement of the form factors in the decay lambda+c-->lambda + nue.* Physical review letters, 2005. 94(19): p. 191801.

31. Smyth, M.J., Y. Hayakawa, K. Takeda, and H. Yagita, *New aspects of natural-killer-cell surveillance and therapy of cancer.* Nature reviews. Cancer, 2002. 2(11): p. 850-61.
32. Laperrousaz, S., S. Tiercy, J. Villard, and S. Ferrari-Lacraz, *HLA and non-HLA polymorphisms in renal transplantation.* Swiss Med Wkly, 2012. 142: p. w13668.
33. Vivier, E., D.H. Raulet, A. Moretta, M.A. Caligiuri, L. Zitvogel, L.L. Lanier, W.M. Yokoyama, and S. Ugolini, *Innate or adaptive immunity? The example of natural killer cells.* Science, 2011. 331(6013): p. 44-9.
34. Kim, S., J. Poursine-Laurent, S.M. Truscott, L. Lybarger, Y.J. Song, L. Yang, A.R. French, J.B. Sunwoo, S. Lemieux, T.H. Hansen, and W.M. Yokoyama, *Licensing of natural killer cells by host major histocompatibility complex class I molecules.* Nature, 2005. 436(7051): p. 709-13.
35. Sun, J.C., *Re-educating natural killer cells.* The Journal of experimental medicine, 2010. 207(10): p. 2049-52.
36. Fernandez, N.C., E. Treiner, R.E. Vance, A.M. Jamieson, S. Lemieux, and D.H. Raulet, *A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules.* Blood, 2005. 105(11): p. 4416-23.

37. Raulet, D.H., R.E. Vance, and C.W. McMahon, *Regulation of the natural killer cell receptor repertoire.* Annual review of immunology, 2001. 19: p. 291-330.
38. Anfossi, N., P. Andre, S. Guia, C.S. Falk, S. Roetynck, C.A. Stewart, V. Breso, C. Frassati, D. Reviron, D. Middleton, F. Romagne, S. Ugolini, and E. Vivier, *Human NK cell education by inhibitory receptors for MHC class I.* Immunity, 2006. 25(2): p. 331-42.
39. Yu, J., G. Heller, J. Chewning, S. Kim, W.M. Yokoyama, and K.C. Hsu, *Hierarchy of the human natural killer cell response is determined by class and quantity of inhibitory receptors for self-HLA-B and HLA-C ligands.* Journal of immunology, 2007. 179(9): p. 5977-89.
40. Kim, S., J.B. Sunwoo, L. Yang, T. Choi, Y.J. Song, A.R. French, A. Vlahiotis, J.F. Piccirillo, M. Cella, M. Colonna, T. Mohanakumar, K.C. Hsu, B. Dupont, and W.M. Yokoyama, *HLA alleles determine differences in human natural killer cell responsiveness and potency.* Proceedings of the National Academy of Sciences of the United States of America, 2008. 105(8): p. 3053-8.
41. Bashirova, A.A., M.P. Martin, D.W. McVicar, and M. Carrington, *The killer immunoglobulin-like receptor gene cluster: tuning the genome for defense.* Annual review of genomics and human genetics, 2006. 7: p. 277-300.
42. Barao, I. and W.J. Murphy, *The immunobiology of natural killer cells and bone marrow allograft rejection.* Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation, 2003. 9(12): p. 727-41.
43. Rajalingam, R., *Human diversity of killer cell immunoglobulin-like receptors and disease.* The Korean journal of hematology, 2011. 46(4): p. 216-28.
44. Owen M. Siggs, E.M.Y.M., Nora G. Smart, Beutler B. *Record for Unnatural*. MUTAGENETIX (TM) 2013 Dec 12, 2013; Available from: http://mutagenetix.utsouthwestern.edu/phenotypic/phenotypic_pdf.cfm/mutagenetix-Unnatural.pdf?pk=391.
45. Vilches, C. and P. Parham, *KIR: diverse, rapidly evolving receptors of innate and adaptive immunity.* Annu Rev Immunol, 2002. 20: p. 217-51.
46. Wagtmann, N., S. Rajagopalan, C.C. Winter, M. Peruzzi, and E.O. Long, *Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer.* Immunity, 1995. 3(6): p. 801-9.
47. Hsu, K.C., X.R. Liu, A. Selvakumar, E. Mickelson, R.J. O'Reilly, and B. Dupont, *Killer Ig-like receptor haplotype analysis by gene content: evidence for*

genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. Journal of immunology, 2002. 169(9): p. 5118-29.

48. Cook, M.A., D.W. Milligan, C.D. Fegan, P.J. Darbyshire, P. Mahendra, C.F. Craddock, P.A. Moss, and D.C. Briggs, *The impact of donor KIR and patient HLA-C genotypes on outcome following HLA-identical sibling hematopoietic stem cell transplantation for myeloid leukemia.* Blood, 2004. 103(4): p. 1521-6.
49. Parham, P., *MHC class I molecules and KIRs in human history, health and survival.* Nature reviews. Immunology, 2005. 5(3): p. 201-14.
50. Kroger, N., T. Binder, T. Zabelina, C. Wolschke, H. Schieder, H. Renges, F. Ayuk, J. Dahlke, T. Eiermann, and A. Zander, *Low number of donor activating killer immunoglobulin-like receptors (KIR) genes but not KIR-ligand mismatch prevents relapse and improves disease-free survival in leukemia patients after in vivo T-cell depleted unrelated stem cell transplantation.* Transplantation, 2006. 82(8): p. 1024-30.
51. McQueen, K.L., K.M. Dorighi, L.A. Guethlein, R. Wong, B. Sanjanwala, and P. Parham, *Donor-recipient combinations of group A and B KIR haplotypes and HLA class I ligand affect the outcome of HLA-matched, sibling donor hematopoietic cell transplantation.* Human immunology, 2007. 68(5): p. 309-23.
52. Valiante, N.M., M. Uhrberg, H.G. Shilling, K. Lienert-Weidenbach, K.L. Arnett, A. D'Andrea, J.H. Phillips, L.L. Lanier, and P. Parham, *Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors.* Immunity, 1997. 7(6): p. 739-51.
53. Shimizu, Y. and R. DeMars, *Demonstration by class I gene transfer that reduced susceptibility of human cells to natural killer cell-mediated lysis is inversely correlated with HLA class I antigen expression.* European journal of immunology, 1989. 19(3): p. 447-51.
54. Pende, D., R. Biassoni, C. Cantoni, S. Verdiani, M. Falco, C. di Donato, L. Accame, C. Bottino, A. Moretta, and L. Moretta, *The natural killer cell receptor specific for HLA-A allotypes: a novel member of the p58/p70 family of inhibitory receptors that is characterized by three immunoglobulin-like domains and is expressed as a 140-kD disulphide-linked dimer.* The Journal of experimental medicine, 1996. 184(2): p. 505-18.

55. Litwin, V., J. Gumperz, P. Parham, J.H. Phillips, and L.L. Lanier, *NKB1: a natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules.* The Journal of experimental medicine, 1994. 180(2): p. 537-43.
56. Fan, Q.R., D.N. Garboczi, C.C. Winter, N. Wagtmann, E.O. Long, and D.C. Wiley, *Direct binding of a soluble natural killer cell inhibitory receptor to a soluble human leukocyte antigen-Cw4 class I major histocompatibility complex molecule.* Proceedings of the National Academy of Sciences of the United States of America, 1996. 93(14): p. 7178-83.
57. Winter, C.C., J.E. Gumperz, P. Parham, E.O. Long, and N. Wagtmann, *Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition.* Journal of immunology, 1998. 161(2): p. 571-7.
58. Locatelli, F., D. Pende, M.C. Mingari, A. Bertaina, M. Falco, A. Moretta, and L. Moretta, *Cellular and molecular basis of haploidentical hematopoietic stem cell transplantation in the successful treatment of high-risk leukemias: role of alloreactive NK cells.* Frontiers in immunology, 2013. 4: p. 15.
59. Jamil, K.M. and S.I. Khakoo, *KIR/HLA interactions and pathogen immunity.* Journal of biomedicine & biotechnology, 2011. 2011: p. 298348.
60. Frohn, C., P. Schlenke, and H. Kirchner, *The repertoire of HLA-Cw-specific NK cell receptors CD158 a/b (EB6 and GL183) in individuals with different HLA phenotypes.* Immunology, 1997. 92(4): p. 567-70.
61. Hsu, K.C., C.A. Keever-Taylor, A. Wilton, C. Pinto, G. Heller, K. Arkun, R.J. O'Reilly, M.M. Horowitz, and B. Dupont, *Improved outcome in HLA-identical sibling hematopoietic stem-cell transplantation for acute myelogenous leukemia predicted by KIR and HLA genotypes.* Blood, 2005. 105(12): p. 4878-84.
62. Grau, R., K.S. Lang, D. Wernet, P. Lang, D. Niethammer, C.M. Pusch, and R. Handgretinger, *Cytotoxic activity of natural killer cells lacking killer-inhibitory receptors for self-HLA class I molecules against autologous hematopoietic stem cells in healthy individuals.* Experimental and molecular pathology, 2004. 76(2): p. 90-8.
63. Khakoo, S.I., C.L. Thio, M.P. Martin, C.R. Brooks, X. Gao, J. Astemborski, J. Cheng, J.J. Goedert, D. Vlahov, M. Hilgartner, S. Cox, A.M. Little, G.J. Alexander, M.E. Cramp, S.J. O'Brien, W.M. Rosenberg, D.L. Thomas, and M. Carrington, *HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection.* Science, 2004. 305(5685): p. 872-4.

64. Momot, T., S. Koch, N. Hunzelmann, T. Krieg, K. Ulbricht, R.E. Schmidt, and T. Witte, *Association of killer cell immunoglobulin-like receptors with scleroderma.* Arthritis and rheumatism, 2004. 50(5): p. 1561-5.
65. van der Slik, A.R., B.Z. Alizadeh, B.P. Koeleman, B.O. Roep, and M.J. Giphart, *Modelling KIR-HLA genotype disparities in type 1 diabetes.* Tissue antigens, 2007. 69 Suppl 1: p. 101-5.
66. Nelson, G.W., M.P. Martin, D. Gladman, J. Wade, J. Trowsdale, and M. Carrington, *Cutting edge: heterozygote advantage in autoimmune disease: hierarchy of protection/susceptibility conferred by HLA and killer Ig-like receptor combinations in psoriatic arthritis.* Journal of immunology, 2004. 173(7): p. 4273-6.
67. Pellett, F., F. Siannis, I. Vukin, P. Lee, M.B. Urowitz, and D.D. Gladman, *KIRs and autoimmune disease: studies in systemic lupus erythematosus and scleroderma.* Tissue antigens, 2007. 69 Suppl 1: p. 106-8.
68. Hiby, S.E., J.J. Walker, M. O'Shaughnessy K, C.W. Redman, M. Carrington, J. Trowsdale, and A. Moffett, *Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success.* The Journal of experimental medicine, 2004. 200(8): p. 957-65.
69. Ruggeri, L., M. Capanni, E. Urbani, K. Perruccio, W.D. Shlomchik, A. Tosti, S. Posati, D. Rogaia, F. Frassoni, F. Aversa, M.F. Martelli, and A. Velardi, *Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants.* Science, 2002. 295(5562): p. 2097-100.
70. Linn, Y.C., C.Y. Phang, T.J. Lim, S.F. Chong, K.K. Heng, J.J. Lee, Y. Loh, W. Hwang, Y.T. Goh, and M. Koh, *Effect of missing killer-immunoglobulin-like receptor ligand in recipients undergoing HLA full matched, non-T-depleted sibling donor transplantation: a single institution experience of 151 Asian patients.* Bone marrow transplantation, 2010. 45(6): p. 1031-7.
71. Ruggeri, L., A. Mancusi, M. Capanni, E. Urbani, A. Carotti, T. Aloisi, M. Stern, D. Pende, K. Perruccio, E. Burchielli, F. Topini, E. Bianchi, F. Aversa, M.F. Martelli, and A. Velardi, *Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value.* Blood, 2007. 110(1): p. 433-40.
72. Ruggeri, L., A. Mancusi, E. Burchielli, K. Perruccio, F. Aversa, M.F. Martelli, and A. Velardi, *Natural killer cell recognition of missing self and haploidentical hematopoietic transplantation.* Seminars in cancer biology, 2006. 16(5): p. 404-11.

73. Moretta, L., F. Locatelli, D. Pende, M.C. Mingari, and A. Moretta, *Natural killer alloeffector responses in haploidentical hemopoietic stem cell transplantation to treat high-risk leukemias.* Tissue antigens, 2010. 75(2): p. 103-9.
74. Giebel, S., F. Locatelli, T. Lamparelli, A. Velardi, S. Davies, G. Frumento, R. Maccario, F. Bonetti, J. Wojnar, M. Martinetti, F. Frassoni, G. Giorgiani, A. Bacigalupo, and J. Holowiecki, *Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors.* Blood, 2003. 102(3): p. 814-9.
75. Pende, D., G.M. Spaggiari, S. Marcenaro, S. Martini, P. Rivera, A. Capobianco, M. Falco, E. Lanino, I. Pierri, R. Zambello, A. Bacigalupo, M.C. Mingari, A. Moretta, and L. Moretta, *Analysis of the receptor-ligand interactions in the natural killer-mediated lysis of freshly isolated myeloid or lymphoblastic leukemias: evidence for the involvement of the Poliovirus receptor (CD155) and Nectin-2 (CD112).* Blood, 2005. 105(5): p. 2066-73.
76. Pende, D., S. Marcenaro, M. Falco, S. Martini, M.E. Bernardo, D. Montagna, E. Romeo, C. Cognet, M. Martinetti, R. Maccario, M.C. Mingari, E. Vivier, L. Moretta, F. Locatelli, and A. Moretta, *Anti-leukemia activity of alloreactive NK cells in KIR ligand-mismatched haploidentical HSCT for pediatric patients: evaluation of the functional role of activating KIR and redefinition of inhibitory KIR specificity.* Blood, 2009. 113(13): p. 3119-29.
77. Stern, M., L. Ruggeri, A. Mancusi, M.E. Bernardo, C. de Angelis, C. Bucher, F. Locatelli, F. Aversa, and A. Velardi, *Survival after T cell-depleted haploidentical stem cell transplantation is improved using the mother as donor.* Blood, 2008. 112(7): p. 2990-5.
78. Davies, S.M., L. Ruggieri, T. DeFor, J.E. Wagner, D.J. Weisdorf, J.S. Miller, A. Velardi, and B.R. Blazar, *Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants. Killer immunoglobulin-like receptor.* Blood, 2002. 100(10): p. 3825-7.
79. Bornhauser, M., R. Schwerdtfeger, H. Martin, K.H. Frank, C. Theuser, and G. Ehninger, *Role of KIR ligand incompatibility in hematopoietic stem cell transplantation using unrelated donors.* Blood, 2004. 103(7): p. 2860-1; author reply 2862.
80. Schaffer, M., K.J. Malmberg, O. Ringden, H.G. Ljunggren, and M. Remberger, *Increased infection-related mortality in KIR-ligand-mismatched unrelated*

allogeneic hematopoietic stem-cell transplantation. Transplantation, 2004. 78(7): p. 1081-5.

81. Leung, W., R. Iyengar, V. Turner, P. Lang, P. Bader, P. Conn, D. Niethammer, and R. Handgretinger, *Determinants of antileukemia effects of allogeneic NK cells.* Journal of immunology, 2004. 172(1): p. 644-50.
82. Hsu, K.C., T. Gooley, M. Malkki, C. Pinto-Agnello, B. Dupont, J.D. Bignon, M. Bornhauser, F. Christiansen, A. Gratwohl, Y. Morishima, M. Oudshoorn, O. Ringden, J.J. van Rood, and E. Petersdorf, *KIR ligands and prediction of relapse after unrelated donor hematopoietic cell transplantation for hematologic malignancy.* Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation, 2006. 12(8): p. 828-36.
83. Cooper, M.A., T.A. Fehniger, A. Fuchs, M. Colonna, and M.A. Caligiuri, *NK cell and DC interactions.* Trends in immunology, 2004. 25(1): p. 47-52.
84. Wang, H., Y. He, W.J. Zhai, M. Wang, Z. Zhou, Y.X. Zhao, S.Z. Feng, and M.Z. Han, *The impact of recipient HLA-Cw and donor killer immunoglobulin-like receptor genotyping on the outcome of patients receiving HLA-matched sibling donor hematopoietic stem cell transplantation for myeloid malignancies.* Swiss medical weekly, 2013. 143: p. w13717.
85. Fischer, J.C. and M. Uhrberg, *Prevention of leukemia relapse by donor activating KIR2DS1.* The New England journal of medicine, 2012. 367(21): p. 2054-5; author reply 2055.
86. Venstrom, J.M., T.A. Gooley, S. Spellman, J. Pring, M. Malkki, B. Dupont, E. Petersdorf, and K.C. Hsu, *Donor activating KIR3DS1 is associated with decreased acute GVHD in unrelated allogeneic hematopoietic stem cell transplantation.* Blood, 2010. 115(15): p. 3162-5.
87. Stringaris, K., S. Adams, M. Uribe, R. Eniafe, C.O. Wu, B.N. Savani, and A.J. Barrett, *Donor KIR Genes 2DL5A, 2DS1 and 3DS1 are associated with a reduced rate of leukemia relapse after HLA-identical sibling stem cell transplantation for acute myeloid leukemia but not other hematologic malignancies.* Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation, 2010. 16(9): p. 1257-64.
88. Cooley, S., E. Trachtenberg, T.L. Bergemann, K. Saeteurn, J. Klein, C.T. Le, S.G. Marsh, L.A. Guethlein, P. Parham, J.S. Miller, and D.J. Weisdorf, *Donors with group B KIR haplotypes improve relapse-free survival after unrelated*

hematopoietic cell transplantation for acute myelogenous leukemia. Blood, 2009. 113(3): p. 726-32.

89. Venstrom, J.M., G. Pittari, T.A. Gooley, J.H. Chewning, S. Spellman, M. Haagenson, M.M. Gallagher, M. Malkki, E. Petersdorf, B. Dupont, and K.C. Hsu, *HLA-C-dependent prevention of leukemia relapse by donor activating KIR2DS1.* The New England journal of medicine, 2012. 367(9): p. 805-16.
90. Tammakorn, C., T. Mongkolsuk, D. Thammanichanond, S. Pakakasama, and P. Kitpoka, *Distribution of killer cell immunoglobulin-like receptor genes in Thai blood donors.* Journal of the Medical Association of Thailand = Chotmaihet thangphaet, 2011. 94(6): p. 738-42.
91. Jiang, K., F.M. Zhu, Q.F. Lv, and L.X. Yan, *Distribution of killer cell immunoglobulin-like receptor genes in the Chinese Han population.* Tissue antigens, 2005. 65(6): p. 556-63.
92. Toneva, M., V. Lepage, G. Lafay, N. Dulphy, M. Busson, S. Lester, A. Vu-Trien, A. Michaylova, E. Naumova, J. McCluskey, and D. Charron, *Genomic diversity of natural killer cell receptor genes in three populations.* Tissue antigens, 2001. 57(4): p. 358-62.
93. Clausen, J., B. Kircher, J. Auberger, P. Schumacher, H. Ulmer, G. Hetzenauer, D. Wolf, G. Gastl, and D. Nachbaur, *The role of missing killer cell immunoglobulin-like receptor ligands in T cell replete peripheral blood stem cell transplantation from HLA-identical siblings.* Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation, 2010. 16(2): p. 273-80.
94. Norman, P.J., C.V. Carrington, M. Byng, L.D. Maxwell, M.D. Curran, H.A. Stephens, D. Chandanayingyong, D.H. Verity, K. Hameed, D.D. Ramdath, and R.W. Vaughan, *Natural killer cell immunoglobulin-like receptor (KIR) locus profiles in African and South Asian populations.* Genes and immunity, 2002. 3(2): p. 86-95.
95. Clausen, J., D. Wolf, A.L. Petzer, E. Gunsilius, P. Schumacher, B. Kircher, G. Gastl, and D. Nachbaur, *Impact of natural killer cell dose and donor killer-cell immunoglobulin-like receptor (KIR) genotype on outcome following human leucocyte antigen-identical haematopoietic stem cell transplantation.* Clinical and experimental immunology, 2007. 148(3): p. 520-8.
96. Bjorklund, A.T., M. Schaffer, C. Fauriat, O. Ringden, M. Remberger, C. Hammarstedt, A.J. Barrett, P. Ljungman, H.G. Ljunggren, and K.J. Malmberg, *NK*

cells expressing inhibitory KIR for non-self-ligands remain tolerant in HLA-matched sibling stem cell transplantation. Blood, 2010. 115(13): p. 2686-94.

97. Chewning, J.H., C.N. Gudme, K.C. Hsu, A. Selvakumar, and B. Dupont, *KIR2DS1-positive NK cells mediate alloresponse against the C2 HLA-KIR ligand group in vitro.* Journal of immunology, 2007. 179(2): p. 854-68.

APPENDIX

Reagent preparation

1. Amplification mixture

Number of reaction	D-mix(ul)	Primer mix(ul)	Taq DNA polymerase(ul)
1	6.9	2	0.1
10	69	20	1
50	345	100	5
96	662.4	192	9.6

2. Hybridization mixture

Number of tests	Hybridization Buffer(ul)	Bead Mixture(ul)
1	16.1	1.9
10	161	19
50	805	95
96	1545.6	182.4

3. SAPE mixture

Number of Tests	100XSAPE(ul)	SAPE Buffer(ul)
1	0.25	24.75
10	2.5	247.5
50	12.5	1237.5
96	24	2376

4. 1 X TBE buffer

Tris Borate EDTA 17 g
Distilled water to 1,000 ml

5. 2% agarose gel

LE agarose 0.8 g
1 X TBE 40 ml
Melt by microwave.

VITA

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