

Killer Cell Immunoglobulin-Like Receptor Genes Polymorphisms in Macedonian Patients with Haematological Malignancies

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Abstract

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Aim: The aim of this study was to examine the gene frequencies of 16 KIR genes and pseudogenes (*KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR2DL4*, *KIR2DL5*, *KIR3DL1*, *KIR3DL2*, *KIR3DL3*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *KIR2DS5*, *KIR3DS1*, *KIR2DP1*, and *KIR3DP1*) and KIR genotypes in Macedonian patients with haematological malignancies, to compare them with the corresponding frequencies of healthy Macedonians and to analyse eventual association of specific genes or genotypes with the studied haematological diseases.

Material and Methods: The study included 63 patients of which 40 had acute myeloblastic leukaemia (63.5%), 11 (17.5%) had chronic myeloid leukaemia, 8 (12.7%) acute lymphoblastic leukaemia, 2 (3.17%) non-Hodgkin Lymphoma, 1 (1.59%) aplastic anemia, and 1 (1.59%) chronic lymphocytic leukaemia.

Results: Comparison of KIR gene frequencies between the 63 patients and healthy Macedonians reveals statistically significant difference for *KIR3DL2* ($F=1$ in the control group, and 0.95 in the patients group, $p=0.001$). Another statistically significant difference was observed for the frequency of Bx3 and Bx439 genotypes both found more often in patients group ($P=0.017$ and $P=0.009$, respectively).

Conclusion: Further analysis, involving larger series of patients and targeted at the ligands of the KIRs are needed in order to determine a certain KIR gene and/or genotype as either predisposing, or protecting factor for haematological malignancy in patients from Republic of Macedonia.

Introduction

The Natural killer (NK) cells are a subset of lymphocytes comprising around 10% of total lymphocytes in peripheral blood [1]. Early recognition and killing of virally infected cells and tumour cells are among the most important roles of the NK cells in the innate immunity [2, 3]. The NK cell activity is regulated in activating or inhibiting manner, through specific receptors present on

their surface, called killer cell immunoglobulin like receptors (KIRs). The KIRs are encoded by the *KIR* gene complex, located on chromosome 19, containing a family of polymorphic and highly homologous members (14 genes and 2 pseudogenes), which can be activating or inhibitory [4]. Based on the gene content, two basic *KIR* haplotypes have been defined, termed A and B. The A haplotype contains a single activating gene, *KIR2DS4*, which is often present in a deleted (non-expressed)

form, while the B haplotype is more variable and contains many activating genes [5, 6].

The different *KIR* haplotypes vary in the number and the type of genes present, but the genes *KIR3DL3*, *KIR3DP1*, *KIR2DL4* and *KIR3DL2* are present on virtually all haplotypes and have therefore been termed framework genes [7]. Population studies performed over the last two decades have revealed extensive diversity at the *KIR* gene locus, which derives from both, its polygenic and multi-allelic polymorphism, whereas on the basis of gene content, haplotype B displays a much greater variety of subtypes [6, 8].

The most important for the triggering or inhibition of the NK cells is the balance between the signals generated by the *KIRs* and their ligands, the HLA molecules [9, 10]. Several recent studies have shown lowered expression of HLA class I molecules in patients with leukaemia [11-13]. It is believed that the down regulation of HLA class I molecules in leukaemia cells releases the inhibitory influence on NK cells, thus permitting the NK cells to activate and lyse the altered target cells. Hypothesizing that NK cells play a major role in the innate immune surveillance of leukaemia cells, several groups have analyzed the *KIR* gene content in leukaemia patients, but have reported contradictory results [14-17].

The aim of this study was to examine the gene frequencies of 16 *KIR* genes and pseudogenes (*KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR2DL4*, *KIR2DL5*, *KIR3DL1*, *KIR3DL2*, *KIR3DL3*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *KIR2DS5*, *KIR3DS1*, *KIR2DP1*, and *KIR3DP1*) and *KIR* genotypes in Macedonian patients with haematological malignancies, to compare them with the corresponding frequencies of healthy Macedonians and to analyse eventual association of

specific genes or genotypes with the studied haematological diseases.

To our knowledge, this is the first study of the association of *KIR* genes with haematological malignant diseases in the Republic of Macedonia.

Material and Methods

Population samples

The study was performed on 63 patients with haematological malignancies hospitalized and treated at the Haematology University Clinic in Skopje, between June, 2001 and September 2009. Distribution according to diagnosis was: acute myeloblastic leukemia 40 (63.5%), chronic myeloid leukemia 11 (17.5%), acute lymphoblastic leukemia 8 (12.7%), non-Hodgkin Lymphoma 2 (3.17%), aplastic anemia 1 (1.59%), and chronic lymphocytic leukemia 1 (1.59%).

KIR genotyping

After signing of written consent, genomic DNA was extracted from the peripheral blood leukocytes using standard phenol/chloroform procedure, described elsewhere [18], and stored in the Macedonian Human DNA Bank (hDNAMKD) [19] until processing. For *KIR* genotyping, commercially available PEL-FREEZ *KIR* genotyping SSP kit (Dynal Biotech, Brown Deer, WI) was used. It is a PCR-based method (using sequence-specific priming approach) designed to detect the presence or absence of 16 *KIR* genes. The presence of each *KIR* gene was determined by the presence of a band of DNA of the expected size. All PCRs contained an internal positive control consisting of an additional pair of primers specific for the growth hormone (GH) gene and a negative control. Individuals were determined

Table 1: Comparison of the observed and estimated *KIR* gene frequencies for Macedonian patients with haematological malignancies (N = 63) and healthy Macedonians (N=214).

| | Frequencies for <i>KIR</i> genes in Macedonian patients with haematological malignancies and Macedonians | | | | | | | | | | | | | | | |
|-------------------|----------------------------------------------------------------------------------------------------------|-----------------|-----------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|---------------------------|-----------------|-----------------|-----------------|-----------------|
| | Pseudogenes | | Inhibitory <i>KIR</i> | | | | | | | | | Non inhibitory <i>KIR</i> | | | | |
| | <i>KIR 2DP1</i> | <i>KIR 3DP1</i> | <i>KIR 2DL1</i> | <i>KIR 2DL2</i> | <i>KIR 2DL3</i> | <i>KIR 2DL4</i> | <i>KIR 2DL5</i> | <i>KIR 3DL1</i> | <i>KIR 3DL2</i> | <i>KIR 3DL3</i> | <i>KIR 2DS1</i> | <i>KIR 2DS2</i> | <i>KIR 2DS3</i> | <i>KIR 2DS4</i> | <i>KIR 2DS5</i> | <i>KIR 3DS1</i> |
| Malignancies (N) | 61 | 63 | 61 | 35 | 58 | 63 | 19 | 60 | 60 | 63 | 29 | 35 | 21 | 62 | 21 | 30 |
| Malignancies (F) | 0.968 | 1 | 0.968 | 0.556 | 0.921 | 1 | 0.302 | 0.952 | 0.952 | 1 | 0.651 | 0.556 | 0.333 | 0.984 | 0.333 | 0.397 |
| Malignancies (GF) | 0.821 | 1 | 0.821 | 0.334 | 0.717 | 1 | 0.165 | 0.781 | 0.781 | 1 | 0.409 | 0.334 | 0.183 | 0.874 | 0.183 | 0.223 |
| Macedonians (N) | 210 | 214 | 201 | 126 | 192 | 214 | 89 | 201 | 214 | 214 | 103 | 122 | 77 | 201 | 64 | 84 |
| Macedonians (F) | 0.980 | 1 | 0.940 | 0.590 | 0.897 | 1 | 0.415 | 0.940 | 1 | 1 | 0.481 | 0.570 | 0.360 | 0.940 | 0.300 | 0.392 |
| Macedonians (GF) | 0.870 | 1 | 0.760 | 0.360 | 0.690 | 1 | 0.230 | 0.800 | 1 | 1 | 0.280 | 0.350 | 0.180 | 0.800 | 0.170 | 0.220 |
| Pearson's p | 0.532 | & | 0.371 | 0.638 | 0.581 | & | 0.102 | 0.695 | 0.001 | & | 0.769 | 0.838 | 0.699 | 0.153 | 0.604 | 0.236 |
| Risk Ratio | 0.99 | 1 | 1.03 | 0.94 | 1.03 | 1 | 0.73 | 1.01 | & | 1 | 0.96 | 0.97 | 0.93 | 1.05 | 1.11 | 1.21 |
| 95% CI | & | & | & | 0.737-1.209 | 0.942-1.118 | & | 0.482-1.091 | 0.950-1.082 | & | & | 0.708-1.293 | 0.759-1.251 | 0.626-1.371 | 1.004-1.097 | 0.743-1.671 | 0.892-1.651 |

N, number of individuals; F, observed frequency was obtained by direct counting; GF, gene frequencies were calculated using the formula $GF=1-\hat{O}(1-F)$; p, statistical significance; &, cannot be calculated because expected <5, c2 test; RR, Risk Ratio; CI, confidence interval.

negative for a particular *KIR* gene when a band of the GH gene. We have used external quality control expected size was absent in the presence of a band for consisting of cell lines from Immunogenetics and

Table 2: *KIR* gene content of Macedonian patients with haematological malignancies (N=63).

| Patient ID | Dg | <i>KIR</i> 3DL1 | <i>KIR</i> 2DL1 | <i>KIR</i> 2DL3 | <i>KIR</i> 2DS4 | <i>KIR</i> 2DL2 | <i>KIR</i> 2DL5 | <i>KIR</i> 3DS1 | <i>KIR</i> 2DS1 | <i>KIR</i> 2DS2 | <i>KIR</i> 2DS3 | <i>KIR</i> 2DS5 | <i>KIR</i> 2DL4 | <i>KIR</i> 3DL2 | <i>KIR</i> 3DL3 | <i>KIR</i> 2DP1 | <i>KIR</i> 3DP1 |
|------------|--------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
| PTH001 | ALL | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH009 | CML | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH014 | AML | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| PTH018 | ALL | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH026 | AML | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH031 | CML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH037 | AML | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH042 | AML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH057 | NHL | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH074 | CML | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH102 | AML | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH106 | CLL | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH112 | AML | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH123 | AML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH135 | CML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH156 | AML | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH168 | CML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH176 | AML | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH179 | AML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH199 | NHL | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH195 | AML | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 |
| PTH235 | LA | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 |
| PTH265 | AML | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH278 | AML | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH294 | AML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH299 | ALL | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH306 | AML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH308 | CML | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH326 | AML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH329 | AML | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH349 | ALL | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH354 | A apl. | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH358 | AML | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH365 | ALL | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH417 | AML | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH455 | AML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH472 | AML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH486 | AML | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH490 | AML | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH493 | ALL | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH526 | AML | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH530 | ALL | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH538 | AML | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH541 | CML | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH544 | AML | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH550 | AML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH554 | AML | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH595 | AML | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH604 | AML | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH693 | AML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH711 | AML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH718 | AML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH724 | AML | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH788 | CML | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH800 | AML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTP946 | ALL | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTP097 | CML | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH224 | AML | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTP251 | AML | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| PTH268 | AML | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTH316 | AML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| PTP370 | CML | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |

AML, acute myeloblastic leukaemia; CML, chronic myeloid leukaemia; ALL, acute lymphoblastic leukaemia; NHL, non-Hodgkin lymphoma; A. apl., aplastic anemia; CLL, chronic lymphocytic leukemia.

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Analyzed samples were assigned as KIR genotype AA (when homozygosity for KIR haplotype A was detected), or as KIR genotype Bx when heterozygosity or homozygosity for KIR haplotype B was typed (KIR genotypes AB and BB).

Statistical analysis

The occurrence of *KIR* genes in individuals (frequency = F) was obtained by direct counting. Gene frequencies (GF) were calculated using the formula $GF=1-\sqrt{1-F}$, being aware of the limitation in its ability to detect KIR genes present at low frequency. For analysis of the molecular polymorphism of the locus studied, the Arlequin software version 3.0 was used. Frequencies for specific genes or genotypes found in the patients group were compared with corresponding frequencies in general Macedonian population, that we have published previously [20]. Comparisons were done using the Fisher's two-sided exact test and Pearson's chi-squared test. Risk ratios or crude odds ratios (OR) were calculated within 95% confidence interval. Statistical significance was set to 0.05.

Results

KIR gene frequencies

The KIR gene content and the diagnosis of the 63 Macedonian patients with haematological malignancies are shown in Table 1.

The frequencies of the 16 *KIR* genes (14 genes and 2 pseudogenes) determined in the 63 haematological patients along with the corresponding frequencies of the 214 healthy Macedonian controls are shown in Table 2. All 16 *KIR* genes were observed in the studied population and framework genes (*KIR3DL3*, *KIR3DP1*, *KIR2DL4*, and *KIR3DL2*) were present in all individuals, except for three patients who lacked the *KIR3DL2* gene.

Comparison of KIR gene frequencies between the 63 patients and healthy Macedonians reveals statistically significant difference for the *KIR3DL2* framework gene (F= 1 in the control group, and 0.95 in the patients group, p=0.001).

Genotype frequencies

If any of the genes *2DL2*, *2DL5*, *3DS1*, *2DS1*,

2DS2, *2DS3*, or *2DS5* was present; the genotype was considered as B. If none of these were present, genotype is considered as AA. We have not attempted to distinguish between AB and BB genotypes and called any of this Bx, nor have we numerated the individual Bx genotypes. Total of 28 different *KIR* genotypes have been determined in the 63 patients (Table 3). We have determined single AA genotype in 14 individuals from the patients group (20.5%) which was comparable with the frequency of AA genotypes in control individuals (21.5%, P=0.902). Statistically significant difference was found for the frequency of the Bx genotype 439, present in two patients and none of the healthy controls (P=0.009), and also for the Bx genotype 3 (present in 5 patients and 4 controls, P=0.017) (Table 3).

Discussion

There are no precise and reliable figures for incidence and survival rates for the different forms of haematological cancer even in highly developed Western European countries and USA. Although the health authorities and their bodies in different regions do publish descriptive statistics, there are many problems with this statistics, such as many cases are never reported to cancer registries, so the actual number of patients could be substantially higher than national figures suggest. Registration figures from United Kingdom for example, suggest around 50 new cases per population of 100,000 [21], but again, those working in the field believe that this number is considerably higher.

Having in mind the different results regarding the distribution of KIR genes reported in patients with certain forms of haematological cancers [14-17], we have analyzed the *KIR* genes distribution in Macedonian patients with haematological malignancies and compared them to healthy Macedonians. Although two recent studies reported higher frequency of activating KIR genes, such as *KIR2DS1*, *KIR2DS3* and *KIR2DS4* in patients with CML and ALL [22, 23], we couldn't reproduce these findings in our cohort. Our findings are thus in agreement with those reported from Babor et al, who also found very similar frequencies of KIR genes among haematological patients and healthy controls with European origin [24]. One significant difference that we have observed exclusively in our study was the one involving the frequency of the framework gene *KIR3DL2* (Pearson's P= 0.001, after applying Yate's correction P= 0.012). However, comparison of frequencies obtained from larger (or META analysis) series would be needed

Table 3: KIR locus haplogroups, genotypes ID and genotype frequency of Macedonian patients with haematological malignancies (N=63) and healthy Macedonians (N=214).

| Haplo group | Geno type ID | KIR 3DL1 | KIR 2DL1 | KIR 2DL3 | KIR 2DS4 | KIR 2DL2 | KIR 2DL5 | KIR 3DS1 | KIR 2DS1 | KIR 2DS2 | KIR 2DS3 | KIR 2DS5 | KIR 2DL4 | KIR 3DL2 | KIR 3DL3 | KIR 2DP1 | KIR 3DP1 | Malignancies No (F) | Macedonians No (F) | Pearson's P |
|-------------|--------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|---------------------|--------------------|-------------|
| AA | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 14 (0,206) | 46 (0,215) | 0,902 |
| Bx | 2 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 (0,016) | 11 (0,051) | 0,178 |
| Bx | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 5 (0,079) | 4 (0,019) | 0,017 |
| Bx | 4 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 4 (0,064) | 27 (0,126) | 0,165 |
| Bx | 5 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 (0,016) | 8 (0,037) | 0,397 |
| Bx | 6 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 3 (0,048) | 8 (0,037) | 0,715 |
| Bx | 7 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 (0,016) | 6 (0,028) | 0,589 |
| Bx | 8 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 (0,016) | 2 (0,009) | 0,660 |
| Bx | 9 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 5 (0,023) | 0,221 |
| Bx | 10 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 (0,016) | 1 (0,005) | 0,356 |
| Bx | 11 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 3 (0,048) | 6 (0,028) | 0,445 |
| Bx | 13 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 6 (0,028) | 0,178 |
| Bx | 14 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 3 (0,014) | 0,345 |
| Bx | 15 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 4 (0,064) | 9 (0,042) | 0,479 |
| Bx | 16 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 18 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 2 (0,009) | 0,441 |
| Bx | 19 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 23 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 28 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 29 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 (0,016) | 0 | 0,065 |
| Bx | 33 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 62 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 3 (0,014) | 0,362 |
| Bx | 63 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 (0,016) | 2 (0,009) | 0,660 |
| Bx | 69 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 2 (0,009) | 0,441 |
| Bx | 70 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 2 (0,009) | 0,441 |
| Bx | 71 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 4 (0,019) | 1 |
| Bx | 72 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 73 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 5 (0,023) | 0,398 |
| Bx | 76 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 2 (0,009) | 0,441 |
| Bx | 87 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 90 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 2 (0,009) | 0,441 |
| Bx | 113 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 (0,016) | 1 (0,005) | 0,356 |
| Bx | 159 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 188 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 (0,016) | 1 (0,005) | 0,356 |
| Bx | 192 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 (0,016) | 0 | 0,065 |
| Bx | 200 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 202 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 3 (0,048) | 3 (0,014) | 0,107 |
| Bx | 205 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,367 |
| Bx | 233 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 (0,016) | 5 (0,023) | 0,716 |
| Bx | 260 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 2 (0,009) | 0,441 |
| Bx | 268 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 276 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 (0,016) | 1 (0,005) | 0,356 |
| Bx | 294 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 (0,016) | 1 (0,005) | 0,356 |
| Bx | 317 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 3 (0,014) | 0,362 |
| Bx | 318 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 319 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 3 (0,048) | 2 (0,009) | 0,045 |
| Bx | 331 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0,098 |
| Bx | 336 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 359 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 363 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 370 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 3 (0,014) | 0,362 |
| Bx | 371 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 372 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 373 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 3 (0,048) | 2 (0,009) | 0,045 |
| Bx | 374 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 375 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 376 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 377 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 3 (0,014) | 0,362 |
| Bx | 378 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 (0,005) | 0,587 |
| Bx | 379 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 380 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 (0,005) | 0,587 |
| Bx | 439 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 2 (0,032) | 0 | 0,009 |
| Bx | new | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 (0,016) | 0 | 0,065 |
| Bx | new | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 (0,016) | 0 | 0,065 |
| Bx | new | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 (0,016) | 0 | 0,065 |
| Bx | new | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 (0,016) | 0 | 0,065 |
| Bx | new | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 (0,016) | 0 | 0,065 |

KIR Genotype [1=Positive, 0=negative]

in order to determine this association as an important protective one in the development of haematological cancers.

As for the comparison of KIR genotype frequencies, we didn't see any significantly different distribution of AA and Bx genotypes between the two groups (P= 0.902). We have observed two statistically significant frequencies for the Bx3 and Bx439 genotypes, both more often present in the patients group (P= 0.019 and P= 0.009, respectively). However, the KIR gene content of the two genotypes is quit different, the former

containing all the known KIR genes except KIR2DS3, and the later lacking 6 genes (both inhibitory and activating) (Table 3), we were not able to draw a conclusion that these might be prototypic KIR genotypes that would increase susceptibility for haematological cancer.

For many of the different forms of haematological cancers, the transplantation of haematopoietic stem cells from bone marrow or peripheral blood either from related or unrelated donors, has become widely recognized as the only curative treatment over the last

three decades. Today, it is generally believed that donors containing at most one activating KIR gene should be avoided if possible, because they could more often lead to severe acute GVHD [25 - 28]. Our next goal would be to analyze the distribution of KIR genes and the success rate in transplanted pairs of patients with haematological cancer and their corresponding bone marrow donor.

We found that KIR gene frequencies between the total 24 donors and healthy Macedonians reveals statistically significant difference for *KIR2DS1* ($F=0.481$ in the controls group, and 0.76 in the patients group, $p=0.004$). This significance is even higher when the frequency of *KIR2DS1* in controls is compared with the frequency in donors from pairs with GVHD ($F=0.923$, $P=0.002$). Another significant difference was observed for the frequency of the full-length allele of *KIR2DS4*001-002*, present in 25.2% of the control individuals, but in as much as 81.8% of the recipients of HSC ($P=0.0005$) and suggest that the *KIR2DS4*001/002* might be a predisposing factor for severe GVHD in sibling HSCT [29].

We investigated the role of KIR genes in some viral infections in the Republic of Macedonia. Comparison of *KIR* gene frequencies between critically ill H1N1/09 Macedonian patients and healthy subjects reveals statistically significant difference for frequency of *KIR2DL1* ($F=1$ in the patients group, and 0.94 in the control group, $p=0.045$). There is evident predominance of *KIR* activating genes in the group of patients with severe disease, compared to the healthy controls [30]. Recently we published that comparison of KIR frequencies between Macedonian patients with West Nile virus infection and healthy Macedonian population reveals several significant differences in the inhibitory group (*KIR2DL2*), and in the non inhibitory group (*KIR2DS1*, *KIR2DS2*, *KIR2DS5*, and *KIR3DS1*). The single most frequent genotypes in the Bx group were genotypes ID71 and ID89 with statistically significant difference compared to healthy Macedonians. Our results suggest that specific *KIR* genotypes could be connected with West Nile virus infection [31].

In conclusion, our results address the distribution of the KIR genes and genotypes in Macedonian patients with haematological cancers. Although we have not determined a certain KIR gene and/or genotype as either predisposing, or protecting factor for haematological malignancy in patients from Republic of Macedonia, we believe that our results could be useful in further (perhaps META) analysis, involving larger series of patients and targeted at the ligands of the KIRs

that might suggest involvement of certain combinations of these genes in the development of haematological cancers.

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