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ICOS gene polymorphisms in B-cell chronic lymphocytic leukemia in the Polish population

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Abstract: There is strong evidence that altered immunological function entails an increased risk of B-cell chronic lymphocytic leukemia (B-CLL). The main mechanism of an anti-tumor response depends on T-cell activation. Unlike the constitutively expressed CD28, inducible costimulatory molecule (ICOS) is expressed on the T-cell surface after activation. ICOS enhances all the basic T-cell responses to a foreign antigen, namely proliferation, secretion of lymphokines, the upregulation of molecules that mediate cell-cell interaction, and effective help for antibody secretion by B cells. ICOS is essential for both efficient interaction between T and B cells and normal antibody responses to T cell-dependent antigens. It does not upregulate the production of interleukin-2, but superinduces the synthesis of interleukin-10. Our previous results indicated the *ICOS* gene has a role as a susceptibility locus to B-CLL. Therefore an extended study was undertaken to evaluate the association between four *ICOS* polymorphisms (which were recently described as functional ones) and susceptibility to B-CLL in the Polish population. A case-control study of 296 individuals, including 146 B-CLL patients, was conducted on four polymorphisms in the *ICOS* gene. Genotyping of the polymorphisms ICOS ISV1+173T>C (rs10932029), ICOSc.1624C>T (rs10932037), ICOSc.2373G>C (rs4675379), and ICOSc.602A>C (rs10183087) was carried out using allelic discrimination methods with the TaqMan® SNP Genotyping Assay. There were no statistically significant differences in the allele, genotype, or haplotype distributions between B-CLL patients and healthy controls for any of the investigated polymorphic markers in the *ICOS* gene. However, we noted that patients carrying genotype ICOS ISV1+173T>C [TT], ICOSc.602A>C [AA], ICOSc.1624C>T [CC], and ICOSc.2373G>C [GG] have a decreased frequency of progression to a higher Rai stage during 60-month follow-up (21.35% vs. 40.8%, p = 0.013) compared to other individuals. This indicates that the investigated polymorphisms do not modulate the risk of B-CLL in the Polish population, but are associated with disease dynamics, in particular with the time to Rai stage progression. (*Folia Histochemica et Cytobiologica 2011; Vol. 49, No. 1, pp. 49–54*)

Key words: ICOS, polymorphisms, B-CLL

Introduction

There is strong evidence that altered immunological function entails an increased risk of lymphoma [1]. The main mechanism of an anti-tumor response depends on T-cell activation [2]. Naive T cells need two signals for full activation. The first antigen-specific signal is provided by the interaction of T cells with a peptide of the major histocompatibility complex (MHC) on antigen-presenting cells (APCs), whereas the second signal is delivered by a T-cell co-stimulatory molecule [3]. CD28 is the primary T-cell co-stimulatory molecule constitutively expressed on the majority of T cells. Upon interaction with its ligands CD80 and CD86, CD28 transduces a signal that enhances the activation and proliferation of T cells [4, 5].

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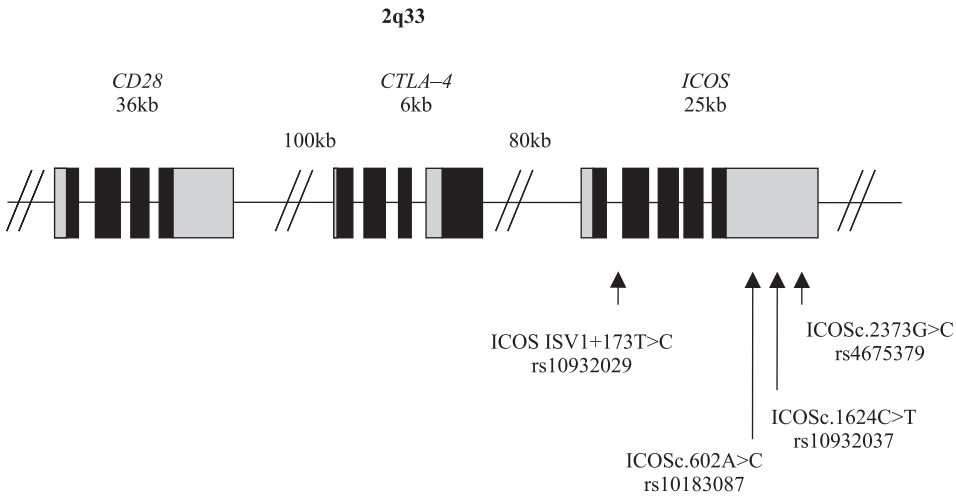

Figure 1. Structure of genes on chromosomal region 2q33 harboring the *CD28, CTLA-4* and *ICOS* genes and location of studied polymorphisms (distances not to scale)

Inducible co-stimulator (ICOS) plays a role in T-cell activation which is critical, independent, and synergic with CD28 signaling. ICOS augments the effector T-cell cytokine response, in particular the production of IL-4, IL-5, IL-10, IFN-alpha, and IFN-gamma [6], but not IL-2 [7]. ICOS is expressed at low levels on resting naive T cells and is rapidly upregulated following TCR ligation and CD28 stimulation [7, 8].

After activation, ICOS is expressed on unpolarized $CD4^+$ cells as well on Th1, Th2, Th17, and Treg subpopulations [8–12]. This co-stimulatory molecule binds the B-7-related protein B7RP-1 [13]. ICOS knockout mice have reduced $CD4^+$ T-cell responses and increased risk of experimental autoimmune encephalomyelitis [14], defects in immunoglobulin class switching, and germinal center formation [15].

Homozygous loss of the *ICOS* gene is the cause of the ICOS deficiency (ICOSD) form of common variable immunodeficiency (CVID), which is characterized by recurrent bacterial infections of the respiratory and digestive tracts characteristic of humoral immunodeficiency, but without other complicating features such as splenomegaly, autoimmune phenomena, or sarcoid-like granulomas and the absence of clinical signs of overt T-cell immunodeficiency [16]. Severe disturbance of T cell-dependent B-cell maturation occurs in secondary lymphoid tissue; B cells exhibit a naive IgD^+/IgM^+ phenotype and the numbers of IgM memory and switched memory B cells are substantially reduced in those individuals [16].

The *ICOS* gene, located on chromosome 2q33 in a region adjacent to the *CTLA-4* and *CD28* genes, contains five exons. Exons 1–4 are parallel to those of *CD28* and *CTLA-4*, where exon 1 encodes the leader peptide, exon 2 the ligand binding domain, exon 3 the transmembrane segment, and exon 4 determines the cytoplasmic tail, while exon 5 encodes an additional fragment of the cytoplasmic tail (Figure 1). Several polymorphisms have been found in the *ICOS* gene: two microsatellite in the fourth intron and 31 single-nucleotide polymorphisms (SNPs) (http://www.hapmap.org). None of the described *ICOS* SNPs leads to changes in amino acid, while a few have been demonstrated to be functional variants [17–20]. The chromosomal region of 2q33 harboring the *CD28, CTLA-4*, and *ICOS* gene family has been described as carrying predisposing genes for several autoimmune diseases [19, 21–23] and, recently, for cancer [24–26]. Our recent results indicate that the *ICOS* microsatellite polymorphism c.1554+4GT(8_15) is associated with susceptibility to B-CLL [24].

This current study investigated the association between ICOS ISV1+173T>C (rs10932029), ICOSc.602-A>C (rs10183087), ICOSc.1624C>T (rs10932037), and ICOSc.2373G>C (rs4675379), which have been recently reported to be functional polymorphisms, with susceptibility to B-CLL. The ICOSc.1624C>T polymorphism was shown to influence *ICOS* mRNA level [17] and the ICOSc.602A>C, ICOSc.1624C>T, and ICOSc.2373G>C SNPs influence functions of the *ICOS* gene [19]. The ICOS ISV1+173T>C polymorphism, located close to the *CTLA-4* gene, has been reported to affect the expression of CTLA-4 isoforms [17].

Material and methods

Study population

The study group comprised 146 B-CLL patients (66 females and 78 males) with a mean age of 66.23 ± 11.14 years. The mean age at diagnosis was 62.23 ± 10.64

years. Diagnosis of B-CLL was established on the basis of standard clinical and laboratory criteria following the National Cancer Institute recommendations. B-CLL stage 0 was found in 43 patients, and stages I, II, III, and IV were diagnosed in 50, 26, 10 and 17 cases, respectively, according to Rai staging [27].

Mean follow-up was 77.6 months (range: 12–180 months) and data was available for 123 B-CLL patients. During 60 months of follow-up, lymphocyte counts doubled in 32 patients in the peripheral blood, and in five patients later. In 38 patients, progression to a higher Rai stage occurred.

Chlorambucil was given as the front-line therapy to all patients requiring treatment and purine analogue-based protocols were used in the treatment of refractory/relapsed cases.

The control population comprised 150 healthy age-matched subjects (70 females, 80 males) originating from the same geographical area as the patients.

Genotyping/determination of polymorphisms

Genomic DNA was isolated using the NucleoSpin®Blood kit (Macherey-Nagel, Germany) from whole frozen blood. Genotyping of polymorphisms was carried out using allelic discrimination methods with the TaqMan® SNP Genotyping Assay: ICOS ISV1+173T> C (rs10932029), assay ID C___430013_10; ICOSc.1624C>T (rs10932037), assay ID C__30981474_10; ICOSc.2373-G>C (rs4675379), assay ID C__27968684_10; and ICOSc.602A>C (rs10183087), assay ID-C__30421029_10 (Applied Biosystems, Foster City, CA, USA).

Statistical analyses

Evaluation of the Hardy–Weinberg equilibrium (HWE) was performed for the whole studied group by comparing the observed and expected frequencies of genotypes using χ^2 analysis. The χ^2 test was used to compare categorical date between patients with B-CLL and controls. Differences were considered statistically significant if the p value was < 0.05. Because of the multiple comparisons of genotype and haplotype frequencies, Bonferroni multiple adjustments were employed to the level of significance. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated using (http://www.quantitativeskills. com/sisa/statistics/twoby2.htm) software [28]. Linkage disequilibrium (LD) coefficients $D' = D/D_{max}$ and r^2 values for the pair of the most common alleles at each locus and haplotype frequency for pairs of alleles was estimated using the SHEsis program (http://202.120.7.14/analysis/myAnalysis.php) [28]. The cumulative probabilities of survival without lymphocyte doubling (lymphocyte-doubling free survival) and of survival without progression to a higher Rai stage (progression–free survival) during 60-month follow-up were calculated according to the Kaplan–Meier method. We used as endpoints the doubling of peripheral lymphocyte count and progression to a higher Rai stage. Kaplan–Meier curves were compared using the log-rank test.

Results

The distributions of the alleles and genotypes of all the studied polymorphisms in the B-CLL patients and the healthy control group are shown in Table 1. Neither in the cases, nor in the controls, was deviation from Hardy–Weinberg equilibrium observed (Table 1). The distributions of the alleles and genotypes for all the studied polymorphisms were similar in the patients and controls (Table 1). No significant prevalence of the ICOSc.2373G>C[G] allele in B-CLL patients compared to controls (89.2 vs. 86.1%) was noted.

The global distributions of the haplotypes did not differ in the cases and controls ($p = 0.5$). The frequency of haplotype ICOS ISV1+173T>C[T], ICOSc.1624-C>T[C], ICOSc.602A>C[A], ICOSc.2373G>C[G] was the highest and similar in both the studied groups (74.0% vs. 71.2%). The haplotype ICOS ISV1+173-T>C[C], ICOSc.1624C>T[C], ICOSc.602A>C[A], ICOSc.2373G>C[G] was overrepresented in the B-CLL patients, but the difference was not statistically significant (5.9% vs. 3.6%, OR 1.6, $p = 0.24$).

The features of the *ICOS* gene polymorphisms were subjected to analysis for correlation with clinical data, i.e. gender, age at diagnosis, peripheral lymphocyte doubling time, and the time to Rai stage progression. No associations were observed between any of the *ICOS* gene polymorphisms and gender, age at diagnosis, survival, or peripheral lymphocyte doubling time. Kaplan–Meier analysis showed that the ICOS ISV1+173T>C and ICOSc.602A>C polymorphisms influenced the time of Rai progression. During 60 months of follow-up, only 24.4% of the patients with ICOS ISV1+173T>C [TT] progressed to a higher Rai stage, compared to 44.5% of the patients carrying [CT] ($p = 0.0248$) (Figure 2A). Additionally, the ICOSc.602A>C polymorphism showed an association with decreased frequency of progression to a higher Rai stage. Progression of Rai stage during the 60 month follow-up occurred only in 21.5% of the patients with ICOSc.602A>C [AA] and in 39.5% of the patients carrying the C allele [CA+CC genotype] ($p = 0.049$) (Figure 2B).

Moreover, homozygosity in the most frequent genotype (AA according to Castelli et al. [19]) ICOS ISV1+173T>C[TT], ICOSc.1624C>T[CC], ICOSc.602A>C[AA], ICOSc.2373G>C[GG] was associated with a significantly decreased frequency

Table 1. ICOS ISV1+173T>C, ICOSc.602A>C, ICOSc.1624C>T, ICOSc.2373G>C genotypes and alleles frequencies in B-CLL patients and controls

			B-CLL n = 146	Controls group n = 150	Global p	OR	95% CI
ICOS ISV1+173T>C	Genotype	T T T C C C	112 (0.778) 32 (0.222) 0 (0.0)	119 (0.815) 26 (0.178) 1 (0.007)	0.46	Reference 1.25 –	 0.70–2.21 –
	Allele	T C	256 (0.888) 32 (0.112)	264 (0.904) 28 (0.096)	0.64	0.88 1.13	0.52–1.52 0.66–1.92
ICOSc.1624C>T	Genotype	CC CT TT	119 (0.826) 24 (0.167) 1 (0.007)	125 (0.850) 19 (0.129) 3 (0.020)	0.43	Reference 1.32 0.35	 0.69–2.53 0.04–3.40
	Allele	C T	262 (0.910) 26 (0.090)	269 (0.915) 25 (0.085)	0.82	0.93 1.07	0.52–1.65 0.69–1.92
ICOSc.602A>C	Genotype	AA AC CC	90 (0.625) 50 (0.347) 4 (0.028)	93 (0.628) 48 (0.324) 7 (0.047)	0.65	Reference 1.08 0.59	 0.66–1.76 0.17–2.08
	Allele	A C	230 (0.799) 58 (0.201)	234 (0.791) 62 (0.209)	0.81	1.05 0.95	0.70–1.57 0.64–1.43
ICOSc.2373G>C	Genotype	G G G C C C	114 (0.792) 29 (0.201) 1 (0.007)	112 (0.757) 31 (0.209 5 (0.034)	0.26	Reference 0.92 0.20	 0.52–1.63 0.03–1.70
	Allele	G C	257 (0.892) 31 (0.108)	255 (0.861) 41 (0.139)	0.26	1.33 0.75	0.81–2.17 0.46–1.24

of progression to a higher Rai stage during 60-month follow-up (21.35% vs. 40.8%, p = 0.013) compared to the other individuals (Figure 2C).

The observed linkage disequilibria between investigated polymorphisms were expressed only by D', but not by r^2, and were as follows: ICOS ISV1+173T>C and ICOSc.1624C>T (D'= 0.929, r^2 = 0.009), ICOSc.1624C>T and ICOSc.602A >C (D'= 0.914, r^2 = 0.302), ICOSc.1624C>T and ICOSc.2373G>C (D'= 1, r^2 = 0.013), ICOSc.602A>C and ICOSc. 2373G>C (D'= 0.883, r^2 = 0.424).

Discussion

The development of B-CLL may be regarded as a failure of immunological surveillance; therefore genes involved in the regulation of the immunological response might be considered as predisposing loci for disease development. Our previous study indicated that CTLA-4g.319C>T, CD28c.17+3T>C, and microsatellite ICOSc.1554+4GT(8_15) polymorphisms are associated with susceptibility to B-CLL [24].

In the present study, we extended our investigation to subsequent *ICOS* gene polymorphisms which were recently described as functional ones [17, 19] and were shown to be associated with susceptibility to multiple sclerosis [19] and outcome after renal [18] or hematopoietic stem cell transplantation [29], but not to melanoma [30].

Kaartinen et al. [17] showed that activated $CD4^+$ T cells from ICOSc.1624C>T [CC] homozygous persons had higher actual levels of *ICOS* mRNA than cells from [TC] heterozygous persons after 1 h and 3 h of activation, after which this difference disappeared. Castelli et al. [19], investigating variations in the *ICOS* gene 3'UTR, showed the presence of three major haplotypes associated with different capacities of expression of ICOS in $CD3^+$ cells and IL-10 secretion. The polymorphisms we chose identify the same haplotypes, since it was shown that SNPs at positions +602, +1564 and +2007 are in complete LD and the same is true for +930, +1495, +1624, and +1862. The AA genotype characterized by the presence of ICOSc.1624C>T [CC], ICOSc.602A>C[AA], and ICOSc.2373G>C[GG] was shown to be associated with the lowest percentage of $CD3^+$ activated cells expressing ICOS and the highest IL-10 secretion. Moreover, according to an Italian study [19], the frequency of the AA genotype was significantly lower in MS patients with relapsing-remitting disease than in controls, and those patients had a lower relapse rate and MS severity score.

The ICOSc.602A>C and ICOSc.1624C>T polymorphisms have been described as susceptibility markers of outcome after renal transplantation [18] and hematopoietic stem cell transplantation [29]. The ICOSc.602A>C and ICOSc.1564T>C SNPs were

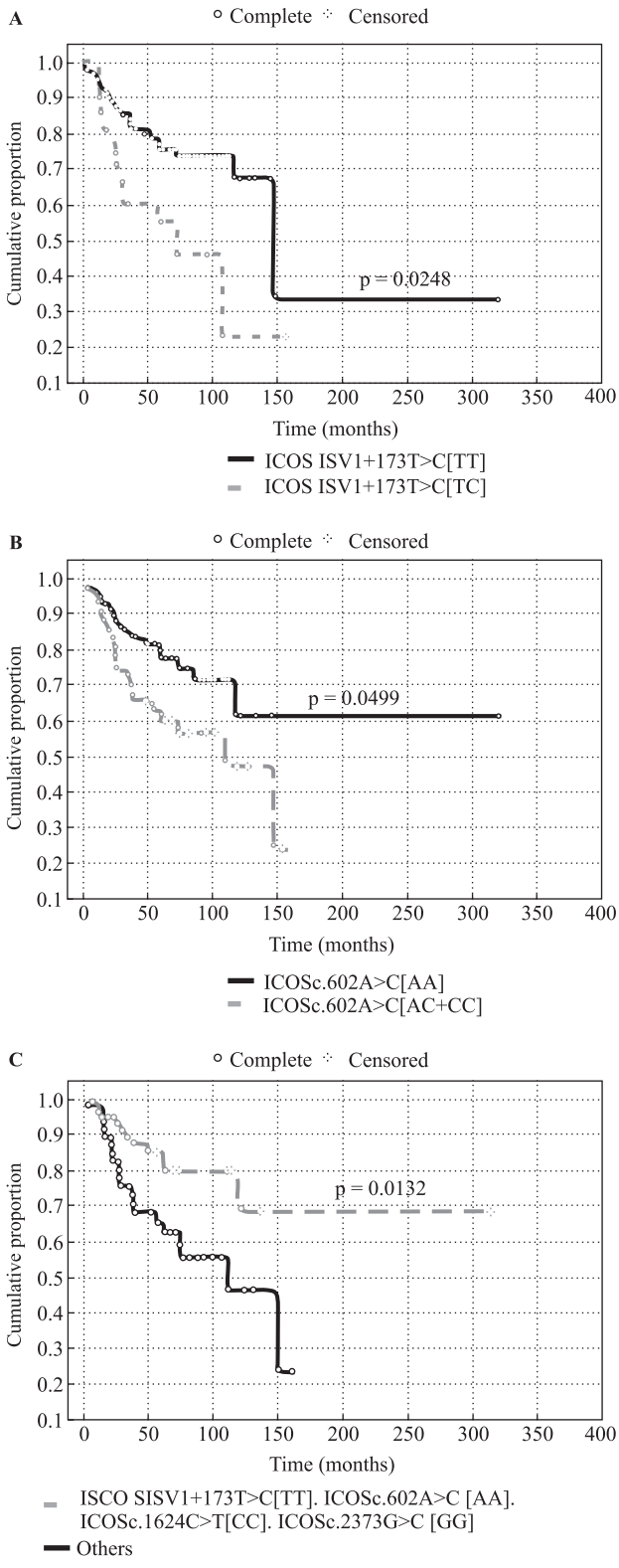

Figure 2. Influence of *ICOS* gene polymorphisms on Rai stage progression. **A** Kaplan–Meier estimate of Rai stage progression according to: **A.** ICOS ISV1+173T>C; **B.** ICOSc.602A>C; **C.** and combined ICOS ISV1+173T>C, ICOSc.602A>C, ICOSc.1624C>T, ICOSc.2373G>C

shown to be associated with delayed graft function and the ICOSc.1624C>T polymorphism with graft survival after renal transplantation. Hematopoietic stem cell transplantation recipients who received a graft from a donor with ICOSc.602A>C [CC] genotype had worse disease-free survival, and recipients of homozygous ICOSc.1624C>T [TT] had worse overall survival.

Additionally, we included the ICOS ISV1+173T>C polymorphism in our study, which does not influence *ICOS* mRNA level in $CD4^+$ cells but, since this polymorphism is located near the *CTLA-4* gene, variations at that position influence CTLA-4 mRNA expression for both CTLA-4 isoforms, the soluble and full length (membrane) [17].

Our study found linkage disequilibria between the polymorphisms ICOSc.602A>C, ICOSc.1624C>T, and ICOSc.2373G>C similar to those described by the Italian group [19]. Weak LD was found between ICOS ISV1+173T>C and ICOSc.1624C>T, while the ICOSc.602A>C and ICOSc.2373G>C genetic markers are not in linkage disequilibrium with the ICOS ISV1+173T>C polymorphism.

We could not find any statistical differences in the frequencies of alleles, genotypes, or haplotypes of any of the investigated polymorphisms between B-CLL patients and controls. However, we noted that ICOS ISV1+173T>C [TT] alone and ICOSc.602A>C [AA] alone and together as part of the genotype AA according to Castelli et al. [19]: ICOS ISV1+173T>C [TT], ICOSc.602A>C [AA], ICOSc.1624C>T [CC], and ICOSc.2373G>C [GG], were associated with a lower rate of disease progression. Only about 20% of homozygous patients carrying the genotype ICOS ISV1+173T>C [TT], ICOSc.602A>C [AA], ICOSc.1624C>T [CC], and ICOSc.2373G>C [GG] increased Rai stage during the 60 months of follow-up, but more than 40% of the patients possessing other genotypes did.

We cannot yet provide an explanation for this finding. As a matter of fact, the AA genotype of the *ICOS* gene is supposed to impair the expression of ICOS molecule [19] and thus impair the T cell-mediated response and T-cell dependent maturation of B-cells. This finding warrants further studies of the role of *ICOS* gene polymorphism in B-CLL dynamics.

To sum up, although the functional genetic markers of the *ICOS* gene ICOSISV1+173T>C, ICOSc.602A>C, ICOSc.1624C>T, and ICOSc.2373-G>C we chose were not found to be associated with B-CLL susceptibility in the Polish population, they were associated with disease dynamics, in particular with the time to Rai stage progression.

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