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Study of *ICOS* gene polymorphisms associations with the risk and clinical course of multiple myeloma

Badanie związku polimorfizmów genu *ICOS* z ryzykiem zachorowania na szpiczaka mnogiego i przebiegiem choroby

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
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Summary

In multiple myeloma (MM) patients, various T-cell abnormalities, such as a marked reduction in the proportions of CD4 and CD8 cells expressing co-stimulatory molecules, signal transduction components, Th1/Th2 imbalance and alterations of regulatory T cells/T helper 17 cells (Treg/Th17 ratio), have been observed. The inducible T-cell co-stimulator (ICOS) has been implicated in the induction and regulation of Th1, Th2, and Treg/Th17 immunity. Therefore, we postulate that variations in *ICOS* gene might be associated with susceptibility and clinical course of MM. We analyzed ICOSISV1+173T>C (rs10932029), ICOSc.1624C>T (rs10932037), ICOSc.602A>C (rs10183087), ICOSc.1564T>C (rs4404254) and ICOSc.2373G>C (rs4675379) polymorphisms in 205 MM patients and 325 controls with TaqMan® SNP Genotyping Assays.

None of the investigated *ICOS* gene polymorphisms were associated with susceptibility to the disease. However, in a multivariate Cox analysis which included the age of diagnosis, ISS, time to the clinical response to the treatment, gender, immunoglobulin classes, *ICOS* gene variations, we found that time to the response to treatment, ISS stage 3 and possessing of ICOSc.2373G>C [C+] allele were independently associated with the overall survival (HR: 1.35, 2.86 and 3.77, respectively).

Although the result of the present study does not confirm the association of the investigated polymorphisms with risk of MM, they indicated that variations in *ICOS* gene might influence overall survival.

Keywords: **ICOS • gene polymorphisms • MM**

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INTRODUCTION

Multiple myeloma (MM) is a malignant disorder characterized by uncontrolled proliferation of clonal plasma cells causing of wide variety complications that lead to organ dysfunction and eventually death. Typical symptomatic presentation of MM includes hypercalcemia, renal failure, anemia, lytic bone lesion, and/or fractures collectively known as "CRAB" [29].

Previous studies reported that MM patients may exhibit a variety of T-cell abnormalities, such as a marked reduction in the proportions of CD4 and CD8 cells expressing co-stimulatory molecules, signal transduction components, and Th1/Th2 imbalance, particularly in advanced stages of MM [2, 5, 10, 26, 27]. The bone marrow microenvironment plays an additional role resulting in the dysfunction of dendritic cell, which is associated with impaired antigen processing and presentation [4].

T cell response begins from T cell activation and is followed by T-cell proliferation and differentiation to the effector cells. The full activation of naïve T-cells requires two independent signals. The first signal is sent via a T-cell receptor (TCR) after the recognition of antigen presented by MHC molecule expressed on antigen presenting cell. The second signal, termed co-stimulation, is critical for allowing full activation, sustaining cell proliferation, preventing anergy and/or apoptosis, inducing differentiation to effector and memory status, and allowing cell-cell cooperation. The primary, constitutively expressed on the majority of T-cells, co-stimulatory molecule is CD28 [38]. The inducible T cell co-stimulator (ICOS) [17] is the next member of the immunoglobulin (Ig) family of co-receptor molecules which play a critical, independent, and synergic with CD28 signaling role in T-cell activation.

Initially, ICOS was identified in humans [17] and, thereafter, in mice [22]. ICOS has significant homology to CD28 and the immune-attenuator cytotoxic T-cell antigen 4 (CTLA-4) [17, 32]. Similarly to CTLA-4, ICOS is not constitutively expressed on resting T cells, but is instead rapidly induced following TCR cross-linking and/or CD28 co-stimulation [17, 44]. Same as CD28 and CTLA-4, ICOS is expressed on activated CD4 and CD8 T cells [39], which suggests that ICOS also regulates the adaptive T cell response. After activation, ICOS is expressed on unpolarized CD4+ cells as well on Th1, Th2, Th17, and Treg subpopulations [1, 6, 25, 28, 37]. This co-stimulatory molecule binds the B-7-related protein B7-H2(ICOSL) [44].

While a number of early studies evaluated the function of ICOS in T cell activation, proliferation, and differentiation, the study on ICOS and ICOS ligand (ICOSL)-deficient mice revealed that this co-stimulatory molecule played a significant role in the generation of class-switched antibodies (Abs) against thymus-dependent (TD) antigens, which was attributed to a reduction in the number and size of germinal centers (GCs) in the spleen in the absence of ICOS signaling. Long-lived plasma cells (LLPCs) and memory B cells (MBCs) that have undergone class-switching recombination (CSR) and somatic hypermutation to increase Ab affinity are products of GC reactions. These cell types and the Abs which they produce are thought to be critical for maintaining life-long protection against pathogens following exposure or vaccination, or for contributing to the development of a number of autoimmune diseases. The recent studies regarding the function of ICOS have focused on how ICOS–ICOSL interactions contribute to GC-derived Abs production [40].

The functional significance of ICOS is confirmed when ICOS deficiency (ICOSD) is considered. Homozygous loss of *ICOS* gene causes this disease, which takes the form of common variable immunodeficiency (CVID). ICOSD disease is characterized by the recurrent bacterial infections of the respiratory and digestive tracts (characteristic for 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 [13]. Moreover, a severe disturbance of T cell-dependent B-cell maturation is present in secondary lymphoid tissue; B cells exhibit a naive IgD+/IgM+ phenotype and the numbers of IgM memory and switched memory B cells are significantly reduced in patients with ICOSD [13].

Multiple myeloma is characterized by increased numbers of malignant plasma cells (PC) in the bone marrow and a high level of monoclonal Abs [21]. MM cells originate from GC plasma cells, since those cells have

mutated variable regions and have undergone CRS. The rearrangement of the developmental process often underlies the process of carcinogenesis and a similar process occurs in myeloma [4].

The next developmental step includes the differentiation of the immature B cells located within germinal center to a matured antibody – secreting cells in bone marrow. This process stops or silences cellular functions unnecessary for antibody production, while simultaneously switching on key programs to make and secrete the antibody. Many molecules/transcriptional factors regulating the differentiation process are of key importance in the pathogenesis of multiple myeloma (reviewed in [4]). Considering the pivotal role of ICOS in generating class-switched antibodies and in reducing the number and size of germinal centers, we have presumed that disturbances in ICOS expression might influence susceptibility to MM. Therefore, we focused our attention on selected single nucleotide polymorphisms (SNPs) in the *ICOS* gene and their influence on disease susceptibility and clinical outcome. The selection of polymorphisms was based on our previous results [20] and the literature data, considering their influence on the level of mRNA expression or the susceptibility to diseases [7, 14, 15, 19].

MATERIALS AND METHODS

Patients

The study enrolled 205 unrelated Polish patients with multiple myeloma from two centers: from the Department of Haematology, Neoplastic Diseases & Bone Marrow Transplantation of the Wroclaw Medical University and from the Department of Haematology of the State Hospital in Opole. The diagnosis of multiple myeloma was based on criteria established by the International Myeloma Working Group [9]. The clinical stage was assessed according to the International Staging System (ISS) for Multiple Myeloma and was determined during enrolment in the study [12], i.e. stage 1 with serum β 2microglobulin less than 3.5 mg/l + serum albumin ≥3.5 g/dl, stage 2 with neither stage 1 nor 3, and stage 3 with serum β2microglobulin level ≤5.5 mg/l. The patients were treated according to the melphalan + prednisone (MP); vincristine, doxorubicin (Adriamycin), dexamethasone (VAD); vincristine, melphalan, cyclophosphamide, prednisone (VMCP); or cyclophosphamide, thalidomide, dexamethasone (CTD) protocol. Table 1 summarizes the clinical characteristics of the MM patients.

The control group included 325 healthy Polish individuals originating from the same geographical area as the patients (182 female and 143 male) with the majority recruited from the blood bank in Wroclaw and others recruited from among employees of the Hirszfeld Institute of Immunology and Experimental Therapy. Genetic homogenity of the Polish population is observed, as reflected in virtually identical frequencies of H-Y polymorphisms in different regions of Poland [31]. All enrolled participants were informed about the study protocols and consent was obtained from each individual. Data on the participation rate was not available. The study was approved by the local ethics committee.

Genotyping/Determination of polymorphisms

Genomic DNA was isolated using the NucleoSpinBlood kit (MARCHEREY-NAGEL, Germany) from whole frozen

Table 1. Patients' characteristics

Number of patients		205
Age (median, st. dev., range)		67±10.7 (33-87)
Age of onset (median, st. dev., range)		63.5±11.2 (32-84)
Gender (female/male)		108/97
International Staging System (ISS)	1	75
	2	68
	3	62
Ig subtype		
	IgG	117
	IgA	45
	IgM	5
	others	38
Light chain type		
	κ	67%
	λ	33%

Table 2. Distribution of alleles and genotypes for the following *ICOS* gene polymorphisms: *ICOS*ISV1+173T>C (rs10932029), *ICOS*c.602A>C (rs10183087), *ICOS*c.1564T>C (rs4404254), *ICOS*c.1624C>T (rs10932037) and *ICOS*c.2373G>C (rs4675379) in MM patients and the controls

ICOS gene polymorphisms			MM N=205	CONTROLS N=325	p	OR [95%CI]
ISV1+173T>C rs10932029	Genotype	T T	166 (81.8)	250 (77.2)	Ref	
		C T	36 (17.7)	72 (22.2)	0.21	0.75 [0.48-1.18]
		C C	1 (0.5)	2 (0.6)	0.82	0.75 [0.07-8.37]
	Allele	T	368 (90.6)	572 (88.3)	Ref	
		C	38 (9.4)	76 (11.7)	0.23	0.78 [0.52-1.17]
c.602A>C rs10183087	Genotype	A A	134 (66.3)	200 (62.1)	Ref	
		A C	63 (31.2)	108 (33.5)	0.48	0.87 [0.60-1.27]
		C C	5 (2.5)	14 (4.3)	0.24	0.53 [0.19-1.51]
	Allele	A	331 (81.9)	508 (78.9)	Ref	
		C	73 (18.1)	136 (21.1)	0.23	0.82 [0.60-1.13]
c.1564T>C rs4404254	Genotype	T T	136 (67.7)	195 (62.5)	Ref	
		T C	60 (29.9)	103 (33.0)	0.36	0.84 [0.57-1.23]
		C C	5 (2.5)	14 (4.5)	0.21	0.51 [0.18-1.46]
	Allele	T	332 (82.6)	493 (79.0)	Ref	
		C	70 (17.4)	131 (21.0)	0.16	0.79 [0.58-1.09]
c.1624C>T rs10932037	Genotype	C C	167 (83.1)	260 (81.0)	Ref	
		C T	34 (16.9)	58 (18.1)	0.70	0.91 [0.57-1.45]
		T T	0	3 (0.9)	0.32	0.22 [0.01-4.33]
	Allele	C	368 (91.5)	578 (90.0)	Ref	
		T	34 (8.5)	64 (10.0)	0.42	0.83 [0.54-1.29]
c.2373G>C rs4675379	Genotype	G G	166 (81.0)	221 (78.6)	Ref	
		G C	38 (18.5)	52 (18.5)	0.91	0.97 [0.61-1.55]
		C C	1 (0.5)	8 (2.8)	0.09	0.17 [0.02-1.34]
	Allele	G	370 (90.2)	494 (87.9)	Ref	
		C	40 (9.8)	68 (12.1)	0.25	0.79 [0.52-1.19]

blood. Genotyping of polymorphisms were done with use of allelic discrimination methods with the TaqMan SNP Genotyping Assay: *ICOSISV1+173T>C* (rs10932029), assay ID C___430013_10; *ICOSc.1624C>T* (rs10932037), assay ID C__30981474_10; *ICOSc.2373G>C* (rs4675379), assay ID C__27968684_10; and *ICOSc.602A>C* (rs10183087), assay ID- C__30421029_10 (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for both studied groups by comparing the observed and expected frequencies of genotypes using χ^2 test. The χ^2 test was used to compare categorical data between MM patients and the controls. 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. Haplotype estimation was carried out using the SHEsis program (http://202.120.7.14/analysis/myAnalysis.php) [34]. The cumulative probabilities of overall survival were performed using the Statistica 13 software. Survival curves were calculated by the Kaplan-Meier method and were compared by the log-rank test. Multivariate analysis of prognostic factors was performed using Cox's regression model. P-values <0.05 were considered to be statistically significant.

Results

The distributions of the alleles and genotypes of all the studied polymorphisms in the group of multiple myeloma patients and the healthy control group are shown in Table 2. Neither in the cases nor in the controls was the deviation from Hardy-Weinberg equilibrium observed (Table 2). The distributions of alleles and genotypes for all the studied polymorphisms were similar in the patients and the controls (Table 2). The global distributions of the haplotypes did not differ in the cases and controls (p=0.56). The frequency of the haplotype, which consisted of wild alleles *ICOSISV1+173T>C[T]/ICOSc.602A>C[A]/ICOSc.1564T>C[T]/*

Table 3. Estimated analysis of haplotype frequencies of SNPs in *ICOS* gene: *ICOS*ISV1+173T>C (rs10932029), *ICOS*c.602A>C (rs10183087), *ICOS*c.1564T>C (rs4404254), *ICOS*c.1624C>T (rs10932037) and *ICOS*c.2373G>C (rs4675379) in the group of patients with MM compared with the controls

Haplotype	MM (freq)	Controls (freq)	p	OR [95%CI]
CATCG	20.4 (5.2)	29.3 (5.3)	0.87	0.95 [0.53-1.71]
CCCCC	13.8 (3.5)	27.2 (4.9)	0.27	0.69 [0.36-1.33]
TATCG	301.7 (77.0)	396.6 (71.3)	0.21	1.21 [0.89-1.65]
TCCCC	21.2 (5.4)	31.1 (5.6)	0.83	0.94 [0.53-1.66]
TCCTG	32.13 (8.2)	52.1 (9.4)	0.45	0.84 [0.53-1.33]

ICOSc.1624C>T[C]/ICOSc.2373G>C[G], was the highest and slight, insignificant overpresence of it was observed in MM patients as compared to the controls (77.0% vs. 71.3%, OR 1.21, 95% CI 0.89-1.65) (Table 3).

The polymorphisms located in 3' UTR gene region were described by Castelli et al. as creating a functional haplotype, which means that they influence ICOS mRNA expression and susceptibility to multiple sclerosis [7]. Therefore, we analyzed whether the distribution of haplotype *ICOSc.602A>C[A]/ICOSc.1564T>C[T]/ICOSc.1624C>T[C]/ICOSc.2373G>C[G]*, which is a part of "Castelli" haplotype, differed between MM patients and controls. We showed that this haplotype was more frequently observed in the MM patients, but the difference did not reach statistical significance (82.1% vs. 76.6%, OR 1.25, p = 0.19) (Table 4).

Separate analyses in men and women were performed in the group of MM patients and the controls. The distribution of alleles, genotypes and haplotypes in women with MM did not differ from that of healthy women. However, in the male subgroup, we noticed a difference in the distribution of the alleles *ICOS*c.1564T>C in MM male patients compared to healthy men (p=0.08). Moreover, we observed that MM male patients with *ICOS*c.1564T>C [TT] genotype were more prone to MM than patients carrying the C allele [TC+CC genotype], but differences did not reach statistical significance (OR 1.63, 95%CI 0.93-2.86, p=0.09) (data not shown).

Then, we analyzed the influence of *ICOS* gene polymorphisms on the clinical outcome of multiple myeloma. We have not observed differences regarding the response to treatment. None of the investigated polymorphisms were associated with a complete or partial remission after first--line therapy as compared to patients in whom the disease remained either stable or progressed (data not show).

The *ICOS* gene variations investigated here together with the age of diagnosis, gender, ISS, time to the clinical response to the treatment, immunoglobulin classes, were employed to univariate and multivariate analyses to assess the independent association with the overall survival.

The results of multivariate analysis showed that a longer response time to the first line therapy, third stage of the disease defined by ISS score and the presence of C allele for *ICOSc.2373G>C* polymorphisms were significantly associated with the risk of death (Table 5). The Kaplan-Meier analysis showed no influence of the *ICOSISV1+173T>C, ICOSc.1624C>T, ICOSc.602A>C* and *ICOSc.1564T>C* SNPs on overall survival.

DISCUSSION

The major abnormalities of T cells are clonal expansions of immunesenescence cells and alterations of regulatory T cells/T helper 17 cells (Treg/Th17 ratio). Therefore, the disturbances in T cell activation process, which might be caused by impaired regulation of this process is of great importance. One of the molecules which regulate the process of T cell activation and differentiation is ICOS. The disturbances in ICOS expression were found in T cell lymphomas, for example in angioimmunoblastic T-cell lymphomas [41]. Moreover, higher ICOS expression is associated with longer survival of melanoma patients, possibly owing to the increased infiltration of memory-effector T cells and elevated anti-tumor T cell activity [3]. We postulated that *ICOS* gene polymorphisms might affect the risk of cancer.

Table 4. Estimated analysis of haplotype frequencies of SNPs in *ICOS* gene: *ICOS*c.602A>C (rs10183087), *ICOS*c.1564T>C (rs4404254), *ICOS*c.1624C>T (rs10932037) and *ICOS*c.2373G>C (rs4675379) (a part of "Castelli" haplotype) in the group of patients with MM compared with the controls

Haplotype	MM (freq)	Controls (freq)	p	OR [95%CI]
ATCG	325.0 (82.1)	425.9 (76.6)	0.19	1.25 [0.90-1.74]
CCCC	36.0 (9.1)	58.3 (10.5)	0.40	0.83 [0.54-1.28]
CCTG	33.0 (8.3)	54.6 (9.8)	0.36	0.81 [0.52-1.28]

Table 5. Cox analysis of the factor influencing overall survival

		Univariate Cox Analysis			Multivariate Cox Analysis		
Variables		p-value	HR	95% CI	p-value	HR	95% CI
Age [years]		0.9867	1.00	0.95-1.05		-	
gender	Female	0.2404	2.18	0.59-8.03	0.1427	2.51	0.73-8.59
Response to the first line therapy (months)		0.1753	1.25	0.91-1.72	0.0368	1.35	1.02-1.79
Immunoglobulin class	IgG	0.3504	1.80	0.52-6.22		-	
Immunoglobulin chain	Lambda	0.0950	2.60	0.85-8.00	0.1493	2.14	0.76-6.05
ISS (1 reference)	2	0.1066	0.46	0.08-2.57	0.1169	0.51	0.10-2.58
ISS (1 reference)	3	0.0145	2.94	0.90-9.57	0.0156	2.86	0.91-9.02
ICOSISV1+173T>C (TT reference)	C+	0.9220	1.09	0.19-6.15		-	
ICOSc.1624C>T (TT reference)	C+	0.3079	4.65	0.24-89.24		-	
ICOSc.602A>C (C+ reference)	A A	0.5353	2.71	0.12-63.13		-	
ICOSc.2373G>C (GG reference)	C+	0.0969	9.47	0.67-134.51	0.0225	3.77	1.21-11.79

HR = Hazard Ratio, CI = Confidence Interval;

ICOS gene is located on chromosome 2q33 in a region neighboring the *CTLA-4* and *CD28* genes. It contains five exons: exons 1-4 are parallel to those of *CD28* and *CTLA-4*, it means 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. The chromosomal region of 2q33 harboring the *CD28*, *CTLA-4*, and *ICOS* gene family has been described as a susceptible region for several autoimmune diseases [7, 11, 14, 18] and, recently, for cancer [8, 30, 36]. Our previous study indicated that the *ICOS* microsatellite polymorphism *c.1554_4GT(8_15)* was associated with susceptibility to B-CLL [36]. Moreover, patients homozygous for *ICOSISV1+173T>C*[TT], *ICOSc.602A>C* [AA], *ICOSc.1624C>T*[CC], and *ICOSc.2373G>C*[GG] have a lower time of progression to a higher Rai stage of disease [20].

The results of this study showed a lack of associations between investigated polymorphisms with the susceptibility to multiple myeloma. The borderline association was found for the c.1564T>C[TT] genotype with the higher risk of the disease in men.

The *ICOS*c.602A>C, *ICOS*c.1564T>C, *ICOS*c.1624C>T, and *ICOS*c.2373G>C SNPs are located in 3'UTR region, which influences mRNA stability [33]. These polymorphisms are a part of SNP sets described by Castelli [7] as creating functional haplotypes of *ICOS* gene. According to this author, the individuals carrying haplotype A (those polymorphisms are the part of A haplotype: *ICOS*c.602A>C[A], *ICOS*c.1564T>C[T], *ICOS*c.1624C>T[C] and *ICOS*c.2373G>C[G]) have higher ICOS mRNA expression, which favors Th2 differentiation and, as a consequence, IL-10 production.

Although we were not able to show the association of the A haplotype with the susceptibility to MM, we noticed an insignificant overrepresentation of this haplotype in MM patients.

Wu at al. reported that *ICOS*c.1564T>C, rs1559931 and *ICOS*c.2373G>C SNPs were associated with susceptibility to colorectal cancer [42]. The authors postulated that the expression of ICOS protein in individuals possessing protective alleles: rs4404254[C], and rs11559931[A], and *ICOS*c.2373G>C [C] is disturbed by the binding of miRNAs (miR-186-5p, miR-1279, miR-2117, miR-3692-3p) with *ICOS* 3'UTR, which results in up-regulation of ICOS receptor on T cells, and consequently protects the individuals from the occurrence of colorectal cancer [23].

Additionally, *ICOS*c.1564T>C was shown to be associated with poorer response to the capecitabine –based chemotherapy in advanced colon cancer [23, 24].

In our work, we found that *ICOS*c.2373G>C [C] allele was associated with a higher risk of death. It might be associated with lower protection of these individuals against infection. In fact, the [CC] genotype of *ICOS*c.2373G>C was more frequently presented in patients with HBV infection than in patients with HBV clearance [16].

We did not find the associations of *ICOSISV1+173T*>C, I*COSc.602A>C*, *ICOSc.1624C>T* with risk and clinical course of MM, although they were described as a functional or as a susceptible locus for different diseases [7, 15, 19, 35].

The *ICOSc.1624C>T* polymorphism was shown to influence ICOS mRNA level [19] and the authors showed that acti-

vated CD4+ T cells from *ICOSc.1624C>T*[CC] homozygous persons had higher actual levels of ICOS mRNA than cells from [CT] heterozygous persons after 1 h and 3 h of activation; then, this difference disappeared.

The *ICOSc.602A>C* and *ICOSc.1624C>T* polymorphisms were described as susceptibility markers of outcome after kidney transplantation [15] and hematopoietic stem cell transplantation [43]. The *ICOSc.602A>C* and *ICOSc.1564T>C* SNPs were 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 *ICOSISV1+173T>C* polymorphism in our study, which does not influence ICOS mRNA level in CD4+ cells, but, since this polymorphism is located near *CTLA-4* gene, variations at that position influence CTLA-4 mRNA expression for both CTLA-4 isoforms, the soluble and full length (membrane) [19].

To sum up, although the functional genetic markers of *ICOS* gene were not found to be associated with multiple myeloma susceptibility, *ICOSc.2373G>C* was associated with overall survival.

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