BJMG 19 (2), 2016 • 51-60
DOI: 10.1515/bjmg-2016-0036

ORIGINAL ARTICLE

POLYMORPHISM OF THE *IL13* GENE MAY BE ASSOCIATED WITH UTERINE LEIOMYOMAS IN SLOVENIAN WOMEN

Krsteski J[1], Jurgec S[1,2], Pakiž M[3], But I[3], Potočnik U[1,2*]

***Corresponding Author:** Professor Uroš Potočnik, Ph.D., Centre for Human Molecular Genetics and Pharmacogenomics, Faculty of Medicine, University of Maribor, Taborska Ulica 8, 2000 Maribor, Slovenia. Tel: +386-2-2345-854, Fax: +386-2-2345-820, E-mail: uros.potocnik@um.si

ABSTRACT

Uterine leiomyomas (ULM) are a common cause of solid pelvic tumors in women. Their etiopathogenesis remains unclear. Interleukins (ILs) and their receptors can influence tumor biology of ULM. The aim of this study was to evaluate single nucleotide polymorphisms (SNPs) exhibited in the genes *IL4* (rs2070874), *IL4R* (rs1801275), *IL12RB1* (rs11575934), *IL12B* (rs6887695), *IL13* (rs 20541) and *IL23R* (rs7517847) as risk factors for ULM in Slovenian women and to identify associations between corresponding clinical parameters and the analyzed SNPs. In addition, solitary and multiple ULM were compared to identify clinical and/or genetic parameters influencing their occurrence. We conducted a case-control study that included 181 women with leiomyomas and 133 control subjects. Genotyping of selected SNPs was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and high resolution melting (HRM) techniques. The TT genotype of rs20541 (*IL13*) was significantly associated with decreased risk of ULM compared to both the CC and CT genotypes [$p = 0.018$; odds ratio (OR) = 0.184; 95% confidence interval (95% CI) = 0.048-0.712]. Using genetic and clinical data to develop a predictive model with logistic regression, we found that adenomyosis, higher age at diagnosis, family history of ULM occurrence, earlier menarche, lower number of pregnancies and lower age at first sexual intercourse, the G allele and genotypes AG and GG of rs1801275 (*IL4R*) were associated with an increased risk of multiple ULM occurrence. We also found an association between rs20541 (*IL13*) and 17β-estradiol serum levels in patients with multiple ULM (p 0.003). Our study showed, for the first time, that rs20541 (*IL13*) may contribute to susceptibility of ULM development and that rs1801275 (*IL4R*) can predispose patients to develop multiple ULM.

Keywords: Genetic risk; Uterine leiomyomas (ULM); Single nucleotide polymorphism (SNP); *IL13* gene.

INTRODUCTION

The most common cause of solid pelvic tumors and an indication for gynecological surgery in women are uterine leiomyomas (ULM), also known as uterine fibroids. Approximately 20.0-40.0% of women during their reproductive period have ULM [1]. These are benign fibrous tumors descendent from a single uterine smooth muscle cell [2]. Clinical problems associated with ULM are excessive bleeding and secondary anemia, increased urinary frequency, pelvic discomfort, bladder and bowel dysfunction, a sensation of pressure in the lower abdomen and pain during intercourse [3]. During the reproductive period of women, infertility and recurrent spontaneous abortions, fetal anomalies and fetal malpresentation have also been associated with leiomyomas [4].

[1] Centre for Human Molecular Genetics and Pharmacogenomics, Faculty of Medicine, University of Maribor, Maribor, Slovenia

[2] Laboratory for Biochemistry, Molecular Biology and Genomics, Faculty for Chemistry and Chemical Engineering, University of Maribor, Maribor, Slovenia

[3] Department of General Gynaecology and Gynaecological Urology, University Clinical Centre Maribor, Maribor, Slovenia

Despite their high prevalence, the etiopathogenesis of the ULM remains unclear. Several predisposing factors, including race, heredity, reproductive factors and lifestyle have been linked to ULM [5]. Furthermore, ULM is a complex disease, which means that interactions between multiple genes, hormones, growth factors, interleukins (ILs), and environment are involved in the tumorigenesis of ULM [6].

Some molecules, including ILs and their receptors, may influence tumor biology, tumor immunology and immuno-surveillance by mediating abnormal cell-cell signaling in the tumor micro-environment [7]. Several studies suggest that ILs and other cytokines are involved in the development of a variety of neoplasms such as glioma [8], gastric cancer [9] and gynecological neoplasms [10]. Moreover, elevated levels of ILs have been found in the uterine cavity of patients with ULM [11].

Interleukin-related single nucleotide polymorphisms (SNPs) might affect IL production and influence the course of the illness, as well as both disease resistance and susceptibility [12]. Thus, certain risk alleles may indicate an individual's degree of genetic predisposition to disease risk. Previous studies have investigated the association between *IL1B*, *IL1Ra*, *IL2*, *IL4*, *IL8*, *IL12*, *IL18* and *IL12RB1* gene polymorphisms and the occurrence of ULM [13-16]. Some of these studies reported a significant association between *IL4*, *IL1B* and *IL12RB1* and ULM development [13-15]. In addition, clinical and genetic differences have been noted between subtypes of solitary and multiple ULM [17,18]. In this study, however, we focused on cytokines derived from T helper type 2 (Th2) cells (IL-4, IL-12 and IL-13), hypothesizing that they might be good candidates as they are involved in the avoidance of tumor immuno-surveillance at the molecular level. The IL-23 and IL-12 are closely associated with Th17-helper phenotype and play a role in suppressing tumor immune response [19]. Furthermore, some functional studies showed effect of SNPs in *IL4* and *IL4R* genes on their expression, particularly SNP in the *IL4* gene correlates with enhanced IL-4 activity [20] and SNP in the *IL4R* gene functionally impacts the signaling and upregulating of the receptor's response to IL-4, which in turn results in activation of the STAT6 pathway [21]. Recently, clinical and genetic differences were noted between subtypes of solitary and multiple ULM [17,18]. The aim of our study was to further evaluate polymorphisms in genes coding for ILs, specifically SNPs with a known functional role in IL expression, including *IL4* and *IL4R*, SNPs with previously contradictory results in different populations, including *IL12RB1*, as well as SNPs previously not yet analyzed for ULM, including *IL12B*, *IL23R* and *IL13*. In addition, we also investigated possible genetic differences between solitary and multiple subtypes of ULM.

MATERIALS AND METHODS

Study Subjects. In this study, we recruited 181 ULM patients and 41 women without ULM as a control group at the Department of General Gynecology and Gynecological Urology (University Medical Centre Maribor, Maribor, Slovenia) as described previously [17]. Additionally, 92 subjects were included to represent the general population. Uterine leiomyomas patients were divided into two subgroups: 85 were patients with solitary ULM and 96 were patients with multiple ULM, with a mean age at diagnosis of 46 ± 11 years. Controls were also divided into two subgroups. The first group (healthy control) consisted of 41 women without ULM but diagnosed with pelvic organ prolapse as described previously [17], with a mean age of 60 ± 11 years, and the second group (normal population) consisted of 92 healthy individuals, with a mean age of 43 ± 12 years. Demographic and clinical parameters of ULM patients analyzed in this study have been described previously [17]. Additionally, quantitative measurements of the 17β-estradiol levels in serum were performed using ARCHITECT i2000SR Immunoassay Analyzer (Abbott Laboratories, Abbott Park, IL, USA) according to the manufacturer's instructions. The study protocol was approved by the Slovenian National Committee for Medical Ethics and the Institutional Review Board (KME 43/10/15).

Selection and Genotyping of *IL4* (rs2070874), *IL4R* (rs1801275), *IL12B* (rs6887695), *IL12RB1* (rs11575934) and *IL23R* (rs7517847) Single Nucleotide Polymorphisms. The rs2070874 (*IL4*) and rs1801275 (*IL4R*) SNPs were selected according to their known functional role in IL expression. Single nucleotide polymorphisms rs 11575934 (*IL12RB1*), rs6887695 (*IL12B*), rs20541 (*IL13*) and rs7517847 (*IL23R*) were previously reported to be associated with other ULM-related immune-mediated diseases, such as inflammatory bowel disease [22], glioblastoma [23], cervical adenocarcinoma and an increased risk of tuberculosis [24].

Genotyping of *IL4* (rs2070874), *IL4R* (rs1801275), *IL12B* (rs6887695, located ~60 kb upstream of the *IL12B* coding region), *IL12RB1* (rs11575934) and *IL23R* (rs 7517847) SNPs was performed by polymerase chain reaction (PCR) followed by the restriction fragment length polymorphism (RFLP) technique. The PCR primers and restriction enzymes were selected using the GeneRunner program (Hastings Software Inc., Hastings, NY, USA). A total of 50 ng of genomic DNA was mixed with oligo-

nucleotide primers in a final volume of 10 µL containing 2 mM MgCl2, 0.2 mM dNTP mix, 10 mM Tris-HCl and 0.25 U Taq polymerase (Fermentas, Waltham, MA, USA). The PCR conditions, primer sequences and the concentration of each primer are shown in Table 1. Polymerase chain reaction amplification was performed in a Professional Standard PCR Thermocycler (TProfessional Basic; Biometra GmbH, Göttingen, Germany). The PCR products were digested with a restriction enzyme at 37 °C overnight. The PCR products were electrophoresed on a 2.0% agarose gel stained with ethidium bromide, and visualized on an ultraviolet transilluminator. Restriction enzymes and fragment sizes after digestion are listed in Table 1.

Genotyping of the *IL13* (rs20541) Single Nucleotide Polymorphism. Genotyping of the *IL13* (rs20541) SNP was performed by high resolution melting (HRM) curve analysis following touchdown PCR amplification. Primers were designed using Primer3 (http://simgene.com/Primer3), and manufactured by Sigma-Aldrich Produktions GmbH, Steinheim, Germany. Primer sequences were as follows: forward 5'-CTG CAA ATA ATG ATG CTT TCG-3' and reverse 5'-ACC TGC TCT TAC ATT TAA AGA AAC TT-3'. The touchdown PCR amplification was performed on a LightCycler®480 detection system (Roche Applied Science, Mannheim, Germany). Samples were amplified in reactions containing 2 µL DNA (2.5 ng/µL), 3 µL of 2X LightCycler® 480 High Resolution Melting Master Mix (Roche Applied Science), 200 nM of each primer, 0.8 µL of $MgCl_2$ (2 mM final concentration) and RNase-free water in a final reaction volume of 10 µL. The touchdown PCR program was as follows: initial denaturation at 95 °C for 10 min., followed by 45 cycles of 10 seconds at 95 °C, 15 seconds at 65 °C (secondary target temperature 53 °C, with 0.5 °C/step) and 10 seconds at 72 °C. The HRM curve analysis was performed with temperature ranges used for the melting curve generation from 65 °C to 95 °C with 25 signal acquisitions per °C.

Statistical Analysis. All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS), version 23 (IBM Corporation, Armonk, NY, USA). Genetic polymorphisms were expressed as bivariate descriptive parameters, either by each of the two SNP alleles or by either of two genetic models assuming a dominant/recessive influence of the non-ancestral allele on a particular phenotype. Appropriate statistical techniques and methods were used, depending on properties of the measured parameter. Association analyses were done by either the two-sided Fisher's exact test, risk determination, Mann-Whitney test or Independent Samples *t*-test. Predictive models for bivariate variables describing phenotype groups were constructed using logistic regression with a stepwise exclusion of parameters (backward: Wald exclusion method with parameter exclusion criteria at $p > 0.05$). Initially included parameters were: all SNPs, adenomyosis, age at diagnosis, family predisposition, menarche, number of pregnancies, parity, body mass index, intake of oral contraceptives, intake of gestagenes, age at first sexual intercourse, and smoking. We did not perform a Bonferroni correction in our statistical analysis to avoid an unnecessary increase of type II errors [25]. Power of statistical association analysis (p) for dominant and recessive genetic models was calculated using G*Power computer software

Table 1. Primer sequences, primer concentrations, annealing temperatures and size of polymerase chain reaction product; restriction enzyme, enzyme concentration and size of DNA fragments after restriction for *IL12B* (rs6887695), *IL12RB1* (rs11575934), *IL23R* (rs7517847), *IL4* (rs2070874) and *IL4R* (rs1801275).

SNP ID (gene)	Primer Sequence (5'-3')	Final Concentration of Primer (nM)	Ta (°C)	PCR Product Size (bp)	Restriction Enzyme	Enzyme Concentration (units)	Allele	DNA Fragment Size After Restriction (bp)
rs6887695 (*IL12B*)	F-TTTCAGCGTGAGACCATTCA R-CCCCTAGGTCACAAGCGTAG	250	55	245	*Hph*I	0.5	C G	207+38 161+46+38
rs11575934 (*IL12RB1*)	F-GGACAATTCTTACGGCCTGA R-TCTAATGCTTGCCCCTGTTC	250	55	203	*Pvu*II	0.5	A G	104+99 203
rs7517847 (*IL23R*)	F-TCTGCCAATTCCCTAAAC R-AAGTAGGTGTGGATTGCC	375	52	259	*Hpy*F3I	0.25	G T	259 168+91
rs2070874 (*IL4*)	F-TAGAGATATCTTTGTCAGCATT R-AGACCCATTAATAGGTGTCG	250	52	188	*Mn*II	0.1	C T	64+30 94
rs1801275 (*IL4R*)	F-TCTCGGCCCCCACCAGTGGCGATC R-GAGGTCTTGGAAAGGCTTATAC	125	60	418	*Pvu*I	0.2	A G	209 186+23

SNP ID: single nucleotide polymorphism identity; Ta: annealing temperature; PCR: polymerase chain reaction; bp: base pair; F: forward; R: reverse.

(Heinrich Heine University, Dusseldorf, Germany). The power of the study was calculated post hoc, using the generally accepted standard error rate $\alpha = 0.05$.

RESULTS

Association Analysis of Selected Single Nucleotide Polymorphisms. We found statistically significant differences between the SNP rs20541 of gene *IL13* and ULM patients. The genotyping of *IL13* and other genes was performed by HRM or RFLP analyses, as described in Materials and Methods and shown in Figure 1. The frequency of the TT genotype of rs20541 was higher in the group of healthy controls (8.7%) compared to all patients with ULM (1.0%) ($p = 0.018$, OR = 0.184, 95% CI = 0.048-0.712) using the recessive genotype model (TT *vs.* CT+CC). We also found a higher frequency of the TT genotype of rs 20541 in all controls (6.8%) compared to patients with multiple ULM, for which the frequency of the TT genotype was 1.0% using the recessive genotype model (TT *vs.* CT+CC) ($p = 0.048$, OR = 0.145, 95% CI = 0.018-1.165). The association was also statistically significant when comparing multiple ULM, where the frequency of the TT genotype was (1.0%) and healthy individuals from the normal population, where the frequency of the TT genotype was (8.7%) ($p = 0.017$, OR = 0.111, 95% CI = 0.014-0.902) using the recessive genotype model (TT *vs.* CT+ CC). Power of association analysis between phenotype (ULM *vs.* all controls) and dominant (CC *vs.* CT+TT) or recessive (CC+CT *vs.* TT) genetic models of rs20541 was $p = 0.381$ and $p = 0.940$, respectively. For other tested SNPs, there were no significant differences in genotype or allele frequencies between patients and controls. Results of association analyses for rs20541 of the *IL13* gene are shown in Tables 2 and 3.

Association Between Evaluated Single Nucleotide Polymorphisms and Clinical Characteristics. When we compared selected SNPs with clinical characteristics of patients within a particular phenotype groups, differences were found between solitary and multiple ULM. In solitary ULM, rs1801275 (*IL4R*) was associated with the age at first sexual intercourse ($p = 0.004$) where median age at first intercourse of patients exhibiting the GG genotype was 19 years and the age of patients exhibiting either the AA or AG genotype was 18. In multiple ULM, rs1801275 (*IL4R*) was associated with the age at diagnosis ($p = 0.003$) where patients with the GG genotype were younger at diagnosis (27.5 years) compared to patients with the AA or AG genotype (45 years).

The SNP rs6887695 (*IL12B*) was associated with parity for solitary and multiple ULM subtypes. In solitary ULM, an association was found using the C *vs.* G allele model ($p = 0.029$) and using the CC *vs.* CG+GG genetic

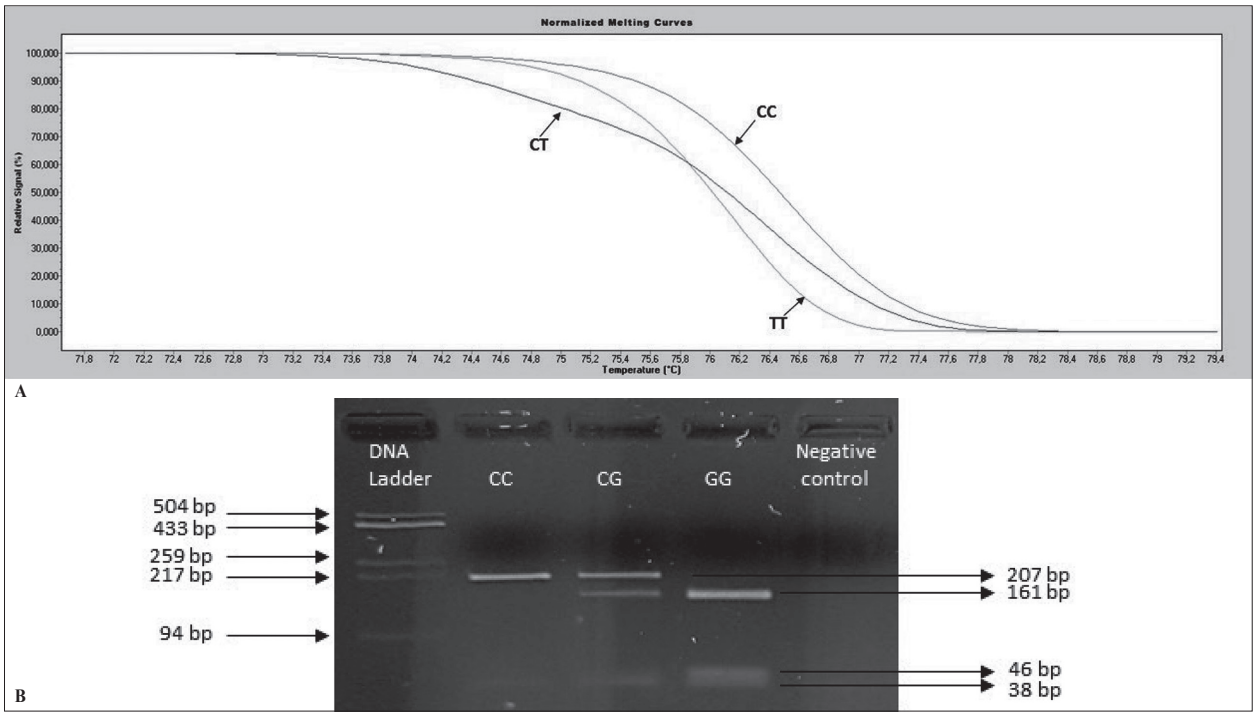

Figure 1. Primer sequences, primer concentrations, annealing temperatures and size of polymerase chain reaction product; restriction enzyme, enzyme concentration and size of DNA fragments after restriction for *IL12B* (rs6887695), *IL12RB1* (rs11575934), *IL23R* (rs7517847), *IL4* (rs2070874) and *IL4R* (rs1801275).

Table 2. Association analysis for *IL13* (rs20541) in women with uterine leiomyomas and controls.

SNP ID (gene)	Genotype	ULM *n*=181 (%)	Normal Population *n*=96 (%)	Healthy Controls *n*=41 (%)	All Controls *n*=133 (%)	Statistical Analyses	ULM *vs.* All Controls		ULM *vs.* Healthy Controls		ULM *vs.* Normal Population	
							CC *vs.* CT+TT	TT *vs.* CT+CC	CC *vs.* CT+TT	TT *vs.* CT+CC	CC *vs.* CT+TT	TT *vs.* CT+CC
rs20451 (*IL13*)	CC CT TT	101 (58.0) 70 (40.2) 3 (1.7)	48 (52.2) 36 (39.1) 8 (8.7)	23 (52.2) 17 (39.1) 1 (8.7)	71 (53.4) 53 (39.8) 9 (6.8)	*p* value OR 95% CI	0.42 1.208 0.767-1.904	0.588 2.5 0.222-28.096	0.862 0.083 0.545-2.151	0.574 0.702 0.071-6.924	0.367 1.268 0.763-2.108	**0.018** 0.184 0.048-0.712

SNP ID: single nucleotide polymorphism identity; ULM: uterine leiomyomas; OR: odds ratio; 95% CI: 95% confidence interval.

Table 3. Association analysis for *IL13* (rs20541) in women with multiple uterine leiomyomas and controls.

SNP ID (gene)	Genotype	Solitary ULM *n*=85 (%)	Multiple ULM *n*=96 (%)	Healthy Controls *n*=41 (%)	All Controls *n*=133 (%)	Statistical Analyses	Multiple ULM *vs.* All Controls		Multiple ULM *vs.* Healthy Controls		Multiple ULM vs. Normal Population	
							CC *vs.* CT+TT	TT *vs.* CT+CC	CC *vs.* CT+TT	TT *vs.* CT+CC	CC *vs.* CT+TT	TT *vs.* CT+CC
rs20451 (*IL13*)	CC CT TT	42 (53.8) 34 (43.6) 2 (2.6)	59 (61.5) 36 (37.5) 1 (1.0)	34 (52.2) 17 (39.1) 1 (8.7)	71 (53.4) 53 (39.8) 9 (6.8)	*p* value OR 95% CI	0.280 1.392 0.816-2.375	**0.048** 0.145 0.018-1.165	0.573 1.248 0.595-2.619	0.511 0.421 0.026-6.899	0.573 1.248 0.595-2.619	**0.017** 0.111 0.014-0.905

SNP ID: single nucleotide polymorphism identity; ULM: uterine leiomyomas; OR: odds ratio; 95% CI: 95% confidence interval.

model ($p = 0.016$). On average, patients with the C allele or CC genotypes had a parity of one and patients with the G allele or the CG or GG genotypes had a parity of two. In multiple ULM, an association was detected for the C *vs.* G allele model ($p = 0.049$) and for the CC *vs.* CG+GG genetic model ($p = 0.048$). On average, patients with the C allele or CC genotype had a parity of two, and patients with the G allele or CG or GG genotypes had a parity of one. In multiple ULM, rs20541 (*IL13*) was associated with 17β-estradiol serum levels in the follicular phase of the menstrual cycle ($p = 0.003$) where patients with the CC genotype had a higher 17β-estradiol levels (0.525 nmol/L) compared to patients with either the CT or TT genotypes (0.130 nmol/L).

In addition, rs11575934 (*IL12RB1*) was also associated with parity in multiple ULM. An association was found for the A *vs.* G allele model ($p = 0.004$) and in AA *vs.* AG+ GG genetic model ($p = 0.009$). On average, patients with the A allele and AA genotypes had a parity of two, and patients with the G allele or AG or GG genotypes had a parity of one.

Logistic Regression Analyses. To further investigate possible parameters that are associated with increased risk of ULM development, we performed logistic regression analyses of selected genetic and clinical parameters in relation to solitary and multiple ULM occurrence (Table 4). For SNP rs1801275 (*IL4R*), association was found in allele and genotype genetic model with the ULM phenotype subgroups, where carrying the G allele (OR = 3.641, $p = 2.19 \times 10\text{-}2$) or genotypes AG or GG (OR = 6.975, p = 7.49× 10-3), presence of adenomyosis (OR = 11.315, $p = 4.55 \times 10\text{-}3$), higher age at diagnosis (OR = 1.086, $p = 3.24 \times 10\text{-}2$), positive family history (OR = 0.152, $p = 3.03 \times 10\text{-}3$), earlier menarche (OR = 0.401, $p = 2.10 \times 10\text{-}4$), lower number of pregnancies (OR = 0.559, $p = 1.99 \times 10\text{-}2$) and lower age at first sexual intercourse (OR = 0.655, $p = 1.81 \times 10\text{-}2$), all increase the risk of multiple ULM development.

DISCUSSION

In the present case-control study, we investigated the associations among six SNPs in selected candidate cytokine genes and the risk of ULM in the Slovenian population. Results of our study suggest that an association of ULM with rs20541 (*IL13*) exists, but for the other analyzed candidate SNPs in selected genes, we could not confirm association with ULM development in Slovenian women. Our study showed that SNP rs20541 in the *IL13* gene is associated with a significantly decreased risk of ULM development. The presence of the TT genotype of *IL13* rs20541 SNP may have a protective role against ULM development in Slovenian women. So far, an association between rs20541 (*IL13*) and some tumors such as renal cell carcinoma [26], bladder cancer [27] and glioblastoma [23] has been reported, but no study to date reports the relationship between rs20541 (*IL13*) and the presence of ULM. Chu *et al.* [26] showed the same protective effect

Table 4. Logistic regression for the prediction of uterine leiomyomas subgroups. [Dependent variable: ULM subtype (multiple *vs.* solitary).]

Independent Variables	Exp (B)	95% CI for Exp (B)		Coefficient *p* Value	Nag. R^2	Accuracy (%)
		Lower	Upper			
rs1801275 (*IL4R*) (G *vs.* A alleles)	3.641	1.206	10.996	2.19×10^{-2}		
Adenomyosis (present *vs.* absent)	7.967	2.668	23.790	2.01×10^{-4}		
Age at diagnosis (years)	1.093	1.036	1.153	1.17×10^{-3}		
Family predisposition (negative *vs.* positive)	0.200	0.087	0.462	1.62×10^{-4}		
Menarche (year)	0.445	0.323	0.613	7.41×10^{-7}	0.485	75.0
Number of pregnancies	0.562	0.405	0.781	5.84×10^{-4}		
Age at first sexual intercourse (year)	0.674	0.533	0.852	9.59×10^{-4}		
Constant	7831870			1.40×10^{-6}		
rs1801275 (*IL4R*) (AG+GG *vs.* AA genotypes)	6.975	1.680	28.958	7.49×10^{-3}		
Adenomyosis (present *vs.* absent)	11.315	2.118	60.454	4.55×10^{-3}		
Age at diagnosis (years)	1.086	1.007	1.171	3.24×10^{-2}		
Family predisposition (negative *vs.* positive)	0.152	0.044	0.528	3.03×10^{-3}		
Menarche (year)	0.401	0.247	0.650	2.10×10^{-4}	0.535	76.6
Number of pregnancies	0.559	0.343	0.912	1.99×10^{-2}		
Age at first sexual intercourse (year)	0.655	0.462	0.930	1.81×10^{-2}		
Constant	55741666			3.98×10^{-4}		

Exp (B): odds ratio, as given by logistic regression in SPSS; 95% CI: 95% confidence interval.

of the rs20541 (*IL13*) TT genotype for renal cell carcinoma development in a Chinese population. The SNP rs20541 (*IL13*) is a functional polymorphism located in exon 4, conferring a change in the amino acid sequence from arginine (Arg) to glutamine (Gln) at codon 130 and it could influence the transcriptional expression of IL-13 [26]. Graves *et al.* [28] demonstrated that the rs20541 (*IL13*) A allele (Gln) compromises IgE production and elevated serum IgE levels in 1399 children from three different populations. Several epidemiological studies suggest an inverse association between IgE levels and the risk of glioma [29,30], non-Hodgkin lymphoma [30] and pancreatic cancer [31]; however, to date, there are no epidemiological studies available on the relationship between IgE levels and ULM. The *IL13* gene encodes IL-13, a pleiotropic Th2 cytokine secreted by many cell types, but especially activated T-cells. Interleukin-13 plays a critical role in many aspects of allergic reaction onset by inducing IgE synthesis [32]. A gene expression study detected over-expression of *IL13* in ULM compared with myometrial tissue [33], which increased the expression of tumor necrosis factor-α (TNF-α), one of the leiomyoma growth factors [34]. The *IL13* gene also caused overexpression and release of matrix metallo-proteinases required for promoting cell proliferation and invasion of tumors [35]. In some tumors, IL-13 inhibits tumor immuno-surveillance by acting as a tumor cell growth factor [36]. Terabe *et al.* [37] demonstrated that IL-13 is necessary for down-regulation of tumor immuno-surveillance in a tumor model through the IL-4R-STAT 6 pathway. Furthermore, natural killer T (NKT) cells in response to tumor growth, could produce IL-13 and induce myeloid cells to secrete transforming growth factor-β (TGF-β) that inhibits tumor immuno-surveillance in several mouse tumor models [38]. Most published association studies in ULM so far used as a control group general population. However, as there are many women in the general population with undetected ULM, estimated to be around 9.0% [39], it is important to include as many women as possible with proven absence of ULM in the control group, as we did in our study. It should be acknowledged that the limitation of the study was the relatively low number of participants because high prevalence of the disease would call for a large sample size. Nonetheless, the strength of the present study was a precise selection of participants with regard to the presence of ULM.

In our study, we also analyzed SNPs in other *IL* genes, *IL4*, *IL4R*, *IL12RB1*, *IL12B* and *IL23R*. However, no statistically significant associations has been found between different genotypes and ULM. The IL-13 and IL-4 have similar immunoregulatory functions and share a common IL-4R chain on their receptors [40].They play a critical

role in the down-regulation of anti-tumor immunity [40]. Both, or at least one copy of the T allele in the rs2070874 (*IL4*) polymorphism is associated with higher IL-4 serum levels [20]. The study by Sosna *et al.* [14] tested the same rs2070874 (*IL4*) polymorphism and they observed an association with the risk for ULM.

An association between rs1801275 (*IL4R*) and a lower age at first sexual intercourse in solitary ULM, and between rs1801275 (*IL4R*) and age at diagnosis in the group of patients with multiple ULM has been found. Likewise, a recent genome-wide association study of 125,667 UK Biobank participants identified 38 loci associated with age at first sexual intercourse with several of these loci having associations with other reproductive and behavioral traits, such as age at first birth, number of children, irritable temperament and risk-taking propensity [41]. Our observations could be explained by the fact that a lower age at first sexual intercourse presents a higher risk of contracting sexually transmitted diseases (STD). Another possibility is that hormonal factors associated with perimenopause and/or the culmination after 20-30 years of stimulation by sex hormones could predispose individuals toward ULM occurrence [6].

For the first time, we provide evidence that rs1801275 (*IL4R*) is a possible contributor to different age at diagnosis of patients with multiple ULM. Our data suggest that carriers of the AA or AG genotypes may exhibit both a later onset of the disease and a higher risk for developing multiple ULM. Additionally, logistic regression confirmed a codependent association of rs1801275 in *IL4R*, adenomyosis, positive family history and an earlier menarche in patients with multiple ULM. Some *in vitro* data suggest a common pathogenetic mechanisms in adenomyosis and ULM [42]. Another well-known risk factor associated with ULM is familial predisposition, which increases the risk of ULM in female relatives of women with ULM [43]. Early onset of menarche increases the overall number of myometrium cell divisions resulting in an increased chance of mutation within some genes expressed in the myometrium [44]. In our study, we showed that in multiple ULM patients, CT and TT genotypes of rs20541 (*IL13*) additionally predispose patients to lower E2 levels compared to the CC genotype. The 17β-estradiol (E2) is an established stimulator of ULM growth through binding to the α and β estrogen receptors subtypes (ER) and upregulates the gene expression of multiple growth factors [45]. Shao *et al.* [18] demonstrated that 17β-estradiol concentrations were lower in multiple ULM compared to solitary ULM.

In conclusion, our study suggests that the polymorphism rs20541 in the *IL13* gene may be associated with a decreased risk for ULM development. Additionally, SNP rs1801275 in the *IL4R* gene predisposes the risk for solitary ULM in patients with a lower age at first sexual intercourse and a risk for multiple ULM in patients with earlier onset of disease. Further research on a larger sample size in ethnically diverse populations, including a subsequent functional evaluation, is warranted. Currently, there are not enough data on ULM in association to the selected genes to perform meta-analysis. However, when more association studies of these candidate SNPs have been accumulated in the future, the meta-analysis will be possible and our study is an important step toward this goal.

ACKNOWLEDGMENTS

We sincerely thank Matjaž Deželak (Centre for Human Molecular Genetics and Pharmacogenomics, Faculty of Medicine, University of Maribor, Maribor, Slovenia) and Katja Repnik and Helena S. Čelešnik (Centre for Human Molecular Genetics and Pharmacogenomics, Faculty of Medicine, University of Maribor, Maribor, Slovenia and Laboratory for Biochemistry, Molecular Biology and Genomics, Faculty for Chemistry and Chemical Engineering, University of Maribor, Maribor, Slovenia) for their valuable help with the manuscript.

Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

REFERENCES

1. Wallach EE, Vlahos NF. Uterine myomas: An overview of development, clinical features, and management. Obstet Gynecol. 2004; 104(2): 393-406.
2. Cramer SF, Patel A. The frequency of uterine leiomyomas. Am J Clin Pathol. 1990; 94(4): 435-438.
3. Marsh EE, Bulun SE. Steroid hormones and leio-myomas. Obstet Gynecol Clin North Am. 2006; 33(1): 59-67.
4. Walker CL, Stewart EA. Uterine fibroids: The elephant in the room. Science. 2005; 308(5728): 1589-1592.
5. Sandberg AA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: Leiomyoma. Cancer Genet Cytogenet. 2005; 158(1): 1-26.
6. Flake GP, Andersen J, Dixon D. Etiology and pathogenesis of uterine leiomyomas: A review. Environ Health Perspect. 2003; 111(8): 1037-1054.

7. Paltridge JL, Belle L, Khew-Goodall Y. The secretome in cancer progression. Biochim Biophys Acta. 2013; 1834(11): 2233-2241.
8. Li S, Jin T, Zhang J, Lou H, Yang B, Li Y, *et al.* Polymorphisms of TREH, IL4R and CCDC26 genes associated with risk of glioma. Cancer Epidemiol. 2012; 36(3): 283-287.
9. Yuan LJ, Jin TB, Yin JK, Du XL, Wang Q, Dong R, *et al.* Polymorphisms of tumor-related genes IL-10, PSCA, MTRR and NOC3L are associated with the risk of gastric cancer in the Chinese Han population. Cancer Epidemiol. 2012; 36(6): e366-e372.
10. Islam MS, Protic O, Stortoni P, Grechi G, Lamanna P, Petraglia F, *et al.* Complex networks of multiple factors in the pathogenesis of uterine leiomyoma. Fertil Steril. 2013; 100(1): 178-193.
11. Malyshkina AI, Posiseeva LV, Sotnikova NY, Antsiferova JS, Suvorkina EE, Arevadze IE. Local immunological markers of different rate of growth of uterine myoma. Russ J Immunol. 2002; 7(1): 58-62.
12. Sano M, Inoue S, Hosoi T, Ouchi Y, Emi M, Shiraki M, *et al.* Association of estrogen receptor dinucleotide repeat polymorphism with osteoporosis. Biochem Biophys Res Commun. 1995; 217(1): 378-383.
13. Hsieh YY, Chang CC, Tsai CH, Lin CC, Tsai FJ. Interleukin (IL)-12 receptor β1 codon 378 G homozygote and allele, but not IL-1 (β511 promoter, 3953 exon 5, receptor antagonist), IL-2 114, IL-4-590 intron 3, IL-8 3'-UTR 2767, and IL-18 105, are associated with higher susceptibility to leiomyoma. Fertil Steril. 2007; 87(4): 886-895.
14. Sosna O, Kolesár L, Slavčev A, Skibová J, Fait T, Mara M, *et al.* Th1/Th2 cytokine gene polymorphisms in patients with uterine fibroid. Folia Biol (Praha). 2010; 56(5): 206-210.
15. Pietrowski D, Thewes R, Sator M, Denschlag D, Keck C, Tempfer C. Uterine leiomyoma is associated with a polymorphism in the interleukin 1-β gene. Am J Reprod Immunol. 2009; 62(2): 112-127.
16. Taghizade-Mortezaee F, Hashemzadeh-Chaleshtori M, Kheiri S, Parvin N, Norbakhsh M, Etemadi S, *et al.* Association of interleukin-1β (IL-1β) gene polymorphisms with uterine leiomyoma. Zahedan J Res Med Sci. 2012; 14(7): 53-56.
17. Pakiz M, Potocnik U, But I. Solitary and multiple uterine leiomyomas among Caucasian women: Two different disorders? Fertil Steril. 2010; 94(6): 2291-2295.
18. Shao R, Fang L, Xing R, Xiong Y, Wang Z. Differential expression of estrogen receptor α and β isoforms in multiple and solitary leiomyomas. Biochem Biophys Res Commun. 2015; 468(1-2): 136-142.
19. Burkholder B, Huang RY, Burgess R, Luo S, Jones VS, Zhang W, *et al.* Tumor-induced perturbations of cytokines and immune cell networks. Biochim Biophys Acta. 2014; 1845(2): 182-201.
20. Cabantous S, Ranque S, Poudiougou B, Traore A, Berbache S, Vitte J, *et al.* Genotype combinations of two IL4 polymorphisms influencing IL-4 plasma levels are associated with different risks of severe malaria in the Malian population. Immunogenetics. 2015; 67(5-6): 283-288.
21. Mitsuyasu H, Izuhara K, Mao XQ, Gao PS, Arinobu Y, Enomoto T, *et al.* Ile50Val variant of IL4R α upregulates IgE synthesis and associates with atopic asthma. Nat Genet. 1998; 19(2): 119-120.
22. Xiao JP, Wang XR, Zhang S, Wang HY, Ye L, Pan HF, *et al.* Association between rs6887695 and 3'-untranslated region polymorphisms within the interleukin-12B gene and susceptibility to autoimmune diseases in Asian and European population: A meta-analysis. Autoimmunity. 2016; 49(4): 277-284.
23. Jin T, Li X, Zhang J, Wang H, Geng T, Li G, *et al.* Genetic association between selected cytokine genes and glioblastoma in the Han Chinese population. BMC Cancer. 2013; 13: 236.
24. Hussain SK, Madeleine MM, Johnson LG, Du Q, Galloway DA, Daling JR, *et al.* Nucleotide variation in IL-10 and IL-12 and their receptors and cervical and vulvar cancer risk: A hybrid case-parent triad and case-control study. Int J Cancer. 2013; 133(1): 201-213.
25. Perneger TV. What's wrong with Bonferroni adjustments. BMJ. 1998;316(7139):1236-1238.
26. Chu H, Wang M, Yan F, Zhong D, Shi D, Ma L, *et al.* Polymorphisms in the IL-13 and IL-4R genes are associated with the development of renal cell carcinoma. Ann Oncol. 2012; 23(8): 2114-2121.
27. Chu H, Ma L, Wang M, Shi D, Qin C, Yuan L, *et al.* The polymorphisms of IL-4, IL-4R and IL-13 genes and bladder cancer risk in a Chinese population: A case-control study. Mol Biol Rep. 2012; 39(5): 5349-5357.
28. Graves PE, Kabesch M, Halonen M, Holberg CJ, Baldini M, Fritzsch C, *et al.* A cluster of seven tightly linked polymorphisms in the IL-13 gene is associated

with total serum IgE levels in three populations of white children. J Allergy Clin Immunol. 2000; 105(3): 506-513.

29. Schoemaker MJ, Swerdlow AJ, Hepworth SJ, van Tongeren M, Muir KR, McKinney PA. History of allergic disease and risk of meningioma. Am J Epidemiol. 2007; 165(5): 477-485.
30. Biggar RJ, Christiansen M, Rostgaard K, Smedby KE, Adami HO, Glimelius B, *et al.* Immunoglobulin subclass levels in patients with non-Hodgkin lymphoma. Int J Cancer. 2009; 124(11): 2616-2620.
31. van Toorenenbergen AW, van Heerde MJ, van Buuren HR. Potential value of serum total IgE for differentiation between autoimmune pancreatitis and pancreatic cancer. Scand J Immunol. 2010; 72(5): 444-448.
32. van der Pouw Kraan TC, van der Zee JS, Boeije LC, de Groot ER, Stapel SO, Aarden LA. The role of IL-13 in IgE synthesis by allergic asthma patients. Clin Exp Immunol. 1998; 111(1): 129-135.
33. Luo X, Ding L, Xu J, Chegini N. Gene expression profiling of leiomyoma and myometrial smooth muscle cells in response to transforming growth factor-β. Endocrinology. 2005; 146(3): 1097-1118.
34. Kurachi O, Matsuo H, Samoto T, Maruo T. Tumor necrosis factor-α expression in human uterine leiomyoma and its down-regulation by progesterone. J Clin Endocrinol Metab. 2001; 86(5): 2275-2280.
35. McCawley LJ, Matrisian LM. Matrix metalloproteinases: They're not just for matrix anymore! Curr Opin Cell Biol. 2001; 13(5): 534-540.
36. Wynn TA. IL-13 effector functions. Annu Rev Immunol. 2003; 21: 425-456.
37. Terabe M, Matsui S, Noben-Trauth N, Chen H, Watson C, Donaldson DD, *et al.* NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. Nat Immunol. 2000; 1(6): 515-520.
38. Berzofsky JA, Terabe M. A novel immunoregulatory axis of NKT cell subsets regulating tumor immunity. Cancer Immunol Immunother. 2008; 57(11): 1679-1683.
39. Zimmermann A, Bernuit D, Gerlinger C, Schaefers M, Geppert K. Prevalence, symptoms and management of uterine fibroids: An international internet-based survey of 21,746 women. BMC Womens Health. 2012; 12): 6.
40. Kobayashi M, Kobayashi H, Pollard RB, Suzuki F. A pathogenic role of Th2 cells and their cytokine products on the pulmonary metastasis of murine B16 melanoma. J Immunol. 1998; 160(12): 5869-5873.
41. Day FR, Helgason H, Chasman DI, Rose LM, Loh PR, Scott RA, *et al.* Physical and neurobehavioral determinants of reproductive onset and success. Nat Genet. 2016; 48(6): 617-623.
42. Pandis N, Karaiskos C, Bardi G, Sfikas K, Tserkezoglou A, Fotiou S, *et al.* Chromosome analysis of uterine adenomyosis. Detection of the leiomyoma-associated del(7q) in three cases. Cancer Genet Cytogenet. 1995; 80(2): 118-120.
43. van Voorhis BJ, Romitti PA, Jones MP. Family history as a risk factor for development of uterine leiomyomas. Results of a pilot study. J Reprod Med. 2002; 47(8): 663-669.
44. Marshall LM, Spiegelman D, Goldman MB, Manson JE, Colditz GA, Barbieri RL, *et al.* A prospective study of reproductive factors and oral contraceptive use in relation to the risk of uterine leiomyomata. Fertil Steril. 1998; 70(3): 432-439.
45. Moravek MB, Bulun SE. Endocrinology of uterine fibroids: Steroid hormones, stem cells, and genetic contribution. Curr Opin Obstet Gynecol. 2015; 27(4): 276-283.