Original Article

Th1/Th2 Cytokine Gene Polymorphisms in Patients With Uterine Fibroid

(uterine fibroid / myoma / cytokine / gene polymorphism)

O. SOSNA[1], L. KOLESÁR[2], A. SLAVČEV[2], J. SKIBOVÁ[3], T. FAIT[1], M. MARA[1], I. STŘÍŽ[4], D. KUŽEL[1]

[1]Charles University in Prague, First Faculty of Medicine and General University Hospital, Department of Gynaecology and Obstetrics, Prague, Czech Republic
Institute for Clinical and Experimental Medicine: [2]Department of Immunogenetics, [3]Department of Medical Statistics, [4]Department of Clinical and Transplant Immunology, Prague, Czech Republic

Abstract. Uterine fibroid or leiomyoma is a frequent non-malignant tumour with unknown aetiology and pathogenesis. The aim of our study was to look for possible genetic markers which could be used as prognostic tools for evaluation of an increased risk for development of uterine fibroid. A large spectrum of Th1/Th2 cytokine gene polymorphisms in 102 patients with uterine leiomyoma was compared with 145 healthy controls. An association between polymorphisms of the *IL4* gene promotor at positions -590 C/T and -33 C/T, and the risk of leiomyoma was observed. The CC genotype of *IL4* -590 and at position -33 was less frequent in the patient group than in the control group (P = 0.03). Besides IL-4, we observed different genotype distribution of the *TNFA* gene -308 A/G. The frequency of genotype AA was higher in the younger (≤ 35 years) patient group (P = 0.02). Our study thus suggests that certain cytokine gene polymorphisms, especially of the *IL4* and *TNFA* genes, may be associated with increased risk for development of uterine fibroid. Further investigation would be needed to elucidate the mechanisms responsible for these associations.

Abbreviations: BMI – body mass index, EGF – epidermal growth factor, HWE – Hardy-Weinberg equilibrium, IFN-γ – interferon γ, IL – interleukin, PCR – polymerase chain reaction, SNP – single nucleotide polymorphisms, TGF-β – transforming growth factor β, TNF-α – tumour necrosis factor α, *TNFA* – gene for TNF-α, UF – uterine fibroid.

Introduction

Uterine fibroid (UF) or leiomyoma is diagnosed in 30–40 % of women in reproductive age. UF becomes symptomatic in one-third of diagnosed patients and is the most frequent indication for hysterectomy (Falcone and Walters, 2008). UF is a benign monoclonal tumour of smooth muscle cells of the uterus and also contains large amounts of extracellular matrix components such as collagen, fibronectin and proteoglycans (Parker, 2007). The clinical symptoms are related to the local mass effect, resulting in pressure upon adjacent organs, excessive uterine bleeding or problems related to infertility and pregnancy. Leiomyomas can be usually diagnosed by gynecologic palpatory examination, transvaginal ultrasonography or hysteroscopy and MRI. Although the tumour represents the most common benign female tumour, its aetiology remains unknown. The influence of several genetic, hormonal, anthropometrical and reproductive factors is supposed (Flake et al., 2003; Stewart and Morton, 2006; Evans and Brunsell, 2007).

The genetic factors contributing to the development of UF are being intensively investigated. The possibility for a role of certain cytokine gene polymorphisms in the pathogenesis of UF has been suggested in several previous works (Hsieh et al., 2004, 2007; Litovkin et al., 2007; Pietrowski et al., 2009). The aim of our study was therefore to examine a large spectrum of Th1/Th2 cytokine gene polymorphisms in patients with uterine leiomyoma and to compare them with healthy controls. Our hypothesis of a possible association between cytokine single nucleotide polymorphisms (SNP) and the incidence of UF was based on the fact that cytokines may enhance elevated production of matrix metalloproteinases, which may further stimulate leiomyoma growth (Inagaki et al., 2003). These polymorphisms may not be directly linked to the disease, but may be useful tools in the study of this multifactorial disorder (Andersen et al., 1994). To our best knowledge, our study is the first to analyse such a wide range of cytokine gene polymorphisms in UF patients.

Material and Methods

Patients

The study group enrolled 102 patients diagnosed with UF between 2007 and 2008 at Charles University in Prague, First Faculty of Medicine and General Faculty Hospital in Prague. The study design and Informed Consent form was approved by the Ethics Committee of the General University Hospital, Prague. Informed consent was obtained from all patients. Ultrasonography targeted on the size and character of the leiomyoma was performed in all patients. Patients also underwent elective surgery such as laparoscopic myomectomy, open myomectomy, transcervical resection of myoma or hysterectomy. Histological verification was performed after surgery. Several undefined cases were excluded, such as leiomyomas with increased cell activity, adenomyosis and one case of stromal sarcoma. Only UF with typical histology were enrolled into the study.

Patients with UF for the statistical analysis were divided into two groups: A) ≤ 35 years of age, BMI < 25, B) > 35 years of age, BMI < 25. None of the patients took any long-term medication and all also had negative oncology history. The diameter of UF was verified by abdominal and transvaginal ultrasonography. The minimal size of UF was set to 30 mm in diameter in case of single leiomyoma or the occurrence of at least four small-sized leiomyomas more than 10 mm in diameter (in case of multiple leiomyomas). The most common symptoms and complaints were pelvic pain and abnormal bleeding.

As a control group we used 145 control samples from female volunteers without leiomyoma and mean age of 45 (20–55 years). No women from the control group took any long-term medication and all had negative oncology history. The absence of UF was verified by abdominal and transvaginal ultrasonography.

DNA analysis

DNA was isolated from whole blood using the QIAamp kits (provided by Qiagen GmbH, Hilden, Germany). The quality of DNA was measured both with gel electrophoresis and by a spectrophotometer. DNA with the ratio of absorbancies A260/A280 between 1.7 and 1.9 was considered for further analysis. A Cytokine Genotyping Kit (Invitrogen, Darmstadt, Germany) was applied to cytokine genotyping. The following cytokine SNPs were detected (Table 1): interleukin (IL)-1α (-889 T/C), IL-1β (-511 T/C and +3962 T/C), IL-1R (pst11970 C/T), IL-1RA (mspa111100 T/C), IL-4Rα (+1902 G/A), IL-12 (-1188 C/A), interferon (IFN)-γ (+874 A/T), TGF-β1 (codon 10 C/T and codon 25 G/C), tumour necrosis factor (TNF)-α (-308 G/A and −238 G/A), IL-2 (-330 T/G and +166 G/T), IL-4 (-1098 T/G, -590 T/C and -33 T/C), IL-6 (-174 G/C and nt565 G/A) and IL-10 (-1082 G/A, -819 C/T and -592 C/A). The principle of SNP detection is based on PCR reaction with sequence-specific primers. Briefly, a master mix was prepared according to manufacturer's instructions – 140 µl reaction buffer, 5.6–9.3 µg DNA, 20 U Taq polymerase (TopBio, Prague, Czech Republic) and PCR water added up to a final volume of 501 µl. Ten microlitres of the master mix were aliquoted to lyophilized primer mixes. The following PCR protocol was run in a Biometra Thermocycler (Schöeller, Göttingen, Germany) with an optimized ramp speed to 1 °C/s. The PCR started with an initial denaturation at 94 °C for 2 min followed by 10 cycles at 94 °C for 15 s and 65 °C for 60 s. The final step involved 20 cycles of 94 °C for 15 s, 61 °C for 50 s and 72 °C for 30 s. PCR products were detected after electrophoresis in 2% agarose gel by staining with GelRed (Biotium, Hayward, CA). The gel was documented with a Gene-Genius Bio-Imaging system (Syngene, Cambridge, UK).

Statistical analysis

Genotype and allele frequencies were determined by direct counting and were compared with the control population by Fisher's exact test or χ^2 test. Statistical analysis was performed using MedCalc statistical software (Mariakerke, Belgium). P values lower than 0.05 were considered significant. Adjusted standardized deviates between observed and expected values for each cell were approximated by Z scores to identify large differences. Bonferroni correction was used for multiple comparisons if more than one SNP at the same locus were assessed.

Results

Hardy-Weinberg equilibrium (HWE) was counted for each locus and none of them reached a statistically significant difference. First, we compared the cytokine gene SNP of the entire patient cohort with the SNP of the control group. The frequencies of the majority of tested cytokine gene SNP in the patient cohort were not statistically different from the cytokine SNP in the control group. However, we found an intriguing difference in the distribution of *IL4* genotypes. The CC genotype of *IL4* -590 and at position -33 was less frequent in the patient group than in the control group (P = 0.03) (Table 1). The power of the test reached 73 % for both polymorphic positions. Furthermore, there is a strong linkage disequilibrium between these two positions (-590 and -33 (D≠0)).

In addition, we compared the cytokine gene polymorphisms of patients with the control group after separation of patients into two cohorts according to age – ≤ 35, and > 35. The same trend of lower frequency of the CC genotype *IL4* -590 and *IL4* -33 in patients compared with controls was preserved in older individuals (> 35 years) (P = 0.09) but not in patients younger than 35 (Table 2). Besides *IL4*, we observed a different genotype distribution of the *TNFA* gene -308 A/G. The frequency of the AA genotype was higher in the younger patient group (P = 0.02) (Table 3), but this result was not conclusive due to the small power of the test (42 %). This difference was not found in the patient group with > 35 years of age.

Table 1. Comparison of genotype frequencies between the patient group and the control group

Cytokine	SNP	Genotype	Controls N (%)	Patients N (%)	P value
IL-1α	-889	CC	69 (48)	51 (50)	0.70
		CT	59 (41)	42 (42)	
		TT	16 (11)	8 (8)	
IL-1β	-511	CC	79 (54)	46 (45)	0.35
		CT	54 (37)	46 (45)	
		TT	12 (8)	10 (10)	
IL-1β	+3962	CC	74 (51)	55 (54)	0.78
		CT	55 (38)	39 (38)	
		TT	15 (10)	8 (8)	
IL-1R	Pst1 1970	CC	55 (38)	40 (39)	0.66
		CT	67 (46)	50 (49)	
		TT	23 (16)	12 (12)	
IL-1RA	Mspa1 11100	CC	13 (9)	13 (13)	0.62
		CT	63 (43)	41 (40)	
		TT	69 (48)	48 (47)	
IL-4Rα	+1902	AA	84 (58)	60 (59)	0.62
		AG	56 (39)	36 (35)	
		GG	5 (3)	6 (6)	
IL-12	-1188	AA	85 (59)	54 (53)	0.24
		AC	55 (38)	42 (42)	
		CC	3 (2)	6 (6)	
IFN-γ	+874	AA	40 (28)	32 (31)	0.85
		AT	70 (49)	47 (46)	
		TT	32 (23)	23 (23)	
TGF-β1	codon 10	CC	25 (17)	13 (13)	0.17
		CT	66 (46)	61 (60)	
		TT	54 (37)	28 (27)	
TGF-β1	codon 25	CC	1 (1)	1 (1)	0.76
		CG	20 (14)	11 (11)	
		GG	124 (86)	90 (88)	
TNF-α	-308	AA	1 (1)	4 (4)	0.14
		AG	29 (20)	25 (25	
		GG	113 (79)	73 (72)	
TNF-α	-238	AA	0 (0)	0 (0)	0.16
		AG	14 (10)	5 (5)	
		GG	129 (90)	97 (95)	
IL-2	-330	GG	15 (10)	12 (12)	0.95
		GT	63 (44)	44 (43)	
		TT	65 (45)	46 (45)	
IL-2	+166	GG	61 (43)	48 (47)	0.76
		GT	62 (43)	42 (41)	
		TT	20 (14)	12 (12)	
IL-4	-1098	GG	0 (0)	0 (0)	0.21
		GT	22 (15)	10 (10)	
		TT	122 (85)	92 (90)	
IL-4	**-590**	CC	112 (78)	64 (63)	0.03
		CT	27 (19)	34 (33)	
		TT	5 (3)	4 (4)	
IL-4	**-33**	CC	112 (78)	64 (63)	0.03
		CT	27 (19)	34 (33)	
		TT	5 (3)	4 (4)	
IL-6	-174	CC	27 (19)	19 (19)	0.23
		CG	72 (50)	41 (40)	
		GG	45 (31)	42 (41)	
IL-6	nt565	AA	25 (17)	18 (18)	0.21
		AG	73 (51)	41 (40)	
		GG	46 (32)	43 (42)	
IL-10	-1082	AA	42 (29)	32 (31)	0.45
		AG	83 (57)	51 (50)	
		GG	20 (14)	19 (19)	
IL-10	-819	CC	74 (51)	56 (55)	0.82
		CT	64 (44)	41 (40)	
		TT	7 (5)	5 (5)	
IL-10	-592	AA	7 (5)	5 (5)	0.82
		AC	64 (44)	41 (40)	
		CC	74 (51)	56 (55)	

Table 2. Genotype distribution of IL-4 -590 and -33 in the two patient groups (≤ 35 years and > 35 years of age); compared with the control group (P = 0.09)

IL-4 -590	CC N (%)	CT N (%)	TT N (%)
Controls	112 (78)	27 (19)	5 (3)
Patient age ≤ 35	24 (63)	12 (32)	2 (5)
Patient age > 35	**38 (61)**	22 (36)	2 (3)
IL-4 -33	**CC**	**CT**	**TT**
Controls	112 (78)	27 (19)	5 (3)
Patient age ≤ 35	24 (63)	12 (32)	2 (5)
Patient age > 35	**38 (61)**	22 (36)	2 (3)

Table 3. Genotype distribution of TNF-α -308 in the two patient groups ≤ 35 years and > 35 years of age); compared with the control group (P = 0.02).

TNF-α -308	AA N (%)	AG N (%)	GG N (%)
Controls	1 (1)	29 (20)	113 (79)
Patient age ≤35	**3 (8)**	5 (13)	30 (79)
Patient age >35	1 (2)	20 (32)	41 (66)

Discussion

Our study was designed to search for possible genetic markers which could be used as prognostic tools for evaluation of increased risk for development of UF. The role of various cytokines in leiomyoma development has been investigated, like that of epidermal growth factor (EGF) (Shimomura et al., 1998), transforming growth factor β (TGF-β) (Arici and Sozen, 2000), chemokines and chemokine receptors (Syssoev et al., 2008). Besides, it is known that leiomyoma growth is dependent on oestrogen and progesterone production (Maruo et al., 2003), which on their part may influence the gene expression of several cytokines and growth factors.

In our study we selected several "candidate" SNPs of pro- and anti-inflammatory cytokine genes which could be associated with UF development. Besides, there is extensive literary data that the studied SNPs may influence the level of gene expression of the corresponding cytokines (Turner et al.,1997; Wilson et al., 1997; Fishman et al., 1998; Smith and Humphries, 2009).

Our study suggests a possible association between the polymorphisms of the *IL4* gene promotor, namely SNP -590 C/T and -33 C/T, and the risk of leiomyoma development. T allele substitution at position -590 increases *IL4* gene expression and is associated with elevation of serum IL-4 concentrations (Rosenwasser and Borish, 1997). On the contrary, the C allele at this position leads to decreased gene expression. It is a matter of speculation what might be the reason for the observed association. As a strong anti-inflammatory cytokine, IL-4 has been shown to modulate the activation of tumour-associated fibroblasts (Blankenstein, 2005). Its role in tumour clearance and reduction of tumour load has also been investigated (Tepper et al., 1989; Golumbek et al.,

1991; Hock et al., 1993, 1994). The study of Hsieh et al. (2007) tested the same *IL4* -590 polymorphisms and other SNPs; however, no association with the incidence of UF was found with the exception of IL-12Rβ1. This contradiction may be caused by differences between the patient groups studied (premenopausal Taiwanese women) or by a race difference as it was previously shown in the study of Skorpil et al. comparing CGP among three European populations (Skorpil et al., 2007).

Our results indicated a possible role of *TNFA* polymorphism on the risk of leiomyoma development. Tumour necrosis factor participates in the induction of inflammatory responses and acute phase reactions, which in turn cause many of the clinical problems associated with various infectious and autoimmune diseases. The effect of the *TNFA* gene on tumour pathogenesis has also been described. There are reports in the literature about the influence of *TNFA* -308 A/G polymorphism on various tumours with a significantly increased frequency of the A allele among patients with malignancies (Chouchane et al., 1997). It has been shown that the substitution of the -308 A to G is a part of haplotype which influences *TNFA* gene expression. The A allele at this position increases cytokine production up to 7-fold (Wilson et al., 1997). However, further studies would be needed to clarify to what extent TNF-α may play a role in the aetiology and pathogenesis of UF. The group of Hsieh et al. (2004) found association of *TNFA* -308 G allele with leiomyoma in premenopausal Taiwan Chinese women. This result, however, cannot be confirmed or rebutted based on our results, but one should take into account the oriental ethnic background, where the G allele is the most frequent at position -308 in Taiwanese women, whilst the A allele is predominant in the European nations.

In conclusion, our study suggests that certain cytokine gene polymorphisms, especially those of *IL4* and *TNFA*, may be associated with increased risk for development of uterine fibroid. Further investigation would be needed to elucidate the mechanisms responsible for these associations.

References

Andersen, T. I., Heimdal, K. R., Skrede, M., Tveit, K., Berg, K., Borresen, A. L. (1994) Oestrogen receptor (ESR) polymorphisms and breast cancer susceptibility. *Hum. Genet.* **94**, 665-670.

Arici, A., Sozen, I. (2000) Transforming growth factor-β3 is expressed at high levels in leiomyoma where it stimulates fibronectin expression and cell proliferation. *Fertil. Steril.* **73**, 1006-1011.

Blankenstein, T. (2005) The role of tumor stroma in the interaction between tumor and immune system. *Curr. Opin. Immunol.* **17**, 180-186.

Chouchane, L., Ahmed, S. B., Baccouche, S., Remadi, S. (1997) Polymorphism in the tumor necrosis factor-α promotor region and in the heat shock protein 70 genes associated with malignant tumors. *Cancer* **80**, 1489-1496.

Evans, P., Brunsell, S. (2007) Uterine fibroid tumors: diagnosis and treatment. *Am. Fam. Physician* **75**, 1503-1508.

Falcone, T., Walters, M. D. (2008) Hysterectomy for benign disease. *Obstet. Gynecol.* **111**, 753-767.

Fishman, D., Faulds, G., Jeffery, R., Mohamed-Ali, V., Yudkin, J. S., Humphries, S., Woo, P. (1998) The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. *J. Clin. Invest.* **102**, 1369-1376.

Flake, G. P., Andersen, J., Dixon, D. (2003) Etiology and pathogenesis of uterine leiomyomas: a review. *Environ. Health Perspect.* **111**, 1037-1054.

Golumbek, P. T., Lazenby, A. J., Levitsky, H. I., Jaffee, L. M., Karasuyama, H., Baker, M., Pardoll, D. M. (1991) Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. *Science* **254**, 713-716.

Hock, H., Dorsch, M., Kunzendorf, U., Qin, Z., Diamantstein, T., Blankenstein, T. (1993) Mechanisms of rejection induced by tumor cell-targeted gene transfer of interleukin 2, interleukin 4, interleukin 7, tumor necrosis factor, or interferon γ. *Proc. Natl. Acad. Sci. USA* **90**, 2774-2778.

Hock, H., Dorsch, M., Richter, G., Kunzendorf, U., Kruger-Krasagakes, S., Blankenstein, T., Qin, Z., Diamantstein, T. (1994) Tumor-cell-targeted cytokine gene transfer in experimental models for cancer therapy. *Nat. Immun.* **13**, 85-92.

Hsieh, Y. Y., Chang, C. C., Tsai, F. J., Lin, C. C., Yeh, L. S., Tsai, C. H. (2004) Tumor necrosis factor-α-308 promoter and p53 codon 72 gene polymorphisms in women with leiomyomas. *Fertil. Steril.* **82 Suppl 3**, 1177-1181.

Hsieh, Y. Y., Chang, C. C., Tsai, C. H., Lin, C. C., Tsai, F. J. (2007) Interleukin (IL)-12 receptor β1 codon 378 G homozygote and allele, but not IL-1 (β-511 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.* **87**, 886-895.

Inagaki, N., Ung, L., Otani, T., Wilkinson, D., Lopata, A. (2003) Uterine cavity matrix metalloproteinases and cytokines in patients with leiomyoma, adenomyosis or endometrial polyp. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **111**, 197-203.

Litovkin, K. V., Domenyuk, V. P., Bubnov, V. V., Zaporozhan, V. N. (2007) Interleukin-6 -174G/C polymorphism in breast cancer and uterine leiomyoma patients: a population-based case control study. *Exp. Oncol.* **29**, 295-298.

Maruo, T., Matsuo, H., Shimomura, Y., Kurachi, O., Gao, Z., Nakago, S., Yamada, T., Chen, W., Wang, J. (2003) Effects of progesterone on growth factor expression in human uterine leiomyoma. *Steroids* **68**, 817-824.

Parker, W. H. (2007) Etiology, symptomatology, and diagnosis of uterine myomas. *Fertil. Steril.* **87**, 725-736.

Pietrowski, D., Thewes, R., Sator, M., Denschlag, D., Keck, C., Tempfer, C. (2009) Uterine leiomyoma is associated with a polymorphism in the interleukin 1-β gene. *Am. J. Reprod. Immunol.* **62**, 112-117.

Rosenwasser, L. J., Borish, L. (1997) Genetics of atopy and asthma: the rationale behind promoter-based candidate gene studies (IL-4 and IL-10). *Am. J. Respir. Crit. Care Med.* **156**, S152-S155.

Shimomura, Y., Matsuo, H., Samoto, T., Maruo, T. (1998) Up-regulation by progesterone of proliferating cell nuclear antigen and epidermal growth factor expression in human uterine leiomyoma. *J. Clin. Endocrinol. Metab.* **83**, 2192-2198.

Skorpil, N., Kolesár, L., Striz, I., Lardy, N. M., Slavcev, A. (2007) Cytokine gene polymorphisms in the Dutch population. *Int. J. Immunogenet.* **34**, 87-90.

Smith, A. J., Humphries, S. E. (2009) Cytokine and cytokine receptor gene polymorphisms and their functionality. *Cytokine Growth Factor Rev.* **20**, 43-59.

Stewart, E. A., Morton, C. C. (2006) The genetics of uterine leiomyomata: what clinicians need to know. *Obstet. Gynecol.* **107**, 917-921.

Syssoev, K. A., Kulagina, N. V., Chukhlovin, A. B., Morozova, E. B., Totolian, A. A. (2008) Expression of mRNA for chemokines and chemokine receptors in tissues of the myometrium and uterine leiomyoma. *Bull. Exp. Biol. Med.* **145**, 84-89.

Tepper, R. I., Pattengale, P. K., Leder, P. (1989) Murine interleukin-4 displays potent anti-tumor activity in vivo. *Cell* **57**, 503-512.

Turner, D., Grant, S. C., Yonan, N., Sheldon, S., Dyer, P. A., Sinnott, P. J., Hutchinson, I. V. (1997) Cytokine gene polymorphism and heart transplant rejection. *Transplantation* **64**, 776-779.

Wilson, A. G., Symons, J. A., McDowell, T. L., McDevitt, H. O., Duff, G. W. (1997) Effects of a polymorphism in the human tumor necrosis factor α promoter on transcriptional activation. *Proc. Natl. Acad. Sci. USA* **94**, 3195-3199.