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Research article

TGF β1 -509 C/T POLYMORPHISM IN UTERINE FIBROIDS

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ABSTRACT: Uterine leiomyomas/fibroids are the most common pelvic tumors of the female genital tract. Transforming growth factor beta (TGF β) family members are multi-functional cytokines that play a key role in cellular growth, proliferation and differentiation. The aim of this study was to investigate whether TGF β1 -509 C/T polymorphism could be used as a susceptibility marker in Uterine fibroids pathogenesis. ARMS PCR was carried out for controls and patients (n=103) to identify the specific genotypes. Genotypes and allelic frequencies in both groups were compared. Proportions of C homozygote, heterozygote and T homozygote for TGF β1gene polymorphisms were 37.9%, 44.7%, 17.5% in the control individuals and 37%, 51.5%, 11.7% in the uterine fibroid patients. There was no significant difference between the controls and the patients thus indicating the tumor suppressor effect of TGF β1 in the early stages of tumor pathogenesis. The study was found to be in association with the earlier reported data wherein TGF β1 acts as tumor suppressor in the early stages and as a tumor promoter in the later stages of tumor pathogenesis.

Keywords: polymorphism, cytokine, extracellular matrix, proliferation, transforming growth factor, uterine fibroids,angiogenesis.

INTRODUCTION

Uterine leiomyomas (fibroids or myomas) are common benign tumors that arise from a single uterine smooth muscle cell and are the most common cause of solid pelvic tumors, found in 20–40% of women during their reproductive years (Wallach and Vlahos, 2004). The common symptoms associated with uterine leiomyoma are irregular and excessive bleeding and anemia, pelvic discomfort, bowel and bladder dysfunctions, pressure sensation in the lower abdomen leading to infertility and recurrent abortion (Marsh and Bulun, 2006; Olive and Pritts, 2010). Leiomyomatous growth is estrogen–dependent, but still the mechanism of how estrogen regulates leiomyoma growth remains elusive (Yangyu *et al* 2007). A distinctive feature of leiomyomata is the presence of abundant extracellular matrix (ECM), TGF-ß1, ß2 and β3 isoforms, as well as their receptors, have all been identified in leiomyoma and myometrium (Dou *et a*l., 1996; Tang *et al*., 1997) and in recent years Transforming growth factor ß (TGFß) has been widely accepted as a key factor in the pathological growth of fibrotic tissue, especially leiomyomata (Dou *et al.,* 1996; Branton and Kopp, 1999; Byung and Nowak, 2001). In early stages of cancer, TGF- ß1 acts as a tumor suppressor inhibiting cellular proliferation and/or promoting cellular differentiation and apoptosis. In the later stages of cancer progression, the role of TGF- ß1 shifts to that of a tumor promoter by stimulating angiogenesis and cell motility, suppressing immune response and increasing the interaction of tumor cells with the extracellular matrix, which leads to progressive invasion and metastasis. The biphasic nature of TGF- ß1 action has also been demonstrated in animal models wherein TGF- ß1 pathway has been implicated. Transforming growth factor β1 (TGFβ1) is the cytokine most causatively associated with disorders characterized by fibrosis. The evaluation of the role of TGFβ1 (-509C/T) gene polymorphism in the pathogenesis of uterine fibroids, is elucidated, as it is the first study to be reported in uterine fibroids to the best of our knowledge.

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MATERIALS AND METHODS

The present study includes 103 uterine fibroid patients referred to Infertility Institute and Research Center, Secunderabad and 103 female control samples without any history of reproductive abnormalities or family history of uterine fibroids from Osmania General Hospital, Hyderabad. Diagnosis of Uterine Fibroids was carried out by transvaginal ultrasound. Blood samples from the patients have been collected at the early stages of the diagnosis.

Methodology

5 ml of blood was collected for genomic DNA isolation from 103 uterine fibroid patients and 103 control group. DNA isolation was carried out following the methodology as described by Debomoy and Nurnberger (1991).

ARMS (Amplified Refractory Mutation System) PCR was carried out to identify the genotypes.

1) Primer details: Primers for the -509C/T polymorphic locus

Common Primer (CF) antisense: 5'-CTACGGCGTGGAGTGCTGAG-3'
C-Primer (RC) Sense: 5'-AAGGGGCAACAGGACACCTGGG-3'
T-Primer (RT) Sense: 5'-AAGGGGCAACAGGACACCTGGA-3'

2) PCR Reaction Details:

The PCR reaction was carried out in 0.2 ml tubes with 25ul of master mix. Each tube containing 100ng of genomic DNA, 50pmoles each of forward and reverse primers, 10mM of dNTP, 1X PCR buffer and water to make up the volume. The conditions applied are: initial denaturation at 94°C for 2 min, followed by denaturation at 94°C for 30 sec, annealing at 61°C to for 20 sec, extension at 72°C for 20 sec and final extension of 3 minutes at 72°C for 30 cycles. Two sets of reaction have been carried out for the same sample each with a common primer (CF) and one of the reverse primer (RC/RT) specific for different alleles of the polymorphic locus. The amplified samples were later checked on 10% acrylamide gels following silver staining protocol for genotype identification.

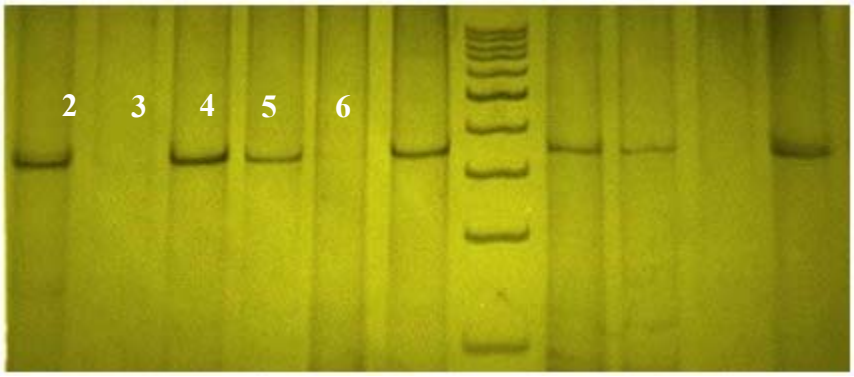

Fig 1: Gel image showing the various genotypes of the -509 C/T polymorphism
Lane 1 & 2 shows CC genotype lane 3 & 4 CT heterozygote lane 5 & 6 TT homozygote.

Statistical analysis

Genotypic and allelic frequencies were calculated followed by computation of Odds ratios with 95% confidence intervals (95% CI) to test for relative risk of specific genotypes to uterine leiomyoma by using *SNPSTAT software.*

RESULTS

The patients and the control DNA samples were genotyped for the SNP-509C/T and the allelic and genotypic frequencies for the polymorphic site is given in Table I.

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Table 1: Allelic & Genotypic Frequencies for the -509C/T polymorphic SNP

Site	Allele	Controls (C)		Patients (P)		Allele Frequency	
						C	P
-509C/T	C	124		129		0.60	0.63
	T	82		77		0.40	0.37
	Genotype	**Controls (C)**		**Patients (P)**		X^2	
		n (103)	**%**	**n (103)**	**%**	**C**	**P**
	C/C	39	37.9	38	37	0.48	1.01
	C/T	46	44.7	53	51.5		
	T/T	18	17.5	12	11.7		

Table 1 – Allelic and genotypic frequencies for the polymorphic site

The frequency of C & T alleles were found to be 0.60 and 0.40 in controls and 0.63 & 0.37 in patients while the proportion of CC genotype was observed to be 37.9% which is similar in both controls and patients. The proportion of CT genotype was observed to be 51% which is higher in patients when compared to the controls 44.7%. The rare homozygotes did not show any significant variation; as the TT genotype was observed to be 11.7% which is lower in patients when compare to the controls 17.5%.

The χ^2 value calculated for the controls and the patients was found to be 0.48 and 1.01 respectively with the distribution being in equilibrium in both controls & uterine leiomyomas at p≤005.

Odds relative risk estimations for -509C/T polymorphic site are given in Table 2.

Table 2: Odds relative risk for -509C/T polymorphic SNP in UL compared to Control group.

	Relative Risk Estimates		
	OR	**CI @95%**	**P**
CC vs CT	1.182	0.624 – 2.244	0.689
CC vs TT	0.684	0.266 – 1.747	0.512
CC vs CT+TT	1.042	0.570 – 1.906	1.000
TT vs CC+CT	1.606	0.685 – 3.796	0.324
CC+TT vs CT	1.313	0.732 – 2.359	0.403
TT vs CT	1.728	0.699 – 4.306	0.276

Table 2 – Odds ratio for the -509C/T polymorphic locus of the promoter region of TGFβ1 gene.

As observed from table II, the relative risk estimates for the genotypes were found to be insignificant. However the frequency of T allele was found to be low in patient samples compared to control samples, indicating the protective nature of T allele encoded protein.

DISCUSSION

Uterine leiomyomas (fibroids; myomas) are the most common benign tumors of the female reproductive tract, leading to reproductive associated problems and hysterectomy. Although the exact cause of these tumors remains unknown, steroid hormones and growth factors and/or their receptors have been reported to play a pivotal role in their development (Moore *et al* 2010). These growth factors either alone or synergistically, appear to regulate the growth of myometrial and leiomyomata smooth muscle cells *in vitro* or possibly influence their differentiation and hypertrophic activities during pregnancy and leiomyomatous growth.

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TGF-βs are recognized for their biological activities influencing stimulation/inhibition of cell growth, differentiation, synthesis and deposition of extra cellular matrix and cellular hypertrophy mediated through their specific receptors. Hence TGF-βs may be the key regulators of various biological activities of smooth muscle cells under both normal and tumor (leiomyoma) conditions (Tang *et al* 1997).

Single nucleotide polymorphisms (SNP) have been observed in various types of human cancers and are reported to be implicated in tumorigenesis. TGFβ1 production is influenced through several SNPs in the structural gene and promoter region. The TGFβ gene, localized to 19q13.1 region may be a candidate locus for susceptibility to numerous diseases (Hsiesh *et al* 2005).

It has been reported that the variant alleles of the TGF-β1 gene appear to be potential risk for cancers of the lung, esophagus, breast, liver, nasopharynx, kidney, prostate, gallbladder and colorectum (Zhang *et al* 2008).

TGF-β signaling is initiated by the binding of TGF-β ligands to type II TGF-β receptors (TGFβR2). Once bound to the ligand, the TGFβR2 recruits and phosphorylates the type I TGF-β receptor (TGFβR1), which stimulates TGFβR1 protein kinase activity. Activated TGFβR1 then phosphorylates two downstream transcription factors, SMAD2 and SMAD3, allowing these proteins to bind to SMAD4. The resulting SMAD complexes translocate into the nucleus and interact with other transcription factors in a cell-specific manner to regulate the transcription of a multitude of TGF-β responsive genes (Elliott *et al* 2005). These pathways regulate SMAD mediated responses and also induce SMAD independent responses (Derynck and Zhang 2003). Some of the downstream targets of TGF-β signaling are important regulators of cell-cycle checkpoint proteins CDKN1A (p21), CDKN1B (p27) and CDKN2B (p15), and their activation leads to growth arrest (Massague *et al* 2000). Therefore, TGF-β serves as a tumor suppressor in the normal cells by inhibiting cell proliferation and inducing apoptosis. Many cells escape the tumor-suppressor effects of TGF-β and become resistant to TGF-β induced growth inhibition thus may also act as a tumor promoter. Prolonged exposure to high levels of TGF-β promotes neoplastic transformation of cells (Yanfei and Boris 2007), however the mechanism by which TGF-β switches its growth inhibitory effect into growth stimulatory effect is still not well understood.

TGFβ has been shown to play an important role in the laying of ECM which is characteristic of uterine fibroids. The ECM of fibroids is not only excessive in amount but is also disordered in orientation and structure. It appears that increased TGF-β signaling leads to increased ECM formation in fibroids, probably through interference with the normal steps in wound healing and fibrosis leading to dysfunctional remodeling, apoptosis and excessive fibrosis. TGFβ therefore appears to play a complex role in conjunction with ECM in the growth of uterine fibroids (Stanley 2008)

The direct effects of TGFβ on the synthesis of ECM components are complemented by its ability to interfere with proteolytic degradation of matrix proteins. This occurs at two levels wherein TGFβ reduces synthesis and secretion of several different proteases that act on ECM and also increases synthesis of specific inhibitors of those proteases especially MMP involved in endometrial remodeling (Godkin and Dore 1998)

Stimulation of DNA synthesis, but not proliferation, of myometrial smooth muscle cells by TGFβ's suggests that the cells receive signals for cell mass and DNA synthesis associated with cell cycle progression. Such a phenomenon often occurs during cellular hypertrophy resulting from incomplete growth stimulation, and is regulated to some extent by growth factors such as TGFβ1 with the ability to arrest cells in the G1/S phase of the cell cycle (Brodsky and Uryvaeva, 1977; Baserga, 1984; Geisterfer *et al.*, 1988; Owens *et al.*, 1988; Turner *et al.*, 1988; Battegay *et al.*, 1990).

Earlier studies conducted by Hsiesh *et al* (2005) the −509C/T promoter polymorphism of TGFβ1 gene have all been associated with endometriosis susceptibility wherein T homozygote and T allele of TGF-B1 are associated with higher susceptibility to endometriosis. In contrast Van Kaam *et al* (2007) demonstrated the non-association of the polymorphic locus with endometriosis.

In the present study, the samples collected are in the early stages of fibroid diagnosis, the activation of cell cycle check point genes which are the downstream targets of TGF β signaling, lead to growth arrest, later triggering promotion. As reported TGF β acts as tumor suppressor by inhibiting cellular proliferation in early stages of tumor pathogenesis. Moreover as the disease progresses it is speculated that TGF β1 may start to influence its matrix expanding effect by taking over the cell's ability to switch the cycle from G1/S phase to the next, thus prolonging the cell cycle leading to muscling up of the tissue instead of cell division.

CONCLUSION

Uterine leiomyomas (fibroids) are benign smooth muscle tumors that often contain an excessive extracellular matrix (ECM). TGFβ1 plays a central role in the regulation of cell and its environment. TGF-β induces the expression of many components of the extracellular matrix (ECM) as well as cellular adhesion factors. TGF- β acts in ECM remodeling through regulation of matrix metalloproteinases (MMPs). Additionally, TGFβ1 acts in a paracrine fashion to regulate stromal cells, blood vessels, and local immune response. The net result of these interactions in malignant cancers is increased tumor cell invasion and angiogenesis.

In the present study genotyping of TGF β1 was carried out to understand its role in Uterine Leiomyomata. Though the present study doesn't show any significant association of TGF β1 with the disease phenotype, yet its role in fibroid proliferation cannot be neglected. The actual role and mechanism of TGF β1 and specific genotype in uterine fibroids, however, remains to be examined. Furthermore, the roles of other TGF subtypes and their receptors as well as other growth factors in the development of uterine fibroids merit further screening and evaluation.

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