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Association of Col6a3 Missense Variants with Uterine Leiomyomata

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***Abstract*-**Uterine fibroids or Uterine leiomyomata (UL), are non-malignant smooth muscle tumors of the uterus, identified in women of child bearing age. They usually develop in the third to fourth decade of life. UL may be asymptomatic or cause heavy and abnormal bleeding, pressure and pain in the pelvic region, difficulty in conceiving or causing repetitive abortions. They tend to shrink in size after menopause indicating that hormones play a role in their aetiology apart from other molecular factors. Missense variants of collagens have been associated with UL. Two polymorphism rs2270669 and rs36117715 of the collagen 6A3 gene have been evaluated in fibroids in this study. DNA samples of women with and without leiomyoma were evaluated for COL6A3 gene polymorphisms. This is the first study indicating susceptibility of South Indian women to UL with missense polymorphisms of COL6A3 gene.

Keywords: COL6A3, Uterine leiomyoma, Missense variants.

Abbreviations: UL- Uterine leiomyoma, ECM- Extracellular matrix, PCR-Polymerase Chain Reaction, RFLP-Restriction fragment length polymorphism, SNV-Single Nucleotide Variation.

I. INTRODUCTION

Uterine leiomyomata (UL) are benign smooth muscle uterine tumors. They are a major cause of morbidity and a public health problem in women of fertility age. The incidence of UL in India is estimated to be 20% - 30% in women; it may be symptomatic or asymptomatic [1]. The distinctive nature of UL is the increased amount and abnormal quality of extracellular matrix components (ECM) [2]. The primary constituents of ECM are collagens, fibronectin, proteoglycans and dermatopontin [3-10]. Structural regeneration and /or repair of the body tissues is stabilized and maintained by the collagens as they are the major part of the ECM. Uterine fibroids or ULs are made up of disordered ECM proteins. It is reported that UL have 50% more extracellular matrix components than its corresponding normal myometrium [11]. UL are a storehouse of chemokines, cytokines, growth factors, angiogenic and inflammatory mediators which respond, and proteases which regulate cell growth, differentiation and ECM output. These molecular factors are important for the growth, as well as regression of UL in post menopause. Several types of collagens are present in UL and are responsible for decreased elasticity and increased firmness of the uterine tissue [12&13].

II. RELATED WORK

Aissani et al [14] reported an association of different members of collagen gene family with risk of developing the UL which were also associated with size of UL. COL6A3 and COL13A1 polymorphisms have been associated with UL in African and European American groups. There are studies on UL from India on other genes such as estrogen, progesterone hormone receptor genes [15,16] and few others, However, there are no studies from India on UL and collagen genes. The COL6A3 gene polymorphisms were c.9034G>C (p. Ala3012Pro) rs2270669 in EA, and c.6653C>T (p. Pro2218Leu) rs36117715 were evaluated in the present study in women from South India with and without UL.

III. METHODOLOGY

Samples: A total of 112 blood samples were collected from 56 healthy women without UL confirmed with ultrasound scan and 56 women diagnosed with UL who had undergone hysterectomy after obtaining approval from institutional ethics committee:
[ECR/139/Inst/AP/2013/RR-16.]

Genomic DNA Isolation
The salting out technique was used to isolate DNA from whole blood published by our group [17]. Briefly 300 µl of blood sample was taken and 900 µl TKM1 and 90 µl 1% Triton-X were added and centrifuged at 8000 revolutions per minute for 10 minutes for the initial RBC lysis, till a white pellet of WBC was seen and 280 µl TKM2 and 60 µl of 10% SDS were added for the WBC

lysis,150 µl of 6M NaCl was added to precipitate proteins and pre-chilled 500 µl Isopropanol was added to obtain DNA which was washed with 70 % ethanol, repeated and the DNA was air dried overnight and TE buffer was added to dissolve DNA and store it at -20^{0}C until future use.

Polymerase Chain Reaction (PCR)

The isolated DNA was amplified with specific oligonucleotide primers in an Applied Biosystems thermal cycler. PCR was carried out using Taq DNA Polymerase (Bangalore Genei, India) by the three-step procedure, followed in our laboratory [18]. The COL6A3 specific genomic primers given below were used for analyzing the two polymorphisms.

(i). rs2270669, exon 41, c.9034G>C, p. Alanine3012 Proline and
(ii). rs36117715**,** exon 28 c.6653C>T, p. Proline 2218 Leucine.

Table 1. Primer, Restriction Enzyme And Genotype Details

FORWARD PRIMER (+):	5'>3' TGGTTGCTTTCCAAGTCCCT		5'>3'ATTTCCTCTCTCGCTCATGC	
REVERSE PRIMER (-):	5'>3' TGTCTCCTTTGTGTCCTATTTGA		5'>3' TGTCTCCTTTGTGTCCTATTTGA3	
PRODUCT LENGTH	202 bp		410 bp	
RESTRICTION ENZYME	Msp1		Blpl	
GENOTYPE	TT	202bp	CC	410bp
	CT	202,129,73bp	GC	410,265,145bp
	CC	129,73bp	GG	265,145bp

Genomic DNA amplification was performed by PCR of 35 cycles each consisting of the following steps:

Table 2.Pcr Conditions

SNV	rs36117715	rs2270669
INITIAL DENATURATION	94^{0}C/ 5 min	94^{0}C/ 5 min
FINAL DENATURATION	94^{0}C/ 30 sec	94^{0}C/ 30 sec
ANNEALING	53^{0}C/ sec	62^{0}C/ sec
EXTENSION	72^{0}C/ 5 min	72^{0}C/ 5 min
FINAL EXTENSION	72^{0}C/ 45 sec	72^{0}C/ 45 sec

IV. RESULTS AND DISCUSSION

Age ranges of patients with UL in this study was from 28yrs - 75 years, whereas healthy controls without UL were in the age range of 52 to 85 years. The respective mean ages of cases were 50.26yrs SD+ 10.33 yrs and those of controls were 69.64yrs SD +8.37yrs.

The amplified PCR products were visualized on 12% Polyacrylamide gel after electrophoresis and the Polymerase Chain Reaction (PCR) products were subjected to Restriction fragment length polymorphism (RFLP) using specific enzymes.

Figure 1 shows Msp1 digested products of rs36117715 polymorphism gave 129,73bp bands for CC genotype and a single band 202bp band for TT genotype. Presence of all three fragments indicates CT genotype.

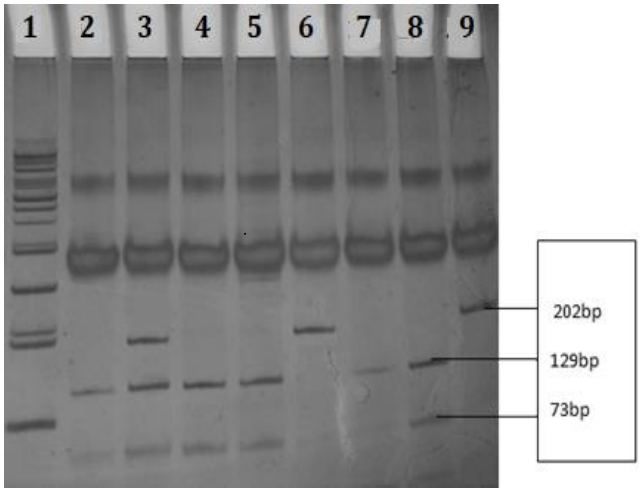

Figure 1. PCR products after RFLP to assess rs36117715 Polymorphism.

Lane 1-Ladder
Lane 2, 4, 5, 7, 8-show 129 and73bp bands indicating CC genotype.
Lane 6 and 9-show 202bp and indicative of TT genotype.
Lane 3-shows 3 bands of 202 ,129 and 73 bp indicating CT genotype.
Figure 2 shows result of Blpl restriction enzyme for rs2270669 in figure 2 shows 410 bp band for CC genotype, 265, 145 bp bands for GG genotype, and similarly all 3 bands indicate GC genotype.

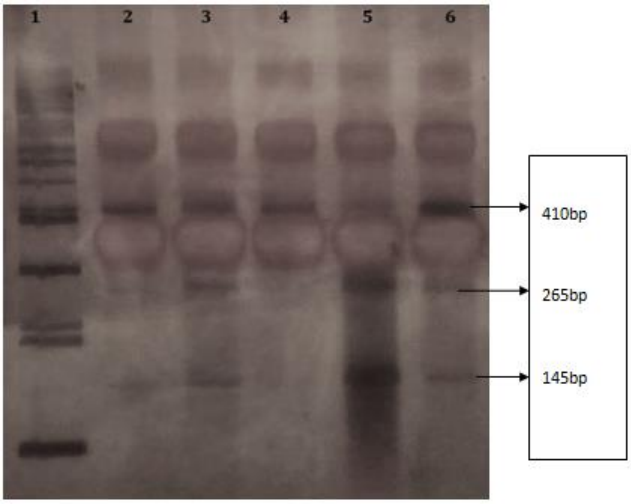

Figure 2. PCR products of RFLP to assess rs2270669 Polymorphism

Lane 1 – Ladder
Lane 4 –shows 410 bp band indicative of CC genotype.
Lane 5 – shows 265 and 145 bp bands indicating GG genotype.
Lane 6 –shows 410, 265 and 145 bp bands indicating GC genotype.

Results of the SNV rs36117715 had a tenfold increased association with CT genotype for uterine leiomyoma when compared to CC or TT genotypes, refer table3(a) and 3(b) individually while SNV rs2270669 showed threefold

likelihood of UL risk for G allele, as per the dominant mode of inheritance it is 5.6-fold. Refer tables 4(a) and 4(b)

COL6A3 EXON 28, rs36117715, c.6653C>T, p. Pro2218 Leu

Table 3(a): Allelic Frequency between Cases and Controls of rs361177715

Alleles	Genotype	Controls	Cases	OR (95%CI)	p-Value
C	CC	53(94.64%)	39(69.64%)	5.17 (1.69-15.82)	p =0.004
C/T	CT	2(3.57%)	16(28.57%)		
T	TT	1(1.78%)	1(1.78%)		

Table 3(b) COL6A3 rs36117715 association with cases and controls

Model	Genotype	OR (95%CI)	P-value	Z-Stats
T Vs C	C/T	5.17(1.69-15.82)	0.004	-
Dominant CT+TT Vs CC	C/T-T/T C/C	7.70(2.11-28.12)	0.002	3.089
Codominant CT Vs CC+TT	C/T C/C T/T	10.80(2.36-50.06)	0.002	3.05
Recessive TT Vs CC +CT	T/T C/C -C/T	1.36 (0.08-22.40)	0.8	-

COL6A3, Exon41, rs2270669, c.9034G>C, p. Ala3012Pro

Table 4(a): Allelic Frequency between Cases and Controls of rs2270669

Alleles	Genotype	Controls n=sample size (%)	Cases n=sample size (%)	OR (95%CI)	p-Value	Z- Stats
G	GG	15 (26.78%)	27(48.21%)	0.31(0.18-0.53)	<0.0001	4.217
G/C	GC	12 (21.42%)	20(35.71%)			
C	CC	29(51.78%)	9(16.07%)			

Table 4(b): COL6A3, rs2270669 association with cases and controls

Model	OR (95% CI)	p-value	Z-Statistics
G Vs C	3.2 (1.8-5.6)	<0.0001	4.217
Dominant GG+GC Vs CC	5.6 (2.8-13.5)	0.0001	3.8
Over dominant GC Vs CC . GG	2.04 (0.88-4.72)	0.1	1.66
Recessive GG Vs GC+CC	2.5 (1.15-5.6)	0.02	2.3

Discussion- UL are considered as the most common benign smooth muscle tumors of the myometrium. They have excessive quantities of ECM including collagen. Extracellular matrix has various functions being a greater component of the cell microenvironment. Every now and then it undergoes remodeling, deposition, degradation and modification. Any abnormality in the ECM properties may lead to removal of restrictions of cell proliferation and invasion, loss of apoptosis and failure of cell differentiation, ultimately causing congenital disorders, tissue fibrosis and tumor development. Lu et al 2011 [19].

Variants of collagen gene family had been implicated in producing components of the ECM that play a role in the aetiology of fibroids [7&8]. Components of the ECM may be important in initiating and developing these tumors. Increase in the density of tissues could be due to increased collagen deposition, reduction in remodeling and heightened post-translational modifications like cross-linking of some ECM components. Increased deposition of Collagens I, II, III, V, and IX is seen in tumor formation. [20]. Development of fibroids could be due to abnormalities in collagen structure, orientation and metabolism. Variant members of the collagen gene family could cause alterations in maintaining, renewing and regulating the extracellular matrix and ultimately leading to the formation of UL.

Ryo et al [21] stated that variations and gene expression of as many as, 120 genes, including alteration of DNA methylation and mRNA gene expression was observed between UL and normal myometrium. Among these were the COL4A1, COL4A2, COL6A3 genes. It had been reported by Demir and Patrizia et al (2002) that mutations in COL6A3 gene cause both mild and severe types of Ulrich congenital muscular dystrophy (UCMD) [22].

Increase in the expression of type I and type V collagen could cause the pathogenesis of uterine leiomyoma was said by Iwahashi and Muragaki in 2011 [23]. A Japanese study reported intermolecular rather than intramolecular cross linking was accelerated in collagen metabolism, this abnormality led to UL development. The study by Aissani et al exome examination and gene expression profiling revealed gross chromosomal changes like chromothripsis causing UL pathogenesis leading to translocations in HMGA2 and RAD51 B loci and chromosomal abnormalities in COL4A5-COL4A6 loci. Further fumarate hydratase gene had germline mutations affecting the highly conserved amino acids. Aissani et al reported that in the European American population significant associations of variant member of COL6A3, c.9034G>C (p. Ala3012Pro) with UL risk and the SNV rs36117715, c.6653C>T, p. Pro2218Leu in AA population had P = 0.029 for UL size.

In our preliminary study CT genotype has shown to pose tenfold higher risk for UL than CC or TT genotypes individually and it is shown to be statistically significant [Odds Ratio (95% CI) 10.8 (2.36-50.06) P=0.002].

The work that we have done has a good result for SNV rs36117715 since the Odds Ratio shows tenfold higher UL risk for women with CT and TT genotype rather than rs2277069 SNV which showed 5.6-fold higher likelihood of UL risk comparatively [Odds Ratio (95% CI) 5.6 (2.3-13.5) P=0.0001]

V.CONCLUSION AND FUTURE SCOPE

COL6A3 gene polymorphisms should be considered as a specific molecular biomarker for identifying women at risk

of developing UL despite the small size of the study. Validating this on a larger sample size from different regions of India is warranted. In this preliminary study bothCOL6A3polymorphisms appear to be significantly associated with UL. The "T" allele of rs36117715 and "G" allele of rs2270669 increase the risk of UL and can be used as biomarkers for identifying women likely to develop UL.

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