Journal of
Cell Science &
Molecular Biology

Open Science Publications

Volume 3, Issue 1 - 2017

www.opensciencepublications.com

IGF2 *ApaI* 'G' Allele, a Significant Risk Factor for Uterine Fibroids with Putative Association with High BMI

Research Article

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Article Information: **Submission:** 05/12/2016; **Accepted:** 02/01/2017; **Published:** 27/01/2017

Abstract

Uterine Leiomyomas (UL's) or fibroids are the most common pelvic tumor in women and the estimated incidence is 20%-40% during their reproductive years. The gene expression profile of UL has been examined, particularly in comparison to adjacent myometrium and several differentially regulated genes were identified, this includes Insulin-like Growth Factor-2 (IGF2), which was found to be upregulated significantly. IGF2 is a well-established cell proliferation factor and a recent study described it as a fibroid specific marker. The IGF2 *ApaI* polymorphism in the gene was evaluated in the present study. The 'G' allele was found to be significantly associated with uterine leiomyomas (OR = 3.56; 95% CI (2.454 - 0 5.172), p < 0.0001). The risk ratio for developing uterine fibroid for individuals with GG genotype was found to be almost four fold when compared to AA or AG genotype in the evaluated population group. Furthermore, 13% (3/23) of obese individuals i.e patients with BMI > 30 kg/m^2 as per WHO criteria were either homozygous or heterozygous for IGF2 'G' allele indicating its possible association with high BMI in UL cases.

Introduction

Uterine leiomyomata (UL), are the most common benign pelvic gynecological tumors. The estimated lifetime risk of uterine fibroids in a woman by the age of 45 years is more than 60% [1]. Based on the fact that 20-30% women in reproductive age group have symptomatic fibroid uterus [2], it is estimated that at least 15-25 million women will be suffering from a fibroid uterus at given time (Ministry of Health and Family Welfare, Govt. of India). Women present with prolonged or heavy menstrual bleeding, pelvic pressure or pain and in rare cases, reproductive dysfunction which requires medical attention [3]. Known risk factors for development of uterine fibroids include nulliparity, early menarche and increased frequency of menses, history of dysmenorrhea, family history of uterine fibroids, ethnicity, obesity and age. To date, the pathogenesis of UL is not well understood because of the disease heterogeneity and multifactorial involvement [4,5]. Earlier work from various groups including ours supports the concept that ovarian sex steroid hormones, growth

ISSN: 2350-0190

factors, apoptosis-related and mitochondrial factors may play critical roles in the regulation of leiomyoma growth [6-10].

Insulin-like Growth Factor 2 (IGF 2), a circulating peptide hormone with high degree of amino acid sequence homology to insulin is well known for its contribution in mammalian cell growth due to its regulatory influence on cell division, differentiation and metabolism [11]. IGF2 may also play a role in intrauterine programming predisposing to different disease in postnatal life [12,13]. IGF2 has been identified as a fibroid-specific marker and its expression levels were found to be increased in leiomyomas when compared to adjacent myometrium [14,15]. According to GWAS studies this region was found to be associated with UL in Asian population [5].

Several reports have shown that specific polymorphisms of IGF2 are associated with weight and obese phenotype [16-20] and are also related to cardiovascular risk factors such as fat mass distribution [21] and hypertension [22,23]. IGF-2 *ApaI* (rs680) polymorphism located in 3' UTR region was demonstrated as a risk factor for renal complications in Diabetes Mellitus cases. Moreover, increased IGF2 expression in association with this polymorphism in obesity and PCOS cases was noted earlier [16,24]. However, its association with risk of uterine leiomyomata remains speculative with contradictory reports [25,26]. Therefore, in the present study, we sought to determine the association of IGF2 *ApaI* to the risk of UL among patients of South Indian population.

Methodology

Subjects and sampling

The present study was carried out on 150 patients (21 to 64 years) diagnosed to have Uterine Leiomyomas (UL's) by clinical and ultrasound examinations and one hundred and fifty age-matched healthy control women (without any evidences of gynecological problems and normal ultrasound). They were enrolled from different hospitals of Hyderabad and Secunderabad cities of South India. The study was approved by the institutional ethical committee prior to its commencement. The patient's clinical and demographic history including age, age at menarche, body weight, height, dietary habits, menstrual history, obstetric history and family history were documented in a specially designed clinical case sheet at the time of sampling. After obtaining informed consent, 2 ml of Na-EDTA blood and leiomyoma and matched myometrium tissue biopsies (0.5-1 cm thickness tissue) in normal saline from hysterectomised uteruses was collected from the study participants to carry out genetic analysis.

Nucleic acid isolation

The DNA isolation from blood and tissue specimens was done following the routine salting-out method described earlier [6].

IGF-2 *ApaI* genotyping

In the present study, 300 DNA samples were processed for IGF2 *ApaI* polymorphism using specific primers, i.e., forward - 5'CTT GGA CTT TGA GTC AAA TTG G 3'; Reverse - 5'GGT CGT GCC AAT TAC ATT TCA. A three-step PCR of 25 µl reaction volume was carried out using thermal cycler (UV Gene, UK). Briefly, the PCR conditions included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 45 sec with a final extension at 72 °C for 5 min [27]. Amplified PCR products of 292 bp were run on a 2% agarose gel, and their band images were analyzed with UV I Tech gel documentation system (Cambridge, UK). Restriction digestion with *ApaI* enzyme was carried out to determine A (not digested by *ApaI*) and G alleles (digested by *ApaI*) by specific banding pattern on the gel i.e., 292 bp and 229 bp, respectively.

Statistical analysis

Statistical analyses were performed with Graphpad quick calc, version 6.0 (GraphPad Software Inc, USA) online statistical software. For each SNP, allele and genotype frequencies were computed in controls. Statistically significant difference in allele and genotypes was determined using Pearson's standard Chi-square test, odds ratio (OR) and 95% confidence interval (CI). Hardy Weinberg equilibrium was tested on a contingency table of observed versus predicted genotype distributions using a chi-squared test with one degree of freedom. A p-value of 0.05 was considered statistically significant.

Multifactor Dimensionality Reduction (MDR) analysis

MDR software application aids to determine which polymorphisms and which environmental factors are associated with common, complex diseases such as Uterine fibroids. This algorithm enables determining and characterizing interactions among multiple factors. In the present study, MDR was applied to demographic characteristics and gene polymorphisms [28].

Results

Clinical analysis

Women with UL were in the age range of 21-64 years and the mean age at hysterectomy was 41.36 ± 7.32 years. For controls, age range was 23-54 years (39.32 ± 6.01 years). Approximately, 80% (120/150) of women diagnosed with UL reported symptoms such as polymenorrhagia, passing clots, pelvic pain and pressure. The average

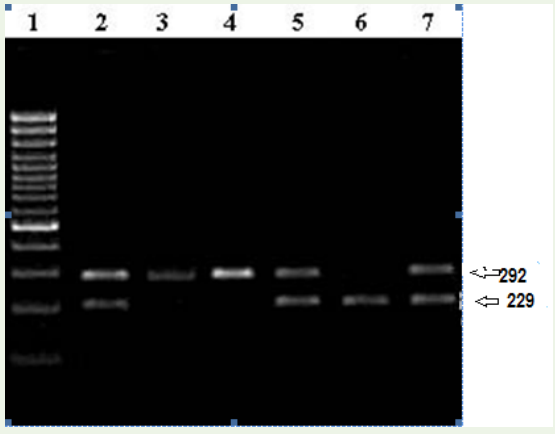

Figure 1: IGF2 PCR products after digestion with ApaI restriction enzyme Lane 1: 100bp DNA ladder. Lanes: 2, 5, 7 exhibits heterozygosity with 292/229 bp digested fragments. Lane 3,4 exhibit homozygosity for 292bp fragment. Lane 6 shows homozygosity for 229 bp fragment.

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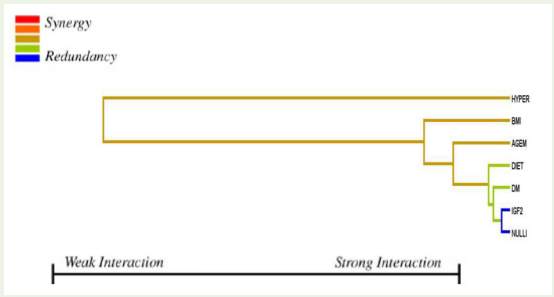

Figure 2: Interaction dendogram showing moderate synergistic interaction between genetic and clinical factors. IGF2 risk allele has shown moderate synergy with BMI.

Table 1: Clinical and anthropometric variables of leiomyoma cases and controls.

Characteristic Feature	Leiomyoma Cases; n=150 (%)	Healthy Controls; n=150 (%)	Statistical Value
Body Mass Index (kg/m²)			
As per Asia-Pacific			
Lean (< 25 kg/m²)	73 (48.5)	76 (50.6)	p = 0.3896
Obese (> 25 kg/m²)	77 (51.5)	74 (49.4)	p = 0.777
As per WHO			
Normal (< 24.99)	73 (48.5)	76 (50.6)	p = 0.3896
Overweight (25-29.99)	54 (36.3)	60 (40)	p = 0.1336
Obese (≥ 30)	23 (15.2)	14 (9.4)	p = 0.0015
Age at menarche (in years)	12.20 ± 1.23	12.8 ± 0.85	p < 0.0001
Type of Leiomyomas			
Intramural	61.16 %	-	-
Intramural + Sub-Serosal	22.40 %	-	-
Sub-Serosal	7.80 %	-	-
Sub-Mucosal	3.89 %	-	-
Nulliparity	22 (14.67)	2 (1.34)	p < 0.0005
Hypertension	22 (14.76)	6 (4)	p < 0.0001
Type 2 DM	19 (12.75)	6 (4)	p < 0.0001
Thyroid dysfunction	11 (7.4)	8 (5%)	P = 0.4777

body weight in cases of UL ranged from 30-95 kg with mean BMI of 25.10 ± 6.17. It was found that obese women according to WHO criteria were more in cases than controls showing the difference to be statistically significant (p = 0.0015). Age at menarche ranged from 8-16 years in women with fibroids and early age at menarche i.e., ≤ 11 years was found in higher number of women with UL when compared to control group whereas later age at menarche (> 14 years) was seen only in cases with none in control group. Thus, both early and late menarche were found to be associated with fibroids. Co-morbidities such as hypertension, Type 2 DM also showed association with UL which was statistically significant. Multiple fibroids occurred in 70.3% patients while 29.7% were solitary tumors and 24.27% of UL cases had large tumors with a size of > 72 mm. Fibroids are of five types based on their location of occurrence in uterus. The present data shows the occurrence of Intramural fibroids (IM) was more common (~ 61%) when compared to sub-serosal (SS), sub-mucosal (SM) or cervical fibroids.

IGF2 *ApaI* genotyping analysis

Of the 150 individuals with uterine fibroids genotyped in the present study 39.3% were A/A, 44% were A/G and 16.7% were G/G while in controls the genotypes were 64% A/A, 33.4% A/G and 2.7% G/G (Table 2). There was an association of UL with 'G 'allele (OR = 3.56; 95% CI (2.454 - 5.172) and G/G genotype (OR = 7.30; 95% CI (2.473 - 21.55) when compared to controls.

MDR analysis of IGF2 *ApaI* polymorphism with clinical variables

The IGF2 *ApaI* A>G polymorphism when evaluated with the demographic and clinical factors like diet, nulliparity, hypertension, BMI, age at menarche, Diabetes Mellitus by MDR analysis has shown more or less independent role of these genetic variants in disease (Figure 2).

IGF2 (AG and GG genotype only) variation was found to have mostly redundant interaction with clinical variables showing negative interaction. IGF2 was showing synergistic interaction in the order of hypertension, BMI, Age at menarche followed by Diet and T2DM and nulliparity was showing the most redundant interaction. The present data corroborates with the finding of putative association of high BMI with G allele of IGF2 *ApaI* polymorphism [16,29] (Figure 2).

Discussion

IGF2 is known to be involved in the pathogenesis of several human tumors. It has proliferative activity in adult muscle, including those arising from smooth muscle [25]. The up-regulation of IGF2 was evident in leiomyomas compared to normal myometrium of uterus in several studies [14,15,28,30-35]. IGF2 shows growth stimulatory effect on Leiomyomas through mediation of ER-α and IGF-1R [36,37]. The 3'UTR region of IGF2 was associated with UL in a previous study [38].

IGF2 gene polymorphism was studied by Gloudemans et al. who were pioneers in this field and they showed differences between allele frequencies in the *ApaI* polymorphism among Dutch patients with

Table 2: Showing the genotyping results of IGF2 ApaI polymorphism.

SAMPLES	AA	AG	GG	A	G
CONTROLS (n = 150)	96 (64%)	50 (33.4%)	04 (2.7%)	242 (80.7%)	58 (19.3%)
CASES (n = 150)	59 (39.3%)	66 (44%)	25 (16.7%)	184 (61.4%)	116 (38.6%)

Minor Allele Frequency (MAF) G vs A: OR = 3.56; 95% CI (2.454 – 5.172), p < 0.0001*

GG vs AG + AA: OR = 7.30; 95% CI (2.473 – 21.55), p < 0.0001*, RR=3.907

GA+GG vs AA: OR = 2.742; 95% CI (1.718 – 4.376), p < 0.0001*, RR=1.649

GA vs GG + AA: OR = 1.571; 95% CI (0.958 – 2.580), p < 0.075*, RR=1.246

G/G genotype individuals in cases are significantly higher compared to controls.

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uterine leiomyomas and a matched unaffected control group [26]. The IGF2 G allele was associated with increased IGF2 mRNA levels in leukocytes compared with the A alleles. It was hypothesized that this functional polymorphism could result in increased IGF2 expression in liver Vafiadis et al. Vu et al. demonstrated a similar correlation in American women with uterine smooth muscle tumors. Based on these data, it was suggested that women homozygous for allele *G* are more prone to develop leiomyomas. In the present study, 'G' allele (presence of *Apa I* site) was found to be strongly associated with uterine leiomyomas showing Minor Allele Frequency G vs. A to be highly statistically significant ($p < 0.0001$). The risk ratio for developing uterine fibroids for GG genotyped individuals was found to be almost seven fold when compared to AA or AG individuals in the evaluated population group (Table 2).

The data from the previous study Rainho et al., suggested the putative association between IGF2 *ApaI* polymorphism and BMI in women with uterine leiomyomas [25]. However, lack of statistical significance provides no support for the hypothesis that obese patients who are carriers of '*G*' allele constitute a group more prone to leiomyoma development. The present study MDR analysis showed moderate synergistic interaction of IGF2 *ApaI* with BMI and 13% (3/23) of individuals who were either homozygous or heterozygous for 'G' allele have a higher BMI (≥ 30 kg/m^2) when compared to AA individuals. The data from 150 patients indicate the possible association between 'G' allele and obesity as per WHO criteria, however, more studies need to be carried out in a large sample size.

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