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Genetics

# INFLUENCE OF +61 G/A POLYMORPHISM ON EGF GENE EXPRESSION DUE TO ALTERED TRANSCRIPTION FACTOR BINDING AND MRNA STABILITY

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| **Ch. Bharathi** | Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Begumpet, Hyderabad, Telangana, India |
| **D. Krishnaveni** | Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Begumpet, Hyderabad, Telangana, India |
| **N. Pratibha** | Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Begumpet, Hyderabad, Telangana, India |
| **Aruna Ramaiah** | Dept. of Gynaecology, Government Modern Maternity Hospital, Hyderabad, Telangana, India. |
| **A. Venkateshwari*** | Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Begumpet, Hyderabad, Telangana, India *Corresponding Author |

**ABSTRACT** **BACKGROUND:** Uterine Leiomyomas are the stable pelvic benign neoplasms characterized by abnormal myometrial cell proliferation initiated by steroid hormones which act on their target tissue through local modulation of growth factors like EGF, TGF, VEGF and PDGF.

**AIM OF THE STUDY:** The present study was conducted to investigate the association of EGF +61 G/A 5' UTR polymorphism with uterine leiomyomas.

**PATIENTS AND METHODS:** A total of 104 women with uterine leiomyomas and an equal number of healthy women without any reproductive abnormalities were included in the present study. Genotyping of the EGF gene (rs4444903) polymorphism was carried out using PCR-RFLP followed by agarose gel electrophoresis.

**RESULTS:** Appropriate statistical methods were applied to test for the significance of the results obtained. The demographic characteristics of patients and controls revealed significant difference with respect to age (p=0.0005), BMI (p=0.00086), age at menarche (p=0.00528), parity (p=0.0078), menorrhea (p=0.00001) and a borderline significance with respect to diet (p=0.06). The genotype frequencies of GG, GA and AA were 18.2%, 68.2% and 13.4% in controls and 23.04%, 56.7% and 20.1% in patients respectively, whereas the frequency of G and A allele among control group were 52.4% and 47.5% and case group was 51.44% and 48.55% respectively. There was no statistical significant difference in the distribution of genotypic or allelic frequencies between the two groups. The bioinformatic results have shown changes in pre-mRNA structure and transcription factor binding site between "G" and "A" alleles.

**CONCLUSION:** The present study suggests that EGF +61 G/A gene polymorphism may not be a major genetic regulator in the etiology of uterine leiomyomas.

**KEYWORDS :** Uterine leiomyoma; Epidermal Growth Factor; Apoptosis; Proliferation; Extracellular matrix.

## INTRODUCTION:

Uterine leiomyomas (UL) (fibroids or myomas) are solid pelvic benign, monoclonal neoplasms of uterus characterised by an extraordinary myometrial or smooth muscle layer growth, apposition of extracellular matrix (i.e., collagen, proteoglycan, fibronectin), commonly found in 20-40% of women of reproductive age[1,2].

Growth of leiomyomas is largely dependent on ovarian hormones i.e., estrogen and progesterone. As fibroids are diagnosed only after menarche and regress on menopause. The growth-promoting effects of estrogen and progesterone upon the myometrium and uterine myomas may be mediated through the mitogenic effects of growth factors produced locally by smooth muscle cells and fibroblasts [3, 4]. In addition, it is well established that ovarian hormones act on their target tissue through local modulation of several growth factors, cytokines and chemokines via autocrine/paracrine signalling pathways, all of which play a vital role in the myometrial cellular transformation and turnover which ultimately leads to leiomyoma formation [5].

Epidermal growth factor (EGF) is a major growth factor among the ligands of EGF receptors (EGFRs) which plays an important role in regulating mitogenesis, angiogenesis, proliferation, survival and differentiation during development, tissue homeostasis and tumourigenesis. It is located on chromosome 4q25 and is 110-kb in length spanning 24 exons which encodes a 4.8-kb mRNA [6-8]. Binding of EGF to its receptor (EGFR) on the cell surface activates a series of intracellular signaling networks including PI3K/AKT, Ras/Erk and JAK/STAT. These networks activate or deactivate some transcription factors like FOXO, ETS-1, C-JUN, C-MYC, CReB, NF-KB and STAT, Sp1 which are responsible for cell proliferation, inhibition of apoptosis, and differentiation [9-11]. Genetic variation located in 5' untranslated region at nucleotide position 61 with a G to A transition is one of the most important polymorphisms in EGF gene [7]. The aim of the present study was to determine the association of EGF (+61 G/A) gene polymorphism in the etiology of uterine leiomyomas as a growth modulator.

## MATERIALS AND METHODS:

**Subjects:** The present case-control study was carried out on the blood samples collected from 104 UL patients referred to Modern Government Maternity Hospital, Petlaburz, Hyderabad India, and an equal number of control samples without prior history of reproductive abnormalities or family history of UL during their reproductive age [25-40 yrs] were considered. The patients and controls were confirmed based on ultrasonography. An informed written consent was obtained from all the subjects and the demographic characteristics such as age, menarche, diet, menstrual history and parity were obtained with the help of a standard proforma. The study was approved by Institutional Ethical Committee of Institute of Genetics, Osmania University, Hyderabad.

**Sample collection:** Five ml of venous blood was drawn from each individual in vaccutainers containing EDTA and stored at 4°C. Genomic DNA was extracted from the peripheral blood using salting out method described by Lahiri et al [12], and stored in TE buffer at -20°C until further use.

**Determination of EGF +61 5' UTR Gene Polymorphism:**
The EGF +61G/A (rs4444903) genotypes were determined as previously described by Shabazi et al (2002) [13] by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) assay. The primers for amplifying the EGF fragment were 5' TGTCACTAAAGGAAAGGAGGT-3' (forward) and 5'-TTCACAGAGTTTAACAGCCC-3' (reverse) [Bioserve, India]. PCR amplification of a 242 bp region of EGF was performed in a 25-µl reaction containing, 10x reaction buffer, 1.5 µM $MgCl_2$, 0.8 mM each

dNTP, 7 μM each primer (forward 5'- and reverse), 1 unit Taq- DNA Polymerase (Bangalore Gene, India) and 40 ng of genomic DNA. The PCR cycling conditions were 95°C for 10 min followed by 35 cycles at 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min followed by a final extension of 72°C for 10 min. An 8-μl aliquot of PCR product was subjected to digestion at 37°C overnight in a 10 μl reaction containing 10 U AluI (Fermentas, USA) and 1x reaction buffer. Later, the products were separated on a 3% agarose gel stained with ethidium bromide. As a result, the +61 G allele produced DNA fragments of size at 15, 34 and 193 bp, while the +61 A allele introduced an additional restriction site after digestion with AluI and produced fragments of size 15, 34, 91, and 102 bp. Whereas the heterozygotes displayed a combination of both alleles of size 15, 34, 91,102 and 193 bp. A total of 10 % of the samples were randomly taken, and the assay was repeated for credibility and found no bias in the genotyping with 100% concordance.

**Statistical Analysis:**
Hardy-Weinberg equilibrium was tested for EGF +61G/A polymorphism, and any other deviation between the observed and expected frequencies was tested for significance using the chi-square test. Differences in age, menarche, BMI, parity, diet, menorrhea was compared between the uterine fibroid patients and controls. Genotype distribution in the control and case groups were compared with values predicted by using $\chi^2$ test of analysis. Odds ratios (OR) and their 95% confidence intervals were computed to measure the strength of association between EGF gene and uterine fibroids.

**Bioinformatics analysis:**
Bioinformatics analysis was carried out to identify the stability of the pre-mRNA secondary structures (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). The changes in the transcription factors binding to the alleles were also identified to infer their role on gene regulation (http://www.gene-regulation.com/pub/programs/alibaba2/ index.html).

**RESULTS:** A total of 104 uterine leiomyoma patients and an equal number of controls without any abnormal reproductive history were included in this study. The demographic characteristics of patients and controls presented in Table 1 revealed a significant difference with respect to age (p=0.0005), BMI (p=0.00086) age of menarche (p=0.00528), parity (p=0.0078), menorrhea (p=0.00001) and a borderline significance with respect to diet (p=0.06).

**Table 1: Demographic Features of uterine Leiomyoma patients and control subjects**

| | Uterine fibroids N% | Controls N% | χ2 | OR (95%CI) | P-value |
|---|---|---|---|---|---|
| **Age (years)** | | | | | |
| <30 | 26 | 51 | | | |
| ≥30 | 78 | 53 | 11.88 | 0.34(0.19-0.62) | 0.0005** |
| BMI* | | | | | |
| Normal | 42 | 67 | | | |
| Over weight | 62 | 37 | 11.1 | 0.3741(0.21-0.65) | 0.00086* |
| **Menarche (years)** | | | | | |
| 10-12 | 68 | 47 | | | |
| 13-15 | 36 | 57 | 7.77 | 2.291(1.31-4.00) | 0.00528* |
| **Parity (n)** | | | | | |
| Parous | 55 | 35 | | | |
| Nulliparous | 49 | 69 | 7.07 | 2.2(1.2-3.8) | 0.0078* |
| **Diet** | | | | | |
| Vegetarian | 13 | 24 | | | |
| Non-Vegetarian | 91 | 80 | 3.28 | 0.47(0.2-0.9) | 0.06 |
| **Menorrhea** | | | | | |
| Eumenorrhea | 38 | 77 | | | |
| Dysmenorrhea | 66 | 27 | 28.08 | 0.21(0.11-0.36) | 0.00001** |

***p<0.05; **p<0.001 *BMI = weight (Kg)/ height2 (m2)**

**Genotyping:**
The genotypic distribution of +61G/A EGF polymorphism is illustrated in Table 2. The frequencies of genotype GG, GA and AA were 18.2%, 68.2% and 13.4% in controls and 23.04%, 56.7% and 20.1% in patients with uterine leiomyoma respectively. The frequency of G and A alleles among control group was 52.4% and 47.5% and in patient group was 51.44% and 48.55% respectively. There was no statistical significant difference found in the distribution of genotypic and allelic frequencies between the two groups. Further the analysis with different models, (test power = 0.66) codominant model: GG vs. AA (OR=0.84, 95% CI=0.34−2.1), dominant model GG vs. GA+AA (OR=1.34, 95% CI=0.68−2.6), overdominant model GA vs. GG+AA (OR=0.6, 95% CI=0.3−1.07), recessive model AA vs. GA+GG (OR= 1.6, 95% CI=0.7−3.4) and G allele vs. A allele (OR=0.96, 95% CI=0.6−1.4) also revealed no significant difference between the patient and control subjects.

**Table 2: Distribution of genotypes and allelic frequencies in patients with uterine leiomyomas and controls**

| Co-dominant | Cases N (%) | Controls N (%) | χ2 | OR (95% CI) | p-Value |
|---|---|---|---|---|---|
| GG | 24(23.04) | 19(18.2) | --- | ---- | --- |
| GA | 59 (56.7) | 71(68.2) | 1.021 | 1.5(0.75-3.0) | 0.314 |
| AA | 21(20.1) | 14(13.4) | 0.0201 | 0.84(0.34-2.08) | 0.88 |
| **Dominant** | | | | | |
| GG | 24(23.07) | 19(18.2) | --- | ----- | ---- |
| GA+AA | 80(76.92) | 85(81.7) | 0.46 | 1.34(0.68-2.6) | 0.493 |
| **Recessive** | | | | | |
| GA+GG | 83(79.80) | 90(86.53) | ---- | ------ | ---- |
| AA | 21(20.19) | 14(13.46) | 1.23 | 1.6(0.7-3.4) | 0.26 |
| **Overdominant** | | | | | |
| GG+AA | 45(43.26) | 33(31.73) | --- | ----- | ---- |
| GA | 59(56.73) | 71(68.26) | 2.482 | 0.6(0.3-1.07) | 0.115 |
| **Allele Frequency** | | | | | |
| G | 107(51.44) | 109(52.4) | ---- | ---- | ---- |
| A | 101(48.55) | 99(47.5) | 0.009 | 0.96(0.6-1.41) | 0.921 |

***p<0.05; **p<0.001**

**Bioinformatics results:**
Secondary pre-mRNA structures were built as per the background algorithm at the http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi web server. Distinct energy changes for the respective alleles were observed.

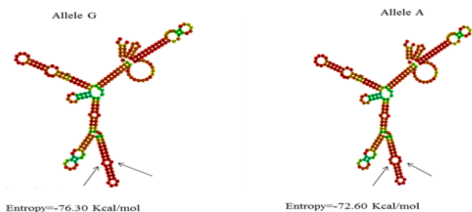


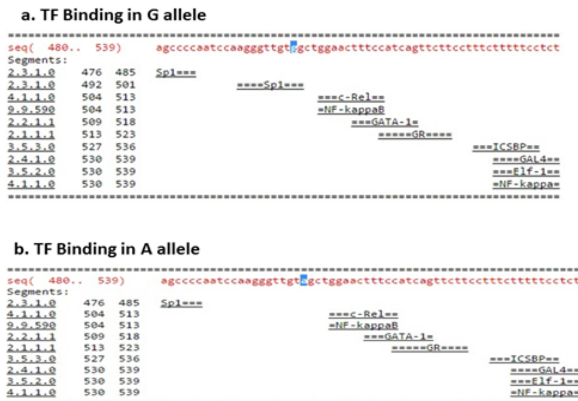
**Fig. 1** Pre-mRNA secondary structures of the individual alleles of EGF+61G/A polymorphism

**EGF+61G/A:**
**mRNA Prediction:**
The pre-mRNA secondary structure prediction of wild and mutant allele (Fig. 1) showed changes in the two alleles of the +61G/A polymorphism (shown by arrows). The allele "A" showed higher entropy (−72.60 kcal/mol) with decreased half-life, when compared to the "G" allele (−76.30 kcal/mol) with increased half life. It can be hypothesised that the stability of the "G" allele's transcript could play a role in disease manifestation by increasing the expression of EGF gene which ultimately results in higher EGF production. Earlier studies reported by Shabzi et al (2002) [13] in malignant melanoma have identified the presence of "G" allele confers higher production of EGF when compared to "A" allele, thus it can be predicted that the presence of "G"allele could play a vital role in leiomyoma formation and progression.

**Transcription Factor Binding Change:**
An additional transcription factor binding site for SP1 (Specificity protein 1) was abolished in the "A"allele when compared to the "G" allele which is indicated in (Fig 2 a & b). SP1 is a zinc finger transcription factor that binds to GC-rich motifs of many promoters, which are involved in many cellular processes, including cell differentiation, cell growth, apoptosis, immune responses, response to DNA damage, and chromatin remodeling. SP1 protein undergoes a number of posttranslational modifications including phosphorylation, glycosylation, and acetylation, enabling fine tuning of the regulation of

gene transcription, which can act as an activator or a repressor and an increase in transcriptional activity and higher protein end product [14].The increased levels of protein may lead to a spike in EGF activity, which may contribute to the pathogenicity of UL.

**Fig 2: Transcription Factor Binding in +61 G/A Allele**




## DISCUSSION:

Uterine leiomyomas are characterized by an increase in smooth muscle cell (SMC) proliferation and excessive deposition of extracellular matrix proteins, primarily collagens type I and III [15]. Growth of leiomyomas occurs as a multistep process, with the involvement of ovarian steroid hormones, cytokines and growth factors. Steroid hormones induce mitogenic effects directly through their receptors or by regulating expression of growth factors such as the epidermal growth factor (EGF) and its receptor [16].

Leiomyomas fail to regress via apoptosis and undergo normal dedifferentiation resulting in dysregulated pattern of cellular differentiation and gene expression [17]. *In vitro* studies carried out by Maruo et al (2000) observed that progesterone up-regulates the expression of EGF in leiomyoma cells, whereas estrogen up-regulates the expression of EGF-R in cells indicating that estrogen and progesterone act in combination to stimulate the proliferative potential of leiomyoma cells through the induction of EGF and EGF-R expression [18]. Harrison-Woolrych et al. (1994) demonstrated that the concentration of EGF mRNA in leiomyomas which is comparable to that of the myometrium during the follicular phase is significantly elevated in leiomyomas during the luteal phase, while the concentration in the myometrium remains essentially unchanged [19]. Yeh et al. (1991) reported that the mitotic activity of leiomyomas is maximal during the luteal phase of the cycle, suggesting that the production of EGF may be one mechanism through which progesterone stimulates mitotic activity in fibroids [20].

The EGF-family of peptide growth factors play an important role as a potent mitogen in the development and progression of diverse benign and non-benign tumors, including leiomyoma, leiomyosarcoma, glioma, malignant melanoma and gastric cancer [16, 21, 22].To the best of our knowledge, this is the first study evaluating the role of EGF polymorphism in relation to uterine leiomyoma from our ethinic group, where in the EGF +61 G/A polymorphism did not reveal any significant difference in the genotype and allele distribution in patients compared to controls, which is consistent with the results reported by Kandace et al (2004) in incident primary melanoma in the Caucasian population [8]. In addition, a study by Goto et al (2005) on gastric cancer in Japanese population [23] also revealed no association of EGF polymorphism with the disease. Though few studies have revealed a significant association between EGF gene [+61 G/A] polymorphism in other diseases like gastric cancer and glioblastoma [24-26]. The preliminary study reported by Shahbazi et al (2002) identified a G to A polymorphism at position 61 in the 5' untranslated region of EGF gene which is correlated with in-vitro production of EGF. Their results showed that individuals with the A/A genotype had lower EGF production *in vitro* than individuals with the G/G or A/G genotypes where the G/G genotype was associated with malignant melanoma. The influence of this polymorphism affecting EGF production is unknown [13]. These results were supported by earlier studies by Michael et al (2004), who reported that EGF polymorphism do not appear to predispose to melanoma but its significant association with tumor thickness implies that it modifies tumor progression [27]. Later Hamai et al (2005) found an association of EGF 61G/A with gastric cancer risk and found that the 61GA/AA genotypes were associated with a significantly decreased risk of gastric cancer [24]. Shujie Wang et al (2010) [25] and Costa et al (2007) reported that the variant genotypes of GA and AA were associated with a significantly increased risk to glioblastoma, when compared to wild-type homozygote GG [26]. A comparision of our SNP study with insilico analysis of the pre-mRNA secondary structure showed a change in minimum free energy this change will cause shift in entropy of the "G" and "A" alleles hence, resulting in the decrease in stability of "A" allele transcript and increasing the stability of "G" allele transcript which leads to higher EGF expression and production by "G" allele. The transcription factor binding site has revealed loss of one of the SP1 binding site in the allele "A" and presence of two SP1 transcription factor binding sites in +61G/A polymorphism thus, indicating a substantial role of the "G" allele in disease pathogenesis and progression. In conclusion, it may be hypothesized that the effect of this polymorphism may have a modifier like effect on leiomyomas rather than an inducer effect.

**CONCLUSION:** The present study suggests that the EGF +61 G/A 5' UTR polymorphism may not be a major genetic regulator in the etiology of uterine fibroids in South Indian population from Telangana.

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**Conflict of interest:** The authors declare that they have no competing interests.

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