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Original Article

Somatic mutational analysis of *MED12* exon 2 in uterine leiomyomas of Iranian women

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Abstract: Uterine leiomyomas are steroid-hormone dependent tumors of myometrial smooth muscle cells that affect numerous women throughout the world. Based on previous studies, we evaluated the mutations of *MED12* gene which encodes a co-activator protein involved in transcription regulation of the vast majority of RNA polymerase II-dependent genes. Exon 2 of *MED12* gene was genotyped by PCR-sequencing method. To determine the proportion of mutation-containing transcripts, RNA was extracted from the tissue samples and the corresponding amplified cDNA was sequenced. We observed 11 mutation positive lesions, 7 of them were located in codon 44. The c.131G >A was found to be the most common somatic mutation in this study. Our investigation also demonstrated two unreported mutations , one large deletion and one insertion. cDNA analyzing revealed that the mutated transcripts were predominantly expressed in almost all changes including the new insertion mutation c.122-123ins15. Our study provides further evidence that the *MED12* somatic mutations occur in a heterozygous manner and are mostly missense mutations in codon 44. The results displayed 47.8% mutation positive lesions in Iranian patients confirming the diversity between the populations.

Keywords: Uterine leiomyomas, MED12, somatic mutation

Introduction

Uterine leiomyomas are myometrial benign tumors that affect women of reproductive age. They originate from uterine smooth muscle tissue and are estrogen and progesterone dependent which explains their relapse at menopause [1].

The main clinical symptoms of the disease is excessive uterine bleeding, although; symptoms depend on the location of the myoma and its size [2]. The tumors appear earlier in black than in white women and are significantly larger with more severe symptoms [3, 4]. This would imply a genetic mechanism of inheritance that is supported by the results of twin's, family and multiple hereditary leiomyoma studies [4] .

Using exome sequencing, Mäkinen *et al.* reported that 70% of the studied uterine leiomyomas have a range of somatic mutations in exon 2 of the mediator complex subunit 12 (MED12) gene [5]. The Mediator complex is a multi-subunit protein that regulates transcription by bridging between DNA regulatory elements and RNA polymerase II initiation complex [6]. Depending on the factors with which it interacts, MED12 operates as both transcriptional activator and repressor [7].

MED12 gene is located on Xq13.1 and contains 45 exons. Germline mutations in *MED12* are responsible for at least two different forms of X-linked dominant mental retardation, Lujan-Fryns and Opitz-Kaveggia syndromes [8]. Previous studies suggest that leiomyoma-linked mutations in *MED12* are involved in the activation of Wnt pathway leading to an impaired regulation of cell growth and tumorigenesis [9, 10]. Recently it has been reported that somatic *MED12* gene mutations lead to the highly specific reduction in CDK activity as a result of impaired MED12/Cyclin C-CDK8/19 interaction [11].

MED12 leiomyoma-linked mutations mostly occur at the nucleotides 130 and 131 of the exon 2, changing a conserved glycine amino acid located at position 44. However the distribution of the mutations is different based on the ethnic groups [12]. To further investigate the frequency of mutation-positive leiomyoma in Iranian women we conducted a somatic mutation detection study to verify if it is comparable to those reached by other studies.

Material and methods

Subjects and tissue sample collection

Twenty three patients were randomly selected from the women referred to the genecology department at Firoozgar general hospital (Tehran, Iran) from June 2013 to March 2014. All the patients were new cases with no medical history of surgery or therapy regarding gynecologic problems. They were first visited by an expert gynecologist and subjected to imaging and laboratory tests according to the standard diagnostic approaches. Each patient contributed to the study signed a written informed consent approved by the ethics committee of Iran university of Medical Sciences. Leiomyoma tissue samples were obtained via surgical operations. In addition, normal matched myometrium tissue was obtained from each patient. Every tissue sample was sectioned into two replicates; one replicate was examined by a pathologist for the evaluation of histopathlogical features. The other replicate was immediately transferred into liquid nitrogen containers and stored until molecular analysis.

DNA extraction and mutation detection

Genomic DNA was extracted from 50 mg of leiomyoma or normal myometrial tissue using DNA Isolation Kit for Cells and Tissues (Roche). Extraction of the peripheral blood genomic DNA was also performed using QIAamp mini kit (Qiagen) and considered as the control genomic DNA. The concentration and purity of the isolated DNA was determined by spectrophotometry (Nanophotometer™, Implen, Germany). High quality DNA samples (A260/280≥1.8) were selected and kept at -20°C. The primers used for the amplification of the *MED12* exon 2 were as previously described [5]. The amplified fragments were sequenced on ABI 3130 Genetic Analyzer instrument. The sequencing data were verified using ChromasPro software and aligned on the BLAST website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All sequence changes were confirmed on both strands.

RNA extraction and RT-PCR

Tissue samples (100 mg) were treated with TRI Reagent® (SIGMA-Aldrich) as recommended by the manufacturer and RNA was extracted using RNeasy mini kit (Qiagen). The RNA concentration and purity were determined by spectrophotometry. High quality RNAs (A260/280≥1.8, A260/A230>1.8) were selected and kept at -80°C until used for cDNA synthesis. Up to 1 µg RNA was converted to cDNA using Quantitect® reverse transcription kit (Qiagen) according to the manufacturer's instruction. To verify the integrity of the cDNA, the RT-PCR experiment was performed by *GAPDH* specific primers. Using the specific primers [5] the cDNA of *MED12* exon 2 was amplified and undergone sequencing to determine the proportion of mutation-containing transcript.

Statistics

Data were analysis by SPSS software (V.18). The differences in genotypes were assessed by standard Chi-squared analysis, with corresponding 95% confidence intervals (CI). p value <0.05 was considered statistically significant.

Results

Clinical characteristics

The mean age of the patients was 45.5±8.05 years old. The distribution of the gravidity and parity were 2.47±1.63 and 2.19±1.47 respectively. The data also revealed that the most common chief complaints of the patients were abnormal uterine bleeding (61.9%) and abdominal pain (32.3%). The main leiomyoma subtype allocated to the intramural with 61.5% of the samples followed by 23.1% of subserous tumors. The tumor size was <30 mm in 33.4%, 30-50 mm in 22.2% and >50 in 44.4% of the cases.

Tissue samples DNA genotyping

To search for *MED12* mutations associated with leiomyoma, we checked the tissue genom-

Table 1. Summary of the observed MED12 exon 2 mutations and patients characteristics

Patients	Age at diagnosis	Type of fibroids	Size	Nucleotide change	Predicted protein change
1	48	Intramural	Multiple	Large deletion	-
7	46	Subserous	Multiple, the biggest one 10-30 mm	c. 131G>A	p.G44D
10	30	Subserous	Multiple, the biggest one 10-30 mm	c. 130 G>T	p.G44C
12	45	Intramural	Multiple, the biggest one 30-50 mm	c. 131G>A	p.G44D
13	36	Intramural	>50 mm	c. 131G>A	p.G44D
15	40	Intramural	Multiple	IVS1-8T>A	p.E33_D34insPQ
16	42	Intramural/Subserous	Multiple	c.107T>G	p.L36R
17	36	Subserous	Multiple, the biggest one 10-30 mm	c. 131G >A	p.G44D
20	50	Submucous	10-30 mm	c.122-123ins15	p.V41_K42insTALNV
21	48	Intramural	<30 mm	c.130G>A	p.G44S
23	37	Intramural/Subserous	Multiple, the biggest one >50 mm	c. 131G >A	p.G44D

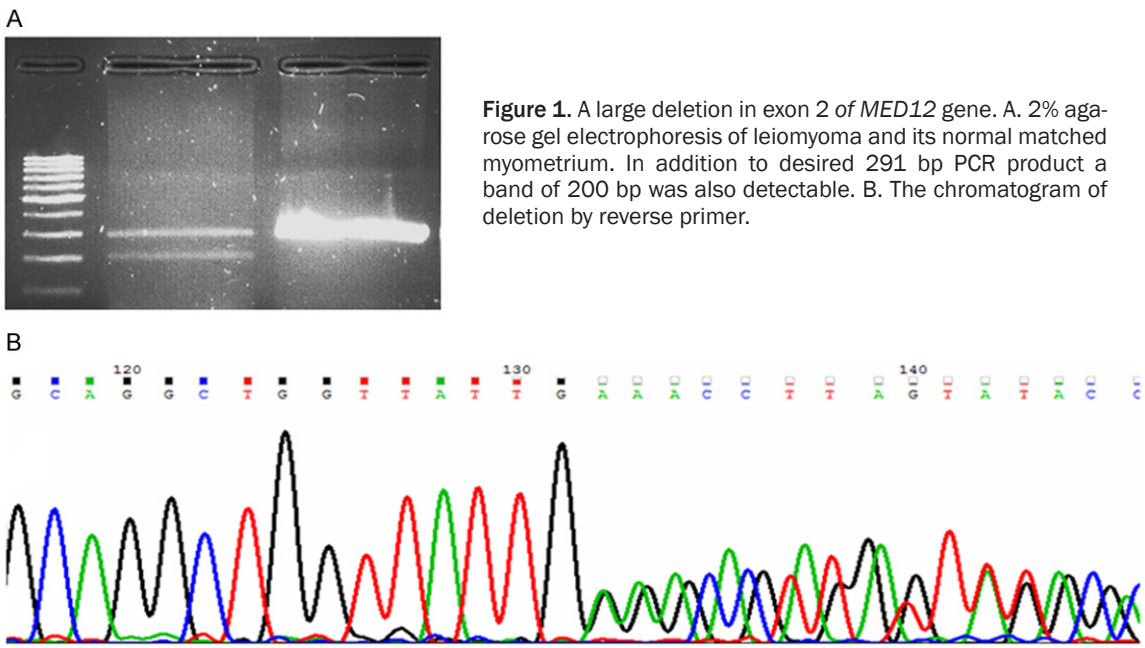

Figure 1. A large deletion in exon 2 *of MED12* gene. A. 2% agarose gel electrophoresis of leiomyoma and its normal matched myometrium. In addition to desired 291 bp PCR product a band of 200 bp was also detectable. B. The chromatogram of deletion by reverse primer.

ic DNA of the tumors and adjacent normal myometrium by Sanger sequencing. **Table 1** shows the summary of the observed *MED12* exon 2 mutations. All of the *MED12* changes that we detected in the uterine leiomyoma patients were heterozygous. In total of 23 samples, 11 samples (47.8%) harboured mutations. Seven of them were located in codon 44 with the frequency of 21.7%, for c.131G>A as the most common alteration.

A large deletion was first revealed by gel electrophoresis (**Figure 1**). Sequencing results showed a 91bp deletion starting from 56bp upstream of the splice acceptor site of exon 2. To the best of our knowledge, this is the largest deletion of *MED12* ever detected in uterine leiomyoma. Additionally, in our study, one novel insertion mutation c.122-123ins15 was identified (**Figure 2A**, **2B**). In the rest of the tissue samples (52.2%), we didn't observed any mutation in exon 2. For all the lesions with *MED12* mutations, normal matched myometrium tissues were examined. None of the samples showed the corresponding variations, confirming the somatic nature of the *MED12* mutations.

To evaluate the association between *MED12* mutation status and clinical characteristics, standard Chi-square analysis was conducted. We examined the age at diagnosis, gravidity, parity, miscarriages, menopausal condition, smoking, type of fibroids, and the size of

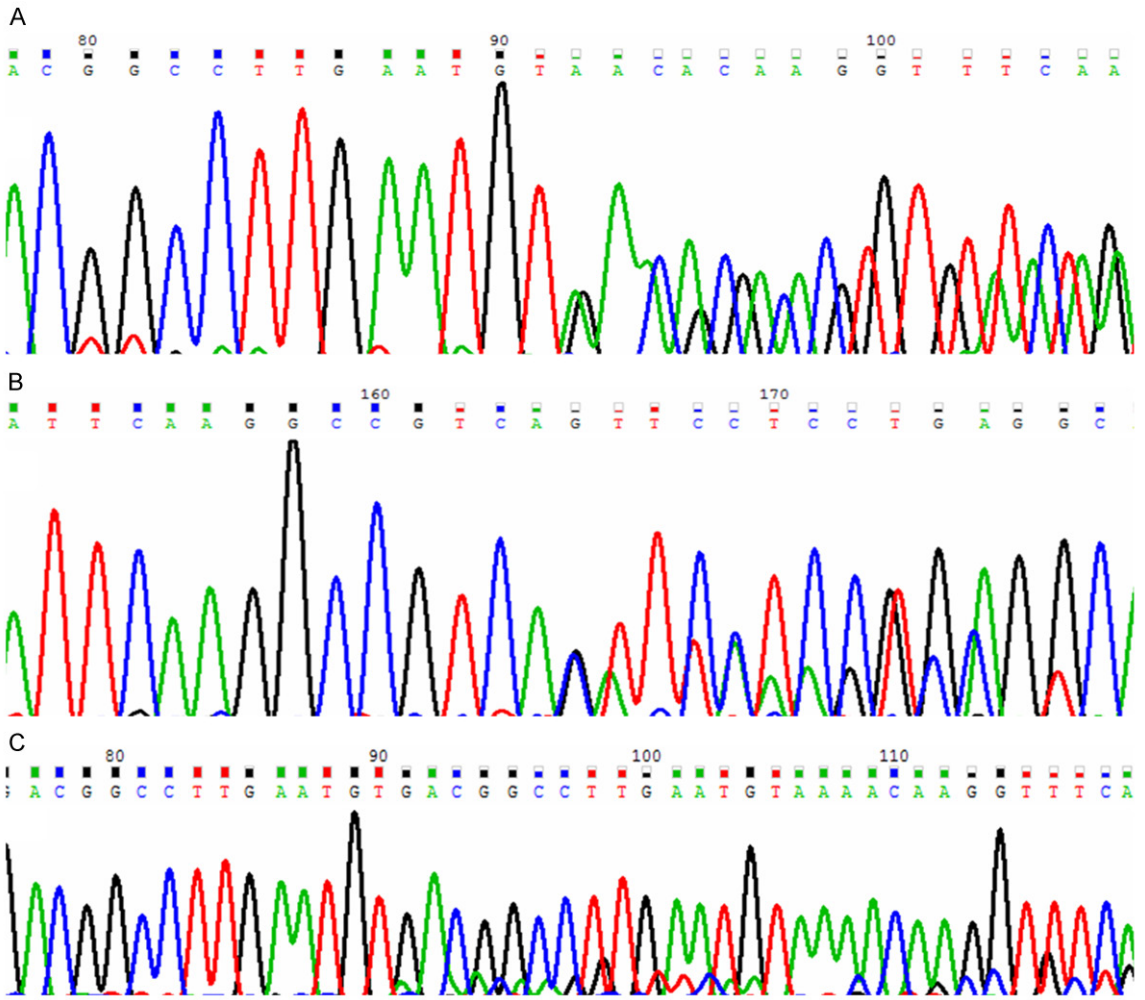

Figure 2. Sequencing of insertion mutation c.122-123ins15 (A), Forward DNA sequencing. (B) Reverse DNA sequencing. (C) Forward cDNA sequencing.

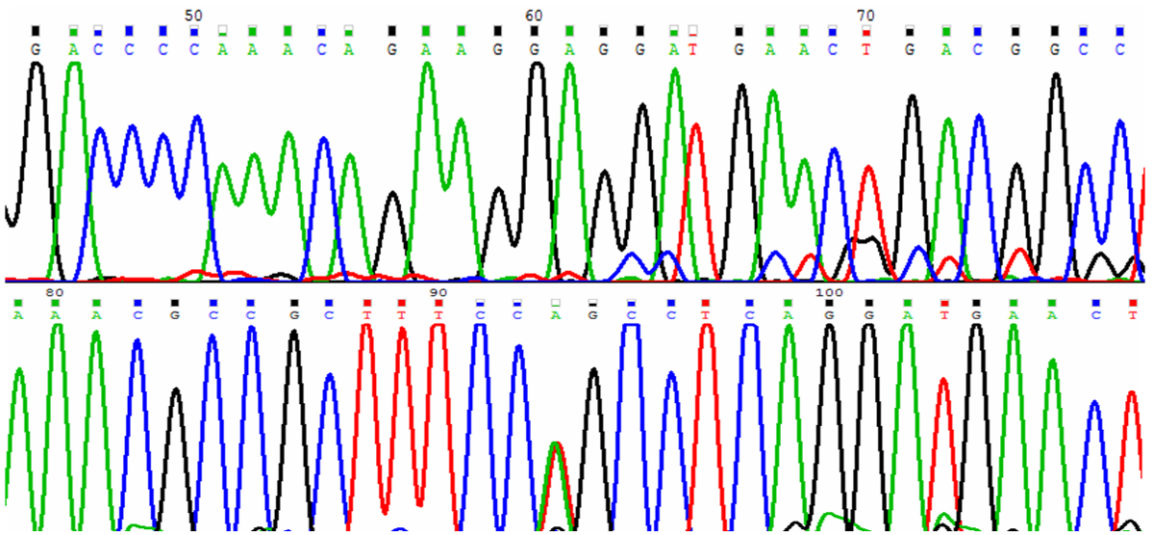

Figure 3. The chromatogram of IVS1-8T>A mutation. Top, leiomyoma genomic DNA sequencing. Down, leiomyoma cDNA sequencing.

tumors. No significant correlation was found between these confounding factors and *MED12* genotype, indicating that *MED12* somatic DNA variants occur independently.

Verification of allele-specific mRNA expression in tissue samples

To prove whether the *MED12* mutant alleles are expressed in the tumors, RNA was extracted from each sample carrying *MED12* DNA variants. cDNA analyzing of these samples revealed that the mutant alleles were mainly present in the cDNA sequence. As indicated in **Table 1**, we found splice site mutation IVS1-8T>A in one sample. This mutation was previously reported by Mäkinen *et al.* [5]. They predicted and confirmed addition of six bases to the transcript by cDNA sequencing. Although we observed this additional six bases in cDNA, but the dominant transcript was coded by the wild type allele (**Figure 3**).

Regarding the new insertion mutation found in our study, the cDNA sequencing revealed that the transcripts carrying c.122-123ins15 are dominantly expressed (**Figure 2C**).

Discussion

To investigate the genetic basis of uterine leiomyomas in Iranian patients, we examined tumor-derived tissues from 23 unrelated patients. By PCR-sequencing method, we found 11 samples representing mutations with 7 different alterations in the exon 2 of *MED12* gene. This study provides further evidence that the mutations occur in a heterozygous manner and are mostly missense mutations in codon 44. As reported by the previous studies, c.131G>A was highly occurred [5, 13, 14]. Besides confirming the results of earlier investigations, our study also demonstrates two unreported mutations , one large deletion and one insertion. In transcript analysis, the sequenced cDNAs showed that mutated transcript was predominantly expressed compared to the wild-type in nearly all mutations including the novel insertion mutation, c.122-123ins15. As an exception, following the splice site mutation IVS1-8T>A, the aberrant transcript was almost not expressed. Although normal tissue contamination couldn't be ignored.

In our study the incidence of *MED12* mutation was found to be 47.8%. As it was mentioned before, the first report of *MED12* somatic changes in leiomyomas of Finnish patients identified that 70% of samples bore mutations [5]. They recently replicated this report by the observation of mutations in 83.0% of the sporadic uterine leiomyomas from the consecutive patient series and 85.5% in unselected sporadic cases [15]. A Japanese series displayed the same incidence of mutation-positive conventional leiomyomas. They found *MED12* mutations in 36 out of 45 (80%) samples [14]. A French study showed an incidence of 66.6% in typical leiomyomas. They also observed MED12 protein expression in all classical types comparing to 40% of atypical tumors and further conclude that MED12 expression could be inhibited in malignant tumors [16]. By Sanger sequencing in 143 samples of North American women, 100 (67%) genotyped leiomyomas were found to be mutated [17].

Besides these reports of high mutation incidence, several other studies showed a rate of about 50% in different ethnic groups. In a set of 28 uterine leiomyomas from black African or coloured South African patients 14 (50%) mutation positive tumors were detected [13]. Prior published results of Korean patients showed 52.2% mutation-positive lesions [18]. Using uterine leiomyomas as a control group of malignant smooth muscle tumors, Markowski and colleagues detected *MED12* mutations in 10/21 (47.6%) of European samples [19]. A recent American study detected aberrant genotypes in 54% (15/28) of classical uterine leiomyomas [20].

As previously described by other researchers, the incidence of *MED12* mutations shows ethnic differences. Our results displayed fewer mutation positive lesions than formerly published data confirming the diversity between the populations. Further studies are necessary in order to conclude whether these differences are consistent across different settings and are clinically meaningful.

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Disclosure of conflict of interest

None.

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