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Detection of exon2-MED12 mutations in uterine leiomyomas from Syrian patients

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Uterine leiomyomas (uLMs) are the most prevalent benign tumors of the female reproductive system. MED12 is one of the mediator complex subunits that has been implicated in uLMs pathogenesis. Somatic mutations in exon2-MED12 have been found in ~70% of uLMs. In this study, we investigated the status of exon2-MED12 in uLMs from Syrian patients. Sixteen leiomyomas from nine patients were assessed. Genomic DNA was isolated from tumors and exon2-MED12 was amplified by PCR and sequenced. Three specimens showed in frame point mutations consisted of missense substitutions in codon 44 (c.130). A novel insertion in codon 35 (c.103insG) was detected in one of the mutated cases and is expected to cause a frameshift in translation and an altered or truncated product. Some of the wild-type uLMs were collected from the same uteri that revealed mutations, which emphasizes the individuality of the uLM lesions and highlights the complexity of uLMs pathogenesis. The study is the first report from Syria on the topic and the second from the Arab world. It indicates genetic heterogeneity and independent clonal origin of the somatic mutations in exon2-MED12. In wild-type uLMs where exon2-MED12 mutations are absent, other players are in place and should be investigated.

Keywords Codon 44, Codon 35, c.103insG, Exon2-MED12, Somatic mutation, Uterine leiomyoma

Abbreviations

FFPE	Formalin fixed paraffin embedded
H&E	Hematoxylin and Eosin
HPF	High power field
Het	Heterozygous
MED12	Mediator Complex Subunit 12
PCR	Polymerase chain reaction
RT	Room temperature
uLM	Uterine leiomyoma

Uterine leiomyomas (uLMs) are the most prevalent benign tumors of the female reproductive system. The prevalence and incidence of uLMs fluctuate among countries and even among ethnicities within the same country. In general, global prevalence is estimated to be 40–60% (in women < 35 years old) and 70–80% (in women > 50 years old) while the incidence is 20–77% worldwide [1–3]. Even though the tumor initiates from the proliferation of an abnormal smooth muscle cell, the microenvironment varies between diverse uLMs [4–6]. Similarly, the mechanisms behind uLMs initiation, uLMs pathobiology, and the required driver and passenger factors for uLMs formation vary [6–10]. Therefore, distinguishing the characteristics of uLM tumors and all etiologic factors contributing to tumor onset and pathobiology is invaluable.

The driving factors behind uLM genesis are being continuously explored and many important alterations have been revealed. One of the most extensively studied driver genes that has been investigated in uLMs from different ethnic groups is the Mediator Complex Subunit 12 (MED12). MED12 gene encompasses 23,899 bp, 6531 of which constitute 45 coding exons and encode for 2177 amino acids. The exon of interest (exon2) has 105 bp and encodes for 35 amino acids [4,11–18]. The Mediator Complex regulates RNA polymerase II activity and affects both transcriptional activation and repression [11]. MED12 is involved in several developmental processes [19] and is frequently mutated in both benign and malignant tumors [11,14,20]. Mutated MED12 is frequently observed in multiple and small uLMs (< 30mm) [10,21] and has been assigned a major functional role, particularly in uLMs

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pathogenesis [14,22]. Somatic mutations in exon2 alone have been found in ~ 70% of uLMs [9,11]. Expressing a common missense variant (c.131G > A, codon 44) in the uterus of a mouse model induces earlier uLMs formation [23]. Furthermore, silencing MED12 expression in cultured uLM cells reduces cell proliferation [24]. Mutated MED12 has been shown to disrupt cyclinC-CDK8/19 activation, change the 3D genome compartmentalization, and drive genomic instability among other functions [10,23,25–27].

Precision medicine conveys a great potential for managing uLMs. Agents that target key drivers such as MED12 and may benefit patients with known driving molecular mechanisms are of particular interest [6,28]. They are anticipated to enable targeting key drivers behind uLMs with minimally invasive approaches. This would be even better if diagnostics were built around driver factors so diagnosing and managing uLMs are based on key drivers in the form of personalized medicine. This of course necessitates knowing the driving mechanisms in patients. Unfortunately, in Syria, no studies have been conducted to date on uLMs to identify driving mechanisms and their frequency. Revealing the driving molecular mechanisms of uLMs in Syrian patients, especially mutated exon2-MED12, seems advantageous as it may enable patients to benefit from emerging diagnostics and targeted therapies in the future.

We conducted this study with the aim of investigating the status of exon2-MED12 in uLMs from Syrian patients. Specifically, we wanted to explore the presence of somatic mutations in exon2-MED12.

Materials and methods

Sample collection

The study was approved by the Institutional Review Board and Ethics Committee on Human Research of Damascus University before its commencement. Sixteen uterine leiomyoma (uLM) tissues were anonymously collected right after surgery from the Department of Pathology at Al-Tawleed University Hospital. ULMs were extracted from the uteri of nine patients, eight of them underwent hysterectomy. All patients had multiple uLMs of various sizes but investigated uLMs in this study were selected particularly small ($\leq$ 25 mm). Multiple uLMs were collected from the uteri of five patients and solitary uLMs were gathered from the uteri of four patients. Sizes of the uLMs ranged 8–25 mm in their largest diameter (average 14.94 mm).

Tissue fixation and preparation

Fresh specimens were immediately fixed in 4–6% buffered formalin for 22 ± 2 h at room temperature (RT) and embedded in paraffin manually: H2O (15 min), 80–100% alcohol (2 h each), Xylene (2x, 2 h each), and paraffin (2x, 2 h each). Eight sections of eight micrometers (8 × 8µm) were cut by microtome (Leica Biocut 2035) and mounted on slides for DNA extraction. Additional five-micrometer sections were stained with hematoxylin (Merck) and eosin (Merck) using a tissue stainer (MEDITE, England) for tissue visualization and diagnosis.

DNA extraction

Genomic DNA was extracted from all tissue samples by using the QIAamp DNA FFPE Tissue Kit (#56404, Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. Concentration and purity (indicated by the 260/280 nm ratio) of the isolated DNA were measured by the Nanodrop (Nanodrop-2000; Thermo Fisher Scientific, Waltham, MA) and 100 ng of genomic DNA templates were used for PCR amplification by thermocycler (Eppendorf Mastercycler).

PCR amplification

Exon2-MED12 amplification was executed using primers (5'-GCCCTTTCACCTTGTTCCTT -3' (forward) and 5'-AAGCTGACGTTCTTGGCACT-3' (reverse) (Vivantis, Malaysia) [25] and PCR One Master Mix (GeneDirex, USA) according to the manufacturer's instructions. Amplification was tested at different annealing temperatures to optimize PCR yield and minimize non-specific bands. The optimized program was as follows: Initial 5 min at 95°C, 35 cycles (30 s at 95°C, 30 s at 65°C, and 30 s at 72°C), 5 min at 72°C, and hold at 4°C. Subsequently, PCR products were separated on 3% agarose gel (GeneDirex, USA) by electrophoresis versus a DNA ladder of 100 bp (Thermo Scientific, GeneRuler). The gel was stained with ethidium bromide (10 mg/ml in H2O, Sigma-Aldrich, USA) and the PCR products were visualized using a Wealtec UV transilluminator and Olympus camera to determine the amplicon lengths. The success of the PCR amplification was judged by the presence of a PCR product band corresponding to the length of 191 bp.

DNA sequencing

200 ng/µl of PCR products and 10 p.mol/ul of forward primer per reaction were sent to Macrogen for sequencing (Seoul, Korea) as per the company's instructions. The sequences were read and analyzed both manually and on a computer using Chromas 2.5. Patient sequences were aligned with the wild-type sequence of exon-2-MED12 available online from NCBI (# NG_012808.1) using Geneious 4.8.4 to differentiate mutations.

Prediction of the pathogenicity of mutations

Pathogenicity of mutations in exon2-MED12 was predicted by using MutationTaster software (https://www.mutationtaster.org/) which evaluates DNA variants for their disease-causing potential. The software uses a Bayes classifier to calculate probabilities for the mutation to be either a harmless polymorphism or a disease alteration. It classifies a variation into one of four possible categories: polymorphism automatic (harmless), polymorphism (probably harmless), disease-causing (probably deleterious), and disease-causing automatic (deleterious).

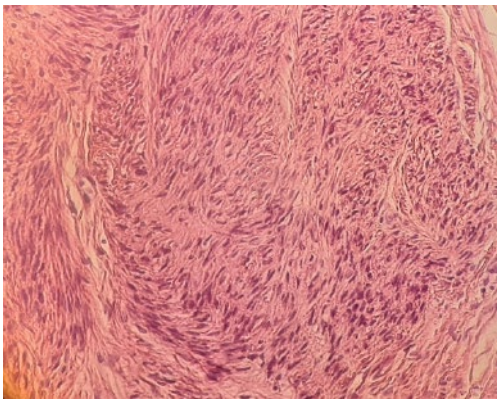

Fig. 1. H&E micrograph illustrating a representative leiomyoma with fascicular spindle cells at 40× magnification (Olympus BX41).

Patient	uLM Specimen ID	Patient age at hysterectomy (years)	ULM diameter (mm)	Nucleotide change	Variant type	Amino acid change	Variant pathogenicity
#1	1	45	9	-	WT	-	-
#2	2	33	14	c.130 G > A Het	Missense	p.Gly44Ser	Disease-causing
#3	3	50	12	-	WT	-	-
	4		15	-	WT	-	-
	5		11	-	WT	-	-
	6		16	c.130 G > C Het	Missense	p.Gly44Arg	Disease-causing
#4	7	44	8	-	WT	-	-
#5	8	51	15	-	WT	-	-
	9		19	-	WT	-	-
#6	10	51	18	-	WT	-	-
#7	11	30	17	-	WT	-	-
	12		18	-	WT	-	-
#8	13 14	45	9 15	- c.103insG c.130G > C Het	WT Insertion Missense	- - p.Gly44Arg	- Disease-causing Disease-causing
#9	15 16	45	25 18	- -	WT WT	- -	- -

Table 1. The status of exon2-MED12 sequences identified in uterine leiomyomas of Syrian patients. The table illustrates sequences from sixteen specimens collected from nine patients. The sequences that did not show mutations in exon2-MED12 were given the term WT. Three uLM specimens (#2, 6 and 14 from patients #2, 3, and 8, respectively) harbor heterozygous point mutations in codon 44 causing missense variants. Additionally, uLM specimen#14 (patient#8) reveals one base (G) insertion in codon 35 causing a frameshift. The variant pathogenicity for the identified mutations (specimens #2, 6 and 14) is indicated.

Results

On gross appearance, all extracted uLMs were solid, homogenous and small (≤25 mm). Under the microscope, specimens showed typical uLM morphology of uniform fascicular spindle cells (Fig. 1). Fourteen uLMs were collected from hysterectomized uteri of eight patients (age 33–51 years) and two uLMs were isolated from the uterus of one patient (#7) without hysterectomy (age 30 years). All patients had multiple uLMs of various sizes but in search for exon2-MED12 mutations investigated uLMs in the study were selected particularly small (≤25 mm). Solitary uLMs were extracted from the uteri of four patients (#1, 2, 4, and 6), two uLMs were extracted from the uterus of four patients (#5, 7, 8, and 9), and four uLMs were extracted from the uterus of one patient (#3) (Table 1).

Fresh uLMs underwent tissue fixation and selection according to our optimized parameters of fixation (concentration and duration) and selection (section quantity and thickness) criteria for the molecular study of the MED12 gene in FFPE-uLM. Briefly, fixation in 4–6% buffered formalin for 22 ± 2 h at RT, manually embedding in paraffin, and slicing 8 × 8µm sections gave DNA yields of good quantity and high quality for PCR amplification and DNA sequencing regardless of uLM cell density [29]. Amplified PCR products were checked on 3% agarose gels (Fig. 2) before being sent to be sequenced at Macrogen (Seoul, Korea). When sequences of exon-2-MED12 were compared to the wild-type sequence (NCBI# NG_012808.1) solitary point mutations in codon 44 were found in two uLMs (#2 and 6) from two patients (#2 and 3) and double mutations (insertion in codons

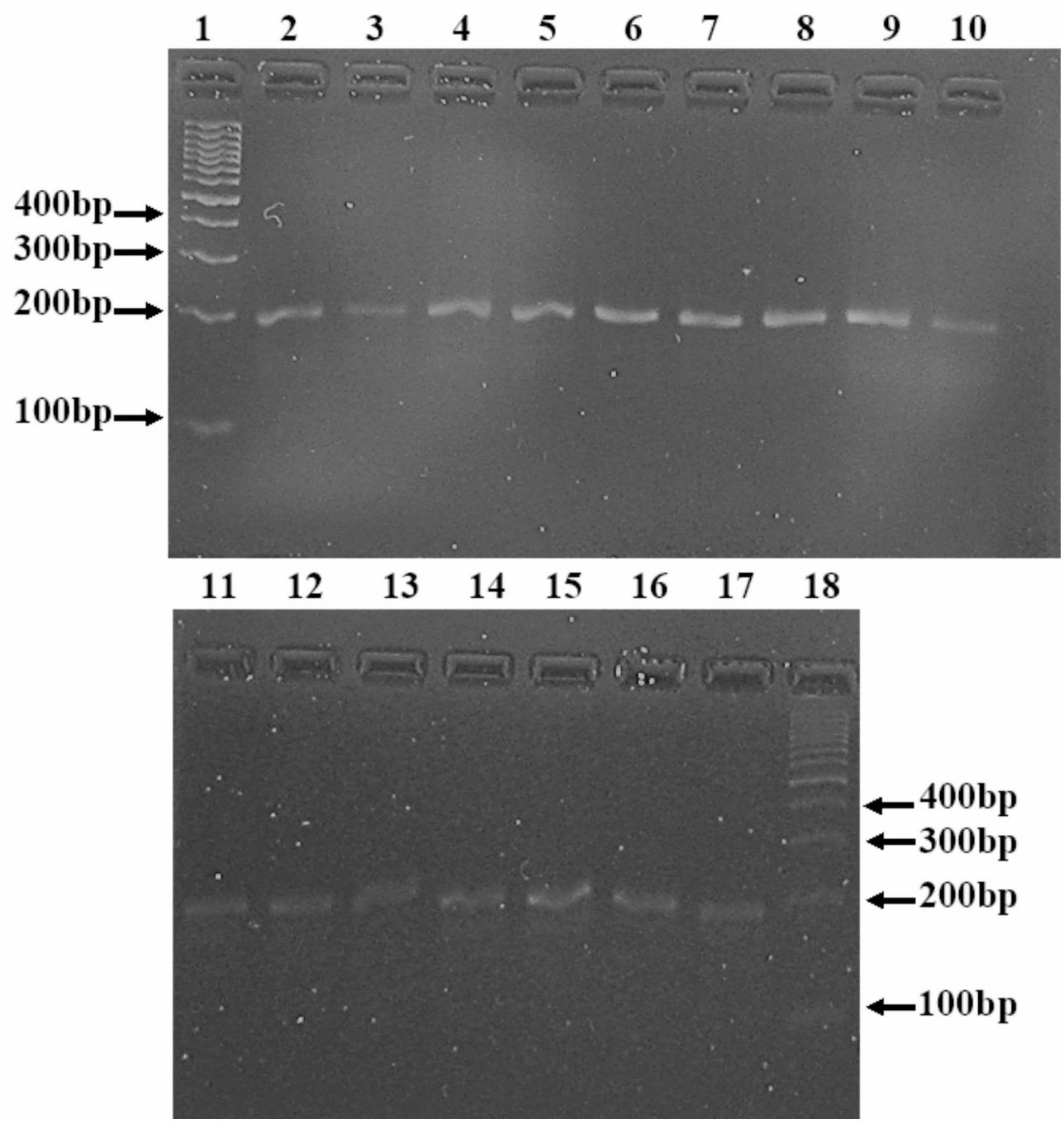

Fig. 2. Image showing agarose gel electrophoresis of the PCR amplified products of exon2-MED12 and its flanking regions (191 bp) from sixteen samples examined in the study using 3% agarose gels. Lanes 1 and 18 correspond to a DNA ladder (100 bp); Lanes 2 through 17 correspond to PCR products of specimens 1 to 16.

35 and point mutation in codon 44) were found in one uLM (#14) from one patient (#8). Variant pathogenicity was predicted with MutationTaster software. Each one of the four mutations was anticipated to be "probably deleterious" and thus referred to as disease-causing (Table 1).

Consequently, exon2-MED12 mutations were found in 33.33% of patients (3/9), and 18.75% of investigated uLMs (3/16) (size 14, 16, and 15 mm). All three point mutations (100%) consisted of missense substitutions in the most commonly mutated codon in exon2-MED12 (codon 44). Two substitutions (66.6%) were G > C (patients# 3 and 8) and one (33.33%) was G > A (patient# 2) (Table 1). Additionally, an insertion of one base (G) at the beginning of codon 35 (c.103) was detected in uLM#14 (patient#8) that is expected to cause a frameshift in translation beginning with codon 35 and afterward (Table 1 and 2, Fig. 3). Sequencing of the amplified exon2-MED12 extracted from the other 13 uLMs in the study did not show any mutations and hence was labeled wild-type (WT). Interestingly, some of the WT uLMs were collected from the same uteri that revealed mutations

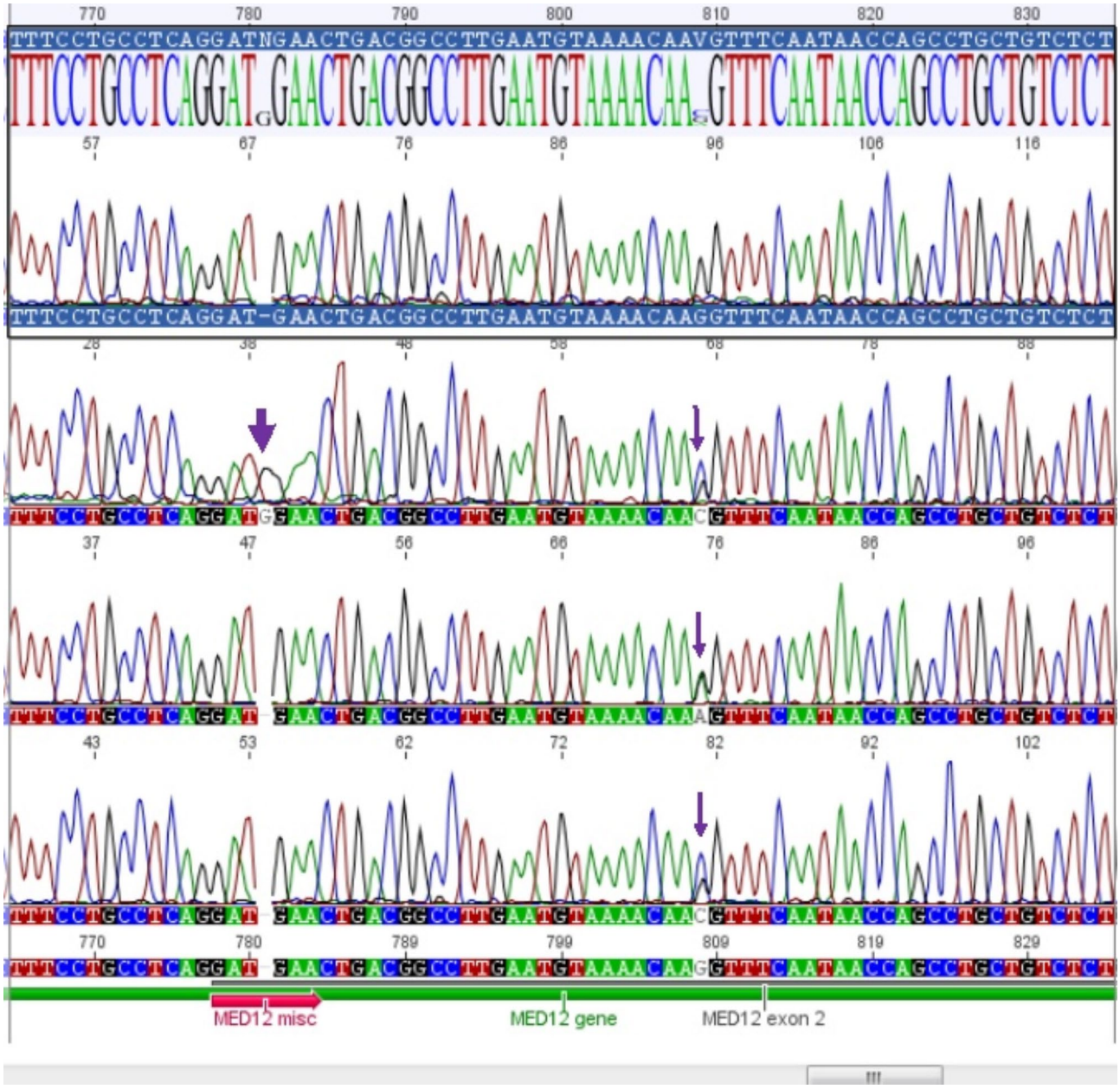

Fig. 3. Representative chromatograms of exon2-MED12 sequences in patient-derived uLMs. Chromatograms are from bottom to top: specimen #6 (c.130G > C, p.Gly44Arg), specimen #2 (c.130G > A, p.Gly44Ser), specimen#14 (c.130G > C, p.Gly44Arg and c.103insG), and representative of WT exon2-MED12 sequences. Long arrows indicate locations of point mutations and the thick arrow indicates the location of insertion.

(patients#3 and 8). This emphasizes the somatic and independent clonal nature of the detected mutations in exon2-MED12 (Table 1).

Discussion

In 2011, an extensive report concluded that the incidence of mutated MED12 gene in uterine leiomyomas (uLMs) was alarmingly high (159 of 225 uLMs, ~71%) [11]. The discovery initiated widespread research around the world on MED12 in uLMs that is still ongoing. However, to date, no studies have been conducted on uLMs in Syria to identify driving mechanisms, including MED12, and their frequency. The current study aimed to explore the status of exon2-MED12 in uLMs from Syrian patients. The majority of MED12 mutations in uLMs have been detected in exon2, especially missense mutations in codons, 35, 43, and 44 [11,25,30]. Accordingly, in our study, we focused our mutational analysis on exon2. Furthermore, it has been shown that mutated MED12 is frequently observed in multiple and small uLMs (< 30mm) [10,21]. Consequently, in our study, we collected small uLMs (≤25mm) from cases with multiple uLMs.

Identifying mutated MED12 in uLMs by pathological microscopic assessment alone is not feasible. Mutated cases usually show typical uLM morphology of uniform spindle cells, no necrosis, low mitotic index (≤ 3 mitoses /10 HPF), varied amounts of degenerative hyaline fibrosis, and absence of unusual subtypes (cellular, epitheloid, myxoid or atypical variant) [11]. Conducting molecular studies, the standard approach to identify mutated MED12, on FFPE specimens can be problematic as well. Archived FFPE-uLMs were not useful in our study due to varied preparation conditions. Extracted DNA was of poor quality and quantity and could not be amplified. Therefore, we collected fresh uLMs, fixed and embedded them according to unified parameters [29], and then conducted the molecular study, which made investigating a large number of uLM specimens very challenging.

The study reveals base changes in three patients (two patients have c.130G > C and one has c.130G > A) giving single nucleotide variants and resulting in missense mutations in codon44-exon2-MED12. The base changes in c.130 accord with changes noted in other studies [25,30,31]. Substitutions at positions 130 and 131 in codon44-exon2-MED12 were observed predominantly and accounted for most of the base changes in studies investigating a larger number of uLMs [11,30,31]. Codon 44 is the most conserved codon of the MED12 gene and holds a pivotal role in the biological process of the MED12 protein [32,33]. Additionally, an insertion of one base (G) at the beginning of codon 35 (c.103) is reported for the first time (registered in GenBank# PP979487). The insertion is detected in one of the mutated cases and is expected to cause a frameshift in translation and a change in amino acid sequence beginning with codon 35.

Exon2-MED12 mutations in uLM lesions were ruled somatic, in previous reports, judged by their presence in uLMs and absence from the respective normal myometrium or blood of the corresponding patients [11,30,31]. Moreover, the somatic mutations in the MED12 gene were of a heterozygous nature [34] and varied in uLMs extracted from the same patient [30]. In accordance with these reports, mutations in codon44-exon2-MED12 in our study were heterozygous and detected in 1/2 and 1/4 uLMs collected from the same uteri (patient #8 and 3, respectively). This indicates an independent clonal origin and suggests other drivers are responsible for the pathology of uLMs in which exon2-MED12 is not involved. Likewise, it emphasizes that the initiation and growth of each uLM is an individual and isolated incidence.

The frequency of mutations in exon2-MED12 in the current study (18.75%, 3/16 uLMs) is the lowest frequency reported so far. Nevertheless, our sample is too restricted to make a conclusion about the incidence of MED12 mutations. The report is preliminary and more studies are needed in which larger groups of specimens should be investigated to reach a prevalence of MED12 mutations or other genes in Syrian patients.

The frequency of mutations in exon2-MED12 in previous studies was much higher and extended from 88% (44/50) [35], 74% (37/50) [31], 71% (159/225) [11], 58.8% (47/80) [30], 44% (34/77) [34], to 31.07% (32/103) [36]. Although the incidence of mutated MED12 in uLMs investigated in these studies is high, the rest of uLMs devoid of mutations is also high, which further accentuates the complexity of uLM pathogenesis.

Deliberating the plausible causes in uLMs devoid of exon2-MED12 mutations implicates other alternations, not related to exon2-MED12 or even MED12 gene. Although very rare, mutations in intron1 and exon1 have been reported in MED12 previously [37]. Other causes include alterations that have been reported in the literature but with much lower incidence than MED12. It has been demonstrated that these alterations are exclusive driving forces in the investigated uLMs. They include overexpression of high mobility group AT-hook 2 (HMGA2), biallelic loss of fumarate hydratase (FH), biallelic mutations in genes associated with neddylation of the Cullin 3-RING E3 ligase (including UBE2M, NEDD8, CUL3, and NAE1), and mutations affecting the genes encoding for members of the SRCAP complex. [7,38,39]. Thus, despite MED12 being the driver alteration in the majority of uLMs in previous studies [8,11], other factors might be the major driver gene(s) in Syrian patients. Revealing which of these driver genes or other newer genetic alterations may require different approaches such as targeted panel sequencing or whole-exome sequencing.

The effect of ethnicity on the outcome regarding driving alterations and their frequencies is significant. In addition to variances in prevalence, lesion characteristics (including size and quantity of uLMs), age at diagnosis, associated symptoms, and age at hysterectomy [40,41], the frequency of mutated exon2-MED12 also varies with ethnicity [11,31,34,36]. Unfortunately, in the Arab world of 22 countries research on MED12 in uLM is scarce making it unfeasible to consult related studies conducted on groups with the same ethnicity. Only one relevant study was identified through a literature search. The study was conducted in Saudi Arabia and the reported frequency of exon2-MED12 mutations was 44% [34].

Anticipating the biological effects of different variants revealed through reading DNA sequencing is very challenging. However, through employing new in silico tools it became conceivable to predict variant pathogenicity. All reported mutations in the current study are anticipated to be disease-causing or pathogenic [42]. This accords generally with other recent studies that have been elucidating the impact of certain mutations in exon2-MED12. By employing algorithm-based-computational tools the studies are investigating the protein structure to anticipate its stability, function and interactions [31,35]. It has been demonstrated that missense mutations (c.130 G > A and c.130 G > C) in codon 44-exon2-MED12 induce conformational changes in the 3D structure leading to deformation and consequent instability and alteration in the biological function of the MED12 protein. The two missense mutations in codon 44 (p.Gly44Arg and p.Gly44Ser) change the protein WT α structure into α β structures [31]. The structural change renders the MED12 translated protein non-functional and emphasizes the two mutations' implication in the pathobiology of uLMs [31,35]. The novel mutation (c.103insG) in codon 35 is expected to result in a frameshift in translation and an altered or truncated product regardless of the alternative ORF scenario (Table 2). Irrespective of the impact of c.103insG mutation and due to the presence of the companion missense mutation (c.130 G > C) in codon 44 (p.Gly44Arg) the consequential protein is ruled non-functional as discussed above.

Research on MED12 is not only affecting our perspective of uLMs but also reforming how we manage uLMs. Recent reports suggest that genetic subclasses should be considered when assessing uLMs therapies as uLM response to treatment is influenced by the molecular subclass. ULMs with mutated-MED12 responded

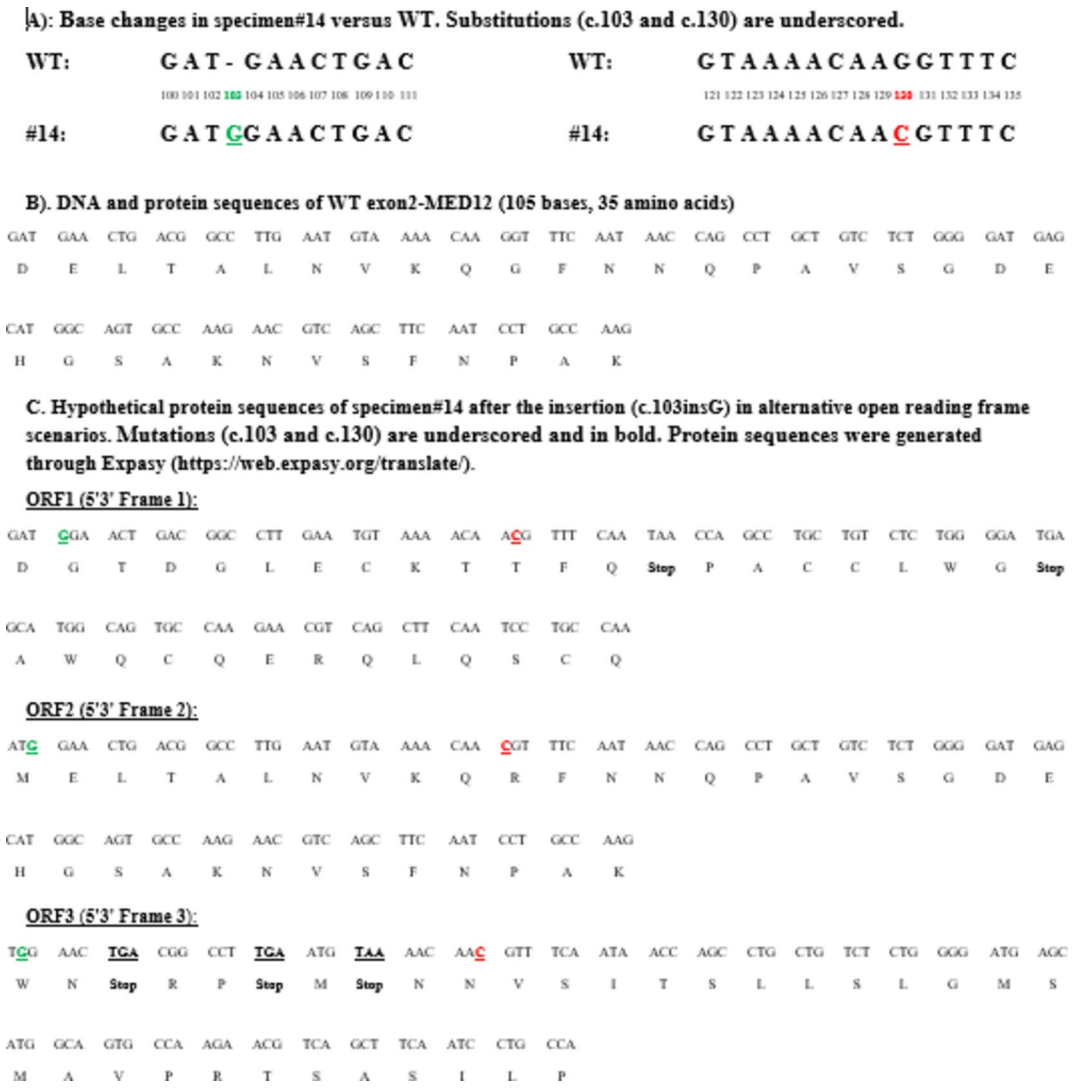

Table 2. Exon2-MED12 sequences in WT (NCBI# NG_012808.1) and specimen#14 (GenBank# PP979487).

by shrinking 4.4 times more compared to other subclasses in response to progesterone receptor modulator ulipristal acetate. The response occurs regardless of the patient's age or parity and regardless of the tumor's total number or initial size [28]. This highlights the importance of identifying driving mechanisms in patients, as precision medicine may hold the key to uLMs noninvasive management. Identifying certain candidate driver genes and biomarkers, such as MED12, may not only help in singling out precise treatment but may further lead to diagnostic and preventive approaches. This emphasizes the importance of the current study as the first attempt to reveal the driving molecular mechanisms and benefit Syrian patients from future diagnostics and targeted therapies. The study calls for a broader investigation to reveal all driving mechanisms critical to uLM pathogenesis in Syrian patients. It will be interesting to examine the presence of other novel mutations and learn whether they are targetable by diagnostic, therapeutic, or preventive approaches.

Strengths and limitations

To the best of our knowledge, this is the first study in Syria that investigates MED12 in uLMs and the first to show that exon2-MED12 is mutated in uLMs extracted from Syrian patients. It is also the second study conducted in the Arab world that explores the status of exon2-MED12 in uLMs. Due to the restricted number of investigated uLMs, our results cannot be seen from a larger perspective or generalized, which is a limitation of the study. Considering the small number of samples, more comprehensive studies employing larger numbers of uLMs are needed to confirm our findings and estimate the contribution of exon2-MED12 mutations to the pathology of uLMs in Syrian patients. Albeit these limitations, the study opens up avenues for recognizing the molecular mechanisms involved in the etiology of uLMs in Syrian patients. It also reports a novel mutation that has not been published before and contributes to the universal body of evidence that MED12 holds a pivotal role in the pathogenesis of uLM.

Conclusions and recommendations

The study is the first report from Syria on mutations in MED12, a gene that has been implicated in the majority of uLMs [9,11,25]. The study reports the presence of missense mutations in the most commonly mutated codon (44) in exon2-MED12 [11,30,31,43]. It agrees with previous reports on the somatic, heterozygous, and independent clonal nature of exon2-MED12 mutations, and the complexity of uLM pathogenesis [11,30,31]. Furthermore, the study reveals a novel mutation (c.103insG) in codon 35 (GenBank# PP979487) that is expected to result in a frameshift in translation and an altered or truncated product. Mutation frequency in the study is the lowest frequency reported so far (18.75%, 3/16). It is lesser than the frequency in all international studies including studies conducted in the Middle East [44% (34/77) [34] and 31.07% (32/103) [36]. One can entertain the idea that even though exon2 is the most commonly mutated exon in MED12 in various studies [11,18,25,43], in our ethnic group other exons might be mutated or other genes are involved that form the driving forces behind uLMs. Nevertheless, the sample size is restricted and much larger studies are needed to determine the generalizability of our findings and examine the other involved players.

Ethics declarations

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board and Ethics Committee on Human Research of Damascus University (#912). Informed consent was obtained from all participants in the study.

Data availability

Sequence data that support the findings of this study have been deposited in the NCBI Genbank with the primary accession code PP979487. All other data generated from the research is included in the manuscript.

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Author contributions

Albitar L. designed and conducted the experiments as a partial requirement for fulfillment of a Master of Science degree in pathology. Al-Chatty E. (major PI) and Ahmad F. (co-PI) substantively revised the work and provided guidance where needed. All authors approved the submitted version and are accountable for their contributions.

Competing interests

The authors declare no competing interests.

Additional information

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