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Optimizing Tissue Fixation Conditions for Molecular Study of The MED12 Gene in FFPE Uterine Leiomyoma Tissues

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Abstract:

Background and aim: Mutations in exon2 of the Mediator Complex Subunit12 gene (exon2-MED12) are major drivers for uterine leiomyomas (ULMs). Proper conditions of fixation are essential for the success of DNA extraction and polymerase chain reaction (PCR) amplification. The study explored whether DNA extracted from preserved formalin-fixed, paraffin-embedded (FFPE) tissues is suitable for PCR amplification of exon2-MED12 and investigated enhancing conditions.

Materials and Methods: The study included 12 ULM-preserved FFPE tissues of unknown fixing and embedding conditions from 12 patients and five fresh tissues extracted from the same hysterectomized patient, fixed in 6% buffered formalin for 22 hours, and paraffin-embedded manually. Section size, quantity, and thickness were comparable among fresh samples. Genomic DNA was extracted and results were judged by the concentration and quality of the isolated DNA and the success of the PCR amplification. Additional sections were stained with hematoxylin and eosin for tissue inspection under the microscope.

Results: PCR amplification and DNA extraction from preserved FFPE-ULM tissues were unsuccessful. However, PCR amplification from newly collected ULMs was successful from DNA as low as 7.7 ng/µl or better. Section size, quantity, and thickness did not correlate with tissue weight or DNA concentration, we investigated the cell density as a plausible cause for the noted variations. The cell density varied indeed in the collected specimens and correlated better with DNA concentration.

Conclusions: Optimization of tissue fixation (duration and concentration) led to successful PCR amplifications. Every ULM sample is unique and tissue histology should be considered for fruitful DNA extraction and PCR results. Rare samples with scarce tissue and minor DNA yields could be useable for PCR amplification and subsequent genetic testing. The study recommends that fixation conditions are documented for FFPE tissues so that educated decisions regarding tissue utilization for DNA extraction and subsequent genetic testing are feasible.

Keywords: FFPE; DNA extraction; PCR; MED12; Uterine leiomyoma.

Abbreviations: FFPE: Formalin fixed paraffin embedded; H&E: Hematoxylin and Eosin; MED12: Mediator Complex Subunit 12; PCR: Polymerase chain reaction; ULM: Uterine leiomyoma.

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أمثلة شروط التثبيت لأجل الدراسة الجزيئية لجين MED12 في أنسجة الأورام العضلية الملساء الرحمية المثبتة بالفورمالين والمدمجة بالبارافين

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الملخص:

خلفية البحث وهدفه: تعد الطفرات في exon2–MED12 من الأسباب الرئيسية لتشكل الورم العضلي الأملس الرحمي. تعد الظروف المناسبة للتثبيت ضرورية لنجاح استخلاص الحمض النووي وتضخيمه بالتفاعل السلسلي للبوليمراز. استكشفت الدراسة ما إذا كان الحمض النووي المستخرج من الأنسجة المحفوظة والمثبتة بالفورمالين والمدمجة بالبارافين مناسبًا لتضخيم exon2–MED12 بالتفاعل السلسلي للبوليمراز وبحثت في الظروف المعززة.

المواد والطرق: شملت الدراسة 12 عينة محفوظة وذات ظروف تثبيت وإدماج غير معروفة لأورام عضلية ملساء رحمية مستأصلة من 12 مريضة بالاضافة لخمسة أورام طازجة تم الحصول عليها من رحم مستأصل وتثبيتها في 6% من الفورمالين لمدة 22 ساعة، ثم دمجها بالبارافين يدويًا. كان حجم المقاطع النسيجية وكميتها وسماكتها مماثلاً بين العينات الطازجة. تم استخلاص الحمض النووي الجينومي والحكم على النتائج من خلال تركيز ونوعية الحمض النووي المعزول ونجاح تضخيم exon2–MED12 بالتفاعل السلسلي للبوليمراز. تم الحصول على مقاطع إضافية لونت بالهيماتوكسيلين والإيوزين لفحص الأنسجة تحت المجهر.

النتائج: لم ينجح تضخيم exon2–MED12 بالتفاعل السلسلي للبوليمراز واستخلاص الحمض النووي من الأنسجة المحفوظة المثبتة بالفورمالين والمدمجة بالبارافين، بينما كان التضخيم من حمض نووي مستخلص من العينات الطازجة وبتركيز منخفض يصل إلى 7.7 نانوغرام/ميكرولتر (أو أكبر) ناجحًا. لم يرتبط حجم المقطع وكميته وسماكته بوزن النسيج أو تركيز الحمض النووي، لذا قمنا بالتحقق من كثافة الخلايا في المقطع كسبب معقول للاختلافات الملحوظة. تباينت كثافة الخلايا بالفعل في العينات وارتبطت بشكل أفضل مع تركيز الحمض النووي.

الاستنتاجات: أدى تثبيت الأنسجة الأمثل (المدة والتركيز) إلى تضخيم exon2–MED12 ناجح. يشكل كل ورم من الأورام العضلية الملساء الرحمية عينة فريدة من نوعها ويجب أخذ هيستولوجية الأنسجة في الاعتبار من أجل استخلاص حمض نووي وتفاعل سلسلي للبوليمراز مثمرين. يمكن أن تكون العينات الإستثنائية التي تحتوي على أنسجة نادرة وحمض نووي قليل التركيز قابلة للتضخيم بالتفاعل السلسلي للبوليمراز والاختبارات الجينية التي تليه. توصي الدراسة بتوثيق شروط التثبيت والإدماج للأنسجة المثبتة بالفورمالين والمدمجة بالبارافين بحيث يمكن إتخاذ قرارات مستنيرة فيما يتعلق باستخدامها لاستخلاص الحمض النووي والاختبارات اللاحقة.

الكلمات المفتاحية: نسيج مثبت بالفورمالين ومدمج بالبارافين؛ استخلاص الحمض النووي؛ التفاعل السلسلي للبوليمراز. جين MED12؛ ورم عضلي أملس رحمي.

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Introduction:

Uterine leiomyomas (ULMs), also known as fibroids, are the most common benign tumors of the female reproductive system. They occur with an incidence rate of 70-80% in females of childbearing age [1]. ULMs are distinctive for their benign nature and low risk of malignancy [2]. Nevertheless, depending on the tumors' sizes, quantities, and locations, ULMs may be accompanied by symptoms that could greatly influence the quality of life of the affected patients. Symptoms may extend from heavy vaginal bleeding, and pelvic pain, to infertility and loss of pregnancy [3][4][5].

ULMs are usually well-rounded white to grayish fascicular nodules (Figure 1) that may look alike to the normal eye but the tumors are indeed far from being homogenous histologically and pathologically [6]. Even though ULMs originate from a single cell and contain mainly smooth muscle cells, fibroblasts and vascular endothelial cells [7], diverse and complicated mechanisms underlie ULMs' pathobiology, onset, and the driver and passenger prerequisites for their formation [6]. One of the extensively studied drivers in ULMs is the Mediator Complex Subunit 12 (MED12) gene [8]. Somatic mutations in exon 2 of MED12 are major drivers for 70% of ULMs [9] by disrupting the activation of cyclin C-CDK8/19 among other functions [10-12].

Molecular genetic analysis of formalin-fixed, paraffin-embedded (FFPE) tissues is indispensable for both diagnostics and research. FFPE is part and parcel of the daily routine in most diagnostic and research pathological laboratories worldwide. FFPE tissue blocks are popular for many sensible reasons especially the opportunity to apply parallel tests (such as immunohistochemistry and polymerase chain reaction (PCR) on the same tissue thus compare results, and the ability to use the blocks over time due to a reasonable preservation of tissue [13].

Optimal tissue fixation, embedding, and selection criteria are essential for the success of the DNA extraction method, PCR amplification, and succeeding procedures such as DNA sequencing [14]. Yet these criteria may vary from one medical center to another and from one type of tissue to another.

In Syria, despite the lack of formal studies, ULMs are frequent, judged by the number of daily ULM specimen extractions and hysterectomies due to ULMs, performed in University Hospitals and received at pathological laboratories [personal communication]. This makes studying ULMs in general and MED12 in particular very important.

We conducted this study with the aims of 1) Examining whether the DNA extracted from preserved FFPE tissues is suitable for PCR amplification of exon2-MED12; and 2) Investigating enhancing conditions by defining preferred parameters for tissue fixing and selection.

Figure (1): The image shows an extracted uterine leiomyoma. The tumor is usually a well-rounded white-grayish fascicular nodule.

Materials And Methods:

Preserved sample collection and handling:

Twelve ULM-preserved FFPE tissue blocks (from 12 patients) were collected anonymously from Al-Tawleed University Hospital and investigated. The blocks were originally used for H&E staining and ULM diagnosis. Tissue fixation and handling conditions were not documented, including the type and percentage of fixative, the duration of fixation, and the embedding conditions. Tissues were sectioned by microtome, mounted on slides (Deltalab, Eurotubo), and DNA was extracted for PCR amplification of exon2-MED12 as below.

Fresh sample collection, fixation, and preparation:

Five uterine leiomyoma tissues were obtained fresh from Al-Tawleed University Hospital anonymously. They were extracted from the uterus of the same patient who underwent a hysterectomy, immediately fixed in 6% buffered formalin at room temperature for 22 hours, and embedded in paraffin at Al-Assad University

Hospital on the following morning. Eight sections of eight micrometers were cut from every ULM block and mounted on slides. Sections from tissue (14±2mm x 1.1±2mm) blocks were collected as follows: Eight sections from every tissue sample were moved to a 1.5 ml tube with a known weight. The previously weighed tube was re-weighed with the tissue and the weight of the tissue was registered. Additional sections of five micrometers thickness were mounted on slides from each FFPE block and stained with hematoxylin and eosin (H&E) for tissue visualization and examination.

DNA extraction:

In preparation for DNA extraction, tissues were first placed in xylene (Riedel-De Hean AG, Seelze-Hannover) to deparaffinize, then in ethanol (Sigma-Aldrich) to remove the xylene residues. Tissues were dried at 37°C to get rid of the ethanol residues before DNA extraction. Genomic DNA was extracted from the ULM tissues using a commercially available DNA extraction kit (QIAamp DNA FFPE Tissue Kit#56404, Qiagen, Dusseldorf, Germany) according to the manufacturer's recommendations. In the final step, DNA was eluted from every sample with equal amounts of buffer (40 µl). Concentration and purity (indicated by the 260/280 nm ratio) of the isolated DNA were measured by Nanodrop (Nanodrop-2000; Thermo Fisher Scientific, Waltham, MA) and 100 ng of the extracted DNA was used for PCR amplification using a thermocycler (Eppendorf Mastercycler).

PCR amplification:

For amplification by PCR, a primer set that amplifies exon2-MED12 (5'-GCCCTTTCACCTTGTTCCTT -3' forward and 5'-AAGCTGACGTTCTTGGCACT-3' reverse, Vivantis, Malaysia) and PCR One master mix (GeneDirex, USA) were used. Amplification was run as follows: Initial 5 minutes at 95°C, 35 cycles (30 seconds at 95°C, 30 seconds at 65°C, and 30 seconds at 72°C), 5 minutes at 72°C, and hold at 4°C. The success of the PCR amplification was judged by the presence of a PCR-product band corresponding to the length of exon 2 (191 bp) versus DNA ladder of 100 bp (Thermo Scientific, GeneRuler) on 3% agarose (GeneDirex, USA) gel stained with ethidium bromide (10 mg/ml in H2O, Sigma-Aldrich, USA). PCR products were visualized using a Wealtec UV transilluminator and Olympus camera.

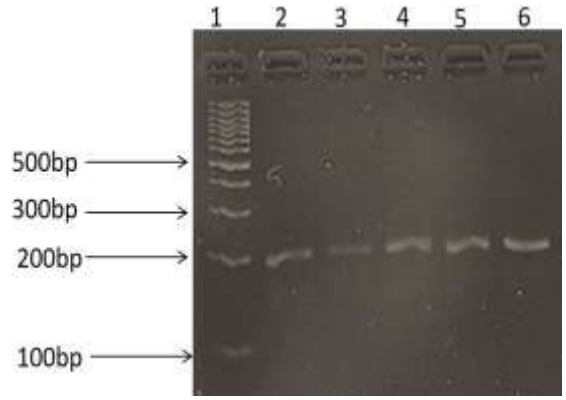
Results:

PCR amplification and DNA extraction from preserved FFPE-ULM tissues with unknown fixing and embedding conditions were unsuccessful. The isolated DNA was poor in quality and/or quantity and did not get amplified for some tissues while the DNA extraction approach failed for other tissues. The tissues did not dissolve in reagents and the extraction protocol couldn't be completed. However, DNA extraction and PCR amplification of exon2-MED12 from newly collected ULMs were successful for all five samples. Amplification was possible from DNA as low as 7.7 ng/µl (Table 1) or higher.

Table (1): The table illustrates selected parameters of section thickness and quantity versus collected tissue weight and the final DNA concentration. DNA concentration does not correlate directly with tissue weight from the collected eight sections.

Sample	Section thickness	Section quantity	Tissue weight (mg)	DNA concentration (ng/µl)
A	8µm	8	5	7.7
B	8µm	8	10	33.2
C	8µm	8	20	26.8
D	8µm	8	20	40.3
E	8µm	8	20	81.3

Equal concentrations of the extracted DNA (100 ng) from newly collected samples were used for PCR amplification and the amplification was carried out on the same day to eliminate day effect or variation. All samples showed bands corresponding to the length of exon 2 (191 bp) on 3% agarose gel indicating fruitful PCR amplification (Figure 2).



Figure(2): Image showing agarose gel electrophoresis of the PCR amplified products of exon 2-MED12 (191 bp) from five fresh samples used in the study. Lanes correspond to the following 1: DNA ladder (100 bp); 2: Sample A; 3:Sample B; 4: Sample C; 5: Sample D; and 5: Sample E (see Table 1). Four microliters of PCR product were loaded on 3% agarose gel versus three microliters of 100 bp ladder.

We optimized first tissue fixation conditions of formalin concentration (6%) and incubation duration (22 hours). The working protocol was then further optimized. The DNA extraction protocol (Qiagen) recommends starting by taking two tissue sections. We did so and found that for the employed kit (#56404) 8x8 µm were good quantity and thickness to give enough DNA yield for exon2-MED12 amplification by PCR. Section size (14±2mm x 1.1±2mm), thickness (8 µm), and quantity (#8) of the tissue sections were comparable among samples, yet weights of the collected tissues varied (5, 10, and 20 mg) (Table 1). At the end of the extraction step, DNA was eluted in the same amount of the buffer (40 µl); however, the concentration of the isolated DNA measured by Nanodrop differed. Even though the minimum DNA concentration corresponded to the lowest tissue weight (Sample A), DNA concentration did not correlate perfectly with the tissue weight for the rest of the samples (Table 1). Interestingly, samples B and D had close DNA concentrations (33.2 and 40.3 ng/µl, respectively) but the starting tissue weight of sample D (20 mg) was twice that of sample B (10 mg). Furthermore, Samples C, D, and E had the same tissue weight (20 mg) yet the DNA concentration of sample E was double the DNA concentration of sample D, and the DNA concentration of sample D was almost double the DNA concentration of sample C.

As tissue surface size, section quantity, and section thickness were comparable among samples but did not correlate well with tissue weight and DNA concentration (Table 1), we investigated the cell density as a plausible cause for the noted variations. The cell density varied indeed in the collected specimens and within the same sample. Low cellularity ULMs were more dominant in tissue blocks A and C. Typical cellularity ULMs were more dominant in tissue blocks B and D. Cellular leiomyoma was more dominant in tissue block E (Figure 3).

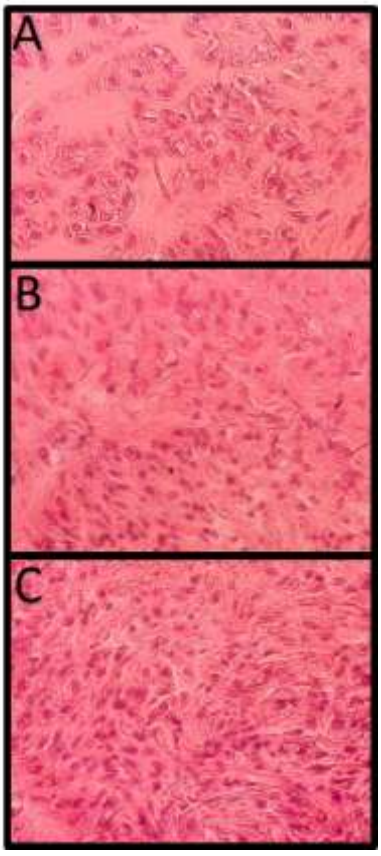


Figure (3): H&E micrographs illustrating the different leiomyoma pathologies in specimens at 40x magnification (Olympus BX41). A. Leiomyoma with low cellularity (representative of cells in samples A and C); B. Leiomyoma with typical cellularity (representative of cells in samples B and D); and C. Cellular leiomyoma (representative of cells in sample E).

Discussion:

Some of the most significant variables influencing the success of molecular testing are tumor quantity and quality. Fixative conditions and tissue handling play major shares in determining tumor quality and eligibility for fruitful molecular testing [15]. This applies to every tissue and uterine leiomyoma (ULM) is not an exception. ULM is responsible for ~70% of hysterectomies forming a major problem for female fertility worldwide. In more recent studies, aberrations in the Mediator Complex Subunit 12 (MED12) gene were presented as some of the main causes for ULMs and have been thoroughly investigated in the literature [10-12].

We started this study with the aims of examining whether the DNA extracted from preserved formalin-fixed, paraffin-embedded (FFPE) tissues is suitable for PCR amplification of exon2-MED12; and defining selecting parameters for successful DNA extraction and PCR amplification. Many factors contribute to the success of DNA extraction and PCR amplification from FFPE tissues including the fixative (strength, pH, temperature and duration), and storage (conditions and time). Failure to apply the right conditions leads to DNA deterioration, DNA destruction in the sample [16] and consequently unsuccessful DNA extraction and PCR amplification.

Initially, twelve tissue ULM blocks were collected from Al-Tawleed University Hospital. Tissue fixation and handling conditions including the type and percentage of fixative, the duration of fixation, and the embedding conditions were not documented. The quality and quantity of the extracted DNA were poor and the PCR amplification was unsuccessful. So, fresh ULMs were obtained from the same hysterectomized patient to reach successful DNA extraction and PCR amplification. Tumors were fixed directly in 6% buffered formalin at room temperature. The fixative percentage (4-10%) and time (less than 24 hours) were suggested previously as optimal fixative conditions [Qiagen, Dusseldorf, Germany] [16]. Usually, the machine embedding protocol is run at the end of the day at Al-Tawleed University Hospital thus the specimens are ready for sectioning on the following morning. However, this routine did not give us enough time for fixation in our study. To handle this obstacle and maintain the time of fixation under 24 hours, tissues were paraffin-embedded manually at Al-Assad University Hospital on the following morning.

Next, different section quantities and thicknesses were tried and we found that 8x8 µm were good starting points to give enough DNA yield and successful PCR amplification using the Qiagen kit (#56404). Though the section quantity and thickness were maintained, the weight of the tissue and the DNA yield differed tremendously. The minimum starting tissue weight and the extracted DNA concentration that gave a PCR product in the current study were 5 mg and 7.7 ng/µl, respectively. We re-examined the H&E stained tissues searching for a reasonable cause for the noted variation. Cell density varied between tissue blocks greatly. The variation in ULM cellularity is common and has been previously reported in the literature [7]. Investigating the cellularity of ULMs is an important step before deciding on using the tissue block for further molecular studies. Another alternative is selecting desired tissue by microarray or similar approaches.

Optimization of the conditions of tissue fixation specifically time and concentration of fixative served the purpose of this study. FFPE-extracted DNA seems appropriate for amplification of exon2-MED12 by PCR under the stated conditions. DNA quality and quantity are critical for the success of the PCR amplification and the subsequent genetic tests such as sequencing.

The study defined some of the most important criteria critical for the success of DNA extraction from ULM-FFPE. The study was cost-effective as it paved the road for future tests dependent on a fruitful amplified PCR product. The study recommends establishing a connection between tissue histology and cell density for ULMs, which might be also necessary for other tissues. The study also emphasizes that rare samples with scarce tissue and a minor yield of DNA may still be useful for PCR amplification and subsequent genetic testing. The study has many limitations though, due to the outrageous cost of reagents the sample size was limited. There is a need to expand the study with more samples to test the conditions in more depth.

Conclusions And Recommendations:

We conclude that DNA extracted from ULM-FFPE tissue is well fit for amplification of exon2-MED12 by PCR under the stated conditions. Every ULM sample is unique thus FFPE tissue weight, section quantity, and section thickness may not reflect specimen cellularity or DNA yield. Tissue histology is indispensable for deciding on a successful choice for PCR amplification.

FFPE tissues are universally used in diagnostics and biomedical research. The study recommends that fixation conditions (especially percentage and duration) are documented for FFPE tissues so that educated decisions regarding tissue utilization for DNA extraction and subsequent genetic testing are feasible.

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