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Detection of the Murine Double Minute2 (MDM2) gen in formalin-fixed tissue samples using PCR

Deteksi Gen Murine Double Minute2 (MDM2) pada Sampel Jaringan yang Terfiksasi Formalin dengan Metode PCR

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ABSTRACT

Background: *Tissue preservation is a crucial procedure in pathological studies to maintain cellular and structural integrity, commonly performed through formalin fixation. However, formalin fixation may cause DNA degradation, posing challenges for molecular analysis. Detection of cancer biomarker genes, such as Murine Double Minute 2 (MDM2), can be performed using Polymerase Chain Reaction (PCR), a technique for amplifying specific DNA fragments.*
Objective: *This study aimed to determine the presence of the MDM2 gene in formalin-preserved tissues stored for different periods using PCR.*
Methods: *A descriptive qualitative design with random sampling was employed. The study was conducted at the Anatomical Pathology and Molecular Biology Laboratories of Sekolah Tinggi Ilmu Kesehatan Nasional. Samples included 10 hair and nail tissues as controls, and formalin-fixed tissues stored for 2 days, 1 month, 2 months, and 8 years. DNA isolation was performed using the Geneaid gSYNC™ DNA Extraction Kit. DNA quality was assessed by 1.5% agarose gel electrophoresis, while concentration and purity were measured using a UV-Vis spectrophotometer. DNA concentrations ranged from 320–420 ng/µL, with purity ratios between 0.8 and 1.125.*
Results: *The results showed that the MDM2 gene (178 bp amplicon) was detectable in tissues stored for up to 2 months but was not detected in tissues stored for 8 years.*
Conclusion: *These findings indicate that storage duration affects the success of gene detection in formalin-fixed tissues.*

Keywords: *Formalin-fixed tissue samples, MDM2, PCR*

ABSTRAK

Latar Belakang: Pengawetan jaringan merupakan prosedur penting dalam studi patologi untuk mempertahankan struktur sel dan jaringan, salah satunya melalui fiksasi menggunakan formalin. Namun, fiksasi formalin dapat menyebabkan degradasi DNA sehingga menjadi tantangan dalam analisis molekuler. Deteksi gen penanda kanker, seperti Murine Double Minute 2 (MDM2), dapat dilakukan menggunakan Polymerase Chain Reaction (PCR), yaitu teknik amplifikasi fragmen DNA spesifik.
Tujuan: Penelitian ini bertujuan untuk mengetahui keberadaan gen MDM2 pada jaringan yang diawetkan dengan formalin dalam periode penyimpanan berbeda menggunakan metode PCR.
Metode: Penelitian ini bersifat deskriptif kualitatif dengan teknik random sampling dan dilakukan di Laboratorium Patologi Anatomi serta Laboratorium Biologi Molekuler Sekolah Tinggi Ilmu Kesehatan Nasional. Sampel terdiri dari 10 jaringan rambut dan kuku sebagai kontrol, serta jaringan terfiksasi formalin selama 2 hari, 1 bulan, 2 bulan, dan 8 tahun. Isolasi DNA dilakukan menggunakan Geneaid gSYNC™ DNA Extraction Kit. Kualitas DNA diuji dengan elektroforesis gel agarosa 1,5%, sedangkan kuantitas dan kemurnian diukur menggunakan spektrofotometer UV-Vis. Konsentrasi DNA berkisar 320–420 ng/µL dengan rasio kemurnian 0,8–1,125.

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Hasil: Hasil menunjukkan bahwa gen MDM2 dengan panjang amplikon 178 bp masih dapat terdeteksi pada jaringan yang disimpan hingga 2 bulan, namun tidak terdeteksi pada masa simpan 8 tahun.
Kesimpulan: Penelitian ini menunjukkan bahwa durasi penyimpanan memengaruhi keberhasilan deteksi gen pada jaringan terfiksasi formalin.

Keywords: Gen MDM2, Jaringan terfiksasi formalin, PCR

INTRODUCTION

Cancer is a pathological condition marked by the uncontrolled proliferation of abnormal cells. The development of cancer cells is associated with genetic and epigenetic alterations that modulate the activity of multiple genes, thereby resulting in a change in phenotype [1]. Tissue samples are preserved using formalin solution following a biopsy procedure. The fixation process, on the other hand, preserves the organ tissue by halting autolysis, a process of decomposition initiated by enzymes, and preventing bacterial degradation. The duration of fixation with formalin has been demonstrated to correlate with alterations in tissue consistency, with prolonged fixation resulting in a hardened and reduced tissue size [2].

The Murine Double Minute 2 (MDM2) gene is a notable cancer marker that can be examined. This gene functions as a significant negative regulator of the P53 tumor suppressor gene [3]. A multitude of studies have revealed that the MDM2 gene has been amplified in various instances of malignancy. The Murine Double Minute 2 (MDM2) gene has been identified in a variety of neoplasms, including lung and breast cancers. Elevated levels of MDM2 expression have been associated with the development of malignancies [4]. The Murine Double Minute 2 (MDM2) gene plays a pivotal role in the cell nucleus, where it functions to inhibit the tumor suppressor protein p53. The Murine Double Minute 2 (MDM2) gene has been shown to regulate and mediate the function of p53 in the transcriptional activity of the plasmid. Furthermore, the MDM2 gene has been demonstrated to mask the activity of the p53 domain from the cellular transcription machinery. The p53 protein's function can be counteracted by the murine double minute 2 (MDM2) gene through two distinct mechanisms: p53 is subject to ubiquitination-mediated degradation and is exported from the nucleus to the cytoplasm. In the context of cancer [5]. In typical circumstances, the expression of this gene is minimal [6]. Gene expression is recognized to increase in the presence of malignancy and can be influenced by multiple factors, including gene amplification, enhanced transcription, and elevated translation processes [7].

The MDM2 gene can be detected through the implementation of a polymerase chain reaction (PCR) method, which is an in vitro technique for DNA synthesis. Polymerase Chain Reaction (PCR) is a molecular technique used to amplify specific DNA sequences through repeated cycles of denaturation, annealing, and extension. The short oligonucleotides, also known as DNA primers, are required to facilitate the initial process of the PCR stage. Primers bind to a single strand of DNA when the temperature is decreased following the separation of the DNA double strands [8]. The principle of PCR is to repeat one cycle of two pairs of opposite oligonucleotide fragments and components of each DNA thread [9]. The PCR product obtained will undergo electrophoresis. The results of this electrophoresis will be analyzed by visually comparing the thickness of the band [10]. This study aimed to detect the Murine Double Minute 2 (MDM2) gene in formalin-fixed tissue samples using the PCR method. This study aimed to detect the Murine Double Minute2 (MDM2) gene in formalin-fixed tissue samples by the Polymerase Chain Reaction (PCR) method.

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METHODS

Study design

This study was a descriptive study conducted between July 2024 and March 2025 at the Anatomical Pathology Laboratory, Molecular Biology Laboratory, and Quantitative Analysis Chemistry Laboratory, Sekolah Tinggi Ilmu Kesehatan Nasional, Sukoharjo Regency, Central Java.

Data source and sampling procedure

MDM2 gene detection was analyzed using formalin-fixed tissue samples collected over various fixation periods in the Anatomical Pathology Laboratory, Molecular Biology Laboratory, and Quantitative Analysis Chemistry Laboratory at Sekolah Tinggi Ilmu Kesehatan Nasional, Sukoharjo Regency, Central Java. The sampling technique used in this study was purposive sampling, taking into account the fixation time of each sample. The sampling criteria used were formalin-fixed tissue samples obtained from the anatomical pathology laboratory. Samples were collected as many as 10 pieces, in the form of 2 pieces within 2 days, 2 within 1 month, 2 pieces 2 months, 2 pieces within 8 years old. 2 controls normal, normal nail tissue (15mg) and normal control hair tissue (10 strands, each 0.5–1 cm). Tissue samples were weighed at approximately 25 mg each (except for controls).

Variable of the study

The variables in this study included the duration of formalin fixation as the independent variable and the quality of DNA amplification of the MDM2 gene as the dependent variable, assessed through PCR visualization.

Data collection

Formalin fixation was reversed through de-formalinization using graded alcohol to prevent interference with DNA isolation.

Measurement and instrument

DNA extraction was performed using the Geneaid gSYNC™ DNA Extraction Kit according to the manufacturer's protocol. DNA quality was assessed using 1.5% agarose gel electrophoresis, while DNA concentration and purity were measured using a UV-Vis spectrophotometer with a 400× dilution. This was followed by temperature optimization and PCR amplification targeting the MDM2 gene using the following primers: forward primer "ATGTTCCTACTTCCGTTTCT" and reverse primer "TCAGCAAGCACATAAATCAG," with an expected amplicon length of 178 base pairs. PCR temperature optimization was conducted prior to amplification. PCR products were visualized using a Bio-Rad UV-Transilluminator Gel Doc instrument after agarose gel electrophoresis.

Ethical considerations

This study was approved by the ethics committee of Universitas Muhammadiyah Purwokerto with registration number KEPK/UMP/136/III/2025.

Data analysis

The data were analyzed based on the results of qualitative and quantitative assessments of DNA amplification visualization using Polymerase Chain Reaction (PCR) techniques, following agarose gel electrophoresis and visualization using the Bio-Rad UV-Transillumination Gel Doc instrument.

RESULTS

In this study, DNA was successfully extracted from all tissue samples fixed with formalin, with results varying based on the duration of fixation. From the gel

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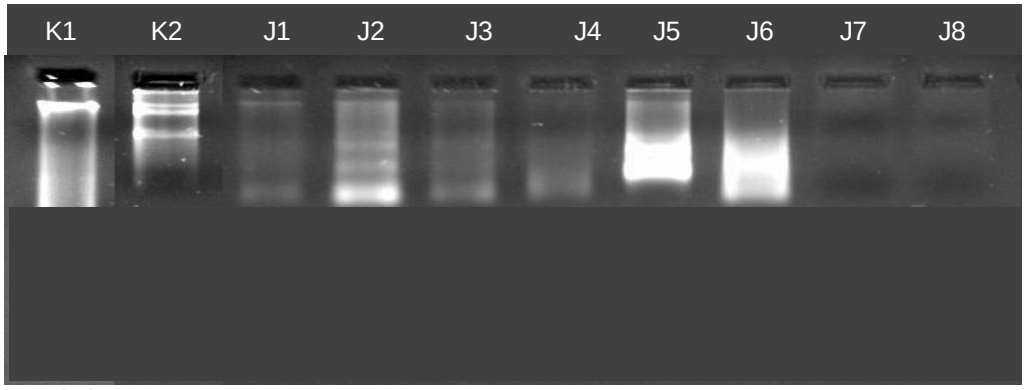
electrophoresis results, the extracted DNA showed visible bands in samples fixed for up to two months, while samples with longer storage periods showed reduced band intensity or no bands at all. The following shows the results of each step of the assessment, from qualitative tests, quantitative tests, and PCR tests on 10 samples.

1. Qualitative Test



Description:
K1 : Nail Control
K2 : Hair control
J1-J2 : 2-day formalin-fixed tissue
J3-J4 : 1 months formalin-fixed tissue
J5-J6 : 2 months formalin-fixed tissue
J7-J8 : Formalin-fixed tissue 8 years old

Figure 1. Gel electrophoresis profile of DNA extracted from formalin-fixed tissue samples

In Figure 1, DNA isolation was tested on 10 samples, 8 formalin-fixed tissue samples, and 2 normal tissue controls. The DNA quality test results showed that the DNA visualized in the 2-day sample had a thin but still visible band intensity, the 1-month sample showed a band pattern that was almost the same as code J1, and the 2-month sample showed a clear and thick band pattern. In the 8-year formalin-fixed tissue sample, codes J7 and J8 did not show any band pattern at all

2. Quantitative Test

Samples with UV-Vis spectrophotometer were tested at a wavelength of 260 nm and 280 nm. Quantitative test results in the form of concentration and purity of DNA isolates can be seen in table 1.

Table 1. DNA Quantitative Test Results

Samples	λ260	λ280	Concentration of DNA	Purity of DNA
K1	0.016	0.020	320	0.8
K2	0.017	0.019	340	0.8947
J1	0.017	0.016	340	1.0625
J2	0.021	0.019	420	1.1052
J3	0.021	0.019	420	1.1052
J4	0.017	0.016	340	1.0625
J5	0.020	0.020	400	1
J6	0.019	0.019	380	1
J7	0.018	0.016	360	1.125
J8	0.018	0.016	360	1,125

*DNA purity limits: 1,8-2,0 ng/µl
*Ideal DNA concentration: >100 ng/µl

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Based on the quantitative DNA test results in Table 1, the DNA concentrations ranged from 320 ng/µL in the nail control sample K1 to 420 ng/µL in samples J2 and J3. None of the samples reached the ideal purity range of 1.8–2.0. The lowest A260/A280 ratio was 0.8 K1, indicating significant protein contamination, while the highest value was 1.125 in samples J7 and J8, which was still below the acceptable purity threshold.

3. PCR temperature optimization

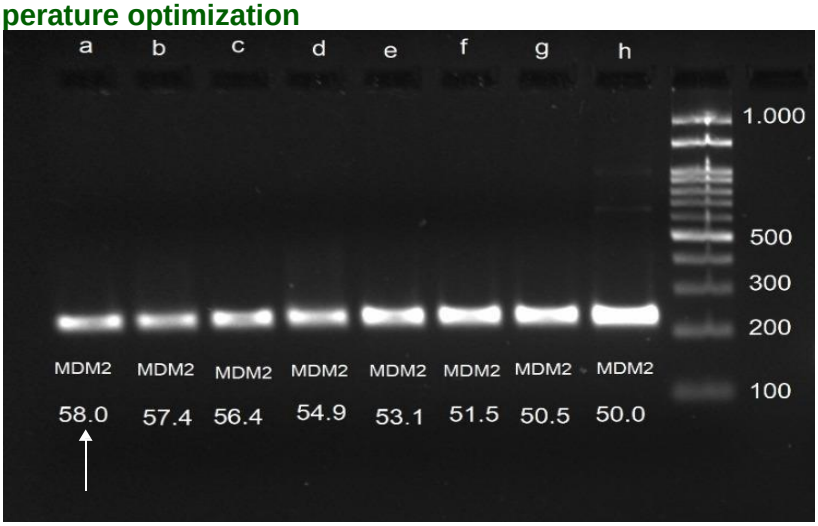

Figure 2. MDM2 Gene Temperature Optimization Results

PCR temperature optimization is carried out to determine the right annealing temperature so that the primers can bind specifically to the DNA target. The temperature was calculated using the formula (Tm Forward Primer-Tm Reverse Primer/ 2) (51.0+50.7/2 = 50.85). Annealing temperature optimization was performed using eight temperature points ranging around 50°C. Based on gel electrophoresis results (Figure 2), 58°C was selected as the optimal annealing temperature, as it produced a single, sharp DNA band of the expected size 178bp, without smears or nonspecific amplification. This temperature was used for subsequent PCR amplification steps. Because the DNA band obtained was a single band, intact, and there were no impurities or smears. According to Setyawati & Zubaidah [11], bands that are thick, clean, and according to the target size, on electrophoresis results are optimal results.

4. PCR Amplification

DNA amplification was carried out using the Polymerase Chain Reaction (PCR) technique using a pair of primers, using the forward primer “ATGTTCCTACTTCCGTTTCT” and the reverse primer “TCAGCAAGCACATAAATCAG” and a target length of 178bp. The next step is visualization with 1.5% agarose gel electrophoresis at 90 volts, 400 mAh, for 90 minutes, and the results are shown in Figure 3.

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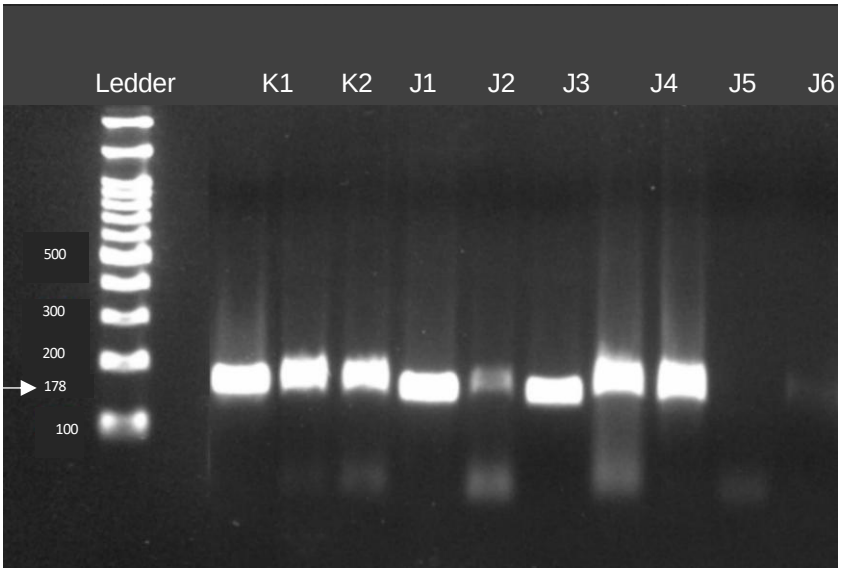

Figure 3. **MDM2 Gene Amplification Test Results (black and white)**

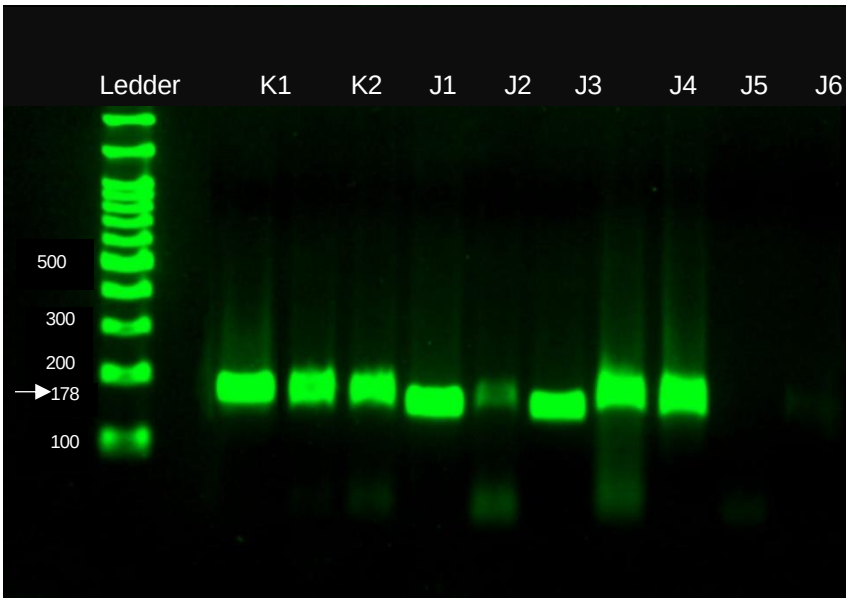

Figure 4. **MDM2 Gene Amplification Test Results (black and green)**

In figure 3 and figure 4, amplification of the Murine Double Minute2 (MDM2) gene resulted in visualized DNA in the control nail and hair samples, as well as in the formalin-fixed samples of 2 days, 1 month, 2 months, and not visualized in the formalin-fixed tissue samples of 8-year-old J7 and J8. DNA band broadening can be caused by degraded DNA, which can affect the results because the DNA does not bind to the primer. Contaminants can also have an effect, as can be seen in purity results that do not fall within the normal range.

DISCUSSION

The isolated samples were then analyzed. Qualitative analysis of DNA isolates was performed using 1.5% agarose gel electrophoresis to evaluate extraction success. As shown in Figure 1, DNA band intensity varied across samples: strong bands were observed in some samples, faint bands in others, and no visible bands in J7 and J8. Good DNA is DNA that shows up as a single band; there is no smear, DNA bands look

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clear and thick, thick bands may indicate high DNA concentration or incomplete migration. The thinness of the DNA bands produced indicates the concentration of the extracted DNA. According to Halimah & Solihat [12], the duration of electrophoresis affects the distance traveled by DNA fragments, whereby the longer the electrophoresis time, the further the distance traveled by the DNA fragments. The possibility of a thick DNA band picture is also influenced by electrophoresis time; the type of sample used can also affect the results of DNA extraction [13]. The DNA bands exhibit signs of fragmentation, as evidenced by their irregular appearance and clear shading [14]. In samples K1-J6, there is a smear. The presence of smears on DNA bands can be attributed to a variety of factors, including contamination of DNA isolates and degradation of DNA. Smearing near or below DNA bands may indicate DNA degradation or contamination [15].

In samples code J7 and J8, there was no DNA band. The longer the formalin exposure or tissue fixation with formalin will result in DNA denaturation. The storage period for sample tissue is 8 years; this can be an indication of not visualizing DNA in samples J7 and J8 [16]. DNA that is not released can be influenced by storage or fixation factors using a formalin solution. Exposure to formalin itself can be an obstacle in DNA analysis because the pH of the formalin solution will decrease over time. This is because formalin will form formic acid, which causes hydrolysis of the N-glycosyl bond, where this is a bond that binds bases, and the hydrolysis that occurs will remove bases that leave the apurinic or apyrimidinic site (AP Site) and will cause DNA fragmentation or damage to the DNA structure [17].

Good DNA purity is 1.8-2.0, and good DNA levels are above 100 ng/µl. In Table 1, it is known that the purity of DNA obtained is less than 1.8-2.0, namely 0.8-1.1. This can be influenced by several factors, among others, the presence of protein contamination, less than optimal conditions during the isolation stage, and poor sample quality [18]. In Table 1, it is known that the concentration of DNA obtained is more than 100 ng/µl, namely 320-420 ng/µl. It is possible that the concentration obtained is not a pure concentration of DNA; some contaminants, such as RNA, that can be read at absorbance 260, will also be counted and cause high DNA concentrations. Dissolved DNA contaminants greatly affect the stability of the resulting DNA concentration [19]. DNA Purity and Concentration
UV-Vis spectrophotometric analysis revealed DNA purity values between 0.8 and 1.1, below the optimal range of 1.8–2.0, possibly due to protein contamination or residual RNA. DNA concentrations range from 320–420 ng/µl but may include contaminants that absorb at 260 nm, affecting stability and quantitative analysis. DNA quality degradation correlates with storage duration and formalin exposure [17].

Visualization of PCR results using 1.5% agarose gel electrophoresis. Wavy DNA bands were obtained; this may be caused by unstable electrophoresis tension conditions that can affect DNA migration in the gel [20]. In Figure 3, there are variations in the results in the form of different band thicknesses in sample J3; thin DNA bands in this sample code can be influenced by work error factors in making improper pipetting. The PCR composition used, such as the number of samples or primers, can also affect the amplification results [21].

Some of the samples, such as J7 and J8, were not detected in this study, and the sample codes J7 and J8 were not visualized from the DNA qualitative test stage. This could be due to several factors, such as poor DNA quality or the length of tissue storage, where these sample codes have been fixed in formalin for 8 years. The more tissue is stored, the greater the possibility of DNA damage due to longer exposure to formalin. According to Kusumadewi [22], samples that have been exposed to formalin have

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obstacles in the success of DNA analysis, this occurs due to a decrease in the pH of the formalin solution which will decrease over time and will form form formic acid and will damage DNA. It is known from testing the pH of the formalin solution that the pH of sample J7 is 5 and the pH of sample J8 is 4. In the visualization results, there is also a smear that can appear due to contamination and degradation of DNA due to fixation factors and the presence of dimers in sample codes K2, J1, J3, and J5. Dimers are non-specific DNA products that appear when the two primers are attached. This is likely to occur because the primer concentration is too high [23].

The results showed that in normal nail and hair tissue controls, formalin-fixed tissue samples of 2 days, 1 month, and 2 months showed the detection of the Murine Double Minute2 (MDM2) gene. The Murine Double Minute2 (MDM2) gene can be detected and visualized qualitatively, but the expression level of the gene cannot be known. According to Hou & Sun [6], in normal circumstances, the expression of this gene will be low, but in cases of malignancy, this gene will have high expression or increased expression. Several studies related to several studies of the Murine Double Minute2 (MDM2) gene show that the Murine Double Minute2 (MDM2) gene will increase in several cancer cases, such as bladder cancer, kidney cancer, leukemia, lymphoma, and sarcoma [24]. This research can be continued with the Real-Time PCR method for further research. According to Gollner & Dorothea [25], the Real-Time PCR method can determine the expression level of the Murine Double Minute2 (MDM2) gene, and this method is more sensitive and precise.

This study successfully detected the MDM2 gene in formalin-fixed tissues but could not quantify gene expression levels due to methodological limits. Future studies employing Real-Time PCR could provide sensitive and quantitative expression analysis. These findings underscore the feasibility of molecular testing on archival formalin-fixed samples, reducing the need for additional sampling.

CONCLUSION

This study demonstrated that the Murine Double Minute2 (MDM2) gene can be detected in control samples and in formalin-fixed tissue samples stored for up to 2 months. In contrast, samples fixed for 8 years did not yield detectable MDM2 amplification, likely due to DNA degradation. Further studies are recommended to explore improved deparaffinization or formalin removal methods for long-fixed tissues, and to apply quantitative PCR (qPCR) methods techniques to assess gene expression levels of MDM2 more accurately.

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