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Detection of The p53 Gene in Formalin Fixed Tissue Archives by Polymerase Chain Reaction (PCR) Method

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Abstract

Formalin fixed tissue archives are tissue biopsy results stored in formaldehyde fixative solution for a long time. The p53 gene is one of the genes that suppresses tumor occurrence and maintains genomic stability to prevent cell mutation and plays a role in the DNA regulation cycle and controls cell proliferation. Polymerase Chain Reaction (PCR) is a nucleic acid amplification technique used to denature and denature DNA or RNA using polymerase enzymes to detect the presence of the p53 gene in formalin-fixed tissue archival samples. This study aimed to detect the presence of the p53 gene in formalin-fixed tissue samples. This study used a descriptive research method with a random sampling technique conducted in the anatomical pathology laboratory and the National College of Health Sciences molecular biology laboratory. The samples in this study consisted of 10 samples consisting of 2 normal tissues used as controls and 8 formalin-fixed tissue samples. In this study, the p53 gene was detected in all samples, both normal tissue samples and formalin-fixed tissue samples after electrophoresis and visualized according to the target of 666 bp (base pair). The presence of the p53 gene qualitatively from DNA isolates can still be detected, but the gene expression level cannot be known in this study.

Keywords: Formalin Fixed Tissue Archive; p53 Gene; PCR.

Abbreviations: Polymerase Chain Reaction (PCR), Deoxyribonucleic acid (DNA), Ribonucleic acid (RNA)

INTRODUCTION

Cancer is an abnormal cell growth in the human body that attacks organs. These abnormal cells are caused by several changes in gene expression, causing an imbalance in the regulation of cell proliferation and cell death. Cancer is known as a chronic disease that needs to be watched out for, because it has a major impact on many people in the world and is one of the leading causes of death worldwide (Prasetyo et al., 2021). According to the Ministry of Health (2023) cancer is the second leading cause of death in the world and is a public health problem both globally and nationally, causing up to 9.6 million deaths each year. About 70% of deaths caused by cancer occur in developing countries, including Indonesia.

Cancer has many types of treatment or therapies depending on the type of cancer and the stage. Some cancer patients undergo only one type of therapy. However, most cancer patients undergo a combination of treatments, such as surgery with chemotherapy and radiation therapy. According to National Cancer (2015), biomarker tests for cancer treatment are a way to look for genes, proteins and other substances such as tumor markers that can provide cancer information. Biomarker tests are different from genetic tests to find out if a person has inherited a mutation that makes them develop cancer. These inherited mutations are mutations that are carried from birth. The cause of cancer is thought to be a mutation in the tumor suppressor gene p53. Mutations in this gene inactivate tumor suppressors and tumor progression. The p53 gene undergoes apoptosis, resulting in increased expression of the p53 gene. The expression of p53 that shows an increase in expression, which is significantly associated with an increase in the rate of invasive cancer tumors (Sadia et al., 2020). The p53 gene is found on chromosome 17p and this p53 gene acts as a tumor suppressor gene. The p53 gene controls the cell cycle before the synthesis phase and plays a role in cell proliferation *(Kisnanto et al., 2015).*

The p53 gene can be detected from frozen biopsy cut tissue organs and formalin fixed tissue archives. Archival tissue samples have enormous benefits as they can be available in large quantities and easily used to study various biomarkers to facilitate diagnostic procedures, target therapies, determine prognosis, and become an available source for research. A biopsy is the act of performing a microscopic examination of tissue from the

human body to establish the diagnosis of a disease process or infection (Erawati et al., 2017).

Sekolah Tinggi Ilmu Kesehatan Nasional has an anatomical pathology laboratory in which formalin-fixed tissue archives are stored for a long time. The tissue archive is used for studies but because it is stored for too long, normal tissue and abnormal tissue are no longer distinguishable. The main challenge in using formalin-fixed tissue archives in genetic research is that it is difficult to obtain quality DNA due to DNA damage caused by the process of tissue fixation with formalin and the influence of the long storage time of tissue archives at STIKES Nasional (Hafy et al., 2018).

Testing that can be used for p53 gene detection use PCR and electrophoresis techniques. Polymerase Chain Reaction (PCR) is a nucleic acid amplification technique used to denature and denature DNA or RNA using polymerase enzymes (Khehra et al., 2024). This study focused on detecting the p53 gene in normal tissue and formalin-fixed tissue samples using the Polymerase Chain Reaction (PCR) method. This study aims to determine the presence of the p53 gene in organ biopsy results stored for a long time in formalin.

MATERIALS AND METHODS

Study Area

This research has passed the ethical review of the Research Ethics Commission of Muhammadiyah Purwokerto University (KEPK-UMP) with the number KEPK/UMP134/VIII/2024. The type of research used descriptive research design, p53 gene detection was carried out using archival samples of formalin-fixed tissues within a certain period of time found in the anatomical pathology laboratory of the National College of Health Sciences. Samples were taken as many as 10 samples consisting of 2 normal tissue samples as controls in the form of hair tissue and nail tissue samples and 8 samples of formalin-fixed tissue archives over a period of 2 pieces of tissue 2 days, 2 pieces of tissue less than 1 month, 2 pieces of tissue 2 months, 2 pieces of tissue more than 5 years.

Procedures

Preparation of Control and archival tissue samples

Samples of 10 hair strands measuring 0.5 - 1 cm in length were taken from the proximal hair tip including the hair follicle and nail samples were taken as much as 15 mg. Archival samples of formalin-fixed tissue that have been cut as much as 25 mg are then removed formalin by being put into alcohol solution which is done in stages from 70% alcohol, 80% alcohol, 90% alcohol in each alcohol solution for 1 hour and absolute alcohol for 2 hours.

DNA Isolation

Preparation of tools and materials to be used include nail control samples, hair control samples and tissue pieces that have been removed formalin which is inserted into a 1.5 ml microcentrifuge tube. Followed by the DNA isolation stage using Geneaid gSYNCTM DNA Extraction through several stages, namely DNA lysis, DNA binding, DNA washing, and DNA elution.

DNA Qualitative Test

The results of DNA isolates were analyzed by DNA qualitative test using 1.5% agarose gel electrophoresis. The components needed in this qualitative test analysis are DNA isolate, gel red, and loading dye. Visualization of DNA isolation results was read using Bio-Rad UV transilluminator Gel-Doc.

DNA Quantitative Test

DNA quantitative test was carried out by making a 400x DNA dilution with a ratio of 10 µl DNA isolate and 3990 µl aquabidest which was put into a 15 ml centrifuge tube and homogenized. DNA purity was measured with a UV-Vis Spectrophotometer at a wavelength of 260 nm and 280 nm.

Optimization of Annealing Temperature

Components needed at the annealing temperature optimization stage in the form of master mix, DNA isolate, forward primer, reverse primer, and NFW are included in the PCR tube. Followed by the Thermal Cyler Machine T100 tool with a temperature range of 50 – 58^{0}C.

Polymerase Chain Reaction (PCR) Test

DNA amplification was performed using Polymerase Chain Reaction (PCR) with 1.5% agarose gel electrophoresis method at 57.40C. The components used in PCR amplification are DNA isolate, gel red, loading dye, and ladder which are electrophoresed on agarose gel in 90 minutes, 90volt, 400 Ampere and read with gel doc.

Data Analysis

The data analysis of this study was taken from the visualization results of the PCR test on normal tissues and tissue archives obtained from formalin-fixed tissue isolation found in the Anatomical Pathology Laboratory of the National College of Health Sciences.

RESULTS AND DISCUSSION

Formalin Removal Process with Graded Alcohol

In the first stage of formalin-fixed tissue archive samples, the process of cutting the tissue weighing 25 mg was carried out, then the process of removing formalin from the tissue using graded alcohol from 70% alcohol, 80% alcohol, 90% alcohol and absolute alcohol. Fixation is the process of soaking the tissue using a fixation liquid such as Neutral Buffer Formalin (NBF) 10% which aims to prevent decomposition. The graded alcohols used in

this process aim to remove water gradually in the tested tissue pieces. (Rahmawanti et al, 2021).

DNA Qualitative Test

DNA isolation is a technique used to obtain pure DNA, which does not contain RNA protein from a cell in tissue (Handayani et al, 2021). Samples that have been formalinized are then subjected to a DNA isolation process using Geneaid gSYNC^{TM} DNA Extraction. The results of DNA isolation that have been obtained are carried out qualitative tests to determine the quality of the isolated DNA. The success of DNA extraction was analyzed qualitatively by visualizing the DNA using agarose gel electrophoresis method. The principle of electrophoresis is the separation of DNA based on molecular weight on agarose gel using an electric charge. DNA will move from negative to positive poles in the electric field (Anissa et al., 2024).

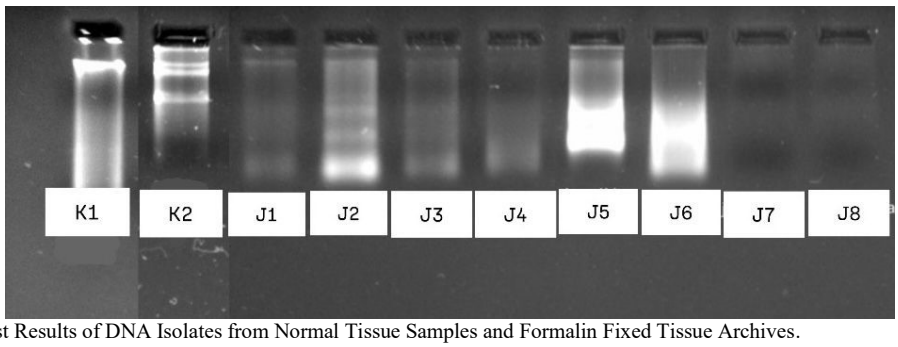

Figure 1. Qualitative Test Results of DNA Isolates from Normal Tissue Samples and Formalin Fixed Tissue Archives.

Description:
K1 : Nail Control
K2 : Hair Control
J1 – J2 : 2 day formalin fixed tissue
J3 – J4 : Formalin fixed tissue <1 month
J5 – J6 : 2 months formalin fixed tissue
J7 – J8 : Formalin fixed tissue > 5 years

Figure 1. shows the results of qualitative tests on K1 and K2. J1, J2, J3, J4, J5, J6 are DNA bands with different thicknesses and smears. The smear formed at the bottom near the DNA band indicates that the isolated DNA is not intact or fragmented and the smear located at the bottom of each lane indicates contamination (Anissa et al., 2024). DNA bands with different thicknesses depend on the weight and amount of DNA. Qualitative test visualization results that do not have DNA bands can be caused by insufficient DNA concentration in the sample (Ramadhani et al., 2024). The more DNA that is successfully extracted, the thicker the DNA band, and vice versa if the less DNA is extracted, the thinner the DNA band will be visualized on the agarose gel. Thin DNA bands can also be caused by contamination. Hidayati et al (2016) added, analysis using qualitative methods, DNA purity is also characterized by the absence of smears that indicate the presence or absence of contaminants such as RNA or protein. Smears formed at the bottom near the DNA band indicate that the isolated DNA is not intact or fragmented and smears located at the bottom of each lane indicate contamination. According to research by Halimah et al (2024), it is known that the length of electrophoresis time affects the distance traveled by DNA fragments, where the longer the electrophoresis time, the farther the distance traveled. DNA qualitative test performed using electrophoresis method through agarose gel is used to separate, analyze, identify, and purify DNA fragments (Anam et al., 2021). However, in samples J7 and J8 there was no DNA band. Sample codes J7 and J8 are formalin-fixed tissue samples of more than 5 years, this could be due to the possibility of DNA damage due to long term formalin fixation. DNA derived from degraded tissue is often fragmented. Exposure to chemicals, pH, temperature, and other exposures can cause DNA damage (Yudianto, 2010).

According to Willianto (2013) cited from Evans (2007) formalin solution is one of the chemical exposures that affect the integrity of DNA strands. Exposure to formalin that induces DNA crosslinks effectively protects the morphology of the cell structure, but at the extreme it is detrimental because DNA crosslinks inhibit denaturation in PCR and the pH of the formalin solution decreases over time due to the formation of formic acid which causes AP Site which ends in fragmentation. In a study (Willianto, 2013) showed that DNA analysis in human soft tissue exposed to 2% to 35% formalin solution for 3 days still obtained visualization of DNA bands, so that it can form a successful process of determining the identification of a person in DNA examination.

DNA Quantitative Test

DNA isolates that have undergone qualitative tests are then subjected to quantitative DNA tests to determine the

purity of DNA in the sample. DNA isolates were tested using a UV-Vis Spectrophotometer at a wavelength of 260 nm and 280 nm and using a 400x dilution with a ratio of 3990 µl of aquabidest and 10 µl of DNA isolate.

Table 1. Results of DNA Quantitative Test Using UV-Vis Spectrophotometer Results of DNA Quantitative Test Using UV-Vis Spectrophotometer.

Sample Code	λ260	λ280	Concentration (λ260 x 50ng/µl x Dilution Factor)	DNA Purity (λ260/λ280)
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 Limit : 1,8 – 2,0
*Ideal DNA Concentration Above 100 ng/µl

Table 4.1 shows the results of quantitative tests which include DNA concentration and purity. The results of the DNA quantity test obtained in all samples tested obtained a fairly good DNA concentration with an average value of more than 100 ng/µl and the results of the DNA purity test obtained an average of less than 1.8. So it can be concluded that the DNA isolate in all samples tested has a good concentration of more than 100 ng/µl but has a low purity value of less than 1.8. DNA concentration was measured with a wavelength of 260 nm. DNA purity test uses the result of absorbance wavelength 260 nm divided by the result of absorbance wavelength 280 nm. The level of DNA purity can also be affected by using DNA isolation kits and different DNA purification solutions. DNA concentration refers to the number of DNA molecules present in one unit volume such as nanograms per microliter (ng/µl). A high DNA concentration indicates many DNA molecules in the sample being analyzed. A high concentration of DNA does not necessarily mean the DNA is pure. DNA samples with high concentrations are likely to contain significant amounts of contaminants. According to Lucena, et al (2016) the presence of RNA can cause an increase in the value of DNA concentration because the UV Vis spectrophotometer at the time of absorbance reading cannot distinguish between DNA and RNA. So it can be said that the wavelength of 260 nm which is the maximum value of DNA absorbing light can be influenced by the presence of RNA which is read at a wavelength of 260 nm as DNA which makes the results higher because the UV-Vis Spectrophotometer cannot distinguish between DNA and RNA. Contaminants can affect the interpretation of the results. DNA purity refers to the extent to which the analyzed DNA sample is free from contamination or other foreign materials. The results of the DNA purity test using the UV-Vis Spectrophotometer found that almost all sample codes had low DNA purity values, with good DNA purity having a ratio of 1,8 – 2,0.

According to Mollah et al (2022) the results obtained on the UV-Vis Spectrophotometer can be influenced by solvent components contained in the DNA solution so that DNA impurities in the form of phenol compounds or contaminants that occur during dissolved DNA extraction greatly affect the stability of the resulting DNA concentration. These contaminants can be in the form of carbohydrates or proteins that cause the 280nm wavelength absorption to increase so that the purity value is influenced by the 280 wavelength value. Good quality DNA has a purity of 1.8 - 2.0 and an ideal DNA concentration above 100 ng/µl. DNA purity values that show numbers below 1.8 indicate the presence of high protein and polysaccharide contaminants, and vice versa if the purity value of DNA is more than 2.0 indicates a fairly high RNA contaminant in the isolation results. added, besides that the low purity of DNA can be influenced by the use of cuvettes that are not clear so that the absorbance of UV light on a UV-Vis Spectrophotometer is disturbed and can also be caused from a technical point of view at the time of measurement, homogenization and improper pipetting processes that cause DNA to break into fragments. The highest concentration of DNA depends on the amount of DNA, sample conditions and several factors at the time of DNA extraction. The cell lysis and precipitation stage requires the removal of supernatant which precipitates DNA in some samples which makes the extraction rate factor an influential factor as well as other factors that can affect the quantity of DNA such as the type of sample used, extraction method, and less sterile environmental conditions. The working principle of UV-Vis Spectrophotometer is based on the absorption of light at a certain wavelength of a sample being analyzed and the results of UV-Vis Spectrophotometer analysis can be used for qualitative and quantitative purposes (Sulistyani et al., 2023). It is necessary to measure quantitative tests with nanodrops but cannot be done in this study due to tool limitations.

Optimization of Annealing Temperature

Before PCR amplification is performed, it is necessary to optimize the annealing temperature. Annealing temperature optimization performed for PCR involves determining the optimal temperature for a particular primer and the DNA target sequence being amplified so that the primer can bind specifically to the target region. This study used a pair of forward primers and reverse primers designed with the National Center for Biotechnology Information (NCBI) website. A good primer is a primer that has characteristics such as primer length between 18 - 30 bases, % GC between 40 - 60%, Tm between 50 – 60^0C and does not have a secondary

structure and dimer. The results of primer design obtained several primer pair options which were then analyzed using netprimer and oligoevaluator software to obtain the best primer results as follows: forward primer 5'CTAAATCCCCAAGACTTCCT-3' and reverse primer 5'ACCAGTTGATTAGCAGAGAA-3' with a target of 666bp. Primers were chosen because they have good primer criteria, namely having no secondary structure, having a rating of 91% and 100% no hairpins.

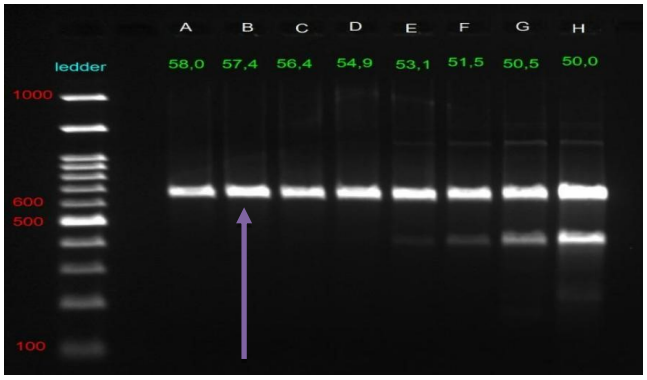

Figure 2. Optimization of Annealing Temperature Results.

Annealing temperature optimization performed for PCR involves determining the optimal temperature for a particular primer and the DNA target sequence being amplified so that the primer can bind specifically to the target region. Temperature optimization in this study was carried out at a temperature range of 50 – 58^{0}C, then the best annealing temperature results were obtained, namely 57.4^{0}C which was amplified at 666 bp because the visualization results at that temperature showed thick and intact DNA bands and the absence of double bands. At 4 temperatures, namely 50.0, 50.5, 51.5, 53.1 there are double bands, this is due to annealing temperatures that are too low so that the primer attaches to non-specific locations on the DNA template. According to Anissa et al (2024) a low annealing temperature causes the primer to bind to an unspecific attachment area which will then result in the amplification of unwanted locus fragments and according to Aulia et al (2021) to obtain thick PCR DNA bands it is necessary to optimize the annealing temperature on the primer to be used Tm will be the basis for determining the annealing temperature or Ta. An annealing temperature that is too high will cause the detachment of the primer attached to the printed DNA so that the PCR product will not be formed, and vice versa if the annealing temperature is too low it will cause non-specific primer attachment to the printed DNA.

The synthesis results for the Tm of forward primer 54.00^{0}C and reverse primer 53.98^{0}C were then calculated to estimate the Tm temperature. PCR temperature optimization with 8 temperatures with a range of 50.0^{0}C – 58^{0}C in accordance with the temperature range of Tm calculation results of primer synthesis. Figure 2. is the result of electrophoresis visualization of the temperature optimization process which shows that the primer amplifies DNA well and there is a DNA band that matches the target at 666 bp. In these results, the temperature selection of 57.4˚C is based on the firm boundaries of the band, the clarity of the band, and the absence of double bands. After determining the optimum temperature, the PCR amplification process was carried out at a pre-denaturation temperature of 95^{0}C for 3 minutes, denaturation 95^{0}C for 30 seconds, annealing 57.4^{0}C for 30 seconds, extention 72^{0}C for 1 minute, followed by electrophoresis and visualization using gel doc. Amplification analysis of isolation results was carried out using specific primers of the p53 gene.

Polymerase Chain Reaction (PCR) Test

DNA amplification was carried out by Polymerase Chain Reaction (PCR) method using a pair of primers, namely forward primer 5'CTAAATCCCCAAGACTTCCT-3' and reverse primer 5'ACCAGTTGATTAGCAGAGAA-3' which has a target length of 666 bp. The PCR program used adjusts the results of annealing temperature optimization that has been done before.

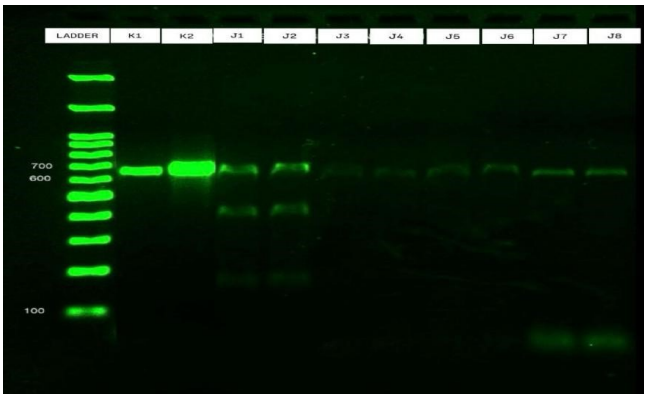

Figure 3. PCR Visualization Results of p53 Gene in Normal Tissue and Formalin Fixed Tissue Archive.

Description:
K1 : Nail Control
K2 : Hair Control
J1 – J2 : 2 day formalin fixed tissue
J3 – J4 : Formalin fixed tissue <1 month
J5 – J6 : 2 months formalin fixed tissue
J7 – J8 : Formalin fixed tissue > 5 years

The visualization of p53 gene detection through the electrophoresis process can be seen in Figure 3. The results show that in all samples there are DNA bands according to the target, namely 666bp with different DNA band thicknesses, there are thick DNA bands and there are thin DNA bands and some have smears and some do not have smears. The absence of smears or impurities indicates that the results of DNA amplification do not contain any residual impurity or contaminant solution. Smear indicates that the results of DNA isolation contain contaminants such as proteins, RNA, degraded DNA, or the isolation solution used. Sample codes J1, J2, J7 and J8 appear DNA bands at a size that does not match the amplification target, which is 666bp,

indicating nonspecific amplification. This is likely caused by primers that attach to non-target DNA sequences. The success of DNA amplification can be influenced by several, namely the concentration of PCR components, purity, annealing temperature which are technical factors and non-technical factors such as contamination (Feranisa, 2020). Primers have a level of specificity to the target DNA. If the primer is not specific, it can cause mispriming or cannot amplify DNA. Low DNA concentration will cause thin and faint DNA bands (Maliza et al., 2021).

According to Exzora & Qurrohman (2024) cited from Aristya et al (2013) stated that DNA that has low purity does not become a nuisance during DNA amplification by Polymerase Chain Reaction (PCR) method if the primer is a specific primer. However, according to Daryono & Yambise (2018) in general, the value of DNA purity is directly proportional to the sensitivity of the PCR test, the higher the purity of DNA, the more PCR sensitivity increases. So it can be concluded that DNA purity affects the efficiency and sensitivity of PCR. However, under certain conditions, PCR can still detect despite low DNA purity, especially if the primers used are highly specific to the DNA target. However, to obtain more optimal results, namely specific amplification and having a good DNA band, it still requires DNA that has purity according to the ideal DNA purity value of 1,8 – 2,0. In addition to DNA purity, DNA concentration is also an important factor to obtain optimal PCR results. Sample codes J7 and J8 in Figure 3. there is a DNA band that was not present in the DNA isolation process there was no DNA band, this is because the PCR technique can amplify DNA segments millions of times in just a few hours so that in sample codes J7 and J8 DNA bands are visualized after the PCR process.

The p53 gene is a tumor suppressor protein that is responsible for the apoptotic mechanism of cancer cells. The mechanism is the regulation of repair of a cancer-induced cell. Specific p53 protein expression occurs in cells that undergo changes from normal cells to abnormal cells and requires regulation of programmed death called apoptosis. The increased expression of the p53 gene, the higher the tendency for apoptosis to occur. In the observation of p53 using immunohistochemical painting method, p53 expression is indicated by brown color in the cell cytoplasm (Prihastuti, 2010). Expression of p53 that shows a significant increase in expression is associated with an increase in the rate of invasive cancer tumors (Sadia et al., 2020).

CONCLUSIONS

Based on the results of this study, it was concluded that the p53 gene was detected in 10 samples consisting of 8 samples of formalin-fixed tissue archives and 2 samples in normal tissue as a control visualized at 666 bp.

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