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The Effect of Fibrosis on DNA Yield from Formalin Fixed Paraffin Embedded Tissues

Muhammad Naveed, Hassan Tariq, Asma Gul, Natasha Arzo, Muhammad Hussain, Sumaira Buksh*

Department of Histopathology, Armed Forces Institute of Pathology/National University of Medical Sciences (NUMS) Rawalpindi Pakistan,
*Department of Histopathology, Combined Military Hospital, Malir/National University of Medical Sciences (NUMS) Rawalpindi Pakistan

ABSTRACT

Objective: To report the effect of fibrosis on yield of Deoxyribonucleic Acid extraction from Formalin-Fixed Paraffin-Embedded tissue.

Study Design: Cross sectional study.

Place and Duration of Study: Department of Molecular Pathology, Armed Forces Institute of Pathology, Rawalpindi Pakistan, Jul to Dec 2021.

Methodology: Fifty-four Formalin-Fixed Paraffin-Embedded tissues having size <2cm containing fibrosis (Mild, Moderate, Severe) were included in the study. Formalin-Fixed Paraffin-Embedded tissues having tissue size > 2cm were excluded. Deoxyribonucleic Acid was extracted and quantified from Formalin-Fixed Paraffin-Embedded blocks.

Results: Nine (17%) Formalin-Fixed Paraffin-Embedded tissues had no fibrosis, 28(51%) had Mild fibrosis, 9(17%) and 8(15%) had Moderate and Severe fibrosis. Measurement scale in 5 different stages for evaluation of our quantified results were used. For specimens with no fibrosis the DNA quantity fell on scale 1, 0(0%); on scale 2, 1(11%); on scale 3, 0(0%); on scale 4, 1(11%); and on scale 5, 7(78%). Deoxyribonucleic Acid yield in moderate fibrosis specimens fell on scale 1, 0(0%)' on scale 2, 7(78%); on scale 3, 0(0%); on scale 4, 1(11%); and on scale 5, 1(11%). Among severe fibrosis the Deoxyribonucleic Acid quantity fall on scale 1, 5(62%); on scale 2, 3(38%); on scale 3, 0(0%); on scale 4, 0(0%); and on scale 5, 0(0%).

Conclusion: Formalin-Fixed Paraffin-Embedded tissues having severe fibrosis resulted in significantly lower Deoxyribonucleic Acid yield as compared to Formalin-Fixed Paraffin-Embedded tissues having no fibrosis

Keywords: Deoxyribonucleic acid, Formalin-fixed paraffin-embedded, Fibrosis.

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INTRODUCTION

Advances in the field of oncology have led to progress in development of multiple new technologies for gene expression and mutation analysis, leading to identification of mutations that are the corner stone of modern personalized medicine and anticancer therapy.[1] The therapeutic and prognostic implications of finding these mutations has made molecular oncology one of the most rapidly evolving fields of medicine.[2] Formalin-fixed paraffin-embedded (FFPE) tissue block is considered as a gold standard for the preservation of human biological tissue specimens, specifically for cancer. The crosslinking ability of formalin to the tissue decreases the larger fragments of DNA (100-200 bps), RNA and protein availability.[1] Embedding in paraffin wax enables thin sections to be cut and the architecture of the tissue to be examined. The important steps that affect high quality DNA from FFPE samples are the pre-extraction steps, such as fixation duration, fixative type, fixative composition (formalin concentration, pH, and salt concentration), tissue type, and temperature 2 and the type of fixative effects the DNA yield. If we compare 10% buffered formalin as a fixative with alcohol, we will examine that with 10% buffered formalin DNA extraction results are better than alcohol fixatives and we can use paraffin wax or celloliden both for impregnation and embedding purpose these both materials will less affect DNA extraction yield. Fresh tissues in 10% buffered formalin fixatives can give us better result the old one.[3] Size of tissue and tumor percentage also effects DNA yield,[4] as FFPE tissues are notorious for producing suboptimal DNA quality and low DNA yield. Differences in pre-analytical capabilities were observed.[5] For good quality results we should standardize the pre analytical factors which can affect DNA yield.

However quantity and quality of nucleic acids extracted from FFPE tissue shows variability and can have significant impact on the results.[6] An irrevocable bond is formed between the nucleic acid during the formalin fixation of tissue, making the DNA highly inaccessible during DNA extraction from FFPE tissue specimen.[7]

Correspondence: Dr Hassan Tariq, Department of Histopathology, Armed Forces Institute of Pathology, Rawalpindi Pakistan
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Reliable assessment of single nucleotide polymorphisms (SNPs) in FFPE biological samples is possible, however, with mandatory precautions.[8] Furthermore, morphological factors like tumor volume, the maximal diameter of the tumor circled area and tumor fraction plays important role in the acquisition of optimal DNA concentration and careful investigations of these factors in routine practice should be incorporated before the isolation of DNA from FFPE tissues.[9]

Apart from local factors tissue factors also affect the DNA yield. Different tissue types (lung, soft tissue, liver ,melanoma) yield different amount of DNA even if they have the same size of tissue.[10] Similarly the amount of tumor compared to normal tissue also has a bearing on DNA yield. FFPE tissues are notorious for producing suboptimal DNA quality and low DNA yield.. Different studies have suggested an adverse outcome of the amount of fibrosis as they are thought to affect DNA extraction subsequently leading to lesser amount of DNA yield. However, no formal research or article stating its comparative effect is found in this country or the region, which forms the rationale for our study.

METHODOLOGY

The cross-sectional study was conducted from June 2021 to December 2021, at Molecular Pathology Department of Histopathology, Armed Forces Institute of Pathology, Rawalpindi, Pakistan after permission was obtained from Institutional Ethical Review Committee (IERB no 638).

Inclusion Criteria: FFPE tissues having tissue size <2cm, mostly having fibrosis were included.

Exclusion Criteria: Over fixed & calcified and tissue having extensive necrosis and tumor volume less than 20% were not included.

FFPE tissue was taken from Adenocarcinoma lung cancer and colon cancer patients after surgery. Sample size was estimated by WHO Calculator using prevalence of colon cancer patients 11%23, which resulted in a sample size of 50. Non-probability convenience sampling technique was used. Informed consent was obtained from patients, keeping data confi-dentialities and anonymities priority of the study. DNA was extracted from FFPE tissue utilizing QIAamp FFPE DNA tissue kit, QIAGEN USA. Qubit fluorometer (1ul of extracted DNA) to quantify DNA in ng/ul via Qubit dsDNA HS Assay kit.

Rotary microtome was used to cut 5 microns of tissues with minimum of 10 sections. Paraffin was removed from these tissue sections using deparaffinization solution according to QIAamp FFPE DNA tissue kit protocol. For Tissue lysis and retrieval used ATL and protienase k in the ratio of 180ul:20ul heated by the help of heat shaker 56o C and 90o C alternatively for 1 hour according to the kit protocol. DNA purified by AL buffer and ethanol with equal quantity 200ul:200ul. DNA attached with silicon membrane with help of micocentrifuge and washed with the help of wash buffer 1 (500ul) and wash buffer 2 (500ul) centrifuged at 8000 RPM for 1 minute for each wash. DNA eluted from silicon membrane in 1.5 ml micro tube with the help of molecular grade water or elution buffer DNA analyzed by Qubit fluorometer (1ul of extracted DNA) to quantify DNA in ng/ul via Qubit dsDNA HS Assay kit Qubit fluorometer (1ul of extracted DNA) to quantify DNA in ng/ul via Qubit dsDNA HS Assay kit. We made stock solution with the help of dye and buffer (1:10). The stock solution was mixed with the help of vortex. Then we added 90ul stock solutions in level 1 labeled tube and added 10ul level 1 solution. We also added 90ul stock solution in tube labeled level 2 and added into it 10ul level 2 solution. Then, the test tube labeled s (sample) was prepared by adding 99ul stock solution and 1ul DNA solution. The DNA concentration was measured with the help of Qubit dsDNA HS Assay.

Data was analyzed by using Statistical Package for Social Sciences (SPSS) version 25. Mean and standard deviation were calculated for quantitative variables and frequency and percentages were calculated for categorical variables.

RESULTS

A total of 54 FFPE tissue samples were analyzed in our study, out of 54 samples 28(51%) showed MILD fibrosis, 9(17%) had moderate fibrosis, 8 (15%) FFPE tissue samples had severe fibrosis and 9(17%) FFPE tissue samples had NO fibrosis (Figure-1). FFPE tissues having MILD fibrosis were significantly higher as compared to those showing other stages of fibrosis.

Measurement scale in 5 different stages for evolution of quantified results were used, (1=0.00ng/ul, 2=0.001ng/ul to 0.99 ng/ul, 3=1.00 ng/ul to 1.99 ng/ul, 4=2.00ng/ul to 2.99ng/ul, 5=>3.00ng/ul). Among tissue specimens with no fibrosis the DNA quantity fell on scale 1, 0(0%); on scale 2, 1(11%); on scale 3, 0(0%); on scale 4, 1(11%); and on scale 5, 7(78%).

Among the tissue with mild fibrosis the DNA quantity fell on scale 1, 0(0%); on scale 2, 11(39%); on scale 3, 4(15%); on scale 4, 2(7%); and on scale 5, 11(39%).

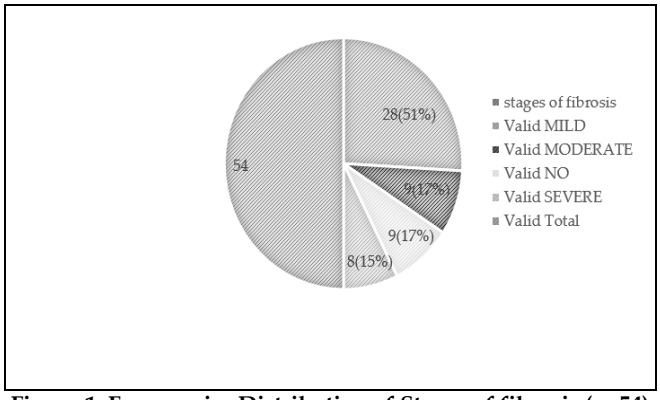

Figure-1: Frequencies Distribution of Stages of fibrosis (n=54)

Among the tissue with Moderate fibrosis the DNA quantity fell on scale 1, 0(0%)' on scale 2, 7(78%); on scale 3, 0(0%); on scale 4, 1(11%); and on scale 5,1(11%)and among severe fibrosis the DNA quantity fall on scale 1, 5(62%); on scale 2, 3(38%); on scale 3, 0(0%); on scale 4, 0(0); and on scale 5, 0(0%) (Figure-2)

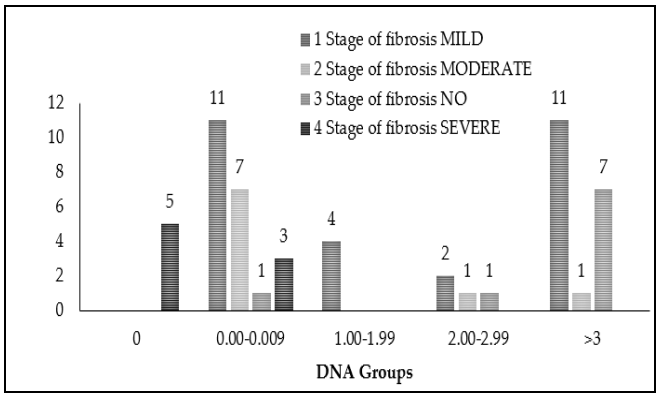

Figure-2: Frequency of Difference Stages of Fibrosis in the DNA Yield Groups (n=54)

DISCUSSION

Our findings provide critical guidance that may significantly enhance the breadth of diseases that can be studied by methylomic profiling. Extracting DNA from formalin-fixed and paraffin-embedded (FFPE) tissue remains a challenge, despite numerous attempts to develop a more effective method.[11,12] Pathological factors (inflammation, necrosis, fungus, tuberculosis) and technical factors (fixatives, timing, temperature, xylene, and the reagents used for impregnation and embedding) can limit performance of genetic analyses and have significant influence on overall study.[13,14]

FFPE tissues are valuable resources to examine the morphology as well as assess the degree of viable tissue along with percentage to tumor in the tissue. It also helps mark the appropriate tumor tissue for microdissection leaving behind the non-tumor tissue for optimal tumor DNA yield. However, FFPE tissues often yield sub optimal for DNA in terms of quality and purity.[15] As our results also indicated the sub optimal FFPE DNA quantity. For downstream processing of DNA and RNA, optimal quantity and purity is an important requirement. Previous studies showed that the preservation of FFPE tissue samples for more than 1 year resulted in lower yield in comparison to fresh tissue.[16] Fresh FFPE tissue samples were included in the study as a number of previous studies presented that old FFPE tissues had effect on their DNA yield.[17,18]

The current study examines the DNA extraction from FFPE tissues and the effect of fibrosis on its yield. Best quality of FFPE DNA can be obtained by tissues which are fixed with 10% neutral buffered formalin for 1 day (24 hours). Other fixatives can minimize the DNA yield. Moreover, increase in fixation time also affects DNA yield negatively. Various studies suggest the use of 10% neutral buffered formalin as a best fixative for FFPE tissues to get optimal DNA yield.[12,13] However, some studies suggested that fixative had fewer effects on DNA yield.[19] Previous studies suggested that paraffin wax also had fewer effects on DNA yield.[20] The condition of 10% neutral buffered formalin fixation was kept constant for the fixation of study to analyze the exact effect of fibrosis on the extraction of DNA. The time and temperature were according to standard time (24 hours, 2 hours) and temperature (22-28o C, 45-55 o C). Therefore, fewer chances to affect DNA yield during our study from technical issues.

Fibrotic tissues have stiff extracellular matrix (ECM). Increase in the collagen as an important part of ECM, usually maintained by the fibrotic cells, enhance the difficulty of DNA extraction, even leading to PCR inhibition.[21] Fibrosis leads to stiffening of the tissue making extraction from it difficult. Several international studies in the past have suggested inverse relation of fibrosis to DNA yield however no analytical review from the country or the region is available. The current study shows that fibrosis effects DNA yield from FFPE tissue. Effect depends upon stages of

fibrosis; severe fibrosis had severe effect mild had mild effect and moderate had moderate effects, as the results indicated. Presence of fibronectin, which is a high molecular weight protein in extracellular matrix also creates difficulty in the extraction of DNA. The increase of fibronectin to support collagen fibrillo-genesisin in fibrotic tissue.[22] Based on the above discussion, the present study will help us in future to better define tissue adequacy for DNA extraction and molecular testing minimize our time and wastage of expensive kits.

LIMITATION OF THE STUDY

The main limitation in our study was that we did not take into consideration other factors like tissue type and calcification which might lead to the interference with DNA extraction.

CONCLUSION

The current study conclusively shows a significant impact of fibrosis on DNA yield with tissues having severe fibrosis yielding a significantly lower DNA yield than tissues having no or little fibrosis.

Conflict of Interest: None.

Authors' Contribution

Following authors have made substantial contributions to the manuscript as under:

MN & HT: Data acquisition, critical review, approval of the final version to be published.

AG & NA: Study design, data interpretation, drafting the manuscript, critical review, approval of the final version to be published.

MH & SB: Conception, data acquisition, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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