Original Article

Unveiling the arcanum of formalin-fixed paraffin-embedded archival tissue blocks: A valuable resource for genomic DNA extraction

Sameera Begum[1], Vishnudas Prabhu[1], Varshasnata Mohanty[2], K. Krishnaraj Upadhyaya[3], Riaz Abdulla[1]

[1]Department of Oral Pathology, Yenepoya Dental College, Yenepoya (Deemed to be University), [2]Center for Systems Biology and Molecular Medicine, Yenepoya Research Centre, Yenepoya (Deemed to be University), [3]Department of General Pathology, Yenepoya Medical College, Yenepoya (Deemed to be University), Mangaluru, Karnataka, India

Abstract

Background: Formalin-fixed paraffin-embedded (FFPE) tissue blocks are routinely preserved after pathological diagnosis and possess tremendous potential for biomarker discovery. These archival samples are prone to degradation on prolonged storage due to the formalin cross-linking.

Aims: This study aimed to evaluate whether the storage period of the formalin-fixed paraffin-embedded tumor blocks had a significant impact on the yield and purity of the isolated DNA archived for 11 years.

Settings and Design: A retrospective study was carried out in the Department of Oral Pathology and Microbiology in accordance with the Institutional Ethics Committee.

Materials and Methods: Genomic DNA extraction was performed using TaKaRa DEXPAT Easy DNA kit from 40 FFPE tissue blocks of oral squamous cell carcinoma archived for 11 years (2006–2017). NanoDrop spectrophotometer was used to determine the DNA yield (A260) and purity (A260/A280 ratio). The quality of DNA fragments was validated using agarose gel electrophoresis.

Statistical Analysis Used: Statistical analysis was obtained by SPSS 22, MS Excel and analyzed using the analysis of variance (ANOVA) test. $P < 0.05$ was set for statistical significance.

Results: There was no statistically significant difference observed both in terms of DNA yield ($P = 0.996$) and purity ($P = 0.997$) of FFPE tumor blocks archived for 11 years among the study groups.

Conclusions: It was concluded that, irrespective of years of storage of the FFPE, it is possible to extract genomic DNA and use it for molecular studies.

Keywords: DNA, formalin-fixed, NanoDrop, oral squamous cell carcinoma, storage, tumor blocks

Address for correspondence: Dr. Riaz Abdulla, Department of Oral Pathology, Yenepoya Dental College, Yenepoya (Deemed to be University), Mangaluru- 575 018, Karnataka, India.
E-mail: rizdent@yenepoya.edu.in

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INTRODUCTION

The tumor tissue serves as a favorable biological material to identify underlying molecular pathways driving a specific tumor; tissue-based biomarker studies are greatly benefited where tissue being the highest reservoir of any tumor-specific markers. Fresh tissue samples or

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snap-frozen samples are considered ideal for molecular genetic analysis, provided their genetic material such as DNA or RNA is well preserved. However, such samples are limited in availability, where their collection is limited to individual centers such as tissue banks and research groups.[1] Alternatively, tissue specimens are routinely fixed in formaldehyde and preserved in paraffin blocks formalin-fixed paraffin-embedded (FFPE).[2] FFPE blocks are inexpensive, easily available and widely stored in pathology departments all over the world. Despite fresh tissue materials are desirable for analysis, FFPE tissue blocks possess tremendous potential for biomarker discovery as it can be routinely preserved and stored after pathological diagnosis and hence serve as an alternative resource for research purposes.

FFPE immobilization preserves the morphological tissue architecture, making it a good source for the extraction of nucleic acids, which can be utilized for translational clinical research and to conduct cancer research projects in future.[3] One of the biggest challenges to extract biological material from FFPE samples is due to formaldehyde cross-linking. Such cross-linking is known to pose harmful effects such as chemical alteration and degradation of the genomic and proteomic content and even causing sequence alterations due to the addition of monomethylol groups to nucleotide base pairs.[4,5] Thus, specific protocols must be ensured for the DNA extraction since the tissue is degraded due to prolonged formalin fixation, which can inhibit the polymerase chain reaction amplification.[6,7] Many researchers have significantly investigated the effect of extracted macromolecules over the storage period from the tumor blocks. Recent advancements in the molecular biology field have facilitated the extraction of DNA, RNA and proteins from FFPE tissues and made it accessible for downstream applications, but the quality remains a concern.[8,9] The extraction of an adequate amount of DNA from FFPE specimen is crucial and challenging. Hence, essential factors need to be considered depending on the requirements of the study to be carried out.[10,11] Thus, the present study was designed to determine whether the storage period of the tumor blocks had a significant influence on the yield and purity of the isolated DNA in a series of archival FFPE blocks.

MATERIALS AND METHODS

Forty FFPE blocks which were histopathologically diagnosed as oral squamous cell carcinoma (OSCC) were retrieved from the archives of the Department of Oral Pathology and Microbiology, Yenepoya Dental College (Mangaluru, India) and divided into four groups comprising 10 samples in each group ranging from years 2006–2008 (Group 1), 2009–2012 (Group 2), 2013–2016 (Group 3) and 2017 (Group 4) archived for 9–11 years, 5–8 years, 1–4 years and <1 year, respectively [Table 1]. The study was approved by the Institutional Ethics Committee (2015/232). The study design was in compliance with the principles of the Declaration of Helsinki. The tissue samples used in this study were fixed in 10% of neutral buffered formalin for 24–48 h, processed and embedded in paraffin and stored at room temperature.

Inclusion criteria of 5 mm × 5 mm size of tissue section were considered. OSCC associated with any other pathology, tissues with inadequate size (< 0.5 cm × 0.5 cm size) were excluded from the study.

DNA extraction from formalin-fixed paraffin-embedded tissue

The DNA extraction from FFPE tissues was done using a commercially available TaKaRa DEXPAT Easy DNA kit (Takara Bio Inc, Shiga, Japan) in accordance with the manufacturer's instructions. Single tissue block was selected, and for each case, 3–FFPE tissue sections of 8 μm thickness were cut and added into a 1.5 ml microcentrifuge tube using sterilized tweezers. Microtome used to cut sections was disinfected with a hydrogen peroxide disinfectant and then wiped with ethanol. 0.5 ml (about 20 drops) of DEXPAT solution was added into the microcentrifuge tube and incubated at 100°C for 10 min in a block heater. The microtubes were placed in a microcentrifuge precooled to 4°C immediately after heat treatment and centrifuged at 12,000 rpm for 10 min at 4°C. The microtubes were removed immediately on completion of microcentrifugation and placed on ice for 5 min. The aqueous layer was collected, and the paraffin thin layer formed at the top was avoided. A gel-like layer above the absorbent resin was formed. This aqueous gel-like layer on top was carefully withdrawn without disturbing the tissue debris and resin.

DNA yield and purity

Following extraction, DNA recovered from the biopsy tissues was immediately assessed using NanoDrop spectrophotometer to determine the yield (A260) and

Table 1: Archival time of formalin-fixed paraffin-embedded blocks

Groups	Year	Archival time (years)
Group 1	2006-2008	9-11
Group 2	2009-2012	5-8
Group 3	2013-2016	1-4
Group 4	2017	<1

purity (A260/A280 ratio) of the isolated DNA for each case and further validated for the quality using agarose gel electrophoresis to check for DNA fragments. 100 ng/μL was considered an optimal cutoff for good DNA yield. Optimal DNA purity was considered in the range of 1.6–1.9.

Quantitative and qualitative analysis using NanoDrop spectrophotometer

The machine was set to blank before measuring concentrations. NanoDrop Arm was lifted and 1.5 μl of blank solution 1x Tris-EDTA (1x TE) were dispensed directly on top of the NanoDrop sensor. The arm was lowered and blank button is clicked at the top left of the computer program. NanoDrop arm was cleaned to remove any remaining solvent and to reduce contamination between samples. 1.5 μl of the sample was loaded onto the NanoDrop sensor and arm was closed. The series of calculations were displayed by the program; the most important was optical density 260/280 calculation for detecting the purity. The data were saved and stored in the folder.

Statistical analysis

The results were obtained by SPSS IBM Chicago version 22, MS Excel and analyzed using the analysis of variance (ANOVA) test. $P < 0.05$ was set for statistical significance.

RESULTS

The mean DNA yield obtained from FFPE tissues for Group 1 (2006–2008) was 88.76 ± 88.45 ng/μL, Group 2 (2009–2012) was 89.84 ± 147.80 ng/μL, Group 3 (2013–2016) was 77.69 ± 93.48 ng/μL and Group 4 (2017) was 85.01 ± 161.23 ng/μL. The mean DNA purity obtained from FFPE tissues for Group 1 (2006–2008) was 0.56 ± 0.27, Group 2 (2009–2012) was 0.54 ± 0.07, Group 3 (2013–2016) was 0.56 ± 0.24 and Group 4 (2017) was 0.56 ± 0.08. While comparing the DNA yield and purity, there were no statistically significant differences observed among the study groups ($P = 0.996$ and $P = 0.997$.[Table 2 and Figures 1, 2]. The spectrophotometric curves obtained by NanoDrop were similar to the typical curve, with some variations [Figure 3]. Agarose gel electrophoresis of 40 DNA extracts from FFPE tissue blocks ranging from 1 to 11 years of storage (2006–2017) showed that DNA was more severely fragmented [Figure 4].

DISCUSSIONS

FFPE archival samples are known to be a poor source for molecular biological assays due to the formalin cross-links formed on prolonged fixation.[4] During sample storage, residual formalin within some FFPE tissues continues to react with DNA. DNA isolation from FFPE tissues is difficult, mainly due to the cross-linking of formalin during fixation, which can cause DNA fragmentation, resulting in poor DNA quality.[12-14] FFPE tissues undergo degradation due to insufficient neutralization of the formalin, resulting in acid depurination of DNA and preventing the amplification of base pairs.[15] The DNA extraction protocol included steps, such as tissue sectioning of the area of interest, deparaffinization, heating, centrifuging and use of a spectrophotometer. These steps were essential to achieve good quality DNA.

In this study, the DNA was isolated from a series of 40 OSCC FFPE samples archived for 11 years (2006–2017). DNA was extracted using commercial DNA kit TaKaRa DEXPAT Easy and analyzed in terms of purity and yield, using a NanoDrop spectrophotometer with an aim to determine whether the storage period of the paraffin blocks had a significant effect on the isolated DNA. For the statistical analysis purpose, the standard deviations and *P* values were generated using ANOVA. The result of this statistical analysis resulted in a *P*value, which indicated that no significant difference exists in this comparison.

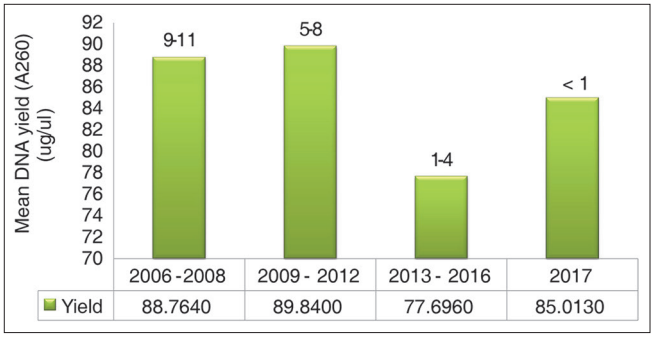

Figure 1: Represents mean DNA yield between groups

Table 2: Represents the characteristics of DNA yield (A260), and purity (A260/A280), the storage period of the tumor block, and *P* value for the study cases (*n*=40 cases)

Group number	Storage year	DNA yield (mean, ng/μL)	Yield (*P*)	DNA purity (A260/A280) ratio	Purity (*P*)
1	2006–2008	88.76±88.45	0.996	0.56±0.27	0.997
2	2009–2012	89.84±147.80		0.54±0.07	
3	2013–2016	77.69±93.48		0.56±0.24	
4	2017	85.013±161.23		0.56±0.08	

Degradation of DNA is more during the embedding of paraffin at high temperatures than during the storage of paraffin blocks.[16] Our results have shown that in terms of yield and purity, the DNA extracted from FFPE blocks stored for up to 11 years is comparable to the DNA extracted from much more recent FFPE tissues stored for 2 or 3 years.

Several studies were conducted to investigate the effect of storage period on the DNA. The results of our study were in accordance with a recent study in which similar DNA concentrations and purity values were obtained from FFPE tissues (corresponding to FFPE blocks stored for up to 8 years) stored for 2–3 years, 4–6 years and 7–8 years, respectively, with no statistically significant differences for both parameters among the study groups ($P = 0.196$ and $P = 0.663$, respectively).[2]

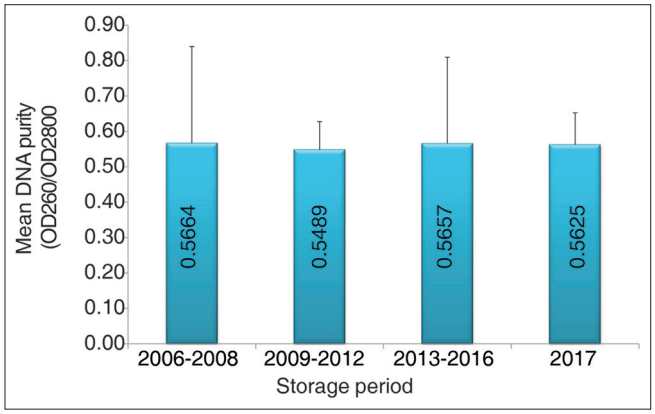

Figure 2: Represents mean DNA purity between groups

Our study was also compatible with a study performed by Kokkat *et al*., in which there were no differences in terms of quality and quantity for all FFPE tissues that they examined (corresponding to FFPE blocks stored for up to 12 years).[8] DNA extracted from FFPE blocks stored for 11–12 years, 5–7 years and 1–2 years, respectively, revealed no differences compared to DNA extracted from FFPE blocks stored for less than a year.

Other groups have reported an increase in DNA yield from new FFPE tissues and decrease DNA yield in older tissues. Moreover, the study of Libório *et al*. on FFPE tissues archived for 40 years has reported that despite degradation, it was possible to extract genomic DNA from paraffin-embedded tissues, and high DNA yield was observed over the last decade.[16]

In our study, the quality of DNA was comparatively less may be due to the incorporation of contaminants while tissue sectioning or while performing DNA extraction. All the samples included in our study group were quantified, and their quality was checked using a NanoDrop spectrophotometer. It was further validated using agarose gel electrophoresis to check for DNA fragments. The agarose gel electrophoresis of 40 DNA extracts from FFPE tissue blocks ranging in age from 1 to 11 years (2006–2017) showed that the DNA was more severely degraded over the years of storage. Agarose electrophoresis of DNA fragments showed that most samples studied presented low-molecular-weight DNA besides good genomic DNA yield.

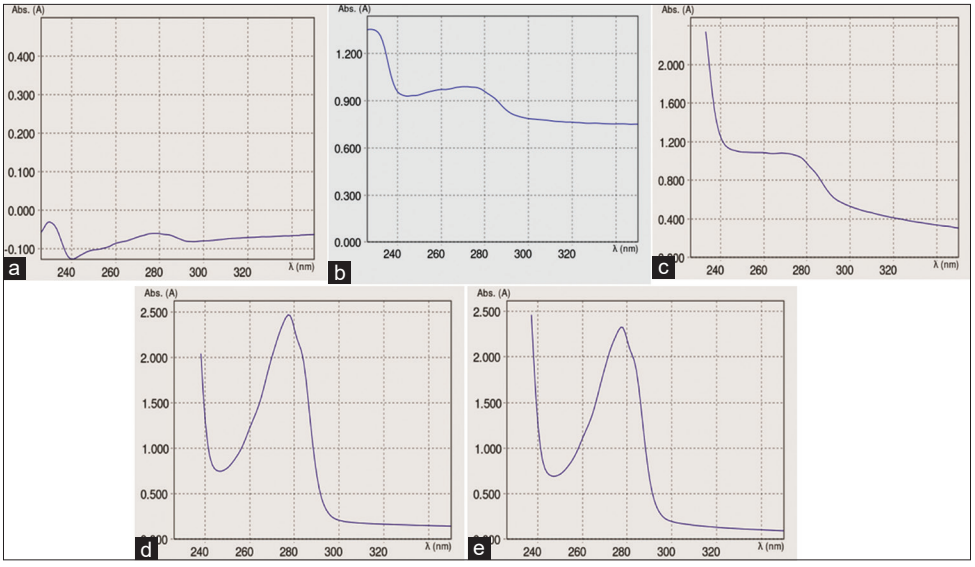

Figure 3: Different aspects of the spectrophotometric curve: (a) a negative extraction control, with very low DNA yield and purity; (b) lower DNA yield (117 ng/μL) but good purity (1.08); (c) one oral squamous cell carcinoma case with very good DNA yield (337.9 ng/μL) and purity (1.21); (d) one oral squamous cell carcinoma case with a very good DNA yield (533.5 ng/μL) but lower purity (0.5); (e) one oral squamous cell carcinoma case with a very good DNA yield (490.9ng/μL) but lower purity (0.48)

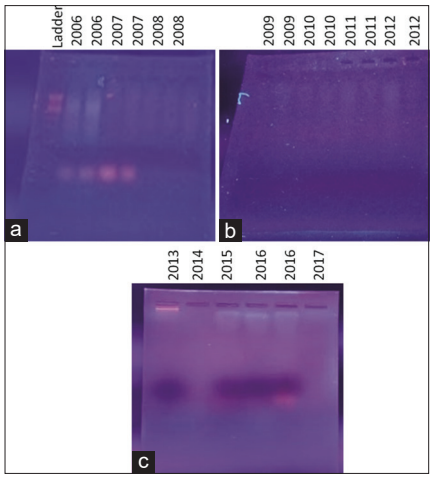

Figure 4: DNA bands visualized under ultraviolet light after agarose gel electrophoresis

The minimum requirement of retaining FFPE tissue blocks according to the College of American Pathologists (CAP) is 10 years.[17] The blocks which are discarded by Pathology laboratories after retaining it for 10 years according to CAP is collected by the surveillance, epidemiology and end results and residual tissue repository program since 2003.[18] Hence, this study was conducted with an idea to utilize these archived FFPE blocks for research, which are usually discarded by pathology centers after retaining for 10 years.

DNA extraction can be done on any other FFPE blocks of different tumors, the reason for the selection of OSCC tissues in this study is due to its availability and relation to Oral Pathology Department which will help us in future to reuse and utilize these FFPE archival OSCC tissues to conduct advanced molecular studies on oral cancer. More recent FFPE tissues (2018–2019) were not selected for the study, as per the laboratory regulatory policy administered by the Clinical Laboratory Improvement Amendments and the need to retain pathology specimen blocks for at least 2 years from the date of diagnosis.[19] The retained FFPE blocks may be required later for legal cases, future diagnostic studies and patient's treatment management.[20]

The study has few potential limitations. Many of the archival tissue blocks are sometimes in a nonretrieval state for any analysis due to improper storage conditions. Therefore a careful selection of the reusable archival FFPE blocks is required before DNA extraction to avoid contamination of samples resulting in poor quality as FFPE blocks are more prone to degradation due to formalin cross-linking and prolonged storage. Incisional biopsies, especially for OSCCs cannot be considered because of the limited size of the tissues, which will hinder the DNA quantity. More studies should be conducted in future on a larger cohort of FFPE tissues stored for longer periods to validate the results and increase the reliability of findings.

CONCLUSIONS

The successful isolation of the genomic DNA from the archival FFPE blocks provided a significant yield and purity for the samples collected for 11 years. The storage period of more than 10 years did not have any significant impact on the extracted DNA yield and purity. This study will serve as a baseline for future research suggesting the importance of archived FFPE blocks for research purposes owing to the limited availability of tissue for biomarker studies.

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Conflicts of interest

There are no conflicts of interest.

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