Original Article

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Pathology Section

Detection of Human Papillomavirus among Cervical Cancer Patients by Qualitative Polymerase Chain Reaction on Formalin-fixed, Paraffin-embedded Tissue: A Retrospective Observational Analysis

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ABSTRACT

Introduction: Cervical cancer is second most commonly diagnosed and third most common cause for cancer death among women in the developing countries. It is now established that Human Papillomavirus (HPV) infection is responsible for pathogenesis of cervical cancer. HPV Deoxyribonucleic Acid (DNA) detection is generally done on cytology specimens to triage women undergoing cervical cancer screening, but testing of Formalin-fixed, Paraffin-embedded tissue (FFPE) is not yet widely used.

Aim: To study the detection of HPV by Qualitative PCR by extraction of DNA from FFPE.

Materials and Methods: This retrospective observational analysis was carried out at Department of Pathology, Armed Forces Medical College, Pune, Maharashtra, India, managed at the centre from 2008 to 2015. The data analysis was done Aug 2013-Oct 2015 on 35 patients of cervical cancer which were reconfirmed by histopatholgical study of sections. Tissue blocks were obtained from the selected subjects and 3-5 micron sections were taken and prepared for Haematoxylin and Eosin (H&E) staining. Qualitative PCR was run on DNA extracted from FFPE tissue for evaluation of HPV. The amplified DNA varied between 230-270 base pairs (bp) and was analysed for oncogenic HPV type 16, 18, 31, 33, 35, 45, 52b and 58 by gel electrophoresis. Data was tabulated in Microsoft excel and mean, frequency and percentages were calculated. Pearson's Chi-square test was used to calculate the significance.

Results: Out of the total 35 samples analysed (mean age: 51.08±10.6 years),15 cases were large cell non keratinising carcinoma, 12 cases of keratinising squamous cell carcinoma, five cases of carcinoma in-situ and three cases were adenocarcinoma. A total of 13 cases out of 35 showed the bands of HPV genomes, indicating either of the HPV stain.

Conclusion: Although molecular diagnostics on FFPE tissue is need of hour, stringent protocols for timing of fixation, technical expertise for extraction of DNA or Ribonucleic Acid (RNA), careful handling of the sample and quality control is of paramount importance. Fragmentation is a problem in DNA extracted from FFPE tissue, so primers having small base pairs should be used to maximise the yield.

Keywords: Amplification process, Carcinoma, Deoxyribonucleic acid, Pathogenesis

INTRODUCTION

Cervical cancer is second most commonly diagnosed and third most common cause of cancer death among women in the developing countries [1]. As India is the second most populous country in the world, one in every five women is suffering from cervical cancer [1]. About 25% cases of cervical cancer death occur in India [2]. It is now well-established that Human Papilloma Virus (HPV) infection is responsible for pathogenesis of cervical cancer. Dr. Haraldzur Hausen was awarded the Nobel prize in 2008 for his discovery [3]. On the basis of histological type, both squamous cell carcinoma and adenocarcinoma have shown strong association with HPV [4,5]. More than 30 different types of HPV virus are known to infect cervical epithelium and among these, HPV 16 and HPV 18 are predominantly associated with cervical carcinoma, squamous cell carcinoma and adenocarcinoma [6]. Apart from HPV infections, factors like inefficiency of immune response, presence of co-carcinogen, multiple sexual partners, a polygamous male partner, young age at first intercourse, high parity, certain HLA subtypes, use of drugs like, oral contraceptives and smoking are responsible for the pathogenesis of cervical cancer [7].

There is increasing interest in the use of nucleic acid amplification testing for HPV identification and typing on FFPE tissues. The detection of high-risk HPV DNA by nucleic acid testing is routinely applied to cytology specimens to triage women undergoing cervical cancer screening but testing FFPE tissues is not yet widely used [8]. This is because nucleic acids from FFPE tissues are much worse templates than those recovered from fresh tissues. It is exceptionally important in an analysis, including PCR. Therefore, a study of evaluation of DNA extraction methods and PCR optimisation on FFPE tissues were conducted which has shown that DNA obtained from FFPE tissue is highly fragmented and can be used for successful amplification [9]. Since, the vast majority of surgical pathology specimens are FFPE tissues, and there are a variety of circumstances in which PCR based detection of HPV in these specimens can be worthwhile, particularly if fresh or frozen tissue is not available [8].

The current study was designed with the objective to extract HPV DNA from FFPE tissue of cervical carcinoma patients and to study the prevalence of high-risk HPV in them using Qualitative PCR.

MATERIALS AND METHODS

This was a retrospective observational study conducted at Department of Pathology, Armed Forces Medical College, Pune, Maharashtra, India, on 35 cases of cervical carcinoma, managed

at the centre from 2008-2015. The data analysis was done Aug 2013-Oct 2015. The Ethical Committee Approval (Approval no. 09/Path/2013; dated 16 Oct. 2013) was taken from the institute.

Inclusion criteria: Data of cases of cervical carcinoma arising from surface epithelium, transitional zone and endocervical region of all age groups were included in the study.

Exclusion criteria: Cases with all non malignant lesions of cervix, other types of malignancy not arising from surface epithelium, transitional zone and endocervical region i.e., arising from mesenchymal component and cases of secondary malignant lesions of cervix like carcinoma of endometrium affecting cervical canal were excluded from the study.

Data of total 35 cases of cervical carcinoma who reported at the Pathology Department of the tertiary care hospital during the specified duration whose tissue blocks have been retained were evaluated after reconfirmation by histopathological study.

Study Procedure

The relevant demographic and clinical data with respect to all the retrospectively selected cases was accrued from the data register in the Pathology Department and from the ward. All the selected cases were reconfirmed by histopathological study of sections. Various criteria of cervical carcinoma [10] were taken into consideration including full thickness epithelial dysplasia, loss of polarity, breach in the basement membrane, desmoplastic reaction of the adjacent stroma, focal conspicuous maturation of the neoplastic squamous epithelium, blurring of stromal epithelial interface and obvious evidence of sheets of neoplastic cell with particular morphology depending upon the type of carcinoma.

Extraction of DNA: Tissue processing along with H&E staining for histopathology was done as per standard operating procedures. Standard paraffin removal procedure was followed for extraction of DNA from FFPE tissue GeNei™ kit, amplification reagents set for HPV was used for isolating DNA from deparaffinised tissue.

Quality of DNA: The quality of DNA was checked by electrophoresis on 1% agarose gel prepared in 1X Tris-acetate-Ethylenediamine tetraacetic acid (EDTA) (TAE) buffer containing ethidium bromide. The high molecular weight genomic DNA appeared as a single band near the well.

Purity and storage of DNA: Purity of DNA was determined by taking the Optical Density (OD) of the samples at 260/280 nm. The ratio of 260/280 was calculated and DNA samples for which the ratio was 1.7 or above was considered satisfactory. The DNA samples were stored at 4°C for a short time but at -20°C for longer duration.

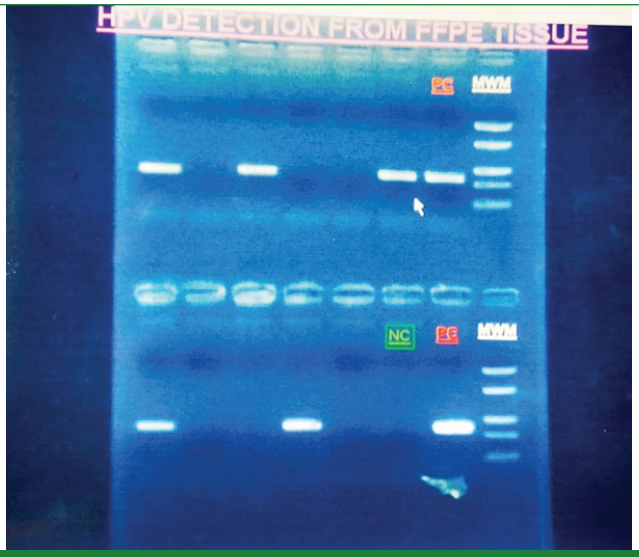
Amplification of DNA: The amplification mix buffered with magnesium chloride ($MgCl_2$), deoxynucleotide triphosphates (dNTPs), and HPV specific primers for type 16, 18, 31, 33, 35, 45, 52b, and 58 were employed in PCR on extracted DNA using the HPV amplification reagent kit from GeNeiTM. The amplified product underwent gel electrophoresis analysis [Table/Fig-1].

STATISTICAL ANALYSIS

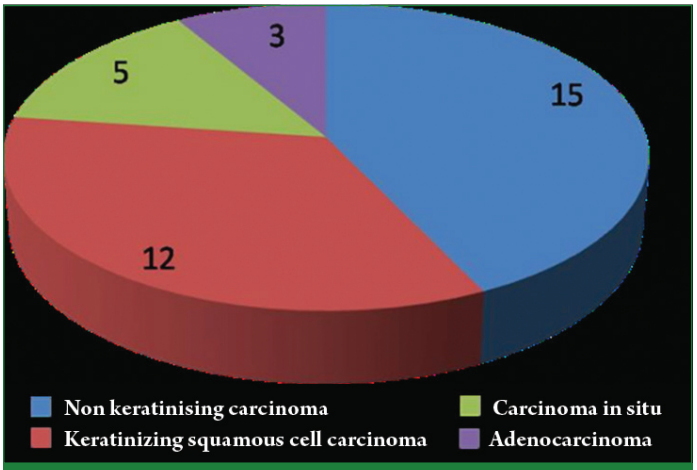
The Statistical software IBM SPSS version 20 was used for statistical analysis. Data was collected in Microsoft excel sheet and frequency (n) and percentages (%) were calculated. The Chi-square test was used for calculating the significance in HPV Present and HPV Absent in all extracted in different types of carcinoma groups.

RESULTS

Total of 35 patients of age range 36-80 years with mean age 51.08±10.6 years were considered in the study. Out of these, 15 cases were large cell non keratinising carcinoma, 12 cases of keratinising squamous cell carcinoma, five cases of carcinoma in-situ and three cases were adenocarcinoma [Table/Fig-2].



[Table/Fig-1]: This image show parallel rows of test samples run along with positive control (PC), negative control (NC) and molecular weight marker/ladder (MWM) for reference. It shows sample 1,3,6 on upper band and sample 1,4 on lower band is positive for HPV.



[Table/Fig-2]: Depicts specific cases of cervical carcinoma included in the study.

While performing the DNA extraction, 25 cases showed single band near the well by electrophoresis on 1% agarose gel. Remaining 10 cases showed smearing of the sample, indicating fragmented nature of the extracted DNA. It appeared that older the tissue more the chances of getting fragmented DNA. However, PCR was run in all cases [Table/Fig-3].

Year of biopsy	Normal DNA	Fragmented DNA	Total
2008	00	01	01
2009	00	00	00
2010	01	02	03
2012	00	03	03
2013	07	02	09
2014	10	02	12
2015	07	00	07
Total	25	10	35

[Table/Fig-3]: Showing the year of biopsy and DNA extraction result.

Evaluation of HPV extracted from FFPE tissue: The amplification product varied between 230-270 bp, which is indicative of an oncogenic HPV type 16, 18, 31, 33, 35, 45, 52b and 58. Out of 35 cases, 13 (37.14%) cases were detected to be positive for HPV. These cases showed the bands indicating either of these (i.e. high risk) HPV infections [Table/Fig-4].

It is seen that all subtype of cervical carcinoma shows presence of HPV. And there is non significant difference between type of carcinoma and HPV detection.

Evaluation of HPV	HPV present	HPV absent	Total	p-value
Large cell non keratinising carcinoma	04	11	15	0.0645
Keratinising squamous cell carcinoma	06	06	12	1.0000
Carcinoma in-situ	02	03	05	0.1768
Adenocarcinoma	01	02	03	0.5951
Total	13	22	35	0.1204

[Table/Fig-4]: Specific cases with HPV evaluation; p-values calculated by Chi-square test.

DISCUSSION

In the present retrospective observational analysis, data and tissue biopsy samples of 35 patients of cervical carcinoma were analysed for association of HPV genomes on cervical carcinoma. HPV genomes were detected in 13 patients 37.14%. HPV belongs to Papillomaviridae family. There are two main genera of HPV i.e.Alpha and Beta papilloma virus [11,12]. Almost 100 different subtypes have been identified based on DNA sequence analysis [13-15]. Beta papillomavirus are typically associated with cutaneous infections [16]. Alpha papillomavirus includes mucosal and genital HPV types. More than 30 different types of HPV virus are known to infect cervical epithelium among these HPV 16 and HPV 18 are predominantly associated with cervical carcinoma, including both squamous and adenocarcinoma [17]. This study also aimed at detecting HPV 16, 18, 31, 33, 35, 45, 52b genomes in all cervical cancer patients.

Several other studies are done for detection of HPV including PCR based studies on fresh frozen tissue, Fluorescence In-situ Hybridisation (FISH), surrogate immunohistochemistry [18,19]. The analysis of archival FFPE tissue samples becomes increasingly important for molecular biologic studies. Optimisation of paraffin removal and DNA extraction procedure from FFPE tissue is paramount. As fixation and paraffin-embedding causes numerous alterations of nucleic acids, DNA must be handled with care. There are more studies comparing the influence of fixative and the time of fixation on the possibility of obtaining DNA suitable for PCR from archival material [20]. Such DNA should not be used as a template for quantitative PCR without reliable control of obtained results [21]. In the present study, protocol provided by the kit with some in-house modification was used, which was standardised as per the requirement.

Extensive and prolong use of xylene at various temperatures was carried out for adequate removal of paraffin wax from tissue. In other studies, they have also used similar protocol for paraffin removal from tissue block [22]. An amount of 30 µL of Proteinase K at high temperature was used for isolation of DNA from de-paraffinised tissue. In one similar study, they have used four different methods for DNA isolation i.e .Qiagen spin column extraction, Phenol/Chloroform Extraction, Protocol by Wickham et al., and Heat treatment protocol which were described in study by Weirich G et al., and they got maximum yield in the heat treatment protocol, which was similar to present study protocol [22].

Although there is increasing need for evaluating range of susceptibility of tumour marker in FFPE tissue, PCR is one of the feasible molecular technique that can be applied on FFPE tissue. However, the efficiency of PCR is influenced by a number of factors including type of fixative as well as time of fixation, the DNA extraction protocol, PCR amplification size, the concentration of the template DNA and optimisation of PCR. Cost-effectiveness and turnaround time are also important factors when used for diagnostic purposes [7,8]. Chloroform extraction among other methods was used in Dehia P et al., study and was found to be very laborious and prone to cross contamination [9]. Whereas, in the present study ,99% ethanol was used for paraffin removal and DNA was isolated from de-parafinised tissue by use of Proteinase K.

In FFPE tissue, the formalin fixation causes cross-linking between nucleic acid strands and DNA adduct with histones. There is also base modification which progress with time [23]. The older the FFPE block, more it is prone to these modifications and liable to poor DNA yield, which is apparent in the present study. The older tissue blocks (i.e., more than three-year-old) yielded fragmented DNA as well as a very low rate of HPV detection. Therefore, it is very important to optimise the method of recovering DNA for better quality and quantity. De-paraffinisation is crucial method for DNA extraction. Xylene although expensive is one of the best wax dissolver. The older tissue gets harden with formation of numerous paraffin crystals which strongly adhere to tissue and prevent easy dissolution. Bielawski K et al., studied three different methods of paraffin removal, depending upon the time and temperature of exposure to xylene and got the similar yield in all three methods. Although one procedure showed highest purity of DNA extracted [24]. Several other studies also tried to extract DNA using different protocols like Alvarez Aldana A et al., (2015) evaluated five different protocol and they found that DNA gained from FFPE tissue was highly fragmented and hence, primers that amplified shorter fragment yield more positive results. They also highlighted that PCR amplification of human genome does not guarantee the successful extraction of viral DNA [25].

One multicentric study done at Italy by Bonin S et al., compared different homemade protocols of different laboratories and grouped them into three major categories. Category 1 and 3 which used purification of extracted material by isopropanol or silica-based adsorption columns, respectively and category 2 included protocols which do not apply any purification method. Surprisingly, they found that DNA yield as well as quality of extracted DNA is highest in category 2 protocols which do not have any purification steps. They also concluded that efficacy of extracting DNA and RNA from FFPE tissue is inversely proportional to the fixation time and, standardised fixation conditions are important. Also, different technical expertise and laboratory instrument variables cannot be entirely excluded from variables [26]. In the present study, fragmented DNA was more in older samples compared to recent samples. Tissue blocks older than three years yielded more fragmented DNA than tissue block of less than three years of age. Present study got six cases of fragmented DNA and one case of normal DNA in tissue blocks older than three years in comparison to four cases of fragmented DNA and 24 cases of normal DNA in tissue blocks less than three years of age. Very few studies detected HPV in DNA extracted from FFPE. In one study done by Bosch FX et al., they were able to detect HPV DNA in 68 cases out of 68 cases. Among these, 47 cases had high risk HPV DNA (HPV 6, 11, 16, 18) [27]. Present study was able to detect (HPV 16, 18,31, 33, 35, 45, 52b) in 13 cases out of 35 cases.

There were various factors for not significant yield of HPV DNA. First, the time of fixation of tissue was not standardised for particular tissue. Second, the fragmented nature of extracted DNA, 10 cases out of 35 cases has fragmented DNA. Present study did not find any correlation of histological type of cervical cancer and detection of HPV DNA, which was in concurrence with others studies showing that all types of cervical cancer are attributed to HPV infection.

Limitation(s)

Firstly, due to non standardised protocol for fixation of cervical biopsies, and secondly fragmented nature of extracted DNA , the prevalence of HPV DNA seemed to be underestimated.

CONCLUSION(S)

The DNA extraction from FFPE tissue requires stringent protocols, technical expertise, careful handling and quality control, since fragmentation is a problem with DNA extraction. The technique of HPV detection on DNA extracted from FFPE tissue is relatively

rare, but it has promising future because of its relative abundance and ease of making FFPE tissue, and its durability as compared to frozen tissue. Further studies on tissue blocks as well as other viral or oncogenic DNA are required for optimising the results.

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