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Usefulness and efficiency of formalin-fixed paraffin-embedded specimens from laryngeal squamous cell carcinoma in HPV detection by IHC and PCR/DEIA

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Abstract: The use of formalin-fixed paraffin-embedded (FFPE) tissues for HPV DNA detection by PCR from biopsy materials is not entirely clear in retrospective studies. The aim of our study was to evaluate the usefulness and efficiency of FFPE tissues from laryngeal cancer (LSCC) in HPV detection by immunohistochemistry reaction (IHC) and PCR-DNA enzyme immunoassay method (PCR/DEIA) and to compare with HPV detection from DFT. HPV-DNA was amplified from 54 FFPE tissues from LSCC specimens by the short PCR fragment (SPF10) primer set using PCR/DNA method and monoclonal anti Human Papillomavirus antibodies in IHC. In the same patients 54 specimens were collected and immediately deep-frozen and stored at (-70°C) to (-80°C). All the FFPE and deep-frozen tissue (DFT) specimens were positive for β-globin amplification. HPV was detected by two methods (SPF10 PCR/DEIA and IHC) in 14 (25.92%) out of 54 specimens from FFPE. Significant differences were found between the HPV detection using PCR/DEIA method and IHC method in FFPE tissues. The comparative analysis of the 54 samples after assuming PCR method in FFPE tissues showed accuracy of 92.6%, sensitivity of 90.5% and specificity of 93.9%. The FFPE tissues method has high sensitivity, specificity and accuracy when used to detect HPV DNA by PCR reaction and it is comparable to DFT results. DNA quality of FFPE samples is adequate and it can be used in HPV-DNA detection and in retrospective studies on LSCC.

Key words: FFPE, HPV, laryngeal cancer, SPF10, PCR/DEIA, IHC

Introduction

Papillomaviruses are members of the papovaviridae family. They are non-enveloped icosahedral DNA viruses, which infect humans as well as a variety of animals. The papillomavirus virion capsid consists of two proteins, L1 and L2. L1 is the major capsid protein and when expressed in eukaryotic expression systems it is able to self-assemble into virus-like particles (VLPs) [1,2]. Expression of L1 protein results in the self-assembly of virus-like particles, which have the size, shape and conformational epitopes of virion capsids. L1 is able to self-assemble into VLPs, morphologically and immunologically very similar to virions that are both necessary and sufficient for binding to the cell surface [1,3]. One hundred and eighteen papillomavirus (PV) types have been completely described and approximately 120 additional isolates represent only partially characterized putative novel genotypes [4]. General or consensus PCR primers have been developed to detect a broad spectrum of HPV genotypes in a single PCR [5-7].

Extraordinary progress in molecular pathology has been made during the last 10 years, and molecular pathology techniques are moving rapidly from the research bench to routine utilization in diagnostic pathology. Several recent reports show that deep-frozen tissue (DFT) is a highly sensitive and specific material which can be used from clinical specimens to PCR detection of the HPV DNA. The majority of studies to date have used high quality DNA from frozen samples however these studies have been restricted due to the small number of samples in their collections. On the other hand, there is a huge resource of FFPE tissues specimens held in histopathology departments around the world. These samples provide an invaluable resource for studying the molecular basis of disease, making it possible to perform large retrospec-

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tive studies correlating molecular features with therapeutic response and clinical outcome.

The need for an effective archived material for the diagnosis of HPV infection in biopsy materials, for retrospective studies or to support histological findings, prompted us to evaluate the use of the 10% formaldehyde-fixed paraffin-embedded tissues.

The aim of our study was to evaluate the usefulness and efficiency of formalin-fixed paraffin-embedded (FFPE) tissues from LSCC in HPV detection by immunohistochemistry reaction (IHC) and PCR-DNA enzyme immunoassay method (PCR/DEIA) and to compare it with HPV detection from deep frozen tissues.

Material and methods

Tissue specimens were obtained from 54 consecutive patients with primary LSCC operated upon by the first author in the Department of Otolaryngology Head and Neck Surgery, Medical University of Lublin in the years 1999-2002. The group consisted of 45 males and 9 females, aged between 32 and 78 years (mean 57.7 years). Patients were treated surgically with or without postoperative radiotherapy depending on the clinical stage of the disease. The patients had neither previous radiotherapy nor chemotherapy.

In order to evaluate the study two specimens from the tumor were collected. For paraffin-embedded technique specimens were collected during a primary diagnostic microlaryngoscopy as a biopsy and they were fixed in 10% buffered paraformaldehyde and paraffin-embedded. Then they were referred to histopathological and virological lab for HPV DNA detection. The second specimens were collected from the excised tumors after total laryngectomy. The samples were immediately deep-frozen and stored at (-70°C) to (-80°C) for future analysis.

Immunohistochemical staining for HPV detection. Paraffin-embedded tissue samples were sectioned serially at 4 µm thickness. After de-paraffinization, sections were incubated with HPV monoclonal mouse antibodies and DAKO® Target Retrieval Solution for 40 min. After washing with TBS samples were incubated with mouse monoclonal anti Human Papilloma virus antibodies Clone K1H8, DAKO (Produktionsvej 42, Glostrup, Denmark) (type: 6, 11, 16, 18, 31, 33, 42, 51, 52, 56, and 58) in dilution 1:50 with DAKO antibody Diluents for 60 min at room temperature. Samples were then incubated for 30 min with DAKO LASB® system at room temperature. Visualization of the reaction was acquired with DAB solution. After that, staining with Mayer's hematoxylin was performed. DAKO positive controls were used as a positive control. In negative controls original antibodies (DAKO) were not added. The detection of antibodies against in the nuclei of proliferating squamous cell carcinoma samples was regarded as a positive reaction.

HPV detection using SPF10 PCR/DEIA method.

DNA Extraction from paraffin sections. Five, 10 µm sections of formalin-fixed, paraffin-embedded tissue were transferred to Eppendorf vials after cutting deep into the block. The microtome blade was changed after each case. DNA extraction from tissue specimens was done using standardization Genomic Mini test for DNA extraction kit (Genomic DNA Prep Plus A&A Biotechnology Gdynia-Poland). After deparaffinization a tissue sample was transferred to consecutive Eppendorf vials and incubated at 50°C with 100 µl Tris buffer, 50 µl universal lyses solution LT and 20 µl proteinase K and it was rotated for complete digest of the tissue sample. After incubation 150 µl LT buffer were added and it was centrifuged for 3 minutes at 10000-15000 rpm and washed twice with 98% ethanol. The precipitated DNA was centrifuged for 1 minute at 10000-15000 rpm and again washed with 70% ethanol. The DNA was precipitated and dried. Dried DNA extracts were incubated for 5 minutes at room temperature with 200 µl Tris buffer (10 mM TRIS.HCI pH 8.5) which had a 75°C temperature and than it was centrifuged for 1 minute at 10000-15000 rpm. The obtained DNA extracts was stored at -25°C for further analysis.

β-globin amplification. To analyze DNA quality PCR amplification of β-globin was performed in a separate reaction using primers PC04 and KM29 with fragment of 205 pb. The sequence of the set primer of PCO4 was d(5'-CAA CTT CAT CCA CGT TCA CC-3') and for KM29 was d(5'-GGT TGG CCA ATC TAC TCC CAG G-3').

HPV DNA detection. HPV DNA was amplified by the short PCR fragment (SPF10) human papillomavirus primer set (Labo Bio-medical Products B.V. – Holland). The SPF10 primers amplify a 65-bp fragment from the L1 region of the HPV genome as described by Kleter *et al.* [6]. The PCR products were analyzed by both 3% agarose gel electrophoresis and HPV DNA enzyme immunoassay (DNA/DEIA). Each run was accompanied by quality control samples. During each PCR run, samples were tested, together with one negative control (distilled H_2O) and one positive control (Caski culture cell line). Amplification products were tested by probe hybridization in a microtiter plate assay to detect the presence of HPV DNA as described earlier by Kleter *et al.* [6]. The assay also included appropriate negative and positive controls.

Control samples. DNA from the Caski cell line was used as a positive PCR control to assess the success of the amplification. PCR reagents lacking DNA (Distilled H_2O) served in each PCR amplification as a negative control. Detection of sufficient quality DNA was performed by β-globin assay.

DNA extraction from frozen tissue. DNA extraction from deep frozen tissue specimens of the tumor and the HPV-DNA detection with nested-PCR method was done using standardized Genomic Mini test for DNA extraction (Genomic DNA Prep Plus A&A Biotechnology Gdynia-Poland) and E6/E7 HPV-16 PCR test (DNA-GDANSK II s.c.-Poland). The amplified products were visualized through 2% agarose gel with ethidium bromide staining. DNA quality control was performed using the β-globin amplification with human β-globin primers KM29 and PC04. Caski culture cell line served as a positive control. The β-globin DNA was amplified in all cases with LSCC.

Control samples. DNA from the Caski cell line was used as a positive PCR control to assess the success of the amplification. PCR reagents lacking DNA (Distilled H_2O) served in each PCR amplification as a negative control.

Ethical issues. The study project was approved by the Local Institutional Review Board .

Statistical analysis. Sensitivity and specificity were assessed by 2×2 contingency tables; Chi-square exact test was performed in order to evaluate the percentage differences between the two assays. Kappa coefficient with 95% confidence limits was also performed to evaluate the correlation between paraffin-embedded specimens and deep-frozen tissue. Statistical significance was defined as $p<0.05$.

Results

The keynote of this study regarding HPV DNA detection was a type of collected specimens for DNA extraction to evaluate the efficiency of paraffin-embedded tissue from LSCC in detection of HPV DNA compared to DFT. Both the histological diagnoses and the evaluation of the

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immunohistochemical reactions were carried out by two experienced pathologists in a blinded fashion. All the FFPE and DFT specimens were positive for β-globin amplification, the internal amplification control, and therefore were considered suitable for HPV analysis by SPF_{10} PCR/DEIA.

Human papillomavirus was detected by two methods (SPF_{10} PCR/DEIA and IHC) in 14 (25.92%) out of 54 specimens from FFPE tissues.

Comparison of HPV detection from FFPE using SPF_{10} PCR/DEIA and IHC methods

Human papillomavirus was detected in 21 (38.8%) out of 54 paraffin-embedded specimens using SPF_{10} PCR/DEIA method and in 14 (25.92%) out of 54 paraffin-embedded specimens using IHC method.

Seven samples from paraffin-embedded tissues were positive in HPV detection using SPF_{10} PCR/DEIA method and negative in HPV detection using IHC method with Mayer's hematoxylin staining but in these patients the deep-frozen specimens were also positive for HPV detection using SPF_{10} PCR/DEIA method. The positive results in HPV detection using SPF_{10} PCR/DEIA method and negative in HPV detection using IHC method resulted from differences in the number of HPV types detected by two methods. The IMH method detect 13 types of HPV and the SPF_{10} PCR/DEIA detect 25 types of HPV.

Characteristically, in all specimens with nuclear negative HPV detection in IHC test HPV was detected in the cytoplasm but it was regarded as negative. Significant differences were found between the HPV detection using PCR/DEIA method and IHC method from paraffin-embedded tissue (χ^2=4.00; p=0.045).

Comparison of HPV detection from FFPE and DFT using SPF_{10} PCR/DEIA method

Using SPF_{10} PCR/DEIA method human papillomavirus was detected in 21 (38.8%) out of 54 paraffin-embedded specimens and in 21 (38.8%) out of 54 deep-frozen tissues. It was the same patients.

Two samples of deep-frozen tissues were positive for HPV detection using SPF10 PCR/DEIA method and negative in paraffin-embedded tissue. This two samples were also negative for HPV using IHC method. No significant correlation was found between HPV detection using PCR/DEIA method from paraffin-embedded tissues and from deep-frozen tissues (χ^2=0.25; p=0.61).

Sensitivity and specificity of HPV detection from FFPE

Overall, in comparison with deep-frozen tissue as the gold standard for PCR reaction, the paraffin-embedded tissue assay showed sensitivity of 90.5% (CI: 72.3-98.3) and specificity of 93.9% (CI: 81.6-98.9). The comparative analysis of the 54 samples after repeat testing of discordants showed accuracy of 92.6%, (κ(Cohen's kappa)=0.84; SE=0.07; 95% CI: 0.71-0.98).

Discussion

The goal is to elevate the quality of FFPE tissue obtained in clinical trials as the basis for discovering and validation of new biomarkers. Central to these efforts is the appreciation that tissue is no longer used only for microscopic interpretation of histopathology, but also for molecular assays of both nucleic acids (DNA), RNA and proteins.

Molecular techniques are rapidly gaining importance as adjuncts to histological tissue assessment. Since disease-related molecules harboring genetic as well as morphological disease characteristics are locked away in the vast collection of formalin-fixed paraffin-embedded tissues stored by the world's pathologists [8,9], it is crucial to evaluate the applicability of new molecular tools for routinely stored human FFPE tissues [8,10]. FFPE tissue samples have been collected throughout decades of routine histopathological examination and are thus the most widely available material in tissue archives around the world [8,9]. Many groups have found improved RNA recovery by disposing of the first sections of the block and using deeper sections for the isolation of RNA. Many laboratories routinely work with 20-year-old material without any problem [11], and it is not unusual to encounter material that is older than 50 years. In fact, nucleic acids have been recovered from pathology museum specimens dating back to the early 20th century (Leeds) and to the 1918 influenza epidemic [12]. The studies were done on much older material delivered from extinct beings including early humanoids. The study material was not necessarily properly stored but found in archaeological excavations (Africa) or simply discovered in hardly accessible regions of Alps or Siberia. Interim approaches based on mtDNA differences between Neandertals and current humans, detection of male contamination through Y chromosomal sequences, and repeated sequencing from the same fossil to detect autosomal contamination allow initial large-scale sequencing of Neandertal genomes. For analyses of other fossil hominins, which may become possible in the future. Green *et al.* [13] suggest a similar 'boot-strap' approach in which interim approaches are applied until sufficient data for more definitive direct assays.

Different materials have been proposed to detect the presence of HPV in tissues. Most of researchers used the deep-frozen tissue as the gold standard of

material to detect HPV DNA using a standardized PCR-based technique. Although frozen tissue is the gold standard for the isolation of biomolecules, the proposal of Medeiros *et al.* [14] for creation of tissue banks with storage at -80°C is impractical for routine clinical care. Storage in mechanical freezers at -80°C is inadequate to maintain RNA integrity for long periods [15]. Storage and retrieval in vapor-phase liquid nitrogen freezers are expensive and technically complicated. These approaches cannot be implemented without significant cost to community hospitals and clinics, where most specimens are obtained.

In this study we compared the usefulness and efficiency of paraffin-embedded specimens from LSCC in detecting the presence of HPV DNA with deep-frozen tissue obtained from the resectable tumor during tumor excision. We demonstrated that FFPE tissue has high sensitivity, specificity and accuracy in comparison with deep-frozen tissue for HPV detection in LSCC. These findings confirm and expand the results of studies of other authors for lung and cervical cancers [16-18]. Farragher *et al.* [16] observed a remarkably high level of present cells in both frozen and FFPE samples. When they examined the overlap between fresh frozen and FFPE tissue expression data, they observed that despite the inevitable loss of information in FFPE tissue, both the specificity and the sensitivity were very high. The sensitivity was 71%, indicating that the transcripts detected in frozen material could be detected in FFPE material and the specificity was 96% with the vast majority of the transcripts detected in FFPE also detected in frozen samples. These results were significantly higher than previously reported [17,18] and provided confidence that reliable high quality data can be derived from FFPE tissue by the Lung Cancer research tool. Bibikova *et al.* [19] found that 90% of the genes detected in fresh frozen samples were detected in FFPE tissue; however, the gene expression proWles from FFPE did not correlate consistently present among technical replicates demonstrated similar and very low levels of noise in the data and equally high levels of correlation on both arrays.

Some authors in search of material to investigation which is simplified and easy in application on delivery applied HPV-DNA assays can use the same specimen as is used for cytological examination, which is an important logistic aspect of routine clinical testing. However, a scrape is only a small sample of the epithelium and sampling errors may influence cytology examination. Only a portion of cervical cell suspension is used for DNA isolation with only a fraction of the isolated DNA being used for specific DNA detection. Therefore, if a specimen contains only a limited number of HPV-DNA copies, sampling errors may produce inconsistencies even in a sensitive assay. Furthermore, the outcome of a HPV-DNA assay can vary depending on the menstrual cycle [20]. This not only has consequences for determining HPV-DNA presence or absence, but also could influence the accuracy of HPV detection, particularly when multiple HPV genotypes are present at different concentrations. Quint *et al.* [21] revealed that analysis of cervical scrapes as well as biopsy specimens from the same patient yielded comparable, but not identical HPV genotyping results. Thus, sampling errors should always be taken into account. Another study has compared biopsy to brush material of a tumor and margin for HPV detection in malignant laryngeal lesions. The results showed a positivity rate of 66.7% from biopsy material compared to 16.7% from brushings of the tumors [22].

The current study revealed that fixed in formalin paraffin-embedded specimens can be collected both for histological examinations and for HPV-DNA detection and these procedures can be performed using the same specimen, which is an important logistic aspect in routine clinical testing. However, the specimens taken from the larynx during the microlaryngoscopy are also small samples of the laryngeal infiltration and sampling errors may influence histological examination. The stability of the sample with 10% buffered paraformaldehyde during transport and storage is also important and give us the possibility of different investigations with nucleic acids for molecular diagnosis even after prolonged storage at ambient temperatures. The study revealed also that DNA quality of 10% buffered paraformaldehyde FFPE samples is adequate and it can be used for HPV-DNA detection and for retrospective researches. A minor limitation is the significant degree of degradation and chemical modification of the nucleic acids recovered from fixed tissues, but the sample collection with 10% buffered paraformaldehyde can reduce this limitation and we can control the quality of DNA using the β-globin test.

To conclude, FFPE tissues have a high sensitivity, specificity and accuracy in detecting HPV DNA infection by PCR reaction and it is comparable to DFT results. DNA quality of 10% buffered paraformaldehyde FFPE samples is adequate and it can be used for HPV-DNA detection and for retrospective researches even after prolonged storage at ambient temperatures.

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