Detection of persistent virus-DNA in Formalin Fixed samples

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The DNA damage and cross-linking induced by formalin fixation can hinder nucleic acid screening. This is of particular concern in the detection of low-abundance targets, such as persistent DNA viruses, present only as a part per million of the total DNA in a sample. We evaluated the analytical sensitivity of the detection of persistent viruses in tissue specimens fixed in formalin for up to 10 days. To this end, we used short amplicon qPCRs and targeted enrichment plus Next-generation sequencing.

virus DNA formalin nucleic acid extraction FFPE qPCR NGS hybridization capture

1. Introduction

The preservation of biological specimens through formalin fixation and paraffin embedding (FFPE) provides an invaluable resource for retrospective molecular and histological investigation. However, although this procedure confers stability to the biomolecules of a sample, it also induces DNA fragmentation, cytosine deamination, and cross-linking [1], all of which can significantly impair nucleic acid-based testing. While cross-links can be partially reversed during extraction [2], the effect on DNA integrity is permanent and directly associated with the duration of fixation, temperature, and tissue type [3].

DNA damage is of particular concern in the detection of low-frequency targets, such as persisting DNA viruses, present normally in scales of parts per million of the total genomic material in a sample [4][5][6]. In virtue of the minute amounts, polymerase chain reaction (PCR)-based methods have been the gold standard for their high sensitivity and specificity; yet, given their constraints to specific targets and intact template areas, these techniques are affected by the chemical and mechanical transformations induced by the fixation [7]. Hence, in recent years, the validation of next-generation sequencing (NGS) to FFPE samples has gained momentum for its versatility and multiplex output [8][9]. However, albeit valuable, most of these validations [8][9] lack paired comparison of fixed and non-fixed tissues from the same individuals.

Most importantly, the analytical sensitivity of detection of low-copy viral nucleic acids in archival and/or highly degraded samples has not been assessed. Indeed, most of the existing reports on FFPE tissue specimens have been PCR-based and focused on oncogenic viruses in tumor samples, in which the viral copies are commonly very high [10][11][12][13][14]. For example, in cervical cancer, human papillomaviruses can have average copies of 10^7/µg in

contrast to 10^2/µg of most persistent viruses in healthy tissues [15]. Yet, given the clinical significance of latent viruses, assessing the sensitivity of detection and identifying optimal methods for detection in FFPE specimens is essential for retrospective analysis of the association to specific disease states.

2. Current Insights

In the present study, we investigated the analytical sensitivity of detection of low-copy viral DNAs in soft tissues, fixed in formalin at a wide range of incubation times. To address this, we systematically compared the prevalences and quantities of persistent DNA viruses in formalin-fixed (±paraffin embedding) vs. frozen lung, liver, and kidney specimens from four deceased individuals.

First, a prerequisite for successful characterization of the low quantities of persistent viral DNAs is efficient isolation of the nucleic acids [16]. We employed two tissue-DNA extraction protocols with similar chemistries, except for the inclusion of de-cross-linking at 90 °C for 1 h in the FFPE protocol. The use of high temperatures during extraction, however, has been suggested to interfere with multiplex qPCR performance [17], and instead, lower de-cross-linking temperatures have been shown to be beneficial in the assessment of somatic variants in cancer patients [18]. The two protocols used in this study have been previously evaluated by Bozic et al. [12] who found comparable detection rates of human papillomavirus in FFPE samples, albeit higher yields of total DNA were obtained with the FFPE kit. This was also the case in our study, where we determined higher ratios of the human single-copy gene RNase P and of viral findings in the samples extracted with the FFPE kit, although the difference to the standard protocol was not significant. The latter was also tested by Lagheden et al. [13], who, using a xylene-free approach (120 °C incubation for 20 min for de-paraffinization) and overnight lysis at 65 °C, reported on optimal DNA yields and sensitivity for HPV testing.

These results highlight the need for in-depth evaluation of different DNA extraction methods as well as de-waxing protocols (mineral oils, microwave irradiation, and alkaline heat retrieval) to maximize the recovery and sensitivity of detection [19][20]. In addition, alternative fixatives (e.g., alcohol-based) should be evaluated in prospective research to control for optimal DNA stability and integrity [20][21].

Using the LabChip analyzer, we evaluated the total-DNA integrity, as the fixation time is a known factor to influence strand damage. We observed an ample degree of fragmentation of the fixed samples that correlated with the duration of formalin incubation, as previously noted by Legrand et al. [22]. The median fragment size in the fixed tissues at day 10 was 200 bp, in contrast to the near intact length distribution of the frozen samples. The degradation was also reflected in the assessment of the RNase P gene, which despite its 84 bp amplicon demonstrated significantly reduced quantities across the incubation days.

We used short amplicon qPCRs (median target size of 90 bp) for the detection of eleven different DNA viruses, including single and double-stranded types. With the viral genoprevalences in the frozen samples as baseline controls, we found the false-negative rates to be directly correlated with longer amplicon sizes (>150 bp) and low copy numbers (<250/million cells). Nevertheless, using short-amplicon qPCRs, most viral DNA findings were

correctly assigned even after 10 days of incubation in formalin. It is also important to note that, some of the false negatives may be due to stochastic variation, given the extremely low copies of our target viruses in the tissues [1].

As a consequence of damage and the fact that qPCR is strictly dependent on intact targets, we also evaluated the detection of viruses using in-solution hybridization coupled with deep sequencing. This method has shown excellent performance in the characterization of highly degraded and fragmented material [23] as well as proven efficacy in the investigation of Merkel cell polyomavirus integration from FFPE samples [24]. Our custom panel and protocol were designed to capture the full-length sequences of 38 virus types [6], including highly divergent reads expected from formalin-induced damage. We found a sensitivity of detection in our virus-enriched libraries of 91.67% compared to 84.62% of qPCR. The higher positivity noted by NGS was prominent in the detection of HHV-7, which had the longest amplicon size (159 bp) and the overall lowest copy numbers in the cohort. In addition, we detected three polyomaviruses not included in our qPCR panel. The remarkable agreement between the two approaches highlights the importance of using short amplicon PCRs (150 bp or less), as well as the need for viral enrichment before sequencing, given the poor analytical sensitivity previously reported by metagenomics [25][26].

Our results emphasize the superiority of fresh samples as starting material, as the median breadth of the assembled genomes was 70% in contrast to 15% in the fixed samples. The suboptimal genome coverage in the FFPE samples may be the result of impaired amplification due to the hindrance of the polymerase binding by cross-linking [27], as well as the read-ahead function of high-fidelity DNA polymerases (such as KAPA used here) that limits the bypassing of abasic or chemically modified sites [1].

These challenges pose obvious limitations to the characterization of full-length viral genomes, particularly in valuable archival materials, in which the validation of variants as opposed to sequence artifacts can be compromised. The latter may be partly compensated by pre-treatment of the FFPE DNA with uracil-DNA glycosylase or the use of repair strategies aiming at sequence authentication.

Our data suggest that low-copy viral DNAs can be satisfactorily investigated from FFPE samples, fixed in formalin for up to 10 days. Clearly, larger cohort sizes and longer follow-ups are granted to validate the search in archival material, particularly when considering that fixation times have not historically been standardized. However, the successful detection of ancient viral nucleic acids of measles and influenza 1918 from FFPE samples [28][29], certainly give exciting grounds for future investigations.

Our data indicates the suitability of viral DNA capture and NGS as a stand-alone procedure for the multiplex screening of FFPE samples, particularly in consideration of scanty histological material and damaged templates that may be missed by qPCR [30].

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