Tissues from routine pathology archives are suitable for microRNA analyses by quantitative PCR

U Siebolts,1,2 H Varnholt,1 U Drebber,1 H-P Dienes,1 C Wickenhauser,1 M Odenthal1

ABSTRACT

Background: MicroRNAs have recently taken centre stage as short non-coding RNAs that regulate mRNA expression.

Aim/Methods: To assess the feasibility of using microRNA techniques on routinely processed tissues, the accessibility of two representative microRNAs was examined by real-time quantitative PCR in 86 human formalin-fixed paraffin-embedded (FFPE) samples from liver, breast, bone marrow, lymphatic tissues and colon. Murine liver was used to analyse the influence of fixation time and different fixatives.

Results: High-quality microRNA was successfully extracted from routinely processed formalin-fixed tissues, resembling PCR amplification results from snap-frozen material analysed in parallel. While fixation time did not affect microRNA accessibility, non-buffered formalin or fixative supplements such as glutaraldehyde influenced PCR results. Storage of human tissues for up to 7 years did not cause a significant deterioration of microRNA. However, microRNA quality in human archival material following routine processing 10–20 years ago was decreased. Oxidation by ambient air during storage and fixation in non-buffered formalin is a possible reason for loss of microRNA quality.

Conclusion: The assessment of microRNAs in readily obtained formalin-fixed paraffin-embedded samples is a highly promising tool in molecular pathology when similarly treated samples are analysed. Therefore, microRNA analyses will gain wider acceptance as an adjunct to morphological tissue assessment in routine pathology and retrospective studies.
Mouse liver tissues

After killing FVB mice, liver samples were punched from one liver segment using a 5 mm dermatological skin punch biopsy instrument (Stiefel Laboratories, Coral Gables, Florida, USA) and either immediately snap-frozen in liquid nitrogen or fixed in 10% neutral buffered formalin for 12, 24 and 72 h and embedded in paraffin. In addition, 10% non-buffered formalin (pH 3) and Schaefer solution25 were used to fix and decalcify the sample for 24 h.

RNA isolation from snap-frozen and FFPE tissues

For total RNA isolation, N 2-frozen tissues (100 mg) were homogenised in 500 µl Trizol reagent using a Precellys 24 tissue homogeniser (Carlsbad, California, USA). Then, total RNA was isolated by Trizol reagent extraction after homogenization, following the instructions of the supplier (Invitrogen, California, USA). The FFPE samples were deparaffinised in xylene by incubation at 65°C for a total of 20 min, substituting xylene twice. After two washes with 100% ethanol, samples were lysed in 200 µl proteinase K buffer (500 µg/ml proteinase K (Invitrogen), 50 mM Tris-HCl pH 7.4, and 5 mM EDTA pH 8) overnight. Total RNA was extracted twice by phenol/chloroform and precipitated with 200 mM sodium acetate and isopropanol.

Reverse transcription and real-time PCR

Extracts of total RNA were resuspended in 20 µl H2O, measured with the ND-1000 NanoDrop spectrophotometer (NanoDrop, Wilmington, Delaware, USA) and then treated with 30 U DNase and 10 U RNase inhibitor, both from Roche Diagnostics (Mannheim, Germany), for 30 min at 37°C in the presence of 1.5 mM MgCl2. A 3 µl volume of the reverse transcription reaction was used in each of the real-time PCR assays by means with the TaqMan MicroRNA assay kit (Applied Biosystems) following the manufacturer’s instructions.

Data normalisation and statistical evaluation

A standard curve of every assay in each run was generated to ascertain the specific amplification efficiency in order to avoid quantification bias. To determine the amount of miR-16 microRNA, a dilution series of total RNA in five steps was performed. Fixation kinetics of mouse livers and experiments with different fixatives were normalised using miR-16 as a reference, and this was followed by calculating the specific calibrated miR-122 microRNA expression of each mouse liver sample. The mean values of normalised miR-122a levels of

![Figure 1](http://jcp.bmj.com/)

**Figure 1** Formalin-fixed paraffin-embedded (FFPE) versus snap-frozen samples of liver tissue. (A) Level of miR-122a microRNA normalised against miR-16 microRNA in snap-frozen (n = 5) and in FFPE mouse liver tissues (n = 5). The mean value of the snap-frozen samples served as calibrator. Error bars indicate SD. (B) miR-122a detection in snap-frozen and FFPE mouse liver tissues, and (C) miR-16 in matched samples of human snap-frozen and FFPE tissues of liver and colon (see also table 1)

### Table 1 Human tissue sample origins and diagnoses

<table>
<thead>
<tr>
<th>miR-16 expression analysis</th>
<th>Description of tissue sample</th>
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<tr>
<td>FFPE versus snap-frozen tissues (see fig 1C)</td>
<td>FFPE human liver tissue (n = 4) Snap-frozen human liver tissue (n = 4) FFPE human colon tissue (n = 3) Snap-frozen human colon tissue (n = 3)</td>
</tr>
<tr>
<td>Length of archival tissue storage (see fig 4)</td>
<td>7 years: human lymph nodes, FFPE (n = 7) 17 years: human lymph nodes, FFPE (n = 7) 27 years: human lymph nodes, FFPE (n = 7) Present day: human lymph nodes, FFPE (n = 11)</td>
</tr>
</tbody>
</table>

DCIS, ductal carcinoma in situ; FFPE, formalin-fixed paraffin-embedded; HBV, hepatitis B virus; HCV, hepatitis C virus; miR-16, miR-16 microRNA.
snap-frozen liver tissues or after 12 h formalin fixation served as calibrators, respectively.

A Student t test was performed for statistical analysis of the data achieved by real time PCR after testing the normal distribution with one-sample Kolmogoroff–Smirnov test. A p value < 0.05 was considered to be statistically significant. Statistical analysis was performed using SPSS 14.0.1 software (SPSS, Chicago, Illinois, USA).

RESULTS
Archival formalin-fixed specimens can be used reliably for microRNA expression studies
In our study, we selected two miRNAs: one that is organ-specific and highly expressed in liver and another one that shows ubiquitous, but just moderate expression. We compared the levels of these representative miRNAs in snap-frozen material with FFPE tissues of mouse and human by real-time PCR. Four snap-frozen murine liver biopsies were compared with four FFPE liver samples from the same FVB mouse, and their miR-122a and miR-16 levels were determined (fig 1).

Similarly, the amount of miR-16 in human matched samples from a total of seven patients was assessed (fig 1C, table 1). miR-122a was chosen as a liver-specific miRNA, while miR-16, known to be ubiquitously expressed, was considered as a representative miRNA of all other organs and tissues. The expression of archival FFPE tissue for both miRNAs closely mimicked that of snap-frozen tissue. Thus, miRNA expression studies can be reliably performed with routinely obtained pathological materials and the results are similar to the yield from snap-frozen tissues.

High quality microRNA can be obtained from FFPE tissues of different origin and pathological diversity
Tissues from different organs in the human body vary to large degrees in their cellularity, infiltration by inflammatory cells, epithelial/mesenchymal ratios, vascularity, fat and extracellular matrix content etc. In order to demonstrate miRNA accessibility in a wide range of tissues, we studied the ubiquitously expressed miRNA-16 in 58 routinely obtained and processed tissues from a variety of organs, consisting of benign and malignant tissues (table 1). PCR analysis revealed some variation in miRNA-16 level; this was expected because of the unique nature of each sample and the wide morphological differences. However, tissues that are traditionally challenging to examine with regards to their nucleic acid contents (ie, bone marrow) showed an acceptable miRNA yield (fig 2). As a consequence, FFPE archival human tissues from many organs and disease processes, including inflammatory and neoplastic, are suitable for miRNA expression profiling.

Effects of different fixatives on microRNA accessibility
Although buffered formalin is currently the most widely used tissue fixative worldwide, some tissues require additional processing steps, such as decalcification of osseous specimens. Since length of tissue fixation in formalin may range from a few hours to multiple days due to departmental work-flow variations, we also compared fixation times of 12, 24 and 72 h in formalin. We showed that fixation in buffered formalin for different time periods does not significantly alter the levels of miRNA expression in the PCR assays (fig 3). Fixation in non-buffered formalin or Schaefer solution resulted in different miRNA yields, but was similar for each fixative and causes only slight but significant variations in relative expression levels compared to buffered formalin fixation (fig 3). Therefore, samples should only be compared with others after treatment with the same fixative.

Effects of length of FFPE tissue storage on microRNA accessibility
Tissue blocks after formalin-fixation and paraffin-embedding are stored in most hospitals worldwide at room temperature with the cut surface of the tissue exposed to ambient air. A loss of miRNA quality during this time is possible. We compared miR-16 accessibility in recently processed human tissues samples with that in tissues that had been routinely processed and stored 7, 17 and 27 years ago (fig 4). We found a decrease of miR-16 accessibility by PCR assays with samples that had been in long-term storage for several decades. However, overall,
miRNA levels were in the satisfactory range for all tissues, even after prolonged tissue storage.

DISCUSSION

Molecular techniques are rapidly gaining importance as adjuncts to histological tissue assessment. Since disease-related molecules harbouring 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, it is crucial to evaluate the applicability of new molecular tools for routinely stored human FFPE tissues.

In the study presented herein, we demonstrate that miRNA accessibility is not affected by prolonged formalin fixation during routinely performed tissue processing, confirming the results of previous studies. Our data reveal that the accessibility of miRNA from FFPE tissue is comparable to snap-frozen material for human and murine samples. These findings confirm and expand the results of studies of other authors, who used fixed cell culture material or mouse tissues. The length of fixation time in formalin varies in most pathology departments due to normal fluctuations of workflow depending on the time of the day or the day of the week that any given specimen reaches the pathology laboratory. Here, we demonstrate that the time of formalin fixation up to 3 days did not significantly alter miRNA detection by real-time PCR, thus allowing miRNA analyses in routinely processed tissues. Fixation in different solutions with or without buffering led to different miRNA yields and slight but significant variations of relative miRNA levels by real-time PCR; these variations should prompt pathologists to compare only those tissues that have been treated with the same fixative. In contrast to total RNA, whose fragmentation has been shown to continue to occur after dehydration and paraffin embedding of the formalin-fixed specimens, miRNA levels of FFPE mice tissues are not affected by a storage time of up to several months. Even routinely processed human FFPE tissues showed only a moderate but not significant loss of miRNA accessibility within 5–7 years. However, stored tissues processed more than 10 or 20 years ago showed nearly a 50% decrease in miRNA accessibility by PCR. Although tissue exposure to ambient air during prolonged storage might be one of the reasons for a loss in miRNA quality, the utilisation of non-buffered formalin at that time might have also contributed to low miRNA yield.

An additional major conclusion of our study is that miRNAs can be assessed reliably by real-time PCR in tissues from various organs and with different diagnoses (normal, neoplastic, inflammatory) regardless of their cellularity, fat content, inflammatory cell infiltrates and degree of stromal fibrosis. Li et al. have suggested in the past that further work may be necessary to determine the precise effects of formalin fixation and paraffin embedding on miRNA expression profiles across different tissue samples. Thus, in the future, miRNA expression profiles of different benign and malignant diseased tissues will be of crucial value to understand disease mechanisms. Reliable miRNA accessibility in tissues of different origin is also of special interest, because a detailed analysis of 345 miRNAs in 40 normal human tissues revealed a number of miRNAs that are specific markers of certain tissue origins. Therefore, miRNA accessibility in a wide spectrum of different FFPE tissues will allow these organ-specific miRNA members to serve as markers of the primary tumour site when metastases of unknown origin are encountered by a pathologist.

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