Unlocking Pathology Archives for MicroRNA-profiling

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Abstract. *Background: MicroRNAs (miRNAs) are ~22 nucleotide long, non-coding RNAs that regulate gene expression by binding to the 3'-untranslated region of target mRNAs and also a variety of cellular processes. It has recently been established that dysregulation of miRNA expression can be detected in the majority of human cancers. A variety of high-throughput screening methods has been developed to identify dysregulated miRNA species in tumours. For retrospective clinical studies formalin-fixed, paraffin-embedded (FFPE) tissue is the most widely used material. Materials and Methods: The miRNA expression profiles of freshly frozen (CRYO) and FFPE tissues of seven tonsil and four liver samples were compared, using a qPCR-based assay, profiling 157 miRNA species. Results: The significance of miRNA-profiles was barely influenced by FFPE treatment in both tissues and the variance induced by FFPE treatment was much smaller than the variance caused by biologically based differential expression. Conclusion: FFPE material is well suited for miRNA profiling.*

Computational predictions have suggested that ~30% of human genes provide targets for microRNAs (miRNAs) in the 3'-untranslated region (3'-UTR) of their respective transcripts (1). In recent years, tremendous progress has been made in elucidating the mechanisms of miRNA-induced post-transcriptional gene silencing (PTGS). Furthermore, it has become evident that miRNAs are involved in biological processes, such as developmental timing (2), left-right asymmetry (3) and programmed cell death (4). Moreover, a large number of miRNAs have been demonstrated to be dysregulated in human tumours (5-7), with putative functions as tumour suppressors and oncogenes (8). Approximately 50% of the currently identified human RNA genes are located at fragile chromosomal sites and in genomic regions involved in cancer (9-11). Thus, miRNAs could be considered as tumour markers and possibly as prognostic markers.

To gain deeper insights into the physiological role of miRNAs in cancer and to establish miRNAs as tumour markers, it is necessary to assess their expression in different cancer types. The success of such a venture depends on methods for high-throughput miRNA expression analysis. The short length of miRNAs and their self-similarity is a challenge for every technical approach to hybridise, amplify or label miRNAs without bias. Ideally, a method for miRNA-profiling should meet the following requirements: high specificity, high sensitivity, detection of mature miRNA only, high-throughput compliance, flexible probe setting, easy procedure and be fast and cheap. To date, many methods have been devised to profile miRNA expression, such as oligonucleotide macroarrays (12), bead-based flow cytometry (7, 13), miRNA microarrays (14-17), RNA-primed, array-based Klenow enzyme (RAKE) assay (18), locked nucleic acid-based miChips (19) and qPCR-based amplification of precursor miRNAs (20) or mature miRNAs (21, 22). While miRNA microarrays have been most broadly applied, the abundance of different techniques may indicate the lack of a current gold standard. There are also disadvantages with the miRNA microarray method, because it measures the miRNA precursor as well as the mature miRNA. Furthermore, the procedure is not suitable for discriminating between miRNA species of high similarity, leading to false-positive results.

The aim of the present study was to compare miRNA-profiles of freshly frozen (CRYO) and formalin-fixed, paraffin-embedded (FFPE) identical tissue samples in order to identify differences induced by the FFPE treatment using a commercially available qPCR-based assay, which met most of the criteria needed for reliable and robust miRNA-profiling as discussed above.

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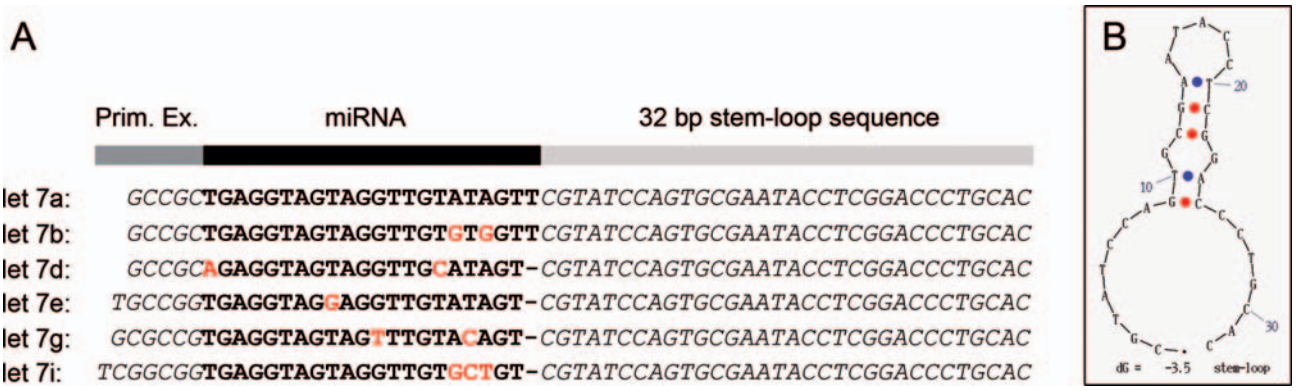

Figure 1. *Accuracy of qPCR-based miRNA amplification. The qPCR products of let-7a, let-7b, let-7d, let-7e, let-7g and let-7i from one of the tonsil miRNA-profiles were cloned and sequenced. A) All let-7 species differ only by a few basepairs from let-7a (red letters). The actual miRNA-sequences were flanked by 5-7 bp upstream and 32 bp downstream, the latter of which comprised the stem-loop (see text). B) Predicted secondary structure of the stem-loop (mfold/http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html).*

Materials and Methods

The expression levels of 157 miRNA species were determined for each sample by qPCR. The CRYO and FFPE material from four liver and seven tonsil samples was analysed, resulting in 22 miRNA-profiles and representing 3,454 individual qPCR reactions. As the template for miRNA-profiling the total RNA was extracted either from four 20 μm sections of FFPE-tissues, using a RecoverAll kit (Ambion, Austin, Texas, USA) or from <50 mg CRYO-conserved tissue using a MirVana kit (Ambion). The human panel of the TaqMan miRNA assay was used for miRNA-profiling (Applied Biosystems, Foster City, California, USA). All the procedures were performed according to the manufacturer's protocol. MiRNA quantification took place in two steps: firstly miRNA-specific reverse transcription (RT), including specially designed stem-loop primers, followed by quantitative PCR using miRNA-specific cDNA as a template. According to the manufacturer and in contrast to hybridisation techniques (such as Northern blots and miRNA arrays), this method can distinguish between miRNA species, which differ in as little as one basepair. In order to test this claim, a complete tonsil miRNA-profile was obtained, including six members of the let-7 family, which marginally differ in sequence. Subsequently, the qPCR reaction products of let-7a, let-7b, let-7d, let-7e, let-7g and let-7i were cloned (TOPO TA Cloning, Invitrogen, Carlsbad, California, USA) and sequenced. Comparisons between samples were made in terms of 'Ct' which was defined as the fractional cycle number at which the fluorescence passed the fixed threshold.

All statistical analyses were based on standard functions available from the R-language (28) (URL http://www.R-project.org). Quantile normalisation was used to normalise the miRNA profiles (modified from Bolstad *et al.* as implemented in the R-package *limma*) (29). Values of Ct=40 (saturation) were deleted before this step and added again afterwards. Differential expression was tested both on a global profile level (30) and for all single miRNA species using Welsh two-sided *t*-tests. Multiplicity was corrected taking a False Discovery Rate (FDR) approach (31).

Results and Discussion

In each case of the let-7 family analysis the expected sequence was found and exactly matched the sequence published in the Sanger database, demonstrating high specificity of the qPCR-based assay (Figure 1A). Furthermore the previously unpublished sequence was identified and the secondary structure of the hairpin included in stem-loop primers was deduced (Figure 1B).

In the individual qPCR reactions Ct values ranging from 25 to 40, reflecting $3.3x10^4$ orders of magnitude were obtained. Analysis of the seven miRNA-profiles from the CRYO samples of tonsils (7x157=1099 qPCR reactions) produced an average Ct of 33.7. Using the identical, but FFPE-treated tonsil tissues, the same experimental set-up resulted in an average Ct of 35.2. The average reduction of the miRNA detection by FFPE compared to CRYO treatment was 1.5 Ct (ΔCt FFPE *vs.* CRYO 1.5). The average Ct for the CRYO-treated liver sample miRNA-profiles was 34.8, while the same, FFPE-treated liver samples yielded an average Ct of 35.8. The ΔCt (FFPE *vs.* CRYO) was 1.0. 12 miRNA species could not be detected in the FFPE samples of the tonsils (Ct=40 in each sample of each tissue), with 10 of the 12 miRNA species also absent in the CRYO samples. Thus, only two out of the 157 miRNA species were detected in the CRYO- but not in the FFPE-treated tonsil tissues (Table I). In comparison, six miRNAs were detectable in the CRYO- but not in the FFPE-treated tissues in the liver samples (*i.e.* 23 CRYO *vs.* 29 FFPE non-detectable miRNAs) (Table I). The higher number of non-detectable miRNA species in liver tissue might be explained by the low overall signal in liver (average Ct liver=34.8 CRYO and 35.8 FFPE *vs.* average Ct tonsil=33.7 and 35.2).

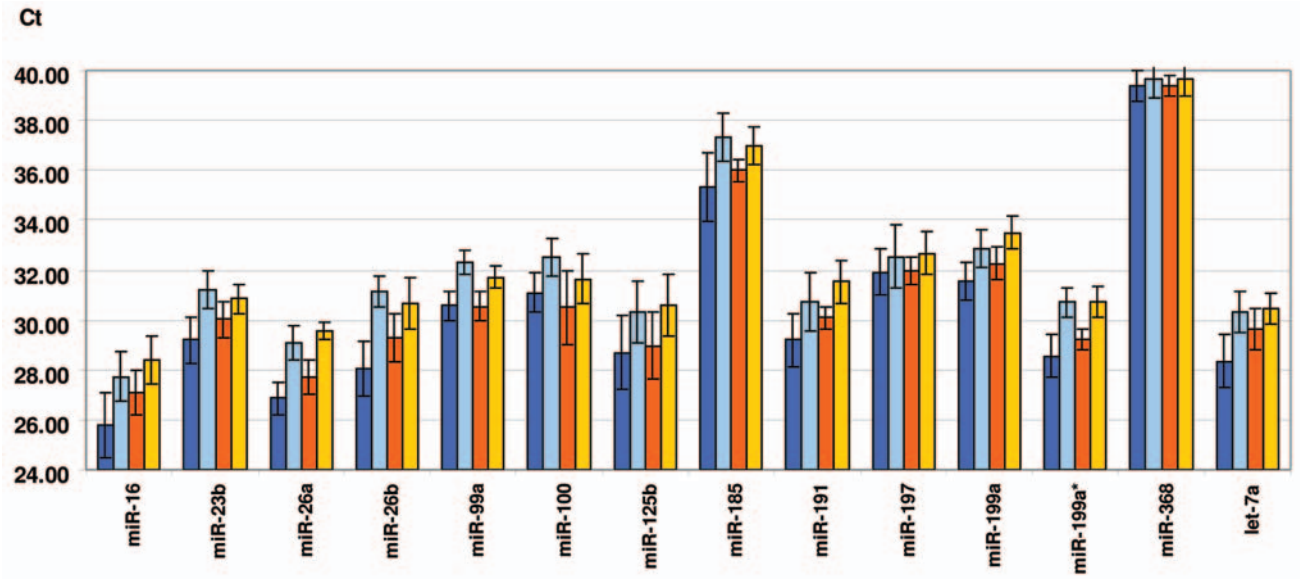

Figure 2. *Comparison of FFPE and CRYO treatment in constantly expressed miRNA species. MiRNA-profiling was carried out on FFPE- and CRYO-conserved tissue of seven tonsil and four liver samples. Selected miRNA species are displayed. A relatively small and constant reduction of miRNA expression was measured after FFPE treatment (detailed in Table I). Dark blue bars: tonsil CRYO; light blue bars: tonsil FFPE; dark orange bars: liver CRYO; light orange bars: liver FFPE. Ct: Fractional cycle number at which fluorescence passed the fixed threshold.*

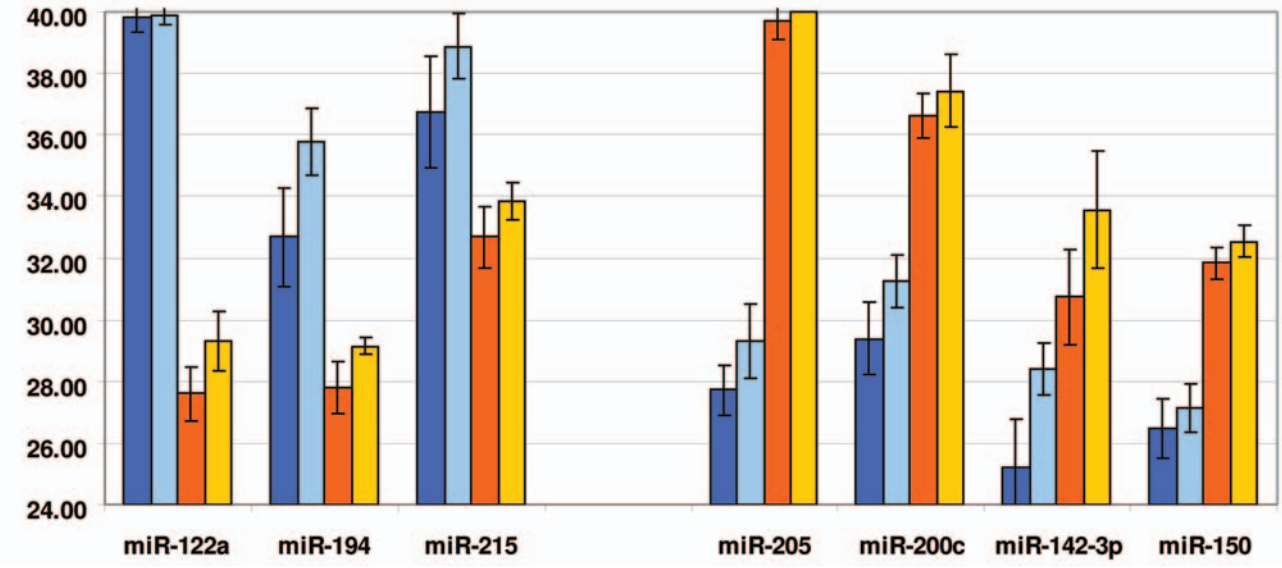

Figure 3. *Comparison of FFPE and CRYO treatment in differentially expressed miRNA species. MiRNA-profiling was carried out on FFPE- and CRYO-conserved tissue of seven tonsil and four liver samples. Selected miRNA species are displayed to demonstrate that differential expression often resulted in marked ΔCt values (for detailed explanations see text). Dark blue bars: tonsil CRYO; light blue bars: tonsil FFPE; dark orange bars: liver CRYO; light orange bars: liver FFPE. Ct: Fractional cycle number at which fluorescence passed the fixed threshold.*

In order to describe the effects of the FFPE treatment for each individual miRNA species the standard deviations were calculated for each tissue, treatment and miRNA species (summarised in Table I). Standard deviation values ranged from 0 $Ct \leq sd \leq 4.4\ Ct$, with an average of $\overline{sd} = 0.9\ Ct$. Importantly, the majority of the miRNA species analysed seemed to be equally affected by the FFPE treatment. A selection of miRNAs, which showed constant expression in all tonsil and liver samples, is depicted in Figure 2. Of note, the profile of miRNA

Table I. *Effects of FFPE and CRYO treatment on miRNA-profiles.*

	Tonsil		Liver	
Treatment	CRYO	FFPE	CRYO	FFPE
miR-species not detected [No (%)]	10 (7.6)	12 (9.6)	23 (16)	29 (19.1)
Average (Ct)	33.7	35.2	34.8	35.8
Average standard deviation (Ct)	1.1	0.8	0.8	0.7

MiRNA-profiling of 157 miRNA species was performed for seven tonsil and four liver tissue samples, each split in half and either formalin-fixed, paraffin-embedded or CRYO-conserved. Ct: fractional cycle number at which the fluorescence passed the fixed threshold.

expression was unchanged, whether it was based on data of the CRYO- or the FFPE-treated samples.

The miRNA expression results from liver and tonsil (CRYO-liver *vs.* CRYO-tonsil and FFPE-liver *vs.* FFPE-tonsil) were compared to determine the differences resulting from genuine differential regulation of miRNAs. The most obvious result was a 3,100-fold overexpression ($2^{\Delta Ct}$) of miR-122a in liver tissue, reflecting a ΔCt of >11.5 (Figure 3). Liver-specific expression of miR-122a has been described previously and is well documented (23-35). Furthermore, miR-194 and miR-215 showed high expression levels in the liver, with miR 194 being 75-fold (ΔCt=6.2) and miR-215 30-fold (ΔCt=4.9) overexpressed. It was recently reported (7) that both miRNAs are strongly expressed in tissues derived from the endoderm (such as the liver). Similarly, some miRNA species have been associated with the development of tissues from the haematopoietic system (such as B-cells of tonsils). As expected, overexpression in tonsils was observed for miR-142-3p (42-fold/ΔCt=5.4) and for miR-150 (34-fold/ ΔCt=5.1) (Figure 3). Thus the key components of the miRNA-regulatory network could be identified regardless of the tissue treatment. The same results would even have been obtained comparing miRNA-profiles of FFPE-treated tonsils and CRYO-conserved liver tissue (and vice versa). The biological variance, as shown by differential miRNA expression, was much higher than the small technical variance introduced by FFPE treatment.

In two lists, ranking the differentially expressed miRNA species (CRYO-liver *vs.* CRYO-tonsil and FFPE-liver *vs.* FFPE-tonsil) (data not shown), the ten most abundant miRNAs were nearly identical (9 out of 10) with ΔCt values ≥4 for all those nine miRNA species (>16-fold change). The variance between both lists increased markedly when the ΔCt values fell below 2 (4-fold change). Since standard deviations ≥ 2 were only measured in 3.5% of the cases, it was concluded that for our experimental set-up ΔCt ≥ 2 strongly indicated differential expression.

A comparison of snap-frozen cells and FFPE cells was recently reported by Li *et al.* (26) who described a generally higher level of miRNA expression in the FFPE treated samples. This was in contrast to our data, but the studies differed since tissue sections and not cells were used for our analysis. Therefore for pathology archives, it is probably appropriate to calculate with a slightly lower miRNA expression in FFPE tissue samples in comparison to fresh frozen tissue.

It has been proposed 'that alterations in miRNA genes play a critical role in the pathophysiology of many, perhaps all, human cancers' (27). What is more, miRNA-profiles 'can be used for the classification, diagnosis, and prognosis of human malignancies' (7, 27). Hence, by showing that FFPE-tissues are well suited for miRNA-profiling, the possibility of using pathology archives has been demonstrated.

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