microRNAs as Biomarkers in Blood and Other Biofluids

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SUMMARY POINTS

microRNAs (miRNA) represent a possible new class of biomarkers for diagnostic applications based upon the following observations:

- The expression levels of miRNAs correlate with disease state (Calin 2002, Lu 2004) and patient prognosis (Takamizawa 2004, Yanaihara 2005),
- miRNAs can be readily recovered from archived FFPE samples and used to identify biomarkers associated with clinical outcome
- miRNAs are present and relatively stable in clinically accessible biofluids such as serum, plasma, urine, and saliva
- Donor-to-donor variation in miRNA expression is extremely low in plasma, suggesting that disease specific miRNAs will be relatively easy to identify
- Highly stable miRNAs have been identified that enable more confident measurements of miRNA expression

INTRODUCTION

Multiple studies have revealed that the expression profiles of miRNAs in cancer samples correlate with prognosis, cancer type, and genetic abnormality (Calin 2002, Calin 2004, Takamizawa 2004, Lu 2005). Because of their small size and inherent stability in clinical samples, miRNAs could be ideal biomarkers for diagnostic assays. These small RNAs can provide patient information that can not be determined by standard pathology, including patient prognosis and response to therapy. Analyses are possible using available surgical samples, FFPE blocks, or readily accessible blood, urine, or saliva samples.

MATERIALS AND METHODS

RNA from frozen tissue samples was isolated using the *mir*Vana™ PARIS™ RNA Isolation kit (Ambion). RNA from FFPE samples was isolated using the RecoverAll™ Isolation kit (Ambion). RNA from biofluids was isolated using a novel glass fiber filter-based protocol for extracting small amounts of RNA from large sample volumes.

The relative abundance of miRNAs in frozen and FFPE tissue samples was measured using *mir*Vana BioArrays. Microarray data was verified by qRT-PCR using TaqMan® assays (ABI) or Asuragen technology as noted. The abundance of miRNAs in biofluid RNA samples was measured by qRT-PCR in the same manner.

Verification of Assay Design

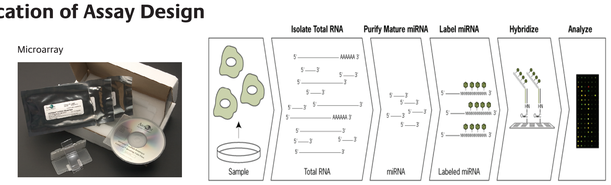


Figure 1. Highly sensitive and quantitative for miRNA Expression Analysis
The *mir*Vana miRNA Bioarray, featuring more than 600 known miRNA's and 150 proprietary human miRNA's, can be used in conjunction with the *mir*Vana miRNA labeling kit (Ambion) to label and quantify miRNAs in RNA samples. To identify miRNAs that are differentially expressed in tumors relative to normal tissues, cancer patient tissue samples were recovered, macro-dissected to separate cancer and normal tissue, and used to recover total RNA. The miRNA in the sample was purified using flashPAGE™ (Ambion) to eliminate precursor miRNAs and other RNAs that might generate false signals on the array. The labeled miRNA samples were hybridized to the array which was then washed and analyzed. Cancer and normal tissue samples were compared and differentially expressed microRNA candidates are identified. The differentially expressed miRNAs were verified by qRT-PCR assays (Asuragen patent pending). As few as 100 copies of a candidate miRNA (per RT reaction) can be accurately quantified using the qRT-PCR method.

RESULTS

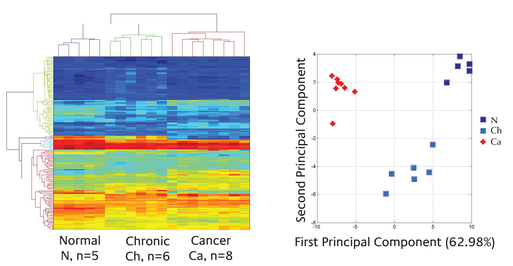


Figure 2: Pancreatic Ductal Adenocarcinoma Study: Array Classification
Total RNA was prepared from frozen pancreatic tumor, chronic pancreatitis, and normal adjacent tissue samples using the *mir*Vana PARIS isolation kit. *mir*Vana Bioarrays were used to profile the miRNA in 10 ug of total RNA that had been processed by flashPAGE. Principal component analysis showed that samples could be sorted by miRNA expression into cancer, chronic pancreatitis, or normal.

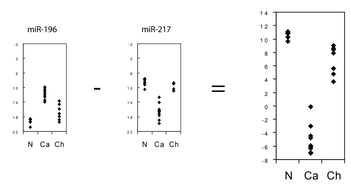
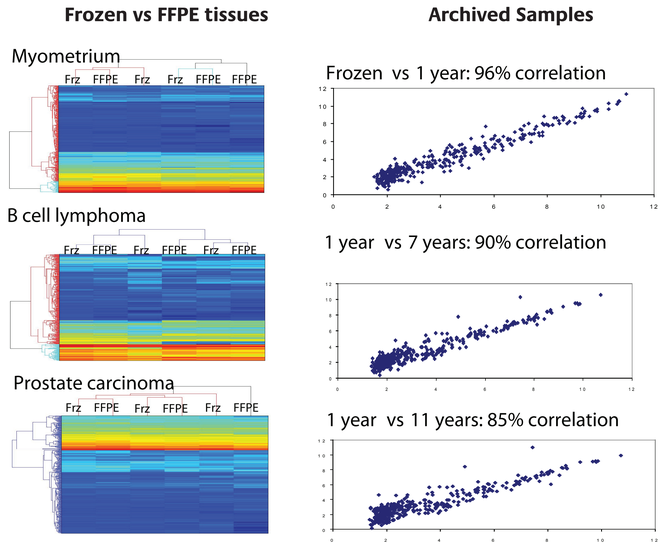
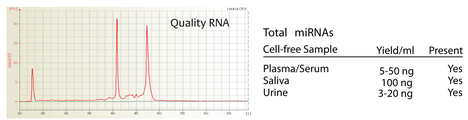


Figure 3: Pancreatic Ductal Adenocarcinoma Study: miRNAs as Potential Diagnostic Analytes
Asuragen's qRT-PCR assays for hsa-miR 194 and hsa-miR 217 were used to compare normal, chronic pancreatitis, and carcinoma samples. These data demonstrate that a combination of two miRNA's could be used to distinguish normal, pancreatitis, and carcinoma samples.



Fixed 4: miRNAs in Fixed Samples
RNA expression for FFPE and frozen samples from three different tissue types as measured to assess miRNA accessibility and stability in archived samples. Tissue samples were stored frozen or as FFPE blocks for 1 to 11 years and processed using *mir*Vana PARIS (frozen samples) or RecoverAll Total Nucleic Acid Isolation Kit (FFPE samples). In each case, the miRNA from 10 ug of total RNA was labeled and analyzed using *mir*Vana Bioarrays. Unlike mRNAs, miRNAs appear to remain relatively intact and compatible with expression analysis even when stored for long periods in FFPE tissue blocks.



Total miRNAs

Cell-free Sample	Yield/ml	Present
Plasma/Serum	5-50 ng	Yes
Saliva	100 ng	Yes
Urine	3-20 ng	Yes

VS.

Figure 5: Characterization of RNA in Biofluid Samples
Expression analysis of hsa-miR 24, hsa-miR 27a, and hsa-miR 98 in different biofluid samples. Total RNA was purified from plasma, serum, urine, and saliva using Asuragen's proprietary isolation procedures. miRNAs were analyzed by qRT-PCR using the Ambion *mir*Vana™ miRNA Detection Kit (urine and Serum) samples, Asuragen's proprietary miRNA detection method (plasma samples), or TaqMan miRNA assays (ABI) (saliva samples). Total input RNAs vary from 250 pg/rxn for plasma to 14 ng/rxn for urine. miRNAs could be readily detected in all biofluid samples tested.

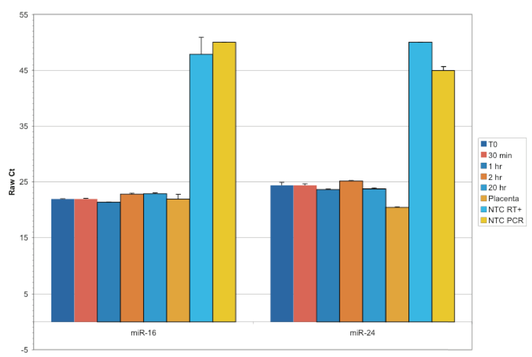


Figure 6: miRNA Stability in Plasma
Plasma from healthy donors was processed immediately or allowed to incubate for extended time periods at room temperature (25°C). miRNAs were analyzed by qRT-PCR using Asuragen's proprietary miRNA detection method. Both hsa-miR 16 or hsa-miR 24 levels remained stable for at least 22 hours.

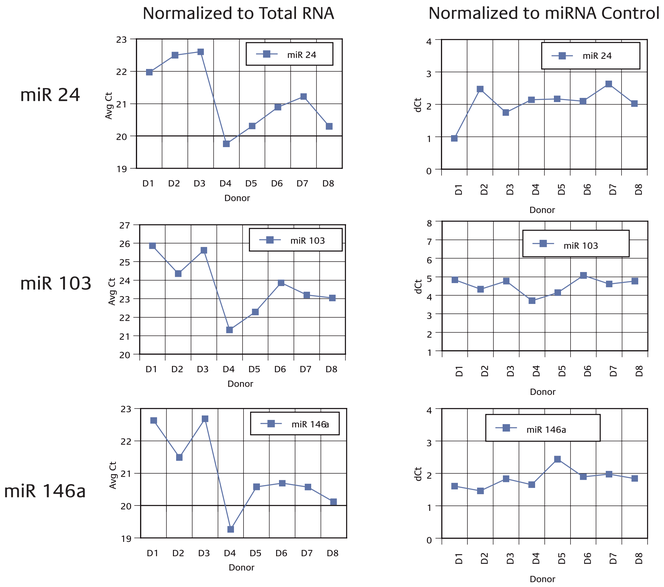
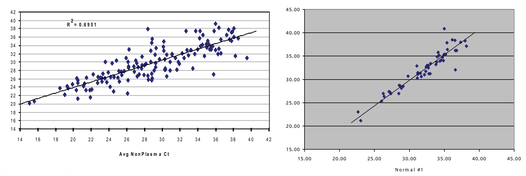


Figure 7: miRNA Normalization
Plasma RNAs isolated from 8 healthy donors were compared in qRT-PCR. A total of 16 miRNAs (250 pg input/rxn) were analyzed using TaqMan miRNA assays (ABI) or Asuragen's proprietary miRNA detection assays. Data was analyzed by geNorm and NormFinder algorithms, which both identified the same miRNA as the most suitable reference RNA for normalization. Representative data for 3 miRNA targets are shown.



	Male #1	Male #2	Female#1	Female#2
Male #1	1	0.983	0.978	0.990
Male #2	0.983	1	0.985	0.978
Female #1	0.978	0.985	1	0.980
Female#2	0.990	0.978	0.980	1

Figure 8: miRNAs in Blood Samples
Whole blood from male and female donors was separated into plasma and cellular fractions. Total RNA was isolated from both fractions and qRT-PCR-based miRNA expression analysis was used to compare the miRNAs in the various samples. The relatively low correlation between the cellular and acellular fractions of blood suggests that the plasma is not simply a repository of miRNAs from blood cells, but that cells from organs in the body might also be contributing to the population of miRNAs in plasma. The levels of miRNAs in plasma from various individuals were surprisingly consistent, suggesting that there is a "normal" amount of cell-free miRNA in blood. This has important implications for diagnostics, since slight variations from normal induced by a disease will be relatively straight forward to identify.

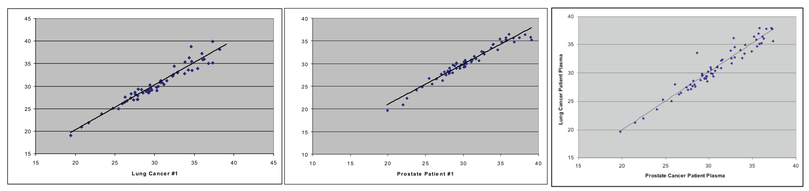


Figure 9: Method for miRNA Expression Analysis
Total RNA was isolated from plasma of prostate and lung cancer patients and miRNA expression profiles were generated by qRT-PCR. Comparing two lung cancer patients or two prostate cancer patients reveals very few significantly differentially expressed miRNAs. While the levels of miRNAs in the plasma of prostate and lung cancer patient samples were also very similar, it is noteworthy that several miRNAs appeared to be present at significantly higher levels in the plasma of prostate cancer patients than in the lung cancer patients.

CONCLUSION

We have used a collection of technologies to isolate and quantify miRNAs from frozen tissues, FFPE samples, serum, plasma, urine, and saliva. The observation that miRNAs are abundant, relatively stable, and easy to recover from patient samples that are readily available in clinical settings suggests that small RNAs might ultimately support diagnostic applications. Combined with the observation that the expression of miRNAs correlates with clinically important factors like prognosis, disease type, and genetic aberration, we believe that miRNAs will ultimately prove to be an ideal analyte for cancer diagnostic assays.

References

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Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H (2004) Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 64, 3753-3756.

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