Microfluidic based multiplex qRT-PCR identifies diagnostic and prognostic microRNA signatures in sera of prostate cancer patients

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ABSTRACT

Recent prostate specific antigen (PSA) based screening trials indicate an urgent need for novel and non-invasive biomarker identification strategies to improve the prediction of prostate cancer behavior. Non-coding microRNAs (miRNAs) in the serum and plasma have been shown to have potential as non-invasive markers for physiological and pathological conditions. To identify serum miRNAs that diagnose and correlate with prognosis of prostate cancer, we developed a multiplex quantitative reverse transcription PCR (qRT-PCR) method involving purification of multiplex PCR products followed by uniplex analysis on a microfluidics chip to evaluate 384 human miRNAs. Using Dgcr8 and Dicer knockout (small RNA - deficient) mouse ES cells (mESC) as the benchmark, we confirmed the validity of our technique, while uncovering a significant lack of accuracy in previously published methods. Profiling 48 sera from healthy men and untreated prostate cancer patients with differing CAPRA (Cancer of the Prostate Risk Assessment) scores, we identified miRNA signatures that allow to diagnose cancer patients and correlate with prognosis. These serum signatures include oncogenic and tumor suppressive miRNAs suggesting functional roles in prostate cancer progression.
INTRODUCTION

One out of six men will be diagnosed with prostate cancer during their lifetime (1). However, using current risk stratification strategies it is still difficult to separate patients with rapidly progressing tumors, which require aggressive treatment, from those with indolent tumors. The result is over treatment, leading to undesirable side affects for a large population of men and unnecessary consequential healthcare costs (2). Therefore, it is essential that new biomarkers are uncovered that better predict outcome of the disease and hence enable better individual treatment decisions.

Cancer biomarkers provide a potentially powerful tool in the diagnosis / prognosis of cancer patients. In prostate cancer, the commonly used biomarker is PSA, a protein antigen specific to prostate tissue, which is measured in the blood and elevated in prostate cancer. Unfortunately, PSA alone is a poor predictor of disease outcome (3). Recently, microRNAs (miRNAs) have been found in serum and have been proposed as potential biomarkers of normal physiology and disease (4). MiRNAs are short (18-24 nucleotides), non-coding RNAs, which regulate gene expression post-transcriptionally by both destabilizing messenger RNAs (mRNA) and inhibiting their translation. Canonical miRNAs derive from longer polymerase II transcripts, called pri-miRNAs. A complex consisting of the proteins DGCR8 and Drosha process the pri-miRNAs to pre-miRNAs, which are then exported to the cytoplasm and cleaved by the protein Dicer to mature miRNAs. Exceptions to this processing include non-canonical miRNAs that bypass DGCR8/Drosha, while still being processed by Dicer. Knockout models of Dgcr8 and Dicer have been developed that remove only canonical miRNAs or both canonical and non-canonical miRNAs respectively (5-7).

For prostate cancer tissue there have been very few studies that compared miRNA expression levels in more than 10 benign and malignant samples (8-11). Noticeably, there is
no overlapping subset between the down- or upregulated miRNAs (12, 13). Mitchel et al. were the first to show a correlation between miRNAs found in plasma and the presence of prostate cancer. They demonstrated that miRNAs originating from human prostate cancer xenografts can enter the circulation, be measured in plasma and robustly distinguish xenografted mice from controls. Furthermore, they found that serum levels of miR-141 distinguished patients with advanced prostate cancer from healthy controls (14). Lodes et al screened 21 sera from 5 different cancer entities (colon, ovarian, breast, lung and prostate). In total 6 prostate cancer patient samples were studied, from which 5 were from patients in advanced prostate cancer stages (3 and 4) and only one sample was from a non-advanced stage. Four of the total 6 prostate cancer patients in this study had received systemic chemotherapy (Taxotere or Lupron with/without the bisphosphonate Zometa). Therefore it remains unclear whether the altered serum miRNA levels are a result of the chemotherapy or reflect the actual advanced cancerous disease (15).

To establish a method for identifying miRNA signatures that could provide diagnostic and prognostic information for prostate cancer patients prior to any treatment, we developed a novel multiplex qRT-PCR method and tested 4 sets of patients (12 patients in each set) with differing risk stratifying CAPRA scores. Due to the inconsistency of previous profiling experiments (12) our multiplex qRT-PCR technique was validated using wild type mESC, Dgcr8 knockout and Dicer knockout mESC as negative controls, showing much improved accuracy relative to currently published protocols. Using this technique on a microfluidic platform we screened patient serum samples for the expression level of 384 miRNAs and uncovered miRNA signatures that diagnosed prostate cancer samples and correlated, but did not exactly match patient CAPRA scores, suggesting that these signatures may have the potential to separate patients within the risk groups and provide additional prognostic information. Many of the miRNAs found to correlate with groupings have been shown to
have oncogenic or tumor suppressive functions in different cancer contexts and, therefore, altered miRNA levels in the serum may reflect functional roles within the tumors.

MATERIALS AND METHODS

ES cell culture

Wild-type, Dgcr8 $^-/-$ and Dicer $^-/-$ mESCs were made and authenticated in the lab by Southern blots, mRNA and microRNA expression analysis as described previously (5, 16).

Patient samples

Human serum samples were obtained with informed consent and studies were performed under the aegis of committee on human research (CHR) approved protocols. Normal serum (500 microliter ($\mu$l)) was obtained from 12 healthy male blood donors. Serum samples (500 $\mu$l) of UCSF prostate cancer patients representing different CAPRA scores were collected from 36 patients before surgery. The CAPRA index is a scoring scheme based on patients age, PSA level, clinical tumor stage, Gleason score and percentage of biopsy cores positive for cancer at diagnosis, which currently provides the best prognostic insight (17). Patients distributed as follows: 12 low-risk (CAPRA-Score 1), 12 intermediate-risk (CAPRA-Score 4) and 12 high-risk (CAPRA-Score $>5$, N+ (regional lymphnodes)) patients. At the time point of blood collection none of the patients had received any therapeutic medication or surgical intervention. Samples were stored at $-80 \, ^\circ \text{C}$ until use.

RNA-Extraction

mESC: total RNA from wild type, Dgcr8 $^-/-$, and Dicer $^-/-$ mESC was extracted using TRIzol® (Invitrogen) and RNA concentration was quantified by the absorbance at 260 nm.

Human serum samples: RNA extraction followed a previously described modified mirVana PARIS® kit protocol (14). After extraction the solution was concentrated (5x) using a centrifugal filter device (Microcon®, Milipore).
miR-specific primer

Design of primers and probes was based on previously published protocol of Tang et al and details are provided in supplementary data (Supplementary Fig.1 online) (18). Libraries for the miRNA-specific reverse stem-loop (RSL-P) and forward primers (FP) as well as for the miRNA specific probes were designed based on the miRNA sequences from the miRBase database (Version 11) (19, 20) and purchased from IDT. The sequences for 677 different primers and probes can be found in supplementary data (Supplementary Table 1 online).

Reverse Transcription

mESC: for each sample, a multiplex RT reaction containing 96 RSL-P was performed. 100 ng total RNA was used for a 5 μl reaction. Final RSL-P concentration: 1 nM. Reaction conditions: 16 °C for 30 min, followed by 60 cycles at 20 °C for 30s, 42 °C for 30s, 50 °C for 1s and finally 85 °C for 5 min to inactivate MMLV-RT.

Human serum samples: for each sample, four independent multiplex RT reactions, each containing 96 unique RSL-P, were performed. Final RSL-P concentration: 2 nM. Reaction conditions were the same as for mESC.

Pre-PCR

mESC: for each sample a multiplex pre-PCR containing 96 FP was performed. All 5 μl RT-Product was used as a template for a 27,5 μl pre-PCR. Final FP concentration: 50 nM. Reaction conditions: 95 °C for 10 min, 55 °C for 2 min, followed by 12 cycles of 95 °C for 1s and 65 °C for 1 min.

Human serum samples: for each sample four independent multiplex pre-PCR reactions, each containing 96 unique FP were performed. Final concentrations and reaction conditions were as for mESC.

Purification of Pre-PCR product
Gel-Purification:

Each pre-PCR product was run through a 10% native polyacrylamide gel. PCR product band was cut out and extracted in 0.3M NaCl. cDNA was precipitated in ethanol, redissolved in nuclease free water and stored at −80 °C until use.

ExoSAP-IT® Purification

5 μl of the pre-PCR product was mixed with 2 μl of ExoSAP-IT® and incubated at 37 °C for 15 min. For inactivation the mix was in a second step incubated at 80 °C for 15 min.

Column Purification:

Quiagen MinElute® spin columns were used to purify fragments ranging from 70 base pairs (bp) to 4 kb after pre-PCR following the manufacturers protocol.

ExoSAP-IT® + Column Purification:

Column purification was performed as described directly after the first step of ExoSAP-IT® purification skipping the inactivation step.

384 well TaqMan qRT-PCR:

1 μl of each purified Pre-PCR product was used in a 10 μl single-plex reaction. All reactions were duplicated. Briefly 2x TaqMan Universal Master Mix (ABI), 1 μM FP, 1 μM Universal Reverse Primer and 0.2 μM TaqMan-Probe was used for each real time PCR. All reactions were run and analyzed on an Applied Biosystems 7900 Real-Time PCR. Reaction conditions: 55 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15s and 55 °C for 1 min.

microRNA Expression Screening using Fluidigm® microfluidic high throughput platform:

Each chip profiled 48 samples in duplicate for the expression level of 96 different microRNAs. 2.25 μl of each purified Pre-PCR product was mixed with loading buffer and 2x TaqMan Universal Master Mix (ABI). For the assays 1 μm FP, 1μm Universal Reverse
Primer and 0.2 μm TaqMan-Probe were combined with loading reagent. Volume per inlet: 5μl. Reaction conditions: 55 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15s and 55 °C for 1 min. The real-time qPCR analysis software provides amplification curves, color-coded heat maps and cycle threshold (Ct). Threshold and linear baseline correction were automatically calculated for the entire chip. Since experimental errors like inaccurate pipetting or contamination can result in amplification curves that look significantly different from a typical amplification curve all amplification plots were checked both computationally and manually.

**Statistical Analysis**

The initial data for statistical analysis were the ∆Ct values for each sample. Seventy out of 384 microRNAs were positive in at least one set of patients and, therefore, went into the final analysis. There were 38 samples analyzed, since some samples had to be excluded due to technical problems and red blood cell lysis. Of these, 9 were healthy controls, 9 had a low-, 11 had an intermediate- and 9 had a high CAPRA score. The remaining data was normalized for every sample and multiplex by subtracting the corresponding median. Complete linkage hierarchical clustering with correlation as the distance metric was performed using Gene Cluster 3.0. To compare expression levels between groups, the t-test was used. To examine trends in expression across more than two groups, linear regression was utilized. Then 12 top candidates from these analyses were validated using single-plex qRT-PCR. To adjust for multiple comparisons, p-values were converted to q-values using the method of Benjamini and Hochberg (21). The cutoff for statistical significance was a q-value less than 0.05. Receiver operating curves were generated using the binormal method and the web based calculator for ROC curves. AUCs were automatically calculated and provided with individual ROCs (22).
RESULTS

Optimization of multiplex qRT-PCR

To identify miRNA signatures that diagnose and correlate with prostate cancer prognosis, we set out to establish a robust profiling method that could be used on patient sera. As the levels of small RNAs in serum are likely to be very low, a multiplex RT-PCR based quantification method was evaluated. RSL-P, FP and TaqMan probes were generated for all annotated human miRNAs (20) (miRBase Version 11). The method involves RT and pre-PCR reactions carried out in a multiplex reaction, dilution of pre-PCR reactions, and finally uniplex qPCR (23) (Fig.1). To test the accuracy of the published method, we performed multiplex qRT-PCR for 57 miRNAs out of a 96-plex reaction that we and others have shown to be expressed in mouse ESC by microarrays and/or deep sequencing (16, 24). Levels in wild-type ESC were compared to levels in Dgcr8 knockout (lacking canonical miRNAs) and Dicer knockout (lacking canonical and noncanonical miRNAs) cells, serving as optimal negative controls. Surprisingly, while many miRNAs showed lower levels in knockout cells as expected, several expressed miRNAs were not detected at all and some showed even increased levels in the knockouts relative to wild-type ESC (Fig.2a). This finding suggested a lack of accuracy in the previously published multiplex qRT-PCR technique.

We hypothesized that this lack of accuracy could be due to carry-over of the many primer sets from the multiplex pre-PCR step to the uniplex qPCR reactions. To test this hypothesis, pre-PCR products were purified away from primers following pre-PCR by size selection on native polyacrylamide gels (Fig.1 and Supplementary Fig.2 online). As expected, bands for the expected product sizes were seen in the wild-type, but not in the Dgcr8 or Dicer knockout ESC, while excessive primers were seen in all backgrounds (Supplementary Fig.2 online). The purified product was then subjected to uniplex qPCR reactions. Resulting quantitation revealed a remarkable improvement in accuracy of the modified protocol over the standard protocol. Of 53 canonical miRNAs, 16 microRNAs were additionally detected, showing
significantly decreased levels in \textit{Dgcr8} knockouts relative to wild-type cells. (Fig.2a). Four miRNAs were not detected independent of the technique used. No canonical miRNAs showed an increase in the \textit{Dgcr8} knockout cells, unlike the standard protocol. All 3 \textit{Dgcr8}-independent, \textit{Dicer}-dependent non-canonical miRNAs (16) (miR-320, -484 and miR-877) showed little change or even increases in the \textit{Dgcr8} knockout cells (Fig.2b). Evaluation of \textit{Dicer} knockout ESCs showed a similar significant decrease of all canonical miRNAs as seen in the \textit{Dgcr8} knockout ESCs (Fig.2c). Unlike the \textit{Dgcr8}-, the \textit{Dicer} knockout ESCs showed greater than 100-fold decrease in the non-canonical miRNAs (Fig.2c). The detection of more miRNAs, the loss of false positive signals and the ability for correct classification of \textit{Dgcr8}-independent, \textit{Dicer}-dependent miRNAs show that adding a purification step in between pre-PCR and qPCR vastly improves robustness of the multiplex qRT-PCR protocol.

\textbf{Alternative purification methods}

To make the purification approach applicable for large sample sets we tested alternative methods of removing carryover of primers. ExoSAP-IT\textsuperscript{®} successfully removed primers, but also led to partial degradation of the PCR product (Supplementary Fig.3a online, lane 2). We hypothesized that degradation occurred during heat inactivation of the exonuclease as the short PCR products would likely begin to partially denature as temperature rose. Therefore, we avoided heat inactivation and directly purified the PCR products from the enzymatic reaction using spin columns (Quiagen MinElute\textsuperscript{®}). Under these conditions, primers were removed without any discernible PCR product degradation (Supplementary Fig.3a online, lane 3). Importantly, qRT-PCR results using the purified product showed similar Ct-values and similar relative expression values of small RNA deficient cells, as well as the ability to correctly classify non-canonical miRNA as seen after gel purification (Supplementary Figs 3b,c online), suggesting equal robustness of this simplified purification approach.
Global profiling of untreated prostate cancer patients with differing prognostic risk

Having established a robust multiplex qRT-PCR method, we initiated profiling experiments on patient sera representing different risks of disease progression. All sera samples (healthy, low-, intermediate- and high-risk patients) were collected prior to any disease related medication or clinical intervention. Patient data is summarized in Table 1.

Isolated serum RNA underwent RT and pre-PCR using conventional PCR machines. After gel purification, quantitative singleplex-PCRs were performed on the Fluidigm® Biomark platform 96x96 arrays. Each of total 4 arrays was loaded with 96 miRNA TaqMan assays (96x4 = 384 assays) and 96 RNA samples (48 patients x 2 = 96 samples) totaling 9,216 reactions per array with each reaction being 6 nanoliters. The ΔCt-values were median normalized before analysis. For each group, 9 samples (11 in intermediate-risk group) went into analysis since some samples had to be excluded due to technical problems and red blood cell lysis. 70 out of 384 miRNAs were present in all sera and therefore comparable between samples. Normalized absolute expression levels of all the expressed miRNAs were used for complete hierarchical clustering using Cluster 3.0. The unsupervised analysis segregated 8 out of 9 healthy patients into one cluster of size 9. There was also a trend toward clustering by CAPRA group (Fig.3a). These findings suggest that miRNA signatures can both diagnose prostate cancer as well as segregate risk for progression.

Trends for individual miRNAs

To identify individual miRNAs from the screen that showed the most significant changes between healthy vs. all malignant and healthy vs. high-risk cancer sera, a pairwise comparisons using the t-test were performed. This analysis identified twelve statistically significant candidates (miR-19a, -20b, -24, -26b, -30c, -93, -106a, -223, -451, -874, -1207-5p and miR-1274a) (expression levels relative to healthy samples shown in Fig.3b). Ten microRNAs (miR-20b, -24, -26b, -30c, -93, -106a, -223, -874, -1207-5p and miR-1274a) were
significantly different between the healthy and all the malignant samples. Four were downregulated in the cancer group (miR-223, -26b, -30c and miR-24) and six were upregulated in the cancer group (miR-20b, -874, -1274a, -1207-5p, -93 and miR-106a). MiR-19a and -451 were significantly different between healthy vs. high-risk. To examine trends across all groups and in cancer patients alone, linear regression was used and identified a significant linear trend for miR-106a and miR-93 across all groups as well as among the cancer patients alone. For miR-24 a significant linear trend among cancer patients was observed, but not across all groups.

All twelve top candidates from the screening were then validated by individual qRT-PCRs and statistically analyzed as the screening results. For miR-19a there was weak correlation between screen and follow-up PCR results. For miR-20b, the expression levels were higher in the cancer group vs the healthy group in the follow-up PCR, but the result was not statistically significant (Supplementary Fig.4 online and Table 2). In ten cases (miR-24, -26b, -30c, -93, -106a, -223, -451, -874, -1207-5p and miR-1274a) Fluidigm® and individual PCR data produced statistically significant changes in the same direction (Supplementary Fig.4 online). Specifically, miR-223, -26b, -30c and miR-24 were downregulated in the cancer group and miR-874, -1274a, -1207-5p, -93 and miR-106a were upregulated.

Among the 10 miRNAs, there were 7 distinct patterns (Figs.3b and 4). Three miRNAs (miR-223, -874, and miR-1207-5p) showed significantly altered expression after the transition from healthy to cancer, but stable expression within the different risk stratified cancer sera. In particular, miR-223 showed a stable decrease, while miR-1207-5p and -874 showed a stable increase in the cancer patients relative to healthy controls (Figs.4a,b). Two miRNAs showed a linear relationship between miRNA levels and cancer risk: miR-24 steadily decreased with risk, while miR-106a steadily increased with risk (Figs.4c,d). Two miRNAs (miR-26b, -30c) were down in the low and intermediate-risk groups relative to both healthy controls and
metastatic disease (Fig.4e). A stepwise increase from healthy to low and intermediate-risk and again to metastatic disease was observed for miR-93 and -1274a (Fig.4f). The final trend was an increase for miR-451 in the patients with the metastatic disease with little to no change in low- and intermediate-risk groups (Fig.4g). P-values were determined and the data was adjusted for multiple comparisons (q-values) (Table 2).

The 10 miRNAs were also evaluated for potential correlations with individual parameters of the CAPRA score among the cancer patient samples including PSA, Gleason, and Age (Supplementary Figs. 5a-c online). While some of the miRNAs showed trends with these parameters, none reached a level of significance.

**Diagnostic utility of miRNA serum profiling**

To further explore the diagnostic ability of the signature receiver operation characteristics (ROC) curves were created for individual miRNAs. Testing the ability to discriminate healthy from cancer serum the binormal method was used to create ROC curves and AUCs were calculated as a measurement of the accuracy. The curves and AUCs (0.778, 0.812, 0.845, 0.876, 0.907, 0.928, 0.928) demonstrated a fine performance of the individual miRNAs to classify healthy and malignant samples (Supplementary Fig.6 online).

**Prognostic utility of miRNA serum profiling**

The uncovered miRNAs showed distinct differential expression levels for the different risk stratified groups (Fig.4). Furthermore the analysis of unsupervised clustering of the individual serum samples also trended to, but not unambiguously differentiated, the individual risk of progression groups (Fig.3, Supplementary Fig.7 online). The strong trends of serum miRNAs to correlate with the different risk groups indicate a promising prognostic potential, however there remained pronounced variability within the different groups (Fig.5). Any marker that could provide additional prognostic information beyond that of the CAPRA score should show
variability within the CAPRA defined groups. It would be most important to be able to identify patients in the low-risk group that are actually at high-risk for progression as well as those in the intermediate-risk group that are actually unlikely to progress. Three miRNAs, miR-93, -106a and miR-24, showed consistently low and high levels among the healthy and metastatic groups, while showing variability within the low and intermediate-risk groups. This in-group variation in the low and intermediate groups may provide additional information to current scoring indexes. However, large-scale longitudinal studies will be required to make a conclusive determination. Such studies would additionally be likely to identify new informative miRNAs.
DISCUSSION

Here we describe an optimized multiplex qRT-PCR approach, which used in combination with a microfluidics platform, enabled us to screen 48 prostate cancer patient sera for alterations in miRNA levels. While previous smaller scale studies suggested the utility of plasma miRNAs in prostate cancer (14, 15), this is the first study to evaluate miRNA levels in an untreated patient set with different risks of progression based on the leading risk indicator currently available. Identifying prostate cancer patients at risk for progression prior to treatment is an essential goal as current measures lead to a over-treatment of some patients and under-treatment of others (2, 3).

MiRNA signatures uncovered in this study not only distinguished sera from healthy and high-risk, but also healthy and curable disease stages (low and medium-risk) sera. Cluster and ROC analyses underscored the diagnostic potential of our serum miRNA signatures. Importantly, the expression levels of the miRNA signatures also trended with CAPRA scores and were able to cluster samples in accordance to their CAPRA score, demonstrating the ability to further separate cancer patients according to their risk of recurrent cancer. Interestingly the in-group variation indicated that serum miRNAs may have the potential to separate patients within the risk categories and may therefore provide additional information. Investigating the potential of serum miRNAs to predict the clinical outcome and cancer behavior of prostate cancer patients in large-scale clinical trials will be required to confirm such a proposition.

Our modified qRT-PCR demonstrates that primer carryover from the multiplex PCR has detrimental effects on miRNA quantification. Purification of the PCR product and therefore removing multiplex primers prior to quantitative PCR led to 1) the detection of more microRNAs (increased sensitivity), 2) loss of false positive signals in Dgcr8−/− and Dicer−/− backgrounds (increased accuracy) and 3) proper categorization of rare Dgcr8-independent
Dicer-dependent small RNAs. Using this technique at nanoliter scale on a microfluidic chip allows for high-throughput multiplex qRT-PCR in a time and cost effective manner. In addition we provide sequences for 677 different miRNA specific primers and probes as well as a detailed description of how primers were designed. Therefore, this approach can be easily adapted by others, allowing for screening of any samples of interest, even when only small quantities of input RNA are available.

The miRNAs found in our study may have functional roles in prostate cancer. Indeed all have been suggested to have either oncogenic or tumor suppressive roles in different settings. For example, miR-24 mediates inhibition of the cell cycle via suppression of cell cycle control genes like E2F2 and Myc (25). MiR-106a, -20b and -93 are part of the miR-17 family, which have been shown to promote tumorigenesis in various models (26-28). Noticeably in accordance to our findings, miR-106a and miR-30c appear to be over expressed in prostate cancer tissue samples compared to healthy prostate tissue (8). Furthermore, miR-93 is upregulated in prostate cancer tissue along with its host gene MCM7 (29, 30). Based on these associations, it will be important to determine the cell source and function of the miRNAs we find elevated and depressed in the sera of prostate cancer patients of different risk for progression.

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The authors declare that they have no competing financial interests.

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REFERENCES

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**Table 1:** Pre- and postinterventional patient data.

LN: Lymphnode
M: Metastases
TABLE 2

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Table 2: Significantly altered individual microRNAs.

The p-values shown above were determined using the t-test for comparison between groups and using linear regression to examine trends. Data was adjusted for multiple comparisons (q-values). * Note that miR-20b is not statistically significant.
**Figure Legends**

**Figure 1.** Diagrammatic step-by-step description of multiplex-qRT-PCR microRNA expression profiling with and without purification after pre-PCR.

Excessive primer from multiplex reverse transcription (step 1) and multiplex preamplification (step 2) are removed before real time detection (step 3), resulting in a purified cDNA template for qRT-PCR.

FP: microRNA specific forward primer, URP: universal reverse primer, R-SLP: microRNA specific reverse stem-loop primer.

**Figure 2: Purification after preamplification greatly improves the accuracy of the standard multiplex qRT-PCR protocol**

a) qRT-PCR data with and without gel based purification. Shown are expression levels of small RNA deficient (*Dgcr8*−/−) relative to wildtype mESCs for 57 miRNAs out of a 96-plex reaction with (black) and without (light grey) an additional purification step after preamplification. Purification results in the detection of more miRNAs as well as in the loss of false positive signals in the *Dgcr8*−/− background.

b) qRT-PCR data for non-canonical (*Dgcr8*-independent) miRNAs with (black) and without (light grey) gel purification. Similar or even increased expression levels in the Dgcr8 knockout background relative to wild type cells show the correct identification of Dgcr8-independent miRNAs. Increased levels are likely secondary to stabilization of non-canonical miRNAs in the absence of canonical miRNAs (16).

c) qRT-PCR data comparing expression levels in *Dgcr8*−/− (black) and *Dicer*−/− (light grey) mESC relative to wildtype after gel purification. Results show a similar decrease for canonical miRNAs in both Dgcr8 and Dicer mESC, the absence of false positive signals and the proper categorization of rare Dgcr8-independent / Dicer-dependent small RNAs.
Figure 3: Linkage hierarchical unsupervised clustering uncovers differentially expressed miRNAs.

Data was median normalized and unsupervised complete hierarchical clustering with correlation as the distance metric was performed using Cluster 3.0 followed by Treeview.

a) Absolute expression levels of miRNAs that were expressed in all samples and therefore were used for sample comparison. The X-axis shows different risk groups individuals and the Y-axis shows miRNAs. 8 out of 9 healthy samples cluster together in one cluster of size 9. There is also a trend toward clustering by Capra group.

b) Expression levels of the 12 most significantly altered miRNAs relative to healthy samples. All candidates were then validated by individual qRT-PCRs on the standard platform (ABI HT-7900) and statistically analyzed as the screening results.

Figure 4: Patterns of significantly altered individual microRNAs.

Candidates were validated by individual qRT-PCR and seven distinct patterns were observed for the significantly altered individual miRNAs. Results are shown relative to expression levels in healthy samples including the standard error. a) Down-regulated in all cancer groups. b) Up-regulated in all cancer groups (Note the discontinuous scale) c) Incremental decrease with increasing risk. d) Incremental increase with increasing risk. e) Down in low and intermediate-risk groups relative to healthy and metastatic. f) Increase in low and intermediate-risk groups and further increase in high-risk group. g) Predominant in metastatic disease. Note that miR-20b is not statistical significant in the follow-up PCR (Table 2).

Figure 5: Inter and intra-group variability

The miRNAs trend to correlate with the different risk groups, however remaining in-group variability could provide additional information to current scoring indexes.
Step 1: multiplex reverse transcription of 96 target microRNAs using 96 different and specific reverse stem loop primers.

Step 2: multiplex pre-PCR using 96 different and specific forward primers and universal reverse primers.

Step 3: singleplex qRT-PCR

A. Without Purification
Preamplification product including carry-over of primer sets and primer-dimers.

B. With Purification
Preamplification product after purifying away excessive primer sets.

12-18 cycles preamplification of 96-plex cDNA library.

Reaction tube content

Schematic of Reaction

B. With Purification
Preamplification product after purifying away excessive primer-sets.

A. Without Purification
Preamplification product including carry-over of primer sets and primer-dimers.

Template for quantitative rt-PCR

Template for quantitative rt-PCR

Template for quantitative rt-PCR
Figure 2
Figure 4
Microfluidic based multiplex qRT-PCR identifies diagnostic and prognostic microRNA signatures in sera of prostate cancer patients

Felix Moltzahn, Adam B Olshen, Lauren Baehner, et al.

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