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MicroRNA profiling in serum from donors with germ cell cancer

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ABSTRACT

MicroRNAs (miRNAs) are short non-coding RNA molecules (~21 bases) that have been identified as important regulators of gene expression at the translational and transcriptional level. They are known to play a crucial role in cell development, differentiation, and disease. Dysregulation of miRNAs has been linked to cancer development and progression. In addition, miRNAs have been identified as cancer classifiers and disease biomarkers. Recent studies have shown that miRNAs are present in body fluids (serum, saliva, semen, and urine) thus providing a non-invasive tool to study and monitor disease states. Earlier research studies identified specific miRNAs as characteristic (serum biomarkers) for germ cell tumors, such as testicular cancer (miR -302 and miR-371/2/3 families (Ref. 2-6)). In this study miRNA Profiling (~760 miRNAs) was performed to identify specific miRNAs as candidate biomarkers in serum samples from germ cell cancer (seminoma and non-seminoma types), and healthy controls. For this research study we used a miRNA specific bead capture system to isolate miRNAs from serum and TaqMan®Array Card platform for profiling. We saw significant differences in miRNA levels for hsa-miR-371, hsa-miR-372, and hsa-miR-302c between tumor and normal sera using this high throughput approach.

MATERIALS AND METHODS

Figure 1. Complete workflow for miRNA profiling

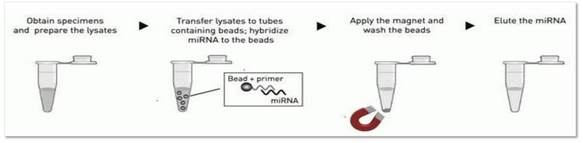

Figure 2. miRNA purification from serum samples using TaqMan® miRNA ABC Purification Kit TaqMan® miRNA ABC Kit Pool A and Pool B contains miRNA specific beads for up to 381 miRNAs per pool (~740 miRNAS total).Only 50ul of serum was needed to isolate miRNAs per pool. 2µl of 1nM spike in control (ath-miR-159a) was added to the lysis reaction to monitor miRNA extraction and reverse transcription reaction.

For Research Use Only. Not for use in diagnostic procedures.

TRADEMARKS/LICENSING

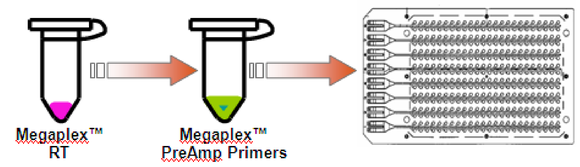

Figure 3. cDNA preparation Following a modified serum protocol (1), 3 µl of purified miRNA was used for each Megaplex™ RT pool A or B reverse transcription reaction followed by pre-amplification with Megaplex™ PreAmp Pools.

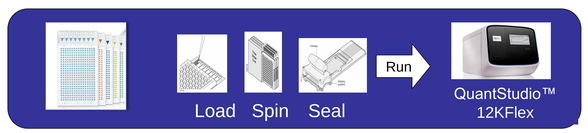

Figure 4. TaqMan® Array Card Workflow Diluted pre-amplification products were loaded on TaqMan Array Card A or B and run on the QuantStudio™ 12KFlex with Universal Master Mix. Primary data analysis was done using ExpressionSuite software.

RESULTS

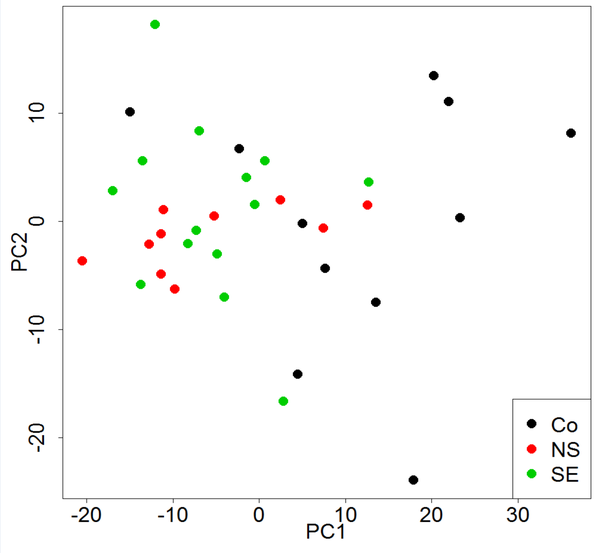

Figure 5. Principle Component Analysis on expression data of all miRNAs There is no global difference in miRNA level profiles between sera from controls and cancer samples or the histological subtypes after batch correction. Co=control sera from healthy individuals, SE=seminoma, NS=non-seminoma. The plotted first 2 principal components (PC) explained 33.76 % of the total variance.

Corrected	Normal	Tumor	Classification Error
Normal	6	5	0.45
Tumor	2	22	0.08

Table 1. Classification A random forest classifier trained on the batch corrected data showed high sensitivity in distinguishing tumor samples from controls. (Out of Bag Error 20%). The specificity was 54% and the sensitivity 92% which is in line with earlier findings in a targeted study (2).

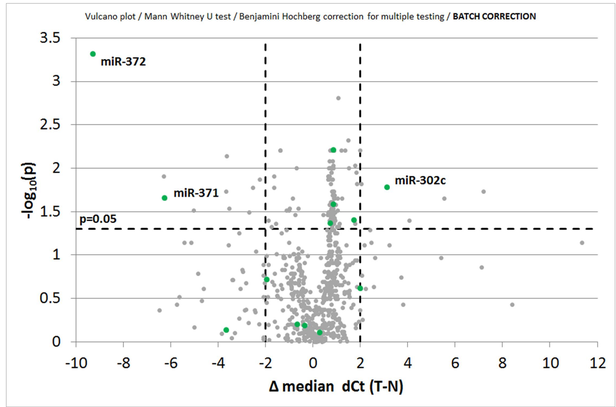

Figure 6. Relevant and significant miRNAs between tumor and normal groups. Difference in median dCt between tumor (SE or NS) and control plotted against the $-\log_{10}$ of the Benjamini-Hochberg adjusted p-value resulting from a Mann-Whitney U test comparing these two groups. Three targets previously identified as germ cell cancer specific were shown to be relevant and significantly differentiating between tumor and control samples (hsa-miR-372 , hsa-miR-302c, hsa-miR-371-3p). An adjusted p<0.05 was considered significant and a difference in median dCt >2 was considered relevant. Batch corrected data was used as input and normalized using global normalization. Grey dots, all miRNAs; green dots are targets of interest (miR-302 a/b/c/d, miR-367, miR-371/2/3).

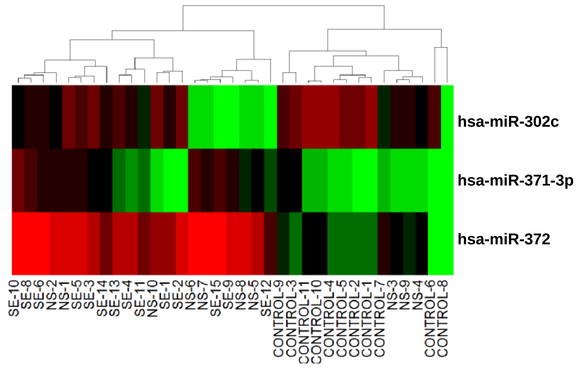

Figure 7. Heat Map Heat map of the differentiating targets identified in Figure 5 and known from previous studies. hsa-miR-372 shows a differentiating pattern of high expression exclusively in tumor samples. Red=high expression. Green=low expression. CONTROL=sera from healthy individuals, SE=seminoma, NS=non-seminoma.

Target (Assay ID)_Pool	dCt median (T-N)	BH adjusted p value
has-miR-372 (000560)	-9.28	0.0005
hsa-miR-511 (001111)	-6.30	0.0123
hsa-miR-371-3p (002124)	-6.25	0.0224
hsa-miR-26b (002444)	-5.04	0.0305
hsa-miR-769-5p (001998)	-3.68	0.0186
hsa-miR-23a (000399)	-3.64	0.0072
hsa-miR-106b (002380)	-3.53	0.0291
hsa-miR-365-(001020)	-2.70	0.0321
hsa-miR-598 (001988)	-2.55	0.0169
hsa-let-7a (000377)	-2.26	0.0135
hsa-miR-875-5p (02203)	2.05	0.0151
hsa-miR-302c (000533)	3.14	0.0169
hsa-miR-1224-3P (002752)	4.06	0.0401
hsa-miR-770-5p (002002)	5.53	0.0224
hsa-miR-520h (001170)	7.21	0.0186

Table 2. miRNA targets. miRNA targets showing relevant and significant differential levels between control and cancer related samples (left and right top areas, p<0.05, dCT >2).

CONCLUSIONS

1. miRNA can be easily and efficiently purified using a miRNA bead specific purification system (Figures 1-4; TaqMan miRNA ABC Kit; Pool A and B, 50ul each) followed by profiling with Megaplex Pools and TaqMan Array cards (~760 miRNAs).

2. miRNA level profiles did not show strong global differences (Figure 5) between cancer and control related serum samples but a random forest classifier was able to distinguish cancer samples with high sensitivity and moderate to low specificity (Table 1).

3. hsa-miR-371-3p, hsa-miR-372 and hsa-miR-302c showed significant and relevant differences between cancer and normal serum samples using a high throughput approach (Figures 6, 7), in agreement with previous reports (Ref 3-6). A number of other differentiating targets was also identified which might be of future interest (Table 2).

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