#1159 Identification Of Potential MiRNA Biomarkers For Breast Cancer Using TaqMan® Pri-miRNA Assays

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ABSTRACT

Identification of cancer biomarkers depends on measuring the differential genotypes and phenotypes between normal and disease tissues. However, cancer is characterized by accumulation of genetic lesions and heterogeneity, and hence great efforts need to be invested to distinguish driver versus passenger abnormalities. Several strategies have been employed to achieve the distinction, including conservation of genetic lesions between mouse models and human diseases, gain of copy number/amplification, deletion/loss of heterogeneity, and epigenetic alterations. Redundancy of some microRNA (miRNA) genes has been an interesting finding and its biological significance is not clear. These redundant miRNA loci have different genomic locations and are transcribed independently, and yet are processed into identical mature miRNA, and these loci are annotated as -1, -2, -3, etc, following the miRNA gene name. Selective expression of these loci could be a regulatory strategy to control expression of other genomically associated genetic elements that are critical to the disease processes, while the required level of mature miRNA can still be maintained by leveraging the redundancy. The proof-of-principle of this rationale was to compare between normal breast and breast cancer cell lines the expression of miRNA genes that have multiple loci and are associated with other genetic elements such as other miRNA genes within the same clusters or their host protein-coding/non-coding genes. This investigation narrowed down to mir-100 gene that is clustered with let-7a-2 gene, both of which lost expression completely in three luminal-type breast cancer cell lines but not in one basal-type cell line and normal breast, whereas expression from let-7a-1 and let-7a-3 remains unchanged between normal breast and cell lines tested. Loss of mir-100 and let-7a-2 expression appears to be caused at least by loss of one copy or both copies of these two loci. Deletion of mir-100 locus can also be detected in clinical specimens. These results suggest that this method might effectively distinguish driver versus passenger differentially expressed genes.

MATERIALS AND METHODS

FirstChoice® total RNAs of normal human tissues and breast cancer cell lines were purchased from Ambion. MCF7 and MDA-MB231 were cultured in 10% FBS-containing DMEM and Leibovitz's L-15 Medium, respectively, under 37ºC and 5% CO2. Total RNA from cell lines was purified using TRI Reagent® (Ambion) followed by genomic DNA (gDNA) clean-up using TURBO DNA-free™ Kit (Ambion). Extraction of gDNA from cell lines used DNeasy Blood & Tissue Kit (QIAGEN). The RT and PCR reactions used the High Capacity RNA-to-cDNA™ kit and the TaqMan® Gene Expression Master Mix (or Universal PCR Master Mix, no No AmpErase® UNG), respectively, quantitated by TaqMan® Pri-miRNA Assays by following manufacturer's protocol using 7900HT (Applied Biosystems). When TaqMan® Pri-miRNA Assays were used to quantitate gDNA, 5 ng/ul of template in a 10 ul reaction was used and started from the PCR part of the original RT-PCR assay protocol. gDNA was fragmented by heating to 95ºC for 15 min; at this temperature most gDNA was fragmented to between 100 and 300bp, and there is no difference in size distribution even though heating prolongs to 30 min, while shorter heating time left detectable unfragmented gDNA. MethylMiner™ Methylated DNA Enrichment Kit (Invitrogen) was used to isolate methylated gDNA fragments, and the sequences encompassing predicted transcription start sites of miRNA genes of interest were amplified by end-point PCR using Phusion® High-Fidelity PCR Master Mix (Finnzymes) with 30 sec at 98ºC, 35 cycles of (10 sec at 98ºC, 10 sec at 59-62ºC, and 15 sec at 72ºC), followed by 5 min at 72ºC. DNA from formalin-fixed paraffin-embedded (FFPE) normal and neoplastic breast samples was extracted by High Pure PCR Template Preparation Kit (Roche Applied Science) from a single 2um section and quantitated by NanoDrop® Instrument. Quantitation of miRNA loci of interest in FFPE samples using TaqMan® Pri-miRNA Assays was performed on LightCycler® 2.0 Instrument (Roche Applied Science) with extended 50 cycles of PCR. Procurement of tissues and experiments using these specimens have been approved by the Ethics and Scientific Committees of the University of Athens.

RESULTS

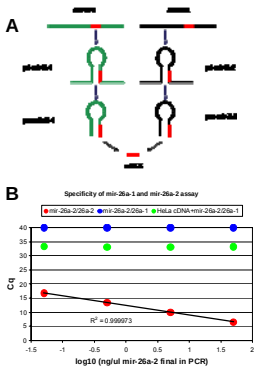

Figure 1. The TaqMan® Pri-miRNA Assays is miRNA locus-specific. The goal of TaqMan® Pri-miRNA Assays is to separately quantitate expression of different miRNA loci, which is particularly valuable for interrogating those being processed into identical mature miRNAs (A). They were designed by our TaqMan® Gene Expression Assay design pipeline that inherits the capability of designing assays based on unique sequences in the genome, and great linearity in assay performance. (B) In a proof-of-principle experiment, a plasmid containing the pri-mir-26a-2 locus was used as a template, quantitated either by assay for pri-mir-26a-2 (red), by assay for pri-mir-26a-1 (blue), or by assay for pri-mir-26a-1 in the presence of HeLa cell cDNA that is known to express low level of pri-mir-26a-1 (green). While pri-mir-26a-2 assays measures the pri-mir-26a-2 template with perfect linearity, no detectable noise was seen when pri-mir-26a-1 assay was used despite increasing amount of template input.

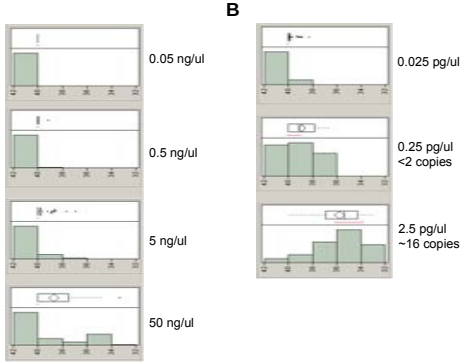

Figure 2. The TaqMan® Pri-miRNA Assays can be used to quantitate primary transcript as well as the DNA copy number of the miRNA gene. (A) When increasing amounts of DNase-treated testis total RNA were evaluated by a panel of 84 pri-miRNA assays using PCR part of the protocol, noticeable background signal started to appear at 50ng/ul, suggesting that 5 ng/ul is the maximal input. The concentrations in each panel indicate the total RNA input in an RT reaction. (B) Detection of increasing background from total RNA might come from non-specific interaction but could also be residual gDNA despite DNase digestion, as a second digestion usually alleviate the background. Using the same panel of 84 pri-miRNA assays to quantitate different amounts of gDNA (with the PCR protocol only) in a 20ul PCR reaction, the data distribution follows the estimated copy number of the spiked DNA, indicating that running the samples with the PCR-only protocol using the pri-miRNA assays to evaluate the gDNA contamination before applying the full RT-PCR protocol is highly recommended. If gDNA does exist, DNase treatment is necessary. X-axis is Cq and Y-axis is the number of assays in the population.

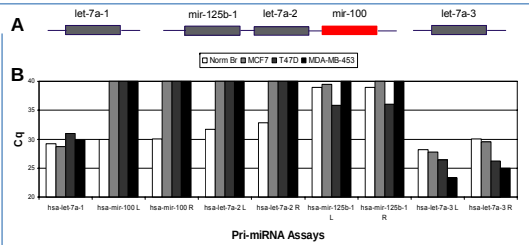

Figure 3. Lack of expression of the miRNA cluster containing let-7a-2, but not the let-7a-1 and let-7a-3 loci, in breast cancer cell lines. (A) The genomic structures of three hsa-let-7a loci. The let-7a-2 locus is in the same cluster with hsa-mir-125b-1 and mir-100 loci. (B) Expression of the five miRNA genes listed in (A) was quantitated using nine assays in normal breast and three breast cancer cell lines. R and L refer to the position of the amplicon targeted by the assay to the right or the left side, respectively, in relation to the miRNA stem loop.

miRNA Gene	Chromosome	Expression of pri-miRNA (Cq)			Relative Copy # to Norm Breast	
		Norm Breast	MCF7	MDA-MB231	MCF7	MDA-MB231
hsa-let-7a-1	9q22.32	28.55	27.28	35.56	1.09	0.87
hsa-mir-1261	11q14.3	31.83	32.91	40	1.51	3.33
hsa-mir-548l	11q21	27.23	27.52	35.93	1.48	3.41
hsa-mir-34b	11q23.1	36.97	38.84	40	0.13	2.33
hsa-mir-34c	11q23.1	37.27	35.66	40	0.36	0.06
hsa-mir-125b-1	11q24.1	26.60	40	31.81	0.80	3.30
hsa-mir-100	11q24.1	29.82	40	31.84	0.60	1.58
hsa-let-7a-2	11q24.1	32.15	40	33.86	0.56	1.70
hsa-let-7a-3	22q13.31	28.09	26.98	32.88	0.46	0.58
hsa-mir-125b-2	21q21.1	27.49	31.02	40	2.04	2.67

Table 1. The let-7a-2 cluster has no detectable expression and has lower gene copy number in MCF7 cells than in MDA-MB231 cells. The let-7a-2 cluster (red, with two other miRNA genes) has intermediate expression in MDA-MB231 cells compared to normal breast, while no expression was detected in MCF7 cells. The other two let-7a loci (let-7a-1 and -3) in MCF7 cells, however, have comparable expression levels as in normal breast. Subsequent interrogation of gDNA from these two cell lines using the same pri-miRNA assays showed that the let-7a-2 cluster has loss of copy in MCF7 cells relative to the normal breast and MDA-MB231, assuming normal breast has two copies of this locus.

ID	Conc (ng/µL)	Subtype	Corrected mir-100 Cq	Copy # Ratio
44371	11.5	Luminal	42.27	0.02
1475782	9.7	Luminal	37.88	0.32
44677	11.9	Luminal A	37.66	0.38
45478	13.1	Luminal A	37.73	0.36
59656	27	Luminal A	34.77	2.79
46306	8.5	Luminal B	32.99	9.58
48790	2.5	HER-2 Enriched	35.21	2.06
53984	24	TNP-Non Basal	41.90	0.02
60644	19	TNP-Non Basal	33.40	7.21
111598	17	Normal	36.25	1

Table 2. Loss of DNA copy of mir-100 locus was detected in clinical breast cancer specimens. Because expression of let-7a and miR-125b could be compensated from other loci away from the let-7a-2 cluster, it suggests that mir-100 might be the true target of the copy number loss and expression suppression. Clinical breast cancer samples in FFPE format were used to interrogate the copy number of mir-100 using pri-miRNA assay and indeed it was observed in about half of the specimens examined compared to normal breast. Cq were corrected by OD concentrations before calculating the copy # ratios.

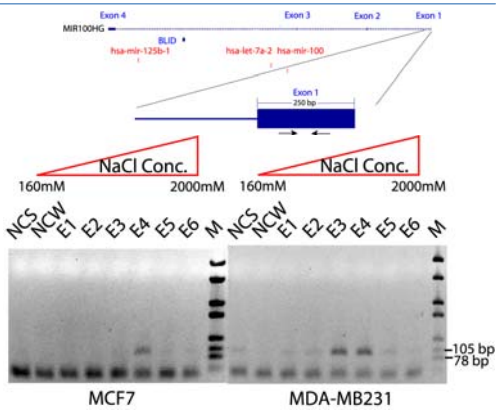

Figure 4. The transcription start site region of the MIR100HG in the genome is more methylated in MCF7 cells than in MDA-MB231 cells. Loss of expression of the let-7a-2 cluster suggested that transcription of the remaining allele is suppressed, probably by methylation. A primer pair was designed (black arrows) to amplified the gDNA fragment encoding the first exon of the MIR100HG transcript immediately downstream to the transcription start site. Fragmented gDNA purified from MCF7 and MDA-MB231 cells was fractionated in elution based on the degree of methylation. Unbound supernatant (NCS) represents un-methylated gDNA, and the bound DNA was subsequently washed (NCW) and eluted (E1 to E6) by a gradient of elevated NaCl concentrations (160 to 2000mM) as the degree of methylation increases. In MCF7 cells, methylated fragments containing MIR100HG exon1 were solely eluted from E4, but in MDA-MB231 cells, there were already detectable un-methylated fragments from exon1, and equal amount of DNA was eluted from both E3 and E4, indicating exon 1 is less methylated in MDA-MB231 cells than in MCF7 cells, possibly explaining the differential expression of the let-7a-2 cluster in these two cell lines. M, DNA size marker.

Conclusion

1. TaqMan® Pri-miRNA Assays can interrogate locus-specific expression and quantitate the miRNA gene from both transcription and gene copy levels.
2. Contamination of gDNA from the total RNA preparation needs to be cautiously monitored.
3. Differential expression of multiple loci that encode transcripts processed into identical mature miRNA can help identify potential biomarkers that are associated with them.
4. Simultaneous examination of both transcription and DNA copy number levels of a miRNA genes increases the likelihood of identifying true biomarkers if both levels are deregulated.
5. miR-100 is under-expressed in at least a subgroup of breast cancer samples probably through the mechanism of methylation in the promoter region, and further validation using larger set of clinical specimens is warranted.

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