Quantitative Analysis of microRNAs by *in situ* Hybridization Reveals Prognostic Value of miRNAs in Melanoma and Breast Cancer

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ABSTRACT

Introduction: Most microRNA (miRNA) measurement methods require total RNA extraction which destroys spatial information and makes standardization a challenge. *In situ* hybridization (ISH) however, allows the direct assessment of miRNA expression levels in tissue and importantly allows for the evaluation of expression in malignant cells as well as stromal cells. We have developed and validated a novel method for the quantitative analysis of miRNA expression by ISH on formalin fixed paraffin embedded tissue microarrays (TMAs) using miR-21, miR-92a, miR-34a, miR-221, miR-205, and Let-7a.

Methods: In order to quantitatively measure miRNA expression by ISH, DIG labeled LNA modified miRNA probes were employed and multiplexed with DAPI and cytokeratin or S100/GP100 immunofluorescence to apply the AQUA® technology. This quantitative approach allows for the measurement of miRNA expression in subcellular compartments. The assay allows standardization of miRNA measurement and potential for rapid large cohort assessment. We also assessed protein targets of each miRNA by AQUA to investigate the hypothesized inverse relationship between miRNAs and target proteins.

Results: In hundreds of tissue samples a broad dynamic range and a variable expression pattern specific to each miRNA was observed. Specificity of the assay was validated with anti-miR and miRNA mimic transfected cell lines, miR-21 knock out mouse tissue, and blocking oligo experiments. The assays were performed on near serial sections and were found to have high reproducibility with R^2>0.8. miR-221 and miR-205 were found to be prognostic in breast cancer and melanoma respectively ($p<0.05$). Finally, the inverse relationship between miRNAs and putative targets were not seen in the large population cohorts.

Conclusions: This specific and reproducible method for the quantitative analysis of miRNA expression provides proof of concept for the use of miRNAs as tissue biomarkers using quantitative ISH. Future studies will further determine the prognostic value of miR-221 and miR-205, and the relationship between miRNAs and target protein expression.

BACKGROUND

miRNAs are a class of small, non-coding RNAs of 18-25 nucleotides that post-transcriptionally regulate protein expression by base pairing with target mRNAs (1). This interaction then represses translation of the mRNA or induces deadenylation and degradation of the mRNA. miRNAs play major roles in almost every cellular process where it has been estimated that up to 60% of all protein coding genes are regulated by miRNAs (2, 3). Not surprisingly, the improper regulation of miRNAs can have severe consequences and is associated with many human pathologies including cancer (4). In cancer, miRNAs can function as tumor suppressors or oncogenes (oncomirs) depending on their target mRNAs, an active oncomir targets and downregulates tumor suppressing proteins, whereas a tumor suppressing miRNA targets primarily oncogenes (1). Since their discovery the detection of miRNAs has been difficult with most methods requiring total RNA extracts. In tissue samples, such RNA extracts are contaminated with heterogeneous cell types such as normal, stromal, and infiltrating immune cells. In situ hybridization however, allows one to visualize the expression of a miRNA in individual cells and as such allows one to determine the cell type contribution of expression changes being studied. Recent advances using EDC fixation, LNA modified and DIG labeled probes allow for the detection of miRNAs in FFPE tissue (5). The miRNA ISH is multiplexed with Dapi and immunofluorescence of a tumor type specific protein marker (cytokeratin for breast and S100/GP100 for melanoma) to apply the AQUA technology for quantitative analysis.

MATERIALS & METHODS

The Yale Breast Cancer Cohort TMA consists of 642 breast cancer patients who underwent surgery at Yale-New Haven Hospital between 1962 and 1983. The Yale Melanoma Discovery Cohort consisted of 192 patients who underwent resection of a primary invasive cutaneous melanoma at Yale-New Haven Hospital between 1959-1994, the array also contained 274 metastases, lymph node metastases, or visceral metastases. The Yale Validation Cohort consisted of 315 patients with invasive cutaneous malignant melanoma with excisions from 1995-2005 at Yale-New Haven Hospital, the array also contained 19 metastatic melanoma control spots.

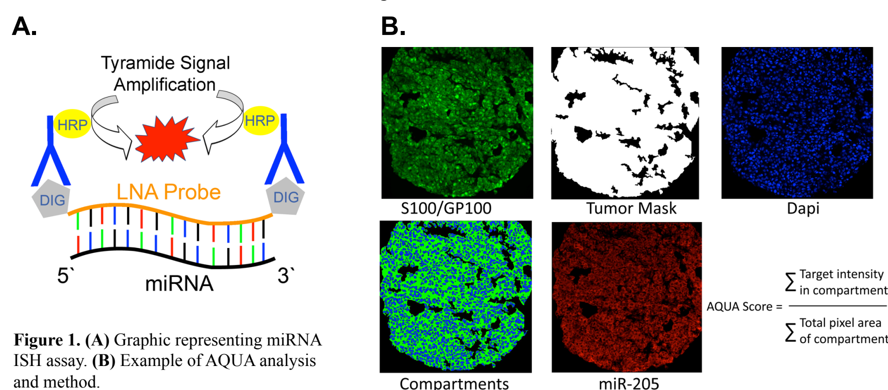

Figure 1. (A) Graphic representing miRNA ISH assay. **(B)** Example of AQUA analysis and method.

RESULTS

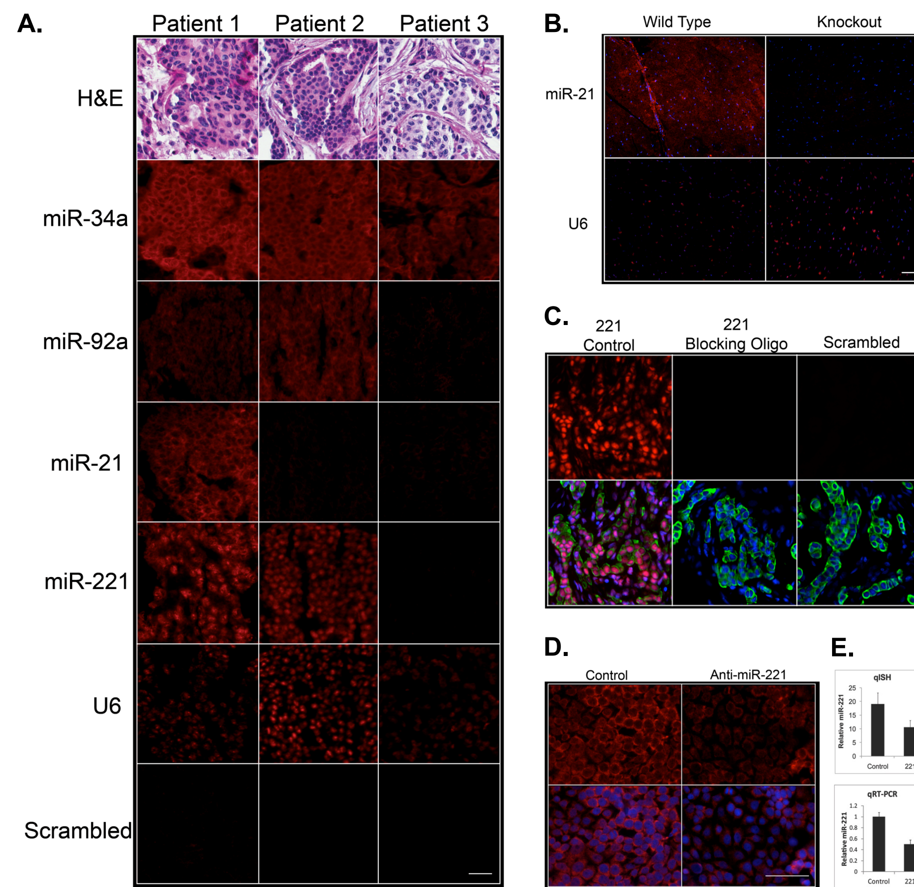

Figure 2. (A) Representative examples of miR-34a, miR-92a, miR-21, miR-221, U6 positive control, and Scrambled probe negative control are shown in three patient samples on breast TMA along with an H&E stained section. The scale bar represents 50μm. **(B)** miR-21 and U6 ISH (red) on miR-21 knockout or wild type mouse heart tissue merged with DAPI (blue). **(C)** Representative examples of miR-221 and Scrambled probe ISH performed on TMAs with and without the miR-221 blocking oligo (Cy5, Red) merged with DAPI (blue) and Cytokeratin (green). **(D)** miR-221 ISH (red) performed on MCF-7 cells transfected with control (anti-miR negative control) or anti-miR-221 inhibitor and merged with DAPI (blue). **(E)** Quantification of miR-221 knockdown in MCF-7 cells by qISH normalized by scrambled probe from 24 random fields, or qRT-PCR normalized by U6.

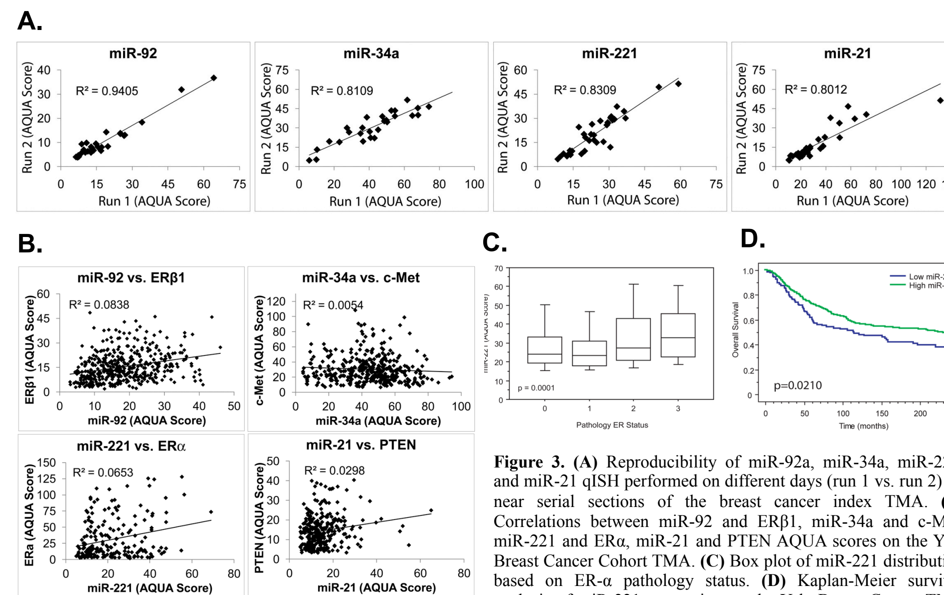

Figure 3. (A) Reproducibility of miR-92a, miR-34a, miR-221, and miR-21 qISH performed on different days (run 1 vs. run 2) on near serial sections of the breast cancer index TMA. **(B)** Correlations between miR-92 and ERβ1, miR-34a and c-Met, miR-221 and ERα, miR-21 and PTEN AQUA scores on the Yale Breast Cancer Cohort TMA. **(C)** Box plot of miR-221 distribution based on ER-α pathology status. **(D)** Kaplan-Meier survival analysis of miR-221 expression on the Yale Breast Cancer TMA (n=473). The comparison of breast cancer specific survival of the highest three quartiles (green, n=354) with the lowest quartile (blue, n=119) shows a statistically significant better prognosis for high expression of miR-221 (p value calculated by log-rank test).

RESULTS

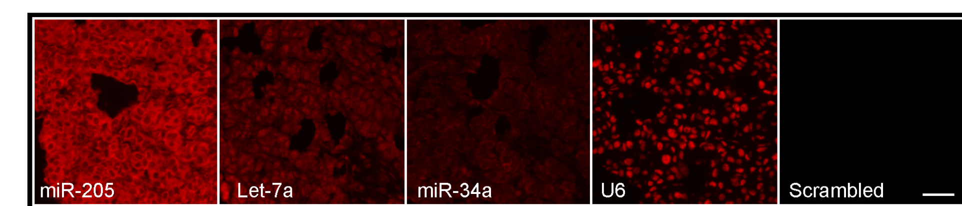

Figure 4. Representative examples of miR-205, let-7a, miR-34a, U6 positive control, and Scrambled probe negative control for a primary melanoma patient.

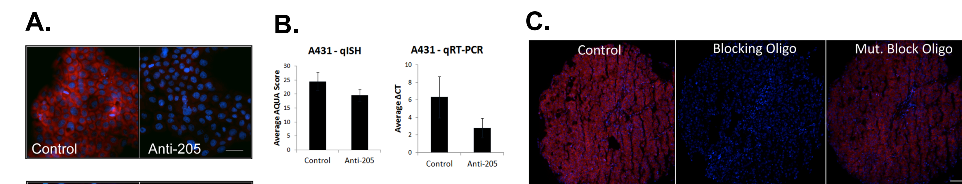

Table 1. Univariate and multivariate analysis for 10 year melanoma specific mortality

Discovery Cohort						
	Univariate Analysis			Multivariate Analysis		
n=105	HR	95% CI	p-value	HR	95% CI	p-value
Breslow						
<2.0 mm	1			1		
>2.0 mm	1.931	1.003-3.721	**0.0491**	1.828	0.922-3.626	0.0843
Age, years	1.019	0.997-1.041	0.0917	1.022	0.997-1.047	0.0796
Sex						
Male	1			1		
Female	0.907	0.495-1.662	0.752	1.085	0.575-2.047	0.802
Stage						
Localized	1			1		
Regional	2.596	1.062-6.157	**0.0363**	2.561	1.045-6.299	**0.0398**
Distant	3.205	1.242-8.268	**0.016**	4.169	1.574-11.039	**0.0041**
miR-205 Expression						
Low	1			1		
High	0.534	0.288-0.990	**0.0463**	0.4	0.207-0.773	**0.0064**
Validation Cohort						
	Univariate Analysis			Multivariate Analysis		
n=192	HR	95% CI	p-value	HR	95% CI	p-value
Breslow						
<2.0 mm	1			1		
>2.0 mm	3.014	1.489-6.101	**0.002**	2.603	1.186-5.714	**0.0171**
Age, years	1.022	1.000-1.045	0.052	1.031	1.005-1.057	**0.0169**
Sex						
Male	1			1		
Female	0.53	0.262-1.069	0.076	0.557	0.263-1.177	0.125
Stage						
Localized	1			1		
Regional	2.686	1.316-5.484	**0.0066**	2.189	1.017-4.713	**0.0452**
Distant	7.726	1.80-33.102	**0.0059**	28.238	5.52-144.422	**<0.001**
miR-205 Expression						
Low	1			1		
High	0.506	0.266-0.965	**0.0386**	0.511	0.262-0.997	**0.0489**

D. Validation Cohort Reproducibility
y = 0.9984x + 0.5346
R² = 0.8596

E. p=0.024
p=0.044
Nevus n=13
Primary n=113
Metastasis n=292

F. Discovery Cohort
Low miR-205 (n=35)
High miR-205 (n=75)
p=0.0289

G. Validation Cohort
Low miR-205 (n=93)
High miR-205 (n=113)
p=0.0386

Figure 5. (A) Representative example of miR-205 ISH (red) performed on A431 cells transfected with the control miRNA inhibitor or miR-205 inhibitor merged with DAPI (blue), and Mel-501 cells transfected with the mimic control or miR-205 mimic. **(B)** Quantification of miR-205 expression by qISH from 24 random fields or by qRT-PCR normalized by U6. **(C)** miR-205 ISH specificity in presence of miR-205 specific blocking oligo or mutated blocking oligo. **(D)** Reproducibility of miR-205 ISH on serial sections of the Yale Validation cohort. **(E)** Box plots of miR-205 expression in nevi, primary, and metastatic melanoma. **(F)** Kaplan-Meier survival analysis of miR-205 expression on the Discovery Cohort and **(G)** Validation Cohort using the same cutoff score (p-value calculated by the log-rank test).

CONCLUSIONS

- We have developed a quantitative ISH method multiplexed with cytokeratin and DAPI for AQUA analysis.
- The miRNA ISH is a sensitive, specific, and reproducible method for quantitative analysis of miRNA expression.
- The specificity of the assay is shown using blocking oligos, antagomiR/mimic transfected cell lines, and knockout mouse tissue.
- Relationship between miRNA and protein targets difficult to discern in large population cohorts.
- miR-221 and miR-205 may be valuable prognostic biomarkers in breast cancer and melanoma respectively.

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