Loss Of miR34a as Measured by Quantitative *In Situ* Hybridization on a Tissue Microarray is Associated with Poor Outcome in Breast Cancer

Seema Agarwal*, Jason A. Hanna and David L. Rimm

Department of Pathology, Yale School of Medicine, New Haven, CT 06510, USA

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ABSTRACT (UPDATED)

INTRODUCTION: MicroRNAs (miRNAs) have emerged as key regulators in the pathogenesis of cancers as either oncogenes or tumor suppressors. It has been shown that the loss of miR34a leads to tumor progression and metastasis using cell lines and a limited number of patient samples by RT-PCR. Lack of a robust, reproducible and quantitative method has limited large scale analysis and assessment of miR34a as tumor suppressor marker in cancer. Herein, we have developed and validated a method for the quantitative analysis of miRNA expression by *in situ* hybridization (qISH) allowing for the direct assessment of tumor epithelial expression of miR34a in breast cancer.

METHOD: Expression level of miR34a was measured in a retrospective breast cancer (n=461) cohort with more than 20 year follow-up using quantitative immunofluorescence (AQUA) technology for ISH in a tissue microarray (TMA) format. The assay was performed in two-fold redundancy with a 40 case index TMA for reproducibility and standardization. Averaged AQUA scores for miR34a were correlated with clinical and pathological characteristics and 20 year disease-free survival in this cohort. An independent breast cancer cohort (n=279) was used as a validation cohort.

RESULTS: Since miR34a is a tumor suppressor, we determined the threshold at which no specific hybridization was seen (AQUA score =24.0). Reproducibility between two different builds (cores) of the TMA was R^2 = 0.59. Using overall survival as an endpoint in Kaplan Meier analysis with the threshold of expression as the cutpoint, the group with loss of miR34a had significantly worse survival in training cohort (log rank p = 0.0188) and in validation cohort (log rank p = 0.0024). Cox multivariate analysis including age, nuclear grade, nodal status, ER, PR and Her2 showed miR34a is an independent marker (p=0.0435 and 0.0452) in both training and validation cohorts respectively**.**

CONCLUSIONS: The microRNA miR34a has been proposed to be a tumor suppressor using mechanistic and functional data. This result, showing loss of miR34a is associated with worse outcome in a large breast cancer cohort provides clinical evidence of the tumor suppressor function of miR34a.

BACKGROUND & METHODS

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). In cancer, miRNAs can function as tumor suppressors or oncogenes (oncomirs) depending on their target mRNAs (1). The miR34a has been shown to be a tumor suppressor in many cancer types (2,3). Reduced expression level of miR34a is associated with a variety of cancer types. Our newly developed qISH method allows one to visualize the expression of a miRNA in individual cells and with in tumor epithelial compartment (4). The miR34a expression was quantified using AQUA on two breast cancer patient cohorts from Yale. YTMA 49 (training cohort) consists of 649 patients diagnosed between 1962-1989 and YTMA 201 (validation cohort) consists of 536 patients diagnosed between 1976-2005.

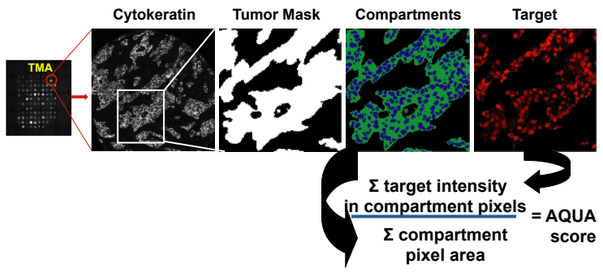

Figure 1. Illustration of AQUA. Pan-cytokeratin marks breast tumor epithelium and DAPI enables subcellular compartmentalization within the tumor mask. The target of interest, ER shown here, can then be measured quantitatively within the desired compartment using the depicted algorithm.

BACKGROUND & METHODS

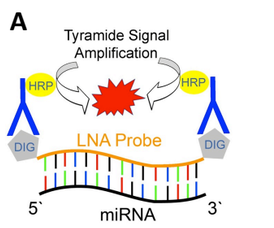

Figure 2. **(A)** Schematic representation of qISH assay. **(B)** qISH assay reproducibility using serial sections of a breast index TMA.

Table 1. Clinicopathological Characteristics of Training and Validation Breast Cancer Cohorts

Parameter	*Training cohort* N (%)	*Validation cohort* N (%)
All patients	461	279
Age (y)		
<50	127 (27.5)	94 (33.7)
≥50	334 (72.5)	185 (6603)
unknown	0	0
Nodal Status		
positive	218 (47.3)	57 (20.4)
negative	243 (52.7)	154 (55.2)
unknown	0	68 (24.4)
Tumor Size		
<2 cm	138 (30)	140 (50.2)
2-5 cm	232 (50.3)	76 (27.2)
≥5 cm	53 (11.5)	3 (1.1)
unknown	38 (8.2)	60 (21.5)
Nuclear Grade		
1	68 (14.6)	NA
2	235 (51)	NA
3	124 (26.9)	NA
unknown	34 (7.4)	279 (100)
ERα (IHC)		
positive (1-3)	239 (51.8)	156 (55.9)
negative (0)	209 (45.3)	95 (34.1)
unknown	13 (2.8)	28 (10)
PR (IHC)		
positive (1-3)	221 (47.9)	113 (40.5)
negative (0)	211 (45.8)	92 (33.0)
unknown	29 (6.3)	74 (26.5)
HER2 (IHC)		
positive (2-3)	79 (17.1)	31 (11.1)
negative (0-1)	363 (78.7)	202 (72.4)
unknown	19 (4.2)	46 (16.5)
Follow-up (m)		
median (range)	105.46 (2.39–498.03)	121 (7-385)
Treatment		
Tamoxifen Only	NA	76 (27.2)
Chemotherapy Only	NA	54 (19.4)
Tamoxifen+Chemo	NA	37 (13.3)
None	NA	64 (22.9)
Unknown	461 (100)	48 (17.2)

RESULTS

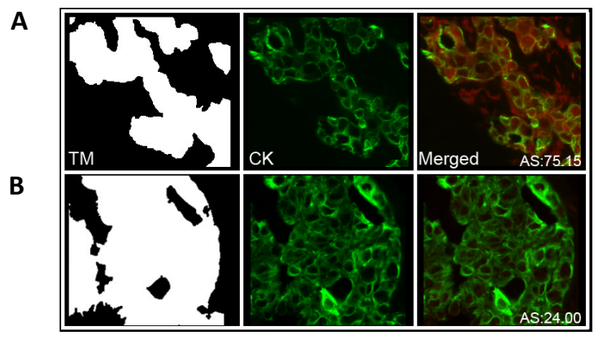

Figure 3. Representative images for high **(A)** and low **(B)** miR34a expression. Panels are TM, (tumor mask), CK (cytokeratin), Merged images of CK (green) and miR34a (red), and AS (AQUA score) are shown in the images.

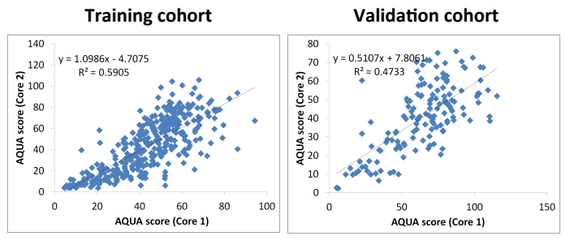

Figure 4. Heterogeneity and expression levels of miR34a expression on both cohorts.

RESULTS

Analysis of miR34a in Two early Breast Cancer Cohorts

Disease specific survival was used as a surrogate marker for metastasis. In addition to Kaplan-Meier survival curve analysis, a multivariate analysis was performed for miR34a using Cox model with other clinical and Pathological variables. The optimal cutpoint to define low and high miR34a expressers was chosen training cohort and this cutpoint was then applied to median normalized AQUA scores in an independent validation cohort.

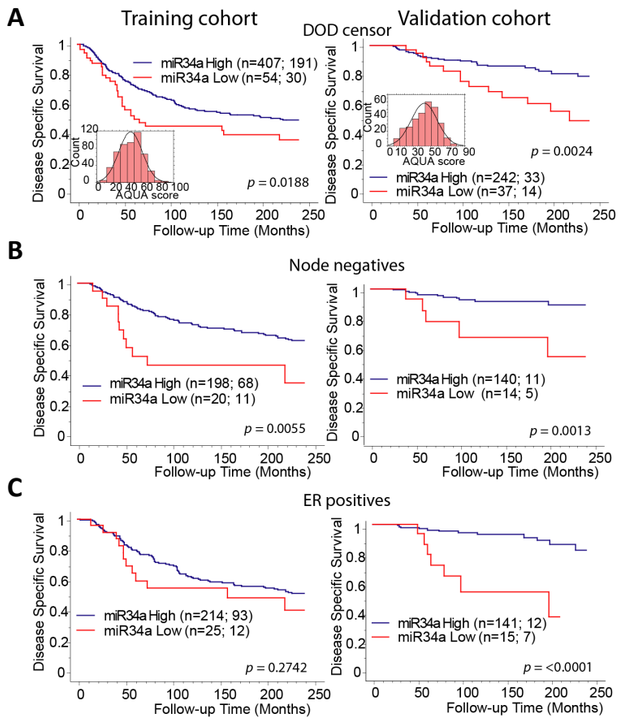

Figure 5.: Kaplan-Meier analysis for both cohorts. **(A)** DOD censor for all patients with 20 years follow up. **(B,C)**: stratified for node negative and ER positive patient subgroups. Log Rank p-values were calculated. For each group analysis number of patients and number of events are stated. Inset in Panel A shows distribution of AQUA scores in both cohorts.

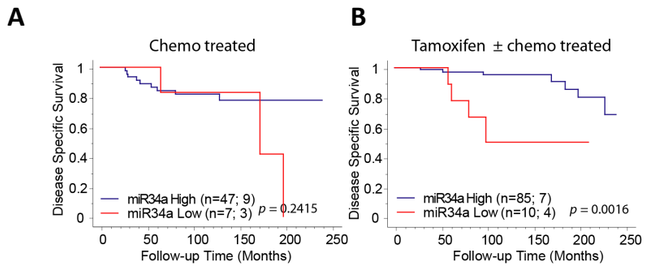

Figure 6. Kaplan-Meier analysis on validation cohort stratified for chemo **(A)** and ER positive patient subgroups treated with tamoxifen ± chemo **(B)** Log Rank p-values were calculated. For each group analysis number of patients and number of events are stated.

RESULTS

Table 2. Multivariate Analysis of miR34a in Two Cohorts

	Training cohort (n = 378; 183)			Validation cohort (n=148; 20)	
Variable	**HR (95% CI)**	**p-Value**		**HR (95% CI)**	**p-Value**
Age					
<50	0.728 (0.51-1.04)	0.0783		1.599 (0.61-4.23)	0.3441
>50	1.00			1.00	
Tumor Size					
<2 cm	0.289 (0.18-0.45)	**<0.0001**		0.395 (0.14-1.12)	0.0794
2-5 cm	0.573 (0.39-0.84)			1.00	
>5 cm	1.00				
Nuclear Grade				ND	ND
low	0.737 (0.54-1.01)	0.0556			
high	1.00				
Nodal Status					
Positive	1.00			1.00	
Negative	0.552 (0.40-0.77)	**0.0004**		0.503 (0.20-1.30)	0.1563
ER					
Positive	1.00			1.00	
Negative	1.418 (1.03-1.96)	**0.0349**		1.143 (0.43-3.03)	0.7878
PR					
Positive	1.00			1.00	
Negative	1.121 (0.83-1.52)	0.4592		2.641 (0.92-7.61)	0.072
Her2					
Positive	1.00			1.00	
Negative	0.764 (0.51-1.14)	0.1862		0.651 (0.23-1.88)	0.4281
miR34a					
Low	1.00			1.00	
High	0.650 (0.43-0.99))	**0.0435**		0.344 (0.12-0.98)	**0.0452**

ND: not determined

CONCLUSIONS

- We have developed a reproducible qISH assay (based on the AQUA technology) to quantify miR34a expression.
- Low miR34a is associated with poor disease specific survival outcome in two independent early breast cancer cohorts.
- miR34a expression stratifies patients among node negative (both cohorts) and ER positive groups (only in validation cohort).
- Loss of miR34a may be associated with poor prognosis in Tamoxifen ± chemo group (ER positives patients only) than chemo only treated group.
- miR34a expression is independent of other clinicopathological variables in both cohorts.

REFERENCES

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