MicroRNA Signature for HER2-positive Breast and Gastric Cancer

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Abstract. *Background/Aim: The molecular mechanism for aggressive clinical behaviour related to v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (ERBB2) amplification is not fully-understood. In particular, little is known about microRNAs in the human epidermal growth factor receptor 2 (HER2) signaling network. Patients and Methods: Using microRNA microarray, the microRNA profiles of 16 HER2-positive breast carcinomas were compared with those of five luminal-type breast carcinomas. Additionally, two frozen, ERBB2-amplified gastric carcinomas were compared with their adjacent normal tissue samples. MicroRNAs that were differentially expressed according to the HER2 status in breast and gastric carcinomas were identified as the HER2 microRNA signature. Results: MiR-337 and miR-302f were commonly overexpressed in HER2-postive breast and gastric cancer. MiR-139 and miR-129 were commonly underexpressed in HER2-positive breast and gastric cancer. A concordant pattern of microRNA expression was noted between discovery sets and the majority of candidate microRNAs (two out of three) in three validation sets. Conclusion: Our study identified novel microRNAs that were differentially expressed according to the HER2 status across different tumor types.*

v-erb-b2 Avian erythroblastic leukemia viral oncogene homolog 2 (*ERBB2*) is amplified in 20% of breast carcinomas (1). Human epidermal growth factor receptor-2 (HER2)-positive breast cancer is a distinct clinical entity, with aggressive biology, chemotherapy resistance, and high propensity to visceral metastasis. *ERBB2* gene amplification is present in 8.9-11.7% of gastric cancer cases (2). *ERBB2* gene amplification is more frequent in intestinal-type and gastroesophageal-junction tumors than in diffuse-type and distal gastric cancer (2, 3). Liver metastasis is frequently associated with metastatic HER2-positive gastric cancer. HER2 positivity, therefore, represents an aggressive and metastatic phenotype both in breast and gastric cancer.

The molecular mechanism for aggressive clinical behavior related to *ERBB2* amplification has not been fully-elucidated. In particular, little is known about whether and how HER2 and microRNA interaction may contribute to the aggressive biology of HER2-positive breast and gastric cancer, although recent data suggest that down-regulation of miR-139 may mediate HER2 signaling pathways in gastric cancer (4). Therefore, we undertook a microRNA profiling study to identify microRNAs in the HER2 signaling network.

Materials and Methods

Discovery sets. Discovery set samples were collected at the time of surgery from patients with cancer at the National Cancer Center, Keimyung University Dongsan Hospital, and Asan Medical Center in Korea from 2001 to 2012. Specimens were collected with Institutional Review Board approval (NCCNCS12581) and all patients gave informed consent, signing Institutional Review Board approved forms. A 10 μm-thick top slide was stained with hematoxylin and eosin. Guided by this top slide, remaining tissue was macrodissected to trim non-tumorous stromal components. Macrodissected, frozen tissue sample was mechanically crushed in liquid nitrogen and subjected to total RNA and genomic DNA isolation using mirVana™ Kit (Ambion, Austin, TX, USA) and DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA), respectively. DNAse I-treated RNA (500 ng) was poly-A tailed, and FlashTag Biotin HSR Labeling Kit (Genisphere LLC, Hatfield, PA, USA) was used to join a biotin-labeled dendrimer molecule to the 3' end using DNA ligase. Labeled samples were hybridized to GeneChip miRNA 2.0 microarrays

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Table I. *Clinicopathological characteristics*.

	Discovery set			Validation set	
	Breast cancer		Gastric cancer	Gastric cancer	
	HER2+ve (n=16)	HER2-ve (n=5)	HER2+ve (n=2)	HER2+ve (n=1)	HER2-ve (n=13)
Female (%)	16 (100%)	5 (100%)	2 (100%)	1 (100%)	4 (31%)
Median age (years)	46	40	38	68	57
Range	(33-61)	(36-67)	(38-38)		(40-70)
Subtype					
ER-ve	6 (38%)				
ER+ve	10 (62%)				
Luminal A (Ki67<15%)		4 (80%)			
Luminal B (Ki67≥15%)		1 (20%)			
Diffuse			2 (100%)	0	8 (62%)
AJCC stage					
IA	2 (13%)				
IB	0				
IIA	4 (25%)	1 (20%)			
IIB	4 (25%)				
IIIA	4 (25%)	4 (80%)			
IIIB	0				
IIIC	1 (6%)		1 (50%)		
IV	1 (6%)		1 (50%)	1 (100%)	13 (100%)

ER, Estrogen receptor; HER2, human epidermal growth factor receptor 2; AJCC, American Joint Committee on Cancer (7th Edition).

Table II. *MicroRNAs differentially expressed according to the human epidermal growth factor receptor-2 (HER2) status in breast and gastric carcinomas in the discovery set*.

		Breast cancer			Gastric cancer	
	p-Value	HER2	Luminal	Ratio[1]	*p*-Value	Ratio[2]
Overexpressed in HER2-positive tumors						
miR-337-5p	0.025	21	6	3.6	0.027	1.2
miR-302f	0.030	2	2	1.1	0.022	1.4
Underexpressed in HER2-positive tumors						
miR-129-3p	0.031	2	3	0.8	0.033	0.5
miR-139-5p	0.024	66	165	0.4	0.039	0.6

[1]Ratio of HER2-positive to HER-negative breast cancer; [2]ratio of *ERBB2*-amplified gastric cancer to adjacent normal tissue.

(Affymetrix, Santa Clara, CA, USA) based on miRbase version 15. All cell files were robust multiarray average-normalized. After filtering out star-form microRNAs, we subjected 913 human microRNAs to further analyses for this study. BRB-Arraytools software (version 4.3; National Cancer Institute, Bethesda, MD, USA) was used to carry out unsupervised and supervised microRNA analyses. Principal component analyses were performed using 1−correlation as a distance metric. Copy number states of gastric carcinomas were determined using SNP6 arrays and Genotyping Console 4.1 (Affymetrix). In breast carcinomas, HER2 immunohistochemistry results were scored as 0-3+ according to the method recommended for the HercepTest (Dako, Glostrup, Denmark) (5). Breast carcinomas with scores of 3+ or *ERRBB2* gene amplification by fluorescence *in situ* hybridization were identified as being HER2-positive.

Validation sets. To validate these candidate HER2-related microRNAs in breast carcinomas, we downloaded microRNA datasets for HER2-positive breast cancer from the gene expression omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/; GSE40525 and GSE39543). ArrayTools was used to normalize the data to the median array and to evaluate the differential expression of

Table III. *Average expression levels of human epidermal growth factor receptor 2 (HER2) microRNA signature in the three validation sets.*

	Breast (GSE40525)			Breast (GSE39543)			Gastric[1]		
	HER2+ve (n=6)	HER2-ve (n=114)	Ratio	HER2+ve (n=13)	HER2-ve (n=32)	Ratio	HER2+ve (n=1)	HER2-ve (n=13)	Ratio
Overexpressed in HER2-positive tumors									
miR-337-5p	18	21	0.84	1,160	134	8.7	0.4	0.2	2
miR-302f	N/A			N/A			N/A		
Underexpressed in HER2-positive tumors									
miR-129-3p	12	13	0.97	557	220	2.5	0.3	0.4	0.77
miR-139-5p	9	33	0.29	121	191	0.63	2.1	0.4	5.87

Ratio, Ratio of HER2-positive to HER2-negative cancer; [1]median expression level; N/A, the microRNA probe was not included in dataset.

the *HER2* microRNA signature in each GEO dataset. Since there were no public microRNA datasets for HER2-positive gastric cancer, we used our own microRNA data from endoscopic biopsy of 14 patients with metastatic gastric cancer (6). These microRNA microarray data were generated using custom-synthesized 8×15k microRNA microarrays containing 4,361 microRNAs (Sanger miR 9.0 database) (Agilent Technologies, San Jose, CA, USA). A mixture of total RNA isolated from three gastric cancer cell lines (SNU-601, SNU-638, and AGS; Korean Cell Line Bank, Seoul, Korea) was used as the reference RNA for competitive hybridization. Copy number status of the 14 gastric cancer samples was previously assessed using Agilent 4×44k HD-CGH microarray (6). Based on the ADM-2 algorithm of Agilent's CGH Analytics software, the aberration filter was set at a minimum of five probes in the region, and a log ratio that was equal to the DLRSpread (the spread of the ratio differences between consecutive probes) of each sample (7).

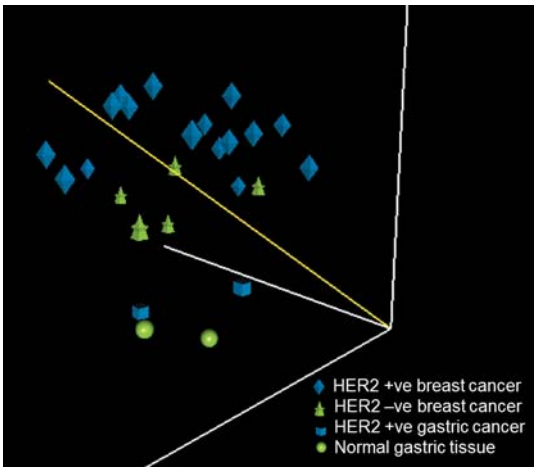

Figure 1. *Principal component analysis of the discovery set. Three axes represent the first three principal components that are orthogonal linear combinations of microRNAs. Each sphere represents each sample. '1−correlation' is used as a distance metric. Samples clustered with those with the same tissue origin. Within the breast cancer group, human epidermal growth factor receptor 2 (HER2)-positive breast carcinomas (green) clustered separately from HER2-negative breast tumors (blue). Within the gastric sample group, samples clustered according to the HER2 status.*

Results and Discussion

This study used 21 frozen, breast cancer tissue samples (16 HER2-positive and five luminal-type) (Table I). In addition, paired tumor and normal samples were collected from two patients with *ERBB2*-amplified gastric cancer (copy number state of 4). According to unsupervised principal component analyses, breast and gastric carcinoma data were clustered separately (Figure 1). This result suggests that the difference in tumor type, rather than HER2 status, primarily affects the global microRNA profile. Within breast tumors, samples clustered by HER2 status.

At feature selection of $p<0.05$, 83 microRNAs were differentially expressed between the HER2-positive and luminal-type breast carcinomas, designated the *HER2* breast microRNA signature. Thirty-seven microRNAs were overexpressed in HER2-positive breast cancer and 46 microRNAs were underexpressed in HER2-positive breast cancer. There were 42 microRNAs that were differentially expressed between two pairs of *HER2*-amplified gastric carcinomas and adjacent normal tissue at $p<0.05$, designated the *HER2* gastric microRNA signature. Four microRNAs overlapped between *HER2* breast and *HER2* gastric microRNA signatures: miR-337-5p, miR-302f, miR-129-3p, and miR-139-5p, designated the *HER2* microRNA signature (Table II). miR-337-5p and miR-302f were commonly overexpressed in HER2-postive breast and gastric cancer. miR-129-3p and miR-139-5p were commonly underexpressed in HER2-positive breast and gastric cancer. Underexpression of miR-129 and miR-139 in HER2-positive breast cancer was brought to our attention, because these were reported to suppress metastases (8-11), and may therefore account for the

metastatic propensity of HER2-positive breast cancer. MiR-129 suppresses cell proliferation and migration by down-regulating the proliferation and survival genes, such as cyclin-dependent kinase 6 (*CDK6*) and SRY-related HMG-box (*SOX4*), in various tumor types (8-10). miR-139 is a negative regulator of metastatic pathways in breast cancer (11). Recently, Bao *et al*. reported that HER2 interacts with CD44 to up-regulate chemokine (C-X-C motif) receptor 4 (*CXCR4*) *via* epigenetic silencing of miR-139 in gastric cancer cells (4). Thus, it is tempting to speculate that HER2 may enhance metastatic potential, partly through down-regulation of miR-129 and miR-139.

We validated the differential expression of these candidate *HER2*-related microRNAs in two GEO datasets (GSE40525 and GSE39543) and our own microRNA datasets for endoscopic biopsy of 14 patients with metastatic gastric cancer. One of 14 gastric carcinomas had an average log2 ratio of 2.2 at the *ERBB2* locus according to array CGH analysis, and was identified as HER2-positive gastric cancer. A concordant pattern of microRNA expression was noted between discovery sets and the majority of candidate microRNAs (two out of three) in three validation sets (Table III). Thus, our study suggested a list of candidate microRNAs in the HER2 signaling network. Further studies are warranted to evaluate whether and how these microRNAs interact with HER2 in breast and gastric carcinogenesis.

Disclosure

The Authors have nothing to disclose in regard to this article.

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