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Cancer Genetics and Cytogenetics 189 (2009) 15–23

Cancer Genetics and Cytogenetics

An investigation of microRNAs mapping to breast cancer related genomic gain and loss regions

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Received 3 June 2008; received in revised form 4 September 2008; accepted 12 September 2008

Abstract Various regions of amplification or loss are observed in breast tumors as a manifestation of genomic instability. To date, numerous oncogenes or tumor suppressors on some of these regions have been characterized. An increasing body of evidence suggests that such regions also harbor microRNA genes with crucial regulatory roles in cellular processes and disease mechanisms, including cancer. Here, we investigated 35 microRNAs localized to common genomic gain and/or loss regions in breast cancers. To examine amplification or loss of these microRNAs as a result of genomic instability, we performed semiquantitative duplex polymerase chain reaction in 20 breast cancer cell lines, 2 immortalized mammary cell lines, and 2 normal DNA controls. A comprehensive DNA fold number change data for 35 microRNA genes on chromosomal gain/loss regions are presented in breast cancer cells. A 23% (8/35) of the investigated microRNAs showed significant fold number increases (greater than fourfold) compared to *GAPDH* in one or more of the breast cell lines. Although no homozygous deletions were detected, fold number decreases indicating potential loss regions were observed for 26% (9/35) of the investigated microRNAs. Such fold number changes may point out some of these microRNAs as potential targets of the genomic instability regions as oncogene and tumor suppressor candidates.

1. Introduction

Genomic instability is commonly seen in breast cancers. So far, various potent oncogenes and tumor suppressors located on some of these common instability regions have been identified. In addition to the coding genes, microRNA genes are also found to be located on genomic instability regions and chromosomal fragile sites [1,2]. Moreover, numerous murine microRNA loci have been indicated as common retroviral integration sites [3].

MicroRNAs are about 18–24 nucleotide transcripts that can bind to the 3' untranslated regions (UTR) of target mRNAs and either prevent their translation or cause degradation [4–7]. Recent research suggests important regulatory roles of such noncoding RNA in various processes, such as those directing pluripotency, differentiation, morphogenesis, cell cycle regulation, metabolism, and immune system pathways [8,9].

A growing body of evidence suggests deregulated expression of microRNAs in various tumors. Abnormal microRNA expression is, therefore, linked to altered levels of proteins that may have significant roles during the initiation or maintenance of the neoplastic phenotype in different cancer types. In fact, deregulated microRNA expression profiles have been shown to effectively differentiate normal breast tissue from breast tumors [10]. As evidence continues to build up linking microRNAs to cancer, hsa-mir-21, mapped on an amplicon region (17q23), was found to be overexpressed in breast cancers, glioblastomas, pancreatic cancers, hepatacellular cancers, cholangiocarcinomas, ovarian, and colorectal cancers [10–18]. So far, identified targets of hsa-mir-21, *PTEN*, *PDCD4*, and *TPM1* are known to be involved in cell survival and transformation processes [14,17,19]. Therefore, identification of deregulated microRNAs and their target mRNAs provides new avenues toward understanding the tumorigenesis processes.

Based on the fact that breast cancers demonstrate high genomic instability phenotypes, we investigated the status of microRNA genes on such gain/loss regions in breast cancer cell lines to pioneer further expression and functional analysis for microRNA genes that may be targets of amplification or regions of loss. While the majority of microRNA genes had various degrees of fold number increases or

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0165-4608/09/$ – see front matter
doi:10.1016/j.cancergencyto.2008.09.009

decreases, 23% (8/35) and of the investigated microRNA genes showed significant fold number increases (more than fourfold) compared to *GAPDH* in one or more of the breast cell lines. No homozygous deletions were detected, but fold number decreases indicating potential loss regions were observed for 26% (9/35) of the investigated microRNAs.

2. Materials and methods

2.1. Cancer cell line DNA

Twenty breast cancer cell lines [BT20, BT474, BT549, CAL51, DU4475, Hs578T, MCF7, MDA-MB157, MDA-MB231, MDA-MB361, MDA-MB435 (breast origin questioned by [20]), MDA-MB468, SUM52, SUM102, SUM149, SUM159, SUM185, SUM229, SK-BR3, and T47D] and 2 immortalized, nontumorigenic mammary cell lines (HPV4-12 and MCF10) DNA samples were kindly provided by E.M. Petty (University of Michigan, Ann Arbor, MI).

2.2. Selection of common genomic gain/loss regions

Publications on homozygous deletion (HD), loss of heterozygosity (LOH), and amplification regions in breast cancer cells were screened to build a list of frequent and common genomic gain/loss regions in breast cancer. Two approaches were used to find microRNAs mapping to these regions. First, boundaries for these regions were extracted from the UCSC Genome Browser (Genome Bioinformatics Group of UC Santa Cruz, Human Genome assembly, May 2004) using single-nucleotide polymorphism (SNP) marker information, when available. microRNA genes located within these boundaries and/or chromosomal bands were then identified from the miRBase database (version 7.1; Welcome Trust Sanger Institute). Second, all known microRNA sequences were combined back to back in FASTA format and were blasted against the selected genomic gain/loss regions to localize any microRNA genes (Tables 1 and 2).

2.3. Semi-quantitative duplex PCR and densitometry analysis

Primer sets for microRNA genes on selected chromosomal regions were designed by using the Primer3 program (version 0.2; Whitehead Institute for Biomedical Research, Cambridge, MA; Table 3). MicroRNA genes were co-amplified with an internal control gene, *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), by semiquantitative duplex PCR, which was optimized and performed as described previously [21]. Optimization of PCR in control DNA samples involved two major steps: (1) coamplification of microRNA gene primers with alternating *GAPDH* primer concentrations to obtain similar-intensity bands and (2) optimization of PCR cycle number for each reaction so as not to observe saturated bands for densitometric

Table 1
List of microRNA genes on genomic instability regions

	Loci	Gain / loss	Samples	References	microRNA genes
1	2q31~q32	Gain	MDA-MB-231	[47]	hsa-mir-10b
	2q	Loss	BT	[48]	
2	3p21	Loss	BCCL and BT	[49–51]	hsa-mir-135a-1, let7g,
	3p	Loss	BT	[52]	hsa-mir-191, hsa-mir-138-1, hsa-mir-425
3	3q	Gain	BT	[53,54]	hsa-mir-15b, hsa-mir-16-2
4	3q13.3	Loss	MCF7	[23]	hsa-mir-198
5	5q33	Loss	BT	[28,51,55]	hsa-mir-143, hsa-mir-145
6	8p11~p12	Gain	BCCL and BT	[56]	hsa-mir-486
	8p11~p21	Loss	BT	[57]	
7	8p21	Loss	BT	[52,57]	hsa-mir-320a
8	8p21~p23	Loss	BCCL and BT	[51,52,58,59]	hsa-mir-383
		Gain	BT	[51]	
9	11q23~q24	Gain	BT	[60]	hsa-mir-34b, hsa-mir-34c,
		Loss	BT	[60–62]	hsa-mir-100, hsa-let7a-2, hsa-mir-125b-1
10	13q14	Loss	BCCL and BT	[23,52,63,64]	hsa-mir-15a, hsa-mir-16-1
11	13q31	Gain	BCCL	[23,65,66]	hsa-mir-17, hsa-mir-18a, hsa-mir-20a, hsa-mir-19a, hsa-mir-19b-1, hsa-mir-92a-1
12	17q22~q24	Gain	BCCL and BT	[67–69]	hsa-mir-301a, hsa-mir-142
13	17q23	Gain	BCCL and BT	[38,70–72]	hsa-mir-21, hsa-mir-633
14	20p	Gain	BCCL and BT	[73]	hsa-mir-103-2
15	21q21	Loss	BT	[39]	hsa-mir-125b-2
16	Xq21	Loss	BCCL	[23]	hsa-mir-384, hsa-mir-325,hsa-mir-361

Abbreviations: BCCL, breast cancer cell lines; BT, breast tumors.

Table 2
Chromosomal coordinates of microRNAs according to UCSC Genome Browser

	microRNAs	Chromosomal coordinates
1	hsa-mir-10b	2: 176,723,277-176,723,386 [+]
2	hsa-mir-138-1	3: 44,130,708-44,130,806 [+]
3	hsa-mir-425	3: 49,032,585-49,032,671 [−]
4	hsa-mir-191	3: 49,033,055-49,033,146 [−]
5	hsa-let-7g	3: 52,277,334-52,277,417 [−]
6	hsa-mir-135a-1	3: 52,303,275-52,303,364 [−]
7	hsa-mir-198	3: 121,597,205-121,597,266 [−]
8	hsa-mir-15b	3: 161,605,070-161,605,167 [+]
9	hsa-mir-16-2	3: 161,605,227-161,605,307 [+]
10	hsa-mir-145	5: 148,790,402-148,790,489 [+]
11	hsa-mir-143	5: 148,788,674-148,788,779 [+]
12	hsa-mir-383	8: 14,755,318-14,755,390 [−]
13	hsa-mir-486	8: 41,637,116-41,637,183 [−]
14	hsa-mir-320a	8: 22,158,420-22,158,501 [−]
15	hsa-mir-34c	11: 110,889,374-110,889,450 [+]
16	hsa-mir-125b-1	11: 121,475,675-121,475,762 [−]
17	hsa-let-7a-2	11: 121,522,440-121,522,511 [−]
18	hsa-mir-100	11: 121,528,147-121,528,226 [−]
19	hsa-mir-16-1	13: 49,521,110-49,521,198 [−]
20	hsa-mir-15a	13: 49,521,256-49,521,338 [−]
21	hsa-mir-17	13: 90,800,860-90,800,943 [+]
22	hsa-mir-18a	13: 90,801,006-90,801,076 [+]
23	hsa-mir-19a	13: 90,801,146-90,801,227 [+]
24	hsa-mir-20a	13: 90,801,320-90,801,390 [+]
25	hsa-mir-19b-1	13: 90,801,447-90,801,533 [+]
26	hsa-mir-92a-1	13: 90,801,569-90,801,646 [+]
27	hsa-mir-142	17: 53,763,592-53,763,678 [−]
28	hsa-mir-301a	17: 54,583,279-54,583,364 [−]
29	hsa-mir-21	17: 55,273,409-55,273,480 [+]
30	hsa-mir-633	17: 58,375,308-58,375,405 [+]
31	hsa-mir-103-2	20: 3,846,141-3,846,218 [+]
32	hsa-mir-125b-2	21: 16,884,428-16,884,516 [+]
33	hsa-mir-384	X: 76,056,092-76,056,179 [−]
34	hsa-mir-325	X: 76,142,220-761,42,317 [−]
35	hsa-mir-361	X: 85,045,297-85,045,368 [−]

analysis. The optimized PCR products were run on ethidium bromide–stained 2% agarose gels, visualized, and documented under ultraviolet light. Band intensities for the microRNA and *GAPDH* PCR products were quantified by using the densitometric image processing and analysis program Scion Image (version beta 3b; National Institutes of Health, Bethesda, MD) and by visual inspection. Ratio of band intensities for the microRNA and *GAPDH* were calculated. Cancer cell line ratios were normalized using the mean of the signals from normal DNA samples (N1, N2). Fold changes of 35 microRNA genes compared to *GAPDH* in 20 breast cancer cell lines (BCCL) and two immortalized cell lines were calculated as follows;

$$\text{Fold change} = \frac{\text{BCCL}(\text{microRNA}/\text{GAPDH})}{\text{AVG}(\text{N1},\text{N2})(\text{microRNA}/\text{GAPDH})}$$

Fold changes for each microRNA in cancer cells versus controls were then classified with the following cut-off values; less than 0.3-fold (loss), 0.3–2.0-fold (no significant change), 2.0–4.0-fold (gain), and ⩾ 4-folds (and/or saturated bands due to significant amplification) (significant gain). For reverse-transcription polymerase chain reaction (RT-PCR), DNase-treated RNA (1 μg) was used to synthesize cDNA using both oligodT primers and random hexamers.

3. Results and Discussion

Common breast cancer–related genomic gain/loss regions were selected from the literature. Screening for microRNA genes located within the boundaries of these chromosomal regions resulted in the identification of more than 30 known microRNA genes (Table 1).

Primers were designed to investigate genomic loss or gain of microRNA genes located on breast cancer–related genomic regions. Thirty-five microRNA genes were successfully co-amplified with *GAPDH* primers in 20 breast cancer cell lines, 2 immortalized mammary cell lines, and 2 normal DNA controls. Ratios of the microRNA and *GAPDH* PCR product bands were then detected by densitometry for cancer cell lines and compared to the average values of the two normal control DNA samples. To minimize insensitivity of semi-quantitative PCR results and to be stringent about calling a region lost, only microRNA/*GAPDH* ratios less than 0.3-fold compared to normal samples were considered as losses and increases of more than 2-fold were indicated as gains (Fig. 1). No HD were observed for any of the microRNA genes in the examined cell lines. Taking limits of duplex PCR into account, we focused on consistent patterns of amplification or loss of microRNA genes across several cell lines, whereas no significant fold number changes were detected for some microRNAs, such as hsa-mir-486 and hsa-mir-10b. Hsa-mir-10b, on 2q31.1, was recently reported to be highly expressed in metastatic breast cancer cells (e.g., MDA-MB-231) contributing to invasion and metastasis [22]. We did not detect any significant amplification in any of the cell lines for this microRNA gene, therefore DNA amplification may not be a common reason of overexpression of this microRNA in breast cancer cells.

Among the selected breast cancer–related genomic regions, some were defined by broad chromosomal boundaries, so it was not unusual to find different studies reporting the same region to harbor either genomic loss or gain. In breast cancer cells, we also observed that some microRNA located on regions of loss showed fold number increases, whereas some others located on gain regions showed fold number decreases. For example, hsa-mir-384 resides on Xp21.2, which was indicated as a region of loss [23]. While we detected loss for Hs578T, MDA-MB-231 demonstrated a threefold increase in the microRNA PCR product compared to *GAPDH* and normal DNA samples (Fig. 2A). While some of these similar results may be partly explained by the insensitivity of semi-quantitative PCR, it may also suggest how complicated genomic instability may manifest in breast cancers.

Table 3
Primer sequences for microRNA genes and *GAPDH*

microRNA	Primer sequences (5' → 3')
hsa-mir-10b	F: TAATAAAGCCGCCATCCTTG, R: CTGGCTATTCCGAAGAAACG
hsa-mir-let7g	F: GGTTTCCCAGAGATGAGCAG, R: AGCCTCTGCTGTGAGGATGT
hsa-mir-135a-1	F: CTGTCCTGCCTCCTTTTGAG, R: GAAGAAGTGCCTGCAAGAGC
hsa-mir-138-1	F: AGCAGCACAAAGGCATCTCT, R: CTCTGTGACGGGTGTAGCTG
hsa-mir-425	F: CCACCCCCATTCCTTTTAAT, R: CAGGTCATGCACCTTCAGAAT
hsa-mir-191	F:AAGTATGTCTGGGGGTCAGG, R: ACAACCTACTCCCGGGTCTT
hsa-mir-198	F:GCCGGAGGTTAAACATGAAA, R: CCCAGCCTACCAATATGCTC
hsa-mir-15b	F: AGAACGGCCTGCAGAGATAA, R: CGTGCTGCTAGAGTGGAACA
hsa-mir-16-2	F: TGTTCGTTTTATGTTTGGATGA, R: AGTGGTTCCACCAAGTAAGTCA
hsa-mir-145	F: GGCTGGATGCAGAAGAGAAC, R: CAGGGACAGCCTTCTTCTTG
hsa-mir-143	F: CCCTCTAACACCCCTTCTCC, R: AACTTCCCCAGCATCACAAG
hsa-mir-486	F: CCTGGGGTGTGAATGGTAAC, R: ATCTCCAGCAGGTGTGTGTG
hsa-mir-320	F: GAGGCGAATCCTCACATTG, R: GGGACTGGGCCACAGTATTT
hsa-mir-383	F: AGTCCACCAAATGCAGTTCC, R: ACTTCAGAATCTCCCCGTCA
hsa-mir-34c	F: TTGAGCTCCAACTCAACCAA, R: GATGCACAGGCAGCTCATT
hsa-mir-125b-1	F: ACCAAATTTCCAGGATGCAA, R: CGAACAGAAATTGCCTGTCA
hsa-mir-let-7a-2	F: ATAGGGAGAAAAGGCCTGGA, R: ATGGCCCAAATAGGTGACAG
hsa-mir-100	F: AGGTCTCCTTCCTCCACCTC, R: GTCACAGCCCCAAAAGAGAG
hsa-mir-16-1	F: TGAAAAAGACTATCAATAAAACTGAAAA, R: CCATATTGTGCTGCCTCAAA
hsa-mir-15a	F: TACGTGCTGCTAAGGCACTG, R: ATTCTTTAGGCGCGAATGTG
hsa-mir-19a	F: TGCCCTAAGTGCTCCTTCTG, R: CCAGGCAGATTCTACATCGAC
hsa-mir-20a	F: CGATGTAGAATCTGCCTGGTC, R: GGATGCAAACCTGCAAAACT
hsa-mir 19b-1	F: GCCCAATCAAACTGTCCTGT, R: ACCGATCCCAACCTGTGTAG
hsa-mir-17	F: CCCCATTAGGGATTATGCTG, R: CCTGCACTTTAAAGCCCAACT
hsa-mir 18a	F: GGCACTTGTAGCATTATGGTGA, R TGCAAAACTAACAGAGGACTGC:
hsa-mir-92-1	F: CCATGCAAAACTGACTGTGG, R: CAGTGGAAGTCGAAATCTTCAG
hsa-mir-142	F: CAGGGTTCCACATGTCCAG, R: CTGAGTCACCGCCCACAAG
hsa-mir-301	F: CTCATTTAGACAAACCATAACAACTT, R: CATCAATAAGCAACATCACTTTGA
hsa-mir-21	F: CCATTGGGATGTTTTTGATTG, R: TCCATAAAATCCTCCCTCCA
hsa-mir-633	F: AGGACTGGGTTTGAGTCCTG, R: TTAGACATTCCTCCTGGTGAA
hsa-mir-103-2	F:CCCTAGGGAGGAATCCAGAG, R: AGCCATAAGCTGCACCAACT
hsa-mir-125b-2	F: TCGTCGTGATTACTCAGCTCAT, R: CAGGGATCAGCTGGAAGAAG
hsa-mir-384	F: TGGCCAGTTAGCATCTTGAA, R: TCAGGCCTGCAGAAATAGTG
hsa-mir-325	F: TCCTTTTCACCCCTCAACAC, R: GGATTCAAGTCCACAGAACCA
hsa-mir-361	F:GGAGCTCAACCATACCAGGA R: TTGGGCATATGTGACCATCA
GAPDH	F: TGCCTTCTTGCCTCTTGTCT, R: CTGCAAATGAGCCTACAGCA

Similarly, hsa-mir-191 on chromosomal band 3p21 mostly showed fold number increases in SUM185, CAL51, and DU4475 cells. Although 3p21 is usually indicated as a loss region in breast and lung cancers [1], amplification of this microRNA gene was consistent with recent reports suggesting hsa-mir-191 overexpression in breast cancer cell lines [24] and colon cancers [25]. Moreover, according to a recently generated comprehensive copy number variation (CNV) map of the human genome [26], chromosomal bands 3p21 and 8p21 were denoted as CNV loci. Chromosomal bands 3p21 and 8p21 harbor hsa-mir-138-1 (Fig. 2B), hsa-let-7g (overexpressed in colon cancers [27]), and hsa-mir-320a, respectively. Therefore, for the microRNA on these chromosomal bands, fold number increases and/or decreases in different cells may also be an indication of CNV. Targets of these microRNA will be interesting to evaluate the role of CNV that may be seen on these chromosomal bands. Other chromosome 3q microRNAs [hsa-mir-198 and hsa-mir-15b, 40 megabases (Mb) apart from each other] demonstrated low to significant fold number increases in more than two cell lines. Hsa-mir-15b demonstrated the following fold number increases: 2.6 for MCF7, 2.5 for T47D, and 2 for both BT474 and MDA-MB-468. Therefore, gains were indicated for these cell lines in Fig. 1 because they were above the threshold of twofold. Hsa-mir-16-2 [60 base pairs (bp) away from hsa-mir-15b] also demonstrated a similar amplification pattern (1.2 for MCF7, 1.7 for T47D, 1.4 for BT474, and 1.6 for MDA-MB-468), but since all were below the threshold value of twofold, no gain was indicated for these cell lines in Fig. 1.

Surprisingly, hsa-mir-145 on 5q, a region to harbor multiple loss of heterozygosity regions [28], was amplified in 13 cell lines, including MCF10 and HPV4-12, whereas no significant fold number decrease was observed. Hsa-mir-145 is known to be down-regulated in different tumors such as breast [10,24] and ovarian [15]. However, when compared to the SNP array–based LOH and copy number analysis data from Cancer Genome Project (CGP) of the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/genetics/CGP), some breast cancer cell lines (e.g., MCF7, MDA-MB-157, and BT549) were indeed reported to harbor small regions of low amplifications around the

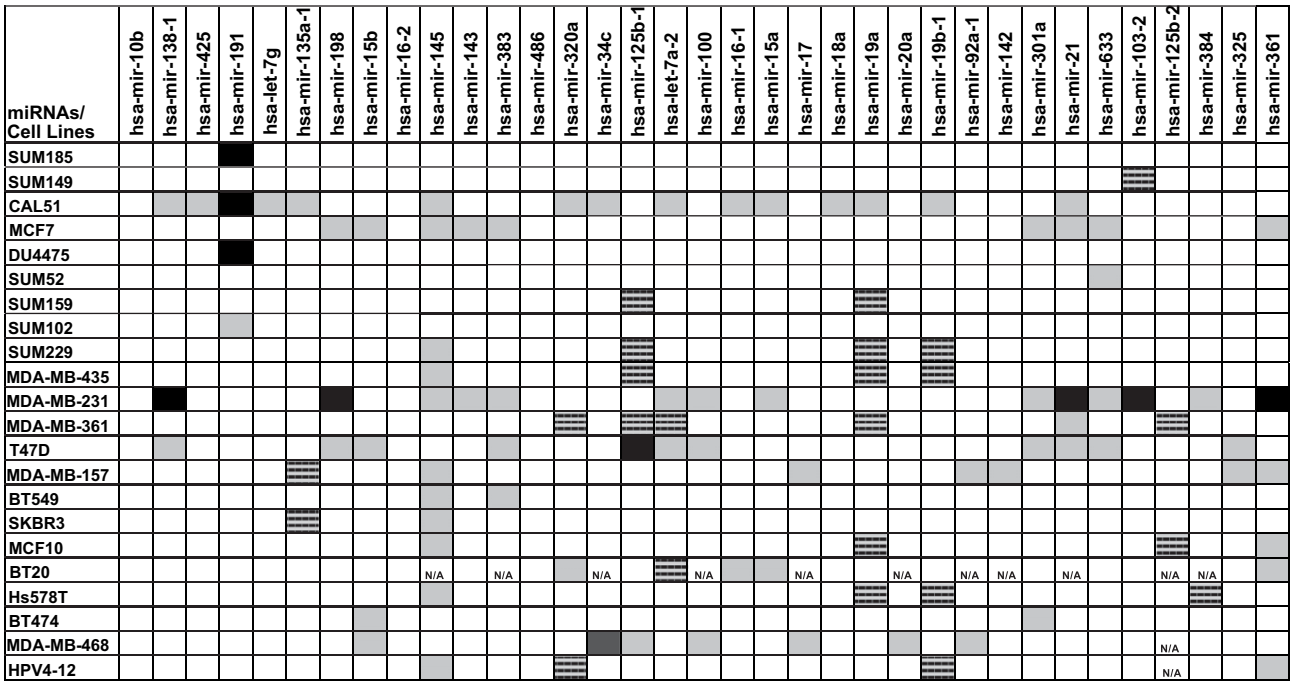

Fig. 1. Fold number changes for the 35 microRNA genes mapping to selected genomic gain/loss regions in breast cancers. Densitometric analysis results of microRNA and *GAPDH* PCR products of cancer cell lines were compared to that of normal DNA.Fold changes for each microRNA in cancer cells versus controls were classified with the following cut-off values; less than 0.3-fold (loss, ▤), 0.3- to 2.0-fold (no significant change, □), 2.0–4.0 fold (gain, ▢), and 4-fold or higher (and/or saturated bands due to significant amplification; significant gain, ■).

140- to 50-Mb region of chromosome 5q32. Both data suggest transcriptional regulation to be a more plausible mechanism than genomic loss for the down-regulation of hsa-mir-145 in breast cancer cells.

Hsa-mir-34b and hsa-mir-34c on 11q23 are recently shown to be normally up-regulated in response to DNA damage and oncogenic stress in a p53-dependent manner and to decrease p53-mediated cell death when hsa-mir-34 function was reduced [29]. We detected low to moderate fold number increase of hsa-mir-34c in two breast cell lines, whereas we failed to generate a PCR product for hsa-mir-34b. According to these results, transcriptional regulation seems to be a more plausible explanation than genomic loss for these microRNA. Expression status of these microRNA genes will be crucial in revealing their roles in breast tumorigenesis.

Consistent with the finding that hsa-mir-125b-1 on 11q24 is downregulated in ovarian cancers [15], we observed a fold number decrease in four cell lines. Surprisingly, two others, especially T47D, showed a fold number increase compared to controls. Hsa-let-7a-2 and hsa-mir-100, separated from each other by 5 kilobases, also demonstrated a conserved significant amplification pattern in MDA-MB-231 and T47D cells. Interestingly, in *Drosophila*, mir-100, mir-125, and let-7 are all clustered within an 800-bp region on chromosome 2L and up-regulation of these miRNA and the down-regulation of mir-34 requires the hormone ecdysone during development [30]. Thus, expression and functional analysis of 11q24 microRNA and possibly mir-34 family members in breast cancers may potentially help to establish such a co-operative role in mammalian cells.

Hsa-mir-16-1 and hsa-mir-15a have been indicated as tumor suppressor genes in leukemia [31]. We did not observe any fold number decreases, to indicate LOH or HD in any of the breast cancer cell lines. The roles of these microRNAs in breast tumorigenesis are yet to be established, but if their deregulated expression contributes to breast tumorigenesis as it does in leukemia, genomic loss does not seem to be common, at least among cell lines. Interestingly, low to moderate fold number increases were observed for some cells (e.g., CAL51) for both hsa-mir-16-1 and hsa-mir-15a. While this result may be due to the semi-quantitative nature of our approach, it is also possible that expression data may also not correlate with genomic level gains or losses.

Chromosomal band 13q31.1, harboring the microRNA-17-92 cluster, was also analyzed in breast cancer cell lines. This cluster is known to be overexpressed in lung cancers and lymphomas [32–34]. We did not detect a significant genomic amplification pattern for the 17-92 cluster in breast cancer cell lines. Especially for hsa-mir-19a and hsa-mir-19b-1, the microRNA PCR product bands were not even as significantly amplified as the *GAPDH* bands in SUM229 and MDA-MB-435 cell lines (Fig. 3), suggesting that amplification of these microRNA genes may not be a common event in breast cancer cell lines, if this cluster also harbors potent oncogenes with roles during breast tumorigenesis. Interestingly, when compared to the CGP of the Wellcome Trust Sanger Institute, we noticed

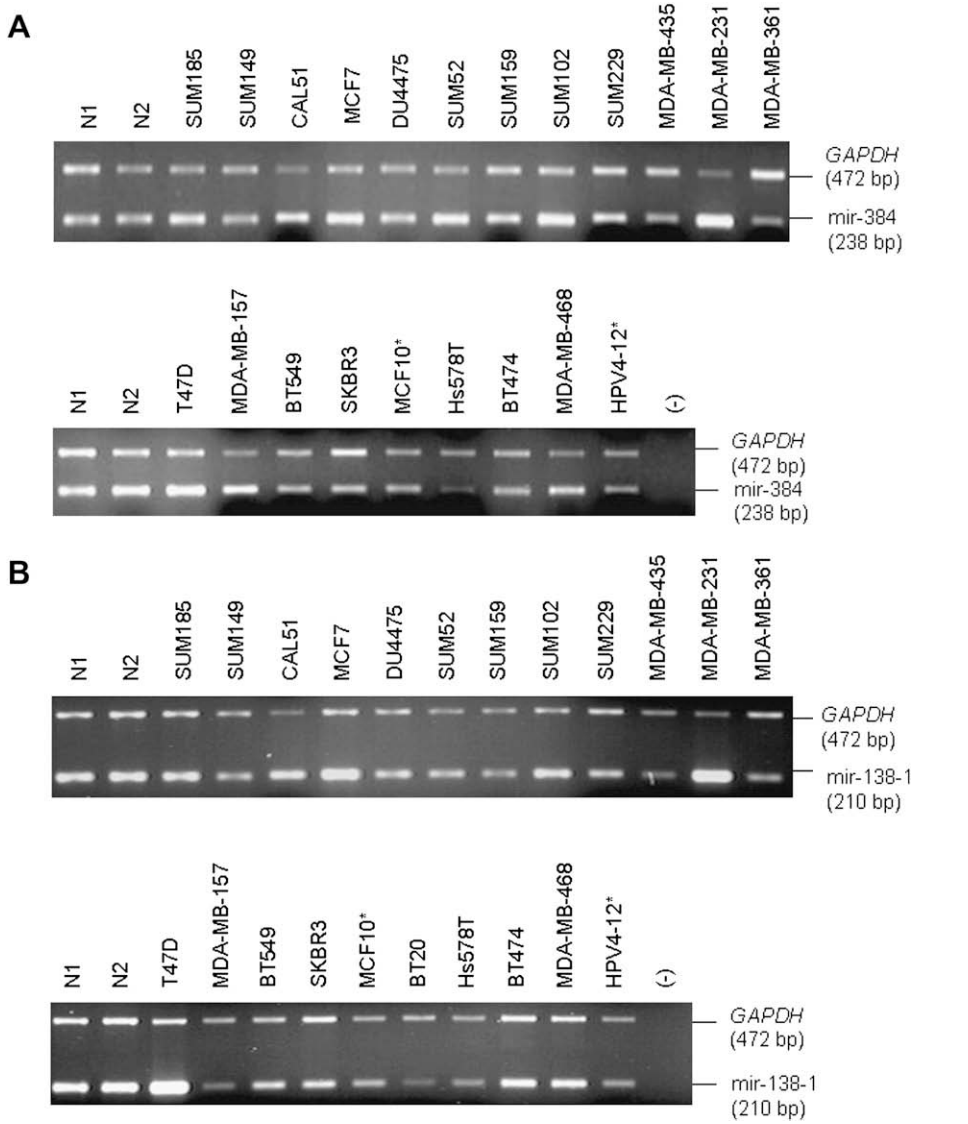

Fig. 2. Genomic gain or loss of microRNA genes detected by semi-quantitative duplex PCR. (A) Hsa-mir-384 (chromosome Xq21.2) and *GAPDH* primers were co-amplified in 20 breast cancer cell lines, 2 immortalized mammary cell lines (denoted by asterisk), and 2 normal DNA controls (N1 and N2). MDA-MB-231 showed more than 2-fold increase (Fig. 1, ▢), whereas Hs578T demonstrated a 0.3-fold decrease (Fig. 1, ▤) in comparison to *GAPDH* and normal control DNA samples. (B) Hsa-mir-138-1 (chromosome 3p21) and *GAPDH* primers were co-amplified in 20 breast cancer cell lines, 2 immortalized mammary cell lines (denoted by asterisk), and 2 normal DNA controls (N1 and N2). Hsa-mir-138-1 was found to be amplified more than twofold in CAL51 and T47D cells (Fig. 1, ▢) and more than fourfold (Fig. 1, ■) in MDA-MB231 cells, compared to *GAPDH* and to normal control DNA samples.

potential LOH and HD of the 90-Mb region of chromosome 13 in some of the cell lines such as MDA-MB-231, MDA-MB-157, and Hs578T. In agreement with this, Eiriksdottir et al. [35] reported 13q31~qq34 as one of the three LOH regions on 13q in breast cancer. Moreover, Hossain et al. [36] reported hsa-mir-17, a member of the 17-92 cluster, as a tumor suppressor in breast cancer cells because expression of hsa-mir-17 was low in breast cancer cell lines and down-regulation of *AIB1* (Amplified in Breast Cancer 1) by hsa-mir-17 resulted in decreased proliferation of breast cancer cells. In the same study, hsa-mir-17 was also shown to abrogate the insulin-like growth factor 1–mediated, anchorage-independent growth of breast cancer cells. Zhang et al. [37] further indicated that 13q31~q34 microRNAs were among the 24 that showed copy number losses in ovarian, breast, and melanoma cells.

A significant amplification pattern was observed for microRNA genes on the 17q22~q23 region. Hsa-mir-301a on 17q22 showed amplification in 18% (4/22) of cell lines. Hsa-mir-21 and hsa-mir-633, located on 17q23, showed consistent and significant amplification in MCF7, MDA-MB-231 (more than fourfold), and T47D. These findings were in concordance with the previous data on 17q23, a frequently amplified chromosomal area that harbors amplified oncogene candidates in breast cancers [21,38]. In addition to coding genes in this known amplicon, amplified microRNA genes are also interesting oncogene candidates, including hsa-mir-21.

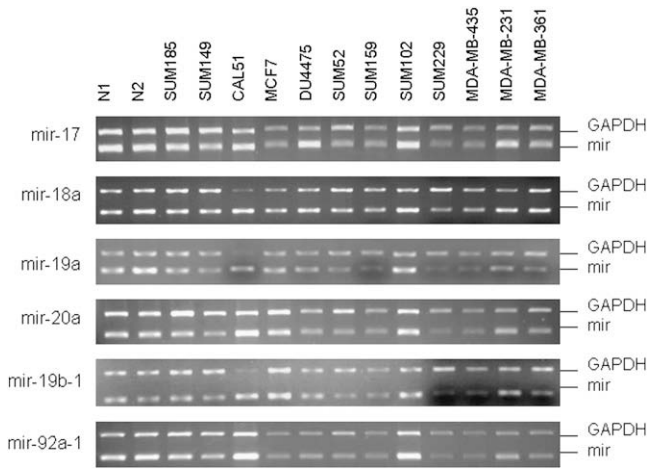

Fig. 3. Semi-quantitative PCR for the microRNA-17-92 cluster. No significant amplification was detected for this microRNA cluster.

Hsa-mir-125b-2 maps to the 16-Mb region of chromosome 21. A 6-centimorgan deletion interval of 21q21 between loci D21S1432 (16 Mb) and D21S1437 (20 Mb) was reported previously [39], with the possibility that one or more tumor suppressor genes associated with breast cancer may exist between these markers. Therefore, consistently decreased fold numbers for this microRNA in two cell lines could be significant and may point out this microRNA gene as a potential tumor suppressor gene. Deletion regions at chromosomal band 21q21 are also reported for other malignancies, such as lung cancers [40] and neuroblastomas [41]. Consistent with our results, hsa-mir-125b-2 was specifically mapped to a HD region of chromosomal band 21q11~q21 in lung cancers [42].

On Xq21, we detected mostly low gain for hsa-mir-384, hsa-mir-325, and hsa-mir-361. The CGP of the Wellcome Trust Sanger Institute data suggest the presence of heterogeneous amplification and regions of loss on this chromosome for the investigated cell lines. Roles and targets of these microRNAs in neoplastic growth are yet to be investigated.

We also chose a representative microRNA (hsa-mir-383) that resides on a chromosomal area (8p21~8p24), which was reported to be mostly lost in tumors but also amplified in some others (Table 1), and performed RT-PCR. As the DNA fold number data did not suggest any loss for this microRNA (suggested low gains), the RT-PCR results were in agreement with the DNA data, showing similar levels of the precursor structure compared to a normal breast cDNA sample (RNA from Ambion, Austin, TX; Fig. 4).

Throughout the study, two immortalized mammary cell lines (MCF10 and HPV4-12) also demonstrated fold differences compared to the control DNA samples for some microRNA genes. For MCF10, we detected fold number increases (2.3-fold for hsa-mir-145 and 2.7-fold for hsa-mir-361) and fold number decreases ($<$ 0.3-fold) of hsa-mir-19a and hsa-mir-125b-2. For the other immortalized cell line, HPV4-12, we detected fold number decreases for hsa-mir-320a and hsa-mir-19b-1. Such genomic changes may

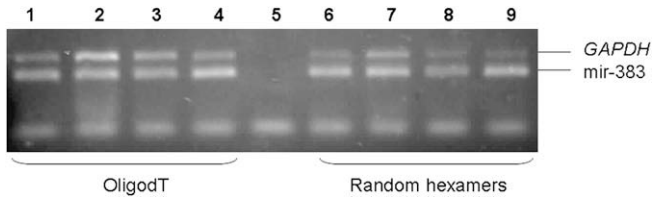

Fig. 4. Duplex RT-PCR for hsa-mir-383 and *GAPDH*. 1. MCF7, 2. MDA-MB-231, 3. HeLa, 4. Normal Breast cDNA, 5. No cDNA, 6. MCF7, 7. MDA-MB-231, 8. HeLa, 9. Normal Breast cDNA. cDNA samples in 1–4 were prepared using oligodT primers and those in samples 6–9 by random hexamers. No significant difference was detected among samples by densitometry.

be expected to result from immortalization and continuous passaging in the laboratories [43].

The internal control of choice in this study, *GAPDH*, resides on the chromosome 12p13 band, which was indicated as a rare amplicon region in breast cancer cells [44]. *GAPDH*, however, resides 3 Mb away from the amplicon boundaries mentioned. Consistent with this, we did not observe a continuous *GAPDH* PCR product amplification pattern in any specific cell line.

In summary, our results suggest that a significant number of microRNA genes on genomic gain/loss regions in breast cancers indeed have DNA level amplification or losses. It should be emphasized that the aneuploidy status of these cell lines may affect the results, and that fold number changes may not always reflect the copy number changes of these microRNA genes. It should also be noted that DNA level copy changes may or may not correlate with the expression data. Another issue even becomes more important for microRNA expression studies when the precursor microRNA levels and mature microRNA levels may not linearly correlate with each other as Lin 28, an RNA-binding protein, acts as a negative regulator and blocks let-7 microRNA processing into the mature form in embryonic cells [45,46].

Such a DNA fold number profile of microRNAs on genomic gain/loss regions may also be useful for delineating boundaries of certain instability regions, as well as for identifying microRNA genes as potential oncogene and tumor suppressor targets of the genomic instability regions, along with protein coding genes. Given the number of target mRNAs that a microRNA can bind, deregulated expression of microRNAs can alter multiple pathways that are important during the initiation or maintenance of the neoplastic growth of cells.

Acknowledgments

We thank Dr. Elizabeth M. Petty from University of Michigan for cell line DNAs, Serkan Tuna for his help in densitometry, and Dr. Sreeparna Banerjee for helpful comments on the manuscript. This work was supported by Turkish Academy of Sciences, GEBIP 2006 and M.E.T.U. internal funds.

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