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Single nucleotide polymorphisms in miRNA binding sites and miRNA genes as breast/ovarian cancer risk modifiers in Jewish high-risk women

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We hypothesized that aberrant gene silencing by miRNA may affect mutant *BRCA* penetrance. To test this notion, frequency of single nucleotide polymorphisms (SNPs; $n = 42$) within predicted miRNA binding sites or miRNA precursors were determined and compared in 363 *BRCA1* mutation carriers: asymptomatic ($n = 160$), breast cancer ($n = 140$) and ovarian cancer ($n = 63$) patients, and in 125 *BRCA2* mutation carriers: asymptomatic ($n = 48$), breast cancer ($n = 58$) and ovarian cancer ($n = 19$) patients. Overall, 16 of 42 SNPs were polymorphic, 11 had a minor allele frequency greater than 5% and 9 of them maintained the Hardy-Weinberg Equilibrium. Based on Cox regression and Kaplan–Meier analyses, statistically significant differences were noted in *BRCA2* mutation carriers by health status in 3 SNPs: CC homozygosity at rs6505162 increased ovarian cancer risk (RR 2.77; $p = 0.028$; 95% CI, 1.11–6.9); heterozygote SNP carriers of rs11169571 had an ~2 fold increased risk for developing breast/ovarian cancer, whereas heterozygotes of the rs895819 SNP had an ~50% reduced risk for developing breast/ovarian cancer. This study provides preliminary evidence for another regulatory level of penetrance of deleterious mutations in cancer predisposition genes.

Carriers of germline *BRCA1* (MIM# 113705) or *BRCA2* (MIM# 600185) genes' mutations are at a substantially increased risk for developing breast and ovarian cancer: the estimated lifetime risk of ~80% for developing breast cancer and ~50% for ovarian cancer.[1,2] However, the considerable variability and the incomplete penetrance in *BRCA1/2* mutation carriers may imply that modifier factors—genetic and environmental—are operative in *BRCA1/2* mutation carriers to affect penetrance. A host of environmental and genetic factors has been evaluated as putative modifiers of *BRCA1/2* mutations.[3,4] Despite these extensive studies, few factors emerged as "true modifiers" by virtue of reproducibility and independent validation.

Key words: miRNA binding sites, miRNA precursors, BRCA1/2 genes, modifier genes, penetrance, SNPs
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Although modification of cancer risk in *BRCA1/2* mutation carriers by other genes has been investigated, the putative role that aberrant gene silencing by microRNAs (miRNA) plays in affecting mutant *BRCA* allele penetrance has not been reported.

miRNA are single-stranded 21–23-nucleotide long RNA molecules involved in regulating the expression of other genes. miRNAs are encoded by genes but not translated into protein. miRNAs are transcribed as pri-miRNA, processed to short stem-loop structures, pre-miRNA in the cell nucleus,[5] which are then processed to mature miRNAs in the cytoplasm to form the RNA-induced silencing complex.[6–8] The miRNA is guided to target sequence at the 3′-UTRs of mRNAs and annealing of miRNA to mRNA inhibits protein translation.[9] Accumulating evidence revealed that 7 nucleotides at the 5′-terminus of miRNAs from position 2 to position 8, called "seed" region, and are essential for their function in miRNA–target binding.[10]

miRNA regulation has a major impact on cellular proliferation and differentiation.[11] There is also evidence that the expression level of several genes and proteins somatically in tumors is also partially regulated by miRNA. Let-7, targeting the oncogene RAS, is downregulated in lung cancers,[12] and miR-15 and 16, targeting the antiapoptotic factor BCL2, are downregulated in chronic lymphatic leukemia (CLL).[13] Furthermore, a germline mutation in the *pri-miR-16-1/15a* precursor was found to cause its reduced transcription in a patient with familial CLL.[14]

A single nucleotide polymorphism (SNP) located in the miRNA binding site of a miRNA target may disrupt miRNA–target interaction, resulting in the deregulation of target gene expression, as was shown in non–small cell lung cancer.[15] Alternatively, a SNP within miRNA precursor may alter miRNA processing and ultimately cause a change in the mature miRNA level, as was recently shown for miR-146a in papillary thyroid carcinoma.[16] Such effects on miRNA levels and function may hypothetically modify cancer risk in *BRCA1 BRCA2* mutation carriers.

The aim of this study was to test and further extend that notion by genotyping SNPs in miRNA binding sites located to selected genes in *BRCA1* and *BRCA2* mutation carriers.

Material and Methods

Study population

The study population was recruited from among individuals counseled and tested at the Oncogenetics Unit, Sheba Medical Center, since January 1, 2000, and from an ongoing study targeting all consecutive breast cancer patients diagnosed and treated at the Sheba medical center from 2002 onwards. All participants recruited from the Oncogenetics unit were high-risk individuals based on well-practiced criteria,[17] and all study participants were unrelated to each other (*i.e.*, only 1 patient per family was included). DNA and relevant clinical demographic and pathological data are already available from these individuals, and the study was approved by the institutional review board, and each patient gave an informed consent. Eligible women were genotyped and found to harbor one of the predominant mutations in Jewish Israeli women in either *BRCA1* (185delAG, 5383InsC and Tyr978X) or *BRCA2* (6174delT and 8765delAG). Genotyping schemes for these predominant mutations were performed as previously described.[18–20] The study population encompassed *BRCA1* mutation carriers and *BRCA2* mutation carriers. Each of these 2 categories was further divided by health status.

1. *BRCA1* mutation carriers—$n = 363$: asymptomatic ($n = 160$), affected with breast cancer ($n = 140$) and affected with ovarian cancer ($n = 63$).
2. *BRCA2* mutation carriers—$n = 125$: asymptomatic ($n = 48$), breast cancer ($n = 58$) and ovarian cancer ($n = 19$).

SNP selection

SNPs within miRNAs binding sites (some experimentally validated) and on miRNA precursors were selected using a bioinformatic approach. Initially, manual compilation from the literature published in peer-reviewed journals of a list of 66 genes that are related to either *BRCA1* or *BRCA2* genes and/or proteins was performed. These are genes encoding for proteins that are that are known to physically interact with either or both proteins (*e.g.*, ATF1[21]) or genes that share a common pathway with BRCA1/2 (*e.g.*, RAD51[22]). After selection of the genes, potential miRNA binding sites within the 3′ UTRs of the genes' transcripts was performed as follows: the Refseq sequences of the transcripts of the candidate genes were downloaded, and the existence of miRNA binding site was subsequently predicted if 6 consecutive nucleotides within the 3′ UTR were complementary to positions 2–7 of a given miRNA, namely, the "seed" region, which is the critical area for miRNA binding to its target gene.[23] This process yielded ~17,500 predicted miRNA binding sites within the 66 BRCA1/2-related genes. Next, we searched for significant SNPs from dbSNP125 (and some SNPs from higher versions) within the predicted miRNA binding sites. We hypothesized that the critical position of such SNPs may alter the binding affinity of a given miRNA to its target gene. This process yielded ~1,000 SNPs within predicted miRNA binding sites. Next, we searched for SNPs within predicted binding sites of miRNAs that are known to be expressed in breast cancer (miRNAs that are not expressed in breast cancer would be less relevant). For this purpose we used published miRNA expression profiles from small RNA libraries[24] of breast cancer tissues and cell lines and unpublished microarray experiments of 24 breast cancer tissues. We compiled a list of >100 miRNAs that were either sequenced in at least 1 clone in breast cancer small RNA library or had a median signal in breast cancer tissues that is higher than the background signal in the microarray experiments. Only 61 SNPs were found within predicted binding sites of miRNAs expressed in breast cancer. We selected 39 SNPs from this list and supplemented these with additional SNPs that were either: (*i*) within *validated* miRNA binding sites within the BRCA1/2-related genes (2 SNPs); (*ii*) within miRNA precursor sequences of miRNA that are expressed in breast cancer (8 SNPs); or (*iii*) within a more reliable predicted binding site (conserved in vertebrates, 7 nts match) than the basic predicted binding site (unconserved, 6 nts match), which is outside the seed-complementary region but is involved in miRNA–mRNA base pairing (1 SNP). Eight SNPs of the resulting 50 SNPs were excluded because they did not fit iPLEX scanning. The final result was a list of 42 SNPs (Table 1) that were selected for genotyping among BRCA1/2 mutation carriers. The flow diagram for SNP selection is shown in Figure 1.

Genotyping using the Sequenom iPLEX™ Assay

SEQUENOM® (La-Jolla, CA) iPLEX™ assay for SNP genotyping allows performing 24-plex reactions on MassARRAY® System (http://www.biocompare.com/newtech.asp?id=1676). Polymerase chain reaction (PCR) primers were designed in a region of ~100 bp around the SNP of interest for first PCR reaction, and an extension primer was designed immediately adjacent to the SNP for the second step of extension PCR during which addition of 1 base, according to the SNP, will occur (primer sequences are available from the authors on request). The assay design was performed in a highly automated fashion by the AssayDesigner Software module. http://www.sequenom.com/Genetic-Analysis/Applications/iPLEX-Genotyping/iPLEX-Overview.aspx. To ensure the accuracy of the results obtained by the sequenom technology, each

Table 1. SNPs chosen for genotyping

	Gene name	SNP name	Chromosome	Observed polymorphism	Validation of SNP	microRNA
SNPs located over microRNA binding sites in genes related to breast cancer						
1	CDKN1A	rs10046116	6	A/C	Unknown	hsa-miR-491
2	IGFBP6	rs1053149	12	A/C	Unknown	hsa-miR-145, hsa-miR-199a
3	IGF1R	rs1065305	15	A/T	Unknown	hsa-miR-449, hsa-miR-34a
4	ATF1	rs11169571	12	C/T	By frequency	hsa-miR-320
5	CTSD	rs11555041	11	C/G	Unknown	hsa-miR-103, hsa-miR-107
6	IGFBP5	rs11575213	2	C/T	By cluster, by frequency	hsa-miR-92
7	MRE11A	rs13447754	11	C/T	Unknown	hsa-miR-181a, hsa-miR-181b, hsa-miR-181c
8	PCNA	rs14453	20	A/T	Unknown	hsa-miR-200c, hsa-miR-200b
9	MRE11A	rs1805361	11	A/G	By cluster, by frequency	hsa-miR-494
10	IGF1R	rs28457673	15	C/G	Unknown	hsa-miR-16, hsa-miR-497, hsa-miR-15b, hsa-miR-107, hsa-miR-103
11	PCNA	rs3626	20	C/G	By frequency, by 2hit-2allele, by hapmap	hsa-miR-92
12	INS	rs3842753	11	A/C	By cluster, by frequency, by 2hit-2allele	hsa-miR-491
13	RB1 (RB)	rs4151631	13	A/T	Unknown	hsa-miR-92
14	RB1 (RB)	rs4151634	13	C/T	Unknown	hsa-miR-494
15	PCAF	rs4858770	3	C/T	By 2hit-2allele	hsa-miR-30d, hsa-miR-30e-5p
16	IGFBP6	rs6413499	12	A/G	By frequency	hsa-miR-141
17	POLR2K	rs7924	8	C/G	By frequency, by submitter, by hapmap	hsa-miR-106b, hsa-miR-106a
18	CTSD	rs8839	11	A/C	By frequency, by 2hit-2allele	hsa-miR-193a
19	BAP1	rs9855479	3	A/G	Unknown	hsa-miR-125a, hsa-miR-125b
20	POLR2K	rs14960	8	A/C	Unknown	hsa-miR-1
21	POLR2K	rs11555067	8	C/T	Unknown	hsa-miR-1
22	ATM	rs227091	11	C/T	By cluster, by frequency	hsa-miR-425-3p
23	KPNA2	rs1059406	17	C/T	Unknown	hsa-miR-106b, hsa-miR-20a, hsa-miR-17-5p, hsa-miR-93, hsa-miR-106a
24	BRCA1	rs4986854	17	C/T	By frequency, by hapmap	hsa-miR-25, hsa-miR-92, hsa-miR-92b, hsa-miR-32
25	BRCA1	rs1799966	17	A/G	By frequency, by submitter, by 2hit-2allele, by hapmap	hsa-miR-326
26	BRCA1	rs4986852	17	A/G	By cluster, by frequency	hsa-miR-149
27	BRCA1	rs4986848	17	C/T	By frequency, by hapmap	hsa-miR-105
28	BRCA1	rs799917	17	C/T	By frequency, by 2hit-2allele	hsa-miR-191
29	BRCA1	rs28897677	17	A/G	Unknown	hsa-miR-574
30	BRCA1	rs28897676	17	C/T	Unknown	hsa-miR-574
31	MRE11A	rs13447758	11	C/T	Unknown	hsa-miR-324-5p
32	NBN (NBS1)	rs11987887	8	A/C	By cluster, by frequency	hsa-miR-186
33	TP53 (P53)	rs916132	17	C/T	Unknown	hsa-miR-29b, hsa-miR-29a, hsa-miR-29c
34	TP53 (P53)	rs916131	17	A/G	Unknown	hsa-miR-151
SNPs located in microRNA precursors that are expressed in breast cancer						
35	hsa-mir-196a-2	rs11614913	12	C/T	Unknown	hsa-miR-196a
36	hsa-mir-92-1	rs9589207	13	A/G	By cluster, by frequency	hsa-miR-92
37	hsa-mir-140	rs7205289	16	A/C	Unknown	hsa-miR-140
38	hsa-mir-423	rs6505162	17	A/C	By cluster, by 2hit-2allele	hsa-miR-423
39	hsa-mir-27a	rs895819	19	C/T	Unknown	hsa-miR-27a
40	hsa-mir-27a	rs11671784	19	A/G	Unknown	hsa-miR-27a
41	hsa-mir-125a	rs12975333	19	G/T	Unknown	hsa-miR-125a
42	hsa-mir-149	rs2292832	2	C/T	By frequency, by 2hit-2allele, by hapmap	hsa-miR-149

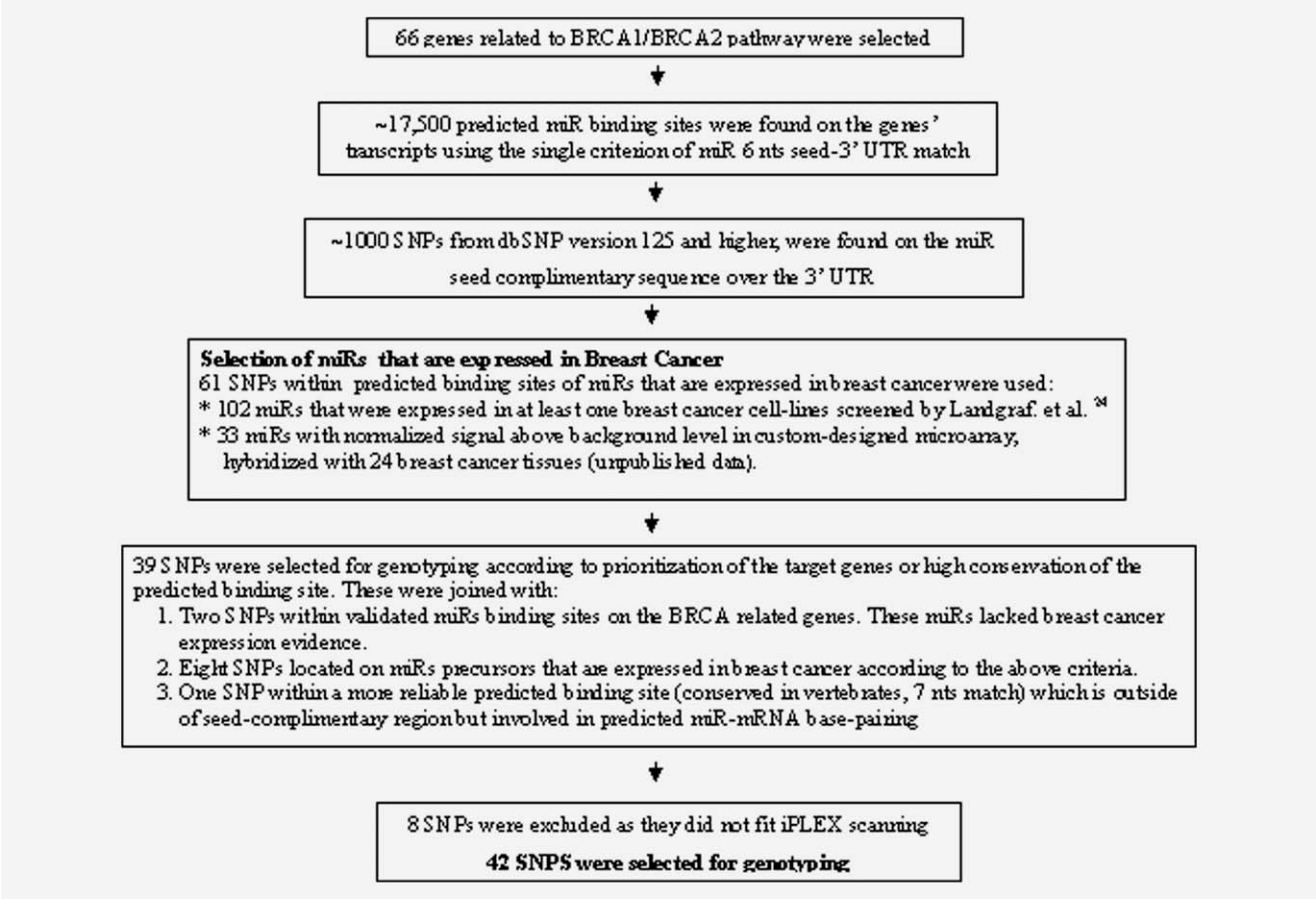

Figure 1. A flow diagram for selecting the SNPs analyzed in this study.

pattern of each SNP (homozygous to either allele and heterozygote) was sequenced in 2–3 samples (data not shown).

Statistical analyses

SNP genotype and allelic distribution were determined by direct counting in the samples. The Arlequin software package (http://lgb.unige.ch/arlequin)[25] was used to detect significant departure from Hardy-Weinberg equilibrium.[26]

Kaplan–Meier test was used to compare age at diagnosis of breast or ovarian cancer between each SNP genotype strata. Cox regression analysis was performed to further investigate the relationship between age at diagnosis of breast or ovarian cancer and SNP genotypes. Both assays were performed using the SPSS program using the V. 15 software. Fisher exact test was used for allele frequency comparisons between group pairs (http://www.quantitativeskills.com/sisa/statistics/twoby2.htm). The Bonferroni corrections (http://home.clara.net/sisa/bonfer.htm) and the false discovery rate (FDR) estimations[27] were applied to individual test statistics.

Results

Patients Characteristics

1. BRCA1 mutation carriers—n = 363; mean age at diagnosis of breast/ovarian cancer or counseling was 44.6 ± 11.7 years (range, 12–91 years). Of these, 279 were carriers of the 185delAG * BRCA1mutation, 3 carriers of the TYR978X * BRCA1 mutation and 81 carriers of the 5382insC *BRCA1 mutation. Ethnic distribution of these Jewish carriers was Ashkenazim, 318; Iraqi, 37; Moroccan, 1; Syrian, 1; Iranian, 6. Health status of these BRCA1 mutation carriers was as follows
   - Asymptomatic *BRCA1* mutation carriers—$n = 160$; mean age at counseling 42 ± 11 years (range, 12–91 years).
   - Breast cancer-affected *BRCA1* mutation carriers—$n = 140$; mean age at diagnosis 44.4 ± 11.5 years (range, 19–82 years).
   - Ovarian cancer-affected *BRCA1* mutation carriers—$n = 63$; mean age at diagnosis 51.7 ± 11.36 years (range, 26–85 years).The differences between the age at diagnosis for breast cancer and age at counseling for asymptomatic cases were not statistically significant.
2. *BRCA2* mutation carriers—$n = 125$; the mean age at diagnosis of breast/ovarian cancer or counseling was 46.86 ± 11.1 years (range, 21–74 years). One hundred twenty-five carriers of the 6174delT * *BRCA2* mutation, all were of Ashkenazi origin.

Table 2. SNP genotype distribution in the BRCA1 and BRCA2 carrier samples[1,*]

SNP (alleles)	Breast cancer			Ovarian cancer			Asymptomatic			Total		
BRCA1												
rs3842753 (C, A)	71	57	10	35	26	3	79	64	20	185	147	33
rs11169571 (T, C)	66	58	12	32	19	11	78	60	12	176	137	35
rs1799966 (G, A)	54	54	24	19	39	5	40	88	21	113	181	50
rs11614913 (T, C)	30	54	48	9	23	29	31	66	59	70	143	136
rs2292832 (T, C)	19	20	47	11	10	13	35	28	43	65	58	103
rs799917 (T, C)	50	58	27	1	10	8	42	94	21	108	191	53
rs3626 (C, G)	1	31	97	2	15	20	6	33	108	9	79	225
rs7924 (C, G)	76	52	10	27	35	3	102	56	3	205	143	16
rs8839 (C, A)	12	59	62	8	25	27	18	71	68	38	155	157
rs6505162 (C, A)[2]	46	59	28	22	21	17	46	76	34	114	156	79
rs895819 (T, C)[2]	61	62	9	35	27	1	75	61	13	171	150	23
BRCA2												
rs3842753 (C, A)	32	23	3	6	12	1	28	18	3	66	53	7
rs11169571 (T, C)	25	26	4	12	6	1	23	22	4	60	54	9
rs1799966 (G, A)	7	24	24	0	9	8	5	23	21	12	56	53
rs11614913 (T, C)	8	27	22	6	6	7	8	22	19	22	55	48
rs2292832 (T, C)	8	8	22	2	2	5	4	2	10	14	12	37
rs799917 (T, C)	10	23	24	16	39	5	5	23	22	16	56	54
rs3626 (C, G)	2	9	44	1	3	13	2	8	39	5	20	96
rs7924 (C, G)	37	17	4	15	4	0	33	15	2	85	36	6
rs8839 (C, A)	24	26	5	10	6	1	25	20	4	59	52	10
rs6505162 (C, A)[2]	22	29	6	9	5	5	9	26	15	40	60	26
rs895819 (T, C)[2]	37	16	2	8	7	2	26	21	2	71	44	6

[1]Only polymorphic SNPs with MAF > 0.05 are included in the table. The cell triads are frequencies of homozygotes at the first allele, heterozygotes and homozygotes at the second allele, respectively. [2]SNPs located in the microRNA precursors sites.
*The total number of genotyped individuals per SNP may be less than the total number of stated patients, as a result of genotyping failures.

- Asymptomatic *BRCA2* mutation carriers—$n = 48$; mean age at counseling 42.8 ± 9.7 years (range, 21–61 years).
- Breast cancer-affected *BRCA2* mutation carriers—$n = 58$; mean age at diagnosis 45.8 ± 9.1 years (range, 25–63 years).
- Ovarian cancer-affected *BRCA2* mutation carriers—$n = 19$; mean age at diagnosis 60.2 ± 10.4 years (range, 39–74 years).

The differences between the age at diagnosis for breast cancer and age at counseling for asymptomatic cases were not statistically significant.

Genotyping results

Overall, 42 SNPs were genotyped in all study participants, 16 exhibited a polymorphic pattern, 11 had a minor allele frequency ≥ 0.05 and 9 of them maintained the Hardy-Weinberg Equilibrium (Table 2).

Three of these 9 *bonafide* polymorphic SNPs showed association with the various health status categories in *BRCA2* mutation carriers: rs6505162, rs11169571 and rs895819. Only 1 SNP (rs3842753) had a significant effect on disease morbidity in *BRCA1* mutation carriers. The SNP associations were detected by both Kaplan–Meier and Cox regression analyses (see below). The frequency of the SNP genotypes in the different test groups is shown in Table 2. Noteworthy, Fisher exact test comparisons not censoring for age at diagnosis did not achieve statistical significance (Table 3).

Breast and ovarian cancer risk in BRCA2 carriers

rs6505162. Kaplan–Meier and Cox regression analyses showed that this SNP affects both ovarian cancer risk and age at diagnosis of breast cancer in *BRCA2* carriers: AA homozygotes developed ovarian cancer at an older age compared with AC/CC carriers. By Cox regression analysis, AC compared with AA carriers had a relative risk (RR) of 2.84 for developing breast cancer ($p = 0.021$; 95% CI, 1.17–6.85);

Table 3. Allele frequency comparison between the cancer-affected individuals and asymptomatic carriers for the relevant SNPs

		rs3842753			rs11169571			rs6505162[1]			rs895819[1]		
Mutation	Status	NN	MAF[1]	p^2	NN	MAF	p	NN	MAF	p	NN	MAF	p
BRCA1	Breast cancer	138	0.28	0.24	136	0.30	0.69	133	0.43	0.36	132	0.30	0.62
	Ovarian cancer	64	0.25	0.17	62	0.33	0.81	60	0.46	0.53	63	0.23	0.19
	Asymptomatic	163	0.32		150	0.28		156	0.46		149	0.29	
BRCA2	Breast cancer	58	0.25	0.54	55	0.31	0.54	57	0.36	0.26	55	0.18	0.19
	Ovarian cancer	19	0.37	0.89	19	0.21	0.26	19	0.40	0.45	17	0.32	0.79
	Asymptomatic	49	0.25		49	0.31		50	0.44		49	0.26	

[1]MAF[] = allele[nucleotide] of minimal frequency. [2]The p value for the same or a stronger association according to Fisher exact test.

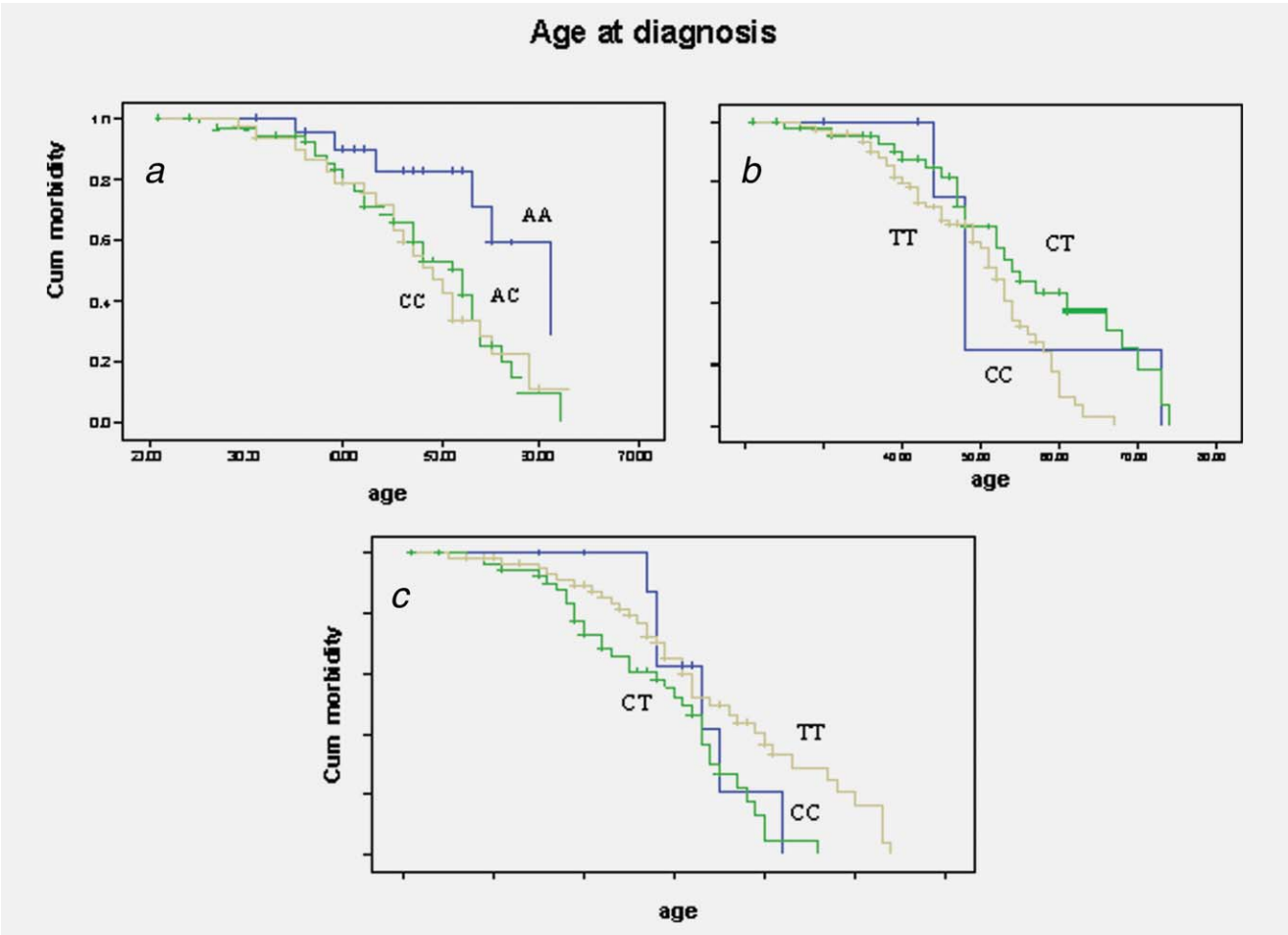

Figure 2. (*a–c*) Kaplan-Meier curves for each of the statistically significant SNPs. Kaplan-Meier results for statistically significant SNPs. (*a*) Results for rs6505162 in *BRCA2* mutation carriers. Median ages at diagnosis of ovarian cancer are AA (blue line) 61 years; AC (yellow line) 51 years; CC (green line) 49 years ($p = 0.017$ AC vs. AA and $p = 0.016$ CC vs. AA). (*b*) Results for rs895819 in *BRCA2* mutation carriers. Median age at diagnosis of breast/ovarian cancer are CT (green line) 53 years; CC (blue line) 57 years; TT (yellow line) 50 years ($p = 0.007$ for CT vs. TT) (*c*) Results for rs11169571 in *BRCA2* mutation carriers. Median ages at diagnosis of breast/ovarian cancer are CC (blue line) 53 years; CT (green line) 49 years; TT (yellow line) 56 years ($p = 0.005$ CT vs. TT). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CC compared with AA homozygotes had a RR of 2.77 for developing ovarian cancer ($p = 0.028$; 95% CI, 1.11–6.9). The results of the Kaplan–Meier analysis are shown in Figure 2*a*.

rs895819. The common allele in our population (primarily Ashkenazi Jews) is the T allele, a finding that is not reported for non-Jewish populations, where the C allele was reportedly the more common allele (http://www.ncbi.nlm.nih.gov/SNP/

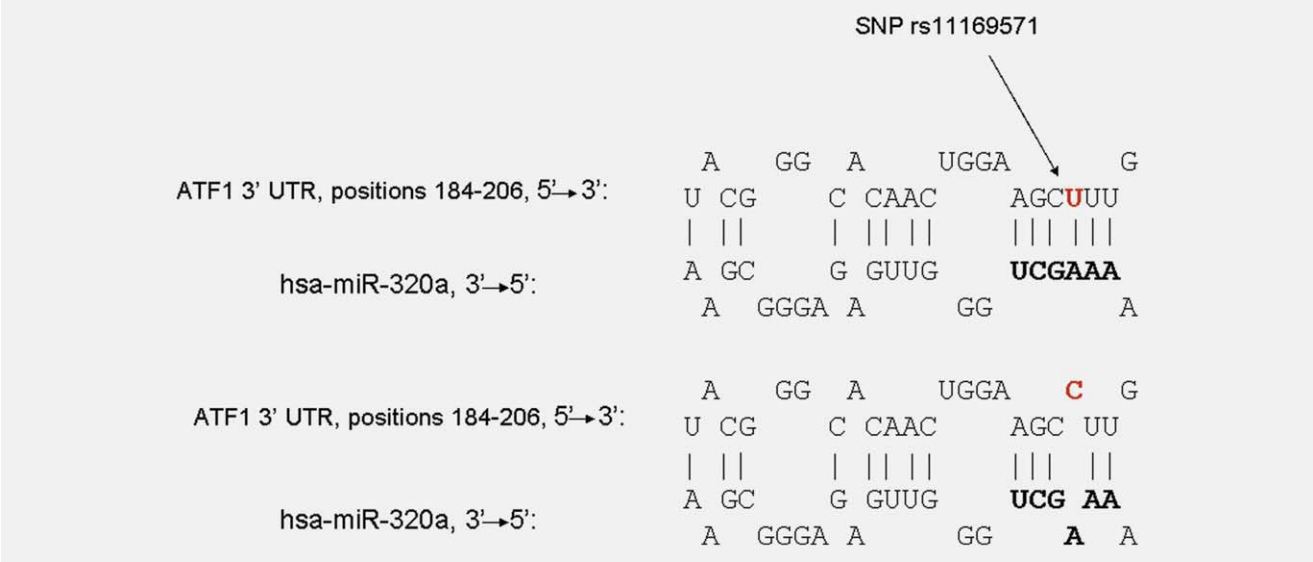

Figure 3. Predicted effect of SNP rs11169571 on miR-320 binding to ATF1 3′ UTR. hsa-miR-320a and its predicted binding site over ATF1 3′ UTR are illustrated one below the other. The bases which are involved in base pairing are illustrated with a vertical line between them. The rest of the bases are illustrated in the upper and lower rows for the 3′ UTR and the microRNA, respectively. hsa-miR-320a is a representative of miR-320a-d microRNA family whose members all share a common seed region: AAAGCU, here in bold. SNP rs11169571 is colored in red. In case the SNP variant is 'U' (corresponding to T in the DNA), then the microRNA seed is in perfect match with the 3′ UTR. Otherwise, there is a mismatch between the seed region and its complimentary sequence. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

snp_ref.cgi?rs=895819). For this latter SNP, by Cox regression analysis, *BRCA2* mutation carriers had a significantly higher risk for developing breast/ovarian cancer in TT homozygous carriers compared with T/C heterozygous carriers: RR 1.96 ($p = 0.013$; 95% CI, 1.16–3.33). Homozygous TT carriers also had higher risk for cancer compared with CC carriers, but this was not statistically significant ($p = 0.36$; 95% CI, 0.56–5.00), possibly because of the small sample size ($n = 6$ of 125). The results of the Kaplan–Meier analysis are shown in Figure 2*b*.

rs11169571. Cox regression analysis showed that among *BRCA2* mutation carriers, being CT heterozygote was associated with a RR of 2.05 for developing breast/ovarian cancer compared with being TT homozygote for the frequent T allele ($p = 0.005$; 95% CI, 1.24–3.39). Carriers of the CC homozygote also had higher risk for cancer that homozygous wild-type TT carriers, but this increased risk did not reach statistical significance ($p = 0.45$; 95% CI, 0.58–3.4), possibly because of the small number of carriers of that genotype (9 of 125). Kaplan–Meier results are shown in Figure 2*c*.

Ovarian cancer risk and age at diagnosis in BRCA1 carriers

rs3842753. Both Kaplan–Meier and Cox regression analyses showed that homozygosity for the A allele at this SNP affects ovarian cancer risk as well as age at diagnosis of ovarian cancer in *BRCA1* carriers: AA carriers develop ovarian cancer at an older age compared with either AC or CC carriers. Cox regression analysis showed that AC heterozygotes compared with AA homozygotes had a RR of 4.78 for developing ovarian cancer ($p = 0.015$; 95% CI, 1.36–16.86), and CC homozygotes have a RR of 3.35 for developing ovarian cancer compared with AA homozygotes ($p = 0.051$; 95% CI, 0.99–11.29).

Discussion

In this study, 4 SNPs located at sites involved in miRNA regulatory action were found to have a statistically significant effect on breast/ovarian cancer risk in *BRCA1/2* mutation carriers: rs6505162, rs895819, rs11169571 and rs3842753.

Noteworthy, that both miRNA precursor SNPs (rs6505162 and rs895819) were involved in the associations. The FDR of this result was estimated to be no more than 0.052, taking into account that the tests were performed in 2 *BRCA1* and *BRCA2* samples. The strong association of the miRNA binding SNP rs11169571 was preserved after Bonfferoni correction taking into account the number of SNPs tested (data not shown).

The SNP rs6505162 is located within hsa-mir-423 miRNA precursor, miRNA whose activity was reportedly increased somatically in breast cancer.[28] A specific genotype within this SNP was associated with decreased risk to developing breast cancer in *BRCA2* mutation carriers. The mechanism by which the SNP affect cancer risk is elusive for several reasons: the SNP is located outside the mature miRNA sequence, either sequence variant of the SNP is not predicted to affect RNA secondary structure by RNAfold program, and the specific genes regulated by this miRNA are not known.

BRCA2 mutation carriers who were also heterozygote SNP carriers (C/T) of rs11169571, a SNP located at ATF1 gene predicted binding site of hsa-miR-320 family (a-d) seed, had about a 2-fold increased risk for developing breast/ovarian cancer. ATF1 (activating transcription factor 1) is a gene that

encodes for a DNA binding protein and cAMP-inducible transcription factor (cAMP-responsive enhancer-binding protein, such as CREB).[29] miR-320,[30] colocalizes to genomic areas displaying DNA copy loss in ovarian cancer, breast cancer and malignant melanoma.[31] Blenkiron *et al.*[32] reported an association between breast cancer and low levels of miR-320 expression.

The exact mechanistic biological effect of the rs11169571 SNP on miRNA binding or on gene regulation is not known. However, because SNP rs11169571 is located on a binding site of miR-320 seed, it is possible that the SNP over the ATF1 binding site interferes with the annealing of mir-320 to ATF1 mRNA, thus affecting regulation of the gene and promoting uncontrolled cellular proliferation. According to this scenario, the "T" allele is part of a functional miRNA binding site, whereas the "C" allele causes nonfunctionality of the miRNA binding site (Fig. 3). Several other miRNAs may bind to this ATF-1 3′ UTR area according to seed base-pairing criterion, including miR-524-5p, miR-9*, miR-330-3p, and miR-520d-5p, but miR-320 is known to be expressed in breast cancer[23,32] and therefore might be more relevant to ATF1 regulation. Although miR-320 predicted binding site is not conserved beyond primates, the existence of yet another miR-320 seed complimentary site upstream on the ATF1 3′ UTR (positions 90–95 over the 3′ UTR) improves reliability of the target prediction.

In this study, *BRCA2* mutation carriers who were heterozygous (C/T) to SNP rs895819 had a significantly lower risk to develop breast/ovarian cancer compared with homozygous (T/T) mutation carriers. The SNP is located in the miRNA hsa-mir-27a precursor. hsa-mir-27a is reportedly downregulated in breast, colon, lung, pancreas, prostate and gastric cancer[33,34] and upregulated in head and neck cancer cell lines.[35] Mechanistically, it is plausible (yet unproven) that the heterozygote SNP is affecting the ability of the premature miRNA to mature and become a functioning miRNA. Noteworthy, either SNP variants of rs895819 (as well as those for rs6505162) are not predicted by the RNAfold program to have an effect on the secondary structure of the miRNA (data not shown). However, these sequence variants may affect miRNA processing by altering protein binding to miRNA precursor. Such a mechanism has been demonstrated for hnRNP A1.[36]

In support of the notion that miRNAs may play a role in determining breast cancer risk in *BRCA1/2* mutation carriers, Shen *et al.*[37] have recently reported that 2 rare sequence variants in the precursors of miR-30c-1 and mir17 have an functional *in vitro* effect on binding to their binding sites, residing in the 3′ UTR of the *BRCA1* gene, and that they are exclusively detected in non-*BRAC1* carriers. Similarly, a sequence variant in the 3′UTR of the ESR1 gene that serves as miRNA binding site for mir-453 was associated with premenopausal and familial breast cancer risk.[38] In a large case–control study from China, SNPs within miRNA precursors (hsa-mir-196a2 and hsa-mir-499) were associated with significantly increased risks of breast cancer.[39] Finally, SNPs within miRNA binding sites in one of the integrin genes (ITGB4) were reported to be associated with disease outcome in Swedish breast cancer cases.[40]

The limitations of the study should be pointed out. The number of affected and asymptomatic individuals was limited, especially for *BRCA2* carriers. Most individuals were of a genetically homogeneous population, Ashkenazim, and most carried 1 of 3 mutations. The extent to which these data could be validated in a larger group of genetically heterogeneous mutation carriers with a diversity of germline *BRCA1 BRCA2* mutations remains unknown. The association of the SNPs to miRNA expression or function should also be validated experimentally.

In conclusion, this preliminary study shows that carriers of a deleterious *BRCA2* mutation who carry an additional SNP that possibly affects miRNA regulatory actions have a modified risk for developing cancer than identical mutation carriers who are wild type for that specific SNP. Taken together with the recent data on other populations, and if confirmed and validated by additional, independent studies, encompassing more carriers or diverse ethnic origins, then this provides evidence for another regulatory level of penetrance of deleterious mutations in cancer predisposition genes.

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