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# **Genetic variants within miR-126 and miR-335 are not associated with breast cancer risk**

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## Abstract

MicroRNAs (miRNAs) are 20~22 nt non-coding RNAs which promote the degradation of target mRNAs or repression of the translation of mRNAs by sequence specific targeting. Many miRNAs are considered as oncogenes or tumor suppressors. MiR-126 and miR-335 play roles in the suppression of breast cancer metastasis by inhibiting tumor growth, proliferation and cell invasion. The effects of SNPs within the two miRNAs are still unknown. In our study, we analysed two SNPs, rs4636297 within miR-126 and rs41272366 within miR-335, in three study populations for a putative association with breast cancer risk. We compared the genotype and allele frequencies of rs4636297 and rs41272366 in 2854 cases versus 3188 controls of the three study populations independently and combined. None of the performed analyses showed statistically significant results. In conclusion, our data suggest that the two genetic variants within miR-126 and miR-335 are not associated with breast cancer risk.

## Introduction

MicroRNAs are a cluster of small endogenous non-coding RNAs (20~22 nt) that can bind to the 3' untranslated region of their target messenger RNAs (mRNAs) and lead to degradation or translation suppression of mRNAs [1, 2]. A single miRNA can repress the expression of hundreds of proteins [3]. MiRNAs are involved in the regulation of several cellular pathways including pathways important for cancer development such as immune system regulation, haematopoiesis, angiogenesis, cell proliferation, differentiation and apoptosis [4-9]. Elevated or decreased expression of miRNAs has been found in various tumor types including breast cancer [10, 11]. Some miRNAs thereby have been considered as tumor suppressors or oncogenes [12-14]. They have been shown to be involved in cancer initiation and progression by suppressing the expression of cancer-related genes [15]. Notably, miRNA processing defects also increase the risk of tumorigenesis [16]. Furthermore, miRNA expression profiles can be molecular markers for cancer diagnosis and therapy [17, 18].

Breast cancer accounts for one fourth of all female cancers, making it by far the most common cancer in women worldwide, and after lung cancer the second most frequent cancer (10.4 % of all cancers) in the world [19]. Taking the U.S. female population as an example, approximately one in eight women will develop breast cancer at some time in their lives [20]. Unfavourable combinations of polymorphic genetic variants in low-penetrance susceptibility genes contribute to breast cancer risk. Most of these susceptibility genes have not been discovered yet [21, 22].

Tavazoie and co-workers reported miR-126 and miR-335 as metastasis suppressor genes. They found that restoring the expression of miR-126 and miR-335 in malignant breast cancer cells can suppress the metastasis to lung and bone [23]. MiR-126 inhibits the tumour growth and proliferation, whereas miR-335 suppresses metastatic cell invasion [23]. Mutations and single nucleotide polymorphisms (SNPs) in miRNAs or miRNA target sites have been shown to be associated with the risk of various cancers [15, 24-28]. Some SNPs located in the miRNAs or their flanking regions are reported to have potential impact on the maturation of miRNAs [29]. In order to study the potential association of SNPs within miR-126 and miR-335 with breast cancer risk, we sequenced the miR-126 and miR-335 and their flanking regions ( $\pm 200$  bp) and verified the presence of three SNPs, rs4636297, rs3807348 and rs41272366. In our previous study, rs3807348 was genotyped, whereas rs4636297 and

rs41272366 were not investigated as they could not be analysed by the TaqMan genotyping assay [27]. Here, we have investigated rs4636297 and rs41272366 by the MALDI-TOF mass spectrometry (Sequenom, CA) in three different study populations for an association with breast cancer risk.

## Materials and methods

### Study populations

#### **The familial breast cancer study cohort of the German Consortium of Hereditary Breast and Ovarian Cancer (GC-HBOC):**

Genotyping was performed on genomic DNA of *BRCA1/2* mutation-negative index patients from 1217 German breast cancer families, comprising a subset of 115 bilateral breast cancer cases, versus 1422 unrelated healthy German women. *BRCA1/2* mutation-negativity in the unrelated breast cancer cases had been tested by applying mutational screening of all exons by denaturing high performance liquid chromatography (DHPLC), and followed by direct sequencing of conspicuous exons [30]. Breast cancer samples were collected during the years 1997–2007 by seven centres of the GC-HBOC (centres of Heidelberg, Wuerzburg, Cologne, Kiel, Duesseldorf, Munich and Berlin, see authors' affiliations). German index patients were first diagnosed with breast cancer and then referred to a family registry. All the samples from the breast cancer patients were obtained around the diagnosis. The informed consent for the study was given to all breast cancer patients.

The control population included unrelated healthy female blood donors collected by the German Red Cross Blood Service of Baden-Wuerttemberg-Hessia and the Institute of Transfusion Medicine and Immunology (Mannheim), sharing the same ethnic background with the breast cancer patients (Caucasian/German population). All control individuals were healthy when donating their blood. None of the control individuals had a reported family history of breast cancer. Age distributions in controls and breast cancer cases were similar (controls: mean age 45.8 years old, median age 49 years old, range from 18 to 68 years old; cases: mean age 46.2 years old, median age 46 years old, range from 19 to 87 years old). The controls were randomly selected during the years 2004–2007 for this study and no further inclusion criteria were applied during recruitment. The study was approved by the Ethics Committee of the University of Heidelberg (Heidelberg, Germany). According to the German

guidelines for blood donation, all blood donors were examined by a standard questionnaire. The informed consent for the study was given to all participants.

### **The Breast Cancer study of the University Clinic Heidelberg (BSUCH):**

Genotyping was performed on genomic DNA of from 1115 German breast cancer patients recruited at the Department of Obstetrics and Gynecology of the University Hospital in Heidelberg, Germany, between 2006 and 2009. All patients gave informed written consent. The study was approved by the Ethics Committee of the University of Heidelberg (Heidelberg, Germany) and all patients gave informed written consent. The control population included 1236 unrelated healthy female blood donors collected by the German Red Cross Blood Service of Baden-Wuerttemberg-Hessia and Institute of Transfusion Medicine and Immunology (Mannheim), as described above. The controls were randomly selected during the years 2006–2008 for this study. Age distributions in controls and breast cancer cases were similar (controls: mean age 52.5 years old, median age 56 years old, range from 30 to 69 years old; cases: mean age 54.8 years old, median age 56 years old, range from 21 to 88 years old).

### **The ESTHER/VERDI Study:**

Genotyping was performed on genomic DNA of from 513 German breast cancer patients, recruited in the German Federal state of Saarland between 1997 and 2003, versus 520 unrelated healthy German female controls recruited from the Saarland's general population in 2000-2002. Recruitment of cases was performed by all hospitals providing primary treatment for breast cancer patients in Saarland as a part of the VERDI study [31] and the ESTHER II study [32]. 94 % of the samples from the breast cancer patients were obtained after the surgery. Controls were recruited by general practitioners in Saarland during a general health screening examination in the context of the ESTHER I study [33]. For this analysis, 520 women, frequency matched by age to the cases, were randomly selected from ESTHER I. The studies were approved by the Ethics Committee of the University of Heidelberg (Heidelberg, Germany) and of the Medical Association of Saarland (Saarbruecken, Germany). Informed consent was obtained in each case. Age distributions in controls and breast cancer cases were similar (controls: mean age 62.3 years old, median age 63 years old, range from 49 to 75 years old; cases: mean age 60.5 years old, median age 62 years old, range from 30 to 79 years old).

### **SNP verification and sequencing**

In order to verify annotated SNPs from the SNP database (NCBI) and to identify potential new SNPs, miR126 and miR335 genes, including pre-miRNAs and about  $\pm 200$  bp flanking regions, were amplified by standard PCR (in order to unfold the secondary structure of miRNAs, 5 % DMSO was added to the PCR buffer). PCR products were purified with the ExoSAP-IT purification kit (USB Corp.) and were sequenced in one of the directions by the 3130XL Genetic Analyzer (Applied Biosystems). Sequencing results were analyzed by the Sequencing Analysis 5.2 software (Applied Biosystems). As a result, three SNPs, rs4636297, rs3807348 and rs41272366, were verified in these segments.

### **Genotyping by MALDI-TOF mass spectrometry**

We investigated the genotype frequencies of rs4636297 and rs41272366 in this study by the MALDI-TOF mass spectrometry (Sequenom MassArray, San Diego, CA). rs3807348 has already been examined in our previous study by a TaqMan genotyping assay.

Assays were designed by the Sequenom Assay Design software v2.0.7.0. The whole process followed the protocol of multiplexed genotyping reactions using iPLEX Gold for MassArray. At the primary PCR step, DNA was amplified under following conditions: 95 °C for 2 min, then 45 cycles of (95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min), and a further extension at 72 °C for 5 min before storage at 4 °C. After purification by the SAP kit, the PCR products were treated by the extend reaction with iPLEX Extend Cocktail (following the instruction of Sequenom). The microplate was thermocycled as follows: 94 °C for 30 s, then 50 cycles of (94 °C for 5 s then 4 cycles of ( 52 °C for 5 s, 80 °C for 5 s )), a final extension at 72 °C for 3 min and stored at 4 °C. After cleaned by CLEAN Resin, the samples were dispensed to a 384 SpectroCHIP by the Nanodispenser. The chip was analyzed by a Bruker Autoflex Mass Spectrometer system. Data were collected by the SpectroACQUIRE v3.3.1.3 software and visualised by the MassArray Typer v4.0 TyperAnalyzer software. Sequences of primers are available upon request.

### **Statistical analysis**



The Hardy–Weinberg equilibrium [32] test was applied using the chi-square ‘goodness-of-fit’ test offered as a tool by the Institute of Human Genetics, Technical University Munich, Munich, Germany (<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>). Genotype-specific odds ratios (OR), 95 % confidence intervals (CI) and *P* values were computed by unconditional logistic regression with SAS version 9.1 (SAS Institute Inc., Cary, NC). Age, treated as a continuous variable was included in the regression as a covariate. Study population, treated as a discrete variable was included in the regression as a covariate. *P* values were calculated using two-sided chi-square test. The meta analysis was done by STATA 9.0. Power was calculated using the power and sample size calculation software PS version 2.1.31 (<http://www.mc.vanderbilt.edu/prevmed/ps/index.htm>) [34].

## Results

We sequenced the DNA segments including the pre-miR-126, pre-miR-335 and their respective flanking regions ( $\pm 200$  bp) in more than 100 randomly chosen familial breast cancer cases. Using this approach three SNPs, rs4636297, rs3807348 and rs41272366, were verified in these segments. We were able to localize rs4636297 12 bp downstream of pre-miR-126 and rs41272366 20 bp downstream of pre-miR-335. Neither these two SNPs themselves nor their linkage SNPs ( $r^2 \geq 0.8$ ) had been analysed, so far, by the Cancer Genetic Markers of Susceptibility genome wide association study (CGEMS) (<https://caintegrator.nci.nih.gov/cgems/browseSetup.do>). The CGEMS is a whole-genome association study conducted by the National Cancer Institute (NCI) enterprise to identify breast cancer susceptibility genes using Illumina HumanHap550 assays on approximately 1200 breast cancer patients and 1200 controls. The additional two annotated SNP in pre-miR-126 (not validated SNPs in dbSNP), rs34051017 and rs7030829, could not be verified in our sample set possibly due to wrong annotations or very low frequency.

Genotype analysis of the two SNPs was performed on genomic DNA of three breast cancer study populations (GC-HBOC, BSUCH, ESTHER/VERDI, see materials and methods). All of the samples previously genotyped by sequencing were re-genotyped by the MALDI-TOF mass spectrometry assay and attained a concordance rate of 100 %. The MALDI-TOF mass spectrometry genotyping results were validated by re-genotyping 5 % of all the samples attaining concordance rates of 100 % for all the investigated SNPs. Genotype distributions in controls and cases were consistent with the Hardy-Weinberg equilibrium [32].

The results were adjusted by age and studies with the SAS software version 9.1. Allele and genotype frequencies of rs4636297 and rs41272366 were comparable in breast cancer cases and controls. rs4636297: ([A] vs [G], OR = 1.01, 95 % CI 0.94-1.10,  $P = 0.73$ ; [GA] vs [GG], OR = 1.01, 95 % CI 0.89-1.13,  $P = 0.93$ ; [AA] vs [GG], OR = 1.04, 95 % CI 0.87-1.23,  $P = 0.69$ ;  $P_{\text{trend}} = 0.73$ , Table 1) and rs41272366: ([A] vs [T], OR = 0.90, 95 % CI 0.69-1.17,  $P = 0.43$ ; [AT] vs [TT], OR = 0.88, 95 % CI 0.68-1.15,  $P = 0.35$ ;  $P_{\text{trend}} = 0.43$ , Table 1), showing no association with breast cancer. The associations of the two SNPs with breast cancer were also analysed after stratification by age (age < 50 years old and  $\geq 50$  years old). However, none of the subgroups showed any significant association with breast cancer risk. The associations of the two SNPs with breast cancer were also analysed by single study population separately, but no significant association was observed. Meta-analysis did not indicate any between-study heterogeneity (for rs4636297,  $P_{\text{heterogeneity}} = 0.528$ ; for rs41272366,  $P_{\text{heterogeneity}} = 0.275$ , Figure 1).

## Discussion

More and more miRNAs are reported to be involved in the process of cancer [17]. MiR-126 and miR-335 have been reported as tumor suppressor genes [23]. They can inhibit tumor development and metastasis through negatively regulating a set of oncogenes. MiR-126 is involved in the angiogenesis. It binds to the 3' untranslated region of VEGF and subsequently negatively regulates the VEGF pathway [35, 36]. MiR-335 suppresses metastasis and migration through targeting the progenitor cell transcription factor SOX4 and extracellular matrix component tenascin C [23]. Expression of miR-126 and miR-335 is lost in the majority of primary breast tumors from patients who relapse, and the loss of expression of either miRNA is associated with poor distal metastasis-free survival [23].

Our work focused on the SNPs in the breast cancer related tumor suppressor genes, miR-126 and miR-335. As it has been reported that a mutation or a SNP in a miRNA gene can affect the transcription and/or process of the pri- and pre-miRNAs [29], we have studied not only the SNPs in the pre-miRNAs but also vicinal SNPs in miRNA flanking regions (about  $\pm 200$  bp). Given our sample size, we had a power of 80 % ( $\alpha = 0.05$ ) to detect an OR of 1.18 for SNP rs4636297 and an OR of 0.66 for SNP rs41272366.

Both SNP rs4636297 and rs41272366 are located in the flanking region of pre-miRNAs, their effects on miRNA secondary structures and free energy is uncertain. We used the RNAfold

program [37] (<http://www.bioinfo.rpi.edu/applications/hybrid/>) to predict the most stable secondary structure and free energy of both wild-types and variants. Comparing to the wild-type [G] allele, the [A] variant of rs4636297 has no effects on the secondary structure of pre-miR-126 itself, but effects the secondary structure of flanking region. Meanwhile, the free energy  $\Delta G$  is increased from -52.7 kcal/mol of the wild-type to -51.2 kcal/mol of the [A] allele variant. The effect of [A] allele in rs41272366 is similar and has no impact on the secondary structure of pre-miR-335. The [A] allele increases the free energy  $\Delta G$  from -54.6 kcal/mol of the wild-type to -54.2 kcal/mol of the variant. However, neither of the two SNPs affects the secondary structure of pre-miRNAs. Therefore, it is very likely that neither rs4636297 nor rs41272366 has effect on the miR-126 and miR-335 maturation and subsequently are not associated with breast cancer risk.

Recently, some polymorphisms in miRNAs have been reported to be associated with metastasis or survival of cancer patients [38-40]. Since miR-126 and miR-335 have been reported to be involved in tumor development, angiogenesis and distant metastasis, it is possible that these SNPs affect breast cancer prognosis such as metastasis and overall survival. Unfortunately, these data is not available for us from all study cohorts.

In conclusion, by investigating three study populations, we have not found an association between the SNPs rs4636297 and rs41272366 and breast cancer risk. All the three different studies consistently showed negative results. However, it would be interesting to investigate the association between these SNPs and breast cancer prognosis in the future.

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## Table

**Table 1. Genotype frequencies of rs4636297 and rs41272366 in the GC-HBOC, BSUCH and ESTHER study populations**

SNP	Genotypes	Case (%)	Control (%)	OR <sup>a</sup>	95 % CI	<i>P</i> <sup>b</sup>
miR-126 rs4636297	GG	957 (39.5)	1093 (39.9)	1.000		
	GA	1136 (46.9)	1287 (47.0)	1.005	0.893-1.132	0.9278
	AA	327 (13.5)	361 (13.2)	1.036	0.871-1.232	0.6925
	[A]vs[G]			1.014	0.936-1.099	0.7327
						<i>P</i> <sub>trend</sub> = 0.7315
miR-335 rs41272366	TT	2283 (95.7)	2580 (95.2)	1.000		
	AT	102 (4.3)	130 (4.8)	0.880	0.675-1.149	0.3478
	AA	1 (0)	0 (0)	-	-	0.9665
	[A]vs[T]			0.901	0.694-1.170	0.4342
						<i>P</i> <sub>trend</sub> = 0.4307

<sup>a</sup> chi-square test for trend.

<sup>b</sup> adjusted for age and study populations; all analyses done with SAS Version 9.1 Proc Logistic.

## Figure

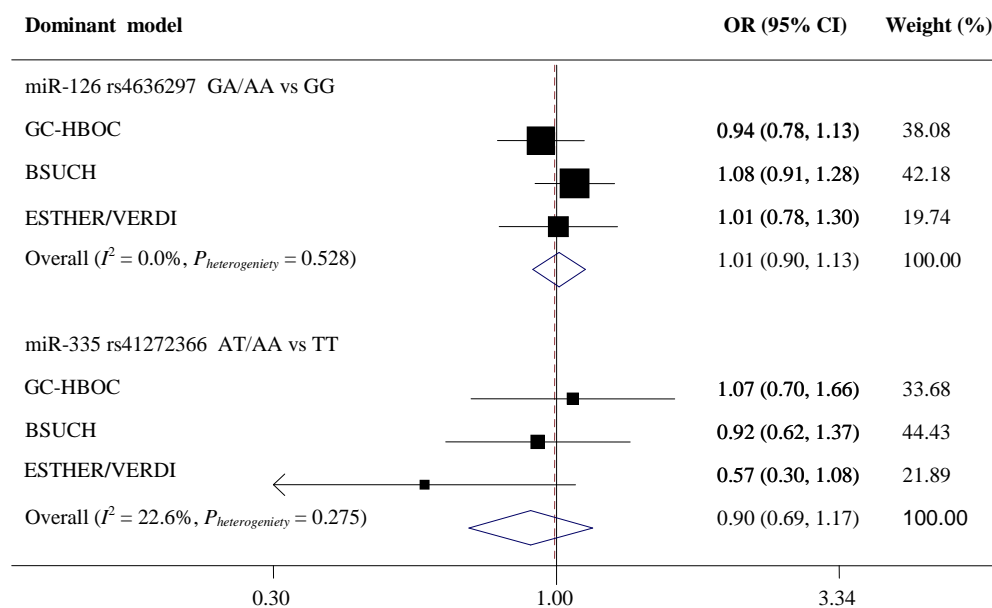


Figure 1. ORs (log scale) of rs4636297 and rs41272355 for variant genotypes compared with the wild type genotypes in dominant genetic model.