Genetic Polymorphism of *miR-196a* as a Prognostic Biomarker for Early Breast Cancer

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Abstract. *Background: As microRNAs (miRNA) may play important roles in tumorigenesis by regulating the expression of proto-oncogenes or tumor suppressor genes, the present study analyzed single nucleotide polymorphisms (SNPs) located in miRNA and miRNA-binding sites of various genes and their impact on prognosis for 452 patients with early breast cancer. Materials and Methods: Three SNPs of miR-196a (rs3746444, rs11614913, and rs1044129) were selected using in silico analysis and genotyped using the Sequenom MassARRAY. Results: The median age of patients was 48 years, and 283 (62.6%) were estrogen and/or progesterone receptor (ER/PgR)-positive, 86 (19.0 %) had human epidermal growth factor receptor 2 (HER2)-overexpressing, and 77 (17.0%) had triple-negative early breast cancer. During the median follow-up of 6.9 years, 67 (14.8%) relapses and 55 (12.2%) deaths were recorded. Among the three polymorphisms, the C allele of miR-196a rs11614913T>C was significantly associated with worse disease-free (DFS) and distant DFS (DDFS) when adjusted for clinical and pathological parameters. In particular, the prognostic impact of rs11614913 was limited to the hormone receptor-expressing subtype, where the patients bearing the CC genotype showed worse survival in terms of DFS and DDFS compared with the patients with the TT or TC genotype as a recessive model (hazard ratio=2.610, p=0.003 for DFS; hazard ratio=2.730, p=0.013 for DDFS). Conclusion: The current study provides evidence that the miR-196a rs11614913T>C polymorphisms are possible prognostic biomarker for patients with hormone receptor-expressing early breast cancer.*

Breast cancer is a common malignant tumor, affecting women at an increasing rate of incidence in many countries and is mostly diagnosed at an early stage *via* the widespread use of screening. Although several prognostic criteria have already been introduced to assist management after curative surgery for early breast cancer (EBC), the need for molecular markers has always been strongly suggested in order to discriminate individual variability and thus predict relapse or survival in patients with similar clinical status, especially when considering that adjuvant regimens containing more toxic chemotherapeutic agents, such as anthracylines, are acknowledged for their efficacy over survival in patients with EBC (1).

MicroRNAs (miRNAs) are a class of small, endogenous, non-coding RNAs able to regulate gene expression by translational repression or mRNA degradation of the target, thereby affecting critical functions in various physiological processes, ranging from cell proliferation to apoptosis (2, 3). Interestingly, most miRNAs are down-regulated in cancer cells and miRNA repression enhances cellular transformation and tumorigenesis, meaning that altering the function of miRNAs can affect tumor development and progression (4). Moreover, recent studies have demonstrated a relationship between the aberrant expression of miRNAs and cancer susceptibility, prognosis, and responsiveness to treatment (5-7). Most miRNAs bind to target sequences located within the 3'-untranslated region of mRNAs, resulting in the cleavage of the target mRNAs or repression of their translation (8). Thus, polymorphisms of miRNAs or within the miRNA-binding sites of the target genes that are implicated in cancer or that function as tumor suppressors or oncogenes could contribute to carcinogenesis or progression by altering the miRNA–mRNA interaction and thereby affecting the expression of the miRNA targets, as shown in previous studies (9-11).

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Polymorphisms in miRNA genes and their target sites are rapidly being identified and investigated in human cancer as a novel class of variation. However, most studies have focused on how their de-regulation can lead to cancer in terms of risk of cancer development. Accordingly, this study investigated whether three target variants of miRNAs or miRNA binding sites, selected using web-based data, were associated with the prognosis for Korean patients with EBC who underwent curative surgery.

Materials and Methods

Patients' characteristics. Four hundred and fifty-two female patients who underwent surgery for EBC at Kyungpook National University Hospital (KNUH) between June 2000 and August 2008 were enrolled for evaluation in the current study. Patients with ductal carcinoma *in situ*, lobular carcinoma of the breast, or who underwent any type of neoadjuvant treatment prior to surgery were excluded. The patient data was obtained from the KNUH breast cancer registry and patient files. The tumors were classified and staged according to the WHO classification (12) and TNM staging system (13). The present study was approved by the local Ethics Committee of KNUH (No. 08-1008).

Selection of polymorphisms. This study selected three previously identified single nucleotide polymorphisms of *miR-196a* (SNPs; rs3746444, rs11614913, and rs1044129) that exhibited a potential association with breast cancer in previous studies and passed the selection criteria of a minor allelic frequency of more than 0.01 based on SNP databases (HapMap data, http://hapmap.ncbi.nlm.nih.gov).

Genotyping of polymorphisms. The genomic DNA of fresh-frozen breast tissue taken at the time of surgery was extracted using a Wizard genomic DNA purification kit (Promega, Madison, WI, USA). The three selected SNPs including two miRNA variants (rs3746444 and rs11614913) and one miRNA-binding site variant (rs1044129) were determined using the Sequenom MassARRAY (Sequenom Inc., San Diego, CA, USA) as described in detail in our previous publication (14) and the genotyping analysis was performed blindly as regards the subjects. The selected PCR-amplified DNA samples (n=2, for each genotype) were also examined using DNA sequencing to confirm the genotyping results.

Statistics. The SNP genotype was analyzed as a three-group categorial variable (referent model), and also grouped according to a dominant and recessive model. The Hardy–Weinberg equilibrium for each polymorphism was analyzed using a χ^2-square test. For survival analysis, relapse was confirmed by biopsy, when possible, and categorized as local, regional, or distant; however, contralateral breast cancer during the follow-up period was not considered a relapse in this study. Disease-free survival (DFS) and overall survival (OS) was defined as the time from the date of surgery to the date of relapse or death from any cause or the date of the last follow-up, respectively. The cumulative incidence of relapse was defined as the time from the date of surgery to the date of the first event, where the curves were constructed based on the Kaplan–Meier method and analyzed using a log-rank test according to possible clinical (age, menopausal status, and use of adjuvant therapies) and histopathological risk factors [tumor size, number of involved lymph nodes, histological grade, and immunohistochemical expression of estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2)], and the genotype of the variants. The hazard ratio (HR) and 95% confidence interval (95% CI) for the genotypes of selected variants were calculated from a Cox regression analysis adjusted to age, stage, histological grade, and ER/PgR and HER2 status. In the multivariate analysis, the possible clinical and pathologic risk factors and SNPs significantly associated with survival in the adjusted univariate analysis were then analyzed as prognostic factors of relapse or survival for operated invasive ductal breast cancer. The differences in the continuous variables were compared using Student's *t*-test or an ANOVA test, while a χ^2-test was used for the categorical variables. The statistical analyses were all performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Patients' characteristics and genotype frequency. For the 452 patients, median age was 48 years (range, 19 to 79 years) at the time of diagnosis, where 35.8% and 74.8% were node-positive and ER/PgR-expressing, respectively. The other basic clinical and pathological characteristics of the patients are listed in Table I. After curative surgery, 89.4% and 36.1% received adjuvant chemotherapy and radiotherapy, respectively, plus adjuvant hormonal treatment with tamoxifen or an aromatase inhibitor was also given if required, except for one patient. The median follow-up for the patients alive at the last follow-up was 7.3 years, at which point 67 (14.8%) had experienced relapse, including 17 locoregional and 50 distant relapses, where 12 distant relapses were identified after a locoregional relapse. In addition, 55 (12.2%) patients had died from breast cancer. The frequencies of each genotype are shown in Table II, and conformed to the Hardy–Weinberg equilibrium ($p>0.05$).

Survival analysis. At a median follow-up duration of 7.3 years, the estimated 5-and 10-year DFS, distant DFS, and OS were 87.8%, 91.0%, and 94.2%, and 81.0%, 87.0%, and 82.1%, respectively. Among the three target variants, miR-196a rs11614913 was significantly associated with DFS and distant DFS in the univariate analysis. Moreover, the multivariate analysis revealed that patients carrying the CC genotype for this polymorphism had a poor DFS and distant DFS when compared to patients with the T allele (DFS: HR=2.354, 95% CI=1.348-4.111, $p=0.003$; distant DFS: HR=2.399, 95% CI=1.236-4.656, $p=0.010$) regardless of patient and tumor characteristics (Figure 1 and Table III), yet there was no statistical association with OS (HR=0.942; $p=0.865$). In particular, the prognostic impact of *miR-196a* rs11614913 was only statistically associated with survival in patients with hormone-responsive breast cancers, based on the ER/PgR and HER2 immunohistochemistry (HR=2.610 and 2.730, $p=0.003$ and 0.013 for DFS and distant DFS, respectively; Figure 2 and Table IV). Moreover, no

Table I. *Patients' characteristics and clinical outcomes*.

Characteristics	Total (%)
Age, median, range (years)	48.0, 16-83
Mean±SD	49.8±11.15
≤50 years	253 (56.0)
>50 years	199 (44.0)
Menopausal status	
Premenopausal	196 (43.4)
Postmenopausal	256 (56.6)
Histological grade†	
1	55 (12.2)
2	258 (57.1)
3	132 (29.2)
Not assessed	7 (1.5)
Site	
Left	192 (42.5)
Right	248 (54.9)
Both	12 (2.7)
Tumor size (T), pathological‡	
1	248 (55.1)
2	187 (41.4)
3	16 (3.5)
Nodal involvement. (N), pathological‡	
0	290 (64.2)
1	110 (24.3)
2	52 (11.5)
Stage, pathological (AJCC)‡	
I	174 (38.5)
IIA	168 (37.2)
IIB	54 (11.9)
IIIA	56 (12.4)
Estrogen receptor (ER)	
Negative	120 (26.5)
Weak	78 (17.3)
Strong	248 (54.9)
Unknown	6 (1.3)
Progesterone receptor (PgR)	
Negative	157 (34.7)
Weak	107 (23.7)
Strong	182 (40.0)
Unknown	6 (1.3)
HER2	
Positive	87 (19.2)
Negative	348 (79.9)
NE	4 (0.9)

Characteristics	Total (%)
Tumor type§	
Hormone-responsive	283 ((62.6)
HER2-overexpressed	86 (19.0)
Triple-negative	77 (17.0)
Unknown	6 (1.3)
Surgery	
Mastectomy	338 (74.8)
Breast-conserving	114 (25.2)
Adjuvant chemotherapy	
None	48 (10.6)
CMF	231 (51.1)
Anthracycline without taxane	44 (9.7)
Anthracycline with taxane	129 (28.5)
Adjuvant hormonal therapy	
None	118 (26.1)
Tamoxifen	183 (40.5)
Aromatase inhibitor (AI)	107 (23.7)
Tamoxifen followed by AIs	44 (9.7)
Adjuvant radiotherapy	163 (36.1)
Relapse	67 (14.8)
Locoregional recurrence	17 (3.7)
Distant	38 (8.4)
Locoregional→ distant	12 (2.7)
Death	55 (12.2)
Cause of death	
Breast cancer	46
Pneumonia	1
Urosepsis	1
Other malignancies	2
Liver cirrhosis	1
Unknown	4

SD, Standard deviation; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2; CMF, cyclophosphamide/methotrexate/5-fluorouracil; AC, adriamycin/cyclophosphamide ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2. †Modified Scarff-Bloom-Richardson grading system. ‡American Joint Committee on Cancer (AJCC) staging system. §According to immunohistochemical staining of ER, PgR, and HER2.

significant difference in the clinicopathological parameters was found according to the genotype or allele of *miR-196a* rs11614913.

Discussion

Although an association between the SNPs in protein-coding genes and the risk or prognosis of cancer has been introduced, only a few studies have been reported on the SNPs of miRNAs. Thus, based on several recent publications linking miRNA polymorphisms and susceptibility to breast cancer, the present study hypothesized that theses polymorphisms may be associated with the prognosis for early breast cancer. Among the three selected variants of miRNAs or miRNA-binding site previously identified as potential biomarkers, it was found that *miR-196a* variant rs11614913 may be a prognostic factor for estimating recurrence after curative surgery in patients with EBC.

miRNAs have already been shown to play an important role in a variety of carcinogenesis or tumor progression by

Table II. *Allelic frequencies and p-values for Hardy–Weinberg equilibrium (HWE) for selected single nucleotide polymorphisms (SNPs).*

Type	SNP	Related miRNA	Related gene	Genotype	N	%	HWE *p*-Value	Minor allelic frequency
miRNA	rs3746444	*miR-499a*	Variable	TT	291	64.4	0.876	0.197
				TC	144	31.9		
				CC	17	3.8		
	rs11614913	*miR-196a*	Variable	TT	144	31.9	0.056	0.454
				CT	203	44.9		
				CC	103	22.8		
miRNA binding site	rs1044129	*miR-367*	*RYR3*	AA	133	29.4	0.123	0.470
				AG	205	45.4		
				GG	106	23.5		

miRNA, MicroRNA; *RYR3*, Ryanodine receptor 3.

Table III. *Multivariate analysis for survival.*

		DFS			Distant DFS			OS		
Variables		HR	95% CI	*p*-Value	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Age (years), > 50	≤50	1.544	0.876-2.721	0.133	2.069	1.059-4.042	0.033	2.016	1.063-3.825	0.032
Histological grade†, 2 or 3	*vs.* 1	2.415	0.791-7.366	0.121	1.687	0.488-5.828	0.408	2.756	0.769-9.877	0.120
Stage, pathological‡			-	0.003		-	<0.001		-	<0.001
IIA	*vs.* I	2.763	1.18-6.468	0.019	3.829	1.235-11.873	0.020	5.327	1.748-16.234	0.003
IIB	*vs.* I	3.076	1.098-8.616	0.033	5.438	1.46-20.256	0.012	9.810	2.625-36.668	0.001
IIIA	*vs.* I	6.682	2.382-18.743	<0.001	16.132	4.346-59.876	<0.001	23.011	6.037-87.716	<0.001
Tumor type§			-	0.403		-	0.052		-	0.253
HER2 overexpressing	*vs.* hormone responsive	1.675	0.836-3.355	0.146	2.879	1.365-6.069	0.005	2.057	1.014-4.173	0.046
Triple negative	*vs.* hormone responsive	1.295	0.436-3.848	0.641	2.543	0.769-8.403	0.126	2.182	0.647-7.361	0.209
Unknown	*vs.* hormone responsive	2.507	0.294-21.413	0.401			0.971			0.981
Surgery, mastectomy	*vs.* breast-conserving	0.819	0.377-1.781	0.615	1.061	0.427-2.636	0.898	0.900	0.366-2.214	0.818
Adjuvant chemotherapy			-	0.186		-	0.643		-	0.001
CMF	*vs.* none	1.395	0.315-6.174	0.661	1.154	0.243-5.467	0.857	0.141	0.053-0.374	<0.001
Adriamycin-based	*vs.* none	0.995	0.169-5.855	0.996	0.813	0.119-5.569	0.833	0.160	0.041-0.631	0.009
AC4→T4	*vs.* none	2.414	0.503-11.58	0.271	1.574	0.293-8.459	0.597	0.164	0.052-0.521	0.002
rs11614913, CC	*vs.* TT or TC	2.354	1.348-4.111	0.003	2.399	1.236-4.656	0.010	0.942	0.472-1.878	0.865

DFS, Disease-free survival; OS, overall survival; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2; CMF, cyclophosphamide/methotrexate/5-fluorouracil; AC, adriamycin/cyclophosphamide; T, taxane. †Modified Scarff-Bloom-Richardson grading system. ‡American Joint Committee on Cancer (AJCC) staging system. §According to immunohistochemical staining of ER, PgR, and HER2.

altering the expression of oncogenes or tumor-suppressor genes. Previous studies have demonstrated an association between rs11614913 in *miR-196a* and cancer risk by alteration of the mature miR-196a expression and its binding to target mRNA (15-19). In particular, the C allele of rs11614913 was recently identified as a risk factor for breast cancer in Asians and Caucasians (16, 20), which is comparable with the present findings, although the impact of SNPs on cancer risk does not always correlate with their impact on prognosis. While the exact biological role of *miR-196a* and its variants has not yet been elucidated, Hoffman *et al.* demonstrated that the rs11614913 variant led to more efficient processing of the miRNA precursor to its mature form, this being more than two-times higher in breast cancer cells transfected with pre-miR-196a-C compared to cells transfected with pre-miR-196a-T (20). Furthermore, an Ingenuity Pathway Analysis network analysis revealed that several cancer-relevant transcripts known as prognostic indicators for poor clinical outcome in breast cancer including tumor protein-63 (*TP63*), inhibin beta-B (*INHBB*), the tumor suppressors growth arrest and DNA damage-inducible, gamma (*GADD45G*), and the S100 family of genes (A8 and A9) were significantly altered following the introduction of pre-miR-196a-C (20-23). Thus, when

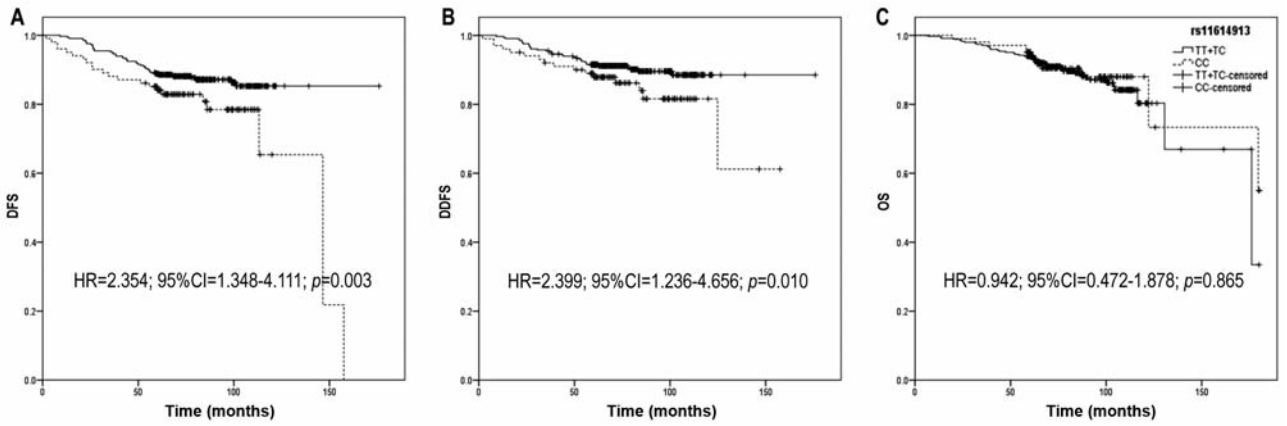

Figure 1. *Survival according to the genotype ofmiR-196a rs11614913: A. disease-free survival (DFS); B. distant DFS (DDFS); C. overall survival (OS).*

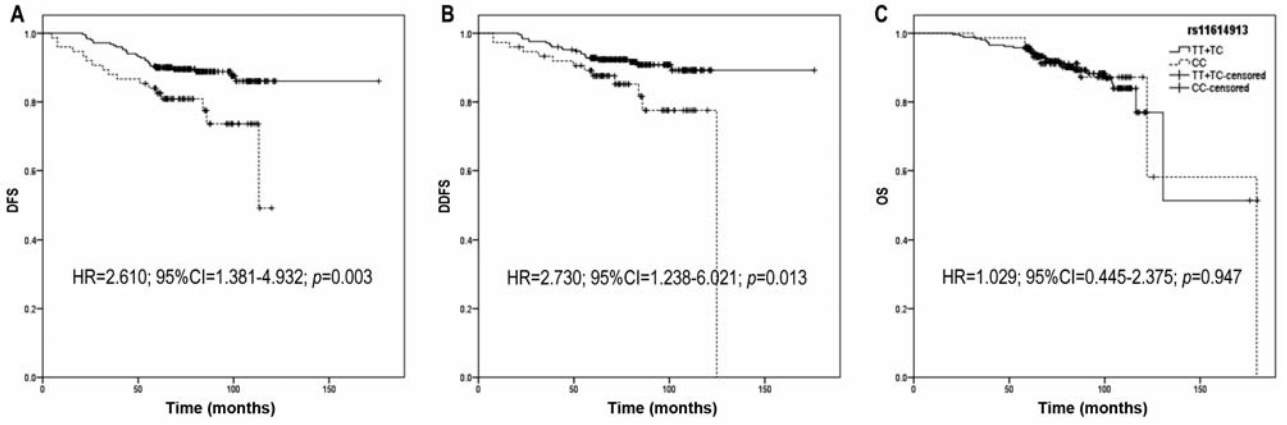

Figure 2. *Survival according to the genotype of miR-196a rs11614913in patients with hormone-responsive breast cancer: A. Disease-free survival (DFS); B. distant DFS (DDFS); C. overall survival (OS).*

combined with epidemiological evidence, the rs11614913C allele appears to be associated with susceptibility to breast cancer in relation to increased mature miR-196a levels influencing genes with relevance for breast tumor development and progression.

Although there are no data regarding the prognostic role of this variant in breast cancer, consistent results associating the C allele of rs11614913 with an increased risk of cancer, including breast, gastric, head and neck, and non-small cell lung cancer, would seem to support this variant as a potential prognostic marker (16, 18, 20, 24, 25). Notably, *miR-196a* rs11614913 was only associated with survival in patients with the ER/PgR-positive and/or HER2-overexpressing breast cancer type. Therefore, rs11614913 could be a promising prognostic factor for patients with ER-positive EBC. Prognostic markers for EBC are crucial due to the ongoing controversies regarding adjuvant management. In particular, for patients with ER-positive EBC, the addition of chemotherapy remains contentious due to the use of highly toxic chemotherapeutic agents, such as anthracyclines. Therefore, the current findings are clearly important for this patient group with regard to determining the use of adjuvant chemotherapy after surgery. Meanwhile, several biological markers, such as Ki-67 (26, 27), and gene signatures (28-30) have recently been identified and introduced as important prognostic and predictive markers for hormone-responsive EBC, pending the validation results of ongoing clinical trials. However, the current study did not evaluate the association between rs11614913 and these recently discovered biological markers, thereby warranting further studies.

Notwithstanding, while SNPs are thought to be attractive biomarkers as they are stably-inherited, highly abundant and

Table IV. *Multivariate analysis for survival in patients with hormone-responsive (HR) early breast cancer.*

		DFS			Distant DFS			OS		
Variables		*p*-Value	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value	HR	95% CI
Age (years), > 50	≤50	0.622	1.192	0.593-2.396	0.177	1.796	0.768-4.197	0.049	2.253	1.004-5.058
Histological grade†, 2 or 3	*vs.* 1	0.225	2.126	0.628-7.194	0.399	1.743	0.479-6.347	0.080	3.405	0.864-13.42
ER expression		0.967		-	0.764		-	0.928		-
Weak	*vs.* negative	0.797	0.802	0.149-4.311	0.563	1.985	0.195-20.204	0.908		
Strong		0.808	0.817	0.159-4.184	0.703	1.561	0.159-15.356	0.910		
PgR expression		0.645		-	0.885		-	0.846		-
Weak	*vs.* negative	0.564	0.766	0.31-1.892	0.638	1.312	0.423-4.072	0.590	1.323	0.479-3.655
Strong		0.351	0.662	0.279-1.573	0.653	1.295	0.42-3.999	0.606	1.296	0.484-3.475
HER2 overexpressed	*vs.* none	0.120	1.750	0.864-3.545	0.003	3.132	1.457-6.73	0.078	1.923	0.929-3.98
Stage pathological‡		0.029		-	0.002		-	<0.001		-
IIA	*vs.* I	0.023	3.097	1.172-8.186	0.064	3.201	0.933-10.984	0.026	3.841	1.175-12.559
IIB	*vs.* I	0.007	5.194	1.574-17.137	0.002	10.353	2.391-44.832	<0.001	12.848	3.064-53.865
IIIA	*vs.* I	0.004	6.333	1.814-22.111	<0.001	18.433	3.928-86.506	<0.001	25.505	5.583-116.52
Surgery, mastectomy	*vs.* breast-conserving	0.673	0.832	0.354-1.955	0.521	1.385	0.513-3.74	0.971	1.019	0.362-2.87
Adjuvant chemotherapy		0.220		-	0.486		-	0.007		-
CMF	*vs.* none	0.853	1.160	0.244-5.521	0.896	1.118	0.209-5.97	0.001	0.156	0.051-0.481
Adriamycin-based	*vs.* none	0.357	0.385	0.051-2.935	0.322	0.298	0.027-3.26	0.013	0.106	0.018-0.624
AC4→T4	*vs.* none	0.645	1.483	0.278-7.921	0.941	0.931	0.141-6.14	0.002	0.107	0.026-0.44
Adjuvant hormonal therapy		<0.001		-	0.002		-	0.027		-
Aromatase inhibitor (AI)	*vs.* Tamoxifen	0.001	0.211	0.084-0.533	0.002	0.162	0.05-0.524	0.060	0.440	0.187-1.036
Tamoxifen→AIs	*vs.* Tamoxifen	0.012	0.154	0.036-0.659	0.026	0.099	0.013-0.76	0.031	0.187	0.041-0.86
rs11614913, CC	*vs.* TT or TC	0.003	2.610	1.381-4.932	0.013	2.730	1.238-6.021	0.947	1.029	0.445-2.375

DFS, Disease-free survival; OS, overall survival; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2; CMF, cyclophosphamide/methotrexate/5-fluorouracil; AC, adriamycin/cyclophosphamide; T, taxane. †Modified Scarff-Bloom-Richardson grading system. ‡American Joint Committee on Cancer (AJCC) staging system.

show diversity within and among populations, the application of individual SNPs is limited due to their penetrance and the difficulty involved in identifying their effects. Thus, until the present results are confirmed by replication studies with different populations, caution is warranted in terms of drawing definite conclusions from the current study. Furthermore, the current study found that rs11614913 CC was significantly associated with a poor DFS (HR=2.354; p=0.003) and distant DFS (HR=2.399; p=0.010), while no significant association was identified for OS (HR=0.942; p=0.865). This could be explained by the relatively short follow-up duration and thus small number of cases who died from breast cancer as disease in most patients was at an early stage (64.2% node-negative and 75.7% stage I or IIA). In addition, the diverse treatment modalities given after recurrence, including metastasectomy and active chemotherapy or hormone treatment, might have weakened the impact of this variant on OS, as previously described in various studies of recurrent or metastatic cancer.

In summary, rs11614913 is a possible prognostic factor for patients with ER/PgR-positive EBC after complete surgery, regardless of the clinical or pathological characteristics. However, since the exact mechanism and tumor specificity of the rs1044129 variant have not yet been defined and genetic polymorphisms often vary between different ethnic groups, the present findings need to be confirmed in further studies with other populations of patients with breast cancer to clarify the association between these polymorphisms and the prognosis of breast cancer. Moreover, the present findings warrant a further study to explore rs11614913 as a predictive marker of treatment, such as chemotherapy and hormonal therapy for EBC or as a novel therapeutic target for the management of breast cancer.

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