Promoter methylation of *BRCA1* is associated with estrogen, progesterone and human epidermal growth factor receptor-negative tumors and the prognosis of breast cancer: A meta-analysis

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Abstract. Aberrant methylation of the breast cancer susceptibility gene 1 (*BRCA1*) promoter is a mechanism for its functional inactivation. It may potentially be used as a prognostic marker in studies for patients with breast cancer and plays an important role in tumorigenesis. Numerous studies have suggested that the methylation of the *BRCA1* promoter is associated with the prognosis of breast cancer. However, the prognosis of *BRCA1* promoter methylation in breast cancer patients of different ethnicities remains ambiguous. The present meta-analysis was performed to adjust and augment a previously published study, which estimated the correlations between promoter methylation of *BRCA1* and the clinical outcomes of breast cancer patients. These results indicated that *BRCA1* methylation was significantly correlated with a poor prognosis of breast cancer, particularly for Asian patients, but the correlation was over-estimated in the previous study. The combined hazard ratios (HRs) in the present study were 1.76 (1.15-2.68) and 1.97 (1.12-3.44) for univariate and multivariate analysis of overall survival, which were different from 2.02 (1.35-3.03) and 1.38 (1.04-1.84) in the previous study. For studies of disease-free survival, the univariate and multivariate analyses also have different pooled HRs: 2.89 (1.73-4.83) and 3.92 (1.49-10.32) in the previously published study and 1.28 (0.68-2.43) and 1.64 (0.64-4.19) in the present study. In addition, the *BRCA1* promoter regions used to detect the hypermethylation were different. All the studies using the Baldwin's primer reported that breast cancer patients with *BRCA1* promoter methylation had a better prognosis. There were also correlations between *BRCA1* promoter methylation and receptor-negativity of the estrogen receptors, progesterone receptor, human epidermal growth factor receptor 2 and a triple-negative status. Patients with the estrogen, progesterone and epidermal growth factor-related receptor-negative status were more likely to be negative for the BRCA1 protein.

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Introduction

Breast cancer is the most frequently diagnosed cancer in women worldwide (1,2). It has an increasing mortality and morbidity rate in women <45 years. Every year in China, ~1.6 million women are diagnosed and ~1.2 million people succumb to breast cancer. Breast cancer results from the accumulation of abnormal genetic and epigenetic changes in tumor-suppressor genes and proto-oncogenes (3). Genes, such as p53, *ATM* and human epidermal growth factor receptor 2 (HER2), are involved in different types of tumors. Breast cancer susceptibility gene 1 (*BRCA1*) is another specific gene, which was identified as a genetic cause of hereditary breast cancer.

BRCA1 is located on chromosome 17q12-21 (2). It is an important tumor-suppressor gene associated with human breast cancer (4). The BRCA1 protein plays an important role in DNA repair of double-strand breaks (5), transcriptional regulation, ubiquitinylation, as well as other functions (6,7). The hypermethylation of the *BRCA1* promoter has been considered as an inactivating mechanism of *BRCA1* expression (8). This low expression or non-expression of *BRCA1* may not be adequate for repairing DNA damage that further promotes the accumulation of mutations in cell growth and division. Certain results suggest that *BRCA1* promoter hypermethylation is associated with poor clinical outcomes. In the present study,

the source data used by the study of Wu *et al* (9) was adjusted and augmented to investigate the association between *BRCA1* methylation and the outcome of breast cancer.

The therapeutic targets for breast cancer are the receptors. The progesterone receptor (PR) is a nuclear receptor located inside cells. PR is encoded by chromosome 11q22 in humans. Estrogen receptors (ER) are receptors that are activated by the hormone estrogen (10). HER2 is a member of the epidermal growth factor receptor (EGFR/ERBB) family. In recent years these proteins have been used as therapy targets in <30% of breast cancer patients (11). Triple-negative breast cancer is defined as the absence of ER, PR and HER2 (12). The present treatment for triple-negative breast cancer is a type of chemotherapy and often has a poor outcome. Therefore, it is essential to find new and alternative therapeutic strategies. In the present meta-analysis, the correlations between therapy target-related negative-receptors and *BRCA1* promoter methylation were also studied. To ensure the quality of analysis, the Begg's test, χ^2-based Q test, Egger's test, sensitivity analysis and publication bias analysis were used.

Materials and methods

Literature search. Two investigators independently conducted a literature search using PubMed, Embase and Google scholar (last search updated on September 14, 2014). The keywords used included: *BRCA1*, breast carcinoma, breast cancer, methylation, prognosis and survival. In addition, the PubMed additional function: Related citations; and the references of the selected studies were scrutinized to identify additional studies.

Eligibility criteria. Studies were included in the meta-analysis only if they had met the following criteria: i) Evaluated prognostic risk of patients with *BRCA1* methylation; ii) provided overall survival (OS) or disease-free survival (DFS); iii) hazard ratios (HR) or odds ratios (OR) with its 95% confidence intervals (CIs); iv) published in English; and v) data from human subjects. In addition, studies were excluded if: i) Data was from reviews or animal studies; and ii) studies had the same population resources or overlapping datasets.

Data extraction. Following the exclusions, 9 studies met all the criteria. Two investigators independently extracted the following data from each study: First author's last name, year of publication, population, number of study subjects, effects on clinical outcomes (OS and DFS), and the number of methylated and unmethylated patients with a different status of ER, PR, HER2 and triple-negative receptors. OS is a term that denotes the chances of remaining alive for a group of individuals suffering from a type of cancer. At a basic level, the OS is representative of cure rates. DFS was defined as the chances of staying free of disease following a particular treatment for a group of individuals suffering from a type of cancer. It is an indication of how effective a particular treatment is.

Statistical analysis. Random effects and subgroup meta-analysis were performed according to the DerSimonian Laird method (13), due to the existence of heterogeneity between studies. The data were divided into two groups by population: European and Asian. The HRs were used to estimate the pooled

Table I. Characteristics of the included studies.

				OS		DFS		
				Univariate analysis	Multivariate analysis	Univariate analysis	Multivariate analysis	
First author, year	Population	Patients, n	Primer	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)	(Refs.)
Sharma, 2014	European	39	Esteller	3.37 (0.23-50.02)	6.2 (2-19.4)	N/A	3.5 (1.3-9.8)	(20)
Ignatov, 2013	European	65	Baldwin	N/A	N/A	0.325 (0.16-0.662)	0.224 (0.092-0.546)	(21)
Krasteva, 2012	European	135	Baldwin	0.47 (0.14-1.54)	0.91 (0.24-3.41)	N/A	N/A	(22)
Xu, 2009	European	851	Others	1.72 (1.06-2.79)	1.67 (0.99-2.81)	N/A	N/A	(23)
Xu, 2013	Asian	1,163	Esteller	1.29 (0.96-1.73)	N/A	1.34 (1.06-1.71)	N/A	(24)
Hsu, 2013	Asian	139	Esteller	N/A	16.38 (1.37-195.45)	N/A	12.19 (2.29-64.75)	(25)
Sharma, 2009	Asian	101	Esteller	5.06 (1.58-16.22)	2.12 (0.47-9.63)	3.88 (2.05-7.34)	2.03 (0.96-4.29)	(26)
Chen, 2009	Asian	536	Esteller	1.56 (1.02-2.37)	1.27 (0.81-1.99)	1.45 (1.01-2.09)	1.23 (0.84-1.8)	(27)
Jing, 2008	Asian	102	Esteller	6.4 (2.0-20.5)	N/A	N/A	N/A	(28)

OS, overall survival; DFS, disease-free survival; HR, CI hazard ratio; CI, confidence interval.

Table II. Distribution of the *BRCA1* methylation status with different hormone and epidermal growth factor receptors.

		BRCA1 methylation, n (total)				*BRCA1* non-methylation, n (total)				
First author, year	Population	ER negative	PR negative	HER2 negative	Triple-negative	ER negative	PR negative	HER2 negative	Triple-negative	(Refs.)
Ignatov, 2013	European	NA	NA	NA	43 (86)	NA	NA	NA	22 (46)	(21)
Krasteva, 2012	European	11 (23)	11 (23)	14 (17)	NA	48 (112)	11 (23)	37 (53)	NA	(22)
Xu, 2009	European	89 (372)	135 (372)	NA	NA	127 (320)	135 (372)	NA	NA	(23)
Xu, 2013	Asian	109 (285)	149 (282)	220 (279)	64 (282)	295 (830)	149 (282)	654 (810)	142 (817)	(24)
Hsu, 2013	Asian	30 (77)	36 (77)	55 (77)	16 (77)	21 (61)	36 (78)	91 (138)	5 (61)	(25)
Sharma, 2009	Asian	22 (27)	21 (27)	18 (27)	15 (27)	35 (74)	21 (27)	60 (74)	25 (74)	(26)
Chen, 2009	Asian	55 (138)	80 (137)	98 (135)	31 (136)	127 (383)	80 (137)	298 (378)	64 (382)	(27)
Jing, 2008	Asian	21 (33)	26 (33)	NA	NA	127 (168)	26 (33)	NA	NA	(28)

Total refers to the total number of identified samples in *BRCA1* methylation or non-methylation with the corresponding hormone receptor: ER, PR and HER2. Triple-negative, lacks expression of ER, PR and HER2 amplification. ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; *BRCA1*, breast cancer susceptibility gene 1.

effect of *BRCA1* methylation on the prognosis of patients with breast cancer, and the ORs were pooled to estimate the strength of the association between *BRCA1* hypermethylation and the risk of three negative statuses of receptors (ER, PR and HER2). When HRs (95% CIs) were shown only in the figure of survival curves, the authors were contacted for the exact value or the investigators estimated them according to the methods provided by Tierney *et al* (14). The Cochran Q (significant cut-off point: P=0.10) and I^2 (I^2>50%, strong heterogeneity) statistics (15,16) were used to assess heterogeneity between studies. The Galbraith plot (17) was used to detect the potential sources of heterogeneity from the meta-analysis. Publication bias was assessed by funnel plot and the test of Egger *et al* (18). Sensitivity analyses were performed by the trim-and-fill method (19). All the analyses and graphs were obtained using STATA 11.0 software (StataCorp LP, College Station, TX, USA).

Results

Characteristics of studies. Fig. 1 summarizes the process of identifying eligible studies. Following the screening by two investigators independently, according to the inclusion criteria there were 9 studies with 3,131 study subjects entered into the meta-analysis (20-28). The characteristics of these studies are listed in Tables I and II. There were 4 studies from Europe and 5 studies from Asia.

As shown in Table II, there were 8 studies that met the inclusion criteria and were included in the present meta-analysis. The studies involved 337 *BRCA1* promoter hypermethylations with an ER-negative status, 458 with a PR-negative status, 405 with an HER2-negative status, 169 with triple-negative receptors, and 780, 458, 1,140 and 258 controls without *BRCA1* promoter methylation but with a negative status, correspondingly.

When the same investigators reported the results obtained from the same cohort of patients in several studies, only the largest series was included in the analysis. A cohort of patients was excluded due to duplicate studies.

Due to insufficient data, HRs on OS could be extracted from 7 studies for univariate analysis and 6 studies for multivariate analysis. According to DFS analysis, there were 4 studies with available data for univariate analysis and 5 studies with available data for multivariate analysis.

Association of BRCA1 methylation with OS and DFS of patients with breast cancer. Considering the significant heterogeneity among studies (P=0.017, I^2=61.1%), the random-effect model was used and a subgroup analysis was performed by considering different ethnicities or the population of the participants to estimate the combined effect of *BRCA1* methylation. *BRCA1* methylation was significantly associated with a poor OS and DFS of breast cancer in the univariate and multivariate analysis (Figs. 2 and 3 and Table III). The combined HR was 1.76 (1.15-2.68) and 1.97 (1.12-3.44) for univariate analysis and multivariate analysis of OS, respectively. For studies of DFS, the pooled HR was 1.28 (0.68-2.43) and 1.64 (0.64-4.19), respectively. The combined HRs (95% CIs) on OS by univariate

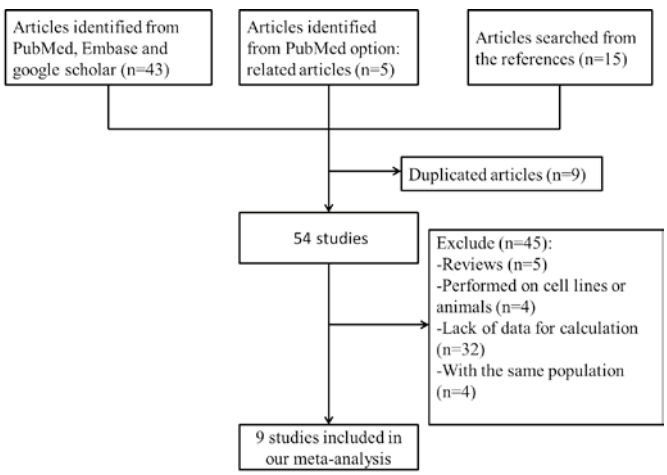

Figure 1. Study selection flow chart for the meta-analysis.

Table III. HRs and 95% CI for the association between BRCA1 methylation and OS or DFS.

Analysis	Population	Survival	Pooled HR (95% CI), P-value	Heterogeneity test P-value	Egger's test P-value	Begg's test P-value
Univariate	European	OS	1.206 (0.444-3.276), 0.713	0.120	0.797	0.602
		DFS	-	-	-	-
	Asian	OS	2.164 (1.212-3.863), 0.009	0.011	0.004	0.174
		DFS	1.795 (1.109-2.907)	0.009	0.236	0.117
	Overall	OS	1.758 (1.154-2.679), 0.009	0.017	<0.001	0.393
		DFS	1.284 (0.679-2.430), 0.442	<0.001	1.000	0.885
Multivariate	European	OS	2.107 (0.827-5.368), 0.118	0.061	0.830	0.602
		DFS	0.876 (0.059-12.953), 0.923	<0.001	-	0.317
	Asian	OS	2.177 (0.711-6.663), 0.173	0.120	0.256	0.117
		DFS	2.250 (0.911-5.562), 0.079	0.021	0.074	0.117
	Overall	OS	1.966 (1.124-3.438), 0.018	0.058	0.019	0.460
		DFS	1.635 (0.638-4.191), 0.306	<0.001	<0.001	0.064

HR, hazard ratio; CI, confidence interval; BRCA1, breast cancer susceptibility gene 1; OS, overall survival; DFS, disease-free survival.

Table IV. Odds ratios and 95% CI for *BRCA1* promoter methylation and breast cancer subtype.

			Heterogeneity test		
Factors	Pooled OR (95% CI)	P-value[a]	χ^2	P-value	I^2 (%)
ER negative					
Asian	1.329 (1.084-1.630)	0.006	9.41	0.052	57.5
European	1.040 (0.736-1.471)	0.824	0.14	0.704	0.0
Overall	1.247 (1.045-1.487)	0.014	10.83	0.094	44.6
PR negative					
Asian	1.459 (1.195-1.782)	<0.001	15.62	0.004	74.4
European	1.010 (0.740-1.379)	0.948	0.28	0.599	0.0
Overall	1.311 (1.108-1.550)	0.002	19.30	0.004	68.9
HER2 negative					
Asian	2.834 (2.277-3.528)	<0.001	2.98	0.394	0.0
European	2.881 (2.322-3.574)	<0.001	3.65	0.456	0.0
Triple-negative					
Asian	1.557 (1.210-2.002)	0.001	2.81	0.422	0.0
European	1.494 (1.178-1.896)	0.001	3.61	0.461	0.0

[a]P-value for the association from the meta-analysis. CI, confidence interval; OR, odds ratio; ER, estrogen receptor; *BRCA1*, breast cancer susceptibility gene 1; HER2, human epidermal growth factor receptor 2.

analysis was 2.16 (1.21-3.86) for the Asian population, which was significantly higher than 1.21 (0.44-3.28) for the European population. All the combined HR scores in the present study are different from the study of Wu *et al* (9). They over-estimated the risk of fatality by using the OR instead of HR value from the Karray-Chouayekh study (29) and using the incorrect value of OS in the Xu *et al* study (23).

Primer for identifying BRCA1 promoter methylation. The majority of the studies identified that *BRCA1* promoter methylation is correlated with poor survival, as shown in the present meta-analysis. However, an opposing opinion remains as *BRCA1* promoter methylation is a protective factor in 2 studies [Krasteva *et al* (22) and Ignatov *et al* (21)]. To explore this difference, we identified that they used different primers. In the Krasteva *et al* (22) and Ignatov *et al* (21) studies, which found a better clinical prognosis in patients with *BRCA1* promoter methylation, they used Baldwin's primer (30) while others used Esteller's primer (31). The two primer sequences were blasted to the human genome in the NCBI database to

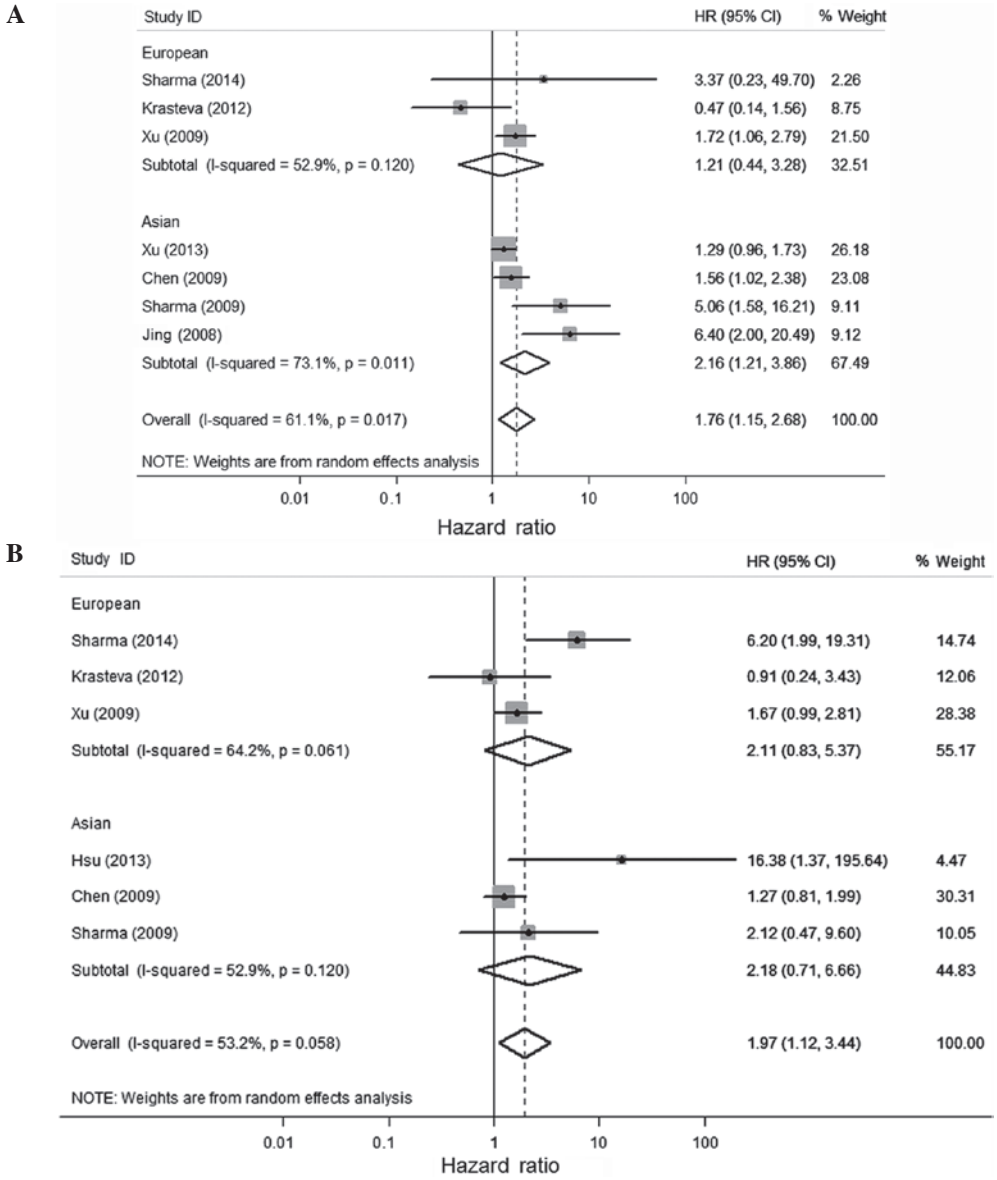

Figure 2. Forest plot for the association between breast cancer susceptibility gene 1 (*BRCA1*) promoter methylation and the overall survival (OS) using univariate (A) and multivariate (B) analysis. The values of the last line in (A and B) are the combined hazard ratio (HR).

find the amplified regions. The two primers are for different regions where there was a different concentration of GC-rich regions. The Baldwin primer exhibited a larger amplification that contained more GC-rich regions and included the transcriptional start point (Fig. 4). Therefore, the difference of *BRCA1* promoter methylation in the region of chr17: 43, 125, 429-43, 125, 541 (GRCh38 assembly) may affect the different prognosis. However, all of these require more testing by molecular-biological experiments to confirm.

Hormone receptor-negativity correlation analysis. The number of patients who carried *BRCA1* methylation and one of the negative statuses of ER, PR, HER2 or triple-negative were extracted for the cases, as well as the number of patients without promoter methylation of *BRCA1* correspondingly for the controls. The correlations between *BRCA1* methylation and the negative status of different breast cancer-related receptors were meta-analyzed separately. The results of the association between hormone receptors that were negative, *BRCA1* promoter methylation and the heterogeneity test are shown in Table IV. The overall results suggested that, particularly for the Asian populations, all the receptors negativity listed in Table II are associated with the *BRCA1* promoter methylation. Patients with those estrogen, progesterone and epidermal growth factor-related negative receptors were more likely to be negative for the BRCA1 protein (OR>1.247, P<0.014). However, further molecular-biological experiments are required to determine whether the correlation is spurious, as the receptor and *BRCA1* promoter methylation are associated with breast cancer.

Sensitivity analysis and publication bias. The Patsopoulos method (32) was used to test if an individual study affected the heterogeneity in the OS and DFS analysis. As a result, if the Jing *et al* (28) and Sharma *et al* (26) studies were removed the heterogeneity disappeared (I^2=17.3%, P=0.30). Funnel

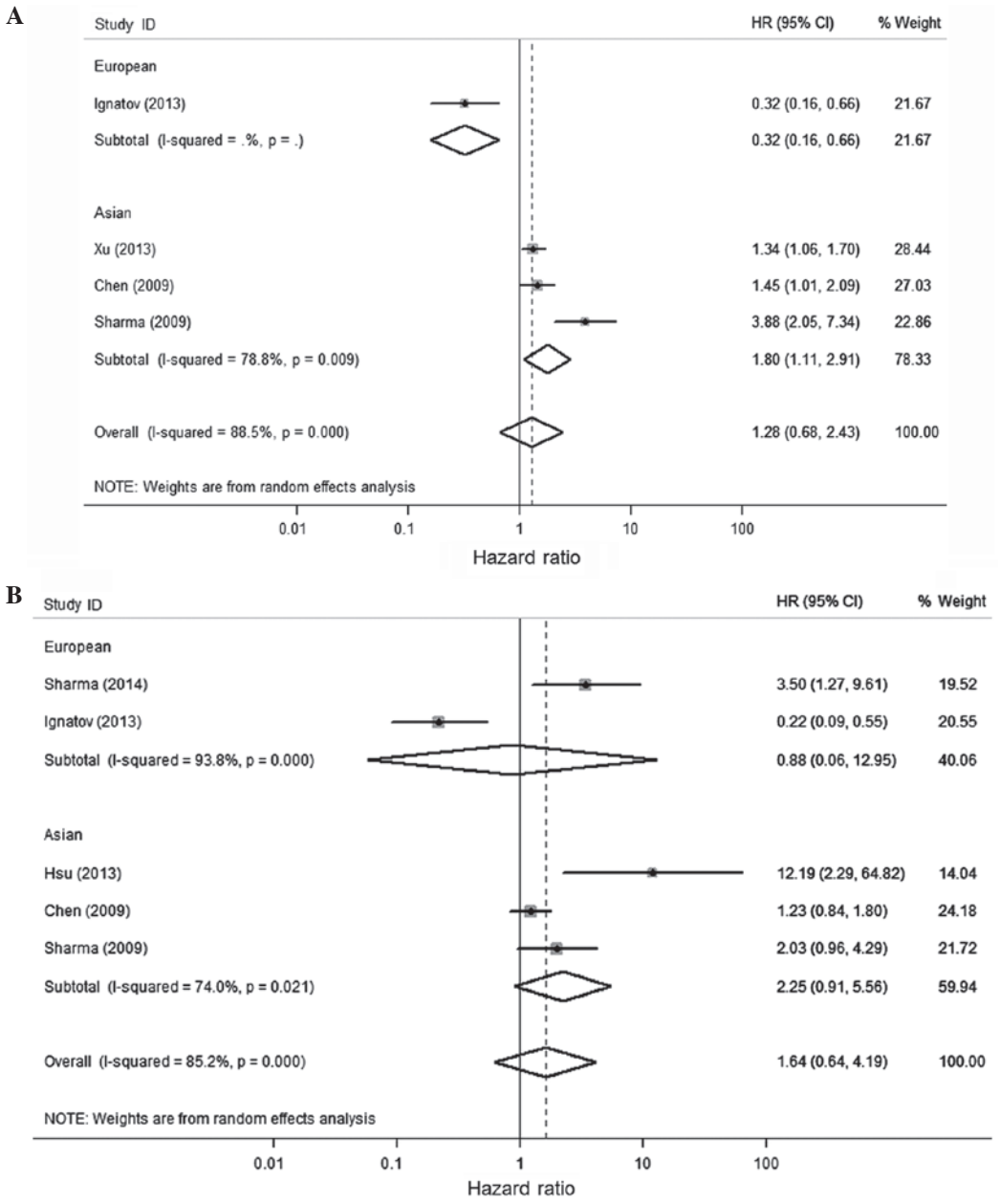

Figure 3. Forest plot for the association between breast cancer susceptibility gene 1 (*BRCA1*) promoter methylation and disease-free survival (DFS) using univariate (A) and multivariate (B) analysis. The values of the last line in (A and B) are the combined hazard ratio (HR).

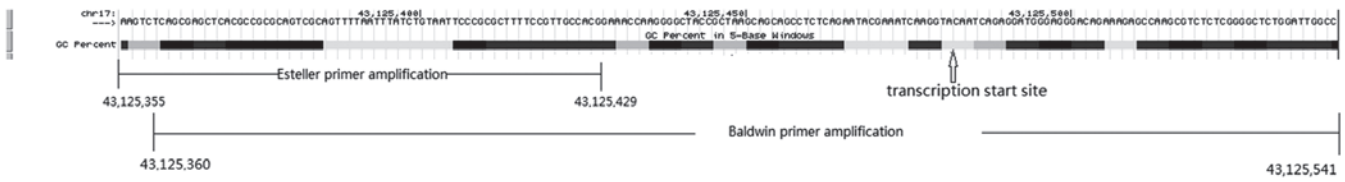

Figure 4. Schematic illustration of the breast cancer susceptibility gene 1 (*BRCA1*) promoter region of methylation-specific polymerase chain reaction (PCR) by two different primers. The view of the genomic context was adapted from UCSC Genome Browsers. The black bar shows the predicted GC-rich region in the promoter region. The amplification region for Esteller and Baldwin primers are chr17: 43125355-43125429, chr17: 43125360-43125541, respectively (assembly version: GRCh38/hg38).

plot and Begg's test were performed to check the publication bias, and they suggest the absence of publication bias. The Begg's test for OS and DFS were P=0.187 and P=0.625, respectively.

Discussion

The present meta-analysis suggested that patients with *BRCA1* promoter methylation had a more significant OS and DFS

disadvantage than those without the methylated status, similar to the previous meta-analysis by Wu *et al* (9), which showed that breast cancer patients with hypermethylation in the promoter of *BRCA1* exhibited poor survival. However, three problems were identified in the Wu *et al* (9) study, which were adjusted in the present study: i) A meta-analysis was performed for the 5 adjusted HR scores of DFS, but one was an OR (20.7) score instead of HR, [from the Karray-Chouayekh study (29)] and was much larger than the others; ii) in the meta-analysis for the 5 adjusted HR scores of OS, the value observed for all-cause mortality instead of fatality from breast cancer in the Xu *et al* (23) study was used; iii) 3 of the 9 HRs from the Sharma *et al* (20,26,33) studies published in 2009 and 2010 for meta-analysis were from the same subjects. Following adjustment for these problems and the addition of a study, the present meta-analysis suggested that the previous study had over-estimated the risk of fatality from breast cancer. The pooled HRs in the present study and the Wu *et al* (9) study were 1.28 vs. 2.89 and 1.64 vs. 3.92 for univariate and multivariate analysis of DFS, respectively.

The present meta-analysis additionally assessed the associations between the promoter methylation of *BRCA1* and breast cancer-related receptors and the results showed that there are significant correlations between them. This suggested that potential interactions may exist between the hypermethylation of the *BRCA1* gene and the negative status of ER, PR and HER2 receptors through complex regulation pathways on tumor progression. However, further studies are also required to explore the correlations in order to assist in finding a therapeutic target for breast cancer.

The consistency of two studies that reported the protective effect for patients with hypermethylation in the promoter of *BRCA1* was found to be due to the different primers used. Therefore, further studies are required to test whether the different regions of the promoter methylation of *BRCA1* exhibit different clinical outcomes.

However, certain limitations of the study should be considered. First, the number of studies contained in the present meta-analysis is relatively small, particularly in non-African populations, and the results should be confirmed in large samples. Second, although the Egger's test did not have statistical significance, the publication bias may still exist and influence the results. Asymmetrical appearance of the funnel plot could be caused by heterogeneity, smaller studies and other factors. Considering the limitations of the study, the associations among *BRCA1* promoter methylation, prognosis of patients and the negative status of the breast cancer-related therapeutic target receptors should be further investigated.

In conclusion, the results revealed that breast cancer patients with *BRCA1* promoter methylation had lower OS and DFS and had significant correlations with the negative status of the ER, PR and HER2 receptors. Hypermethylation in different regions of the *BRCA1* promoter may exhibit a different clinical performance.

Acknowledgements

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