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ORIGINAL ARTICLE

Association of CCND1 Gene c.870G>A Polymorphism with Breast Cancer Risk: A Case-Control Study and a Meta-Analysis

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Abstract Cyclin D1 (CCND1) plays an essential role in regulating the progress of the cell cycle from G1 to S phase. There is a common c.870G>A polymorphism in the CCND1 gene. The aim of this study was to investigate the association of CCND1 gene c.870G>A polymorphism with breast cancer risk in a case-control study, which followed by a meta-analysis and an in silico analysis. Three hundred and thirty-five subjects composed of 174 women with breast cancer and 161 healthy controls were included in the case-control study. CCND1 gene c.870G>A genotyping was performed by PCR-RFLP. Meta-analysis was done for 14 studies composed of 7281 cases and 6820 controls. Some bioinformatics tools were applied to investigate the effects of c.870G>A on the mRNA splicing and structure. Our data obtained from case-control study revealed that GA genotype (OR: 1.89, 95%CI: 1.12–3.17, $p = 0.017$), AA genotype (OR: 1.95, 95%CI: 1.08–3.53, $p = 0.027$), and A allele (OR: 1.44, 95%CI: 1.06–1.95, $p = 0.019$) were significantly associated with breast cancer risk. The results of meta-analysis showed a significant association between CCND1 c.870G>A polymorphism and breast cancer risk, especially in Caucasian population. In silico analysis revealed that c.870G>A transition affect CCND1 mRNA splicing and secondary structure.

Keywords Breast cancer · CCND1 gene · Genetic polymorphism · c.870G>A · Meta-analysis

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Introduction

Breast cancer is one of the most common malignancies in women worldwide, and each year more than one million new cases is diagnosed [1]. The genetic susceptibility, dietary preferences, lifestyle, and environmental factors play main roles in the risk of breast cancer [2–4]. Genetic polymorphism is one of the causes of individual differences in the incidence of cancer [5].

CCND1 gene is located on chromosome 11 (11q13), and it encodes cyclin D1 as a key cell cycle regulatory protein. This protein composed of 5 following domains [6, 7]: 1- retinoblastoma protein (pRb) binding motif; 2- cyclin box domain; 3- LxxLL binding motif; 4- PEST sequence; 5- threonine residue (threonine 286). Cyclin D1 controls the transition from G1 to the S phase during cell division [8, 9]. There is a common single nucleotide polymorphism (c.870G>A, with ID: rs603965) in exon 4 of CCND1 gene. Although c.870G>A polymorphism is a synonymous transition, but it will alter the splice site in the transcript of CCND1 [10]. After c.870G>A transition, a different transcript without PEST motif will be produced. This motif marks the cyclin D1 for degradation, therefore the transcript without PEST displays to have a longer half-life than the wild-type transcript [11]. The aim of this study was to investigate the association of CCND1 gene

c.870G>A polymorphism with breast cancer in a case-control study which, is followed by a meta-analysis.

Materials & Methods

Subjects

In a case control study, 174 women (mean age 56.32 ± 9.11 years) with sporadic breast cancer and 161 healthy women (with mean age 57.40 ± 4.66 years) were included. The cases and controls were selected from women referred to the Shahid Beheshti hospital (Kashan, Iran). Diagnosis of breast cancer was confirmed histologically for case subjects. Only cases with newly incident disease were included. Control subjects were participating in a local screening program, and they did not show positive results. Also, all of control subjects were lack of history of oncological disease. Finally 3 mL blood was collected from all of the subjects. All the participants' informed written consent. In addition, this study confirmed by the principles outlined in the Declaration of Helsinki and approved by the Hospital's Ethics Committee.

SNP Genotyping

Firstly, genomic DNA was isolated from blood samples by QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). CCND1 gene c.870G>A genotyping was done by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Specific primers for CCND1 gene were designed by Oligo7 software. The sequence of sense and antisense primers were S: 5′-CGGATCACGGGGGCCCTGAG AG-3′ and A: 5′-CGGCAAGGCTGCCTGGGACATC-3′, respectively. To amplify CCND1 fragment, PCR was carried out within total 25 μl volume containing 0.35 μl of each primer, 2.5 μl of 10X PCR buffer, 0.2 μl of *Taq* DNA polymerase, 1 mM dNTPs, and 60 ng DNA template (All of PCR agents were purchased from Fermentas Company). The PCR was performed in a thermal cycler (Peqlab, Erlangen, Germany) with following the program: 10 min at 94 °C followed by 30 repetitive cycles of 45 s at 94 °C, 45 s at 60 °C, and 45 s at 72 °C with a final extension at 72 °C for 7 min. About 5 μl (~0.1 μg) of the PCR products were digested with 5 units *Msp*I restriction enzyme (Fermentas) by incubation at 37 °C for 16 h. After digestion, the enzymatic mixtures were electrophoresed in 8% polyacrylamide gel and visualized by silver nitrate (AgNO3) staining. The digested samples showed 3 different patterns; genotype GG, demonstrating 213 bp and 36 bp fragments, genotype AA with 249 bp fragment and genotype GA with 249 bp, 213 bp, and 36 bp fragments.

Meta-Analysis

On February 2015, a systematic literature search was done by utilizing of PubMed, ScienceDirect, and Google Scholar databases for the following terms: "breast cancer", "CCND1", "polymorphism", and "c.870G>A". The following inclusion criteria were applied to a selection of articles: 1- investigation of CCND1 gene c.870G>A polymorphism with breast cancer risk; 2- case-control design; 3- human beings; and 4- sufficient data to estimate the odds ratios (ORs) and 95% confidence intervals (95%CIs). We extracted the following information from all eligible articles: the first author, publication year, ethnicity of the study population, frequencies of genotypes and alleles for cases and controls, and genotyping method. Ethnic subgroups were categorized as Caucasian and Asian. All of these data were extracted by two students, independently. The differences were resolved by discussion. The extracted data from included studies in meta-analysis are presented in Table 1.

Statistical Analysis

We used the Chi-squared test to evaluate Hardy-Weinberg equilibrium (HWE), and we considered $p < 0.05$ as a significant deviation from HWE. In case-control study, OR with 95%CI was calculated for all of genotypes and alleles in case and control groups. The Chi-square test was applied to assess the differences between the case and control groups. A two-tailed *p*-value less than 0.05 was considered as statistically significant. These statistical analyses were performed by SPSS Statistical software version 16. In meta-analysis, the pooled ORs with 95% CIs were calculated for the five following genetic models: A vs. G (allelic model), 2- AA vs. GG (Codominant model), 3- GA vs. GG (Codominant model), 4- GA + AA vs.GG (Dominant model), and 5- AA vs. GG + GA (Recessive model). The heterogeneity assumption was evaluated by using Chi-squared based on Q test, and $p < 0.1$ was considered as a significant difference [25]. In the lack of significant heterogeneity, the fixed effect model was used for calculation of the pooled OR of all studies [26], otherwise the random effect model was applied in combination of the data [27]. Also, a sensitivity analysis was performed by excluding each study, one at a time, and recalculating the ORs and 95% CIs to evaluate the effects of each study on the pooled ORs. Then we performed an estimate of potential publication bias by using the funnel plot and Egger's test [28, 29]. The meta-analysis, performed by utilizing Open Meta-analyst and Comprehensive Meta analysis software.

Bioinformatics Tools

Both ASSP (Alternative Splice Site Predictor) web server (http://wangcomputing.com/assp/) [30] and NetGene2 online

Table 1 Characteristics of included studies in meta-analysis

Ethnicity	Allele frequencies				Genotype frequencies						*P* HWE[a]	Genotyping method	Author, Year (Reference)
	Case		Control		Case			Control					
	G	A	G	A	GG	GA	AA	GG	GA	AA			
Caucasian	338	316	360	318	90	158	79	92	176	71	0.436	SSCP	Grieu et al. 2003 [12]
Caucasian	471	523	497	499	112	247	138	116	265	117	0.152	PCR-RFLP	Krippl et al. 2003 [13]
Caucasian	234	212	328	268	59	116	48	91	146	61	0.862	PCR-RFLP	Forsti et al. 2004 [14]
Asian	209	301	557	775	57	95	103	124	309	233	0.230	TaqMan PCR	Ceschi et al. 2005 [15]
Asian	987	1273	1040	1352	213	561	356	250	540	406	0.005	PCR-RFLP	Shu et al. 2005 [16]
Caucasian (Ontario)	1243	1201	780	658	335	573	314	217	346	156	0.412	TaqMan	Onay et al. 2008 [17]
Caucasian (Finland)	713	713	724	620	179	355	179	195	334	143	0.999	TaqMan	Onay et al. 2008 [17]
Asian	763	1221	801	1119	156	451	385	192	417	351	0.001	RT-PCR	Yu et al. 2008 [18]
Asian	219	241	206	194	58	103	69	54	98	48	0.787	PCR-RFLP	Naidu et al. 2008 [19]
Caucasian	472	492	163	207	113	246	123	42	79	64	0.069	MALDI-TOF MS	Justenhoven et al. 2009 [20]
Caucasians	38	38	67	61	15	8	15	18	31	15	0.816	PCR-RFLP	Yaylim-Eraltan et al. 2009 [21]
Caucasian	717	821	667	723	178	361	230	171	325	199	0.096	Multiplex PCR	Jeon et al. 2010 [22]
Caucasians	67	89	86	82	10	47	21	21	44	19	0.659	PCR-RFLP	Canbay et al. 2010 [23]
Asian	143	159	61	105	33	77	41	7	47	29	0.047	PCR-RFLP	Wasson et al. 2014 [24]
Asian	163	185	180	142	38	87	49	56	68	37	0.069	PCR-RFLP	This study

HWE Hardy-Weinberg equilibrium, *PCR* polymerase chain reaction, *RFLP* restriction fragment length polymorphism

[a] Hardy–Weinberg equilibrium in the control group (groups with p-value < 0.05 did not satisfy the Hardy-Weinberg equilibrium)

software (http://www.cbs.dtu.dk/services/NetGene2/) [31] were applied to find the influence of c.870G>A on splice site pattern of CCND1. Web application called RNAsnp (http://rth.dk/resources/rnasnp/) which predicts the effects of SNPs on mRNA structure was used to determine possible effects of the CCND1 gene c.870G>A polymorphism [32]. Finally, the String online server (http://string-db.org/) [33] was used to obtain the network of gene-gene interaction for CCND1.

Results

Distribution of c.870G>A Polymorphism in Cases and Controls

Genotype distribution for CCND1 gene c.870G>A polymorphism was in Hardy-Weinberg equilibrium for case ($\chi^2 = 0.003$, $p = 0.958$) and control ($\chi^2 = 3.308$, $p = 0.069$) groups. Frequencies of alleles and genotypes in cases and controls are shown in Table 2. Frequencies of GG, GA, and AA genotypes in cases were 21.84%, 50.00%, and 28.16%, respectively. While these ratios in the control group were 34.78%, 42.24%, and 22.98%, respectively. Compared with GG genotype, the GA genotype and AA genotype significantly increased breast cancer risk with ORs of 1.89 (95%CI: 1.12–3.17) and 1.95 (95%CI: 1.08–3.53), respectively. The A carriers (GA + GG) were also at a high risk for breast cancer (OR: 1.91, 95%CI: 1.18–3.10, $p = 0.009$). Allele analysis revealed a significant association of A allele with breast cancer risk (OR: 1.44, 95%CI: 1.06–1.95, $p = 0.019$).

Association Results in Meta-Analysis

After the search procedure, 86 published reports were recognized. But after the screening, 13 association studies included in our meta-analysis [12–24]. Also, the data from our case-control study was added to meta-analysis (Fig. 1). These studies were contained a total of 7281 cases and 6820 controls. The genotype distribution in the control groups of three studies was deviated from HWE [16, 18, 24]. Six studies were performed in Asian populations, but nine studies were conducted in Caucasian populations. Our meta-analysis showed that there is a significant association between CCND1 gene c.870G>A and breast cancer risk in A vs. G (OR: 1.08, 95%CI: 1.01–1.15, $p = 0.020$) and AA vs. GG (OR: 1.168, 95%CI: 1.03–1.33, $p = 0.020$) models (Fig. 2). The subgroup analysis of Caucasian studies indicated that four genetic models were significantly associated with breast cancer risk (A vs. G, OR: 1.10, 95%CI: 1.03–1.17, $p = 0.005$; AA vs. GG,

Table 2 Genotype and allele frequencies of G870A in cases and controls

Genotype/Allele	No. and Percentage		OR (95% CI)	*p*-value
	Control (*n* = 161)	Case (*n* = 174)		
GG	56 (34.78%)	38 (21.84%)	–	–
GA	68 (42.24%)	87 (50.00%)	1.89 (1.12–3.17)	**0.017**
AA	37 (22.98%)	49 (28.16%)	1.95 (1.08–3.53)	**0.027**
GA + AA	105 (65.22%)	136 (78.16%)	1.91 (1.18–3.10)	**0.009**
G	180 (55.90)	163 (46.84%)	–	–
A	142 (44.10%)	185 (53.16%)	1.44 (1.06–1.95)	**0.019**

Significant differences between the case and control groups are bolded

OR odds ratio, *CI* confidence interval

OR: 1.20, 95%CI: 1.06–1.36, p = 0.005; GA + AA vs. GG, OR: 1.11, 95%CI: 1.00–1.23, p = 0.041; AA vs. GG + GA, OR:1.14, 95%CI: 1.03–1.26, p = 0.014) (Fig. 2). We also performed a meta-analysis in Asian subgroup. Our data revealed that there is no association between CCND1 gene c.870G>A and breast cancer risk in all of the five genetic models within Asian populations. All association results of meta-analysis are summarized in Table 3.

Heterogeneity, Sensitivity, and Publication Bias

A significant heterogeneity was found in total population, especially in two of the genetic models (GA + AA vs. GG, I^2 value: 45%, p value: 0.030; and AA vs. GG + GA, I^2 value: 41%, p value: 0.049). In addition, we found a significant heterogeneity in Asian studies in four of the genetic models (A vs. G, I^2 value: 63%, p value: 0.020; AA vs. GG. I^2 value: 65%, p value: 0.014; GA vs. GG, I^2 value: 75%, p value: 0.001; GA + AA vs. GG, I^2 value: 73%, p value: 0.003). Therefore, we applied the random effects models to these groups. While we didn't observe any significant heterogeneity in Caucasian studies, and therefore we applied the fixed effects models to the meta-analysis (Table 4). Also, we conducted a sensitivity analysis to evaluate the stability of the results by sequentially removing each study from our meta-analysis. The data from sensitivity analysis revealed that none of the studies changed the pooled OR, and it shows that the meta-analysis is stable (The data not shown). Exclusion of studies with deviation from HWE did not change the conclusion of the overall meta-analysis, significantly. But, the data from a recalculation of meta-analysis in an Asian population after exclusion of studies with deviation from HWE, revealed a significant association with AA vs. GA + GG model (OR: 1.29, 95%CI: 1.04–1.61, p = 0.021, p *of* heterogeneity: 0.959, I^2: 0%, p of egger: 0.385). Begg's funnel plot and Egger's test were used to evaluate the publication bias in meta-analysis. The shape of funnel plots did not show any evidence of asymmetry (Fig. 3). Also, the Egger's linear regression test did not reveal publication bias (p = 0.85 for A vs. G and p = 0.687 for AA vs. GG in the total population; p = 0.813 for A vs. G, p = 0.978 for AA vs. GG, p = 0.808 for GA + AA vs. GG and, p = 0.883 for AA vs. GG + GA in Caucasian).

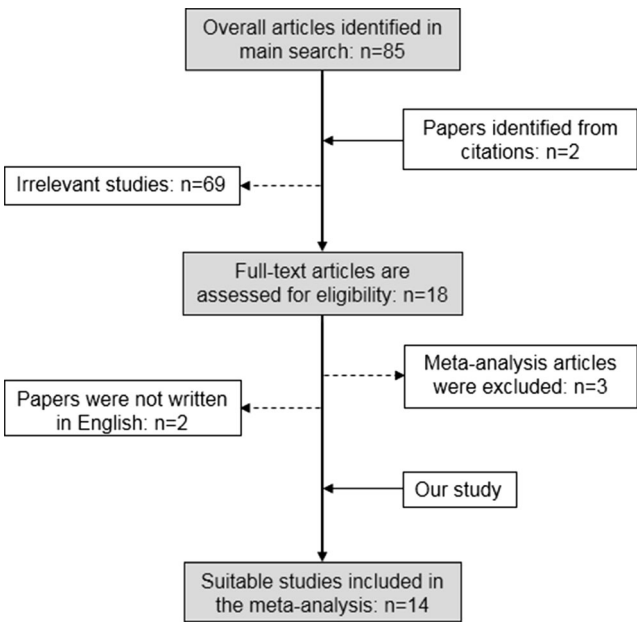

Fig. 1 Flowchart of the study selection

In Silico Analysis

The ASSP online web server revealed that the c.870G>A transition changes splice site pattern of the CCND1 gene (Fig. 4). The data showed that there were three and two constitutive acceptor splice sites for 870GG and 870AA genotypes, respectively (Table 5). NetGene software also approved the results of ASSP and predicted an alteration in splicing scheme of CCND1 gene due to c.870G>A transition (Fig. 4). In addition, the data which obtained from RNAsnp

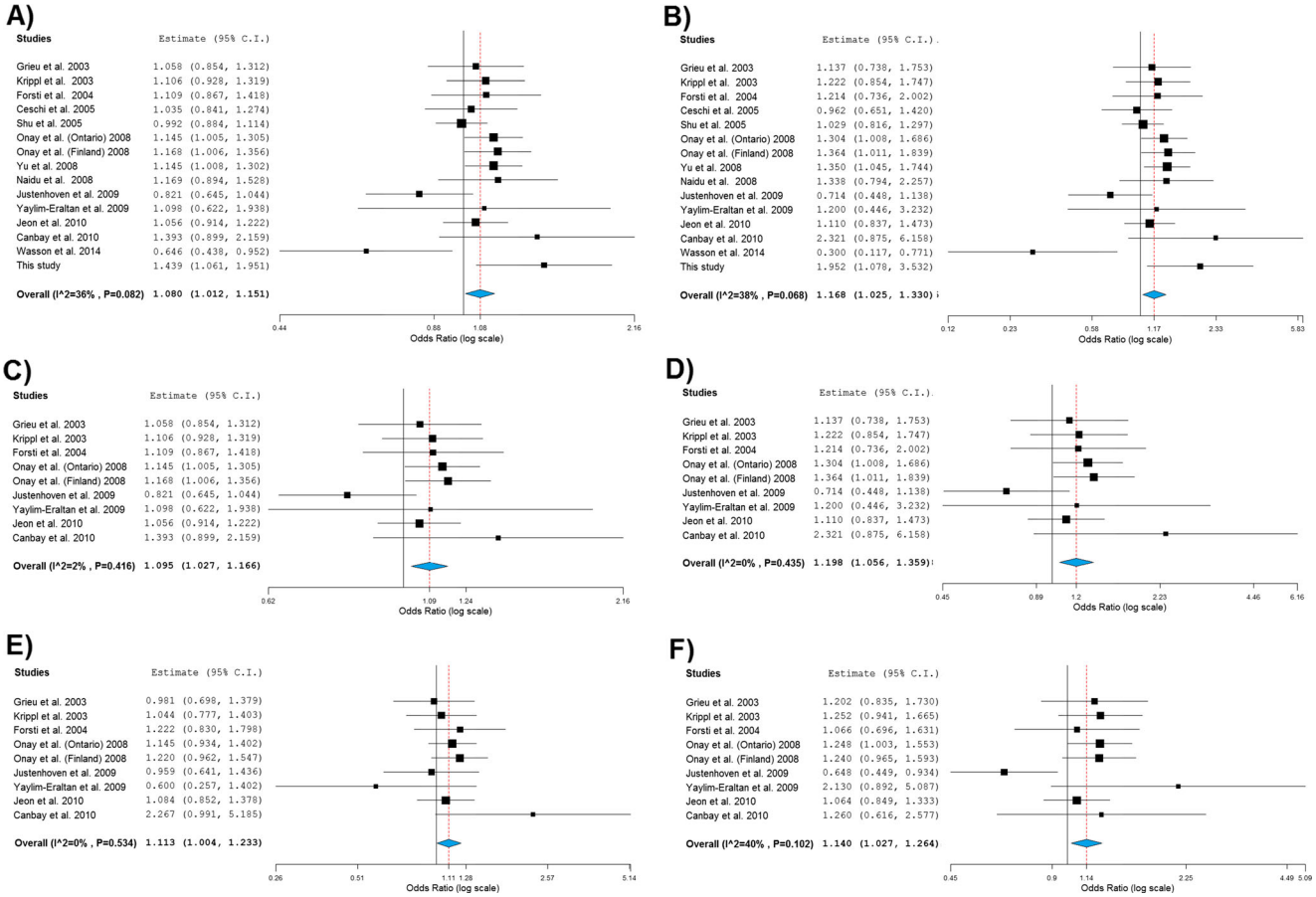

Fig. 2 Forest plot for the association of CCND1 gene c.870G>A polymorphism and breast cancer risk. **a** Results of quantitative data synthesis under A vs. G in total population; **b** AA vs. GG in total population; **c** A vs. G in Caucasian; **d** AA vs. GG in Caucasian; **e** GA + AA vs. GG in Caucasian; and **f** AA vs. GG + GA in Caucasian

showed that CCND1 gene c.870G>A polymorphism is deleterious for the structure of mRNA (p = 0.0548; p < 0.2 is a significant). The minimum free energy of mRNA is equal −138.70 for genotype GG that this amount increases to −136.40 kcal/mol for AA genotype (Fig. 5). Finally, we applied the String online server to obtain the gene-gene interaction network for CCND1. The data from String revealed that this gene interacts with 10 other genes (Fig. 6).

Discussion

In this study, we investigated the association of CCND1 gene c.870G>A polymorphism with breast cancer risk. The data from a case-control study showed that GA genotype, AA genotype, and A allele was significantly associated with the risk of breast cancer (p < 0.05). Previous studies showed inconsistent results. For instance, Yu et al. 2008 [18] and Yaylim-Eraltan et al. 2009 [21] reported a significant association

Table 3 Association results in the meta-analysis

Group	A vs. G		AA vs. GG		GA vs. GG		GA + AA vs. GG		AA vs. GG + GA	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Total	1.08 (1.01–1.15)	**0.020**	1.17 (1.03–1.33)	**0.020**	1.07 (0.93–1.23)	0.339	1.11 (0.99–1.25)	0.082	1.10 (0.99–1.23)	0.068
Asian	1.07 (0.93–1.22)	0.360	1.12 (0.85–1.48)	0.421	1.04 (0.76–1.41)	0.815	1.09 (0.82–1.44)	0.555	1.04 (0.94–1.16)	0.466
Caucasian	1.10 (1.03–1.17)	**0.005**	1.20 (1.06–1.36)	**0.005**	1.07 (0.96–1.20)	0.218	1.11 (1.00–1.23)	**0.041**	1.14 (1.03–1.26)	**0.014**

Significant differences between the case and control groups are bolded

OR odds ratio, *CI* confidence interval

Table 4 Results of heterogeneity and publication bias in the meta-analysis

Group	A vs. G			AA vs. GG			GA vs. GG			GA + AA vs. GG			AA vs. GG + GA		
	Ph	I^2	Pe	Ph	I^2	Pe	*Ph*	I^2	Pe	Ph	I^2	Pe	Ph	I^2	*Pe*
Total	0.082	36%	0.850	0.068	38%	0.687	0.006	55%	0.226	0.030	45%	0.385	0.049	41%	0.460
Asian	0.020	63%	0.928	0.014	65%	0.646	0.001	75%	0.302	0.003	73%	0.474	0.106	45%	0.574
Caucasian	0.416	02%	0.813	0.435	0%	0.978	0.238	23%	0.692	0.534	0%	0.808	0.102	40%	0.883

*P*h, *P*heterogeneity ($P < 0.1$ was considered as a significant difference). *P*e, *P*egger ($P < 0.05$ was considered as a significant difference)

between CCND1 gene c.870G>A polymorphism and breast cancer risk. While, Krippl et al. 2003 [13] and Jeon et al. 2010 [22] found no significant association between the polymorphism and risk of breast cancer. To achieve more accurate results, we performed a meta-analysis of all existing studies. The data from our meta-analysis revealed that there are significant association between c.870G>A polymorphism and the risk of breast cancer in both A vs. G and AA vs. GG genetic models ($p < 0.05$). The inconsistent results in different studies may arise from geographic, ethnic, and environmental variations. For example, ethnicity-stratified subgroup showed different results. The results of meta-analysis in Caucasian studies showed that there was a significant association between CCND1 gene c.870G>A polymorphism and breast cancer risk in A vs. G, AA vs. GG, GA + AA vs. GG, and AA vs. GG + GA genetic models. This despite the fact that, our data

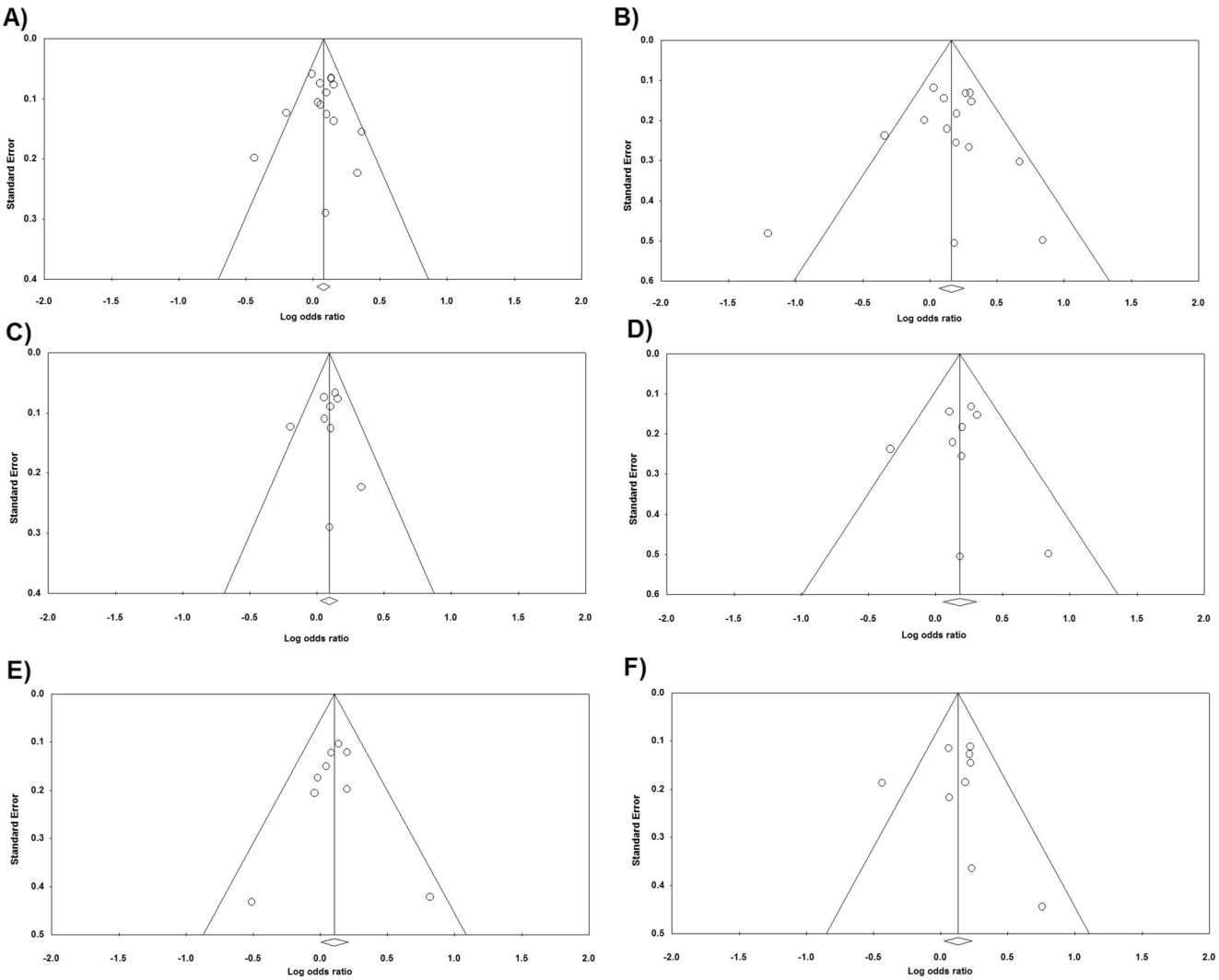

Fig. 3 Funnel plot of breast cancer risk associated with CCND1 gene c.870G>A polymorphism. **a** Results of under A vs. G in total population; **b** AA vs. GG in total population; **c** A vs. G in Caucasian; **d** AA vs. GG in Caucasian; **e** GA + AA vs. GG in Caucasian; and **f** AA vs. GG + GA in Caucasian

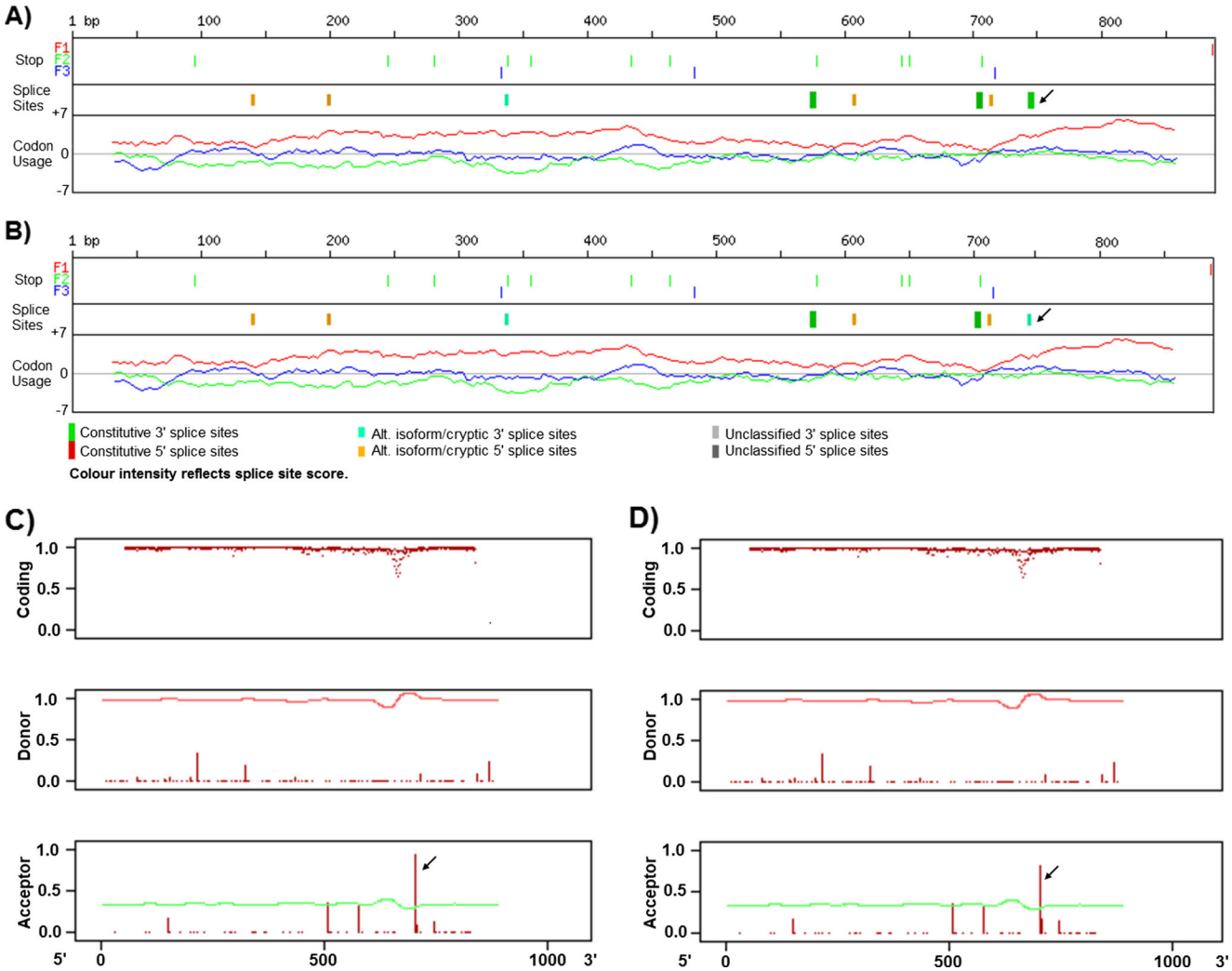

Fig. 4 Prediction results of ASSP and NetGene2. ASSP pattern of splice sites when nucleotide G locates in the position 870 (**a**); ASSP splice sites pattern after A substitution in the position 870 (**b**). NetGene2 prediction of splice sites when nucleotide G locates in the position 870 (**c**); NetGene2 splice sites pattern after A substitution in the 870 (**d**). A change was observed in acceptor splice site after substitution. (The difference between the patterns after substitution are shown by arrowhead)

revealed no association between CCND1 gene c.870G>A and breast cancer risk in all of five genetic models within Asian populations.

CCND1 is a gene with clinical potential which is amplified in 5–20% of breast cancer subjects, but it is deleted in 5–9% of cases [34, 35]. Also, this gene has some potential oncogenic feature by influencing the regulation of cell cycle at the transition of G1/S phase [36, 37]. It is reported that cyclin D1, the product of CCND1 gene, is overexpressed in over 50% of breast cancer cases [35, 38]. As a functional polymorphism of the CCND1 gene, c.870G>A transition, may play a key role in the development of breast cancer [20, 39]. Cyclin D1 mRNA displays two different transcripts (a and b) by alternate splicing. Transcripts a and b produce different proteins in their C-terminal domains which the possible function of these two variants may be different. Transcript a is normally spliced molecule, whereas transcript b losses exon 5 [10, 40, 41]. This alternative splicing modulates by c.870G>A transition at codon 242 of exon 4 of the CCND1 gene. The 870G allele tends to create the complete transcript, while the mutant 870A allele generates the shortened transcript [41, 42]. Previous studies revealed that individuals with 870A allele tend to have enhanced alternative splicing than the 870G allele carriers. But, the results of previous studies are contradictory [10, 18, 40, 43]. Bioinformatics tools can be helpful to determine SNPs effects on the gene expression, protein and mRNA structure and function [44–48]. Therefore, we performed an in silico analysis to find the influence of c.870G>A transition on mRNA secondary structure and mRNA splicing. Our in silico analysis showed that not only c.870G>A transition affect mRNA splicing but also it affects mRNA secondary structure of CCND1. It is reported that different SNPs may affect the interaction of several molecules with mRNA and influence the mRNA maturation, transport, translation or degradation,

Table 5 ASSP prediction results for 870GG and 870AA genotypes

Positio (bp)	Putative splice site	Sequence	Score[a]	Intron GC[b]	Activations[c]		Confidence[d]
					Alt./Cryptic	Constitutive	
A) 870GG Genotype							
139	Alt. isoform/cryptic donor	TACTTCAAATgtgtgcagaa	5.245	0.629	0.946	0.038	0.960
198	Alt. isoform/cryptic donor	GATGCTGGAGgtctgcgagg	7.743	0.600	0.820	0.135	0.836
337	Alt. isoform/cryptic acceptor	ggcctctaagATGAAGGAGA	4.089	0.629	0.668	0.320	0.522
575	Constitutive acceptor	tgtgccacagATGTGAAGTT	7.943	0.586	0.281	0.703	0.601
606	Alt. isoform/cryptic donor	GCCCTCCATGgtggcagcgg	5.650	0.657	0.854	0.112	0.869
705	Constitutive acceptor	tcctctccagAGTGATCAAG	8.895	0.586	0.113	0.881	0.872
713	Alt. isoform/cryptic donor	GAGTGATCAAgtgtgacccg	5.095	0.686	0.860	0.105	0.878
745	Constitutive acceptor	ggcctgccagGAGCAGATCG	4.756	0.629	0.481	0.502	0.042
B) 870AA Genotype							
139	Alt. isoform/cryptic donor	TACTTCAAATgtgtgcagaa	5.245	0.629	0.946	0.038	0.960
198	Alt. isoform/cryptic donor	GATGCTGGAGgtctgcgagg	7.743	0.600	0.820	0.135	0.836
337	Alt. isoform/cryptic acceptor	ggcctctaagATGAAGGAGA	4.089	0.629	0.668	0.320	0.522
575	Constitutive acceptor	tgtgccacagATGTGAAGTT	7.943	0.586	0.281	0.703	0.601
606	Alt. isoform/cryptic donor	GCCCTCCATGgtggcagcgg	5.650	0.657	0.854	0.112	0.869
705	Constitutive acceptor	tcctctccagAGTGATCAAG	8.895	0.586	0.123	0.870	0.859
713	Alt. isoform/cryptic donor	GAGTGATCAAgtgtgacccg	5.095	0.671	0.881	0.089	0.899
745	Alt. isoform/cryptic acceptor	ggcctgccagGAGCAGATCG	4.756	0.614	0.504	0.478	0.051

[a] Scores of the preprocessing models reflect strength of splice site

[b] Intron GC values refer to 70 nt of the neighboring intron

[c] Activations are output values for the networks of backpropagation used to classification

[d] Confidence expresses the differences between output activations. Confidence ranges between 0 (undecided) to 1 (perfect classification)

subsequently [49]. Also, Wang et al. reported that some SNPs change the splicing of mRNA [50]. With all these interpretations, it could be suggested that c.870G>A transition may affects CCND1 mRNA structure apart from its effect on splicing.

Three Meta-analyzes have been conducted about the association of CCND1 gene c.870G>A polymorphism and breast

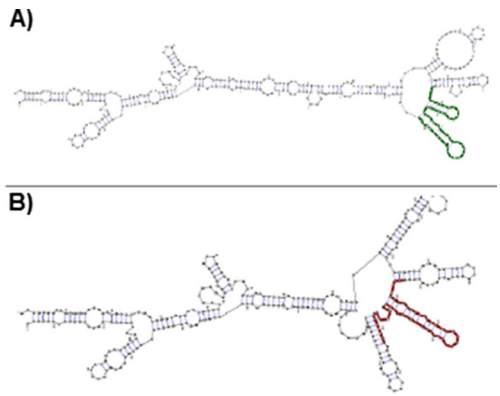

Fig. 5 Secondary structure of mRNA of CCND1. Optimal secondary structure of global sequence (highlighted from 524 to 888 nt) in the genotype GG with minimum free energy = −138.70 kcal/mol (**a**) and genotype AA with minimum free energy = −136.40 kcal/mol (**b**)

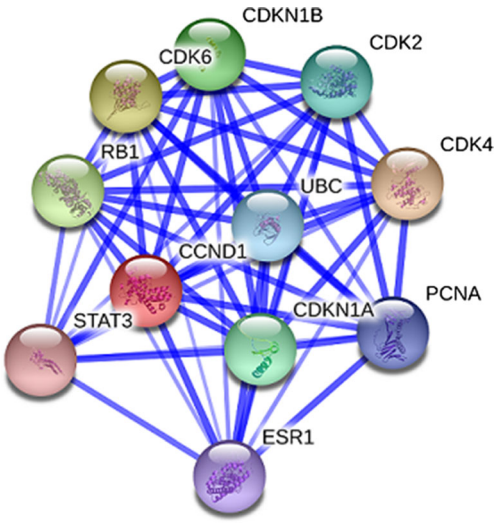

Fig. 6 Human CCND1-interactions network was deduced from String server. The CCND1 gene interacts with 10 other genes. CDKN1A: cyclin-dependent kinase inhibitor 1A; CDKN1B: Cyclin-dependent kinase inhibitor 1B; CDK2: Cyclin-dependent kinase 2; CDK4: Cyclin-dependent kinase 4; CDK6: Cyclin-dependent kinase 6; PCNA: Proliferating cell nuclear antigen; ESR1: Estrogen receptor 1; STAT3: Signal transducer and activator of transcription 3; RB1: Retinoblastoma 1; and UBC: Ubiquitin C

cancer risk [51–53]. The study of Cui et al. 2012 [53] is more complete than the other two studies. However, there were some errors in Cui study. For example, they reported the data of Justenhoven et al. (2009) study, incorrectly. Also, the *p*HWE in control groups of three studies [12, 20, 21] was reported wrong in the study of Cui et al. 2012 [53]. Nevertheless, there are some limitations in our meta-analysis that must be mentioned. First, we don't have access to original data of the included studies, then we couldn't evaluate some possible interactions such as gene-environment and gene-gene, which may modulate the risk of cancer. Second, no data from African or Latino populations included in the meta-analysis. Third, a possibly language bias may arise from the restriction of our study to English language papers.

There are different molecular subtypes of breast cancer: Luminal A, Luminal B, HER2 over-expression, Basal, Normal-like forms and recently established claudin-low subtype [54, 55]. Cyclin D1 is known to be expressed at variable levels across cell lines and subtypes of breast cancer [56]. The pattern of cyclin D1 overexpression in tissues along the spectrum from normal epithelium to invasive breast cancer suggests the involvement of cyclin D1 in the earliest stages of mammary carcinogenesis [57]. Therefore, because of involvement of cyclin D1 in earliest stages of all subtypes; it seems logical to say that if a mutation affects cyclin D1 function or expression, all subtypes of breast cancer will be affected. Also, if a mutation couldn't affect the function of molecule, then it is probable that none of cancer subtypes could be identified as affective. Here, we showed a significant association between CCND1 c.870G>A polymorphism and breast cancer risk and according to above mentioned facts, all subtypes of breast cancer could be affected from this polymorphism.

It is shown that inhibition of endogenous cyclin D1 expression by siRNA resulted in accumulation of cells in G1. Then, pharmacological inhibition of cyclin D1 proposed as a useful strategy to inhibit the growth of tumors [58]. A number of therapeutic agents have been shown to induce cyclin D1 degradation. The therapeutic ablation of cyclin D1 may be useful for the prevention and treatment of cancer [59]. If a polymorphism in cyclin D1 facilitates the function of siRNA or degradation enzymes activity, it will be helpful. Indeed, there are some structural polymorphism that enhance degradation of proteins. For example, T341C (Ile114Thr) polymorphism significantly reduces cytosolic NAT2 immunoreactive protein through enhanced protein degradation [60]. We couldn't find a report about the effect of polymorphisms on cyclin D1 degradation or inhibitory drugs impacts. But, theoretically it is plausible that polymorphisms could modify cyclinD1 activity or sensitivity for inhibitory drugs.

In conclusion, our case-control study and meta-analysis suggest that the CCND1 c.870G>A polymorphism is associated with the increased risk of breast cancer especially in Caucasian population. However, further studies with more subjects are required to approve our results. In addition, association of other SNPs in CCND1 gene and possible haplotypes with breast cancer risk should be studied. Finally, as depicted in Fig. 6 the CCND1 gene interacts with 10 other genes, so the study of this interactions and their role in breast cancer risk would be useful.

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Compliance with Ethical Standards

Conflict of Interest There are no conflicts of interest related to this project for any of the authors.

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