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The polymorphism of CAG repeats in the androgen receptor gene and breast cancer mortality

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Abstract.
BACKGROUND: The polymorphic CAG repeats of the androgen receptor (AR) gene have been suggested to affect the risk of breast cancer, but the results are controversial. In addition, the relationship between patients' CAG genotype and the prognosis has not been investigated.
OBJECTIVE: The purpose of this study is to access the association between the polymorphic CAG repeats and the incidence and prognosis of breast cancer.
METHODS: One hundred and fifty-six breast cancer cases and 108 healthy controls from Taipei Veteran General Hospital were enrolled. The length of CAG repeats was analyzed among by means of PCR amplification. The logistic regression model was used for cross-sectional analyses of prevalent breast cancer.
Furthermore, we categorized the cases according to the average length of both CAG alleles (CAGn $\geqslant 23$ *versus* < 23). Outcomes were disease-free survival and mortality. The Cox proportional hazards model and Kaplan-Meier estimate were used for survival analysis.
RESULTS: The median age was 56 (51–64) and 46 (37–52) in breast cancer patients and healthy controls, respectively. The median of CAGn was 22.5 (21.5–24) in study group and 23 (21.5–24) in controls. Our study showed the length of CAG repeats did not contribute to breast cancer or benign breast tumors (HR 1.01; 95% CI, 0.90–1.13). In the median follow-up of 6.59 years, we found the CAGn $\geqslant 23$ ($n = 75$) could be a poor prognosis (adjust HR, 3.08; 95% CI, 1.42–6.67, $p = 0.004$).
CONCLUSION: The CAG polymorphism is not associated with development of breast cancer, but patients with more CAG repeats of the AR gene are prone to poor prognoses.

Keywords: Prognostic factor, CAG repeat, CAG polymorphism, androgen receptor, breast cancer

1. Introduction

Sex hormones play a certain role in the development of breast cancer. Unlike in prostate cancer, androgen in the development of breast cancer is controversial. Some evidence suggests that androgen may have a synergic effect with estrogen on tumor proliferation, but other studies have proposed that androgen

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protects the breast cancer [1]. Androgen can bind with the androgen receptor (AR) and activate the transcription of androgen-related genes. For example, breast cancer susceptibility gene 1 (BRCA1) is one of the cofactors and can interact with the AR [2]. In addition, the AR also regulates the cell cycle from the G1 to the S phase [3].

The AR gene with eight exons is located on the X chromosome. A series of CAG trinucleotide repeats in exon 1 codes a polyglutamine tract at the N-terminal domain of the AR. The length of CAG repeats varies individually and is believed to affect the activity of the AR [4,5]. The polymorphism of CAG repeats on breast cancer has been investigated. Although the majority of these studies reveal no correlation between lengths of CAG repeats and breast cancer, both positive and inverse results have been reported. In addition, the relationship between the polymorphism of CAG repeats and the incidence of benign breast tumors has not yet been explored

Recently, it has been reported that the AR expression ($> 1\%$ tumor cell nuclei staining) in the cancer tissue is a good prognostic factor among estrogen receptor (ER) positive breast cancer patients and triple negative breast cancer patients [6,7]. These results indicate that the AR could be a novel target for breast cancer treatment. Thus, we hypothesize that the patients' CAG genotype of the AR may affect not only breast cancer incidence but also cancer survival.

Therefore, we designed a case-control study to investigate the correlation between the polymorphism of CAG repeats and the incidence of breast cancer and benign breast tumors. Furthermore, we examine the relationship between the polymorphism of CAG repeats and breast cancer mortality.

2. Patients and methods

2.1. Study population

One hundred fifty-six patients, who were referrals from primary or secondary health professionals or firstly diagnosed at Taipei Veterans General Hospital, with pathologically confirmed breast cancer were enrolled in this study. One hundred eight healthy women receiving physical check-up at Taipei Veterans General Hospital were selected as controls. Informed consents were signed by all participants. All analyses of CAG repeats were performed in 2009. The basic characteristics of controls and cases were collected. Cases' clinical information, expression of ER, expression of human epidermal growth factor receptor 2 (HER2), tumor stage, therapeutic strategies, and comorbidity were assessed. All study cases were followed until either death, loss of follow-up or the recent data retrieved date on June 30, 2014.

2.2. Analysis of CAG repeats

DNA samples were extracted from whole blood via a DNA isolation kit (Puregene Gentra Systems, Minneapolis, MN), according to the manufacturer's instructions. The primers for CAG polymorphism of AR were designed as follows: forward ($5' \rightarrow 3'$, TCCAG AATCTGTTCCAGAGCGTGC) and reverse ($5' \rightarrow 3'$, GCTGTGAAGGTTGCTGTTCCTCAT). PCR amplifications were processed in a 12.5 μL of buffer containing 10 ng of template DNA, 10 mM Tris-HCl, 50 mM potassium chloride, 1.75 mM magnesium chloride, 200 μM dNTP, 0.2 μM of each primer, and 0.1 U of Ampli Taq Gold DNA polymerase (Applied Biosystems, Foster City, CA). Amplification profile was performed as follows: initial denaturation at 95°C for 11 minutes and 30 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for one minute and extension at 72°C for one minute. Electrophoresis was performed with an ABI 310 Genetic Analyzer (Applied Biosystems Inc.) in which 1 μL of PCR product was mixed with 25 μL Hi–Di formamide and 0.5 μL of the GeneScan-500ROX size standard. GeneScan Analysis Software (Version 3.7) was used to analyze the data. Allelic typing was based on the genotypes sequenced from three commercially available cell lines: K562 (Promega, Madison, WI), 9947A and 9948 (Applied Biosystems).

2.3. Outcome and statistical analyses

The polymorphism of CAG repeats was obtained from each study case and the controls. The average length of both CAG alleles (CAG_n), length of CAG repeats in the long allele and length of CAG repeats in the short allele were compared between cases and controls by Mann-Whitney U test. The only male patient was categorized into the patients with homozygous alleles. A logistic regression test was conducted to estimate the odds ratio (OR) between lengths of CAG repeats and breast cancer risk and development of benign breast tumors.

We further performed survival analysis among study group. We chose the median of CAG_n ($\mathrm{CAG}_n \geqslant 23$

Table 1
Baseline characteristics

	Case	Control	p
Number of participants	156	108	
Median age at diagnosis	56 (51–64)	46 (37–52)	< 0.001
Homozygous	31 (19.9%)	15 (13.9%)	0.208
Heterozygous	125 (80.1%)	93 (86.1%)	
Average length of both CAG alleles			
Mean (SD)	22.7 (2.15)	22.7 (2.19)	0.83
Median (interquartile range)	22.5 (21.5–24)	23 (21.5–24)	0.712
Median length of the long allele	24 (23–26)	24 (22–26)	0.377
Median length of the short allele	21 (20–23)	22 (20–22.75)	0.758

versus $\text{CAG}_n < 23$) as the cutoff to dichotomize the case group. Categorical variables were compared by chi-square test. Disease progression and mortality were the outcome. Disease-free survival (DFS) was calculated between the date of diagnosis and the date of local recurrence, radiographic evidence of disease progression or new metastatic lesion, development of second breast cancer or other primary malignancy, death, or the last follow-up visit. Disease progression was defined as relapse, new metastatic lesion, development of second breast cancer or new diagnosis of other primary malignancy during the follow-up period. Survival was calculated from the date of diagnosis to the date of death or the last follow-up visit. For analysis adjustment, relevant risks, such as age, comorbidities, ER, HER2, and cancer stage, were analyzed by Cox proportional hazards regression. The Cox proportional hazards model was used to estimate the hazard ratios (HRs) for survival analysis. OS and DFS were illustrated by means of the Kaplan-Meier estimate. All analyses used IBM SPSS Statistics (version 21).

3. Result

3.1. CAG repeats and breast tumors

One hundred fifty-six breast cancer patients, including one male patient, were enrolled and compared with 108 healthy female controls. The median age was 56 years (interquartile range, 51–64 years) and 46 years (interquartile range, 37–52 years) in the breast cancer group and control group, respectively. The median of CAG_n was 22.5 (21.5–24) among breast cancer patients and 23 (21.5–24) among healthy individuals ($p = 0.712$). For zygosity status, 19.9% of the cases and 13.9% of the control group were homozygous ($p = 0.208$). The median length of CAG repeats on the long allele was 21 in the cases and 22 in the controls ($p = 0.758$) (Table 1).

We found the length of CAG repeats was not a risk factor for breast cancer. The ORs for each unit increased in length for CAG_n, the long allele, and the short allele were 1.01 (95% CI, 0.90–1.13, $p = 0.836$), 0.98 (95% CI, 0.89–1.07, $p = 0.720$), and 1.03 (95% CI, 0.94–1.13, $p = 0.457$), respectively. Furthermore, 39 participants in the control group had benign breast tumors. Compared to normal participants, we found the linkage between the length of CAG repeats and the development of benign breast tumors was not significant (OR, 1.00; 95% CI, 0.83–1.20, $p = 0.959$) (Table 2).

3.2. CAG repeats and survival of breast cancer patients

The basic characteristics of patients with $\text{CAG}_n \geqslant 23$ ($n = 75$) and < 23 ($n = 81$) were similar. The median follow-up time was 6.4 (4.4–8.7) years and 6.7 (4.5–9.7) years, respectively. We observed no association between the length of CAG repeats, ER, HER2 and cancer stage (Table 3).

Within the duration of follow-up, 52 patients died of breast cancer. The risk factors in the univariate analysis included tumor size with T3 or T4 (HR, 6.35; 95% CI, 2.19–18.42), lymph node metastasis (HR, 5.90; 95% CI, 2.07–16.83), and distant metastasis (HR, 7.00; 95% CI, 3.37–14.55). In the multivariable analysis, lymph node metastasis (HR, 4.34; 95% CI, 1.44–13.12), and distant metastasis (HR, 6.25; 95% CI, 2.30–17.01) still independently correlated with cancer-related mortality.

In univariate analysis, the length of $\text{CAG}_n \geqslant 23$ (crude HR, 1.53; 95% CI, 0.85–2.75, $p = 0.150$), length of the long allele > 24 (crude HR, 1.25; 95% CI, 0.69–2.26, $p = 0.457$) and length of the short alleles > 21 (crude HR, 1.30; 95% CI, 0.73–2.32, $p = 0.368$) were not correlated with patients' OS and DFS. After multivariate analysis, the length of $\text{CAG}_n \geqslant 23$ (adjust HR, 3.08; 95% CI, 1.42–6.67, $p = 0.004$) for OS and length of the long alleles > 24 for DFS

Table 2
Cross-sectional association of CAG repeats with malignant and benign breast tumors

	OR (95% CI)	p value
Breast cancer[a]		
Average length of both CAG alleles	1.01 (0.90–1.13)	0.836
CAG repeats in the long allele	0.98 (0.89–1.07)	0.720
CAG repeats in the short allele	1.03 (0.94–1.13)	0.457
Benign breast tumor[b]		
Average length of both CAG alleles	1.00 (0.83–1.20)	0.959
CAG repeats in the long allele	1.05 (0.90–1.22)	0.513
CAG repeats in the short allele	0.96 (0.82–1.11)	0.583

[a]Compared with participants with normal breast tissue and benign breast tumors; [b]Compared with participants with normal breast tissue.

Table 3
Characteristics of patients

	Average length of both CAG alleles		
	$\geqslant 23$	< 23	P
Number of cases (%)	75 (48.1)	81 (51.9)	0.071
Age at diagnosis years (interquartile range)	58 (52–67)	55 (50–61)	0.979
Median of follow-up years (interquartile range)	6.4 (4.4–8.7)	6.7 (4.5–9.7)	0.747
Stage (%)			0.506
1	10 (13.3)	12 (14.8)	
2	24 (32)	28 (41.8)	
3	19 (25.3)	18 (22.2)	
4	10 (13.4)	9 (11.1)	
ER/PR positive (%)	48 (64)	51 (63)	0.751
HER2/NEU positive (%)	21 (28)	16(19.8)	0.127
TNBC positive (%)	11 (14.7)	15 (18.5)	0.608

ER, Estrogen receptor; HER2, Human Epidermal Growth Factor Receptor 2; TNBC, Triple-negative breast cancer.

Table 4
Association of CAG repeats with breast cancer survival

	DFS		OS	
	HR (95% CI)	p value	HR (95% CI)	p value
Average length of both CAG alleles $\geqslant 23$				
Unadjusted	1.17 (0.74–1.87)	0.487	1.53 (0.85–2.75)	0.150
Age-adjusted	1.21 (0.76–1.94)	0.411	1.61 (0.90–2.90)	0.108
Multivariable-adjusted[a]	1.51 (0.86–2.66)	0.145	3.08 (1.42–6.67)[b]	0.004
CAG repeats > 24 in the long allele				
Unadjusted	1.46 (0.90–2.35)	0.120	1.25 (0.69–2.26)	0.457
Age-adjusted	1.48 (0.91–2.39)	0.106	1.25 (0.69–2.13)	0.489
Multivariable-adjusted[a]	1.83 (1.04–3.20)	0.034	1.60 (0.74–3.41)	0.225
CAG repeats > 21 in the short allele				
Unadjusted	1.15 (0.72–1.82)	0.551	1.30 (0.73–2.32)	0.368
Age-adjusted	1.15 (0.72–1.83)	0.539	1.29 (0.72–2.31)	0.375
Multivariable-adjusted[a]	1.63 (0.92–2.89)	0.094	1.26 (0.27–5.81)	0.760

DFS, Disease free survival; ER, Estrogen receptor; HER2, Human Epidermal Growth Factor Receptor 2; OS, Overall Survival; TNBC, Triple-negative breast cancer; [a]Adjusted for age, Stage, ER and HER2/NEU and comorbidities; [b]Interaction with ER (+), $p = 0.017$.

(adjust HR, 1.83; 95% CI, 1.04–3.20, $p = 0.034$) were risk factors. The interaction with ER was present ($p = 0.017$). In stratified analysis (ER positive, ER negative, triple-negative breast cancer, and HER2 positive), we found the long $\text{CAG}_n \geqslant 23$ appeared to be a poor prognostic factor for patients with ER negative (adjust HR, 13.0; 95% CI, 2.20–77.2, $p = 0.005$) and triple negative breast cancer (adjust HR, 16.1; 95% CI, 1.41–183, $p = 0.025$). The relationship between CAG repeats and DFS and OS was summarized in Tables 4 and 5. Kaplan-Meier curve were also shown in the Fig. 1 ($p = 0.001$) and Fig. 2 ($p = 0.052$).

4. Discussion

To the best of our knowledge, this is the first study to explore the role of patients' CAG genotype on breast

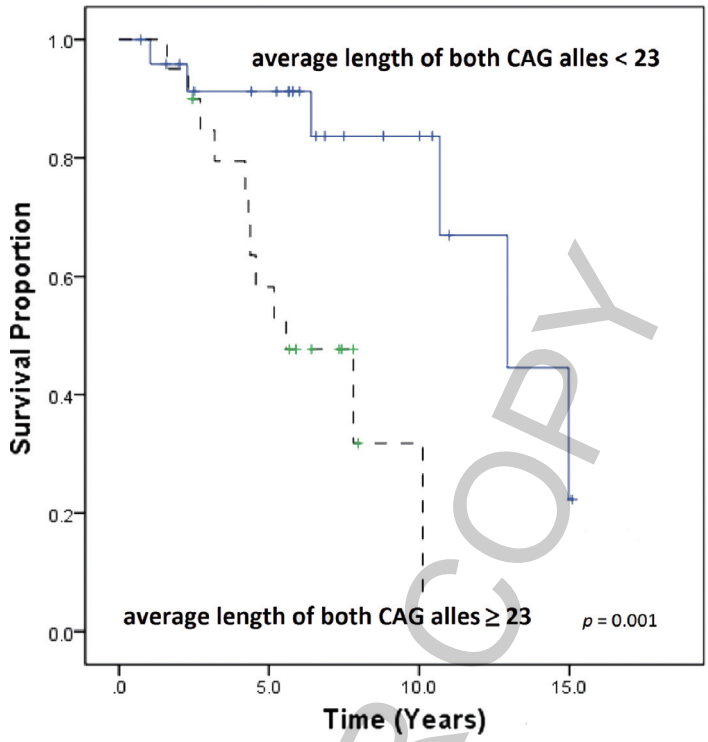

Fig. 1. Survival curve for patients with ER (-). (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/CBM-150525)

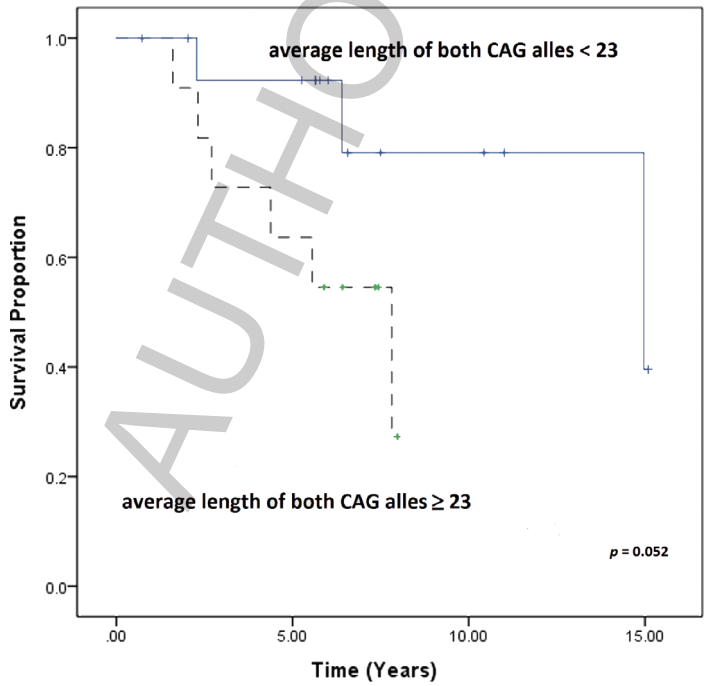

Fig. 2. Survival curve for patients with triple-negative breast cancer. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/CBM-150525)

Table 5
Stratified analysis

	ER (+)				ER (-)			
	DFS		OS		DFS		OS	
	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
Average length of both CAG alleles								[a]
Unadjusted	1.07 (0.95–1.21)	0.253	1.12 (0.94–1.35)	0.190	1.29 (1.03–1.61)	0.022	1.43 (1.12–1.82)	0.004
Age-adjusted	1.07 (0.95–1.22)	0.242	1.16 (0.96–1.41)	0.113	1.32 (1.05–1.64)	0.014	1.43 (1.12–1.83)	0.004
Multivariable-adjusted[a]	1.12 (0.97–1.30)	0.114	1.30 (1.03–1.65)	0.024	1.35 (1.03–1.77)	0.025	1.52 (1.14–2.03)	0.004
Average length of both CAG alleles $\geqslant 23$								
Unadjusted	1.04 (0.59–1.85)	0.876	0.99 (0.46–2.12)	0.994	2.29 (0.97–5.37)	0.057	6.44 (1.76–23.5)	0.005
Age-adjusted	1.05 (0.58–1.90)	0.857	1.23 (0.55–2.72)	0.605	2.37 (1.00–5.67)	0.048	6.29 (1.70–23.2)	0.006
Multivariable-adjusted[b]	1.35 (0.67–2.71)	0.400	8.51 (0.68–106)	0.096	2.06 (0.60–7.05)	0.246	13.0(2.20–77.2)	0.005
	HER2 (+)				TNBC			
	DFS		OS		DFS		OS	
	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
Average length of both CAG alleles[a]								
Unadjusted	1.27 (1.00–1.62)	0.042	1.53 (1.11–2.14)	0.011	1.12 (0.81–1.53)	0.477	1.22 (0.85–1.76)	0.265
Age-adjusted	1.27 (1.00–1.62)	0.043	1.53 (1.08–2.15)	0.015	1.15 (0.83–1.58)	0.388	1.24 (0.85–1.79)	0.254
Multivariable-adjusted[a]	1.29 (0.93–1.79)	0.123	1.50 (1.03–2.19)	0.037	1.21 (0.81–1.81)	0.350	1.49 (0.93–2.39)	0.096
Average length of both CAG alleles $\geqslant 23$								
Unadjusted	1.79 (0.67–4.78)	0.444	8.12 (1.01–65)	0.048	1.94 (0.59–6.38)	0.275	4.31 (0.86–21.4)	0.074
Age-adjusted	1.77 (0.66–4.73)	0.256	7.30 (0.87–60.6)	0.066	2.08 (0.63–6.92)	0.228	4.28 (0.85–21.4)	0.076
Multivariable-adjusted[b]	0.49 (0.07–3.41)	0.474	5.00 (0.13–182)	0.380	5.39 (0.68–42.3)	0.025	16.1 (1.41–183)	0.025

DFS, Disease free survival; ER, Estrogen receptor; HER2, Human Epidermal Growth Factor Receptor 2; OS, Overall Survival; TNBC, Triple-negative breast cancer; [a]As a continuous variable; [b]Adjusted for age, Stage, ER and HER2/NEU and comorbidities.

cancer mortality. Our results show patients' polymorphic CAG repeats and zygosity status are not associated with the risk of breast cancer. For the cases, tumor size, lymph node metastasis, distant metastasis, ER expression and HER2 expression were not correlated with the length of CAG repeats. In the survival analysis, a trend showed that patients with $CAG_n \geqslant 23$ would have higher mortality, especially among patients with ER negative or triple negative breast cancer.

Several previous studies have reported an irrespective relationship between polymorphic CAG repeats of the AR and the incidence of breast cancer. The largest participants enrolled from Harvard Nurses' Health Study and Women's Health Study indicated no association between CAG polymorphism and the risk of breast cancer [8]. As BRCA1 was a cofactor of the AR, Kadouri et al. proposed that CAG polymorphism might have an impact on BRCA1 carriers. However, they found a not significant result (Relative Risk 1.05; 95% CI 0.97–1.17) after comparing 122 patients bearing BRCA1/2 mutations with 66 BRCA1/2 carriers [9]. In addition, a study on Taiwanese subjects also displayed no linkage between the length of CAG repeats and breast cancer risk [10].

However, some conflicting results exist about breast cancer incidence. For example, González et al. conducted a study including 257 Spanish cases and 393 Spanish controls. They reported that women with an average length of both CAG alleles > 22 had an increased risk of breast cancer (OR 1.49; 95% CI 1.06–2.09) [11]. Similar results were observed by Mehdipour and colleagues (OR 2.03; 95% CI 1.56–2.60) [12]. A recent meta-analysis displayed an opposite point and showed that long CAG repeats might contribute to protective effects on breast cancer [13].

Regarding tumor size, lymph node metastasis, distant metastasis, clinical stage at diagnosis and presentation of ER and HER2, our study does not show a significant relationship to the length of CAG repeats. Nevertheless, pathological features, including histologic grade, were not available for all patients in our study.

In the survival analysis, our results showed a trend that patients with the average length of both CAG alleles $\geqslant 23$ could have poor prognosis, but the probability was not in statistical significance. It may be related to the limited case numbers. Further enrolling more cases to analyze the probability will give definite conclusions. The CAG polymorphism influencing the prognosis or carcinogenesis of breast cancer is probably via changing the efficiency of the AR. In humans, it has been demonstrated that the length of CAG repeats is inversely associated with the transactivation function of the AR [5]. Recently, some selective androgen receptor modulators have been investigated to increase muscle mass against cachexia [14]. In addition, the polymorphism of CAG repeats has been reported to affect mus-

cle mass [15]. Thus, the activity of the AR perhaps influences patients' physical function and prognosis indirectly.

In cancer cell biology, AR signaling might be associated with the growth of breast cancer cells [7]. About 85% of primary breast cancer and 45% of triple negative breast cancer express the AR, and the AR expression is well known as a good prognostic factor [6,16]. However, no data has provided the linkage between patients' AR genotype and AR expression in the tumor cells. In addition, the AR gene is located on chromosome X. Women have two X chromosomes, and the mechanism behind which X chromosome are expressed or activated in cancer cells is still unclear. As a result, the variation of CAG repeats between normal tissue and cancer cells may be dissociated [17].

Our study has several limitations. First of all, the AR expression on tumor cells was limited and we could not confirm the link with CAG polymorphism. Second, all participants were volunteers and we did not enrolled adequate controls resulting in unfitted match. Third, some clinical information, such as exposure to oral contraceptives, body mass index, X-chromosome inactivation, menarche and gestation, were not available for analysis. Finally, carcinogenesis is a long process; therefore, a longer follow-up time is necessary for the development of breast cancer.

In conclusion, our study shows that the long length of CAG repeats was not a risk for development of breast cancer. In survival analysis, the CAG polymorphism was not a prognostic factor on patients with breast cancer. In subgroup analysis, the average length of both CAG alleles $\geqslant 23$ has a trend to be a poor prognostic factor among patients with ER negative or triple negative breast cancer. The association is of borderline significance. Further investigations are needed to determine the role of patients' CAG genotype on cancer-associated mortality.

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Conflict of interests

The authors declare that there is no conflict of interests.

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