Report

Shorter CAG repeat length in the androgen receptor gene is associated with more aggressive forms of breast cancer

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Summary

The androgen receptor (AR) is a transcription factor mediating the action of androgens. The AR gene is localized on chromosome X and it contains a series of CAG trinucleotide repeats. The length of the CAG repeats varies among individuals and this polymorphism is believed to be related to AR transcriptional activity. Studies have shown that fewer CAG repeats are associated with an increased risk as well as more aggressive forms of prostate cancer. Although AR is expressed in breast cancer and the impact of androgen and AR on breast cancer has been recognized, the role of the CAG repeats in breast cancer remains unknown. In this study, we measured the CAG repeats in breast cancer tissue using a PCR-based method. Of the 133 patients with primary breast cancer, 102 were heterozygous and 31 were homozygous. The mean CAG repeat number for homozygous women was 21; for heterozygous women the repeat number mean was 20 for the short allele and 24 for the long allele. The length of CAG repeats either in one allele or in both alleles was inversely correlated with the histological grade of breast cancer ($r = -0.23$ or -0.26, respectively, $p < 0.05$). An association between positive lymph nodes and fewer CAG repeats in both alleles was also suggested ($p = 0.06$). Furthermore, survival analysis indicated that the total number of CAG repeats in both alleles was associated with patient overall survival. With every CAG repeat increase, there was a 6% reduction in the risk of death ($RR = 0.94$, $p = 0.03$). The association remained significant after controlling for the homozygous and heterozygous status ($RR = 0.92$, $p = 0.01$). The association became no longer significant when clinical and pathological variables were adjusted in the analysis but this could be due to the reduction of sample size in the multivariate analysis. CAG heterozygosity and difference in number of CAG repeats between the two alleles were not associated with either disease features or patient survival. Our results suggest that longer CAG repeats may occur more frequently in less aggressive cancer and that the CAG repeats may play a role in breast cancer progression.

Introduction

The androgen receptor (AR) binds specifically to androgen and mediates androgen action by activating the transcription of androgen-regulated genes. Thus, the activity of AR is critically involved in the action of androgen. AR is a protein of 910 amino acids, and the AR gene resides on chromosome X [1]. In the amino terminus of AR, there exists a glutamine stretch that is encoded by a series of CAG trinucleotides, known as CAG repeats. The length of CAG repeats in the AR gene varies considerably among individuals. This polymorphic variation is believed to be related to the transcriptional activity of the AR and consequently, it could affect the potency of androgen action [2, 3]. Recently, several studies have suggested an inverse

association between the length of CAG repeats and the risk of prostate cancer, that is, fewer CAG repeats being associated with an increased risk [4–7].

The impact of androgen and AR on breast cancer has been recognized for many years [8]. AR is expressed in breast tissue [9, 10], and the expression of AR in breast cancer is correlated with estrogen receptor (ER) and progesterone receptor (PR) expression [11, 12]. The presence of these steroid receptors in breast cancer is associated with well-differentiated cancer cells. Therefore, receptor status has clinical implications for prognosis of breast cancer as well as for prediction of response of tumor cells to endocrine treatment. Androgen itself has been used effectively in treating some breast cancer patients, and the average response rate is about 20% [13, 14]. Androgen has also been suspected to play a role in breast cancer development [8, 15]. Findings from some epidemiological studies did suggest such a role, indicating that high plasma levels of testosterone are associated with an increased risk of breast cancer in women [15-17].

To further explore the role of AR in breast cancer, we analyzed the CAG repeats of the AR gene in tumor tissues from 133 breast cancer patients and examined associations of the CAG repeats with clinical and pathological features of breast cancer. Survival analysis was also performed to assess the potential impact of the CAG repeats on breast cancer progression.

Materials and methods

Tissue specimens and breast cancer patients

One hundred fifty-one consecutive patients with histologically confirmed primary breast cancer were selected for this study. These patients underwent either radical or modified mastectomy at the Department of Gynecologic Oncology, University of Turin, Italy. Fresh tissue specimens were snap frozen in liquid nitrogen immediately after surgical resection and stored at –80°C until analysis. The age of the patients ranged from 25 to 93 years with a mean of 55. Tumor size was known for 148 patients and ranged between 0.8 and 7 cm with a mean of 2.7 cm. Of the 150 patients who had information on clinical stage, 30% had a stage I disease, 58% had stage II, 4.7% had stage III and 7.3% had stage IV. Information of histological grade was available for 107 patients. Among them, six (5.6%) had grade 1, 57 (53.3%) had grade 2 and 44 (41.1%) had grade 3 disease. Sixty-two percent of patients had tumor that had infiltrated the lymph nodes.

Sixty-seven percent and 64% of the patients were ER and PR positive, respectively. Follow-up information was available for 148 patients. The median follow-up time was 64.5 months. Prostate-specific antigen (PSA) concentrations in the tumor tissue extracts, measured previously by an ELISA method [18], were also available for this study.

Specimen preparation and PCR analysis of CAG repeats

Method

DNA from breast tumors was extracted by using the QIAamp kit, following the manufacturer's recommendations (Qiagen, Chatsworth, CA, USA). The method for determining the CAG repeat length in the AR gene is described in detail elsewhere [19]. Briefly, we amplify by PCR a region of exon 1 of the AR gene that contains the CAG repeat, using one primer labeled with Cy5.5 fluorescent dye. The PCR product is then resolved in a polyacrylamide gel using the DNA sequencer MicroGene Blaster^{TM} from Visible Genetics Inc., Toronto, Ontario, Canada. Accurate sizing with a resolution of ± 1 base is accomplished routinely by comparison to molecular weight standards. With this method, CAG sizing is relatively simple and highly reproducible. An example of analyzing three female samples (one homozygous and two heterozygous) is shown in Figure 1.

Statistical analysis

The CAG repeat data were analyzed in four categories, three of which were continuous variables including the number of CAG repeats in allele 1, total number of CAG repeats in both alleles, and the difference in number of CAG repeats between alleles. One categorical variable included the CAG repeat status, being either homozygous or heterozygous. For heterozygous samples allele 1 was the allele with the fewer number of CAG repeats. The total number of CAG repeats in both alleles was the number of CAG repeats in allele 1 plus the number of CAG repeats in allele 2 for the heterozygous samples, and was the number of CAG repeats multiplied by 2 for the homozygous samples. The difference in number of CAG repeats between alleles was 0 if the samples were homozygous.

Correlations of the three numerical CAG variables among themselves and with other numerical variables were analyzed with either Pearson or Spearman correlation coefficients. Mean CAG repeats in allele 1
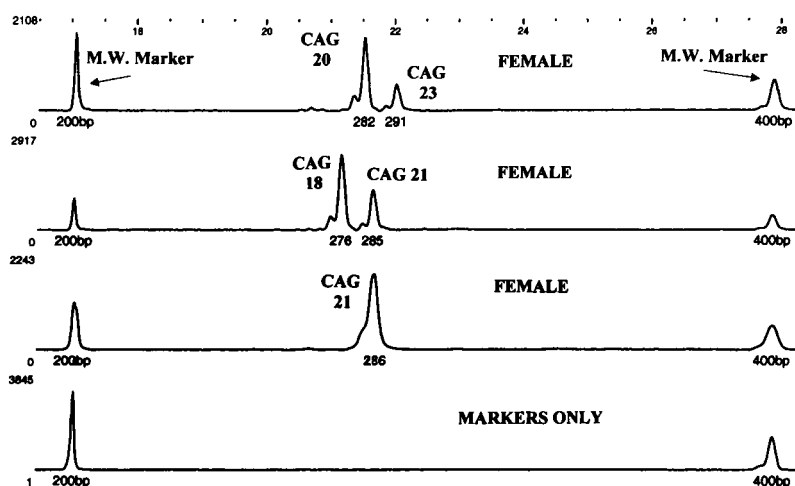
Figure 1. Fragment analysis of CAG repeats in the androgen receptor (AR) gene of three DNAs from females. In the first lane, the sample is a heterozygote with CAG repeat lengths of 20 and 23. The length of the PCR products is shown in bases, under each peak. Lane 2: Another heterozygote sample. Lane 3: A homozygote sample. The fourth lane contains molecular weight markers only (length 200 and 400 base pairs). The method is described in detail elsewhere [19].

or in both alleles and mean CAG differences between alleles were compared among various categories of clinical and pathological variables with use of the analysis of variance (ANOVA). The three numerical CAG variables were also classified into dichotomous groups using the mean of each variable as a cutoff point. Associations between the categorical CAG variables and clinical or pathological variables were examined with the use of the chi-square test. The Cox proportional hazards regression analysis was performed to determine the relative risks of relapse and death in association with the CAG variables, including the three continuous variables and the one categorical variable. Disease-free survival and overall survival were the outcome variables in the Cox regression model. The model was developed at both univariate and multivariate levels, and the interaction between two variables was assessed in the model using the product of two given variables. All *p* values were calculated based on two-sided statistical tests.

Results

CAG repeats in breast tissue

Of the 151 patients included in the study, 133 had DNA specimens available for the analysis of CAG repeats. The results of the DNA analysis indicated that 102 patients (77%) were heterozygous and 31 (23%) were homozygous (Table 1). The mean number of CAG repeats was 21 in homozygous women.

Table 1. Androgen receptor (AR) CAG repeats in 133 breast cancer patients

	Homozygous	Heterozygous
Number of samples	31	102
Number of CAG repeats		
Allele 1		
Mean (SD)	21.0 (3.6)	20.4 (2.6)
Median	21	20
Range	15–27	8–28
Allele 2		
Mean (SD)	–	24.3 (2.4)
Median	–	24
Range	–	18–34
CAG repeat difference between alleles		
Mean (SD)	–	3.9 (2.6)
Median	–	3
Range	–	1–15

For heterozygous women, the average number of CAG repeats was 20 in the short allele and 24 in the long allele. Interestingly, it appears that the variation of the number of CAG repeats among individuals was slightly larger in homozygous samples (standard deviation, SD = 3.6) than in heterozygous samples (SD = 2.6 and 2.4 for the two alleles). By Pearson correlation, the number of CAG repeats in allele 1 was positively correlated with the total number of CAG repeats in both alleles ($r = 0.87$, $p = 0.0001$), and was

Table 2. Numer of CAG repeats and clinicopathological features of breast cancer

Feature	Number of patients	CAG repeats[a] in allele 1 (Mean)	CAG repeats[b] in both alleles (Mean)	CAG difference[c] between alleles (Mean)
Stage				
1	36	20.3	43.6	3.0
2	79	20.8	44.3	2.8
3–4	17	19.6	43.5	4.4
p value[d]		0.267	0.737	0.106
Tumor size (cm)				
<2.5	48	20.4	44.1	3.4
≥2.5	83	20.6	44.0	2.8
p value[d]		0.692	0.858	0.254
Grade				
1–2	55	21.1	45.0	2.9
3	37	19.9	42.8	2.9
p value[d]		0.071	0.055	0.933
Nodal status				
Negative	48	21.0	45.0	3.0
Positive	79	20.3	43.7	3.1
p value[d]		0.156	0.143	0.864
ER				
Positive	90	20.5	44.1	3.1
Negative	39	20.2	43.5	3.1
p value[d]		0.580	0.530	0.961
PR				
Positive	82	20.6	44.3	3.2
Negative	47	20.2	43.3	2.9
p value[d]		0.489	0.314	0.640

[a]Mean number of CAG repeats in allele 1.
[b]Mean total number of CAG repeats in allele 1 plus allele 2.
[c]Mean difference in CAG repeats between alleles 1 and 2.
[d]ANOVA.

inversely correlated with the number of CAG repeat difference between alleles ($r = -0.41$, $p = 0.0001$). No correlation was found between the total length of CAG repeats in both alleles and the difference between alleles ($r = 0.09$, $p = 0.29$).

Associations between CAG repeats and clinical and pathological features of breast cancer

Table 2 shows the mean CAG repeats in one allele or in both alleles among various categories of clinical and pathological variables. No significant discrepancy was observed with respect to clinical stage, tumor size, nodal status, and steroid receptors. There was, however, an indication that differences might exist among patients with different histological grade. Patients with lower grade cancer (grade 1 or 2), compared to those with higher grade (grade 3), tended to have slightly longer CAG repeats in both alleles combined (45.0 versus 42.8, $p = 0.055$). This relationship was also suggested for allele 1 (21.1 versus 19.9, $p = 0.071$). In addition, results from Spearman correlation analysis indicated that histological grade was inversely correlated with the length of CAG repeats in either

Table 3. Association of CAG repeats with clinical and pathological features

Feature	CAG repeats in allele 1		CAG repeats in both alleles	
	<21	≥21	<44	≥44
	No. (%)	No. (%)	No. (%)	No. (%)
Stage				
1	15 (23.1)	21 (31.3)	16 (28.7)	20 (26.7)
2	40 (61.5)	39 (58.2)	35 (61.4)	44 (58.7)
3 + 4	10 (15.4)	7 (10.5)	6 (10.5)	11 (14.6)
p value[a]		0.470		0.781
Tumor size (cm)				
<2.5	21 (33.3)	27 (39.7)	20 (35.7)	28 (37.3)
≥2.5	42 (66.7)	41 (60.3)	36 (64.3)	47 (62.7)
p value[a]		0.449		0.849
Nodal status				
Negative	20 (32.8)	28 (42.4)	15 (28.3)	33 (44.6)
Positive	41 (67.2)	38 (57.6)	38 (71.7)	41 (55.4)
p value[a]		0.263		0.062
Grade				
1–2	19 (44.2)	24 (73.5)	15 (40.5)	40 (72.7)
3	36 (55.8)	13 (26.5)	22 (59.5)	15 (27.3)
p value[a]		0.004		0.002
ER				
Positive	42 (65.6)	48 (73.9)	38 (67.9)	52 (71.2)
Negative	22 (34.4)	17 (26.1)	18 (32.1)	21 (28.8)
p value[a]		0.309		0.679
PR				
Positive	40 (62.5)	42 (64.6)	35 (62.5)	47 (64.4)
Negative	24 (37.5)	23 (35.4)	21 (37.5)	26 (35.6)
p value[a]		0.803		0.826

[a]Chi-square test.

allele 1 ($r = -0.23$, $p = 0.03$) or in both alleles ($r = -0.26$, $p = 0.01$). The difference of CAG repeats between alleles did not show much variation among the clinical and pathological categories (Table 2).

The three numerical CAG variables were also analyzed categorically in order to examine their associations with clinical and pathological variables. Table 3 shows the results of analysis of CAG repeats in allele 1 and in both alleles. The cutoff points were the means, 21 for allele 1 and 44 for both alleles. Shorter CAG repeats in allele 1 seemed to be associated with a higher grade of cancer; 26.5% of patients with the CAG repeats ≥21 have grade 3 cancer, compared to 55.8% of the patients with the CAG repeats <21 having grade 3 cancer ($p = 0.004$). A similar finding was also suggested when the number of CAG repeats in both alleles was analyzed. Among patients with CAG repeats ≥44, 27.3% had grade 3 cancers compared to 59.5% of grade 3 cancer in patients with CAG repeats <44 ($p = 0.002$). Associations with clinical stage, tumor size or steroid receptors were not observed. For nodal status, positive lymph nodes seemed to be weakly associated with shorter CAG repeats in both alleles, (71.7% versus 55.4%; $p = 0.062$). For CAG repeats in allele 1, the association was not clear (67.2% versus 57.6%; $p = 0.263$).

Table 4. Association of CAG repeats with breast cancer survival

CAG variable	Disease-fres survival		Overall survival	
	RR[a] (95% CI)[b]	*p* value	RR[a] (95% CI)[b]	*p* value
CAG repeats in allele 1				
Unadjusted (*n* = 130)	0.95 (0.87–1.04)	0.261	0.93 (0.84–1.03)	0.172
Adjusted (*n* = 130)[c]	0.95 (0.87–1.04)	0.282	0.93 (0.84–1.04)	0.198
Adjusted (*n* = 116)[d]	0.99 (0.89–1.10)	0.785	0.98 (0.86–1.12)	0.778
CAG repeats in both alleles				
Unadjusted (*n* = 130)	0.95 (0.90–1.00)	0.072	0.94 (0.88–0.99)	0.033
Adjusted (*n* = 130)[c]	0.95 (0.90–1.00)	0.058	0.92 (0.87–0.99)	0.019
Adjusted (*n* = 116)[d]	0.96 (0.91–1.02)	0.217	0.95 (0.88–1.02)	0.137
CAG zygosity status				
Unadjusted (*n* = 130)	1.15 (0.59–2.24)	0.689	1.35 (0.59–3.09)	0.485
Adjusted (*n* = 130)[e]	2.13 (0.89–5.11)	0.090	1.73 (0.72–4.15)	0.223
Adjusted (*n* = 116)[f]	0.99 (0.47–2.07)	0.980	1.77 (0.68–4.61)	0.243
CAG repeat difference between alleles				
Unadjusted (*n* = 130)	0.95 (0.86–1.06)	0.384	0.93 (0.82–1.06)	0.298
Adjusted (*n* = 130)[e]	0.96 (0.87–1.06)	0.403	0.95 (0.84–1.07)	0.392
Adjusted (*n* = 116)[f]	0.92 (0.82–1.02)	0.097	0.93 (0.81–1.06)	0.278

[a]Relative risk estimated using the Cox proportional hazard regression analysis.
[b]95 percent confidence interval for the estimated relative risk.
[c]Adjusted for CAG zygosity status.
[d]Adjusted for CAG zygosity status, age, clinical stage, tumor size, nodal status, ER and PR status.
[e]Adjusted for CAG repeats in both alleles.
[f]Adjusted for CAG repeats in both alleles, age, clinical stage, tumor size, nodal status, ER and PR status.

CAG repeat zygosity status (homozygous vs heterozygous) and of CAG repeat difference between alleles were also examined in relation to clinicopathological variables. Neither of these two variables was found to be associated with the clinical and pathological variables studied, including clinical stage, tumor size, nodal status, histological grade and steroid receptors (data not shown).

CAG repeats and survival of breast cancer patients

Of the 133 patients who had CAG repeat data, all but three had follow-up information. Follow-up time ranged from 10 to 120 months, and the median was 64.5 months. During the follow-up, 49 patients developed recurrent or metastatic disease, and 34 died. Table 4 shows the results of the survival analysis. In the univariate analysis, the number of CAG repeats in allele 1, CAG zygosity, and CAG repeat difference between alleles, were not associated with either disease-free or overall survival. No interaction was found among these CAG variables (data not

shown). These findings were unchanged after clinical and pathological variables were adjusted in the analyses.

The total number of CAG repeats in both alleles was inversely associated with the risk of death (relative risk, RR = 0.94, *p* = 0.033). With every CAG repeat increase, there was a 6% reduction in the risk of death. The association remained statistically significant when the CAG zygosity status was adjusted in the analysis (RR = 0.92, *p* = 0.019). Interaction between total CAG repeats and CAG status was not found (data not shown). Similar associations were also suggested for disease-free survival (RR = 0.95, *p* = 0.072 without adjusting for CAG zygosity status, and RR = 0.95, *p* = 0.058 after adjusting for CAG status). However, this inverse relationship between the total number of CAG repeats and risk of death became not significant after clinical and pathological variables were adjusted in the model (RR = 0.95, *p* = 0.137). Reduction in the sample size from 133 to 116 might have contributed to the change. Variables that were significant in the multivariate model were patient age,

nodal status, tumor size, and ER status (data not shown).

CAG repeats and PSA levels in breast cancer

Analysis by Spearman correlation indicated that levels of PSA in breast cancer cytosols were not correlated to the length of CAG repeats either in allele 1 or in both alleles ($r = 0.03$ and 0.04; $p = 0.70$ and 0.65, respectively). Also, no correlation was found between PSA and the difference of CAG repeats between alleles ($r = 0.01$, $p = 0.91$).

Discussion

In this study, we analyzed the CAG repeat length of the AR gene in tumor tissue from 133 breast cancer patients, using a PCR-based method. Four CAG repeat variables were used to assess its relationship with breast cancer. The zygosity status (homozygous versus heterozygous) and the difference of CAG repeats between alleles were not associated with any of the clinical or pathological features studied. However, the length of CAG repeats in either one or both alleles was inversely correlated with tumor histological grade. Tumors with higher grade tended to have shorter CAG repeats. An association between positive lymph node status and shorter CAG repeats was also suggested in the study. Survival analysis further indicated that the total CAG repeat length in both alleles was inversely associated with the risks of relapse and death. In the Cox regression model, the number of CAG repeats was a continuous variable. Therefore, although the magnitude of the risk reduction appears seemingly small (about 6%), the accumulated change in risk would be much higher if there were relatively large differences in the length of CAG repeats. An increase of five CAG repeats would result in a 30% reduction in risk, based on the Cox model.

The association between total number of CAG repeats and patient survival was no longer significant when the clinical and pathological variables were adjusted in the analysis. This change may be attributed to the smaller number of patients eligible for analysis. Clinical and pathological information was not available for all the patients, and therefore, there were only 116 patients included in the multivariate analysis. A significant association was already not found in these patients when univariate analysis was performed (RR $= 0.95$, $p = 0.178$). Because of this, we could not include in the multivariate model the variable of histological grade, which was available for only 92 patients who had CAG repeat data, even though the total CAG length was correlated with this variable.

Our observation of a possible association between longer CAG repeats and better prognosis of breast cancer is consistent with the findings in prostate cancer. CAG repeat polymorphism has been investigated extensively in prostate cancer. Studies found that more CAG repeats were associated with a reduced risk of prostate cancer [4–6]. In addition, the length of CAG repeats was associated with several risk factors of the disease, such as race and age of onset. African-Americans who had the highest incidence of prostate cancer tended to have fewer CAG repeats than Asians [20]. The age of patient at diagnosis was inversely correlated with the length of CAG repeats. Fewer CAG repeats occurred more frequently in younger patients [21]. Contrary to the findings in prostate cancer, a recent report describes an opposite association between the age of breast cancer patients and CAG repeat polymorphism [22]. An association of a relatively long CAG repeat and early onset of the disease was suggested in the study, but the observation was made in a special group of patients who had BRCA1 mutation. In our study, correlation between the length of CAG repeats and patient age at diagnosis was not observed. Further, and consistent with our last statement, Spurdle et al. found no evidence for a CAG repeat length association with breast cancer risk in women before the age of 40 years [23]. These studies are different from ours, and our finding of a more aggressive form of cancer in women with shorter CAG repeats, was not examined in the two previous studies.

Results of prostate cancer studies further suggested that CAG repeat polymorphism might play a role in prostate cancer progression [24]. Fewer CAG repeats were linked to higher histological grade, advanced clinical stage as well as higher metastasis and mortality rate of prostate cancer [4, 24]. Our observation of higher tumor grade in association with fewer CAG repeats is consistent with the findings in prostate cancer. Despite no clear association between clinical stage and CAG repeats, a possible association between lymph node status and total CAG repeats was also suggested in our study. Furthermore, the association of CAG repeats with patient survival was also in the same direction as found in prostate cancer.

Prostate-specific antigen (PSA) is an androgen-regulated protein. PSA expression is up regulated by androgen through the AR action in both prostate and

breast tissues. No correlation or association between PSA and CAG repeat polymorphism was observed in prostate cancer [21]. Our study of breast cancer also did not show any relationship between PSA concentration and CAG length.

The AR gene resides on chromosome X. Women, unlike men, have two X-chromosomes. This situation could potentially make our observations more complicated, especially when heterozygosity is a prevalent phenomenon. Based on the findings of our study, most women are heterozygous with regard to their CAG repeats of the AR gene. To our knowledge, this is the first study designed to examine the CAG repeats in women and its relation to breast cancer progression. In order to evaluate which variable would be relevant to the CAG repeats in breast cancer, we created four variables to assess the relationship of CAG repeats with breast cancer. It seems that the total length of CAG repeats in both alleles would be a useful variable in assessing the CAG repeat role in breast cancer progression. However, our current knowledge on this gene is limited. Thus, whether this variable represents a measure of the actual transcriptional activity of the AR gene in women, remains to be determined. A further limitation of our study is that we did not determine which of the two CAG alleles is inactivated in women.

Earlier studies have shown that AR is expressed in most breast cancers, and that the presence of AR is highly correlated with the expression of other steroid receptors, including ER and PR. The percentage of AR-positive breast cancer reported is even higher than the percentage of ER- or PR-positive cancer [9–12]. The impact of AR on breast cancer has been recognized for many years, but the exact role of AR in breast cancer is still controversial. An *in-vitro* study showed that depending upon the cell lines being tested, androgen could either inhibit or stimulate the growth of breast cancer cells [25]. Both the inhibitory and stimulatory effects of androgen were mediated through the AR. Blocking the binding of androgen to AR abolished the action of androgen. The contradictory roles of AR in breast cancer have also been indicated by the fact that both androgens and anti-androgens have been used to treat breast cancer with positive effects in certain groups of patients [13, 14].

In addition to mediating the effect of androgen, AR also interacts synergistically with a number of mitogenic molecules. Peptide growth factors, insulin-like growth factor-I (IGF-I), epidermal growth factor (EGF) and interleukin 6 are among these molecules. In prostate cancer cell lines, both IGF-I and EGF are able to stimulate the action of AR on AR-regulated gene transcription and this stimulation of gene transcription could occur even without the presence of androgen [26–28]. This finding has been interpreted as cross talk between different signal transduction pathways. Despite that no observation has been made in breast cancer, similar findings have been reported between ER and IGFs in breast cancer cell lines [29]. In both laboratory experiments and epidemiological studies, IGFs and their specific binding proteins have been suggested to play an important role in breast cancer development and progression [30, 31]. Knowledge on the interplay between IGFs and AR in breast cancer is limited. Exploring the interaction between AR and other molecules may provide insights into the role of AR in breast cancer.

We believe that our findings, as well as those already reported by other groups on the role of CAG repeat lengths in breast cancer [22, 23], should be further studied with more patients to better understand the role of androgens in the pathogenesis and progression of breast cancer.

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