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Increased risk of breast cancer in women bearing a combination of large CAG and GGN repeats in the exon 1 of the androgen receptor gene

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ARTICLE INFO

Article history:
Received 21 May 2007
Received in revised form 21 June 2007
Accepted 3 July 2007
Available online 28 August 2007

Keywords:
Androgens
Androgen receptor
Breast cancer
CAG polymorphism
GGN polymorphism

ABSTRACT

The exon 1 of the human androgen receptor gene (AR) contains both CAG (polyglutamine) and GGN (polyglycine) repeat length polymorphisms. Large CAG repeats have been related to an increased risk of breast cancer (BC), whereas the influence of the GGN repeats is still unclear. Here, we have studied how the length of CAG and GGN repeats is associated with the risk of BC in a population from Tenerife (Canary Islands, Spain).

The study was carried out on 257 woman diagnosed with BC and 393 controls, nesting in the 'CDC of the Canary Islands' cohort study. The AR CAG and GGN genotyping was performed by means of PCR amplification with specific fluorescently labelled primers followed by a capillary electrophoresis.

The allelic distribution of CAG and GGN polymorphisms was similar in cases and controls. The mean of short and long CAG and GGN alleles did not show differences between cases and controls and the same was true when the average length of both CAG alleles (CAG_n) and GGN alleles (GGN_n) was considered. However, when CAG_n and GGN_n were categorised using 22 and 24 repeats as the cut-off point, respectively, significant differences between cases and controls were observed. The $CAG_n > 22$ repeats were more frequent in cases than in controls, being associated with an increased risk of BC (OR = 1.49; $CI_{95\%}$ = 1.06–2.09; $p = 0.021$). No significant differences were found for categorised GGN_n. For CAG_n/GGN_n combinations, the highest BC risk was found to be associated with the $CAG_n > 22/GGN_n \geqslant 24$ combination (OR = 2.47; $CI_{95\%}$ = 1.37–4.46; $p = 0.003$). In conclusion, our results indicate that longer CAG_n/GGN_n combinations increase the risk of BC and suggest that CAG and GGN AR polymorphisms should be considered in order to assess the BC risk.

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0959-8049/$ - see front matter
doi:10.1016/j.ejca.2007.07.001

1. Introduction

Androgens are the predominant sex steroid hormones in women after menopause, a stage of life during which many breast cancers (BCs) are diagnosed. In normal postmenopausal women, dehydroepiandrosterone-sulphate, androstenedione and testosterone (T) are the main androgens produced by both the ovary and the adrenal gland.[1] Increased plasma androgen levels have been described as a BC risk factor in both premenopausal and postmenopausal women.[2,3]

Aromatase converts androgens (T and androstenedione) into oestrogens (estradiol and estrone, respectively) in a number of tissues and organs, including the breast.[4] Estradiol, in turn, activates the oestrogen receptor (ER), which is mitogenic in breast cells.[5]

Androgens can also act directly on BC cells by binding to the androgen receptor (AR), which mediates androgen action at the transcriptional level.[6] The AR and some androgen-induced proteins, like prostatic-specific antigen (PSA) and gross cystic disease fluid protein-15 (GCPF-15), are co-expressed in about 60% of BC specimens,[7] thus indicating the capability of breast cells to respond to direct androgen stimulation.

Both proliferative and anti-proliferative effects of physiologic levels of T on breast epithelium and BC cells have been described. However, it is still unclear which effect of T is predominant in the female breast, whether a direct anti-proliferative effect or a combined direct/indirect proliferative effect.[8] A genetic, and hence invariant, marker of androgenic action would help to better understand the androgenic activity in human breast tissue, and also to better clarify its possible causal relationship to breast carcinogenesis.

The exon 1 of AR gene contains two trinucleotide repeat polymorphisms, CAG (encoding for polyglutamine) and GGN (encoding for polyglycine). These polymorphisms flank the N-terminal domain of the AR protein, where the transactivation activity resides.[6]

Several studies have observed an association between increasing AR CAG repeat length and a linear decrease in AR transactivation activity.[9,10] Physiological proof of these findings is provided by patients with a rare, neurodegenerative disorder called spinal and bulbar muscular atrophy,[11] which is characterised by 40–66 CAG repeats. These patients develop symptoms of androgen insensitivity, such as gynecomastia, testicular atrophy, oligospermia, azoospermia and elevated serum gonadotropins.

Exon 1 of the AR gene also includes a GGN repeat, that is less polymorphic than the CAG repeat.[12] An inverse relationship between GGN repeat length and AR protein yield has been described. This fall has been attributed to the increased difficulty of AR mRNA translation due to hairpin formation by an increasing number of GGN repeats.[13] A direct association has been found between GGN length and the risk of developing cryptorchidism and penile hypospadias, both conditions considered to be the consequences of low androgenicity.[14]

Some studies have reported an association between these polymorphisms and prostate,[15] endometrial,[16,17] ovarian,[18] colon,[19] esophageal[20] and breast cancers,[21] although discrepant results have also been obtained.

Several studies have related longer CAG repeats with an increase in BC risk,[22–24] whereas others have limited the impact of the AR-CAG repeat on BC.[25–32]

Out of the three studies on the influence of the GGN repeat on BC ,[26,30,33] only one has found an association between short alleles and an increased BC risk in women below the age of 45.[30] To the best of our knowledge, none of the published studies were able to prove that CAG/GGN combinations are associated with BC in the general population. However, several studies have found evidence that some CAG/GGN combinations are of clinical interest in endometrial[17] and esophageal cancers.[20]

Since GGN and CAG repeats, respectively, influence the AR protein yield and its transcriptional activity '*in vitro*', we hypothesised that the given CAG/GGN combinations might influence the risk of BC in the general population. In order to prove such a hypothesis, we designed a case–control study, nested in the 'CDC of the Canary Islands' cohort, to evaluate the effect of the CAG and GGN repeats on BC, evaluating both the individual effect of each repeat and the combined effect of both of them together.

2. Materials and methods

2.1. Study population

Three hundred women with confirmed breast cancer volunteered for this study. All the participants, between the ages of 33 and 74, were patients at the Department of Oncology of NS de La Candelaria University Hospital and residents in the province of Santa Cruz de Tenerife (Canary Islands, Spain). They were diagnosed between 1st January 1990 and 31st October 2005. The median age at diagnosis was 50 (range: 26–74 years).

Data related to risk factors for BC, such as first degree family history of BC, age at menarche, menopausal status, oral contraceptive use, hormone replacement treatment, parity information, smoking history and anthropometric measures, were obtained from each woman through a structured questionnaire and in-person interview from 1st February to 30th May 2005 (Table 1). Clinical and pathological data about tumour size, grade, metastasis, node invasiveness and hormone receptors status were obtained from the patients' medical records (Table 2).

Four hundred and thirty women, between the ages of 32 and 77, residents in the province of Santa Cruz de Tenerife, were randomly selected as controls from the CDC of the Canary Islands cohort for this study. The CDC of the Canary Islands cohort was designed to study the effects of lifestyle on Cancer, Diabetes and Cardiovascular diseases (CDC). This cohort recruited more than 6700 subjects, which were selected from the Canary Islands Primary Health Service Register between the years 2000 and 2005. This register covers 97% of the population of the Canary Islands.

Similar information regarding lifestyle habits and cancer risk factors was obtained for controls (Table 1). Blood collections and interviews were carried out for cases and controls who gave informed consent. The study was approved by the Ethical Committee of the Hospital.

Table 1 – Characteristics of cases and controls

Characteristic	Cases		Controls		p
	n	Mean ± SE (%)	n	Mean ± SE (%)	
Age at study (years)	295	55.6 ± 0.5	427	54.0 ± 0.4	0.025
At least one first degree family history of breast cancer	295	16	427	6	<0.001
Age at menarche (years)	290	12.8 ± 0.1	419	13.3 ± 0.1	0.002
Age at menopause (years)[a]	115	49.8 ± 0.5	207	49.3 ± 0.3	0.309
OCs consumption	289	48	422	49	0.922
OCs duration (years)	140	6.2 ±0.6	206	5.7 ± 0.4	0.558
Number of children	291	2.5 ± 0.1	425	2.7 ± 0.1	0.103
Age at first live birth (years)	256	24.1 ± 0.3	387	23.4 ± 0.2	0.079
Age at last live birth (years)	256	31.1 ± 0.4	387	31.2 ± 0.3	0.834
Breast-fed	247	85	378	85	0.955
Active smokers	295	15	427	13	0.455
Tobacco consumption (years)	119	22.4 ± 1.0	90	20.1 ± 1.3	0.158
BMI (kg/m^2)	285	28.8 ± 0.4	420	29.3 ± 0.2	0.533

a Physiologic menopause (without medical intervention or any other non natural cause).

Table 2 – Clinical characteristics of breast cancer cases

Characteristic	n	%
T (tumour size)		
T1	133	55
T2	91	38
T3 + T4	18	7
N (nodal status)		
N0	176	67
N1	80	30
N2 + N3	3	3
M (metastatic status)		
M0	174	99
M1	2	1
Tumour grade		
1	46	23
2	53	26
3	71	35
4	32	16
Tumor stage		
I	45	38
II	65	55
III	6	5
IV	2	2
Estrogen receptor		
Positive	132	79
Negative	36	21
Progesterone receptor		
Positive	123	74
Negative	44	26

2.2. *Analysis of CAG and GGN tracts*

DNA was extracted from blood samples (200 μl) using High Pure PCR Template Preparation Kit (Roche Applied Science). Fragments of exon 1 containing either the CAG repeat or GGN repeat were amplified using a pair of primers (one of which was labelled with a fluorescent dye) as previously described.[12,34] PCR amplifications were performed in a 25 μL reaction volume, containing approximately 100 ng of genomic DNA, 200 μM of each dNTP, 1× Fast Start *Taq* DNA polymerase buffer (Roche Applied Science), 1× GC-rich solution buffer, 1 U of Fast Start *Taq* DNA polymerase, 2.5 mM $MgCl_2$ and 1.2 μM of each primer.

The conditions for PCR amplifications were: initial denaturation at 95 °C for 1 min followed by 30 cycles of 95 °C for 45 s, 56 °C for 30 s and 72 °C for 30 s for CAG amplification, and 30 cycles of 95 °C for 1 min, 55 °C for 2 min and 72 °C for 2 min for GGN amplification. A final extension was performed at 72 °C for 5 min.

The PCR product was diluted 1:100 in distilled water and 1 μL of the dilution was mixed with 10 μL of formamide and 0.3 μL of GeneScan 500 LIZ Size Standard (Applied Biosystems), denatured at 95 °C for 3 min and cooled on ice. Fragment separation was performed by automated capillary electrophoresis using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems), and the length was determined using Gene Scan Analysis Software (version 3.7) (Applied Biosystems). Genotyping analyses for both polymorphisms were repeated with 70 blind DNA samples and the results were 100% coincident. Fragment size was confirmed by sequencing the 70 DNA samples harbouring alleles of different sizes for both repeats using the Big Dye Terminator Sequencing Kit (Applied Biosystem). Under these conditions, the Gene Scan systematically underestimated the actual length of the CAG and GGN repeats by four and two repeat units. Consequently, the allele size estimation obtained by Gene Scan was corrected for all samples.

2.3. *Statistical analysis*

Each variable was summarised according to its scale and distribution. The Chi-squared Pearson or the Student's *t* test was used for comparison between controls and cases. The risk of CAG and GGN repeats on BC was estimated by binary logistic regression analysis, using the BC condition as outcome and CAG and GGN repeats as predictor, adjusting for suspected or known BC risk factors. Statistical significance was reached in all tests when a two-tailed $p < 0.05$ was obtained. Main analyses were carried out using the SPSS version 13.0 statistical package from SPSS Inc., Chicago, IL, USA™. Hardy–Weinber

equilibrium was calculated using Arlequin version 2.0 software.

3. Results

3.1. Characteristics of the studied population

Table 1 shows the characteristics of the studied population. The number of individuals with first-grade relatives affected by BC was significantly larger for cases compared with controls and the age at menarche was significantly lower in cases than in controls. With the remaining studied variables (age at menopause, oral contraceptives consumption, number of children, age at first and last live birth, breast-feeding, tobacco consumption and body mass index) no significant differences were found between cases and controls.

The cases group includes recently diagnosed patients as well as patients who have been undergoing treatment for a number of years (the mean number of years since diagnosis was 5.6 ± 5.5 years). The percentage of patients at early stages of BC development (T1, N0 and M0) and of those with ER+ and PgR+ tumours is above the average for BC patients treated at this Oncology Department (Table 2). Thus, patients with a better general condition may be overrepresented in this series. In order to minimise that bias, a subgroup of the more recently diagnosed BC patients (⩽3 years) was analysed separately.

3.2. Distribution of the CAG and GGN alleles in cases and controls

Complete information and genotypic data were obtained for 259 BC cases and 393 controls for CAG polymorphism and 257 BC cases and 398 controls for GGN repeats.

The assayed control population showed 23 different CAG alleles, ranging from 11 to 37 repeats and the most frequent alleles were 19, 21 and 24, in both cases and controls. The CAG allelic distribution was near normal. (Fig. 1a). The GGN repeat showed 19 alleles, ranging from 12 to 30 repeats and the most frequent alleles were 23 and 24 in both cases and controls (Fig. 1b). CAG and GGN allelic distribution was similar in both groups.

The CAG allelic frequencies did not deviate from the Hardy–Weinberg equilibrium for either controls ($p = 0.088$) or cases ($p = 0.978$). The GGN allelic frequencies were also in the Hardy–Weinberg equilibrium in both controls ($p = 0.156$) and cases ($p = 0.0763$).

3.3. CAG and GGN polymorphisms and breast cancer

The mean of CAG repeats was not significantly different between controls and cases, neither when the average of both CAG alleles (CAG_n) in each individual was considered (21.6 ± 0.1 controls versus 21.7 ± 0.1 cases; $p = 0.598$) nor when the short and large alleles were considered separately (not shown). The same situation occurred for the average of GGN alleles (GGN_n) (23.1 ± 0.1 controls versus 23.1 ± 0.1 cases; $p = 0.573$) and for both alleles separately (not shown).

A comparison of CAG_n and GGN_n distributions between cases and controls revealed some differences (Fig. 2a and b). Maximum differences between cases and controls were observed when groups above and under 22 repeats for CAG_n and 24 repeats for GGN_n were generated. Thus, we categorised CAG_n and GGN_n, using 22 and 24 repeats as cut-off points ($CAG_n \leqslant 22$, $CAG_n > 22$; $GGN_n < 24$, $GGN_n \geqslant 24$).

The association between CAG_n and GGN_n lengths and BC was estimated by means of binary logistic regression modelling adjusted for known or suspected breast cancer risk factors (age, first-degree family breast cancer history, age at menarche, OC consumption and body mass index) using BC status as outcome. Table 3 shows that $CAG_n > 22$ repeats were more abundant in the group of cases than in controls. Women bearing CAG_n repeats >22 displayed an increased risk of developing BC compared to those in the $CAG_n \leqslant 22$ category (OR = 1.49; $CI_{95\%} = 1.06–2.09$; $p = 0.021$). When the analysis was restricted to cases diagnosed within the last three years, a coincident stronger result was obtained (OR = 1.74; $CI_{95\%} = 1.11–2.70$; $p = 0.016$).

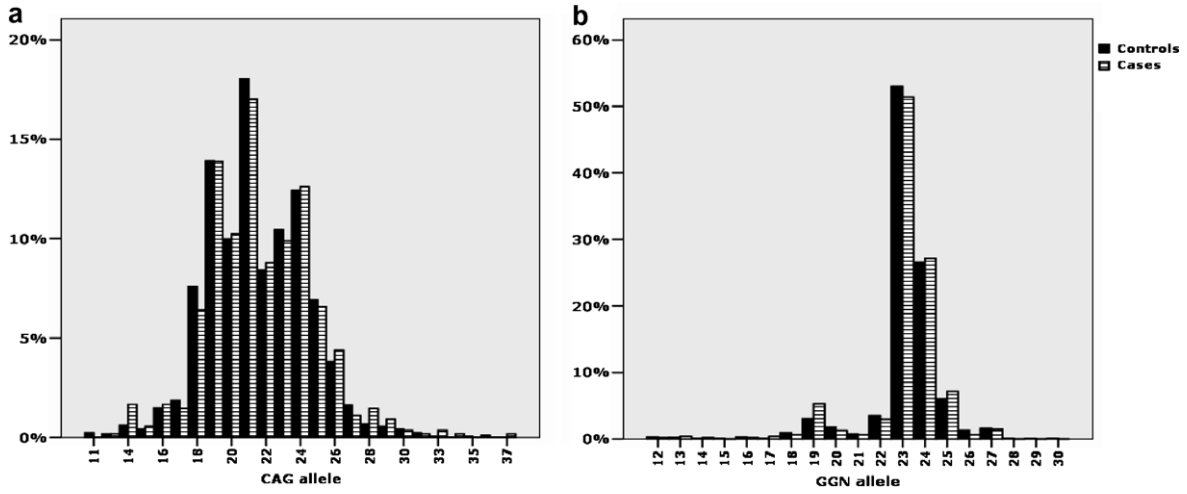

Fig. 1 – Allelic distribution of the androgen receptor CAG and GGN repeats in cases and controls.

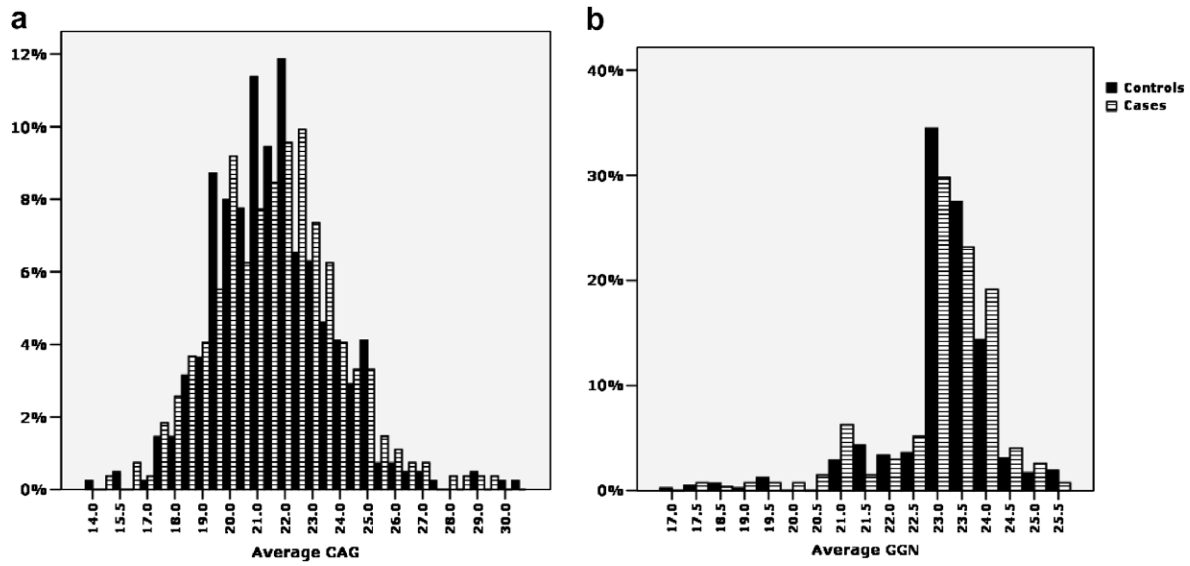

Fig. 2 – Distributions of the average of both CAG alleles and GGN alleles in cases and controls.

Table 3 – Multivariate logistic regression analysis of CAG and GGN average lengths and breast cancer

Group	n		OR[c] (95% CI)	p	n		ORc (95% CI)	p
	Controls	All cases			Controls	Cases ⩽ 3 years[d]		
CAG_n[a] ⩽ 22	270	157	Reference	0.021	270	66	Reference	0.016
CAG_n > 22	123	102	1.49 (1.06–2.09)		123	50	1.74 (1.11–2.70)	
GGN_n[b] < 24	319	188	Reference	0.080	319	81	Reference	0.187
GGN_n ⩾ 24	79	69	1.41 (0.95–2.05)		79	28	1.42 (0.84–2.39)	

a (CAG_n) average length of both CAG alleles.
b (GGN_n) average length of both GGN alleles.
c Adjusted for age, age at menarche, oral conceptive use, family history of breast cancer and body mass index.
d Only cases within the last three years after diagnosis were included.

We did not observe increased breast cancer risk for woman bearing GGN_n < 24 or ⩾24 repeats.

3.4. *CAG/GGN combinations and breast cancer*

We assessed whether the combination of both polymorphisms was a better predictor of breast cancer than each polymorphism independently. Thus, we created four possible combinations of CAG_n and GGN_n categories (CAG_n ⩽ 22/GGN_n < 24; CAG_n ⩽ 22/GGN_n ⩾ 24; CAG_n > 22/GGN_n < 24 and CAG_n > 22/GGN_n ⩾ 24). Logistic multivariate analysis showed an increased risk for the CAG_n > 22/GGN_n ⩾ 24 combination with regard to the CAG_n ⩽ 22/GGN_n < 24 combination (OR = 2.47; $CI_{95\%}$ = 1.37–4.46; p = 0.003) (Table 4).

4. Discussion

The allelic frequencies for CAG in our control sample resemble those reported for other Caucasian populations.[35,36] In the case of the GGN repeat, the most frequent alleles found in this study were the 23 and 24 repeats. This result is in agreement with others previously reported.[14,33]

Table 4 – Multivariate logistic regression analysis of CAG and GGN average length combinations and breast cancer

CAG_n/GGN_n combinations	n		OR[a] (95% CI)	p
	Controls	Cases		
CAG_n ⩽ 22/GGN_n < 24	211	109	Reference	
CAG_n ⩽ 22/GGN_n ⩾ 24	51	35	1.20 (0.72–2.01)	0.491
CAG_n > 22/GGN_n < 24	96	63	1.29 (0.86–1.93)	0.222
CAG_n > 22/GGN_n ⩾ 24	26	31	2.47 (1.37–4.46)	0.003

a Adjusted for age, age at menarche, oral conceptive use, family history of breast cancer and body mass index.

Two main findings central to this discussion can be highlighted from the above presented study: (1) CAG_n repeats >22 were more frequent in BC cases than in control; and, (2) the $CAG_n > 22/GGN_n \geqslant 24$ combination increases the risk of BC.

Our finding that long CAG repeats increase the risk of BC has been previously reported by other authors. In fact, long CAG repeats have been associated with an increase in BC risk in Caucasian[23] and Philippine women.[22] Similar results were described for male BC[37] and for a population of BRCA1/2 mutation carriers,[24] but the latter's results have yet to be further confirmed.[26,32] Other studies have reported a slight, yet not statistically significant, increase in the risk of BC associated with long CAG alleles.[25,27,31,33] Two studies found an increased BC risk in individuals with a first-degree family history of BC bearing long CAG alleles,[28,38] a finding which was not observed in our series. Finally, one study[39] reports that genotypes comprised of one or two short CAG repeats had a higher risk of BC compared to genotypes with two long alleles, which contradicts all previous studies. The bulk of evidence provided by the cited authors, as well as the results described here points to a moderately increased risk of developing BC in women bearing large CAG repeats. These findings support the hypothesis that androgens might play a protective role in BC development.

Also, androgens have been previously reported to inhibit the growth of breast cancer cell lines *in vitro* and longer CAG alleles have been described as having a lower transcriptional activity. The increased risk of breast cancer in women bearing CAG_n repeats >22 could be the consequence of the decreased AR transcriptional efficiency in their breast cells and hence a lower response to circulating androgens.

We have found that GGN_n repeats, considered independently of the CAG_n repeat, have no evident effect on BC risk. One of the previous studies which examined GGN repeat length and BC found an association between short GGN repeats and an increased risk of BC in women diagnosed before the age of 45.[30] Another two studies failed to find any association of the GGN repeat with BC risk in BRCA1/2 carriers[26] or in the general Caucasian population,[33] as is the case in the study described here. Thus, the individual effect of the GGN on BC risk remains to be confirmed by a larger series.

Given the prevalent cases included in the study, a selection bias would have been introduced. To reduce this bias, the analysis was restricted to cases diagnosed within the last three years. The association between long CAG_n and breast cancer risk was then stronger, suggesting that our findings are not a consequence of this selection bias.

None of the above mentioned studies described any association between CAG and GGN combinations and BC risk. We found that women bearing the longest combination ($CAG_n > 22/GGN_n \geqslant 24$) possess the highest risk of developing BC attributable to these polymorphisms. Interestingly, this result can be compared to the results of some studies on prostate cancer, where short CAG and GGN, representative of the more abundant and active AR protein, are reported to increase the risk of prostate cancer.[15,40,41] Taking this data into account, it seems that the opposite effect would occur in prostate cancer. Our finding suggests that long GGN_n increases even further the risk of BC in women bearing long CAG_n.

The effects of CAG length on BC prognosis and progression have also been previously evaluated. One study, carried out in archival BC tissues, found an association of short CAG alleles, representative of higher transcriptional activity of the AR, with more aggressive forms of BC.[42] This result is not in agreement with the observation of increased BC risk in women bearing long CAG alleles.

Women with long CAG repeats have been described as having a decreased risk of endometrial cancer,[16] whereas our laboratory has recently reported that short CAG and GGN tracts are associated with less aggressive endometrial cancer.[17] Again these data are contradictory and there is an apparent disagreement between the CAG/GGN genotype and the risk or prognosis of breast and endometrial cancers.

Data from hormone related cancers in women suggest that genetic markers such as the CAG and GGN repeats have different capabilities of modifying carcinogenesis in androgen responsive tissues. These capabilities might or might not be related to their possible influence on the clinical behaviour of the same cancer type.

When all the data are taken into account, it seems to suggest that a number of combinations of CAG and GGN alleles might influence the AR-mediated response to androgens in different cell types and organs. Interaction of the AR protein is known to be dependent on tissue and promoter context. Our data suggest that a decreased amount of AR protein (long GGN_n) with low transcriptional activity (long CAG_n) in the cell would increase the BC risk. Thus, the results described here are consistent with the hypothesis that androgens have a direct, protective role on BC development. However, the present knowledge on the influence of CAG and GGN repeats on androgen-dependent gene transcription in human cancer cells '*in situ*' is still circumstantial and does not allow us to put forward any solid theory that would integrate all the described observations on BC and other cancer types.

In summary, in the study described here we have confirmed previous findings regarding an increased risk of BC in women bearing CAG_n repeats >22. We have also found evidence that a long combination of $CAG_n > 22/GGN_n \geqslant 24$ is associated with an increased risk of BC. This finding remained when the data were adjusted for established or suspected risk factors of BC, thus suggesting that the AR CAG and GGN should be considered in order to assess BC risk in women.

Conflict of interest statement

None declared.

Acknowledgements

We express our gratitude to all of the women who have participated in this study as well as those who contributed to the enrolment of participants, especially Marta Batista and Marta Rodríguez. We thank Dr. Ana G. Matilla, Dr. Vicente Cabrera, M[a] del Carmen Maeso, Raquel Cruz and M[a] Concepción Vilar for their help.

This study has been sponsored by 'The Canary Islands Cancer Research Institute Foundation', Spain, 'Fundación de Investigación Sanitaria' (Spanish Ministry for Health, FIS-IS-CIII-RTICCC C03/10), 'Fundación Canaria de Investigación Sanitaria' (The Canary Islands Government, FUNCIS), Interreg IIIB BIOPOLIS (European Union funding for the development of the Azores, Madeira and the Canary Islands) and 'Dirección General de Universidades' (Canary Islands Universities Government, to BNDC and JCDC).

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