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Interaction between estrogens and androgen receptor genes microsatellites, prostate-specific antigen and androgen receptor expressions in breast cancer

D. NARITA[1], A. ANGHEL[1], A. M. CIMPEAN[2], D. IZVERNARIU[2], N. CIREAP[3], R. ILINA[3], S. URSONIU[4]

[1]Department of Biochemistry, University of Medicine and Pharmacy "Victor Babes",M.Murgu Square No 2A, 300041 Timisoara, Romania, e-mail: diana_narita@yahoo.com, [2]Department of Histology, University of Medicine and Pharmacy "Victor Babes", Timisoara, Romania, [3]Department of Surgical Oncology, University of Medicine and Pharmacy "Victor Babes", Timisoara, Romania, [4]Department of Public Health, University of Medicine and Pharmacy "Victor Babes", Timisoara, Romania

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The role of estrogen and androgen receptors signaling in breast cancer is widely accepted, but the interrelations between them are not well understood. It was suggested that PSA could be a marker of endogenous balance between androgens and estrogens. In this context, we intended to investigate the potential of relationship between polymorphic tandem repeats (CAG, TA and CA) in AR (androgen receptor), ERα (estrogen receptor alpha) and ERβ (estrogen receptor beta) genes and the immunoexpression of PSA and AR proteins. We assessed also the possible influences of CAG, TA, and CA variables and other available prognostic factors (ER, PR, AR, HER2/neu, PSA expression, and nodal status) on disease-free survival.

We assessed the polymorphic tandem repeats lengths by genotyping, followed by high-resolution denaturing polyacrylamide gel electrophoresis in 163 breast cancers. Immunohistochemistry was performed to assess the expressions of AR, PSA, ER, PR and HER2/neu proteins.

Our results showed that PSA was correlated with the length of CA repeats in the 3'-untranslated region of ERβ, shorter CA repeats being correlated with PSA expression (p=0.03). AR immunoexpression was correlated with CAG repeats on AR gene, higher number of repeats being linked to a higher AR immunoexpression (p=0.04). Performing logistic regression to investigate relationships with prognosis, we observed that PSA immunoexpression (p=0.004), the nodal status (p<0.001) and marginally, longer TA repeats (p=0.05) were correlated with increased disease-free survival. AR expression presented a low statistical value (p=0.054) in predicting evolution and was not entered into the multivariate regression analysis. Altogether, our findings supports the hypothesis that estrogens, through both alpha and beta-receptors variants are mediating the AR signaling pathway.

Keywords: polymorphic tandem repeats, estrogen receptor genes, androgen receptor gene, prostate-specific antigen, breast cancer

Researchers are trying to discover new biomarkers for diagnosis, prognosis, treatment monitoring and to develop new drugs that might work better against breast cancer. An example in this context is the identification of PSA (prostate-specific antigen) in normal and tumoral mammary gland. PSA (hK3), a 240 amino acids 33-kDa single-chain glycoprotein is one of the human kallikreins expressed at high levels in the epithelium of the human prostate gland. PSA gene is the best-characterized AR (androgen receptor) - regulated gene and PSA protein is an important clinical marker used for prostate cancer screening, diagnosis, prognosis and monitoring. Previous studies showed that PSA is expressed in a considerable proportion of female breast cancers, ranging from 9.3% to 49% [1–5], but the functional role and the clinical significance of extraprostatic PSA were not defined yet. PSA, like other proteases can digest insulin-like growth factor-binding proteins, leading to the release of growth factors [6]. Digestion of the basal membrane and extracellular matrix proteins might facilitate cell migration and invasion. However, PSA inhibits the endothelial cell response to angiogenic stimulation by fibroblast growth factor-2 and vascular endothelial growth factor, suggesting that PSA might be an endogenous anti-angiogenic compound [7]. Lai et al. [8] reported that PSA stimulates the conversion of the potent estradiol to the less potent estrone, inhibiting the growth of certain breast cancer cell lines in vitro. This might contribute

to the association between PSA and good prognosis observed in some studies [1–3, 9–10].

AR (androgen receptor) is expressed in the majority of breast cancers, ranging from 70 to 90%, binds specifically to androgen and mediates its action by activating the transcription of androgen-regulated genes [11]. Despite the potential of testosterone and dehydroepiandrosterone to be aromatized to estrogen, it has been shown that androgens exhibit growth-inhibitory and apoptotic effects in some, but not in all breast cancer cell lines, suggesting that testosterone may serve as a natural, endogenous protector of the breast. The differences between cell lines appear to be due to the variations in concentrations of specific co-regulatory proteins at the receptor level [12–14]. A polymorphic polyglutamine stretch in the amino-terminal domain (exon1) of the AR gene (located on chromosome Xq11–12), encoded by the nucleotides cysteine, adenine, and guanine (CAG)n, appears to influence the function of the receptor as a transcription factor, so that relatively long fragments are associated with a low level of receptor function [15]. Previous data suggest that long repeat regions exert inhibitory actions for the interactions with co-activators, which could explain the lower activity of the receptor [16]. Clinical studies support the functional importance of the CAG repeat sequence of the AR gene. Relatively few (<22) (CAG)n repeats in the AR gene were associated with a higher risk of prostate cancer, benign prostate hyperplasia, young-onset rheumatoid arthritis and with a lower risk of infertility. A substantial expansion (40–72 repeats) of the CAG repeat sequence is the cause of a rare X-linked form of motor neuron disorder in men, termed Kennedy's disease (spinal and bulbar muscular atrophy), that is associated with some degree of androgen insensitivity [17–19]. Data on the functional importance of the number of CAG repeats in the AR gene in women suggested associations between this polymorphism and hirsutism, acne, androgenic alopecia [20], bone mass density [21], and breast cancer [22–23].

Estrogen mediates its effects by interacting with specific nuclear receptors – ERα (estrogen receptor alpha) and ERβ (estrogen receptor beta) –, which are ligand-activated transcription factors and now are serving as a basis for many therapeutic interventions. The two ERs (estrogen receptors) are encoded by separate genes, ESR1 and ESR2 located on chromosomes 6q 25.1 and 14q 23-24.1, respectively. The ERα gene contains in its promoter region a polymorphic TA repeat has been associated with coronary heart disease and anxiety in men and with bone mineral density, and endometriosis in women [24–28]. A polymorphic dinucleotide CA repeat in the untranslated 3'-region of the ERß gene was identified and has been suggested to be associated with bone mineral density in women and with the serum levels of androgen and SHBG (steroid hormones binding globulin) in premenopausal women [29–32].

In a previous study [33–34], we investigated the prevalence of PSA in consecutive series of breast carcinomas using immunohistochemical techniques in tissue sections from paraffin-embedded material. We detected PSA in 44.5% of breast cancers, 35.5% of normal tissues and 42% of benign lesions adjacent to breast cancers. 54% of PSA-positive breast carcinomas were simultaneous AR-positive, while 80% of PSA-negative carcinomas did not express AR. Hall et al. [35] reported an even stronger association of AR and PSA expression, 98% of breast carcinoma that expressed PSA being AR positive. PSA was significantly associated with pathological parameters that are known to be associated with a better prognosis, like AR and PR (progesterone receptor) and it was inversely correlated with HER2/neu overexpression and G3, known to count for a worse prognosis. On the other hand, the detection of PSA in breast cancer metastases was in contrast with these results [34]. In another work [36], we performed a case-control study to analyze the potential link between breast cancers and three tandem repeats (CAG, TA and CA) in the AR, ERα and ERβ genes, and we concluded that the combination of longer CAG, shorter TA and CA repeats may represent a characteristic genetic profile for breast cancers.

In order to further investigate these associative actions and according to the supposition that PSA could be a marker of endogenous hormone balance between androgens and estrogens, we started to investigate the potential relationships between these polymorphic tandem repeats (CAG, TA and CA) in the AR, ERα and ERβ genes and the immunoexpression of PSA and AR proteins. We assessed also possible influences of CAG, TA, and CA variables and other available prognostic factors (ER, PR, AR, HER2/neu, PSA expression, and nodal status) on disease-free survival.

Patients and methods

Patients. We studied one hundred sixty-three surgical specimens from female patients with breast cancer. Clinical characteristics and follow-up data were retrieved from the files of surgical oncology departments from Timisoara. The maximum follow-up period was 99 months. We selected only patients who followed standardized treatment protocols. All patients gave informed consent and the study was approved by the Ethics Committee of our University. The mean age at diagnosis was 57 years (range between 23 and 88). The pathological diagnosis and grading were repeated on hematoxylin-eosin-stained samples in order to confirm the cancer histopathological type described in patient's records. The clinical and histological characteristics of breast cancer patients are presented in Table 1.

Immunohistochemistry was performed to study PSA, AR, ER, PR and HER2/neu expressions in breast cancers using standardized automated procedures (Dako, Glostrub, Denmark). We used the following clones: polyclonal PSA, clone AR441 for AR, 1D5 for ER, PgR636 for PR, and HercepTest all from Dako. The detailed methodology is described in another publication [34]. For semiquantitatively evaluation of PSA, AR, ER, and PR immunoreactivity we considered the percentage of positive nuclei; samples were considered positive when at least 10 % of nuclei were immunoreactive, independently of the intensity of the immunostain [37]. Positive controls included normal breast tissue surrounding the tumors for steroid receptors, cases of prostate adenocarcinoma and prostate benign hyperplasia for AR and PSA, and control slides from Dako for

Table 1. Clinical and histopathological characteristics of studied breast cancers

Characteristic	Cases (n=163)
Age at diagnosis	
Mean age	55.76
Median age	56
Range	23 – 80
Follow-up period (months)	
Mean	37.49
Median	34
Range	0 - 99
Evolution	
Favorable	120
Unfavorable (death or recurrences)	43
Histopathological type	
Invasive ductal carcinoma	109
Other types	54
Grade	
1	9
2	93
3	59
Tumor size	
<5cm	136
≥5cm	27
Lymph node metastasis	
Present	73
Absent	90
Distant metastasis	
Present	6
Absent	157
TNM stage	
0	3
I	26
II	104
III	26
IV	4
Estrogen receptor status	
Positive	73
Negative	90
Progesterone receptor status	
Positive	80
Negative	83
HER2/neu	
Overexpression (+2,+3)	45
Negative (0,+1)	118
Prostate-specific antigen status	
Positive	66
Negative	97
Androgen receptor status	
Positive	130
Negative	33
Chemotherapy	
Yes	132
No	31
Radiotherapy	
Yes	98
No	65
Hormone therapy	
Yes	97
No	66

HER2/neu. As negative controls, we used additional sections incubated without primary antibodies.

DNA extraction was performed from paraffin-embedded samples with RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Ambion), according to the manufacturer's protocol.

PCR amplification for CAG repeats in the AR gene was performed in a total volume of 25 μL containing 50 ng DNA, 1.5 mmol/L MgCl2, 0.625 U HotStar TaqDNA polymerase (Qiagen, Valencia, CA, USA), and 0.2 mmol/L of each of the primers (5'-GTG CGC GAA GTG ATC CAG A-3' and 5'-GTT TCC TCA TCC AGG ACC AGG TA-3'). Thermal cycling was performed in an ESCO PCR System with the following temperature profile: 95° C for 5 min, followed by 35 cycles of 95° C for 30 s, 57° C for 30 s, 72° C for 30 s, and a final incubation at 72° C for 7 min.

The PCR for TA repeats of the ERα gene was performed in a total volume of 15 μL containing 50 ng DNA, 1.5 mmol/L $MgCl_2$, 1 U HotStarTaq polymerase (Qiagen), and 0.3 μmol/L of each of the primers. We used pairs of primers with the same sequences as described by Westberg and co-workers [30]: the forward primer 5'-AGA CGC ATG ATA TAC TTC ACC-3') and the reverse primer 5'-GTT CAC TTG GGC TAG GAT AT-3'. The temperature profile was 95° C for 15 min, followed by 35 cycles of 95° C for 30 s, 60° C for 30 s, 72° C for 30 s, and a final incubation at 72° C for 7 min.

For the CA repeats polymorphism of the ERß gene, PCR was performed in a total volume of 25 μL containing 50 ng DNA, 1.5 mmol/L $MgCl_2$, 1 U HotStarTaq DNA polymerase, and 0.3 μmol/L of each of the primers: the forward primer 5'-GGT AAA CCA TGG TCT GTA CC-3' and the reverse primer (5'-AAC AAA ATG TTG AAT GAG TGG G-3'). The temperature profile was 95° C for 12 min, followed by 35 cycles of 95° C for 30 s, 60° C for 30 s, 72° C for 30 s, and a final incubation at 72° C for 7 min.

Electrophoresis assay. PAGE gel-electrophoresis was performed to separate PCR products and, in order to determine allele size, pGEM DNA markers (Promega) were run together with the PCR products. Briefly, following steps were performed: PAGE gels (6%) preparation; pre-run in order to prepare the gels for the migration; samples preparation - we prepared a mix containing 1 μl sucrose, 2,5 μl 2x loading solution containing bromophenol blue and xylen cyanol as staining solution (Promega) and 2,5 μl amplified DNA; after homogenization, the mix was denatured through thermic shock; migration for 2 hours at 10 W; gel fixation with 10 % acetic acid for 20 min.; washing three times for 2 min with ddH_2O; staining with $AgNO_3$ solution; washing with ddH_2O to remove the excess of nitrate; for visualization, on the washed gels we added sodium carbonate solution. The gels were scanned with an imaging densitometer (Bio-Rad) followed by sizing with the Quantity One-The Discovery Series v.4.0.1.software, taking the pGEM DNA markers (containing polynucleotides between 50 and 500 bp) as standards. Electrophoresis and allele sizing were performed twice to ensure consistency of the results. Examples of tandem repeats length interpretation are shown in Fig. 1.

*Statistical analysis***.** The repeats data for CAG, CA and TA were analyzed in three categories: the number of repeats in

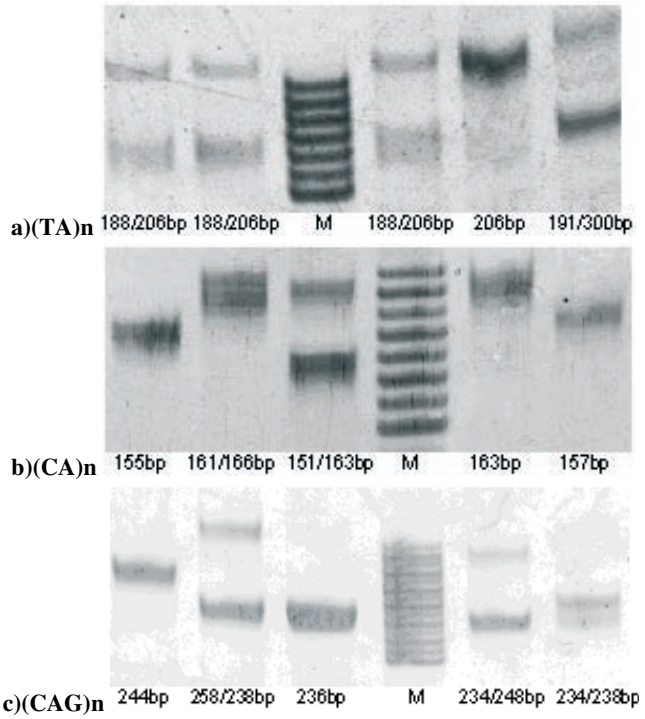

Figure 1. Interpretation of (TA)n, (CA)n and (CAG)n number of tandem repeats in ERα (estrogen receptor alpha), ERβ (estrogen receptor beta) and AR (androgen receptor) genes. PCR products were separated by PAGE gel-electrophoresis, scanned and sized with an imaging densitometer and Quantity One-The Discovery Series v.4.0.1. software. In order to determine allele size, the DNA marker (M) (pGEM DNA marker containing polynucleotides between 50 and 500 bp with 4bp differences between markers) was run together with the PCR products. In the exemplified figures, there are shown from the left lane: a) (TA)n: two heterozygous with amplicons of 188bp and 206 bp length, the marker, another heterozygous of 188 and 206 bp, a homozygous of 206 bp and a heterozygous of 191 and 300bp amplicons. b) (CA)n: a homozygous of 155bp, two heterozygous of 161 and 166bp and respectively of 151 and 163 bp; after the marker, there are two homozygous of 163bp and 157bp. c) (CAG)n: a homozygous of 244bp, a heterozygous of 258 and 238bp, and a homozygous of 236 bp; after the marker, two heterozygous of 234 and 248bp and respectively of 234 and 238bp.

Table 2. The number of the tandem repeats on each allele in breast cancer cases

	CA1	**CA2**	**CAsum**
Range	14-27	16-28	17-54
Median	22	23	45
Average	22.20	22.50	44.50
SD	2.14	1.91	4.18
	TA1	**TA2**	**TAsum**
Range	13-26	12-35	26-52
Median	17	17	36
Average	17.77	18.65	36.33
SD	3.37	4.09	6.25
	CAG1	**CAG2**	**CAGsum**
Range	9-27	11-27	20-54
Median	21	21	42
Average	20.22	20.79	41.02
SD	4.23	4.22	8.23

Abbreviations: *CA1 and CA2 – number of tandem repeats on each allele of estrogen receptor β gene; TA1 and TA2 – number of tandem repeats on each allele in the promoter of estrogen receptor α gene; CAG1 and CAG2 – number of polymorphic tandem repeats on each allele in androgen receptor gene; CAsum, TAsum and CAGsum – the sum of repeats on both alleles; SD – standard deviation.*

allele 1, the number of repeats in allele 2 and the total number of repeats in both alleles. For heterozygous, allele 1 was considered as the allele with the fewer number of repeats. The numerical variables were classified into dichotomous groups using the median of each variable as cut-off point. Patients were dichotomized in PSA-positive and PSA-negative, and in AR-positive and AR-negative groups, respectively. Associations between variables (number of CAG repeats, TA and CA repeats on each and both allele), prostate-specific antigen, AR immunoexpression and between variables and disease evolution were assessed using univariate COX regression. We assessed also possible correlations between the other available potential prognostic factors (ER, PR, AR, HER2/neu, PSA expression, nodal status) and disease-free survival. The independent statistically significant variables were entered into multivariate logistic regression models. Using backward elimination procedures, the most parsimonious multivariate logistic model was produced for predicting disease evolution.

We considered a favorable evolution when none event (death, metastasis or recurrences) was observed during the follow up period. Crude ORs (odd ratios) were then adjusted for age, radiotherapy, chemotherapy and hormone therapy as potential confounders. The p values were calculated based on two-sided statistical tests and we set statistical significance at $P<0.05$. All analyses were conducted using SPSS v.10 software or STATA v.9.2.

Results

Out of 163 cases, 66 cases (40.5%) expressed PSA; 44.78% (73 out of 163) were ER positive, 49.08% (80 out of 163) were PR positive, 79.75% (30 out of 163) expressed AR and 27.6% (45 out 163) overexpressed HER2/neu. The histhopathological and clinical characteristics of the patients are presented in Tab. 1.

Polymorphic tandem repeats lengths were assessed by genotyping, followed by high-resolution denaturing polyacrylamide gel electrophoresis. Each polymorphic repeat length was dichotomized into two allele categories taking the medians as cut-off points (22 for CA, 17 for TA and 21 for CAG). The number of repeats on each allele and the sum of each type of polymorphic repeat on both alleles are presented in Tab. 2, together with their means and medians.

Table 3. Relationships between the PSA and AR immunoexpression and the number of CA, TA and CAG repeats.

Variables	PSA negative	PSA positive	P value (Pearson's)	AR negative	AR positive	P value (Pearson's)
CA1						
<22	37	14		8	43	
≥22	63	49	***0.04***	25	87	0.33
CA2						
<22	31	11		9	33	
≥22	69	52	***0.054***	24	97	0.82
***CAsum**						
11	51	45		5	21	
12	30	11	***0.03***	7	34	
22	19	7		21	75	0.806
TA1						
<17	47	30		17	60	
≥17	53	33	0.93	16	70	0.58
TA2						
<17	49	25		17	57	
≥17	51	38	0.24	16	73	0.43
***TAsum**						
11	36	25		12	37	
12	32	21		10	43	
22	32	17	0.78	11	50	0.67
CAG1						
0	10	4		0	14	
<21	46	25		20	51	
≥21	44	34	0.41	13	65	***0.031***
CAG2						
0	10	4		0	14	
<21	38	22		19	41	
≥21	52	37	0.6	14	75	***0.008***
***CAGsum**						
0	10	4		0	14	
11	35	19		17	38	
12	12	7		3	16	
22	43	33	0.65	13	62	***0.044***

*CAsum: 11 – both alleles <22; 12 – one allele <22 and the other one ≥22; 22 – both alleles ≥22; *TAsum: 11 –both alleles <17; 12 – one allele <17 and the other one ≥17; 22 – both alleles ≥17; *CAGsum: 0 = unknown; 11 – both alleles <21; 12 – one allele <21 and the other one ≥21; 22 – both alleles ≥21. The number of repeats on each allele and the sum of repeats on both alleles are represented.

Abbreviations: *PSA – prostate-specific antigen; AR - androgen receptor; CA1 and CA2 – number of tandem repeats on each allele of estrogen receptor β gene; TA1 and TA2 – number of tandem repeats on each allele in the promoter of estrogen receptor α gene; CAG1 and CAG2 – number of polymorphic tandem repeats on each allele in androgen receptor gene; CAsum, TAsum and CAGsum – the sum of repeats on both alleles*

$P < 0.05$ was set as statistic significant.

Statistical analyses between the length of genotyped polymorphic repeats and immunohistochemical and clinical available characteristics. We compared the short/long allele frequency for each tandem repeat in both alleles and the sum of repeats/genotype in the PSA, respectively AR positive breast cancers versus PSA/AR negative cases by univariate logistic regression and the results are showed in *table 3*. In summary, the immunoexpression of PSA was significantly correlated only with the length of CA repeats in the 3'-untranslated region of ERβ, genotypes with longer CA repeats being associated with PSA negativity (p=0.04 for CA1, p=0.05 for CA2 and p=0.03 for CAsum) (*table 3*). The other genotyped repeats were not associated with PSA expression. AR immunoexpression was correlated with the length of CAG repeats on the AR gene, higher number of CAG repeats being linked to a higher AR immunoexpression (p=0.03 for CAG1, p=0.008 for CAG2 and p=0.04 for CAGsum on both allele) (Tab. 3). Neither CA nor TA repeats were correlated with AR immunoexpression. We checked also for associations between the number of each repeats and the other available parameters, but we did not find significant correlations. For example, for metastasis and CAGsum, CAsum and TAsum *p values* were 0.15, 0.11 and 0.68, respectively; for the nodal status and the same repeats, *p* were 0.32, 0.59 and 0.79; for ER expression, *p* values were 0.95, 0.84 and 0.59; for PR expression and number of CAGsum, CAsum and TAsum repeats, *p values* were 0.17, 0.94 and 0.73, respectively.

Table 4. Univariate COX regression analyses for associations between genotypes with long/ short CA, TA and CAG repeats and disease-free survival

Variables	SE	df	P	HR	95% CI for HR
CA		2	0.289		
CA(11)				1(ref)	
CA(12)	0.496	1	0.345	1.597	0.604 – 4.223
CA(22)	0.465	1	0.868	0.926	0.372 – 2.305
TA		2	0.091		
TA(11)				1(ref)	
TA(12)	0.355	1	0.974	0.988	0.493 – 1.981
TA(22)	0.411	1	***0.043***	0.435	0.195 – 0.973
CAG		2	0.306		
CAG(11)				1(ref)	
CAG(12)	0.558	1	0.591	0.741	0.248 – 2.212
CAG(22)	0.356	1	0.125	0.579	0.289 – 1.163

For CA – both alleles (11 – ref - both<22, 12 –one<22 and another one≥22, 22 - each≥22; *For TA - both alleles* (11 – ref - each allele<17, 12 –one<17 the another one≥17, 22 – each allele≥17; *For CAG - both alleles* (11 –ref- each<21, 12 –one<21 the other≥21, 22 - each≥21. Abbreviations: *HR – hazard ratio; CI- confidence interval; SE – standard errors; df – degree of freedom; CA – tandem repeats in the promoter of estrogen receptor β gene; TA – tandem repeats in estrogen receptor α gene; CAG – polymorphic tandem repeats in androgen receptor gene; P < 0.05 statistic significant.*

Univariate and multivariate COX regression analyses for disease-free survival prediction. When we performed univariate logistic regression between the short/long allele frequencies in the group with events (recurrences, death) versus the group without events during the follow up period, we observed a significant correlation for the TA repeats in the ERα gene (p=0.043), the longer repeats being associated with evolution without events. For the other repeats, we did not observe significant associations. These results are displayed in Tab. 4.

Regarding the other potential prognosis parameters, we observed a significant correlation between PSA expression (Yates' corrected P=0.004), nodal status (Yates' corrected P=0.001) and disease-free survival, respectively. We did not obtain significant associations between disease-free survival and the other checked variables: G (P=0.304), ER (P=0.12), PR (P=0.08), HER2/neu (P=0.065) and AR (P=0.054). It can be observed that AR expression presents a marginal statistic value.

The independent variables that were statistically significant were entered into multivariate logistic regression for predicting disease-free survival. The crude ORs were adjusted for age, radio-, chemo-, and hormone therapy as possible confounders. The multivariate logistic regression data after adjustments are presented in Tab. 5, and showed that PSA and the nodal status were statistically associated with the disease-free survival. The frequencies of short/long TA repeats carried out a marginal statistical significance (p=0.05) in predicting disease evolution after adjustments. Model chi-squared test value was 42.36 (P<0.001) with chi-square overall score of 63.745 (P<0.001) and

Table 5. Multivariate logistic regression analyses for associations with disease-free survival in breast cancer cases

Variables	SE	P	HR*	95% CI for HR
PSA	0.46	0.004	0.261	0.105 – 0.649
TA	0.36	0.055	0.497	0.243 – 1.015
N	0.415	<0.0001	5.928	2.629 – 13.367

* Adjusted for age, radio-, chemo-, and hormone therapy
Abbreviations: *PSA – prostate-specific antigen; N – nodal status; TA – polymorphic tandem repeats in the promoter of estrogen receptor α gene; HR – hazard ratio; CI - confidence interval; SE – standard errors; P < 0.05 statistic significant.*

the power for cohort studies based on normal approximation with continuously correction was 95.36%.

Discussions

Although AR, ERα and ERβ tandem repeats were studied in breast cancer, we did not find papers that compared these polymorphic genotypes with the expression of AR or PSA proteins. In this context, we intended to study the relationships between CAG, TA and CA polymorphic repeats and the immunoexpression of AR and PSA and to check possible links between repeats, the other available known prognosis factors and breast cancer survival.

DNA sequencing confirmed that no mutations were present in the coding region of PSA gene in breast tumors, but multiple polymorphisms were detected in the promoter and enhancer region. These polymorphisms in proximal ARE (androgen response element), particularly the G/A – A/AA and A/A – AA/AA genotypes were associated with increased transcriptional activity of PSA, and less aggressive forms of breast cancer [38–39]. In this context, it might be assumed that PSA could be a pathway through which the protective effects of AR operates, at least for a subset of breast carcinomas.

The idea that estrogens may influence serum androgen levels in women gains support from the reduction in serum levels of ovarian and adrenal androgens observed in women receiving estrogen replacement therapy during menopause or oral contraceptives [40]. The involvement of the alpha and beta-receptor subtypes in this effect of estrogens on androgen levels is, however, not known. Westberg et al. [30] suggest that the ERß gene influences androgen serum levels, women with short CA repeat region of the ERß gene displaying higher serum levels of total and free testosterone than women with long CA repeats. Also, association between a low number of CA repeats in the ERß gene and low levels of SHBG suggests that free testosterone levels is rather associated with this gene than total levels of testosterone. They did not observe associations between ERα TA polymorphism and sex steroids serum levels. These findings would suggest that this variant of the ERβ gene leads to a less active receptor, which contrasts

with the fact that CAG short repeats of the AR leads to a more active receptor.

Investigations of the relationship between AR CAG polymorphism and breast cancer have been conflicting, some reports associated short repeats with decreased risk [36, 41–44], other studies reported no association [45–47], or contrarily reported increased breast cancer risk [48], shorter survival [23], or increased risk and earlier diagnosis with long allele in BRCA1 carriers [22, 49].

Estrogen is essential for normal growth and differentiation in the mammary gland, but also, it supports growth of approximately 50% of breast cancers. ERα and ERβ have distinct cellular distributions, whereas ERβ is found in both ductal and lobular epithelial and stromal cells of the rodent, ERα is only found in the ductal and lobular epithelial cells and not in stroma [50]. We also know that ERβ is widely expressed in both the normal and malignant breast and there are proliferating cells in the breast which express ERβ. They regulate separate sets of genes and can oppose each other's actions on some genes. ERα appears to play a predominant role in cell proliferation, and ERβ is suggested to be antiproliferative, being a possible tumor suppressor gene. It was suggested the possibility that ERβ-selective ligands may represent a useful class of pharmacological tools, targeting proliferating cells expressing ERβ [51]. On the other hand, PSA expression is regulated by androgens through the activation of androgen receptor; however, in the absence of androgens, PSA gene expression can be overexpressed. This fact suggests that either the AR can be activated in the absence of androgen to elevate PSA gene expression and/or another transcription factor is acting on the PSA promoter. In this regard, use of siRNAs confirmed involvement of ERβ together with AR in hormone-induced PSA production and supports involvement of both AR and ERβ in mediating DHEA-, DHT-, and E(2)-induced PSA expression in prostate cancer cells [52].

Our work underline that PSA expression is correlated with shorter CA repeats in ERβ gene (p=0.03 for the sum of CA repeats on both allele) and, taken in account that shorter CA repeats were associated with higher ERβ transactivation and PSA expression was correlated with AR expression [33–35], our findings are in accordance with aforementioned previous study [52].

On the other hand, in our study, AR expression was correlated with the length of CAG repeats on the AR gene, higher number of CAG repeats being linked to a higher AR immunoexpression (p=0.03 for CAG1, p=0.008 for CAG2 and p=0.04 for CAGsum on both allele). Considering the feedback mechanisms, it can be assumed that this higher AR immunoexpression could be correlated with a less active receptor and respectively with a degree of insensitivity of the receptor to androgens. Likewise, in multivariate logistic regression, PSA expression (p=0.004), together with the absence of nodal metastasis (p<0.001) and longer TA repeats (p=0.05) were correlated with increased disease-free survival.

In summary, our study could suggests that longer TA repeats and therefore a less active ERα and a higher PSA expression implying a more active ERβ, together with a higher AR immunoexpression could be correlated with a better prognosis in breast cancer. Further studies are needed to confirm these suppositions, but, altogether, our findings supports the hypothesis that estrogens, through the both alpha and beta-receptors variants are mediating the AR signaling pathway.

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