ORIGINAL RESEARCH ARTICLE

Oligoadenylate synthetase 1 (OAS1) expression in human breast and prostate cancer cases, and its regulation by sex steroid hormones

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Abstract: Oligoadenylate synthetase 1 (OAS1) is an interferon-induced protein characterised by its capacity to catalyse the synthesis of 2’-5’-linked oligomers of adenosine from adenosine triphosphate (2-5A). The 2-5A binds to a latent Ribonuclease L (RNase L), which subsequently dimerises into its active form and may play an important role in the control of cell growth, differentiation and apoptosis. Previously, our research group identified OAS1 as a differentially-expressed gene in breast and prostate cancer cell lines when compared to normal cells. This study evaluates: i) the expression of OAS1 in human breast and prostate cancer specimens; and ii) the effect of sex steroid hormones in regulating the expression of OAS1 in breast (MCF-7) and prostate (LNCaP) cancer cell lines. The obtained results showed that OAS1 expression was down-regulated in human infiltrative ductal carcinoma of breast, adenocarcinoma of prostate, and benign prostate hyperplasia, both at mRNA and protein level. In addition, OAS1 expression was negatively correlated with the progression of breast and prostate cancer. With regards to the regulation of OAS1 gene, it was demonstrated that 17β-estradiol (E2) down-regulates OAS1 gene in MCF-7 cell lines, an effect that seems to be dependent on the activation of oestrogen receptor (ER). On the other hand, 5α-dihydrotestosterone (DHT) treatment showed no effect on the expression of OAS1 in LNCaP cell lines. The lower levels of OAS1 in breast and prostate cancer cases indicated that the OAS1/RNaseL apoptotic pathway may be compromised in breast and prostate tumours. Moreover, the present findings suggested that this effect may be enhanced by oestrogen in ER-positive breast cancers.

Keywords: OAS1; oestrogen; androgen; breast cancer; prostate cancer


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Introduction

Breast and prostate cancers are among the most commonly-diagnosed malignancies in women and men worldwide with approximately 1.7 million and 1.1 million new cases in 2012, respectively[1]. Carcinogenesis is a multifactorial process, and it is believed that several genetic alterations are needed to convert normal cells into carriers of a malignant phenotype[2]. The identification of genetic alterations in cancer cells is crucial for the development of more effective methods for early diagnosis, as well as to provide targets for treatment and therapeutic interventions. The analysis of the similarities and differences between breast and prostate cancers provides

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a fascinating picture of the natural history, biological basis and clinical features of these two endocrine-related cancers and suggests that breast and prostate cancer may represent, in some aspects, homologous cancers in females and males. Indeed, several studies have shown that a few genes which have been widely accepted to be breast cancer-specific (e.g. BRCA1, BRCA2 and HER2) are also associated with prostate cancer, and vice-versa (e.g. PSA).

As preliminary results (unpublished data), our research group has identified 2'-5'-oligoadenylate synthetase 1 (OAS1) as a differentially-expressed gene in human breast (MCF-7) and prostate (LNCaP) cancer cell lines. The human OAS gene family contains four genes, namely OAS1, OAS2, OAS3 and OAS4. Two isoforms of OAS1 protein with the molecular weight of 40 and 46 kDa, respectively, have been identified as a result from alternative splicing. All OAS, except OASL, are interferon-induced proteins characterised by their capacity to catalyse the synthesis of 2'-5'-linked oligomers of adenosine from adenosine triphosphate (2-5A). The 2-5A binds to a latent Ribonuclease L (RNase L), which subsequently dimerises into its active form. It was shown that the suppression of RNase L activity in cancer cell lines dramatically blocked cell death, which supports its role as a strong inducer of apoptosis. Furthermore, the OAS/RNase L pathway has been associated with the inhibition of cell growth and promotion of apoptosis in human breast and prostate cancer cell lines. No studies have been conducted to characterize the clinical expression of OAS1 in human breast and prostate cancer specimens.

Both oestrogens and androgens play an important role in the proliferative and apoptotic events of normal physiological processes in the mammary gland and prostate, in which pathway deregulation of these sex hormones may lead to the development of carcinogenesis in both tissues. Several studies indicated that OAS1 gene expression and activity are under the control of 17β-estradiol (E2) in a few tissues, but the role of androgens in the regulation of OAS1 remains unexplored. This study focused on the analysis of OAS1 expression in human breast and prostate cancer specimens, and its regulation by E2 and 5α-dihydrotestosterone (DHT) in human breast (MCF-7) and prostate (LNCaP) cancer cell lines, respectively.

Materials and methods

Cell lines and human samples

The human breast cancer cell line MCF-7 (ECACC 86012803) and prostate cancer cell line LNCaP (ECACC 89110211) were purchased from the European Collection of Cell Cultures (Public Health England, UK). The MaxArray™ Human Breast Carcinoma Tissue Microarray Slides (Zymed, Ref. 75-4043) containing samples of infiltrating ductal carcinoma (IDC, N = 40) and normal breast cancer (N = 1), and the MaxArray™ Human Prostate Cancer & BPH Tissue Microarray Slides (Zymed, Ref. 73-5063) containing samples of adenocarcinoma of prostate (N = 19), benign prostatic hyperplasia (BPH, N = 16), and normal prostate cancers (N = 1) were purchased from Invitrogen (Karlsruhe LMA, Germany).

Human breast cancer tissue sections (IDC, N = 3) were obtained from Centro Hospitalar Cova da Beira (CHCB, Portugal). Breast tumour specimens and normal tissue adjacent to these tumours (N = 6) were obtained from patients of Salamanca University Hospital who had not received preoperative chemotherapy or any other treatment. These samples were either frozen at −80°C for RNA extraction, or fixed in formaldehyde for histopathology analysis. These samples were graded according to tumour differentiation (I to III), mitotic index, and nuclear pleomorphism as previously proposed by Elston and Ellis.

Prostate adenocarcinoma paraffin embedded tissue sections (N = 23) and BPH (N = 1) were obtained from Porto University Hospital, and classified according to Gleason’s classification. All tissue samples were obtained with informed consent of the patients, and in accordance to the local ethical committee’s guidelines.

Immunohistochemistry staining analysis

OAS1 protein of human breast IDC (N = 43), normal breast (N = 1), adenocarcinoma of prostate (N = 42) and normal prostate (N = 1) were detected by immunohistochemistry (IHC) in formalin-fixed, paraffin-embedded (FFPE) 3-µm tissue sections as described previously. OAS1 immunoreactivity was assessed semi-quantitatively by IHC scores. This system was based on the intensity and percentage of stained cells. Briefly, the staining intensity was divided into moderate (score 1) and strong (score 2), with the percentage of stained tumour cells divided into three occasional scores, i.e., less than 1/3 (score 1), more than 2/3 (score 2) and almost all (score 3). Low immunoreactivity indicates that up to 1/3 of the neoplastic cells were moderately stained (score 1 = 1 × 1), moderate immunoreactivity indicates strong staining of up to 2/3 of the neoplastic cells (score 2 = 1 × 2 or 2 × 2) or moderate staining in almost all neoplastic cells (3 × 1), and high immunoreactivity indicates that...
almost all the neoplastic cells were strongly stained (score 6 = 3 × 2).

Cell culture and hormonal treatment

MCF-7 and LNCaP cell lines were cultured in DMEM or RPMI medium (Invitrogen), respectively, supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin.

For hormonal treatment experiments, approximately 4 ×10³ MCF-7 or LNCaP cell lines were plated in 6 multiwell plates. Before treatment, cells were grown in phenol red-free and serum-free medium for two days to reproduce the complete oestrogen- or androgen-free environment, and minimize the gene expression levels. After an additional two days, MCF-7 and LNCaP cell lines were exposed to 0 nmol/L and 10 nmol/L of E₂ (Sigma, Saint Louis, USA) or DHT (Sigma), respectively, for 6, 12, 24 and 48 h. After adjusting the exposure time, the assay with MCF-7 cell lines was repeated using 0 nmol/L and 10 nmol/L E₂, 10 nmol/L E₂ plus 10 nmol/L of oestrogen receptor (ER) antagonist ICI 182,780 (Tocris Cookson, Bristol, UK), 10 nmol/L E₂ plus 10 μg/mL of protein synthesis inhibition cycloheximide (Chx) (Sigma), 10 nmol/L of ICI 182,780 alone and 10 μg/mL of Chx alone. ICI 182,780 and Chx were added 1 h before hormone stimulation. All experiments were carried out in triplicate.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from IDC of breast, normal adjacent tissue to IDC, MCF-7 and LNCaP cell lines using TRI reagent (Ambion, UK) in accordance with the manufacturer’s instructions. Total RNA integrity was assessed by agarose gel electrophoresis and quantified by the OD260/280 (Pharmacia Biotech, Ultraspec 3000). cDNA was synthesized using 2 μg of total RNA. After denaturation at 65 °C for 5 min, 500 μmol/L de-oxyribonucleotide triphosphates (Amersham, Uppsala, Sweden) and 250 ng of random primers (Invitrogen), together with total RNA, were reverse-transcribed at 37 °C for 60 min in a 20 μL reaction volume, which contained reverse transcriptase buffer (50 mmol/L Tris–HCl, 75 mmol/L KCl, 3 mmol/L MgCl₂ and 0.1 mmol/L DTT), 10 mmol/L DTT, 60 U of RNaseOUT (Invitrogen), and 200 U of M-MLV RT (Invitrogen). The reaction was stopped at 75 °C for 15 min.

Quantitative real-time PCR

The OAS1 mRNA gene expression in human breast specimens and in MCF-7 and LNCaP cell lines was evaluated by quantitative real-time PCR (qPCR). Specific primers of human OAS1 (sense: 5’ GAG CCT CAT CCG CCT AGT CAA G 3’; antisense: 5’ TCC AAG ACC GTC CGA AAT CCC 3’) were used to amplify an OAS1 fragment of 161 bp. Human β-2-microglobulin (sense: 5’ ATG AGT ATG CCT GCC GTG TG 3’; antisense: 5’ CAA ACC TCC ATG ATG CTG CCT AC 3’) and human GAPDH (sense: 5’ CGC CCG CAG CCG ACA CAT C 3’; antisense: 5’ CGC CCA ATA CGA CCA AAT CCG 3’) primers were used as internal controls to normalize OAS1 gene expression.

qPCR efficiency for OAS1, β2M and GAPDH primers in all sample reactions was determined using serial dilutions of cDNA (1:1, 1:5 and 1:25). The qPCR reactions were carried out using 1 μL of cDNA in a 20 μL reaction volume containing 10 μL of SYBR Green Mix (Biorad) and 300 nmol/L of OAS1, β2M or GAPDH primers. After an initial denaturation at 95 °C for 5 min, the PCR conditions were set to 40 cycles as follows: denaturation at 95 °C for 10 s, annealing at 62 °C for 30 s and extension at 72 °C for 10 s. The specificity of the amplified fragments was checked by melting curve analysis. Reactions were heated from 55 to 95 °C with 10 s hold at each temperature (0.05 °C/s). Moreover, PCR products were cloned into pGEM-T easy vector (Promega) and sequenced to confirm the identity of the amplicons. Samples for each qPCR assay were run in triplicate. The fold differences were calculated by following the mathematical model proposed by Pfaffl using the 2^{ΔΔCt} formula.[29]

Statistical analysis

All experimental data were shown as mean ± standard error of mean (SEM). The statistical significance differences in OAS1 mRNA gene expression among the experimental groups were assessed by paired t-test or by ANOVA, followed by the Dunnett’s test. Significant distributions among the parameters analysed by IHC were determined using the chi-square test. Significant differences were considered when p < 0.05. The SPSS software was used in all the statistical analyses.

Results

Gene expression of OAS1 in breast cancer specimens

In order to examine whether the OAS1 gene is differentially-expressed in breast cancer cases, we used fresh tissue from 6 cases of IDC and normal tissue adjacent to these tumours. qPCR analysis showed that OAS1 mRNA
levels were reduced by 67% (4 out of 6 cases) in breast cancer cases compared to normal epithelial tissue adjacent to tumours (p < 0.05, paired t-test) (Figure 1).

Samples of IDC of breast, adenocarcinoma of prostate and BPH were classified positive for OAS1 immunoreactivity according to a score scale established for this parameter, which takes into account the intensity and extension of the signal. A statistically significant negative correlation was found between OAS1 immunoreactivity and breast cancer grading, or prostate cancer cellular differentiation (Table 1 and Table 2, respectively). Low-immunoreactivity of OAS1 was detected with grade I in 38% of breast tumours, grade II in 53% of tumours and grade III in 75% of tumours. Regarding the adenocarcinoma of the prostate, low-OAS1 immunoreactivity was found in 27% of moderately-differentiated tumour cells and 52% of poorly-differentiated tumour cells, whereas none was found in well-differentiated tumour cells. In contrast, BPH samples showed 18% of low-immunoreactivity of OAS1, whereas 35% and 47% displayed moderate-immunoreactivity and high-immunoreactivity of OAS1, respectively. Representative of each tissue sections with low, moderate and high OAS1 immunoreactivity in breast and prostate samples are shown in Figure 2.

Table 2. Comparative analysis between OAS1 immunoreactivity in adenocarcinoma of prostate and cellular differentiation

<table>
<thead>
<tr>
<th>Histological diagnostic</th>
<th>Cellular differentiation</th>
<th>Number of samples</th>
<th>OAS1 immunoreactivity</th>
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<tbody>
<tr>
<td>Non-malignant tissue</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>BPH</td>
<td>17</td>
<td>18% (3/17)</td>
<td>35% (6/17)</td>
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<tr>
<td></td>
<td></td>
<td>47% (8/17)</td>
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<tr>
<td>Malignant tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate adenocarcinoma</td>
<td>42</td>
<td>41% (17/42)</td>
<td>38% (16/42)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21% (9/42)</td>
<td></td>
</tr>
<tr>
<td>Poorly</td>
<td>25</td>
<td>52% (13/25)</td>
<td>40% (10/25)</td>
</tr>
<tr>
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<td>27% (4/15)</td>
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<tr>
<td></td>
<td></td>
<td>33% (5/15)</td>
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<td>0% (0/2)</td>
<td>0% (0/2)</td>
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<td></td>
<td></td>
<td>100% (2/2)</td>
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</table>

The expression of OAS1 was significantly different between non-malignant tissue and malignant tissue (p < 0.01, chi-square test), and between OAS1 immunoreactivity and cellular differentiation of prostate adenocarcinoma (p < 0.01, chi-square test). The number of samples in each group is indicated in parenthesis.

Down-regulation of OAS1 expression by E2 in MCF-7 cell lines

In order to evaluate OAS1 levels in response to oestrogen and androgen presence in human breast and prostate cancer cell lines, both MCF-7 and LNCaP cell lines were treated with E2 and DHT, respectively. The response of OAS1 mRNA to E2 and DHT was analysed by qPCR. The results showed that E2 down-regulated OAS1 gene expression in MCF-7 cell lines, except when hormone incubation was allowed to proceed for 48 h (Figure 3A). On the other hand, DHT did not seem to regulate OAS1 expression in LNCaP cell lines (Figure 3B). In order to elucidate the mechanism underlying the response of OAS1 to E2 in MCF-7 cell lines, the hormonal stimulation was repeated for 6 h in the presence of ICI 182,780 and Chx (Figure 3C). The E2-induced down-regulation of OAS1 mRNA expression was inhibited in the presence of ICI 182,780. Moreover, MCF-7 cell lines

![Figure 1. Comparison of OAS1 mRNA expression between IDC of breast and normal adjacent epithelial tissue (N = 6). Samples were obtained from the Salamanca University Hospital, and mRNA expression was determined by qPCR after normalization to GAPDH and β2M expression. The experiment was performed in triplicates. *p < 0.05 (One Way ANOVA followed by Dunnett’s test).](Image 1 to 278x686)

![Table 1. Comparative analysis between OAS1 immunoreactivity in IDC of breast and histological grading](Image 67x490 to 278x686)
Figure 2. Immunohistochemical staining of OAS1 protein in human breast and prostate cancer. (A) Normal breast. (B, C and D) Infiltrative ductal carcinoma of the breast with high, moderate and low OAS1 immunoreactivity, respectively (IDC of grade I, II and III, respectively). (I) Normal prostate. (J, K and L) Adenocarcinoma of the prostate with high, moderate and low OAS1 immunoreactivity, respectively (well, moderately and poorly cellular differentiation, respectively). Negative controls obtained by omission of anti-OAS1 polyclonal antibody were carried out for IDC of breast (E-H) and adenocarcinoma of prostate (M-P). Arrows indicated OAS1 staining of epithelial cells.

Figure 3. Time-course analysis of OAS1 mRNA expression in (A) human MCF-7 and (B) LNCaP cell lines in response to E2 and DHT stimulation, respectively. MCF-7 and LNCaP cell lines were exposed to 0 and 10 nmol/L of E2 or DHT for 6, 12, 24 and 48 h and mRNA expression was determined by qPCR. (C) OAS1 mRNA expression in MCF-7 cell lines in response to 0 and 10 nmol/L of E2, 10 nmol/L E2 plus 10 nmol/L ICI 182,780, 10 nmol/L E2 plus 10 μg/mL cycloheximide (Chx), 10 nmol/L of ICI 182,780 alone and 10 μg/mL of Chx alone in MCF-7 cell lines for 6 h of treatment. OAS1 expression was normalized to GAPDH and β2M expression. *There was statistically significant difference between control and treated group (p < 0.05; One Way ANOVA followed by Dunnett’s test)
stimulated with the combination of E2 and Chx showed down-regulation of OAS1 mRNA levels when compared to controls. No significant differences were observed using ICI 182,780 or Chx alone.

**Discussion**

Considering that OAS1 gene expression was under-expressed in MCF-7 and LNCaP cell lines in comparison to normal cell lines (data unpublished), we hypothesized that OAS1 gene expression was found to be differentially expressed in human breast and prostate cancer specimens. In order to attain this goal, the mRNA expression of OAS1 was determined in matched tumor-normal adjacent tissue pairs. Our results showed that OAS1 gene expression was under-expressed in IDC of breast specimens when compared to normal adjacent tissue. This differential expression was confirmed by IHC in several cases of breast cancer. It should be noticed that a negative correlation was found between OAS1 protein expression and tumour grading, suggesting that OAS1 expression decreased in aggressive tumours. Considering that OAS1 mRNA levels were lower in LNCaP cell lines when compared to normal prostate cell lines (data not shown), the OAS1 protein levels in samples of adenocarcinoma of prostate and BPH were also investigated by IHC. The results suggested that OAS1 protein expression was lower in malignant lesions. In addition, a negative association between OAS1 protein expression and tumour progression was found, i.e., well-differentiated tumours showed no reduction of OAS1 expression in contrast to tumours with moderate or poor cellular differentiation.

Taking into account that OAS1 levels were lower in both breast and prostate cancers than in normal tissue, that aggressiveness of the tumours correlated positively with lower levels of OAS1 expression, and considering the role of OAS1 in apoptosis, it is liable to speculate that apoptosis induced by the OAS/RNase L pathway may be compromised in these cancers. Recently, several number of polymorphisms in OAS1 gene were identified to be associated with prostate cancer, also suggesting that RNase L can be compromised, and consequently, changing the cell growth and apoptosis rate.

Both oestrogens and androgens play crucial roles in the proliferative and apoptotic events in the normal physiological states of mammary gland and prostate, as well as in pathological conditions. It leads us to study the effects of E2 and DHT on OAS1 expression in MCF-7 and LNCaP cell lines, respectively. Our results indicated that OAS1 mRNA expression in MCF-7 cell lines achieved maximum reduction by E2 between 6 h and 12 h of stimulation, showing that reduction of OAS1 mRNA expression was time-dependent in MCF-7 cell lines. The presence of ICI 182,780 abrogated the down-regulatory effect of E2 on OAS1 expression, indicating that this effect was mediated by a nuclear ER. Moreover, in order to investigate whether protein synthesis was needed for the modulation of gene expression, we analysed the OAS1 mRNA levels in MCF-7 cell lines following E2 treatment in the presence of Chx. The results demonstrated that the inhibition of protein synthesis had no effect on E2-induced down-regulation of OAS1 expression, suggesting that the downstream pathways were not involved in this regulation. Besides the classical interactions with oestrogen-response-elements (ERE), ERs also regulate gene expression by interacting directly with the AP-1 protein complex, the Sp1 protein and the NF-kB protein. Using the genomatix software, it was possible to identify two EREs binding sites, seven AP-1 binding sites, five Sp1 binding sites, and three NF-kB binding sites in the 10 kb upstream region of the OAS1 gene promoter. These data suggested that multiple mechanisms may be involved in the regulation of OAS1 protein by ER-ligand complexes. The OAS/RNase L system is associated with the inhibition of cell growth and promotion of apoptosis. Our results suggested that E2 may enhance the reduction of OAS1 expression in breast tumours, which may further compromise the OAS/RNase L apoptotic pathway. Nonetheless, further studies are required to support this hypothesis. Our group previously demonstrated that OAS1g, the most abundant OAS1 gene expressed in rat mammary gland, was up-regulated by E2 in this tissue. The finding was in contrast with the results obtained in the present study, but after 48 h of stimulation of MCF-7 cell lines with E2, the OAS1 mRNA expression levels were equal to the controls. Thus, it is possible that long-time exposure to E2 may increase the OAS1 mRNA expression as seen in the in vivo studies, in which the rats were exposed to E2 for 7 days. As a matter of fact, the former study was carried out in normal rat tissues and there is high possibility that OAS1 may be differently regulated in normal and tumour breast tissues. Alternatively, human and rat mammary gland may respond differentially to E2 stimulation. Further studies using several cell lines with non-malignant and malignant phenotypes are required to better understand these differences. The results obtained with the DHT stimulation of LNCaP cell lines suggested that OAS1 mRNA expression was androgen-independent in human prostate. Overall, the results suggested that OAS1 gene may be involved in the onset and/or progression of breast and prostate cancer, and it deserves further investigation as a putative target.
for breast and prostate cancer management.

**Conclusion**

In conclusion, the OAS1 gene was under-expressed in both breast and prostate cancer, which seemed to be associated with tumour aggressiveness. Moreover, OAS1 expression was down-regulated by E2 in human breast cancer cell lines, but DHT showed no effect in the regulation of OAS1 expression in human prostate cancer cell lines. Although more studies are required, our data supported the hypothesis that genetic changes in OAS1 gene could be involved in the progression of breast and prostate cancer.

**Author contributions**

Maia CJ performed the collection, analysis and interpretation of the data, besides being involved in the writing and approval of the final manuscript. Rocha SM contributed to the analysis and interpretation of data as well as writing of the manuscript. Socorro S designed the conceptual study, contributed to the analysis and interpretation of data and also performed the critical reading of the manuscript. Schmitt F helped in the analysis and interpretation of the data, besides contributing to the critical reading of the manuscript. Santos CR designed the conceptual study, contributed to the analysis and interpretation of data, and also involved in the critical reading of the manuscript.

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**Conflict of interest**

The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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