**JDP1 (DNAJC12/Hsp40) expression in breast cancer and its association with estrogen receptor status**

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**Abstract.** The members of the DnaJ/Hsp40 proteins are highly conserved through evolution, expressed in several tissues and act as co-chaperone regulating protein folding, transport, translational initiation and gene expression. Recently, using cDNA microarray we identified differences in the expression of the JDP1 (DNAJC12) gene, a member of the DnaJ/Hsp40 family, between ER-positive and ER-negative breast tumours. In this study, using quantitative real-time PCR (qPCR) we evaluated the expression pattern of the JDP1 gene in a series of 72 primary breast tumours and investigated the effects of 17ß-estradiol on the expression of the JDP1 in MCF-7 breast cancer cells. Three patterns of JDP1 mRNA expression were identified in the primary breast tumours analysed: normal expression was found in 14% of the cases, under-expression in 50%, and over-expression in 36% of the cases. High levels of JDP1 mRNA expression were significantly associated with estrogen receptor-positive status (p=0.02). No relationship was found between JDP1 mRNA expression and any other clinicopathological characteristics of the patients. Sequence analysis of the promoter region of the JDP1 gene revealed the presence of potential estrogen response elements (EREs), suggesting it to be under the control of estrogen action. We also assessed the effects of 17ß-estradiol (10 nM) on JDP1 mRNA expression in MCF-7 breast cancer cells. The JDP1 transcripts were found to be up-regulated in a time-dependent fashion in MCF-7 cells exposed to 17ß-estradiol treatment. Here we show for the first time that JDP1 is an estrogen target gene and that its expression might be used as a marker of the ER transactivation activity and may have a predictive value for response to hormonal therapy.

**Introduction**

Lifetime exposure to estrogens is considered one of the main risk factors for breast cancer development (1,2). Most of the biological effects of the estrogens are mediated by the estrogen receptors, ERα and ERß, members of the steroid hormone receptor superfamily, which are ligand induced transcription factors (3,4). Similar to other members of the steroid hormone receptor superfamily, ER forms large heterocomplexes with members of the heat shock proteins (Hsp90, Hsp70) and immunophilins (5). In the classic mechanism of estrogen action, the hormone binding to the ER leads to Hsp90 dissociation resulting in an estrogen-ER complex that directly interacts with estrogen response elements (EREs) in the promoter region of estrogen regulated genes (3,4). The heterocomplex formed between the ER and Hsp90, Hsp70 and other co-chaperone proteins is important for the maintenance of the molecular conformation of the receptor and is required for an appropriate and efficient hormone binding to the receptor (6,7).

Using cDNA microarray analysis we found a linear correlation between the expression of the DNAJC12 gene (also named JDP1; J domain containing protein 1) and the presence of estrogen receptor in breast tumours (8). The JDP1 gene is located at chromosome 10q22.1 and encodes for the J domain containing protein 1, a member of the small-molecular-weight heat shock proteins (DnaJ/Hsp40) family, which has essential co-chaperone activity with other heat shock proteins (9). The members of the large family of DnaJ/Hsp40 proteins are evolutionarily conserved and characterized by the presence of one or more J-domains in their N-terminal region (10,11). The J domain, composed by three coiled coil helices spanning approximately 70 amino acids with the exposure of a tripeptide histidine-proline-aspartate (HPD) is important for the Hsp40-Hsp70 interaction and ATPase activity stimulation (12-14). There is also evidence that eukaryotic DnaJ homologue can interact physically and functionally with the major cytoplasmatic molecular chaperone Hsp90 (15,16). In addition, in yeast, Ydj1/Hsp40 mutants displayed high basal levels of ER activity in the absence of estrogen suggesting that Ydj1/Hsp40 is important for the receptor regulation by the Hsp90 folding pathway (17).
In the present study, in order to better understand the possible role played by the *JDP1* in breast cancer, we investigated the expression levels of the *JDP1* transcripts in a series of primary breast tumours using quantitative real-time PCR (qPCR). Additionally, we examined the potential promoter region of the *JDP1* gene for the presence of ERE binding sites and investigated the effects of 17ß-estradiol on the expression of the *JDP1* transcripts in MCF-7 breast cancer cells.

Materials and methods

**Samples.** Seventy-two primary breast tumour samples and 20 adjacent normal tissues were obtained from 72 breast cancer patients at Hospital do Câncer, A.C. Camargo, São Paulo, Brazil. The age of the patients at the time of operation ranged from 23 to 93 years (median 54 years). Tumour samples were dissected to remove residual normal tissue before freezing and storage in liquid nitrogen. The largest diameter of the tumours was recorded. The number of lymph node metastases was determined by microscopic examination of an average of 24 lymph nodes per patient. Tumour metastasis at lymph nodes was detected in 47 patients. All the cases were submitted to a histopathological review of tumour slides in order to confirm diagnosis. All tumours were classified according to the WHO Histological Typing of Breast Tumours (WHO, 1982). The tumours studied were infiltrating ductal carcinomas. The clinical stage of the patients was determined according to the UICC TNM (tumour, nodes, metastases) staging system (UICC, 1978). The institutional Ethics Committee approved this study, and all subjects provided informed consent. Estrogen and progesterone receptor binding assays were performed by the dextran-coated-charcoal method (DCC) as previously described (18).

**Cell lines and culture conditions.** The human breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (ATCC). The cells were cultured at 37°C in an atmosphere of 5% CO₂, 95% air in RPMI (phenol red-free) supplemented with 10% FCS and 1% penicillin-streptomycin (Gibco). Before the treatments with 17ß-estradiol and 4-hydroxytamoxifen, the cells were washed twice with PBS to remove residual serum and grown in media without phenol red, containing 5% of charcoal-stripped FCS (CS-FCS) for 48 h. After hormone deprivation, the cells were incubated with 17ß-estradiol 10 nM for 2, 6 and 24 h or 4-hydroxytamoxifen 1 μM for 24 h. The cells maintained in media supplemented with 10% FCS and 1% penicillin-streptomycin (Gibco). The cells were cultured at 37°C in an atmosphere of 5% CO₂, 95% air in RPMI (phenol red-free) supplemented with 10% FCS and 1% penicillin-streptomycin (Gibco). Before the treatments with 17ß-estradiol and 4-hydroxytamoxifen, the cells were washed twice with PBS to remove residual serum and grown in media without phenol red, containing 5% of charcoal-stripped FCS (CS-FCS) for 48 h. After hormone deprivation, the cells were incubated with 17ß-estradiol 10 nM for 2, 6 and 24 h or 4-hydroxytamoxifen 1 μM for 24 h. The cells maintained in media without phenol red and 5% FBS (C) were used as controls.

**RNA extraction.** Tissue specimens were pulverized under liquid nitrogen using a frozen tissue pulverizer (Termovac). The cells, after the treatments, were washed twice with PBS and harvested. For RNA extraction tissue powder or the harvested cells were homogenized in a solution containing guanidine isothiocyanate and extracted as described by Chomczynski and Sacchi (19). The quality of the RNA samples was determined by 1% agarose gel electrophoresis and ethidium bromide staining. All RNA samples were treated with DNaseI for 30 min at 37°C to eliminate genomic DNA contamination.

**Quantitative real-time PCR (qPCR).** qPCR was performed by using the GeneAmp 5700 sequence detector (PE Applied Biosystems). cDNA was generated using the High Capacity cDNA Archive kit (Applied Biosystems). Each cDNA sample was analysed in duplicate. PCR reactions were carried out in a total volume of 50 μl according to the manufacturer’s instructions for Platinum SYBR Green qPCR SuperMix UDG (Invitrogen). The PCR primers used were as follows: *JDP1* (DNAJC12), forward primer 5'-CAGACAAGCATCCGAAAACC-3' and reverse primer 5'-TCGCCAGTGCTCATAGCGGGC-3' (this set of primers spans exons 2 and 3 amplifying a 110 bp fragment common to variants 1 and 2 of the *JDP1* gene); *GAPDH*, forward primer 5'-CCTCCATACCAATTCAAGTGGGGGCG-3' and reverse primer 5'-GGGGCAATCAGTGAGGACCCTT-3'. The relative gene expression was normalized using *GAPDH* expression as an internal control. The average value of two pools composed of 10 normal tissue samples each served as calibrator sample for the tumours. MCF-7 cells maintained in medium without phenol red and supplemented with 5% FCS served as calibrator samples for hormone treated cells. The results were expressed as n-fold differences in gene expression of the target gene relative to the expression of the *GAPDH* gene and calibrator sample. The relative expression was calculated by 2-ΔΔCT (CT = fluorescence threshold value; ΔΔCT = CT of the target gene - 364
CT of the reference gene (GADPH); \( \Delta \Delta CT = \Delta CT \) of the target sample - \( \Delta CT \) of the calibrator sample) (20).

Statistical methods. Analyses of statistical significance between the JDP1 expression levels and the demographic and clinico-pathological characteristics of the patients were performed by the Chi-square test and Fisher exact test for frequency data in contingency tables and considered statistically significant at \( p<0.05 \). The statistical analysis were performed using SPSS software 10.0 (SPSS Inc., Chicago, IL).

Results

The pattern of JDP1 (DNAJC12) mRNA expression was determined in a series of 72 primary breast tumours by quantitative real-time PCR reaction. The relative expression of the target gene was determined in n-fold differences relative to the normalized calibrator samples (two pools of normal breast tissue samples). Breast tumours displayed variable patterns of JDP1 mRNA expression relative to the normal breast samples. The JDP1 transcripts was normally expressed in 10 tumours (14%), under-expressed in 36 tumours (50%; >2-fold decrease) and over-expressed in 26 tumours (36%; >2-fold increase). We correlated the expression levels of the JDP1 transcripts in the breast tumours with the clinicopathological characteristics of the patients, such as, clinical stage, tumour size, nodal status, ER and PR status, overall and disease-free survival. No statistically significant differences were observed, except for the ER status. We found a relationship between the JDP1 mRNA expression levels and the estrogen and progesterone receptor status. The expression levels of the JDP1 transcripts were statistically significantly higher in the group of ER-positive breast tumours compared to the group of ER-negative breast tumours (\( p=0.02 \)) (Fig. 1). In addition, the highest levels of JDP1 mRNA expression were displayed by ER+/PR+ breast tumours (\( p=0.035 \)) (Fig. 2).

We further evaluated the potential promoter region of the JDP1 gene for the presence of ER-binding sites. The databases at the NCBI (www.ncbi.nlm.nih.gov) and USCS Genome Bioinformatics (www.genome.uscs.edu) were used to annotate the position of the JDP1 in the genome and to extract sequences 2 kb upstream and 2 kb downstream of the transcription start site for binding-site analyses. The search for EREs and other binding-sites was performed using several publicly available programs (Transfac 6.0, www.gene-regulation.com; and Dragon Genome Explorer, http://research.i2.a-star.edu.sg/promoter/). The potential promoter region of the JDP1 contains 7 imperfect palindromic EREs, several widely spaced 1/2 EREs, and binding sites for several transcription factors (Fig. 3).

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The promoter region of the JDP1 gene showed a high potential to be a target of the ER action. Therefore we further investigate the effect of 17ß-estradiol and tamoxifen on JDP1 mRNA expression in MCF-7 cells, a hormone-responsive breast cancer cell line, using quantitative real-time PCR. For
Heat shock proteins are highly conserved and widely expressed chaperones that act in several biological processes preventing the accumulation of improperly folded proteins and promoting proper refold of damaged proteins (21). Members of the high- and low-molecular-weight heat shock proteins, such as Hsp90, Hsp70 and Hsp40 take part in the steroid hormone receptor heterocomplex to assist proper protein folding and activation by hormone binding (5). JDP1 is a member of the DnaJ/Hsp40 family (9). The members of this small-molecular-weight heat shock proteins have been shown to direct interact with the Hsp70 and Hsp90 as a co-chaperone protein (14,21). In addition, in yeast, Ydj1/Hsp40 mutants displayed high basal levels of ER activity in the absence of estrogen suggesting that Ydj1/Hsp40 is important for the receptor regulation by the Hsp90 folding pathway (17). These data suggest that the co-chaperone activity of the DnaJ/Hsp40 in the Hsp90 pathway might also be important for the integrity and functionality of ER in the mammary normal gland and breast tumour tissue. The association observed here between high levels of JDP1 transcripts and the ER positivity may suggest that the JDP1 expression might be a marker of the ER transactivation activity and may have a predictive value for response to hormonal therapy.

Substantial evidence indicates that altered expression of members of the heat shock protein families, such as Hsp90, Hsp70 and Hsp27 play important roles in breast cancer (22-25). There is also experimental evidence showing that Hsp90, Hsp70 and Hsp27 are regulated by estrogen via ER in mice uterus, breast cancer cells and in cells from other tissues (26-31). The relationship observed between the JDP1 mRNA expression and ER status in the clinical specimens lead us to speculate that the JDP1 could be under the control of ER transcriptional transactivation. The mechanism of estrogen action is complex and depends on several factors, such as the availability of the amount and subtype of the ER, amount and time of the estrogen exposure and presence of different co-regulators (32). However, the genomic effects of the estrogen is thought to be mediated by the interaction between the estrogen receptor with specific DNA binding sites named estrogen response elements (EREs) or by the interaction with other transcription factors, such as AP1 and SP1 (3,33,34).

Recently we identified by cDNA microarray a set of differentially expressed genes in breast tumours and showed a direct association between JDP1 mRNA expression and the presence of estrogen receptor in breast tumours (8). In the present study, examining the JDP1 expression in a larger series of primary breast tumours, we confirmed our previous results showing that JDP1 mRNA expression is direct associated with the ER status in breast tumours. However, no relationship was observed between the expression levels of the JDP1 and any other clinicopathological characteristics.

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We identified and analysed the promoter region 5’ up-stream of the JDP1 gene for the presence of potential ERE binding sites. We were able to identify seven imperfect palindromic EREs and several widely spaced 1/2 EREs in the potential promoter region of the JDP1 gene, suggesting that it might be transcriptionally regulated by estrogen. So far, this was confirmed by preliminary experiments in MCF-7 breast cancer cells upon 17β-estradiol treatments. In MCF-7 cells exposed to 10 nM of 17β-estradiol the JDP1 transcripts were up-regulated in a time-dependent fashion. The levels of the Hsp90 transcripts are also regulated by 17β-estradiol in a time-dependent fashion in uterus of ovariectomized mice, the Hsp90 mRNA levels reach a maximum in 2-6 h and decline to control baseline value after 24 h after estradiol administration (26,35). Although further functional studies are required to determine the role and possible cooperativity of the potential EREs observed in the promoter regions of the JDP1 gene to confer estrogen responsiveness, this is the first report identifying the JDP1 as a novel estrogen target gene. It has been proposed that some members of the DnaJ/Hsp40 proteins, which interact and cooperate with Hsp70 as a co-chaperone are required to assist the assembly and maintenance of a functional aporeceptor complex (36). The estrogen induction of different heat shock proteins, such as Hsp90, Hsp70 and also the JDP1 (DnaJ/Hsp40) that are components of the

Discussion

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receptor heterocomplex may work as a positive feedback for the appropriate supply of proteins required to maintain proper receptor folding and signal transduction.

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