Silencing of LRRC49 and THAP10 genes by bidirectional promoter hypermethylation is a frequent event in breast cancer

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Abstract. Previously we found that levels of LRRC49 (leucine rich repeat containing 49; FLJ20156) transcripts were elevated in ER-positive breast tumors compared with ER-negative breast tumors. The LRRC49 gene is located on chromosome 15q23 in close proximity to the THAP10 (THAP domain containing 10) gene. These two genes have a bidirectional organization being arranged head-to-head on opposite strands, possibly sharing the same promoter region. Analysis of the promoter region of this gene pair revealed the presence of potential estrogen response elements (EREs), suggesting the potential of this promoter to be under the control of estrogen. We used quantitative real-time PCR (qPCR) to evaluate the expression of LRRC49 and THAP10 in a series of 72 primary breast tumors, and found reduced LRRC49 and THAP10 expression in 61 and 46% of the primary breast tumors analyzed, respectively. In addition, the occurrence of LRRC49/THAP10 promoter hypermethylation was examined by methylation specific PCR (MSP) in a sub-group of the breast tumors. Hypermethylation was observed in 57.5% of the breast tumors analyzed, and the levels of mRNA expression of both genes were inversely correlated with promoter hypermethylation. We investigated the effects of 17ß-estradiol on LRRC49 and THAP10 expression in MCF-7 breast cancer cells and found both transcripts to be up-regulated 2- to 3-fold upon 17ß-estradiol treatment. Our results show that the transcripts of LRRC49/THAP10 bidirectional gene pair are co-regulated by estrogen and that hypermethylation of the bidirectional promoter region simultaneously silences both genes. Further studies will be necessary to elucidate the role of LRRC49/THAP10 down-regulation in breast cancer.

Introduction

Closely located gene pairs organized in a head-to-head orientation sharing a bidirectional promoter are a relatively common feature of the human genome (1). Computational genome-wide analysis showed that more than 10% of the human genes are organized in this bidirectional fashion with <1.0 kb between their transcript units (2). The transcriptional regulation of genes sharing a bidirectional promoter is complex, and involves co-regulation, anti-regulation and tissue-specific independent regulation (1-3). Several bidirectional gene pairs consist of genes associated with human diseases such as BRCA1/NBR2 (4), ATM/NPAT (5), DHFR/MSH3 (6) and SERPINII/PDCD10 (7).

Altered patterns of DNA methylation, which can lead to tumor suppressor gene inactivation, have been implicated in the development and progression of multiple types of tumors (8). Recent studies have explored the hypothesis that hypermethylation silencing of bidirectional promoters could play a role in cancer progression. Shu et al (9) demonstrated that hypermethylation of the promoter region of WNT9A/CD558500 and CTDSP2/BC040563 bidirectional genes is a frequent event in colon cancer and acute lymphoid leukemia, and leads to silencing of both transcriptional units from both gene pairs. More recently, Agirre et al (10) showed that loss of PARK2/PACRG expression due to aberrant promoter methylation is associated with the pathogenesis of acute lymphoblastic leukemia.

Our previous study using microarray analysis revealed that expression of the LRRC49 (Leucine rich repeat containing 49; FLJ20156) gene is elevated in ER/PR-positive primary breast tumors compared with ER/PR-negative tumors (11). Furthermore, analysis of the LRRC49 human genome reference sequence (NM_017691.2) using the BLAT algorithm (genome.uscs.edu) showed that LRRC49 is located close to the THAP10 (THAP domain containing 10) gene on chromosome 15q23. The LRRC49 and THAP10 genes are a bidirectional gene pair; the transcripts are separated by less than 1.0 kb and are head-to-head on opposite strands. To date, little is known about the biochemical and biological functions of the proteins encoded by LRRC49 and THAP10 or their role in tumorigenesis.

In the present study, we assessed the levels of LRRC49 and THAP10 transcripts in a series of primary breast tumors. 

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using quantitative real-time PCR (qPCR). MSP analysis was employed to examine the status of DNA methylation of the bidirectional LRRC49/THAP10 promoter region. Additionally, we investigated the effects of 17ß-estradiol on the expression of the LRRC49 and THAP10 transcripts in MCF-7 breast cancer cells. Our results show that the transcripts of the LRRC49/THAP10 bidirectional gene pair are co-regulated by estrogen and that hypermethylation of the bidirectional promoter region simultaneously silences both genes.

Materials and methods

Tissue samples. Seventy-two primary breast tumor samples and 20 adjacent normal tissues were obtained from 72 breast cancer patients at the Hospital do Câncer, A.C. Camargo, São Paulo, Brazil. The age of patients at the time of operation ranged from 23 to 93 years (median 54 years). Tumor samples were dissected to remove residual normal tissue prior to freezing and storage in liquid nitrogen. The largest diameter of the tumors was recorded. The number of lymph node metastases was determined by microscopic examination of an average of 24 lymph nodes per patient. Tumor metastases at lymph nodes was determined in 47 patients. All cases were submitted to a histopathological review of tumor slides in order to confirm diagnosis, and the tumors were classified according to the WHO Histological Typing of Breast Tumors (WHO, 1982). The tumors included in this study were infiltrating ductal carcinomas. The clinical stage of the patients was determined according to the UICC TNM (tumor, nodes, metastases) staging system (UICC, 1978). The institutional Ethics Committee approved the study, and all subjects provided informed consent. Estrogen and progesterone receptor binding assays were performed by the classical dextran-coated-charcoal method (DCC) as previously described by Brentani et al (12).

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 in RPMI supplemented with 10% FCS and 1% penicillin-streptomycin (Gibco). Prior to treatments with 17ß-estradiol, 4-hydroxytamoxifen, or ICI 182.780, the MCF-7 cells were washed twice with PBS to remove residual serum and cultured in media without phenol-red supplemented with 5% FCS served as a control 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 GAPDH gene and GAPDH gene (14). Briefly, 2 μg DNA was extracted using phenol-chloroform and precipitated with ethanol. For total RNA extraction tissue powder or harvested cells were homogenized in a solution containing guanidine isothiocyanate and extracted as described by Chomczynski and Sacchi (13). The quality of the RNA samples was determined by 1% agarose gel electrophoresis and ethidium bromide staining. All RNA samples were treated with DNasel for 30 min. at 37°C to eliminate DNA contamination.

RT-PCR. cDNA was generated from 10 μg of total RNA treated with DNasel using the High Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer's instructions. RT-PCR was performed using 1 μl of cDNA (diluted 1:10), and PCR reactions were carried out with 30 cycles using gene-specific primers. GAPDH cDNA amplification was used as an internal control. PCR products were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining.

Quantitative real-time PCR (qPCR). qPCR was performed using the GeneAmp 5700 sequence detector (PE Applied Biosystems). cDNA was generated using the High Capacity cDNA Archive kit (Applied Biosystems), and each cDNA sample was analyzed in duplicate. PCR reactions were carried out with Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA) in a total volume of 5 μl according to the manufacturer's instructions. The PCR primers used were as follows: LRRC49 (FLJ20156), forward primer 5'-CCC GTTCAGATGGAGAAGC-3' and reverse primer 5'-TGGCTCCTGTACAGCCCCCA-3' and reverse primer 5'-TGGGCCCTTCTTCACAGTGAT-3' GAPDH, forward primer 5'-CCTCCAAATACGACGGGGCGC-3' and reverse primer 5'-GGGGGCCAGAGATGATGACCCTT-3'. The relative gene expression was normalized using GAPDH expression as an internal control. The average value of two pools of 10 normal tissue samples each served as a calibrator sample for the tumors, and MCF-7 cells maintained in medium without phenol-red and supplemented with 5% FCS served as a 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 (ΔΔCT = fluorescence threshold value; ΔCT = CT of the target gene - CT of the GAPDH reference gene; ΔΔCT = ΔCT of the target sample - ΔCT of the calibrator sample).

Detection of the DNA methylation status by MSP and DNA sequencing. Aberrant methylation in the promoter region of LRRC49/THAP10 was determined by MSP (methylation-specific PCR) analysis. Genomic DNA was first treated with sodium bisulfite, which converts unmethylated cytosine to uracil, and subjected to PCR amplification using primers sets specific for unmethylated and methylated templates. The chemical modification of genomic DNA by sodium bisulfite was performed as described by Herman et al (14). Briefly, 2 μg of DNA in 50 μl was denatured by NaOH (final concentration of 0.2 N) at 37°C for 10 min, followed by treatment with 520 μl of 3 M sodium bisulfite and 10 nM hydroquinone at 50°C for
The modified DNA samples were purified using the DNA Clean Up kit (Promega Corp., Madison, WI, USA) following the manufacturer's instructions. NaOH (final concentration of 0.3N) was added to the modified DNA (eluted in 50 μl of water) and incubated at 37°C for 15 min, followed by the addition of ammonium acetate (final concentration of 2.5 M) and ethanol precipitation. The modified DNA was resuspended in 15 μl of water and used immediately or stored at -20°C. Genomic DNA from normal mammary tissue was treated with SssI methylase (New England Biolabs).
and used as positive control for methylation. Hot-started PCR amplification was performed using primers specific for unmethylated and methylated templates (primers were designed using MethPrimer; http://urogene.org/methprimer). Primers used to detect methylated sequences for the LRRC49/THAP10 MSP-PCR were 5'-GTTGTAACGATTGGTTTTATC-3' (forward) and 5'-TTAAATCTACCTTCCAAATAACGCT-3' (reverse); primers to detect unmethylated sequences were 5'-TGTAATGATTGTGTGGTTTTATTGA-3' (forward) and 5'-TTAAATCTACCTTCAAATAACGCT-3' (reverse). PCR products were analyzed by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. Bisulfite-modified genomic DNA was also amplified using two universal primer pairs, one set that amplified a 740-bp DNA sequence near the 5' region of THAP10: 5'-TCCCAAGGTGACACTAAGTGG-3' (forward); 5'-GAGGAGGAGGAAGATATCAAAG-3' (reverse); and a second set that amplified a 690-bp DNA sequence near 5' region of LRRC49: 5'-CTTTGATATCTTCCTCCTCCTC-3' (forward); 5'-TCCTGGACAAGACTTGAGTA-3' (reverse). PCR products were cloned into the pCR® 4-TOPO vector using the TOPO TA Cloning® Kit for Sequencing (Invitrogen). The individual clones were subjected to PCR using M13 primers, the products were sequenced using the ET Dye Terminator Kit (Amersham Biosciences) and analyzed with a MegaBACE automated sequencer (Amersham Biosciences).

Loss of heterozygosity (LOH) analysis. Loss of heterozygosity analysis was performed using primers that amplified a dinucleotide repeat marker mapped on chromosome 15q23 and intragenic to LRRC49/THAP10. Primers used for the LOH analysis were 5'-AAGCAACTCACTACTCTGCAGGAC-3' (forward) and 5'-AGCTAATTCTCTTAAACTGCTCC-3' (reverse). PCR reactions were carried out in 25 μl volumes using 50-100 ng of genomic DNA template, 1 μM of each primer, 1.5 mM MgCl₂, 200 μM of each deoxynucleotide triphosphate, 50 mM KCl, 10 mM Tris-HCl pH 8.0, and 0.5 U of Taq DNA polymerase (Pharmacia, NJ, USA). The reactions were performed in an automated Thermal Cycler - Perkin-Elmer 9600 (Applied Biosystems). The PCR products were diluted 2:1 in a loading buffer containing 0.3% bromophenol blue, 0.3% xylene cyanol, and resolved by electrophoresis on 12% polyacrylamide gels, which were stained with ethidium bromide and visualized under UV light.

Statistical methods. Analyses of statistical significance were performed by the χ² test, Fisher’s exact test and Student’s t-test using the SPSS software 10.0 (SPSS Inc., Chicago, IL); p<0.05 was considered statistically significant.

Results

The genomic position of the LRRC49 gene was determined using the databases at the NCBI (www.ncbi.nlm.nih.gov) and USCU Genome Bioinformatics (genome.uscs.edu); sequences 2 kb upstream and 2 kb downstream of the start sites were extracted and used for transcription factor binding-site analysis. The putative transcription units of LRRC49 and THAP10 are organized in a head-to-head orientation separated by an intergenic region of 622 bp and the DNA sequence of the potential promoter region included LRRC49 exons 1 and 2 and THAP10 exon 1. Notably, a 413 region spanning the first exon of THAP10 gene was found to contain 69% C and G, 20% of which were CpG sites. 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 - research.i2.a-star.edu.sg/promoter). The potential bidirectional promoter region for the LRRC49/THAP10 genes contains one perfect palindromic ERE separated by 7 bases, 3 imperfect palindromic EREs, several widely spaced EREs, 3 FOXA1 sites, 2 SP1 sites and binding sites for several transcription factors (Fig. 1).

The levels of LRRC49 and THAP10 mRNA expression in a series of 72 primary breast tumors were determined by qPCR (Fig. 2). The relative level of expression of the target gene was determined in n-fold differences relative to normal breast tissue samples using GAPDH as a calibrator gene. Down-regulation of the LRRC49 and THAP10 transcripts of more than 2-fold was detected in 61 (44/72) and 46% (33/72)
of primary breast tumors, respectively. We did not find any statistically significant associations between LRRC49 and THAP10 mRNA expression and patient characteristics, such as age, clinical stage or tumor size. However, we found a relationship between the level of LRRC49 mRNA and the status of estrogen receptor. The expression levels of the LRRC49 transcripts were statistically significantly higher in the group of ER-positive breast tumors compared to the group of ER-negative breast tumors (p=0.008) (Fig. 3).

We next examined the methylation status of the LRRC49/THAP10 promoter region by MSP in 40 of the primary breast tumors for which DNA were available. Overall, 57.5% (23/40) of the breast tumor samples showed hypermethylation (Fig. 4). Furthermore, altered methylation status of the LRRC49/THAP10 promoter correlated with the loss of expression of both transcripts. Matched normal and tumor DNAs from these patients were also examined for loss of heterozygosity (LOH) using a dinucleotide repeat marker intragenic to LRRC49/THAP10. Eighty percent of the cases were informative, but none of the cases showed LOH (Fig. 5).

The correlation between ER-positive tumors and elevated LRRC49 transcripts suggested the possibility of the bidirectional promoter of LRRC49/THAP10 to be a target of the ER activity. Therefore, we investigated the effect of 17ß-estradiol and the anti-estrogens, tamoxifen and ICI 182.780, on LRRC49 and THAP10 mRNA expression as determined by qPCR in MCF-7 cells, a hormone-responsive breast cancer cell line. As shown in Fig. 6, both the LRRC49 and THAP10 transcripts were induced by 17ß-estradiol. The LRRC49 transcripts showed a higher induction (3-fold) after 6 h of estrogen exposure compared to the control cells. THAP10 was induced more than 2-fold after 2, 6 or 24 h of estrogen treatment. MCF-7 cells were also treated with 1 μM of Tamoxifen or ICI 182.780 for 24 h. Tamoxifen affected only LRRC49 expression, and the pure anti-estrogen ICI 182.780 affected both LRRC49 and THAP10 mRNA levels.

Discussion
Here we show that the LRRC49 and THAP10 genes, mapped on chromosome 15q23 organized in a head-to-head orientation,
sharing promoter sequences are down-regulated in breast cancer due to DNA hypermethylation. We further provide preliminary data that the LRRC49/THAP10 promoter is estrogen responsive. Bidirectional pairs of genes arranged in a head-to-head orientation are relatively common in the human genome, and 90% of the shared bidirectional promoters show transcriptional activity in both directions; in most cases, both transcripts are co-expressed (2).

The expression patterns of LRRC49 and THAP10 transcripts were highly concordant, as both transcripts were down-regulated in a large proportion of the breast tumors analyzed. MSP analysis revealed that aberrant DNA methylation of LRRC49/THAP10 promoter region that reduces the expression of both genes may be a common event in breast cancer.

To date, the biochemical and biological functions of LRRC49 or THAP10 or their role(s) in tumorigenesis is unknown. The protein encoded by the THAP10 gene is a member of a family of cellular factors containing an evolutionary conserved motif known as the THAP domain (Thanatos-associated protein) (15). THAP domains are short evolutionary conserved motif known as the THAP domain (15). THAP domains are short sequences (approximately 90 amino acids long) located at the N-terminus of the proteins that form a zinc finger C2CH structure related to DNA-binding domains (15,16). The nuclear pro-apoptotic factor THAP1, death associated protein DAP4/p52rIPK and the transcriptional repressor THAP7 members of this family are well characterized (17-19). DAP4/p52rIPK, a member of the LRP superfamily, is down-regulated in astrocytomas and glioblastomas and its expression mediates growth suppression in the U251 glioblastoma derived cell line through the inhibition of AKT/NF-κB signaling in an ERK-dependent fashion (23).

Here we demonstrate for the first time that LRRC49 and THAP10 are down-regulated in breast cancer, suggesting a potential of these genes as candidate marker associated with the tumorigenesis of the breast. However, further studies will be required to elucidate the role(s) played by LRRC49/THAP10 down-regulation in breast cancer.

The mechanism of estrogen action is complex and depends on several factors, such as the amount and subtype of the ER isoforms (ERα and ERβ), the amount and length of estrogen exposure and presence of different co-regulators (24). The genomic effects of the estrogen are mediated either by the interaction between the ER with specific estrogen response element (ERE) DNA binding sites (classical mechanism of action) or by interaction with other transcription factors, such as AP1 and SP1 (non-classical mechanism of action) (25-27). The relationship between LRRC49 mRNA expression and ER status in the clinical specimens and the presence of potential ERE binding sites in the LRRC49/THAP10 bidirectional promoter lead us to speculate whether these genes could be under the control of ER transcriptional transactivation. This hypothesis has been confirmed thus far, by preliminary experiments in MCF-7 breast cancer cells subjected to 17β-estradiol treatment, in which exposure of cells with 17β-estradiol led to up-regulation of both LRRC49 and THAP10 transcripts. Although further functional studies are required to determine the role of the putative EREs in the LRRC49/THAP10 promoter in conferring estrogen responsiveness, this is the first report identifying the LRRC49/THAP10 bidirectional pair of genes as a novel estrogen target.
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