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.

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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

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 metastasis 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% CO_2, 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, and containing 5% charcoal-stripped FCS (CS-FCS) for 48 h. After hormone deprivation, the cells were incubated with 10 nM 17ß-estradiol for 2, 6 and 24 h, 1 μM 4-hydroxy-tamoxifen for 24 h or 1 μM ICI 182.780 for 24 h. Control cells were maintained in media without phenol-red supplemented with 5% FBS.

DNA and RNA extraction. Tissue specimens were ground to powder under liquid nitrogen using a Frozen Tissue Pulverizer (Termovac). The cells, before and after treatment, were washed twice with PBS and harvested. A portion of the tissue powder or harvested cells was resuspended in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA and 0.6% SDS) and 100 μg/ml proteinase K, and incubated at 37˚C overnight for DNA extraction. High molecular weight 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 DNaseI for 30 min. at 37˚C to eliminate DNA contamination.

RT-PCR. cDNA was generated from 10 μg of total RNA treated with DNaseI 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 Platimum SYBR Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA) in a total volume of 50 μl according to the manufacturer's instructions. The PCR primers used were as follows: *LRRC49* (FLJ20156), forward primer 5'-CCC TGTCAGATTGATGGAAGC-3' and reverse primer 5'-TGC TCCTGAGCCATATAGG-3'; *THAP10*, forward primer 5'-GTCTCCTGTACACGCCCCCGA-3' and reverse primer 5'-TGGGCCTTCTTCACAGTGAGT-3'; *GAPDH*, forward primer 5'-CCTCCAAAATCAAGTGGGGCG-3' and reverse primer 5'-GGGGCAGAGATGATGACCCTT-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 $2^{-\Delta\Delta CT}$ (CT = fluorescence threshold value; ΔCT = CT of the target gene - CT of the *GADPH* reference gene; $\Delta\Delta$CT = 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

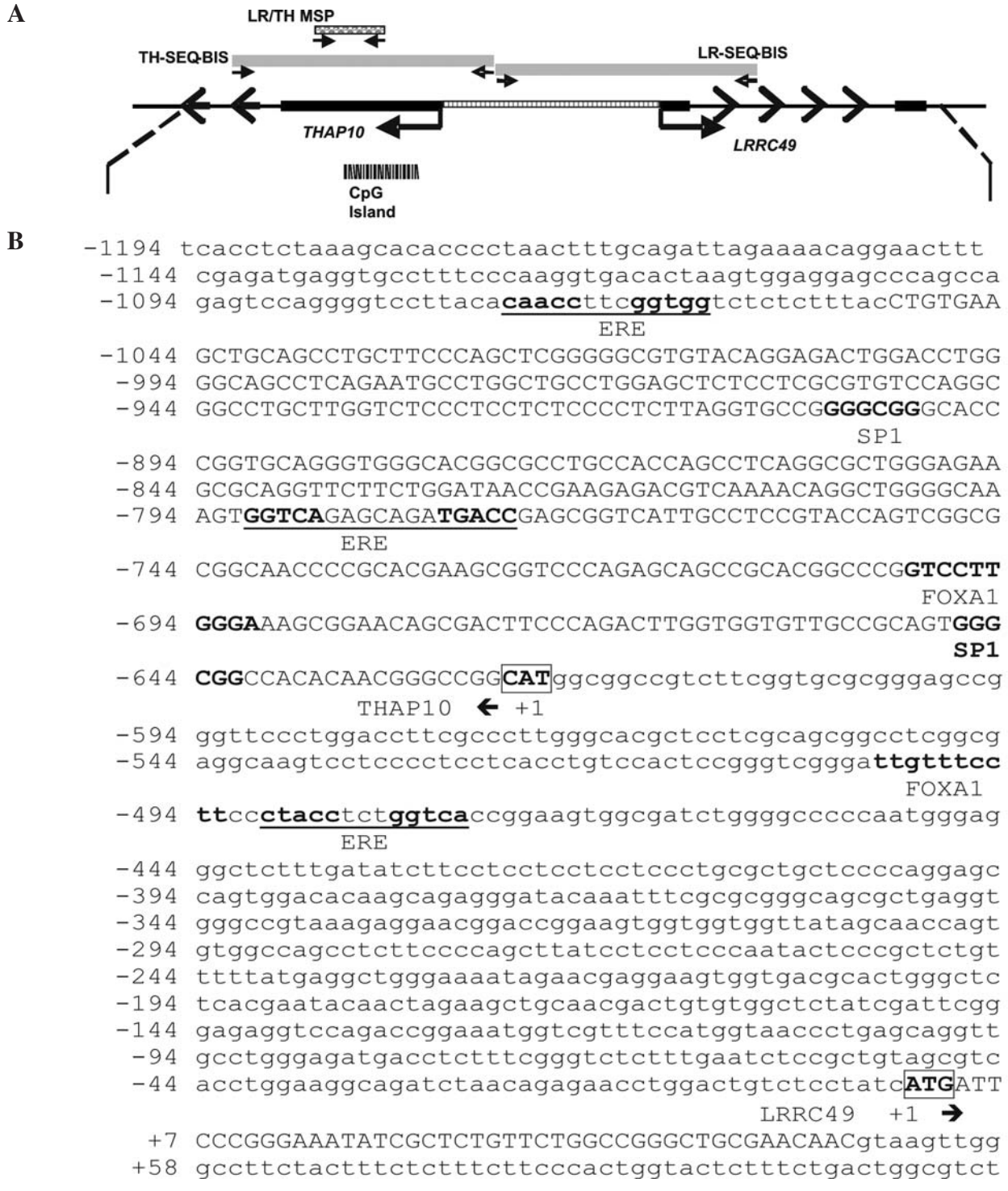

Figure 1. Structure and nucleotide sequence of the bidirectional promoter region of the *LRRC49* and *THAP10* genes. A, The *LRRC49* and *THAP10* transcription units are organized in a head-to-head orientation on chromosome 15q23 (http://genome.uscs.edu). The hatched box corresponds to the 622-bp intergenic sequence; LR/TH-MSP, 109 bp PCR product for MSP analysis; TH-SEQ-BIS, 740 bp PCR product for sequencing; LR-SEQ-BIS, 690 bp PCR product for sequencing. B, Partial nucleotide sequence of the 4-kb region analyzed. The numbers shown to the left indicate the nucleotide position relative to the transcription start site (+1) of the *LRRC49* gene. The black arrows indicate transcription direction. The exons are shown in capitals and the non-coding sequences are represented in small cases. The potential estrogen response elements (EREs), the EREs, and the FOXA1 binding sites are shown in bold.

16 h. 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)

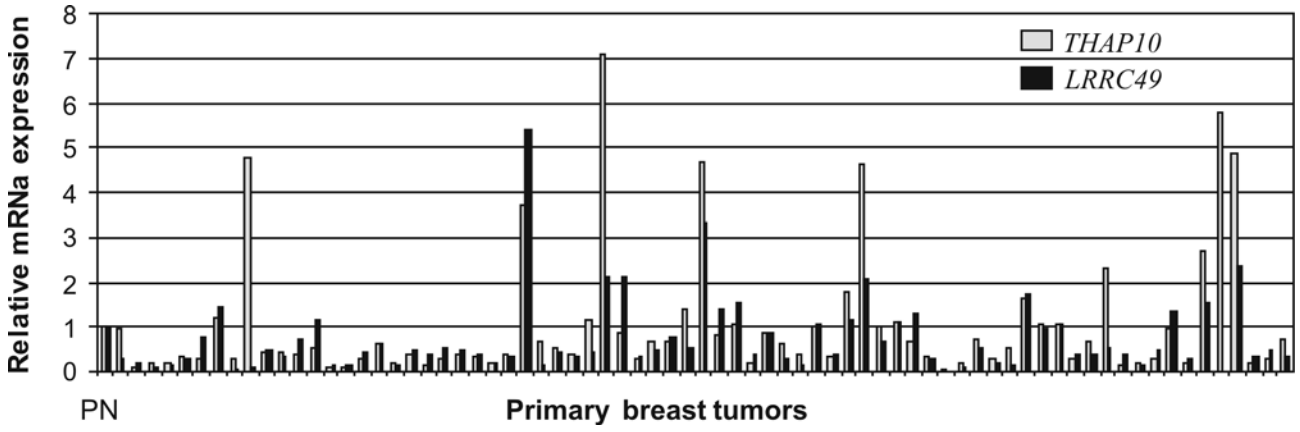

Figure 2. Expression analysis of the *LRRC49* and *THAP10* genes in primary breast tumors. The relative expression was determined by qPCR, normalized to GAPDH as the reference gene. Height of the bars represents the relative gene expression for individual tumors taking normal breast tissue as calibrator samples.

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'-GTTGTAACGA TTGTGTGGTTTTATC-3' (forward) and 5'-TTAAATCTAC CTTCCAAATAACGCT-3' (reverse); primers to detect unmethylated sequences were 5'-TGTAATGATTGTGT GGTTTTATTGA-3' (forward) and 5'-TTAAATCTACCTTC CAAATAACGCT-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'-CTTTGATATCT TCCTCCTCCTC-3' (forward); 5'-TCCTGGACAAGACTT TGAGTA-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'-AAGCAACTCACTACTCTGCAG GAC-3' (forward) and 5'-AGCTAATTCCTTCTAACTGC TCC-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 $MgCl_2$, 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 χ^2 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.edusg/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)

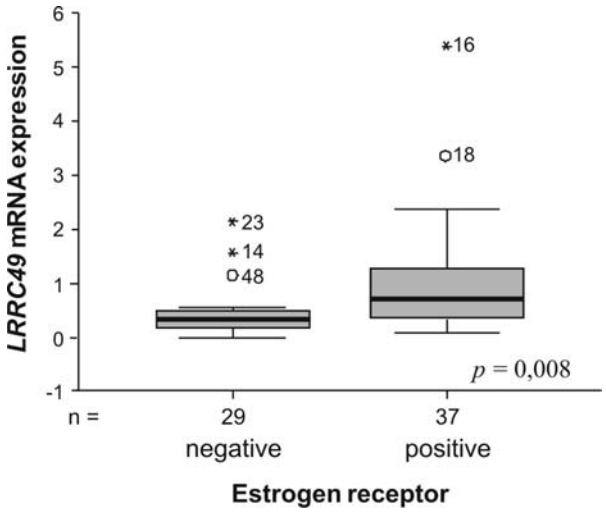

Figure 3. *LRRC49* mRNA expression in primary breast tumors according to estrogen receptor status. The box plot shows the data distributed in relation to median values of *LRRC49* mRNA expression in 72 primary breast tumors stratified by the ER status (p=0.008).

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.

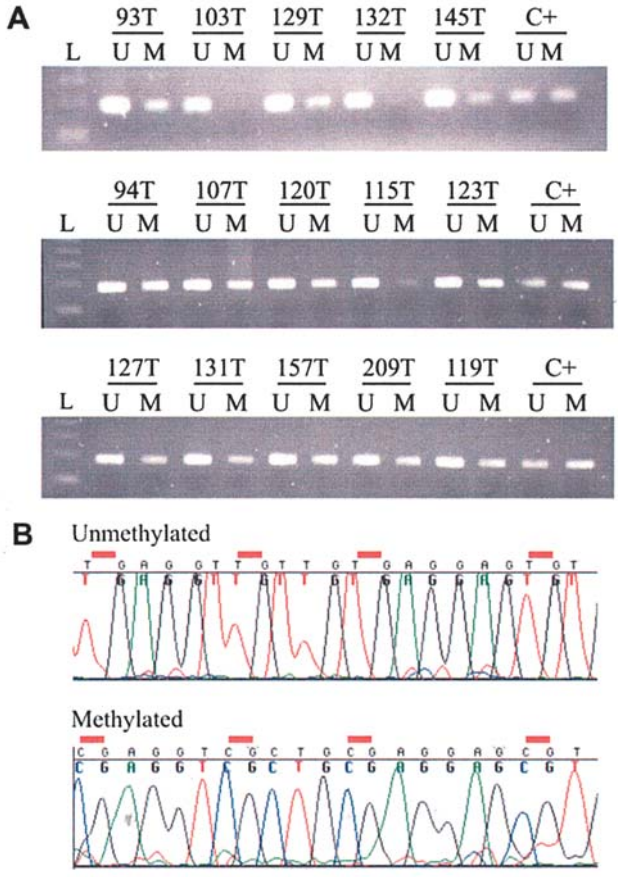

Figure 4. Methylation status of the *LRRC49/THAP10* promoter region in primary breast tumors. A, MSP analysis: genomic DNA from normal breast tissue methylated with SssI methylase was used as positive control for methylated DNA; U, unmethylated; M, methylated. B, Bisulfite DNA sequencing analysis of MSP-negative (Unmethylated) and MSP-positive (methylated) tumor samples amplified for DNA sequence near 5' *THAP10* (TH-SEQ-BIS).

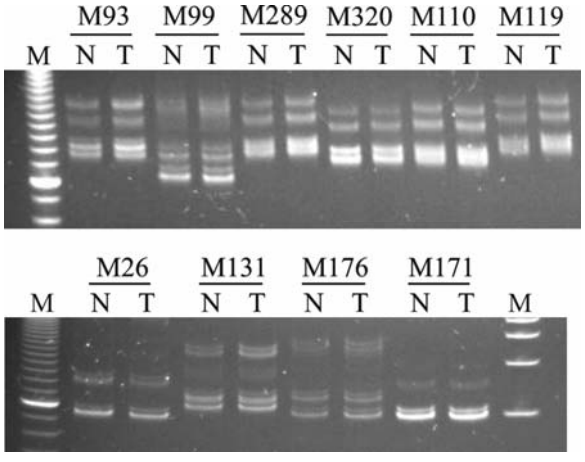

Figure 5. Agarose gels representative of the loss of heterozygosity analysis (LOH) for a dinucleotide repeat on chromosome 15q23 intragenic to *LRRC49* gene in primary breast tumors. N, normal tissue DNA, T, tumor tissue DNA.

Discussion

Here we show that the *LRRC49* and *THAP10* genes, mapped on chromosome 15q23 organized in a head-to-head orientation,

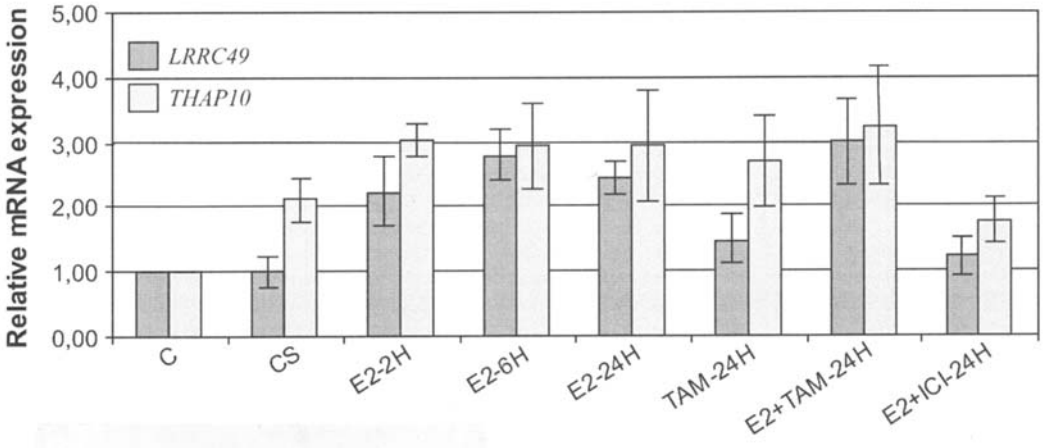

Figure 6. Effect of 17ß-estradiol (E_2) and the anti-estrogens, Tamoxifen (Tam) and ICI 182.780 (ICI), on *LRRC49* and *THAP10* mRNA expression in MCF-7 breast cancer cells. Syncronized MCF-7 cells were in: C, medium supplemented with 5% fetal calf serum (FCS); CS, medium supplemented with 5% charcoal-stripped FCS; E_2 2 h, exposed to 17ß-estradiol (10 nM) for 2 h; E_2 6 h, exposed to 17ß-estradiol (10 nM) for 6 h; E_2 24 h, exposed to 17ß-estradiol (10 nM) for 24 h; Tam 24 h, exposed to Tamoxifen (1 μM) for 24 h; E_2+Tam 24 h, exposed to Tamoxifen (1 μM) plus 17ß-estradiol (100 nM) for 24 h; or E_2+ICI-24 h, exposed to ICI (1 μM) plus E_2 (10 nM) for 24 h. Data are expressed as mean ± SD of three experiments.

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 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). Recently, Macfarlan *et al* (19) demonstrated that THAP7 recruit TAF-1ß (template activation factor 1ß) to specific DNA regions and inhibit histone acetylation. Interestingly, TAF-1ß binds to ERα (estrogen receptor α), inhibit p300-mediated histone and ERα acetylation and represses ERα transactivation activity (20).

The *LRRC49* (leucine rich repeat containing 49) gene encodes a protein containing 7 conserved leucine-rich repeat motifs (LRR). The LRR motif is composed of 20-29 amino acids and has been identified in a large number of proteins with diverse functions and cellular localizations that are members of the LRP (leucine-rich repeat protein) superfamily (21). LRR containing proteins are generally involved in protein-protein interactions and known to bind a variety of unrelated ligands (22). *LRRC4*, 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 ERß), 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 17ß-estradiol treatment, in which exposure of cells with 17ß-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.

Acknowledgments

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