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RESEARCH

p53 Binds to *Estrogen Receptor 1* Promoter in Human Breast Cancer Cells

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Abstract p53 is a tumor suppressor protein that regulates *estrogen receptor 1* (*ESR1*) expression. To investigate the mechanism of *ESR1* gene regulation by p53, chromatin immunoprecipitation was applied to assess the binding of p53, DNMT1, HDAC1 and MeCP2 to both silenced *ESR1* promoter in MDA-MB-468 cells and active *ESR1* promoter in MCF-7 breast cancer cells. The results of chromatin immunoprecipitation experiments showed that p53 protein binds to both unmethylated CpG island of the *ESR1* promoter in the ER-positive MCF-7 and the hypermethylated *ESR1* promoter in the ER-negative MDA-MB-468 cells. However, repression complex including DNMT1, HDAC1 and MeCP2 is only associated with silenced *ESR1* in ER-negative MDA-MB-468 human breast cancer cells. In addition, ectopically expressed wild type p53 failed to reactivate the *ESR1* gene in these cells. These results suggest that specific p53 mutations may contribute to loss of estrogen receptor α expression in breast tumors and also support the hypothesis that mutant p53 is likely to impact DNA methylation.

Keywords Breast cancer · *Estrogen receptor 1* · Methylated promoter · p53 · Epigenetic

Abbreviation

ESR1	*estrogen receptor 1*
DNMT1	DNA methyltransferase 1
HDAC1	histone deacetylase 1
MeCP2	methyl-CpG-binding protein
ER	estrogen receptor
ChIP	Chromatin immunoprecipitation
RT-PCR	reverse transcription-PCR
siRNA	small interfering RNA

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Introduction

As a tumor suppressor protein, p53 functions are based on its ability to up- or downregulate the expression of many genes involved in cell growth, cell cycle progression, DNA repair, cellular senescence, autophagy, metabolism and p53 regulation [1, 2]. These functions are essential in preventing malignant transformation of cells. p53 predominantly functions as a sequence-specific DNA-binding transcription factor to activate transcription following the appropriate signals [3, 4]. However, it can also repress gene expression indirectly by association with histone deacetylases or the basal transcriptional machinery and interference with transcriptional initiation [5, 6]. Inactivating mutations in the *p53* gene allows the cells to evade pro-apoptotic signals, thus promoting tumorigenesis [7]. Mutations in the *p53* gene most often occur in its sequence-specific DNA-binding region [8].

One of the genes which are regulated by p53 is the *estrogen receptor 1* (*ESR1*) gene [9]. *ESR1* transcriptional regulation is poorly defined. It has been shown that p53 upregulates, *ESR1* gene expression by increasing its transcription [9]. Numerous studies have demonstrated that hypermethylation of CpG islands located in the promoter regions of tumor suppressor genes including *ESR1* is an important mechanism for gene inactivation in breast cancer [10–12]. Gene encoding estrogen receptor α (ERα) in humans (designated *ESR1*) is involved in cell signaling

[12]. Therefore, CpG island methylation of *ESR1* gene promoter disrupts its cell growth regulatory effect in breast cancers. *ESR1* hypermethylation occurs early in tumorogenesis that results in disruption of pathways that may predispose the cells to malignant transformation [13]. It is known that both promoter methylation and histone deacetylation is required to reach estrogen receptor silencing. Several studies have demonstrated the role of DNA methyltransferase 1 (DNMT1), histone deacetylase 1 (HDAC1) and certain methyl binding proteins to the methylated CpG dinucleotides such as MeCP2 in the repression of *ESR1* transcription in ER- negative breast cancer cell lines [14–16]. Intriguingly, most of the breast tumors with *p53* mutations are ER- negative [17]. These studies suggest that specific *p53* mutations may play a role in the progression of breast cancer from ER-positive to ER-negative and hormone-independent tumors. Loss of ERα expression in ER-negative tumors means that breast cancer cells cannot be stopped by hormone therapy which results in aggressive tumor and poor prognosis [18]. How breast cancer cells with *p53* mutation acquire such epigenetic changing of *ESR1* promoter is not clear.

In this study, we examined the association of p53, DNMT1, HDAC1 and MeCP2 proteins with *ESR1* promoter in two breast cancer cell lines, MDA-MB-468 with and MCF-7 without methylated *ESR1* promoter. Then, we studied whether ectopically expressed wild type p53 is capable of reactivating the *ESR1* gene in ER-negative MDA-MB-468 cells.

Materials and Methods

Cell Culture

The ER- negative MDA-MB-468 cells and ER-positive MCF-7 cells were obtained from national cell bank of Iran (Pasture Institute, Iran). The human breast cancer cell lines, MDA-MB-468 and MCF-7, were grown in RPMI 1640 (Biosera, UK) supplemented with L-glutamine to 2 mM and 10% fetal calf serum (Cinagen, Iran) and subcultured 2–3 times weekly.

Chromatin Immunoprecipitation (Chip) Assay

ChIP assays were performed on MDA-MB-468 and MCF-7 cells using the ChIP kit (ab500; Abcam, Canada). 1×10^6 cells were grown on 10 Cm plates and formaldehyde was added directly to culture medium to a final concentration of 1% and incubated for 10 min at 37°C to cross-link histones to DNA. The reaction was stopped by adding glycine (0.125 M). After washing and lysing cells as instructed by kit manufacture, mixtures were sonicated on ice three times for 10 s each to shear genomic DNA to an optimal size of 0.2–1 kb. The cell debris was pelleted by centrifugation (14000×g for 5 min at 4°C). The supernatants were diluted in ChIP dilution buffer and supplemented with protease inhibitors according to the manufacturer's instructions. ChIPs were carried out overnight at 4°C with rotation using 5 μg of anti-p53 (ab17990; Abcam, Canada), anti-DNMT1 (ab13537; Abcam, Canada), anti-HDAC1 (ab51846; Abcam, Canada) and anti-MeCP2 (ab2828; Abcam, Canada) polyclonal antibodies. Anti-histone H3 (ab1791; Abcam, Canada) antibody was used as a positive control. The antibody/DNA-histone complex was collected with 42 μl of protein A-agarose beads for 2 h. After washing the beads, DNA was decross-linked and purified according to the manufacturer's recommendation. Immunoprecipitated DNA was analyzed by PCR for the presence of *ESR1* gene exon 1 sequence. PCR reactions were carried out in a total volume of 25 μl containing 2 μl of immunoprecipitated or total input, 0.5 μM of each primer (Cinnagen, Iran), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotid triphosphate mixture (Fermentas, Iran), 1× PCR buffer and 1.25 U of *Taq* DNA polymerase (Fermentas, Iran). PCR primers were blasted for *ESR1* gene (GenBank accession number NC_000006) amplified a region encompassing the *NotI* site within the *ESR1* CpG island at ±311 bp relative to transcription start codon: 5'- TGA ACC GTC CGC AGC TCA AGA TC-3' and 5'-GTC TGA CCG TAG ACC TGC GCG TTG-3' [16]. Amplification of this gene was performed under the following condition: 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 50 s, and a final extension of 10 min at 72°C. The PCR product of 150 bp was electrophoresed on 1.5% agarose gel and analyzed by ethidium bromide staining. All ChIP assays were performed at least twice with similar results.

Western Blot Analyses

The MCF7 and MDA-MB-468 cells which had been solubilized in a lysis buffer containing 10 mM Tris–HCl pH 7.4, 0.825 M NaCl and 1% NP-40 were sonicated and cleared by centrifugation. Fifty-microgram protein samples including the cell lysate proteins were electrophoresed on 12% SDS–PAGE gel and electrophoretically transferred to a nitrocellulose membrane (Amersham Biosciences, US) following the standard protocol. Immunoblotting was performed with anti-p53 antibody (ab17990; Abcam, Canada), anti-DNMT1 (ab13537; Abcam, Canada) and anti-actin (ab1801; Abcam, Canada). A horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Sigma, USA) and chemiluminescence substrates (ECL; Amersham Bioscience AB) were used to detect the immuno-labeled bands.

Plasmid and siRNA Transfections and Reverse Transcription-PCR (RT-PCR) Analysis

A human pcDNA-p53 was kindly provided by Dr. A. Turnell (University of Birmingham, UK). The plasmid contains the full-length coding region of the wild type p53 cDNA (1.3 kb, exons 2–11). MDA-MB-468 cells were seeded at a density of 1×10^6, trasfected with 1 μg of pcDNA3 vector or pcDNA3-p53 vector using lipofectamine (Invitrogen, UK); 48 h after transfection cells were harvested. The presence of exogenous p53 cDNA and its integrity were confirmed by direct sequencing of both strands of the p53 cDNA in total DNA extract by the use of an ABI Prism 3100 genetic analyzer.

siRNA targeting *DNMT1* [19] was used. The siRNA sequences were 5′-CGGUGCUCAUGCUUACAACT-3′ (sence) and 5′- GUUGUAAGCAUGAGCACCGTT-3′ (antisence). A non-silencing siRNA (UUCUCCGAACGU GUCACGUTT), with no known homology to any human gene, was used as a negative control. All siRNAs were obtained from Eurofins MWG Operon, Germany. The sence and antisence oligonucleotides were annealed to make double-stranded siRNA. MDA-MB-468 cells (5×10^5) were transfected with 10 nM of siRNAs by electroporation (975 μF and 220 V in 4 mm cuvettes). The cells were grown and harvested 72 h after transfection. Our previous studies revealed that siRNA transfection by electroporation was more efficient than lipofectamine (data not shown). To ascertain the specificity of siRNA, the target protein was monitored by Western blotting.

Total RNA was extracted using the RNXTM plus solution (Fermetas, Iran), according to the manufacturer's instructions. Three microgram of each RNA were retrotrascribed using RevertAid TM First-Strand cDNA Synthesis Kit using the oligo-d (T) primers (Fermetas, Iran). Semi quantitative PCRs were performed after normalizing all the cDNAs for beta actin control. RT-PCR primers that amplify between exons 7 and 8 of ESR1 (5′-GCTGCTGGCTACATCATC-3′, 5′-AGGACTCGGTGGATATGG-3′) and beta actin (5′-AGTCTTCCTTCCTGGGCAT-3′, 5′-CAGGAGGAGCAAT GATCT-3′) were designed. The annealing temperatures used for the ER and actin primers were 60°C and 64°C, respectively. The PCR product of 150 bp was subjected to electrophoresis on 1.5% agarose gel.

Results

p53 is Associated with *ESR1* Promoter cpG Island along with DNMT1, HDAC1 and MeCP2 in MDA-MB-468 Breast Cancer Cells

The ER- negative MDA-MB-468 cells with a hypermethylated *ESR1* promoter and the ER-positive MCF-7 cells with an unmethylated *ESR1* promoter [20] were used in ChIP assays. Anti-p53, anti-DNMT1, anti-HDAC1 and anti-MeCP2 antibodies were used for immunoprecipitation of formaldehyde cross-linked protein-chromatin complexes from MCF-7 and MDA-MB-468 cells. In parallel, anti-histone H3 antibody which is enriched at chromatin was used as a positive control. Immunoprecipitated DNA was analyzed for the presence of the *ESR1* gene by PCR using primers spanning a CpG island in its first exon region, where it has been revealed that its methylation is most closely associated with *ESR1* gene expression [14, 21]. Figure 1 shows that p53 is associated with both the silenced *ESR1* promoter in MDA-MB-468 cells and active *ESR1* promoter in MCF-7 breast cancer cells. Histone H3 which was used as positive control was observed at the *ESR1* promoter in both MCF-7 and MDA-MB-468 cells. To gain a deeper understanding of the binding proteins along with p53 protein to CpG island of *ESR1* gene, the recruitment of binding proteins that recognize methylated DNA was investigated. As shown in Fig. 1, ChIP experiments with anti-DNMT1, anti-HDAC1 and anti-MeCP2 antibodies showed that those proteins are associated with the silenced ER promoter along with p53 in MDA-MB-468 cells whereas the active ER promoter in MCF-7 cells is not associated with these proteins.

Evaluation of the p53 in MCF-7 and MDA-MB- 468 Cells and Expression of Wild Type p53 in MDA-MB-468 Cells

Lysates of MCF-7 and MDA-MB-468 cells were used for immunoblotting for detection of p53, using anti-p53 antibody (Fig. 2a). A band of approximately 53 kD was observed in MDA-MB-468 cells (Fig. 2a). However, p53 protein was not recognized in MCF-7 cells (Fig. 2a). MDA-MB-468 breast cancer cells contain a hemizygous mutant *p53* gene that overexpresses a mutant p53 protein [22]. But, MCF-7 human breast cancer cells have normal p53 function. Since MCF-7 cells express wild type (wt) p53, the low amount of p53 proteins in these cells is difficult to measure.

Given the importance of the significant role of tumor suppressor function of p53, in this study, we examined that ectopically expressed wt p53 is capable of activating the *ESR1* gene in MDA-MB-468 cells which are hemizygous for a mutant p53 [22]. The full-length coding region of human wt p53 inserted into pcDNA3 and empty vector as a negative control were used for transfection. The cells were examined for ER mRNA expression by RT-PCR; β-actin was used as internal standard. As shown in Fig. 2b, wt p53 did not induce *ESR1* gene expression in this cell line as compared with the negative control. This finding indicates that the exogenous wt p53 did not impair the suppression complex on *ESR1* gene promoter in this cell line.

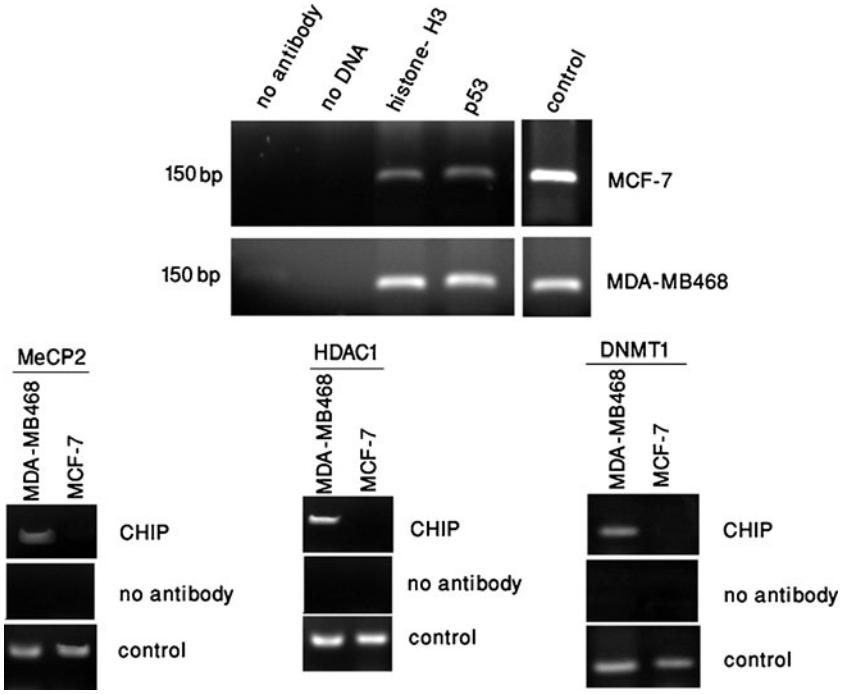

Fig. 1 ChIP analysis for the presence of p53, DNMT1, HDAC1, MeCP2 and histone-H3 on the human *ESR1* CpG island. Cross-linked chromatin-protein complexes from ER-positive MCF-7 and ER-negative MDA-MB-468 human breast cancer cells were immunoprecipitated with antibodies indicated. The immunoprecipitates were analyzed by PCR for *ESR1* promoter CpG island. Negative controls had no antibody; aliquots of chromatin taken before immunopercipitation were used as input controls. Data are representative of three independent experiments

siRNA against DNMT1 Reactivates the *ESR1* Gene in MDA-MB-468 Cells

To study whether demethylation induces the *ESR1* gene in ER negative breast cancer cells, MDA-MB-468 cells were treated with the siRNA against DNMT1 and expression status of *ESR1* gene was analyzed by RT-PCR; β-actin was used as internal standard (Fig. 3). Upon introduction of DNMT1 siRNA, DNMT1 proteins get reduced, resulting in functional analysis of this protein in MDA-MB-468 cells. Cell extracts were monitored for DNMT1 expression by using Western blot analysis (Fig. 3a). As shown in Fig. 3b, RNAi-mediated endogenous DNMT1 knockdown restored the expression of *ESR1* gene. This result confirmed pervious findings [14, 21] that DNA methylation of the *ESR1* gene plays a functional role in silencing of ER expression in ER-negative breast cancer cells.

We next determined how the levels of ER reactivation in MDA-MB-468 cells compared with endogenous ER expression in the ER positive MCF7 cells. DNMT1 siRNA induced ER transcripts in MDA-MB-468 cells to 22% of the expression level found in MCF7 cells. It shows that the effect of RNAi-mediated DNMT1 knockdown on the expression of *ESR1* gene is more than that of using DNMT1 inhibitors such as 5′-aza-2′-deoxycytidine which only induced ER mRNA in MDA-MB-231 cells to 4% of the MCF7 expression levels [23].

Discussion

In an attempt to better understanding of the epigenetic events on *ESR1* promoter, the binding of p53, DNMT1, HDAC1 and MeCP2 proteins to this promoter in the ER-negative MDA-MB-468 cells with a hypermethylated *ESR1* promoter and the ER-positive MCF-7 cells with an unmethylated *ESR1* promoter [20] were examined. Our ChIP data showed that p53 protein binds to *ESR1* promoter CpG island in both the ER-positive MCF-7 and the ER-negative MDA-MB-468 cells. However, DNMT1, HDAC1 and MeCP2 proteins are associated with the silenced ER promoter along with p53 in MDA-MB-468 cells. Western blot analyses revealed that MDA-MB-468 breast cancer cells overexpress the p53 protein, but MCF-7 breast cancer cells express wt p53. To our knowledge, this is the first report on the association of p53 with silent *ESR1* promoter in MDA-MB-468 cell line.

ESR1 promoter is regulated by epigenetic modification and its epigenetic lesion by aberrant methylation is of key importance in the development of breast cancer [10–12]. It has been reported that hypermethylation of CpG island of tumor suppressor genes is not a random event [13] and it may occur by loss of specific protection factor of the CpG islands [24]. p53 is a regulatory protein and elucidates its biological functions as both transcription factor [3, 4] and

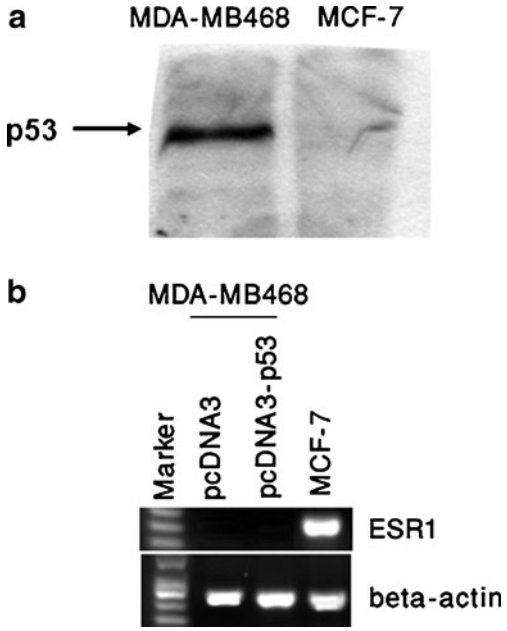

Fig. 2 Endogenous p53 expression and effect of ectopically expressed wt p53 in MDA-MB-468 cells. **a** Evaluation of the p53 protein in MCF-7 and MDA-MB- 468 cells by western blot analysis. 50 μg of whole cell lysates from MCF7 and MDA-MB-468 cells were separated by 12% SDS-PAGE and subjected to Western blot analysis with ab17990. **b** Effect of wt p53 expression on ER expression. Transfections were done with 1 μg of empty and p53 expression vectors and RNA was prepared at 48 h. RNA extracted from MCF-7 cells was used as an ER positive control. RT-PCR analysis of *ESR1* gene and β-Actin internal control was performed

repressor [25–28]. The ability of p53 to regulate gene expression is accompanied by binding to DNA either directly [29, 30] or by its interaction with other transcription factors [31]. One of the genes regulated by p53 is the *ESR1* gene [9]. It has been shown that p53 upregulates the expression of this gene in human breast cancer cell line MCF-7. However, it has been demonstrated that p53 ability to upregulate *ESR1* gene expression is not dependent on its binding directly to DNA but on interaction with other proteins on *ESR1* promoter [9]. These results suggest that specific p53 mutations in breast tumors may contribute to loss of ERα expression and progression of breast cancer to an aggressive tumor [9]. In addition, it has been reported that p53 binds to *ESR1* promoter and inhibits [32] as well as maintains the transcriptional activity of *ESR1* gene in MCF7 cells [33]. A recent experiment has shown that p53 binds to *ESR1* promoter along with CARM1, CBP, c-jun and Sp1 in MCF7 cells and upregulates its expression [33]. Our ChIP experiments confirmed that p53 protein binds to the *ESR1* promoter CpG island in the ER-positive MCF-7 cells. However, we found that this protein also binds to *ESR1* promoter CpG island along with DNMT1, HDAC1 and MeCP2 proteins in the ER- negative MDA-MB-468 cells. This region includes a critical *NotI* site in the *ESR1* gene, the region where it is methylated in multiple ER-negative breast cancer cell lines. *ESR1* gene is unmethylated at the *NotI* site in the CpG island in all ER-positive cell lines [21]. Therefore, the *NotI* site methylation in the *ESR1* gene is most closely associated with ERα gene expression [14]. MCF-7 cells have normal p53 function and are the ER-positive cells with an unmethylated *ESR1* promoter. However, MDA-MB-468 breast cancer cells are hemizygous for p53 gene, containing a single point mutation at codon 273 in the remaining allele and over-expressing a transcribtionaly active mutant p53 protein [22]. These cells are the ER- negative cells with a hypermethylated *ESR1* promoter. We showed that the silenced ER promoter in ER-negative MDA-MB-468 cells has a repressive chromatin structure associated with DNMT1, HDAC1 and MeCP2 proteins. Our results are consistent with in vivo studies demonstrating that the *ESR1* promoter is silenced by recruitment of DNMT1, HDAC1 and MeCP2 proteins in MDA-MB-231 breast cancer cells [14]. These findings also showed that mutation of p53 in

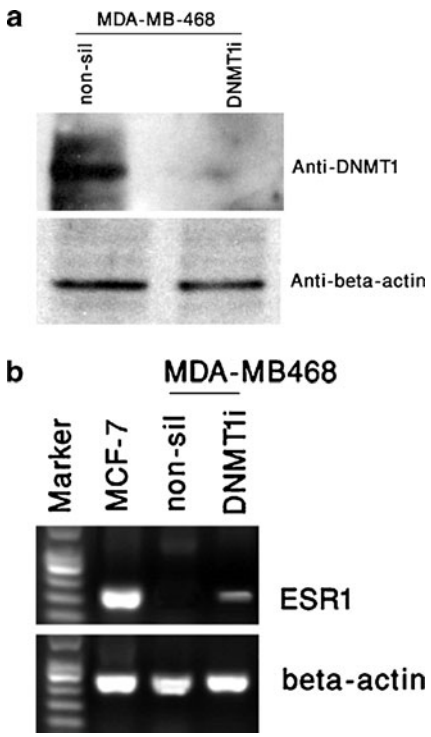

Fig. 3 Effect of DNMT1 siRNA on DNMT1and ER expression in MDA-MB-468 cells. **a** Western blot analysis of DNMT1 protein in siRNAs treated cells. 50 μg of whole cell lysates from non-silencing or DNMT1 siRNA trasfected cells were separated by 8% SDS-PAGE and subjected to Western blot analysis with anti-DNMT1. β-Actin was probed as a protein loading control. **b** ER reexpression is induced by DNMT1 siRNA. RT- PCR analysis showed ER mRNA reexpression after treatment with DNMT1 siRNA. RNA extracted from MCF-7 cells was used as an ER positive control. RT-PCR analysis of *ESR1* gene and β-Actin internal control was performed. Non-sil., non-silencing RNA oligonucleotides; DNMT1i refers to a situation in which DNMT1 expression was ablated by siRNA

MDA-MB-468 cells does not prevent its binding to *ESR1* promoter. However, p53 mutation may change its normal function on this promoter in favor of making different complexes with proteins that result in epigenetic changes on *ESR1* promoter. p53 binding to DNMT1 through amino acids 323–393 [34] would not be affected by p53 mutation in this cell line. Mutation of p53 is common in sporadic breast cancer [35]. Intriguingly, most of the breast tumors with p53 mutation are ER-negative [17] and positive for *ESR1* methylation [36]. But, how p53 mutation can play a role in the progression of breast cancer from ER-positive to ER-negative is unknown. It had been previously shown that both p53 mutation and aberrant DNA methylation silenced gene expression through independent, but interrelated, mechanisms of transcriptional control [37]. In addition, other studies demonstrated that overexpression of mutated p53 is associated with the silencing of CCN5 gene during progression of cancer [38]. Our data support these findings and hypothesize that *ESR1* is one of the genes which are affected by p53 mutation and mutant p53 is likely to impact DNA methylation. However, in an attempt to correct p53 function in MDA-MB-468 cells, ectopically expressed wt p53 did not reactivate *ESR1* gene. It has been also reported that the mutant p53 proteins inhibit wt p53 functions [39]. Nevertheless, further studies are demanded to elucidate the molecular mechanism of p53 mutation in *ESR1* promoter.

It has been reported that treatment of ER-negative cells with inhibitors of DNMT1 and HDAC1 enzymes results in dissociation of these proteins from the *ESR1* promoter and reactivation of this gene [14]. We also demonstrated that RNAi-mediated DNMT1 knockdown restored the expression of *ESR1* gene. This result confirms that DNA methylation of the *ESR1* gene plays a functional role in silencing of ER expression in ER-negative breast cancer cells.

The role of wt p53 in controlling apoptosis in response to genotoxic stress is essential in breast cancer chemotherapy [40]. It has been demonstrated that down–regulation of mutant p53 in MDA-MB-468 cells inhibits cell proliferation [41]. However, introduction of wt p53 into these cells did not result in cellular apoptosis [22]. While mutant p53 proteins which are highly expressed in one third of breast tumors impair the therapeutic response [39], study of other target proteins might provide novel diagnosis and therapeutic insights.

In conclusion, we revealed that p53 protein binds to the promoter of *ESR1* in the ER-positive MCF-7 and along with DNMT1, HDAC1 and MeCP2 proteins in the ER-negative MDA-MB-468 cells. Binding of p53 to *ESR1* promoter of the ER- negative MDA-MB-468 cells can be explained by the fact that mutant p53 protein gains a new function in regulation of *ESR1* promoter. This should be more elucidated in future works.

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Conflict of interest None of authors has any conflict of interest.

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