

Mutant p53 binds to estrogen receptor negative promoter via DNMT1 and HDAC1 in MDA-MB-468 breast cancer cells

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Received: 2 August 2012 / Accepted: 9 December 2012 / Published online: 15 December 2012
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Abstract DNA methylation and histone deacetylation are two epigenetic mechanisms involved in the lack of estrogen receptor (ER) expression. Our previous studies demonstrated that mutant p53 along with repression complex proteins including DNMT1, HDAC1 and MeCP2 is associated with ER-negative promoter in MDA-MB-468 cells. To elucidate the molecular mechanism of *estrogen receptor 1 (ESR1)* gene silencing in these cells, we down-regulated DNMT1 and HDAC1 expression using siRNAs and studied the ability of DNMT1, HDAC1, MeCP2 and p53 in binding to *ESR1* promoter CpG island. Our results showed that DNMT1 or HDAC1 down-regulation disassembled the repression complex proteins and mutant p53 from ER-negative promoter. The partial demethylation of *ESR1* promoter and ER re-expression in down-regulated cells supports these findings. In vivo binding studies demonstrated that mutation of p53 protein in this cell line did not affect its binding capacity to DNMT1, HDAC1 and MeCP2 proteins. Our observations suggest that not only histone deacetylase activity of HDAC1 contributes to inactivation of methylated *ESR1* gene but also HDAC1 presence on *ESR1* promoter is important for assembly of DNMT1 in repression complex. In addition, our data revealed that mutant p53 protein binds to the promoter of *ESR1* through direct interaction with HDAC1 and indirect interaction with DNMT1, MeCP2 proteins in the ER-negative MDA-MB-468 cells.

Keywords p53 · MDA-MB-468 cells · ESR1 · HDAC1 · DNMT1 · MeCP2

Introduction

Estrogen and estrogen receptor (ER)- α play important roles in development and growth of invasive breast cancer [1]. The tumor expression of ER- α is a prognostic factor for hormonal therapies of breast cancer [2]. However, some breast tumors lose ER- α expression during tumor progression or lack ER- α at the time of diagnosis [3]. Many studies have shown that the epigenetic alterations in mammalian cells including hypermethylation of CpG islands within the *estrogen receptor 1 (ESR1)* promoter are involved in its gene silencing [2].

Over the last decade, many studies have reported that a repression complex that includes DNA methyl transferase family (DNMT1 and DNMT3b), histone deacetylase (HDAC1) and methyl CpG-binding proteins regulates *ESR1* promoter transcription in ER-negative breast cancer cells [4, 5]. DNMT1 is the most abundant and catalytically active DNA methyltransferases at silenced *ESR1* promoter [6]. It is a primary maintenance methylase which establishes methylation patterns from the parent strand to the daughter during cell division [7]. But, DNMT3b is a de novo DNA methyltransferase [8]. Down-regulation of DNMT1 by antisense oligonucleotides [6] or treatment with the DNA methyltransferase inhibitor, 5-aza-2 deoxycytidine (5-aza-dC), results in re-expression of ER- α protein in ER-negative human breast cancer cells [9]. HDAC1 is involved in histone modification and binds to the N-terminus of DNMT1, thereby forming a transcriptional repression complex which cooperatively initiates and sustains epigenetic gene silencing [10]. In addition, HDAC inhibitors such as trichostatin A (TSA), scriptaid and

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LBH589 can induce ER- α protein expression [11–13]. In vivo experimental evidence has documented the association of methyl-CpG-binding domain (MBD) proteins such as MeCP2 (methyl-CpG-binding protein) to the *ESR1* promoter in ER-negative breast cancer cell lines [5]. MeCP2 has been identified to recruit histone deacetylases (HDAC1 and HDAC2) for gene silencing [14, 15]. It also binds to N-terminal domain of DNMT1 [16, 17].

Several studies have shown that p53 is also involved in *ESR1* transcription regulation [18]. p53 is one of the most widely studied tumor suppressor proteins due to its crucial role in apoptosis, senescence or a reversible and protective cell cycle arrest [19]. It binds to *ESR1* promoter [20–22] and up-regulates ER- α expression [18, 21]. Since most breast tumors with p53 mutation are ER-negative, it has been suggested that p53 mutations in these tumors may contribute to loss of ER- α expression [18]. Our recent studies have shown that mutant p53 protein interacts with *ESR1* promoter CpG island along with DNMT1, HDAC1 and MeCP2 proteins in ER-negative MDA-MB-468 cells [22]. As mutant p53 proteins which are highly expressed in one-third of breast tumors impair chemotherapy responses [19], study of other target proteins might provide novel diagnosis and therapeutic insights.

To elucidate the molecular mechanism of *ESR1* gene silencing in ER-negative MDA-MB-468 cells, we down-regulated DNMT1 and HDAC1 expression using siRNAs and studied the ability of DNMT1, HDAC1, MeCP2 and p53 in binding to *ESR1* promoter CpG island. In addition, we compared the binding capacity of wild type (WT) p53 in MCF7 cells with mutated one in MDA-MB-468 cells through interaction with DNMT1, HDAC1 and MeCP2 proteins.

Materials and methods

Cell culture and siRNA transfections

The human breast cancer cell lines, MDA-MB-468 cells (ER-negative) and MCF-7 cells (ER-positive) were prepared from national cell bank of Iran (Pasture Institute, Iran). The cell lines were grown in RPMI 1640 (Biosera, UK) supplemented with L-glutamine to 2 mM and 10 % fetal calf serum (Cinagen, Iran). For siRNA transfections, MDA-MB-468 cells at 50–70 % confluence were diluted to the density of 5×10^5 cells and transfected with 10 nM of siRNA by electroporation (975 μ F and 220 V in 4 mm cuvettes). The cells were grown and harvested 72 h after transfection. The siRNAs targeting DNMT1 [23] and HDAC1 [24] transcripts were used. The DNMT1 siRNAs sequences were 5'-CGG UGC UCA UGC UUA CAA CTT-3' (sense), 5'-GUU GUA AGC AUG AGC ACC

GTT-3' (antisense) and the HDAC1 siRNA sense sequence was 5'-AAG CCG GUC AUG UCC AAA GUA TT-3', antisense sequence was 5'-UAC UUU GGA CAU GAC CGG CUU-3'. A non-silencing siRNA (UUCUCCGAAC GUGUCACGUTT), with no known homology to any human gene, was used as the negative control. All siRNAs were synthesized by Eurofins MWG Operon, Germany. The sense and antisense oligonucleotides were annealed to make double-stranded siRNA.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation experiments were performed as previously described, using 1×10^6 cells equivalent of chromatin for each immunoprecipitation [22]. Five micrograms of anti-p53 (ab17990; Abcam, USA), anti-DNMT1 (ab13537; Abcam, USA), anti-HDAC1 (ab51846; Abcam, USA), and anti-MeCP2 (ab2828; Abcam, USA) polyclonal antibodies were applied. In addition, anti-histone H3 (ab1791; Abcam, USA) antibody was used as a positive control. The immunoprecipitated DNA from test and total DNA of input samples were purified and subjected to PCR analysis for the presence of *ESR1* gene exon 1 sequence. 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 [4]. PCR mixture contained 2 μ l of the purified input or test DNA, 0.5 μ M of each primer (Cinnagen, Iran), 1.5 mM MgCl₂, 0.2 mM deoxynucleotide triphosphate mixture (Fermentas, Iran), 1 \times PCR buffer and 1.25 U of *Taq* DNA polymerase (Fermentas, Iran) in a total volume of 25 μ l. The primers 5'-TGA ACC GTC CGC AGC TCA AGA TC-3' and 5'-GTC TGA CCG TAG ACC TGC GCG TTG-3' were used for *ESR1* gene analysis. PCR condition was as previously described [22], 30 cycles of 94 $^{\circ}$ C for 30 s, 56 $^{\circ}$ C for 30 s and 72 $^{\circ}$ C for 50 s, and a final extension of 10 min at 72 $^{\circ}$ C. The PCR products (150 bp) were electrophoresed on 1.5 % agarose gel and analyzed by ethidium bromide staining. All ChIP experiments were performed at least three times.

GST pull-down analysis

The cDNA of WT p53 was sub-cloned from a human pcDNA-p53 (kindly provided by Dr. A. Turnell, University of Birmingham, UK) to a pGEX-4T-1 vector (Amersham Biosciences, UK) for bacterial expression. Glutathione S-transferase (GST) fusion protein was expressed and purified as described previously [25]. The purified proteins were dialyzed against a buffer containing 50 mM Tris-HCl, (pH 7.4), 0.15 M NaCl, 1 mM dithiothreitol, and 10 % (vol/vol) glycerol. The MCF7 and MDA-MB-468

cell lysates were prepared by solubilization in a lysis buffer containing 10 mM Tris–HCl pH 7.4, 0.825 M NaCl and 1 % NP-40 and then clarified by sonication and centrifugation. For GST pull-downs, 50 µg of GST-p53 or GST proteins was mixed with 5 mg of either the MCF7 or MDA-MB-468 cell lysates for 2 h at 4 °C. The pull-downed complexes with GST and GST-fusion proteins were eluted from the glutathione-agarose by 25 mM reduced glutathione in 50 mM Tris–HCl solution pH 8.0. The samples were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then subjected to Western blot analysis with appropriate antibody.

Immunoprecipitation of proteins

The cells were lysed in buffer containing 10 mM Tris–HCl pH 7.4, 0.825 M NaCl and 1 % NP-40. Cell lysates were sonicated and cleared by centrifugation. Immunocomplexes were precipitated from 5 mg of protein lysate, using 5 µg of appropriate primary antibody. After 2 h mixing at 4 °C on a rotator, 30 µl of packed protein G agarose beads (Sigma, UK) was added into the protein-antibody complex and mixed for a further 1 h. Immunocomplexes bound to the beads were then spun and washed, prior to re-suspending in the SDS sample buffer for SDS–PAGE analysis.

Western blotting

Immunoprecipitates or GST pull-down samples with 50 µg proteins of appropriate lysates were electrophoresed on 8 or 12 % SDS–PAGE gels for detection of DNMT1 or ER- α , β -actin, MeCP2 and HDAC1 proteins. Following electrophoresis, cell lysates were transferred to a nitrocellulose membrane (Amersham Biosciences, USA) using the standard protocol. Immunoblotting was performed with anti-DNMT1 (ab13537; Abcam, USA), anti-HDAC1 (ab51846; Abcam, USA), anti-MeCP2 (ab2828; Abcam, USA), anti-ER- α (ab32063; Abcam, USA), anti-p53 (ab17990; Abcam, USA), and anti- β -actin (ab1801; Abcam, USA). 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.

DNA extraction and methylation-specific PCR (MSP)

Genomic DNA was extracted by the standard phenol/chloroform procedure. DNA from MCF-7 and MDA-MB-468 cells as well as siRNAs transfected MDA-MB-468 cells were treated with sodium bisulfate according to a

previously published protocol [26]. Methylation status of bisulfite-modified DNA using primers encompassing nucleotides +44 to +236 bp within the *ESR1* CpG island was characterized by PCR as described previously (25). The following primers were used: *ESR1* unmethylated (U) 5'-TTT TGG GAT TGT ATT TGT TTT TGT TG-3' and 5'-AAA CAA AAT ACA AAC CAT ATC CCC A-3'; *ESR1* methylated (M) 5'-TTT TGG GAT TGT ATT TGT TTT CGT C-3' and 5'-AAC AAA ATA CAA ACC GTA TCC CCG-3' [27]. The amplified products were loaded onto a 2 % agarose gel and visualized under UV illumination. The PCR for those samples demonstrating methylation was repeated to confirm the reproducibility of the results.

Results

Down-regulation of DNMT1 or HDAC1 expression induced ER- α expression

The ER-negative cell line MDA-MB-468 was used as a cell model to investigate whether knock-down of either DNMT1 or HDAC1 expression by RNAi could reactivate ER- α expression. As shown before, 10 mM siRNA against DNMT1 could down-regulate DNMT1 expression and re-express ER- α mRNA [22]. Our Western blot analysis also confirmed that DNMT1 siRNA treatment of MDA-MB-468 cells led to increased ER- α protein expression (Fig. 1a, b). Again, 10 mM siRNA against HDAC1 not only down-regulated HDAC1 expression but also reactivated ER- α protein expression (Fig. 1a, b).

Down-regulation of DNMT1 or HDAC1 expression alters the association of DNMT1, HDAC1 and MeCP2 with *ESR1* promoter.

We next determined the effect of down-regulation of DNMT1 or HDAC1 expression on the binding of DNMT1, HDAC1 and MeCP2 proteins to *ESR1* promoter. We already showed that DNMT1, HDAC1 and MeCP2 proteins are associated with the silenced *ESR1* promoter in MDA-MB-468 breast cancer cell line, whereas they were not observed on active *ESR1* promoter in MCF7 cells [22]. As shown in Fig. 2a, ChIP analysis with anti-HDAC1 and anti-MeCP2 antibodies revealed that down-regulation of DNMT1 expression abolished the association of HDAC1 and MeCP2 with the *ESR1* promoter. Thus, recruitment of HDAC1 and MeCP2 to methylated *ESR1* promoter was not observed upon demethylation of the *ESR1* promoter.

In addition, ChIP assays with anti-DNMT1 and anti-MeCP2 antibodies showed that down-regulation of HDAC1 expression also prevents the association of these proteins with the *ESR1* promoter (Fig. 2b). Therefore, deacetylation of chromatin dissociates DNMT1 and MeCP2 proteins from silent *ESR1* promoter.

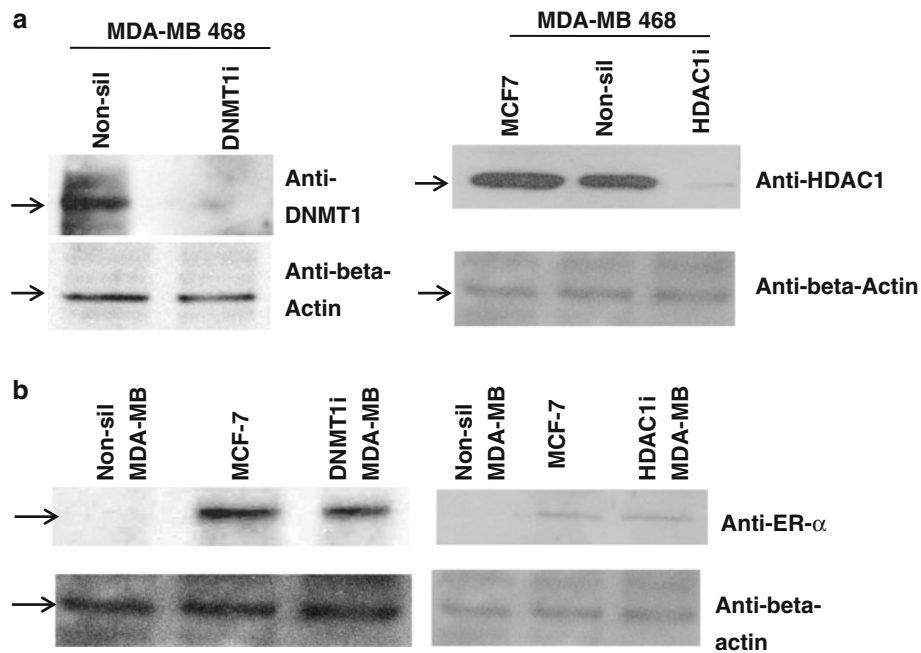


Fig. 1 Requirement for DNMT1 and HDAC1 in *ESR1* repression. **a** Down-regulation of DNMT1 or HDAC1 expression. MDA-MB-468 Cell line was transfected with 10 mM of siRNA against DNMT1 or HDAC1 and their protein expression was analyzed by Western blotting. β -Actin was probed as a protein loading control. **b** Effect of DNMT1 or HDAC1 knock-down upon ER protein expression. Whole

cell lysates from siRNA transfected cells were analyzed for ER- α protein expression by Western blotting. MCF7 was used as a positive control for ER expression. β -Actin expression was used as a protein loading control. *Non-sil.* non-silencing RNA oligonucleotides, DNMT1i and HDAC1i refer to situations where DNMT1 or HDAC1 expression was ablated by siRNAs

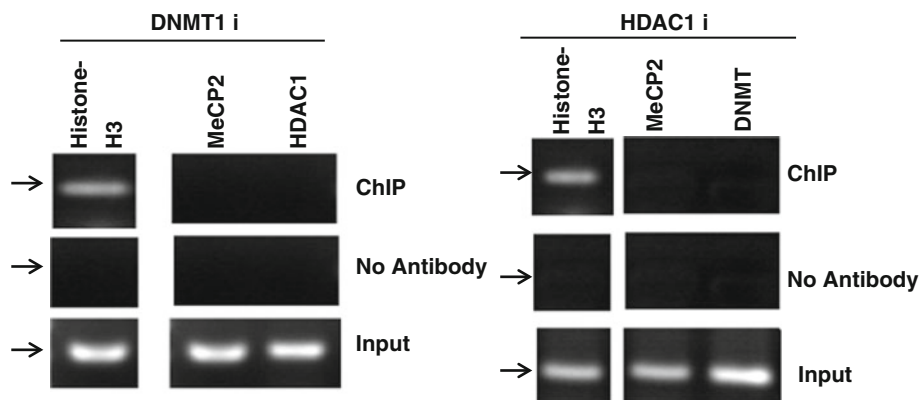


Fig. 2 Dissociation of DNMT1, HDAC1 and MeCP2 proteins from *ESR1* promoter after down-regulation of DNMT1 or HDAC1. ChIP analysis was used for immunoprecipitation of Cross-linked chromatin-protein complexes with indicated antibodies in ER-negative MDA-MB-468 human breast cancer cells. The immunoprecipitates were

analyzed by PCR for *ESR1* promoter CpG island. Negative controls had no antibody; aliquots of chromatin taken before immunoprecipitation were used as input; DNMT1i and HDAC1i refer to situations where DNMT1 or HDAC1 expression was ablated by siRNAs. Data are representative of three independent experiments

Methylation status of *ESR1* gene is changed by siRNAs against DNMT1 or HDAC1

To show whether reactivation of *ESR1* by knocking-down of DNMT1 or HDAC1 expression is associated with demethylation of the promoter, methylation-specific PCR (MSP) analysis was performed on transfected MDA-MB-468 cells with siRNAs against DNMT1 or HDAC1 transcript. As

shown in Fig. 3, the methylated *ESR1* gene promoter was partially demethylated by down-regulation of DNMT1 or HDAC1 expression. The MSP method is a non-quantitative method and it can be seen from Fig. 3 that the non-silenced MDA-MB-468 cells have only methylated band, but down regulation of either DNMT1 or HDAC1 expression made the unmethylated band in addition to the methylated one. MSP analysis was applied using primers encompassing

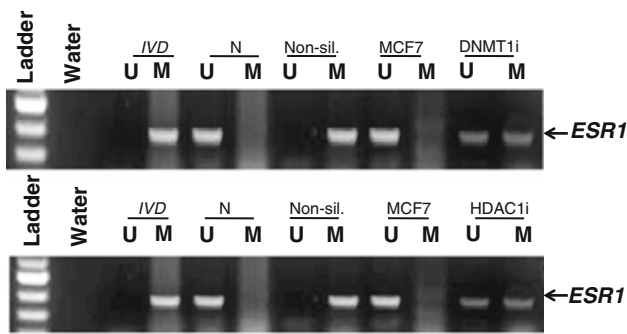


Fig. 3 *ESR1* gene methylation status after siRNA treatment. MSP analysis of *ESR1* CpG island was performed after siRNAs treatments in MDA-MB-468 cells. Normal tissue (N) and ER-positive MCF7 cells line were used as an unmethylated control, in vitro-methylated DNA (IVD) and non-silencing RNA oligonucleotides (*Non-sil.*) treated MDA-MB-468 cells were used as methylated control. U Unmethylated, M methylated, H_2O without DNA, DNMT1i and HDAC1i refer to situations where DNMT1 or HDAC1 expression was ablated by siRNAs

nucleotides +44 to +236 bp related to the exon 1 of *ESR1* gene. The CpG island in this region has been shown to be methylated in MDA-MB-468 cells [4]. These results suggest that down-regulation of DNMT1 or HDAC1 expression activates silenced *ESR1* gene through DNA demethylation and histone deacetylation in the *ESR1* CpG island.

Mutant p53 associates with *ESR1* promoter in the presence of DNMT1 and HDAC1

We showed that DNMT1 and HDAC1 proteins are important in repression of *ESR1* promoter in ER-negative MDA-MB-468 cell line. To elucidate the role of mutant p53 in repression complex on *ESR1* promoter [22], we performed ChIP assays with antibody against p53 in knocking down either DNMT1 or HDAC1 cell lines. Immunoprecipitated complex was examined for *ESR1* gene region in treated and untreated cells by PCR. As shown in Fig. 4, mutant p53 was only found to be associated with the *ESR1* promoter on the untreated MDA-MB-468 cells. Thus, mutant p53 did not interact with *ESR1* promoter in the absence of DNMT1 or HDAC1 protein in ER-negative MDA-MB-468 cell line.

Mutant and WT p53 associate to DNMT1, HDAC1 and MeCP2 in MDA-MB-468 and MCF7 cells

We further tested the ability of mutant p53 for interaction with partner proteins in repression complex of *ESR1* promoters. Immunoprecipitation of mutant p53 from MDA-MB-468 cells was compared to WT p53 in MCF7 cells. Western blotting for bound p53 revealed that p53 mutant similar to WT p53 could bind to DNMT1, HDAC1 and MeCP2 in vivo (Fig. 5a). In vivo binding studies presented

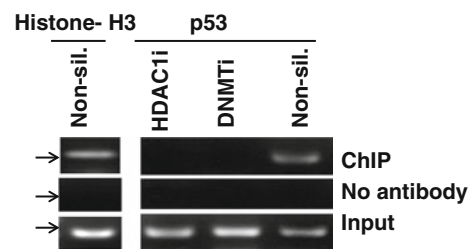


Fig. 4 Effect of DNMT1 or HDAC1 down-regulation upon mutant p53 binding to *ESR1* promoter. MDA-MB-468 cells were treated with siRNAs against DNMT1 and HDAC1. Cross-linked chromatin-protein complex was immunoprecipitated by ChIP with antibodies indicated. *ESR1* promoter CpG island was detected by PCR. Negative controls had no antibody; aliquots of chromatin taken before immunoprecipitation were used as the input; DNMT1i and HDAC1i refer to situations where DNMT1 or HDAC1 expression was ablated by siRNAs. Data are representative of three independent experiments

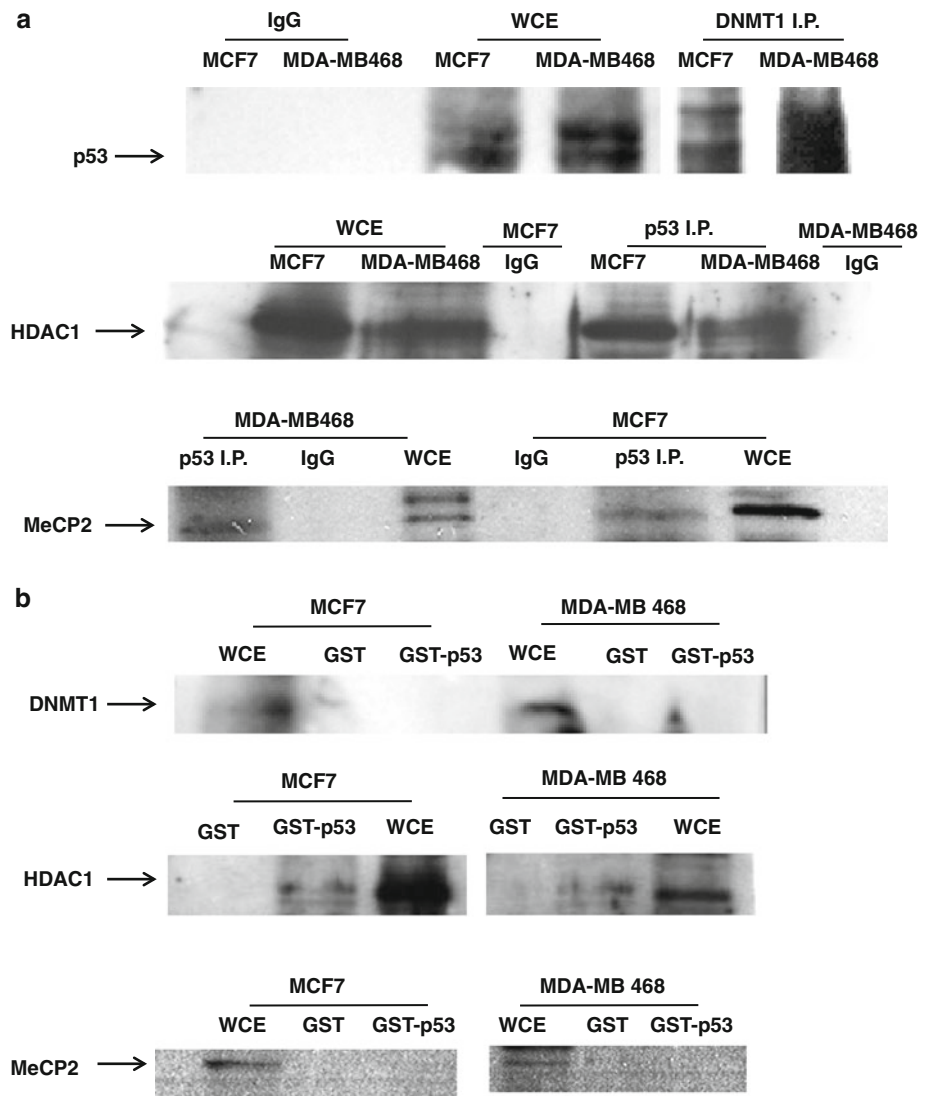
within this report suggested that mutation at codon 273 of p53 protein did not affect its binding capacity to DNMT1, HDAC1 and MeCP2 proteins.

To investigate the in vitro binding capacity of the WT p53 for DNMT1, HDAC1 and MeCP2 proteins, GST-p53 fusion protein was expressed and purified as described in Materials and Methods section. The results from GST-pull down experiments indicated that the GST-p53 fusion protein retained its binding capacity for HDAC1 but could not bind to DNMT1 and MeCP2 in lysates of MDA-MB-468 and MCF7 cell lines (Fig. 5b).

Discussion

In breast cancer, it has been shown that loss of ER- α expression is as a result of the epigenetic changes of CpG island in *ESR1* promoter and its first exon regions [4]. Many studies have documented that aberrant methylation of *ESR1* CpG island is occurred during breast cancer progression [28, 29]. In vivo experiments have shown that the *ESR1* promoter is silenced by recruitment of repression complex proteins including DNMT1, HDAC1 and MeCP2 proteins in MDA-MB-231 and MDA-MB-468 breast cancer cells [5]. Histone deacetylation and DNA demethylation of ER-negative promoter have been used as potential targets in order to re-expression of ER- α protein which is necessary for response to endocrine therapies in treatment of breast cancers [30]. The molecular mechanism which regulates gene silencing of *ESR1* gene and reactivates this gene in ER-negative breast cancers is not understood in detail. In this study, MDA-MB-468 cell line which was previously shown to have densely methylated *ESR1* CpG island [4, 27] and has a hemizygous mutant p53 [31] was used as a model for study of the epigenetic regulation of

Fig. 5 Interaction between p53 and DNMT1, HDAC1 and MeCP2 in vitro and in vivo. **a** Immunoprecipitation of p53 in vivo. p53 complexes were immunoprecipitated from MCF7 and MDA-MB-468 cellular lysates with anti-DNMT1 and anti-p53. The coexistence of p53 with DNMT1, HDAC1 and MeCP2 in the immunocomplex was revealed by Western analysis with anti-p53, anti-HDAC1 and anti-MeCP2. **b** Physical interaction between GST-p53 and DNMT1, HDAC1 and MeCP2. GST-p53 was used to pull-down protein partners in MCF7 and MDA-MB-468 cell extracts. Western blot analysis with anti-DNMT1, anti-HDAC1 and anti-MeCP2 was performed. *WCE* whole-cell extract, *I.P.* immunoprecipitate



ESR1 promoter. To elucidate if DNMT1 and HDAC1 binding to ER-negative promoter are associated together or act separately, we down-regulated DNMT1 and HDAC1 transcripts in MDA-MB-468 cell line. CHIP results indicated that down-regulation of DNMT1 in MDA-MB-468 cells dissociated the repression complex proteins including HDAC1 and MeCP2 at CpG island on *ESR1* promoter. Indeed, depletion of DNMT1 via siRNA not only contributed to DNA partial demethylation but also decreased recruitment of HDAC1 and MeCP2 to methylated *ESR1* promoter. This result was consistent with previous report showing that MDA-MB-231 cells treatment with 5-aza-dC reduced the level of association of DNMT1 and MeCP2 with the ER-negative promoter and decreased the DNMT1 protein expression in MDA-MB-231 cell line [5]. We also obtained the same results in the case of using RNAi-mediated HDAC1 knockdown through depletion of DNMT1 and MeCP2 binding to *ESR1* promoter. Therefore,

deacetylation of chromatin dissociates these proteins from the silent *ESR1* promoter. In contrast, TSA treatment of MDA-MB-231 cells was demonstrated to reduce only MeCP2 binding to the *ESR1* promoter in MDA-MB-231 cells [5].

In addition, our data showed that down-regulation of each of these two proteins resulted in re-expression of ER- α protein by loss of DNA hypermethylation. Partially methylated pattern in RNAi-mediated DNMT1 or HDAC1 cells may be due to contribution of DNMT3a and 3b to the methylation status of the entire human genome [32]. 5-aza-dC or TSA treatment of ER-negative breast cancer cells could lead to partial demethylation and without alteration in the methylation status of the *ESR1* CpG island, respectively [5, 33]. Our results confirm previous findings that DNMT1 and HDAC1 proteins play a functional role in silencing of ER- α expression in ER-negative breast cancer cells [5]. Our observations suggest that there is a

connection between DNMT1 and HDAC1 at CpG island of ER-negative promoter. In addition, our studies showed that not only histone deacetylase activity of HDAC1 contributes to inactivation of methylated *ESR1* gene but also HDAC1 presence on *ESR1* promoter is important for assembly of DNMT1 in repression complex. HDAC1 is a critical component of *ESR1* gene silencing in order to inactivate chromatin structure [5]. It binds to the DNMT1 [14, 16] and MeCP2 at CpG island via a corepressor, mSin3A [34] to sustain its epigenetic silencing. Therefore, we suggest that binding of HDAC1 to DNMT1 is as important as its histone deacetylase activity on ER-negative promoter in MDA-MB-468 breast cancer cells.

Our previous report demonstrated that mutant p53 protein in MDA-MB-468 cells is associated with repressor complex including DNMT1, HDAC1 and MeCP2 proteins on silenced *ESR1* promoter [22]. Many studies have shown that WT p53 is involved in ER- α transcriptional regulation in MCF7 cell line [18, 20, 21]. The WT p53 up-regulates ER- α expression and is associated with activator complex on *ESR1* promoter in ER-positive cell lines [18, 21]. The WT p53 mediates its effects on target genes regulation as both activator and repressor [35, 36]. The role of mutant p53 on silenced *ESR1* promoter in MDA-MB-468 cells is not yet established. We showed that mutant p53 was not able to bind *ESR1* promoter in the absence of DNMT1 and HDAC1 in MDA-MB-468 cells. It implies that mutant p53 in MDA-MB-468 cell line binds to this promoter through interaction with these proteins. WT p53 binds to DNMT1 through amino acids 323–393 [37] and the HDAC1 [38]. To elucidate how mutant p53 protein contributes to the repression complex on ER-negative promoter, we examined the ability of mutant p53 for interaction with partner proteins in repression complex of *ESR1* promoters in ER-negative cell line in comparison to the WT p53 in MCF7 cells. In vivo binding studies suggested that mutation of p53 protein in MDA-MB-468 cells did not affect its binding capacity to studied proteins. In addition, mutant p53 may stabilize the DNMT1-MeCP2-HDAC1-p53 complex on silenced *ESR1* promoter, by the ability of direct interaction with HDAC1 and indirect interaction with DNMT1 and MeCP2 proteins. In vitro studies confirmed that GST-p53 has a strong binding capacity for HDAC1 in extracts of MDA-MB-468 and MCF7 cells. It has been reported that interaction of p53 and HDAC1 deacetylates p53 and, therefore, down regulates its transcriptional activity [38]. Since previous report showed that DNMT1 binds physically to GST-p53 [37], the low level of this protein in our study may be the reason for our in vitro result. While there has been no report on p53 interaction with MeCP2 so far, our data suggested that either the post-translational modification of p53 or an associated protein affect the p53 association with MeCP2 in vivo.

In response to cellular stress and DNA damage, WT p53 is stabilized and accumulated in cells [19]. Thus, it is able to repress some p53-responsive genes upon association with their promoters along with other repressor complex proteins including DNMT1, HDAC1 [37, 39]. It has been reported that WT p53 knockdown induced DNMT1 and HDAC1 proteins as well as increased promoter methylation of some p53-activator genes [40]. On the other hand, mutant p53 may have a dominant negative or a gain of function effect in tumorigenesis [19]. It has been demonstrated that mutant p53 in MDA-MB-468 cells has an active function in mediating the survival of breast cancer cells [41]. Another study also showed that over-expression of mutant p53 is associated with silencing of *CCN5* gene during progression of cancer [42]. Therefore, p53 either by post-translational modification in normal pathways or by mutation in cancer cells is able to bind to repression complex proteins and repress gene expression. These results indicate that specific p53 mutations may stabilize the repression complex on ER-negative promoter and contribute to loss of estrogen receptor α expression in breast tumors and that mutant p53 is likely to impact DNA methylation.

It has been shown that *ESR1* promoter is silenced by recruitment of MBD1, MBD2, DNMT3b as well as the MeCP2, DNMT1 and HDAC1 in MDA-MB-231 cells [5]. WT p53 is able to bind to Sp1 (Sephacryl and phosphocellulose purification 1) and impairs its transactivator function in some promoters [43]. However, Sp1 was reported to be recruited to *ESR1* promoter to up-regulate the ER- α expression in a regulator complex with CARM1, CBP, c-jun and WT p53 in MCF7 cells [21]. The effects of these proteins in binding of mutant p53 to ER-negative promoter in MDA-MB-468 cells should be more elucidated in future studies.

In conclusion, our results confirm previous findings indicating that DNMT1 and HDAC1 play a functional role in silencing of ER- α expression in ER-negative breast cancer cells. We also showed that the binding of HDAC1 to ER-negative promoter is as important as its histone deacetylase activity for *ESR1* gene repression. In addition, our data revealed that mutant p53 protein binds to the promoter of *ESR1* through association with DNMT1, HDAC1 and MeCP2 proteins in the ER-negative MDA-MB-468 cells. Binding of mutant p53 to *ESR1* promoter of the MDA-MB-468 cells may stabilize the repression complex proteins on ER-negative promoter.

Acknowledgments This work was supported by Grants No: 89-5176 and 90-5620 from the Shiraz University of Medical Sciences, Iran for Ms Rita Arabsolghar's thesis and Ms Tayebe Azimi's thesis, respectively. The authors would like to thank the School of Advanced Medical Sciences and Technologies for their support in performing this project.

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

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