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Down-regulation of Tripartite-motif containing 22 expression in breast cancer is associated with a lack of p53-mediated induction

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ABSTRACT

Tripartite-motif containing 22 (*TRIM22*) is a direct p53 target gene and inhibits the clonogenic growth of leukemic cells. Its expression in Wilms tumors is negatively associated with disease relapse. This study addresses if TRIM22 expression is de-regulated in breast carcinoma. Western blotting analysis of a panel of 10 breast cancer cell lines and 3 non-malignant mammary epithelial cell lines with a well-characterized TRIM22 monoclonal antibody showed that TRIM22 protein is greatly under-expressed in breast cancer cells as compared to non-malignant cell lines. Similarly, TRIM22 protein is significantly down-regulated in breast tumors as compared to matched normal breast tissues. Study of cell lines with methylation inhibitor and bisulfite sequencing indicates that TRIM22 promoter hypermethylation may not be the cause for TRIM22 under-expression in breast cancer. Instead, we found that TRIM22 protein level correlates strongly ($R = 0.79$) with p53 protein level in normal breast tissue, but this correlation is markedly impaired ($R = 0.48$) in breast cancer tissue, suggesting that there is some defects in p53 regulation of TRIM22 gene in breast cancer. This notion is supported by cell line studies, which showed that TRIM22 was no longer inducible by p53-activating genotoxic drugs in breast cancer cell lines and in a p53 null cell line H1299 transfected with wild type p53. In conclusion, this study shows that TRIM22 is greatly under-expressed in breast cancer. p53 dysfunction may be one of the mechanisms for TRIM22 down-regulation.

1. Introduction

Tripartite motif-containing protein 22 (TRIM22) is a member of the TRIM protein family, characterized by the conserved domain structure at their N-terminal ends consisting of a RING domain, followed by one or two B-box domains and a Coiled-Coil domain (CC) [1,2]. TRIM22 contains E3 ubiquitin ligase activity that is localized to the RING domain [3]. Like most of the TRIM proteins, TRIM22 has a SPRY-like (RFP-like/B30.2) domain at their C-terminal ends, which exhibit the most variability [1].

TRIM22 was first identified as an interferon (IFN) inducible factor referred to as stimulated trans-acting factor of 50 kDa (Staf50) [4]. Further study demonstrated that TRIM22 is involved in the anti-HIV response induced by type I IFN through disruption of HIV Gag protein trafficking and blocking the budding of HIV virions [5]. While most studies on TRIM22 focus on its antiviral property, several lines of evidence suggest a tumor suppressor role for TRIM22. TRIM22 gene is a direct p53 target in U-937, K562 and MCF7 cells and contains p53 response element in intron 1 [6]. Over-expression of TRIM22 in U-937 cells resulted in reduced clonogenic growth of the cells on soft agar [6]. The tumor-suppressing property of TRIM22 was also suggested in a clinical study, in which TRIM22 gene expression was 10 times lower in relapse patients than that in patients with non-relapse Wilms tumors [7]. Further study validated that down-regulation of TRIM22 expression level in Wilms tumor was associated with poor survival [8].

TRIM22 is also a target gene of the progesterone receptor (PR). TRIM22 expression is drastically up-regulated by progesterone in breast cancer cell lines T47D cells and MDA-MB-231 derived ABC28 cells expressing PR [9]; this up-regulation is associated with progesterone-induced growth inhibition in these cells [10,11]. These results suggest that TRIM22 is involved in breast cancer biology [9].

Interferon and p53 mediate major innate defense mechanisms against tumor. Being a downstream target of both interferon and p53, TRIM22 may have important tumor suppressor roles. We report here that TRIM22 expression is significantly down-regulated in breast cancer cell lines and tissues as compared to the normal. Furthermore, TRIM22 expression is highly correlated

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with p53 levels in normal breast tissue but this correlation is significantly less in breast tumors. The decreased correlation between p53 and TRIM22 in breast tumor suggests defective p53 signaling to TRIM22 induction.

2. Materials and methods

2.1. Reagents

IFN-β and epidermal growth factor (EGF) were purchased from ProSpec-TanyTechnoGene Ltd. (Rehovot, Israel). 5-aza-2′deoxycitidine, mitomycin C, taxol, camptothecin, nocodazole, human insulin, hydrocortisone, cholera toxin were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell cultures

MCF7, MDA-MB-231, T47D, ABC28 and HeLa cells were maintained in DMEM (PAA Laboratories Ltd., Somerset, UK) supplemented with 7.5% fetal bovine serum (FBS) (Sigma–Aldrich). CRL-1500 cells were maintained in RPMI 1640 medium (PAA Laboratories) with 10% FBS. Cama-1, SK-BR-3, MDA-MB-361, MDA-MB-436 cells were maintained in DMEM:F12 (50:50) medium with 10% FBS. MCF10A, MCF-12A and H16N2 cells were maintained in DMEM:F12 (50:50) medium with 5% FBS, 2 μg/ml human insulin, 0.5 μg/ml hydrocortisone, 0.1 μg/ml cholera toxin and 0.01 μg/ml EGF. H1299 cells were maintained in DMEM with 10% FBS, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids (Gibco, Chagrin Falls, OH, USA).

2.3. Plasmid and siRNA transfections

Polyethylenimine (PEI) (Polysciences, Warrington, PA, USA) dissolved in Milli-Q water at 2 mg/ml and pH adjusted to 7.0 was used for the plasmid transfection at a ratio of PEI (1.5 μl):plasmid (1 μg). Flag-TRIM22 plasmid was generated by inserting full length TRIM22 into pXJ-flag vector. TRIM22 knock down was carried out using Lipofectamine2000 from Life Technologies (Carlsbad, CA, USA) and TRIM22 siRNA sequence is 5′GCACCUGCACAUUUAAGAAtt (sense) and 5′UUCUUAAAUGUGCAGGUGCgt (antisense) (Ambion, Austin, TX, USA). Ambion negative control siRNA 1 (Cat No. 4390844) was used for control knock down (sequence unrevealed).

2.4. Cell/tissue lysate collection and Western blotting analysis

Cells were lysed with cold lysis buffer as described in [9]. 63 breast tumors and 63 matching control breast tissue samples were obtained from the Tissue Repository at the National Cancer Centre Singapore (CIRB number 2010/361/B). Tissue mashed in liquid nitrogen were homogenized in the presence of 500 μl of cold lysis buffer and spun down at 13,800 rpm for 12 min at 4 °C. Protein concentration was analyzed using BCA™ Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL, USA). Twenty micrograms of the protein were resolved by SDS–PAGE and transferred to nitrocellulose membranes. Antibodies used are as following: Mouse anti-GAPDH antibody (AmbionInc), mouse anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-p53 antibody (Calbiochem, Billerica, MA, USA), anti-mouse HRP conjugated secondary antibody (GE Healthcare Biosciences, Pittsburgh, PA, USA).

2.5. Generation of TRIM22 monoclonal antibody

Full length His-tagged TRIM22 protein was purified and injected into Balb/C mice as described in [9]. Tail blood was collected from four mice after three boosters and the mouse whole serum that showed the most specific recognition of TRIM22 protein in Western blotting analysis was chosen for monoclonal antibody generation. Splenocytes from the selected mouse were isolated and fused with myeloma to form hybridoma cells. These hybridoma cells were further cultured and their conditioned media were screened by ELISA with TRIM22 specific peptides to select for the clone that produces specific TRIM22 monoclonal antibody.

This study was approved by Nanyang Technological University Institutional Animal Care and Use Committee (IACUC) and mice were handled following the guidelines on the Care and Use of Animals for Scientific Purposes (2004) set by National Advisory Committee for Laboratory Animal Research in Singapore. Balb/c mice were obtained from the Sembawang Laboratory Animals Centre, National University of Singapore.

2.6. Reverse-transcription and real-time PCR

Total RNA was extracted using TRIzol reagent (Life Technologies), chloroform:isoamyl-ethanol (24:1) and phenol:chloroform:isoamyl-ethanol (50:24:1). cDNA was synthesized from 1 to 5 μg of total RNA using random primer (Promega, Madison, WI, USA) and SuperScript II™ reverse transcriptase (Life Technologies). Real-time PCR was performed using KAPA SYBR Green PCR reagents on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's protocol. PCR for each gene was performed in triplicates. The relative amount of PCR products generated from each primer set was determined on the basis of the threshold cycle (Ct) number. Housekeeping gene 36B4 was used as control to normalize the amount of cDNA used. Relative expression = $2^{[[Ct\ (control)\ gene\ X - Ct\ (treatment)\ gene\ X] - [Ct\ (control)\ 36B4 - Ct\ (treatment)\ 36B4]]}$. Primer sequences are available upon request.

2.7. Bisulfite sequencing

Genomic DNA was extracted from cells by protein proteinase K digestion (Fermentas Life Sciences, Burlington, Canada) followed by extraction with phenol:chlorofoam:isoamyl alcohol (25:24:1), and precipitation with 100% ethanol. Bisulfite treatment of genomic DNA was done with CpGenome™ Fast DNA modification kit (Chemicon International, Temecula, CA, USA), and sodium hydroxide (Merck KGaA, Darmstadt, Germany). PCR of bisulfite-treated DNA was carried out with primers (designed using MethPrimer [12]) specific for the 2 CpG islands. The PCR products were then cloned into a pCR®2.1–TOPO® vector from TOPO TA Cloning® Kit (Life Technologies) and sequenced. Primers used are CpG1 forward 5′ ATAGGATTTATGAATAGAAAAGGAGTTGTA, reverse 5′ AAATAACTAAAACCACAAACACCC. CpG2 forward 5′ GGTGGATTATGAGGTTAGGAGATTGAGATT and reverse 5′ CACCACACACAATTTATCACCAAAATAAAA. Analysis of bisulfite sequencing results was done using Quantification tool for methylation analysis (QUMA) [13].

2.8. Statistical analysis

Real-time PCR results were plotted using mean values with standard error mean (SEM) indicated as error bars. Differences in protein expression levels in normal and tumor tissues were compared using paired Student's *t*-test (two-tailed, equal variance) and *p* values less than 0.05 were considered to be significant. Pearson correlation was employed to examine the relationship between p53 and TRIM22 expression.

3. Results and discussion

To facilitate the study on endogenous TRIM22, we generated TRIM22 specific monoclonal antibody using His-tagged full length TRIM22 recombinant protein (498 amino acids, GI: 313760627) as immunogen (Supplementary Fig. 1). The antiserum from mouse 3 detected the TRIM22 protein band that largely disappeared in cell lysates of mammary epithelial cells MCF10A transfected with TRIM22 siRNA (Supplementary Fig. 1A). Accordingly, a clone of hybridoma cells (Clone 8) derived from the spleen of the mouse produced an antibody that recognized the TRIM22 that is absent in samples transfected with TRIM22 siRNA. This TRIM22 monoclonal antibody, named Mab8, was able to co-immunoprecipitate TRIM22 specifically at ~55–60 kD (Supplementary Fig. 1B). Conditioned medium from hybridoma cells negative for TRIM22 antibody was used as control in immunoprecipitation.

3.1. Down-regulation of TRIM22 expression in breast tumors

With this specific TRIM22 antibody, we examined the expression level of TRIM22 in mammary epithelial cell lines and breast tumor tissues. Western blotting analysis performed on a panel of three non-malignant mammary epithelial cell lines and ten breast cancer cell lines revealed that TRIM22 expression is high in non-malignant mammary epithelial cells but greatly down-regulated in malignant breast cancer cell lines (Fig. 1A). Real-time PCR analysis of the TRIM22 transcript levels also showed that TRIM22 mRNA level is 677 and 240 times higher in non-malignant mammary epithelial cells MCF10A and MCF-12A respectively than that in MCF7 breast cancer cells (Fig. 1B), suggesting that this differential TRIM22 expression level is due to the decrease in its transcript level.

We then evaluated if TRIM22 was similarly down-regulated in breast tumors. Breast tumor biopsies were obtained from National Cancer Centre Singapore according to Singapore Guidelines for Good Clinical Practice. Whole protein lysates were collected from 63 pairs of breast tumors and the adjacent normal breast tissues and the protein expressions were analyzed by Western blotting. The whole lysates of 63 pairs of breast tissue samples were analyzed in 16 Western blots. The normal and tumor tissues were analyzed in separate blots because β-actin levels in the normal tissues are much lower than the tumor tissues and it is impossible to obtain optimal X-ray film exposure that can detect the band from normal tissues without over-exposing the bands for tumor samples. To account for the differences among the immunoblots, 20 μg of whole cell lysates from TRIM22 transfected MCF7 cells (MCF7-TRIM22) was loaded in each blot as control. Band intensities for the immunoblots were quantified by densitometry and relative TRIM22 protein expression was obtained by normalization with the level of β-actin followed by normalization with TRIM22 level of MCF7-TRIM22 cell lysate to eliminate variations among immunoblots (Fig. 1C and D). Comparison of the relative TRIM22 level showed that 57 of the 63 pairs of breast tissue have higher TRIM22 protein level in the adjacent normal tissue ($R > 1$, $R = N/T$, Fig. 1E, top chart), 46 of them have at least double the amount of TRIM22 protein in normal tissue than in tumor tissue ($R > 2$, Fig. 1E, top chart). Paired *t*-test shows that this difference in TRIM22 level is statistically significant ($p < 0.00001$); TRIM22 protein level was consistently down-regulated in the breast tumors compared to the adjacent normal tissues.

It is to be noted that, although 20 μg of total tissue lysates were used for normal and tumor tissues, the band intensities of β-actin were considerably lower in the adjacent normal tissues than in the tumor tissues. This was likely due to the high proportion of adipose tissue in the normal tissue, which contains less β-actin compared with the epithelial tissue. Since TRIM22 protein expression is not detectable in adipose tissues [14], the lower band intensity of β-actin and TRIM22 from the normal tissue is due to the high amount of adipose tissue which contributes to total protein amount but little to β-actin and TRIM22 levels. Nonetheless, TRIM22 levels relative to β-actin were still significantly ($p < 0.00001$) higher in normal tissues than that in tumor tissues. De-regulation in the expression levels of several TRIM proteins (TRIM 24, 25 and 27) have been reported in breast cancer [15,16]. This is the first report confirming the differential TRIM22 protein levels in normal and tumor breast tissues. Since TRIM22 levels has been shown to decrease by 41-fold during early transformation of MCF10F cells *in vitro* [17], its level may be important as an indicator to identify early stage breast cancer.

3.2. Weakened correlation between TRIM22 and p53 expression in breast tumors

As TRIM22 is a direct target gene of p53 [6], we investigated if the decreased TRIM22 expression is associated with p53 dysfunction in tumor tissues. Relative p53 protein level after normalization with β-actin is shown in Fig. 1D (bottom chart). Out of 63 pairs of tissue, 51 of them have higher p53 protein levels in tumor tissue than adjacent normal tissue (Fig. 1E, bottom chart) and the difference in p53 protein level between breast tumor tissue and the adjacent normal tissue was statistically significant ($p < 0.00001$), which is consistent with previous reports [18,19]. When TRIM22 expression was plotted against p53 expression, we found that TRIM22 expression is highly correlated (Pearson's correlation $R = 0.79$, $p < 0.001$) with p53 protein expression in normal tissues (Fig. 1F). However, this correlation coefficient dropped to 0.48 in breast tumor tissues that is significantly lower than the correlation between TRIM22 and p53 in the normal tissues (MedCalc [20], $p < 0.002$) (Fig. 1F). The strong positive correlation between p53 and TRIM22 in normal breast tissues suggests that p53 is a major regulator of TRIM22 expression. This notion is further supported by the marked decrease in correlation in cancer tissues which are known to have high p53 mutation rate and hence p53 dysfunction [21].

3.3. TRIM22 gene promoter is hypermethylated in both normal and cancer cells

We next tested if the low level of TRIM22 expression in tumor cells is also associated with epigenetic inactivation of the TRIM22 gene. Treatment of MCF7 cells with methylation inhibitor 5-aza-2′-deoxycytidine (5-aza-dC) increased TRIM22 mRNA level in a time-dependent manner, reached about 300-fold by 120 h (Fig. 2A). Interestingly, 5-aza-dC treatment also increased TRIM22 gene expression significantly in non-malignant mammary cell lines MCF10A, MCF-12A as well as the breast cancer cell line T47D and cervical cancer cell line HeLa (Fig. 2B). These observations indicate that TRIM22 gene promoters are hypermethylated in both the normal and malignant mammary cells except for MDA-MB-231 cells, which did not respond to 5-aza-dC treatment with an increase of TRIM22 expression (Fig. 2B).

To determine if the TRIM22 promoter is indeed methylated, bisulfite genomic sequencing was carried out to determine the methylation status of individual cytosines in two predicted CpG dinucleotides (CpG1 and CpG2) by EMBOSS CpGPlot in the 5 kb genomic sequence upstream of TRIM22 transcription start site (Fig. 2D). Interestingly, all twelve cytosine residues in CpG1 are heavily methylated in both MCF7 and MCF10A cells whereas CpG2 is not methylated in both cell lines (Fig. 2E). This together with methylation inhibitor studies suggests that the down-regulation of TRIM22 expression in cancer cell is unlikely due to promoter hypermethylation.

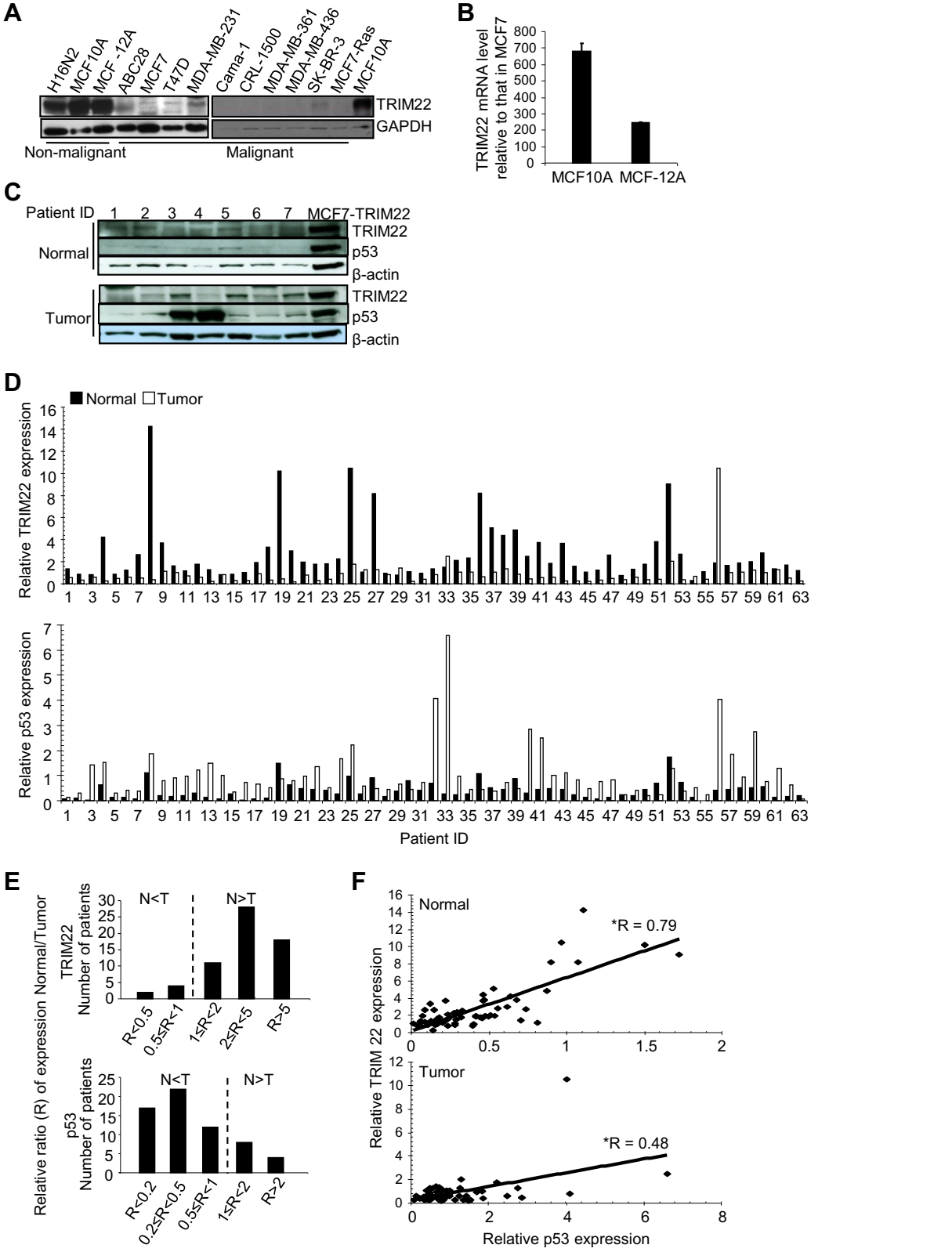

Fig. 1. TRIM22 is under-expressed in both breast cancer cell lines and breast tumors. (A) TRIM22 protein levels in three non-malignant mammary epithelial cell lines and ten breast cancer cell lines were analyzed by Western blotting. (B) Comparison of TRIM22 mRNA levels in MCF7, MCF10A and MCF-12A cells by real-time PCR. (C) A representative Western blot of protein lysates collected from normal and tumor breast tissues. (D) Normalized TRIM22 and p53 protein band intensities for 63 pairs of patient biopsies. (E) Patients were grouped by the relative ratio (*R*) of TRIM22 (top) and p53 (bottom) protein levels in adjacent normal tissue (*N*) vs. tumor tissue (*T*). (F) Correlation between TRIM22 and p53 in normal breast tissues (top) and tumor tissues (bottom).

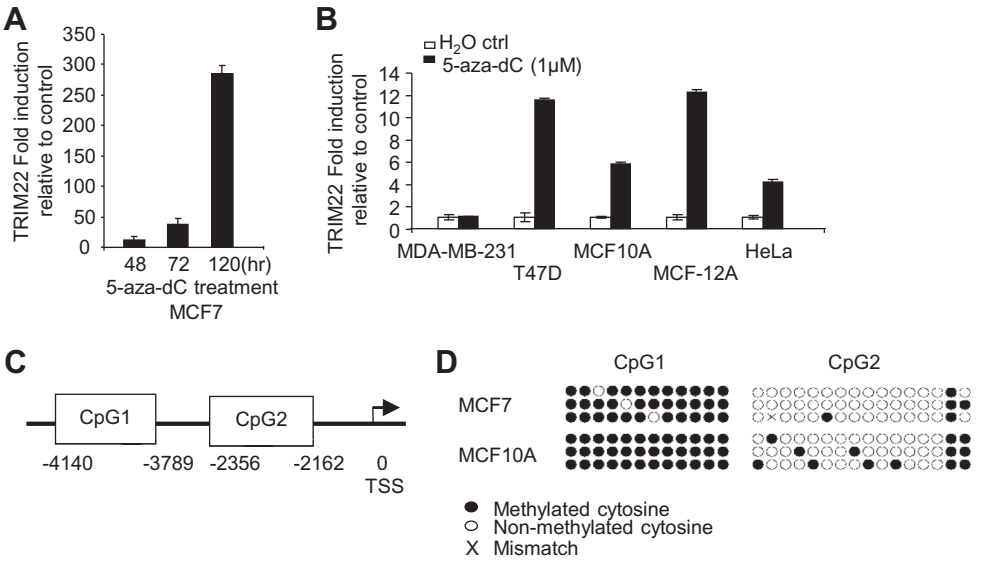

Fig. 2. Up-Regulation of TRIM22 expression by the DNA methylation inhibitor 5-aza-dC. (A) TRIM22 mRNA level in MCF7 with 5-aza-dC treatment. (B) Real-time PCR analysis of TRIM22 expression in MDA-MB-231, T47D, MCF10A, MCF-12A and HeLa cells after 5-aza-dC treatment for 120 h. (C) Predicted location of two CpG islands in the 5 kb genomic region upstream of TRIM22's transcription start site (TSS). (E) Methylation status of the two CpG islands in MCF7 and MCF10A determined by bisulfite sequencing. Each horizontal string represents the methylation pattern of cytosine sequenced from one clone.

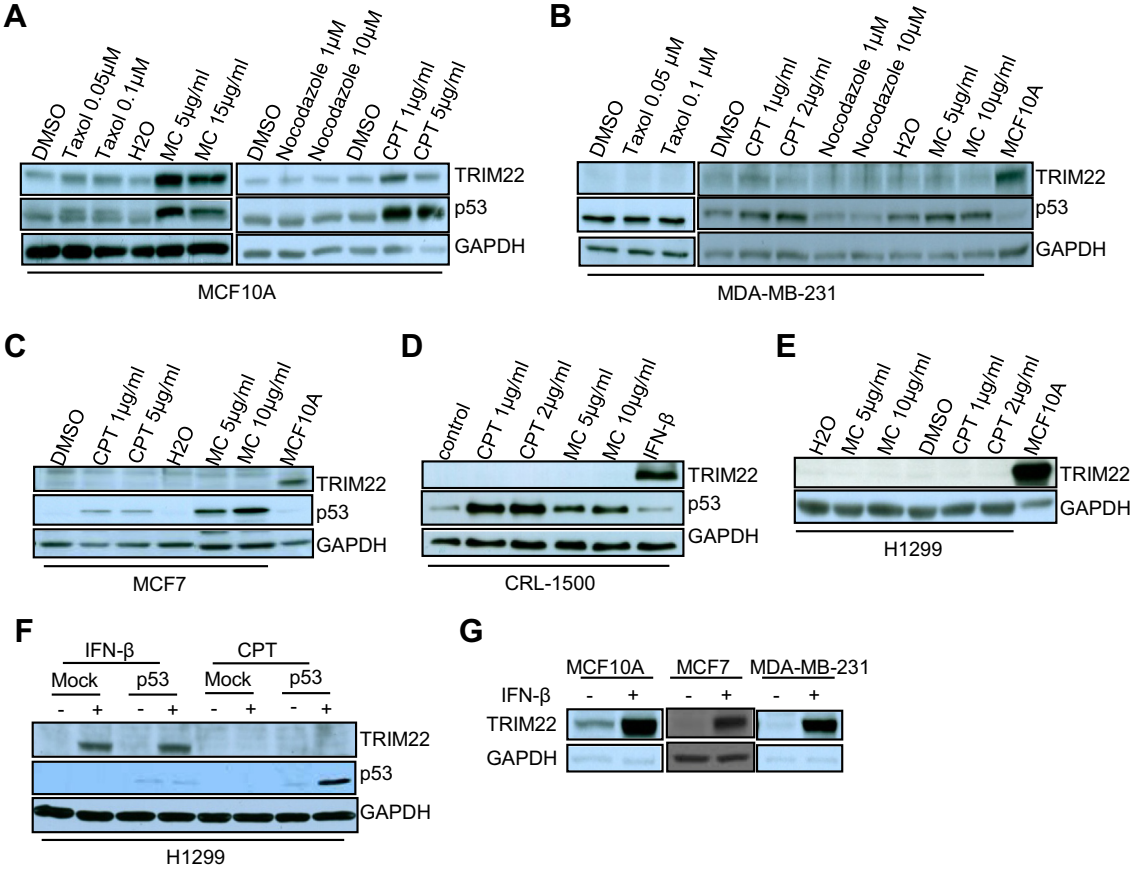

Fig. 3. p53 activating genotoxic drugs were unable to induce TRIM22 in cancer cell lines. (A) MCF10A cells and (B) MDA-MB-231 cells were treated with different dosages of Taxol, mitomycin C (MC), Nocodazole and camptothecin (CPT) for 24 h. TRIM22 and p53 protein levels were analyzed by Western Blotting. (C–E) Western blots for TRIM22 expressions in MCF7, CRL-1500 and H1299 cells treated with MC and CPT. (F) Exogenous p53 expression in H1299 was not sufficient to rescue TRIM22 induction by CPT. (G) Western blot for TRIM22 expression in MCF10A, MCF7 and MDA-MB-231 cells after IFN-β treatment.

3.4. p53-activating genotoxic drugs are unable to induce TRIM22 in breast cancer cells

TRIM22 is a p53 direct target gene. A consensus p53 response element has been identified and characterized in the intron 1 of TRIM22 and p53 binding to this sequence activate downstream reporter gene [6]. The observation that TRIM22 expression is highly correlated with p53 expression in normal breast tissue suggests that p53 is a major regulator of TRIM22 expression in the breast. Since there is a significant decrease in the correlation between TRIM22 and p53 expression in tumor samples, we investigated if the down regulation of TRIM22 in breast tumor cells and tissues is due to p53 dysfunction which occurs in 20–40% of breast cancer [22]. Various cytotoxic chemicals were used for their effects on TRIM22 induction in various cell lines (Fig. 3). In MCF10A cells, which express wild type (wt) p53, DNA cross-linker mitomycin C (MC) and topoisomerase inhibitor camptothecin (CPT) induced a p53 response whereas Taxol (inhibitor of microtubule disassembly) and Nocodazole (inhibitor of microtubule assembly) did not. Accordingly, MC and CPT, but not Taxol and Nocodazle, increased TRIM22 protein levels in MCF10A cells (Fig. 3A). On the other hand, although MC and CPT enhanced p53 protein levels in breast cancer cells (MDA-MB-231, MCF7 and CRL-1500), no change in TRIM22 level could be detected in these cells regardless of their p53 status (Fig. 3BD). Note that MDA-MB-231 cells carry a mutant p53 whereas both MCF7 and CRL-1500 were reported to express normal p53 [23].

While it appears that TRIM22 induction during DNA damage is p53-dependent in non-malignant mammary epithelial cells, wt p53 in breast cancer cells (MCF7 and CRL-1500) failed to induce TRIM22 expression. We further addressed if indeed a wt p53 in cancer cells is unable to induce TRIM22 using p53-null cell line H1299. As is expected, MC and CPT treatments had no effect on TRIM22 levels in H1299 cells (Fig. 3E). More interestingly, introduction of exogenous p53 in H1299 cells was unable to up-regulate the expression of TRIM22 in response to CPT treatment, despite of the fact that p53 protein level is evidently increased due to the increase of p53 stability in response to DNA damaging signals (Fig. 3F). These cell lines studies suggest that p53 signaling to induce TRIM22 expression is defective in cancer cells. In contrast, marked induction of TRIM22 by interferon was observed in all the cell lines examined regardless of p53 status or malignancy (Fig. 3D, F and G), suggesting that interferon signaling to TRIM22 gene regulation in these breast cancer cells is unabated. Recent studies have shown that deregulation in p53 post-translation modifications is often observed in cancers and contribute to tumorigenesis [24]. It is plausible that while there is no genetic mutation in the p53 gene in these cells, p53 protein may be defective as a result of dysregulation of post-translational modifications, leading to dysregulation of some of its downstream genes including TRIM22. Alternatively, there may be a lack of critical factors in p53 pathway for p53 induction of TRIM22 expression in cancer cells.

TRIM family proteins are known to be involved in cancer development through various mechanisms [15,25]. For instance, TRIM19 (also known as PML) fused with retinoic acid receptor α (RARα) by *t*(15; 17) translocation is involved in the development of acute promyelocytic leukemia (APL); TRIM24 fusion with the RET tyrosine kinase domain is found in papillary thyroid carcinoma [26,27]. Deregulation in TRIM 24, 25 and 27 protein levels are associated with breast cancer [15,16,28]. The present study demonstrated that TRIM22 expression is significantly down-regulated in breast cancer cells. This down-regulation in breast cancer cells is at least partly due to defective p53 signaling since wt p53 in cancer cells was unable to induce TRIM22 expression in response to DNA damaging signals. Taking account of its anti-proliferative role in cancer and the drastic down-regulation during mammary cell transformation [6,17], the lack of TRIM22 expression may participate in mammary carcinogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.110.

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