

Gain-of-function mutant p53-R280K mediates survival of breast cancer cells

Yun-Hee Bae · Jong-Myung Shin · Hyun-Joo Park ·
Hye-Ock Jang · Moon-Kyoung Bae ·
Soo-Kyung Bae

Received: 7 October 2013 / Accepted: 18 October 2013 / Published online: 31 October 2013
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Abstract Missense mutations in *TP53* resulting in the expression of p53-R175H, p53-R273H, or p53-R280K are frequently detected in human breast cancer. Currently, the role of mutant p53-R280K in breast cancer is relatively unknown, and therefore, the present study analyzed the function of mutant p53-R280K in breast cancer cell growth. To this end, we used small interfering RNA to study the role of mutant p53-R280K in MDA-MB-231 cells, which endogenously express the mutant protein. We found that curcumin induced apoptosis in MDA-MB-231 cells and downregulated mutant p53-R280K. We also observed that knockdown of mutant p53 by small interfering RNA induced apoptosis in MDA-MB-231 cells. Curcumin-induced apoptosis was further enhanced by the overexpression of wild-type p53, but was decreased by mutant p53-R280K overexpression. Our findings indicate that mutant p53-R280K has an important role in mediating the survival of triple-negative breast cancer MDA-MB-231 cells. Furthermore, this study suggests mutant p53-R280K could be used as a therapeutic target for breast cancer cells harboring this *TP53* missense mutation.

Keywords Apoptosis · Breast cancer cells · Curcumin · Mutant p53

Introduction

Curcumin (diferuloylmethane) is a polyphenol derived from the plant turmeric (*Curcuma longa* Linn) that has antioxidant and anti-inflammatory properties (Menon and Sudheer 2007). In addition, curcumin exhibits anticancer activities in various organs and cell models, including breast cancer cells, by modulating signaling pathways and genes (Kunnumakkara et al. 2008). Many studies, including our own, have demonstrated that curcumin suppresses the activation of nuclear factor kappa B, which decreases breast cancer invasion and induces apoptosis (Kunnumakkara et al. 2008; Kim et al. 2012). Moreover, curcumin has been found to induce p53-dependent apoptosis in various cancer cells, including hepatoblastoma, neuroblastoma, glioma, and breast carcinoma (Jiang et al. 1996; Liontas and Yeger 2004; Banerjee et al. 2010).

TP53 is a tumor suppressor gene that encodes the p53 transcription factor, which is activated in response to a variety of cellular insults such as DNA damage, hypoxic stimuli, and oncogene activation (Horn and Vousden 2007). Activated p53 has been reported to play a very important role in apoptosis, thereby contributing to the prevention of tumor development (Horn and Vousden 2007; Molchadsky et al. 2010). Indeed, mutational inactivation of p53 is frequently observed in >50 % of human cancers (Wijnhoven et al. 2007; Brosh and Rotter 2009). Furthermore, it has been suggested that mutant p53 proteins not only lose the tumor-suppressive activity of wild-type (WT) p53 but also acquire new oncogenic gain-of-function properties, thus contributing to the development and progression of tumors (Brosh and Rotter 2009; Walezych et al. 2012). Previous studies have frequently detected the endogenous gain-of-function p53 mutants, including R175H, R273H, and R280K, in human breast cancers

Y.-H. Bae · J.-M. Shin · H.-O. Jang · S.-K. Bae (✉)
Department of Dental Pharmacology, School of Dentistry, Pusan
National University, 49 Busandaehak-ro, Yangsan 626-870,
South Korea
e-mail: skbae@pusan.ac.kr

H.-J. Park · M.-K. Bae
Department of Oral Physiology, School of Dentistry, Pusan
National University, 49 Busandaehak-ro, Yangsan 626-870,
South Korea

(Fontemaggi et al. 2009; Gurtner et al. 2010; Yeudall et al. 2012). Among them, the mutant p53-R280K is highly expressed in the triple-negative breast cancer cell line MDA-MB-231 (Mehta et al. 2007), which suggests that this mutant may play a role in the development and progression of triple-negative breast cancers.

The purpose of this study was to investigate the role of the mutant p53-R280K in mediating the survival of MDA-MB-231 cells. Here, we report that the level of mutant p53-R280K was downregulated by curcumin, which may contribute to the curcumin-induced apoptosis.

Materials and methods

Materials

Curcumin was purchased from Sigma (St. Louis, MO). Mouse monoclonal anti-p53 and anti- β -actin antibodies were obtained from Calbiochem (San Diego, CA) and Abcam (Cambridge, MA), respectively. Rabbit polyclonal anti-cleaved caspase-3 antibodies were purchased from Cell signaling Technology (Beverly, MA). Rabbit polyclonal anti-poly (ADP-ribose) polymerase (anti-PARP), anti-Bcl-2, and mouse monoclonal anti-caspase-3 were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Santa Cruz Biotechnology. Alexa Fluor 594-conjugated anti-mouse IgG was obtained from Molecular Probes (Eugene, OR).

Cell culture

MDA-MB-231, MCF7, and MCF10A cells were obtained from the American Type Culture Collection (Manassas, VA). MDA-MB-231 and MCF7 cells were cultured in Dulbecco's modified eagle's medium (DMEM; Gibco BRL, Grand Island, NY) containing 10 % heat-inactivated fetal bovine serum (Gibco BRL) and 1 % antibiotics. MCF10A cells were maintained in DMEM/F-12 medium supplemented with 5 % heat-inactivated horse serum (Invitrogen, Grand Island, NY), 10 μ g/mL insulin, 20 ng/mL EGF, 0.1 μ g/mL cholera toxin, 0.5 μ g/mL hydrocortisone, and 1 % antibiotics. Cells were maintained in a humidified 5 % CO₂ atmosphere at 37 °C.

MTT assay

Cells (2×10^4) were seeded into 24-well plates and incubated for the indicated times. After exposure to the indicated doses of curcumin or 0.1 % DMSO (control), cells were treated with MTT solution (0.5 mg/mL) for 4 h at 37 °C in the dark. The supernatant was removed, and

500 μ L of DMSO was added to each well to dissolve the purple formazan salt crystals. The solubilized formazan product was spectrophotometrically quantified using an ELISA reader (TECAN, Salzburg, Austria) at a wavelength of 570 nm.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Apoptotic cells were confirmed using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) in accordance with the manufacturer's instructions. Cells were fixed in 4 % paraformaldehyde for 25 min at 4 °C, and then permeabilized with 0.2 % Triton X-100 for 5 min at room temperature. Free 3' ends of fragmented DNA were enzymatically labeled with the TUNEL reaction mixture for 60 min at 37 °C in a humidified chamber. Labeled DNA fragments were monitored by fluorescence microscopy (Nikon; Instech Co., Ltd., Kanagawa, Japan).

Flow cytometry

Cells were seeded into 100 mm dishes at a density of 1×10^6 cells/dish and incubated for overnight at 37 °C. The cells were transfected with siRNA and 48 h later, washed twice in $1 \times$ phosphate-buffered saline (PBS), and fixed in chilled 70 % ethanol. The cells were stained with 5 μ g/ml propidium iodide at room temperature for 10 min and analyzed by FACSCalibur (BD Bioscience). The cell cycle profile was determined with Modfit LT software.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNAs were isolated from MDA-MB-231 cells using the TRIzol reagent kit (Invitrogen). cDNA synthesis was performed using 2 μ g of total RNA and a RT kit (Promega). The oligonucleotide primers for PCR were designed as follows: β -actin, 5'-GACTACCTCATGAAGATC-3' and 5'-GATCCACATCTGCTGG AA-3'; p53, 5'-GGCCCACTT CACCGTACTAA-3' and 5'-GTGGTTTCAAGCCAGATGT-3'.

Western blot analysis

Harvested cells were lysed in a lysis buffer (40 mM Tris-Cl, 10 mM EDTA, 120 mM NaCl, and 0.1 % NP-40 with a protease inhibitor cocktail [Sigma]). A constant protein concentration (30 μ g/lane) was used. The proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was blocked with 5 % skim milk in PBS containing 0.1 % Tween-20 for 1 h at room

temperature and probed with appropriate antibodies. The signal was developed using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ). β -Actin served as the loading control. Band intensities were quantified using MetaMorph software (Molecular Devices, Union City, CA).

Construction of pCMV-p53 expression vectors and transient transfection

The pCMV-WT p53 expression vector, which harbors WT p53, was purchased from Clontech Laboratories, Inc. Site-directed mutagenesis was performed to construct pCMV-Mut p53, which contained mutant p53-R280K, from pCMV-p53 by using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The oligonucleotide primers used in site-directed mutagenesis were designed as follows: p53-R280K, 5'-TGTGCCTGTCTGGGAAAGACCGGCGCACAGAG-3' and 5'-CTCTGTGCGCCGGTCTTTCCACAGGACAGGCACA-3'. The sequences of the mutant construct were checked by automatic DNA sequencing (Cosmo, Seoul, Korea). Cells were seeded onto 24-well plates and transfected with pCMV-WT p53 or pCMV-Mut p53 by using the X-tremeGene HP DNA transfection reagent (Roche Applied Science, Indianapolis, IN). After 24 h, the cells were incubated in the presence or absence of curcumin (20 μ M) for the indicated times.

Transient transfection of small interfering (si)RNA

We designed and synthesized double-stranded siRNA oligonucleotides (Bioneer, Daejeon, Korea) against p53 (5'-GACUCCAGUGGUAUCUACTT-3' and 5'-GUAGAUUACCACUGGAGUCTT-3'). A non-silencing fluorescein-labeled siRNA (Bioneer, Daejeon, Korea) was used as control for transfection efficiency as well as for monitoring the effect of silencing during all experiments. Transfection of MDA-MB-231 cells with siRNA (200 nM) was performed using Oligofectamine (Invitrogen) according to the recommendations of the manufacturer.

Statistical analysis

Data are shown as the mean \pm standard deviation obtained for at least three independent experiments. Statistical comparisons between groups were performed by one-way analysis of variance followed by the Student's *t* test.

Results

Curcumin induces apoptosis and reduces mutant p53-R280K protein levels in MDA-MB-231 cells

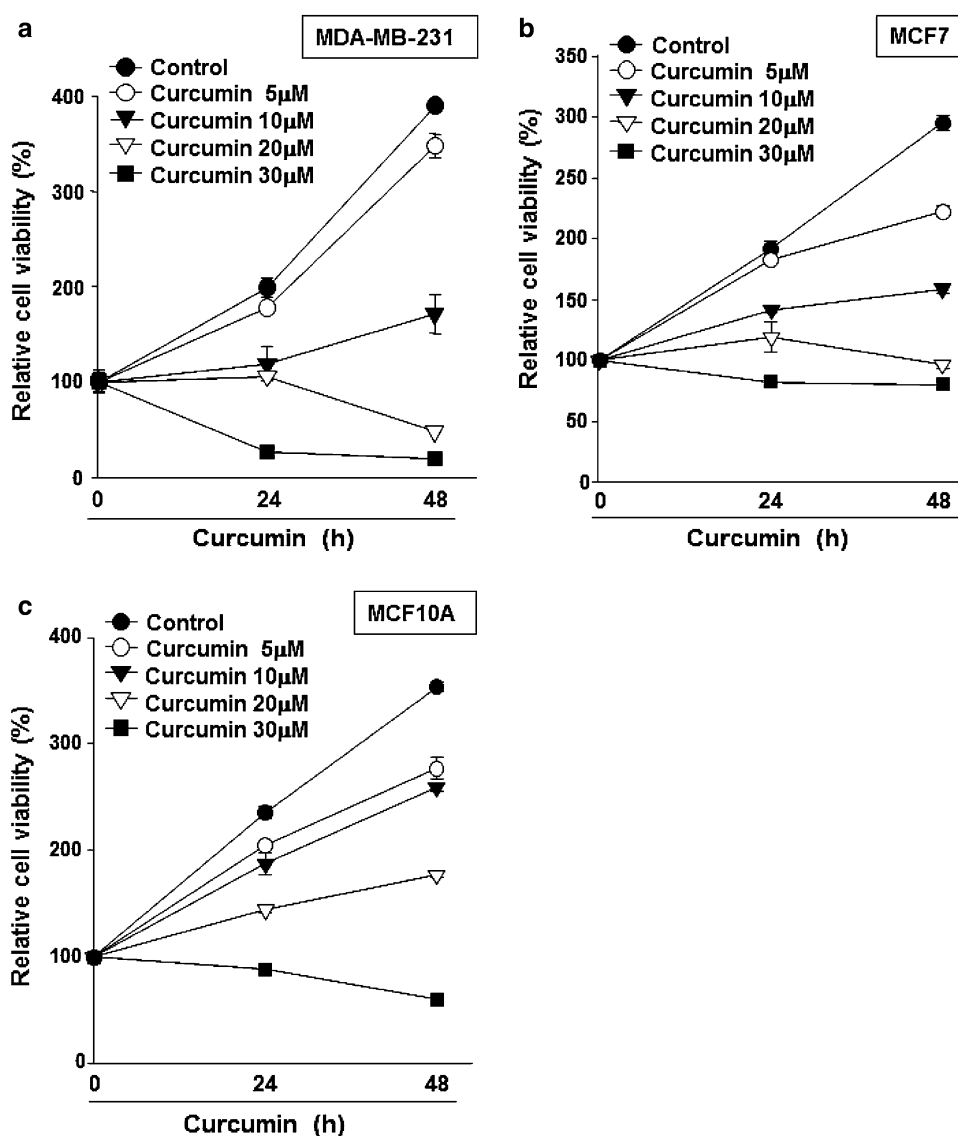
We tested the effects of curcumin on the viability of two human breast cancer cell lines (MDA-MB-231 and MCF7) and a non-transformed mammary epithelial cell line (MCF10A). The cells were treated with different concentrations of curcumin for the indicated times, and the cell viability was measured by MTT assay. Curcumin caused a significant dose- and time-dependent decrease in the viability of the MDA-MB-231 cells. At a dose of 20 μ M curcumin, cellular viability was reduced by \sim 45 % at 48 h of treatment (Fig. 1a). Comparatively, the viability of the MCF7 and MCF10A cells was not reduced by treatment with 20 μ M curcumin for 48 h (Fig. 1b, c). Based on this result, we used MDA-MB-231 cells for further experiments.

To investigate whether MDA-MB-231 cells undergo apoptosis in response to curcumin, we performed TUNEL assays and found that apoptotic cell death occurred when the MDA-MB-231 cells were treated with 20 μ M curcumin for 48 h (Fig. 2a, b). Western blot analysis was then performed to confirm curcumin-induced cellular apoptosis at the molecular level. The results demonstrated increased levels of cleaved PARP and caspase-3, which are the hallmark features of apoptosis in most cells, whereas the levels of the Bcl-2 protein, an anti-apoptotic protein, were reduced in 20 μ M curcumin-treated cells (Fig. 2c). It has been reported that the p53 protein levels are higher in apoptotic cells, and therefore, we examined the levels in curcumin-treated cells. We found that the level of mutant p53-R280K in MDA-MB-231 cells was significantly decreased in the presence of curcumin (Fig. 2c, d), whereas WT p53 level increased in MCF7 cells (Fig. 2e, f).

p53 silencing induces apoptosis in MDA-MB-231 cells in vitro

Because mutant p53-R280K harboring an arginine-to-lysine substitution at codon 280 (R280K) is highly expressed in MDA-MB-231 triple-negative breast cancer cells (Mehta et al. 2007; Walerych et al. 2012), we investigated the role of mutant p53-R280K in MDA-MB-231 cells by performing knockdown experiments with p53-siRNA. As shown in Fig. 3a, b, endogenous mutant p53 expression was successfully silenced by p53-siRNA at both the mRNA and protein levels. The effect of p53-siRNA on cellular apoptosis in vitro was examined by TUNEL assay and indicated that p53 silencing induced apoptotic cell death in MDA-MB-231 cells (Fig. 3c). To

Fig. 1 Effects of curcumin on cell growth. MDA-MB-231 (a), MCF7 (b), and MCF10A (c) cells were treated with different concentrations of curcumin (0–30 μ M) for the indicated times, and cell viability was measured by MTT assay



further examined apoptosis induced by p53 silencing, we performed FACS analysis and found that p53 silencing caused the accumulation of cell population in apoptotic sub-G1 phase, confirming the effect of p53 silencing on cell apoptosis (Fig. 3d). Next, western blot analysis was performed to evaluate the effects of p53 silencing on cellular apoptosis at the molecular level. The results demonstrated that while the levels of cleaved PARP and caspase-3 increased, Bcl-2 protein levels were reduced in p53-silenced cells (Fig. 3d), which were very similar to those observed after curcumin treatment (Fig. 2c).

Curcumin-induced apoptosis is attenuated by mutant p53-R280K

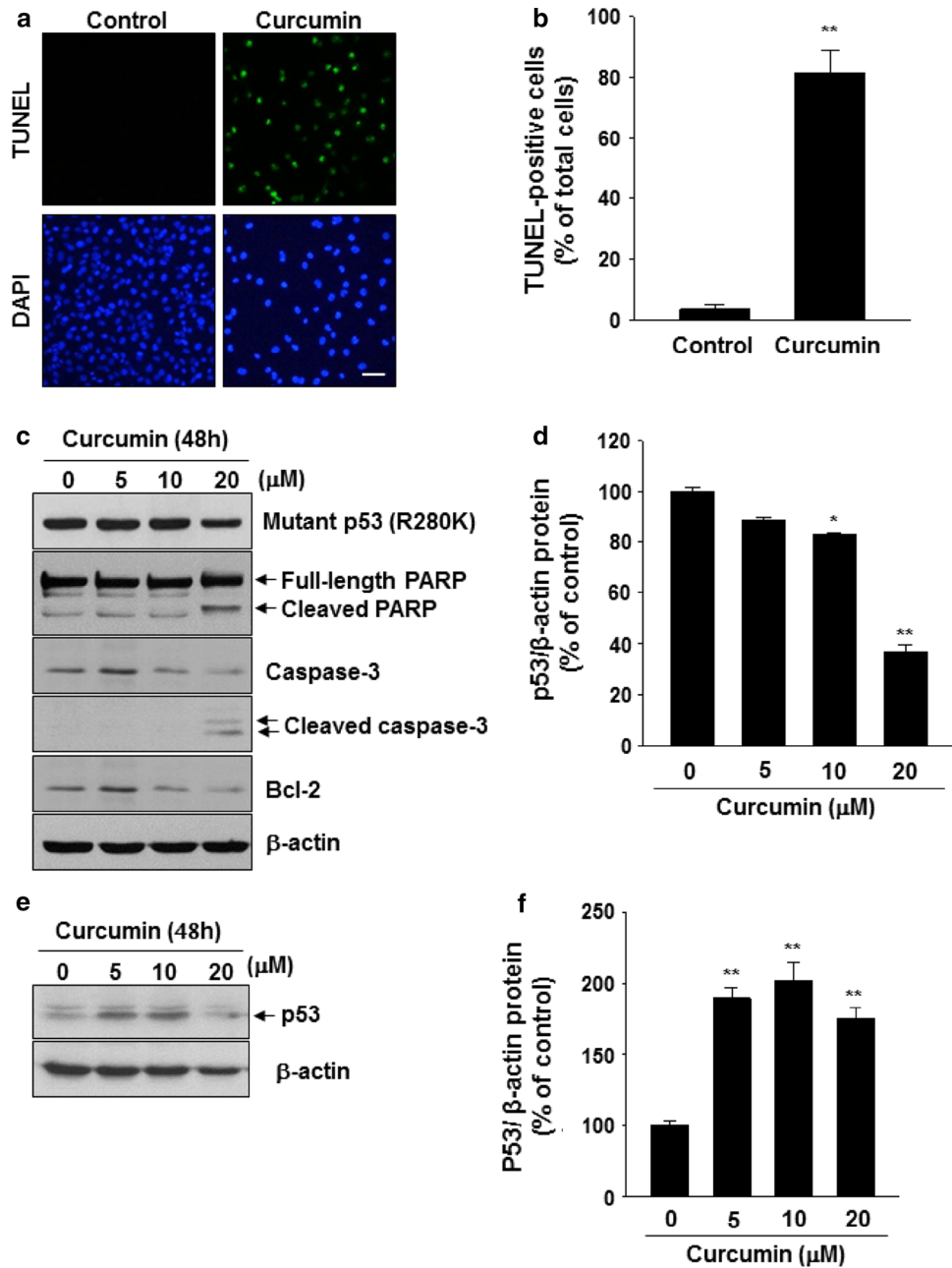
Next, we examined whether an increase in mutant p53-R280K levels can block curcumin-induced apoptosis. WT

p53 overexpression led to increased sensitivity to spontaneous and curcumin-induced apoptosis (Fig. 4a), whereas there was a significant reduction in TUNEL-positive nuclear staining in the curcumin/mutant p53-R280K-overexpressing cells compared with those treated with curcumin alone (Fig. 4a). Moreover, the number of TUNEL-positive apoptotic cells was reduced following mutant p53-R280K overexpression compared with that of curcumin treatment (Fig. 4b).

Discussion

In the present study, we observed the differential responses of mutant p53-R280K-expressing MDA-MB-231 cells and the WT p53-expressing cells MCF7 and MCF10A to curcumin. We found that curcumin induced apoptosis in

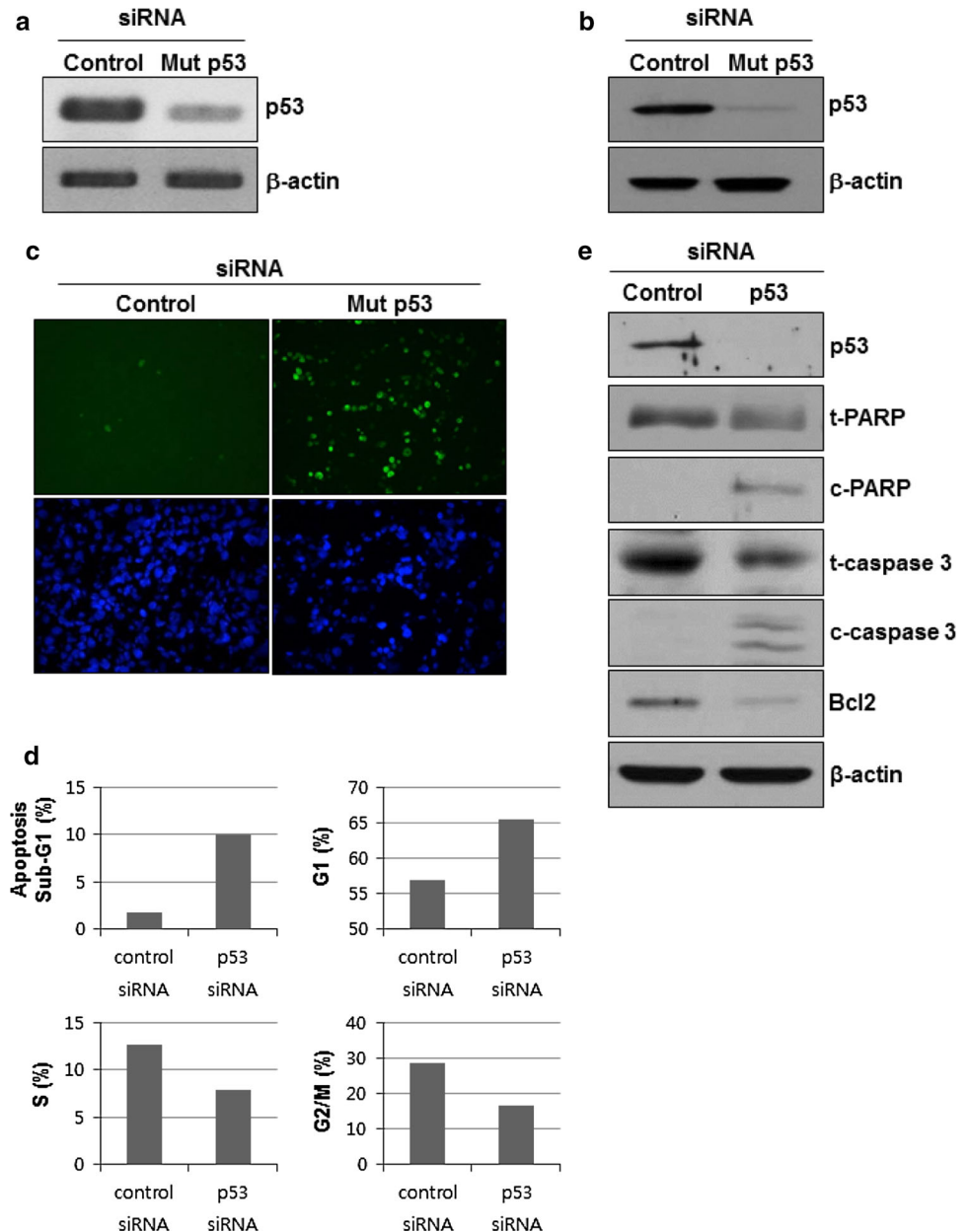
Fig. 2 Effects of curcumin on cellular apoptosis. **a** MDA-MB-231 cells were treated with 20 μ M curcumin for 48 h, and cellular apoptosis was examined by TUNEL assay. DAPI (blue) stain indicates nuclear DNA. Scale bar 50 μ m. **b** The stained cells (green) were counted, and the percentage of positive cells was calculated. $**P < 0.001$ versus control, $n = 3$. **c** MDA-MB-231 cells were treated with the indicated doses of curcumin for 48 h. The protein levels of human p53, PARP, caspase-3, Bcl-2, and β -actin were detected by western blot analysis. **d** The protein levels of human p53 and β -actin were detected by western blot analysis; cumulative densitometric data assessing relative protein expression levels. $*P < 0.01$, $**P < 0.001$ versus control, $n = 3$. **e** MCF7 cells were treated with the indicated doses of curcumin for 48 h. The protein levels of human p53 and β -actin were detected by Western blot analysis. **f** Cumulative densitometric data assessing relative protein expression levels. $**P < 0.001$ vs control, $n = 3$



MDA-MB-231 cells at a higher rate than that observed in MCF7 and MCF10A cells; this seems to be due to the difference in Notch1 levels between cells after curcumin treatment, because Notch1 is able to protect cancer cells from apoptosis (Wang et al. 2006; Alimirah et al. 2007). Previous studies have demonstrated that Notch1 is a transcriptional target of mutant p53-R280K as well as WT p53. WT p53 induced Notch1 gene expression in normal human epithelial cells, including keratinocytes (Yugawa et al. 2007; Lefort et al. 2007). In addition, the overexpression of functional p53 or restoration of WT p53 by etoposide treatment in human cancer cell lines that lack p53 function, upregulated the expression of Notch1 (Alimirah et al.

2007). Furthermore, mutant p53-R280K was also reported to upregulate Notch1 expression in human breast cancer cell line, MDA-MB-231 cells (Bae et al. 2013). Thus, the correlation between p53 and Notch1 suggests that p53-mediated upregulation of Notch1 expression in human cancer cells may counteract p53-mediated proapoptotic functions. Considering that after treatment with curcumin, the protein levels of mutant p53-R280K decreased in MDA-MB-231 cells but the WT p53 level increased in MCF7 cells, it is likely that curcumin-mediated induction of WT p53 in MCF7 may lead to the upregulation of Notch1 gene expression, which causes the MCF7 cells to become more resistant to curcumin-induced apoptosis than

Fig. 3 Effects of mutant p53 silencing on tumor cell apoptosis. MDA-MB-231 cells were transfected with control siRNA or p53-siRNA for 48 h. **a** The mRNA levels of human p53 and β -actin were detected by reverse transcription polymerase chain reaction (RT-PCR) analysis; representative RT-PCR. **b** The protein levels of human p53 and β -actin were detected by western blot analysis; representative western blot. **c** Apoptosis was detected by TUNEL assay. DAPI (blue) stain indicates nuclear DNA. Scale bar 50 μ m. **d** For FACS analysis, nuclei were stained with propidium iodide and the DNA content of the cell nuclei was measured. **e** The protein levels of human p53, PARP, caspase-3, Bcl-2, and β -actin were detected by western blot analysis



MDA-MB-231 cells under curcumin treatment in which Notch1 mRNA and protein levels are reduced; this was probably due to decreased mutant p53-R280K levels as a result of curcumin treatment.

In this report, we observed that curcumin induced apoptosis in MDA-MB-231 cells, which was accompanied by a reduction in endogenous mutant p53-R280K, thereby suggesting the role of mutant p53-R280K in regulating cell survival. Lim et al. (2009) previously reported the role of other p53 mutants, p53-R273H and p53-L194F, in mediating the survival of human breast cancer cell lines, MDA-MB-468 and T47D, respectively. In addition, knockdown of p53-R280T not only induced cell cycle arrest and cell apoptosis in 5,637 bladder cancer cell line, but also

cooperated with cisplatin in the inhibition of 5,637 cells (Zhu et al. 2013). Based on the above findings, it is conceivable that mutant p53-R280K may be involved in regulating spontaneous and curcumin-induced apoptosis. Indeed, we showed that downregulation of endogenous mutant p53-R280K by siRNA promoted MDA-MB-231 cell apoptosis. In addition, while the exogenous expression of WT p53 increased the sensitivity to spontaneous and curcumin-induced apoptosis, the overexpression of mutant p53-R280K rescued this effect to a certain degree. Therefore, these results indicate that mutant p53-R280K mediates the survival of MDA-MB-231 cells. Furthermore, the findings suggest that breast cancer cells expressing high levels of mutant p53-R280K may be more resistant to

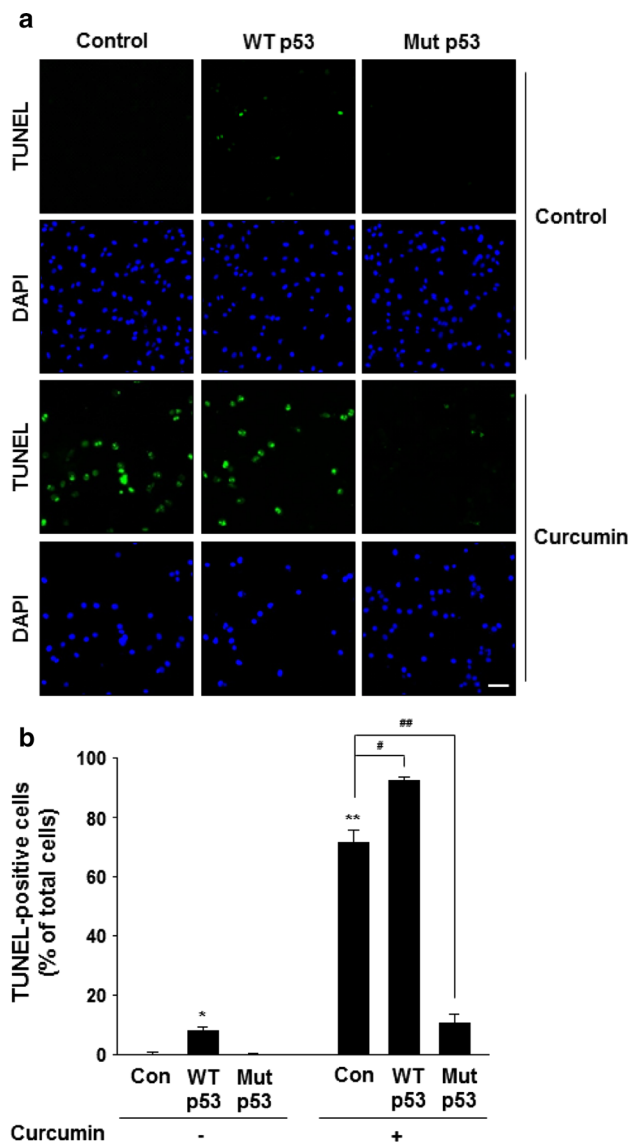


Fig. 4 Effects of mutant p53-R280K overexpression on curcumin-induced apoptosis. MDA-MB-231 cells were transfected with an empty vector control or a mammalian expression vector encoding WT p53 (p53WT) or mutant p53 (p53Mut). After 24 h of transfection, cells were treated with curcumin (20 μ M) and incubated for 48 h. **a** Apoptosis was detected by TUNEL assay. DAPI (blue) stain indicates nuclear DNA. Scale bar 50 μ m. **b** The stained cells were counted, and the percentage of green-positive cells was calculated. * $P < 0.01$, ** $P < 0.001$ versus control without curcumin, # $P < 0.01$, ## $P < 0.001$ versus control with curcumin $n = 3$

curcumin-induced apoptosis than cells expressing the mutant p53-R280K at low levels or WT p53.

In conclusion, our results demonstrated that the mutant p53-R280K participates in the regulation of breast cancer MDA-MB-231 cell growth through its protective effect on apoptosis. Therefore, the mutant p53-R280K may be a possible therapeutic target for management of human triple-negative breast cancer cells.

Acknowledgments This research was supported by the Bio-Scientific Research Grant funded by the Pusan National University (PNU, Bio-Scientific Research Grant; PNU-2008-101-20080596000; to S-K Bae).

Conflict of interest The authors declare no competing financial interests.

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