Constitutively activated ERK sensitizes cancer cells to doxorubicin: Involvement of p53-EGFR-ERK pathway

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The tumour suppressor gene p53 is mutated in approximately 50% of the human cancers. p53 is involved in genotoxic stress-induced cellular responses. The role of EGFR and ERK in DNA-damage-induced apoptosis is well known. We investigated the involvement of activation of ERK signalling as a consequence of non-functional p53, in sensitivity of cells to doxorubicin. We performed cell survival assays in cancer cell lines with varying p53 status: MCF-7 (wild-type p53, WTp53), MDA MB-468 (mutant p53, MUTp53), H1299 (absence of p53, NULLp53) and an isogenic cell line MCF-7As (WTp53 abrogated). Our results indicate that enhanced chemosensitivity of cells lacking wild-type p53 function is because of elevated levels of EGFR which activates ERK. Additionally, we noted that independent of p53 status, pERK contributes to doxorubicin-induced cell death.

[Kumari R, Chouhan S, Singh S, Chhipa RR, Ajay AK and Bhat MK 2017 Constitutively activated ERK sensitizes cancer cells to doxorubicin: Involvement of p53-EGFR-ERK pathway. *J. Biosci.* **42** 31–41]

1. Introduction

A tumour cell frequently responds to chemotherapy, but complete remission is rare and relapses occur frequently after initial positive response. Therefore, it is imperative to search for molecular determinants of chemosensitivity, in tumours.

p53 is one of the most frequently altered genes in human cancers. It has an important role in regulating cell cycle, apoptosis, DNA repair and in maintenance of genomic stability. Loss in p53 function is a common feature of human malignancies and is reported to be involved in the development of resistance to chemotherapy, radiotherapy and increase in tumorigenicity (Coradini *et al.* 2015). On the other hand, some reports indicate that loss of p53 function leads to increased chemosensitivity (Wafik S El-Deiry 2003; Ventura *et al.* 2007; Patricia *et al.* 2014). Therefore, influence of p53 on cellular sensitivity in response to DNA damaging agents is debatable. It has been reported that inactivated and mutated p53 is responsible for malignant transformation because of oncogenic activation of EGFR pathway (Dong *et al.* 2009; Rieber and Rieber 2009). However, significance of this correlation towards sensitivity of cancer cells to chemotherapeutic drugs is not clear.

In this study, we demonstrate the role of activated ERK in sensitivity of cancer cell lines with varying p53 status to DNA damaging agents. For this purpose, we used MCF-7, MCF-7As,

Keywords. Doxorubicin; EGFR; ERK; p53

Supplementary materials pertaining to this article are available on the Journal of Biosciences Website.

DOI: 10.1007/s12038-017-9667-8

Published online: 3 February 2017

MDA MB-468 and H1299 cell lines. MCF-7 cells have functional p53 while MDA MB-468 and H1299 are p53 non-functional cell lines. MCF-7As is an isogenic cell line to MCF-7 in which p53 function is abrogated (Chhipa *et al.* 2007). All these cells were screened for sensitivity towards doxorubicin (DXR), mitomycin c (MMC), 5-fluorouracil (5-FU) and carboplatin (Carb). Among these drugs, doxorubicin is more effective in killing various cancer cells with non-functional p53. Further, we explored the role of activated ERK and EGFR as a consequence of non-functional p53 towards sensitivity to DXR.

2. Materials and methods

2.1 *Cell lines and reagents*

Human breast cancer cell lines, MCF-7 and MDA MB-468, lung cancer cell line H1299, were obtained from American Type Culture Collection (ATCC, VA, USA) and maintained in our inhouse National Centre for Cell Science Repository. MCF-7, MCF-7As, MDA MB-468 cell line and H1299 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) at 37°C with 5% CO_2. The medium was supplemented with 10% heat inactivated serum (Sigma, MO, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Invitrogen CA, USA). Carboplatin (Carb), mitomycin-c (MMC), doxorubicin (DXR) and 5-flourouracil (5-FU) were purchased from Sigma. DXR, 5-FU and Carb were dissolved in sterile glass distilled water to make a stock of 17.2 mM, 100 mM and 25 mM respectively. MMC was dissolved in methanol to make a stock of 5 mM. ERK inhibitor (U0126) and EGFR inhibitor (AG1478) were purchased from Calbiochem and were dissolved in DMSO to make a stock of 50 mM and 168 mM respectively Methylethiazole tetrazolium (MTT) was purchased from USB Corporation, (OH, USA) and reconstituted in DMEM without phenol red to make 1 mg/mL solution. Antibodies against p53, phosphor-ERK (Tyr204), ERK, β-tubulin and EGFR, pAKT, AKT and HRP conjugated secondary antibodies, Ultracruz mounting media and recombinant EGF protein were purchased from Santa Cruz Biotechnology (CA, USA). EGF protein was reconstituted in sterile glass distilled water to make a stock solution of 5 μg/μL.

2.2 *MTT cell survival assay*

To assay cell survival under drug treatment, cells were seeded at a density of 1×10^4 cells/well in 96-well plates and allowed to adhere overnight at 37°C. Treatment with drugs/inhibitors was given as per experimental requirements. At designated time points, MTT was added and cells were incubated in humidified CO_2 incubator for 4 h to allow the formation of formazan crystals. Thereafter, 50 μL of iso-propanol was added to each well. Absorbance was read at 570 nm. Graph was plotted as percentage of value compared with untreated control using Sigma Plot 12.0.

2.3 *Colony formation assay*

One-thousand cells plated in each well of a six-well plate were treated with indicated concentrations of DXR (5 μM) and EGF (100 ng/mL) or inhibitors as per experimental requirements. Cells were washed and incubated in media for 21 days with media change every fourth day. Cells were stained with 0.005% crystal-violet stain for 1 h; images were taken (Olympus, Tokyo, Japan). Densitometric analysis was performed by using Image J software (NIH, USA) and graph was plotted using Sigma Plot 12.0.

2.4 *Western blot analysis*

Whole cell lysates were prepared and blotting was performed as previously described (Chhipa and Bhat 2007). Densitometric analysis was performed by using Image J software (NIH, USA). Band Intensities were normalized by dividing the intensity obtained from each protein band to their corresponding β-tubulin band intensity. Band Intensities of pERK in figure 3 is normalized with respective basal ERK band intensity.

2.5 *Cell transfection*

Wild-type p53 plasmid (pC53-SN3) was a kind gift from Dr. Bert Vogelstein, John Hopkins, Baltimore, MD. For transient transfection, cells were transfected with Lipofectamine2000 reagent as per manufacturer's instruction. Cells were plated in 35 mm tissue culture plates and were transfected with 3 μg of DNA including 1 μg of pEGFPN1 plasmid as an internal control to assess the transfection efficiency. Cells in 96-well plate were transfected with 0.5 μg of pC53-SN3 or EGFP plasmid per well. Plasmid was diluted in 25 μL Opti-MEM per well. Lipofectamine2000 (0.5 μL) was also diluted in 25 μl of Opti-MEM per well. They were incubated at room temperature for 5 min. Then the diluted DNA was mixed with diluted Lipofectamine2000 and co-incubated at room temperature for 45 min to allow the formation of Lipofectamine-DNA complex. One hour prior to addition of Lipofectamine-DNA complex, media was removed and 50 μL Opti-MEM was added to each well. Fifty micro-liters of Lipofectamine-DNA complex was added drop-wise to each well and mixed gently. Cells were incubated at 37°C for 18 h. Thereafter, cells were washed thrice and processed as per experimental requirement.

2.6 *DAPI staining*

Five thousand cells were plated in each well of multi-well slides (MP Biomedicals, OH, USA) and kept for 24 h at

37°C in humidified CO_2 incubator. Medium was removed and fresh media containing indicated concentrations of drugs were added and incubated further for 24 h at 37°C in humidified CO_2 incubator. After 24 h medium was replaced with drug-free medium and cells were allowed to grow for additional 24 h. Cells were fixed and stained with Ultracruz

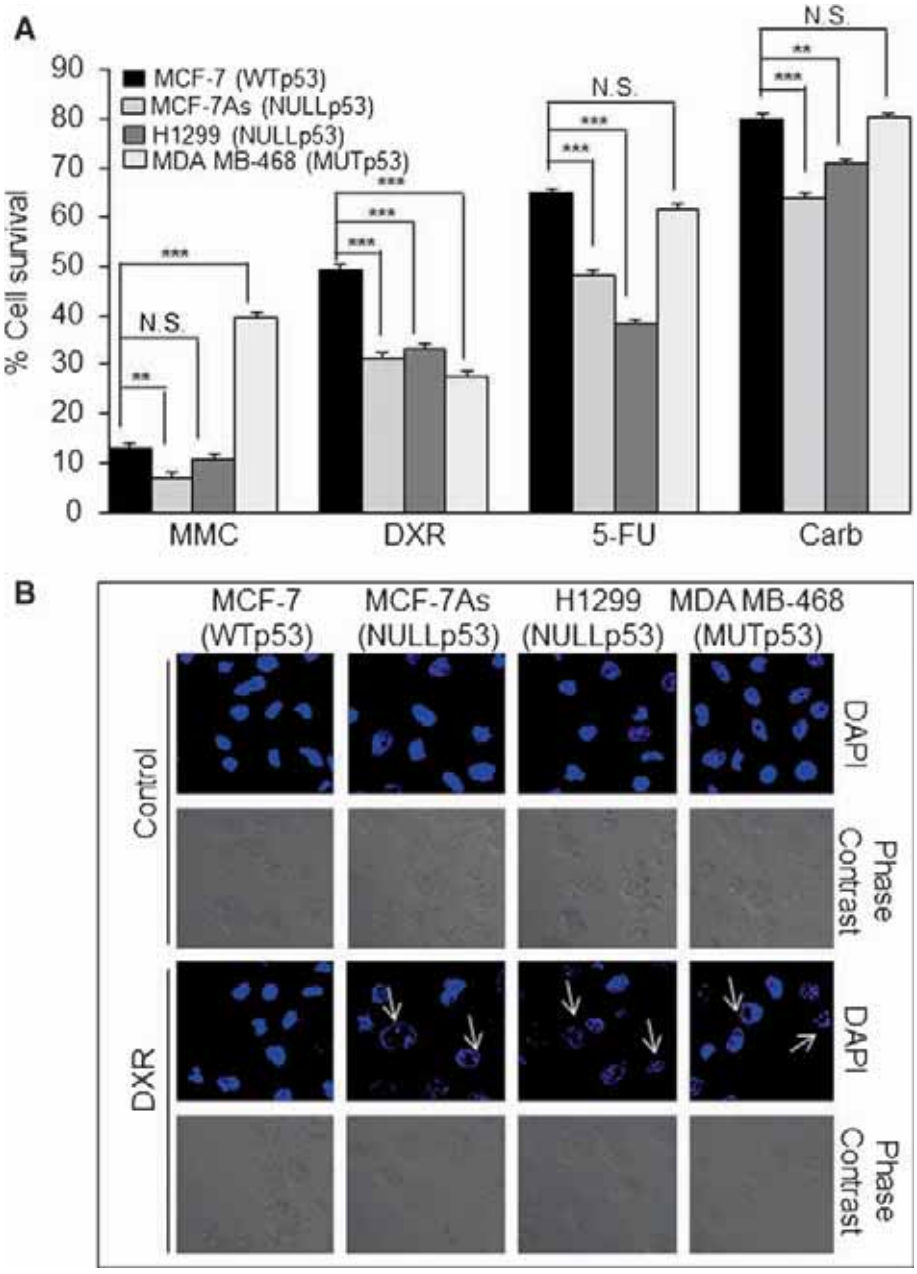

Figure 1. Sensitivity of MCF-7, MCF-7As, H1299, and MDA MB-468 to chemotherapeutic drugs. (**A**) MCF7, MCF-7As, H1299 and MDA MB-468 cells were treated with MMC (5 μM), DXR (10 μM), 5-FU (100 μM) and Carb (100 μM) for 24 h. Cells were grown in drug free medium for additional 24 h and MTT assay was performed. All the bar graphs represent the mean±SD of an experiment done in triplicate. Statistical comparison was performed by Student's two-tailed unpaired *t*-test. The values of $P<0.05$ were considered statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$). Wild-type p53 is represented as WT p53, mutated p53 is represented as MUT p53 and absence of p53 is represented as NULL p53. (**B**) MCF7, MCF-7As, H1299 and MDA MB-468 cells were treated with DXR; MCF-7 (10 μM), MCF-7As (5 μM), H1299 (2.3 μM) and MDA MB-468 (5 μM) for 24 h and processed for DAPI staining and were visualized for nuclear disintegration.

mounting media containing DAPI. The images were captured and processed by Adobe Photoshop software.

2.7 *Statistical analysis*

Statistical analysis was performed using Sigma Plot 12.0 (Systat Software Inc., CA, USA). All data were represented as the mean ± standard deviation (S.D.). In most experiments, bars represent variations within the wells of an experiment and the experiments were repeated at least three times. Statistical comparison was performed by Student's two-tailed unpaired *t*-test. The values of $P<0.05$ were considered statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

3. Results

3.1 *p53 non-functionality enhances sensitivity towards doxorubicin*

To investigate the role of p53 in chemosensitivity, p53 functional cell line, MCF-7 and p53 non-functional cell lines, MCF-7As, H1299 and MDA MB-468 cells were treated with indicated concentrations of MMC, DXR, 5-FU and Carb. Thereafter, MTT assay was performed to assess cell survival. Enhanced sensitivity towards DXR was observed in MCF-7As, H1299 and MDA MB-468 cells. Sensitivity towards MMC, 5-FU and Carb was not dependent on p53 status (figure 1A). Next, MCF-7, MCF-7As, H1299 and MDA MB-468 cell were seeded in multi-well slides and

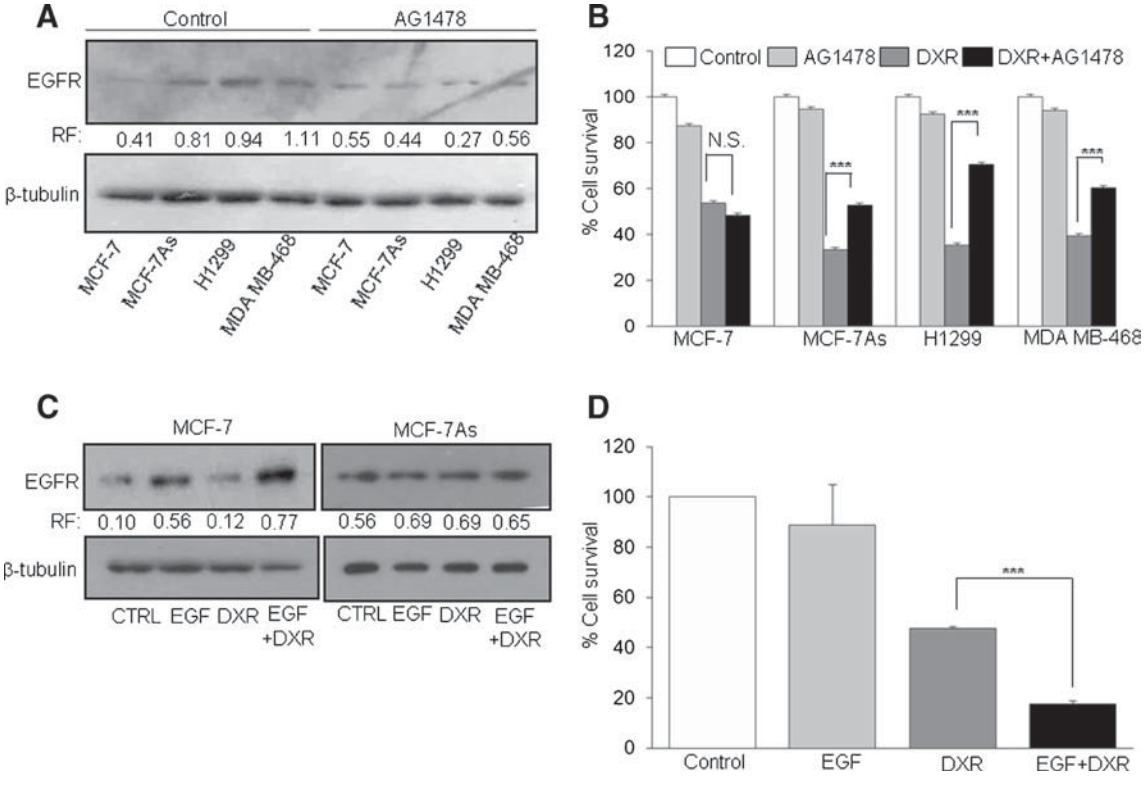

Figure 2. EGFR level in p53 non-functional cells. (**A**) Western blot analysis for EGFR level in the presence of AG1478 (10 μM) in MCF-7, MCF-7As, H1299 and in MDA MB-468 cells. Relative fold value (RF) calculated by densitometric analysis after normalizing with respective β-tubulin. The experiment was repeated twice and data represents western blot from one of the experiment. (**B**) MCF-7, MCF-7As, H1299 and MDA MB-468 cells were pre-treated with AG1478 (10 μM) for 1 h and then treated with DXR; MCF-7 (10 μM), MCF-7As (5 μM), H1299 (2.3 μM) and MDA MB-468 (5 μM) for 24 h. Cells were allowed to grow in drug free medium for additional 24 h then MTT assay was performed. All the bar graphs represent the mean±SD of an experiment done in triplicate (***$P<0.001$). (**C**) MCF-7 and MCF-7As cells were treated with EGF ligand (100 ng/mL) along with DXR (10 μM) for 24 h. Western blot was performed for detection of levels of EGFR protein. Relative fold value (RF) calculated by densitometric analysis after normalizing with respective β-tubulin. (**D**) MCF-7 cells were treated with EGF ligand (100 ng/mL) along with DXR (10 μM) for 24 h. Percentage cell survival was determined by MTT assay. All the bar graphs represent the mean±SD of an experiment done in triplicate. Statistical comparison was performed by Student's two-tailed unpaired *t*-test. The values of $P<0.05$ were considered statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

treated with DXR for 24 h. Cells stained with DAPI were captured and visualized for alteration in nucleus, if any. DXR causes enhanced nuclear DNA disintegration indicative of increased apoptosis (figure 1B).

3.2 *p53 non-functionality and elevated EGFR level contribute to sensitivity towards DXR*

EGFR is one of the upstream signalling molecules of ERK and is down regulated by p53 (Cui *et al.* 2012). Elevated EGFR level was detected in MCF-7As, H1299 and MDA MB-468 cells as compared to MCF-7 cells. Treatment of cells with AG1478, an EGFR inhibitor reduces EGFR level (figure 2A) in cells with non-functional p53. Decrease in EGFR level upon AG1478 has been previously documented (Busse *et al.* 2000). To investigate the role of EGFR on drug sensitivity, cells were treated with DXR in the presence and absence of AG1478. It was observed that, AG1478 treatment causes reduction in DXR-induced cytotoxicity in p53 non-functional cells whereas no changes were detected in MCF-7 cells (figure 2B).

3.3 *Up-regulation of EGFR level enhances sensitivity of MCF-7 cells to DXR*

EGF increases EGFR levels in various cells including MCF-7 cells (Garcia *et al.* 2006; Moerkens *et al.* 2014). Therefore, EGFR status is likely to further enhance the sensitivity to DXR. To ascertain this, MCF-7 and MCF-7As cells treated with EGF were subsequently exposed to DXR and western blotting was performed to detect EGFR level. We observed that EGF treatment increases the level of EGFR in MCF-7 cells, while no change was observed in MCF-7As cells (figure 2C), indicating that EGF causes increase in EGFR level only in p53 functional cells. Upon exposure of MCF-7 cells to EGF and subsequent treatment with DXR, survival was reduced in the cells treated with DXR along with EGF compared to DXR alone (figure 2D). The effective killing by DXR following up-regulation of EGFR pathway is clearly observed by long-term cell survival assay as very few surviving colonies were observed in DXR treated cells in the presence of EGF (supplementary figure I).

3.4 *EGFR activates ERK, which correlates with enhanced sensitivity*

Elevated level of EGFR has been previously reported to enhance pERK level and induction of apoptosis (Dong *et al.* 2009). Therefore, we investigated the role of EGFR in activation of ERK-induced apoptosis. We observed increased level of pERK in p53 non-functional cells compared to MCF-7 cells. Also, it was observed that in the presence of AG1478, pERK levels were reduced in MCF-7As, H1299 and MDA MB-468 cells (figure 3).

3.5 *ERK activation is independent of p53 but EGFR level is dependent on non-functionality of p53*

EGFR is one of the upstream signalling proteins of ERK pathway and is regulated by p53 (Lachat *et al.* 2004) and this correlation between them is not much explored. Therefore, to find an association if any, between wild-type p53 and EGFR levels towards determining the overall survival of cancer cells, MCF-7, MCF-7As and H1299 cells were transfected with wild-type p53 plasmid or pEGFP plasmid as a control. Overexpression of p53 in p53 non-functional cells decreases EGFR protein level. To understand the relationship between wild-type p53 and pERK, we transfected plasmid expressing wild-type p53 in all the cell lines. In the presence of wild-type p53 protein there was no change in pERK level in MCF-7 cells. Surprisingly, p53 non-functional cells transfected with wild-type p53 plasmid did not exhibit any change in pERK level (figure 4A). Densitometric analysis from two independent blots is represented as figure 4B. The three independent blots for the aforementioned experiments are incorporated as in supplementary figure IIa, b, c. These results suggest that ERK is regulated in EGFR dependent and also in EGFR independent manner, in p53 non-functional cells.

Overexpression of wild-type p53 in MCF-7 cells did not affect the sensitivity of cells to DXR. However, it rescued MCF-7As and H1299 cells from DXR-induced death (figure 4C).

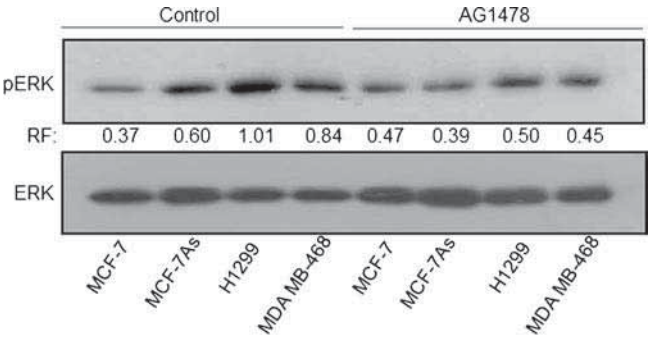

Figure 3. EGFR-dependent activation of ERK. MCF-7, MCF-7As, H1299 and MDA MB-468 cells were treated with AG1478 (10 μM) for 24 h and whole cell lysates were subjected to detection of pERK and ERK proteins by Western blotting. Densitometric analysis was performed and data was plotted as relative fold value (RF) for pERK after normalizing with respective basal ERK levels. The experiment was repeated twice and data represents Western blot from one of the experiment.

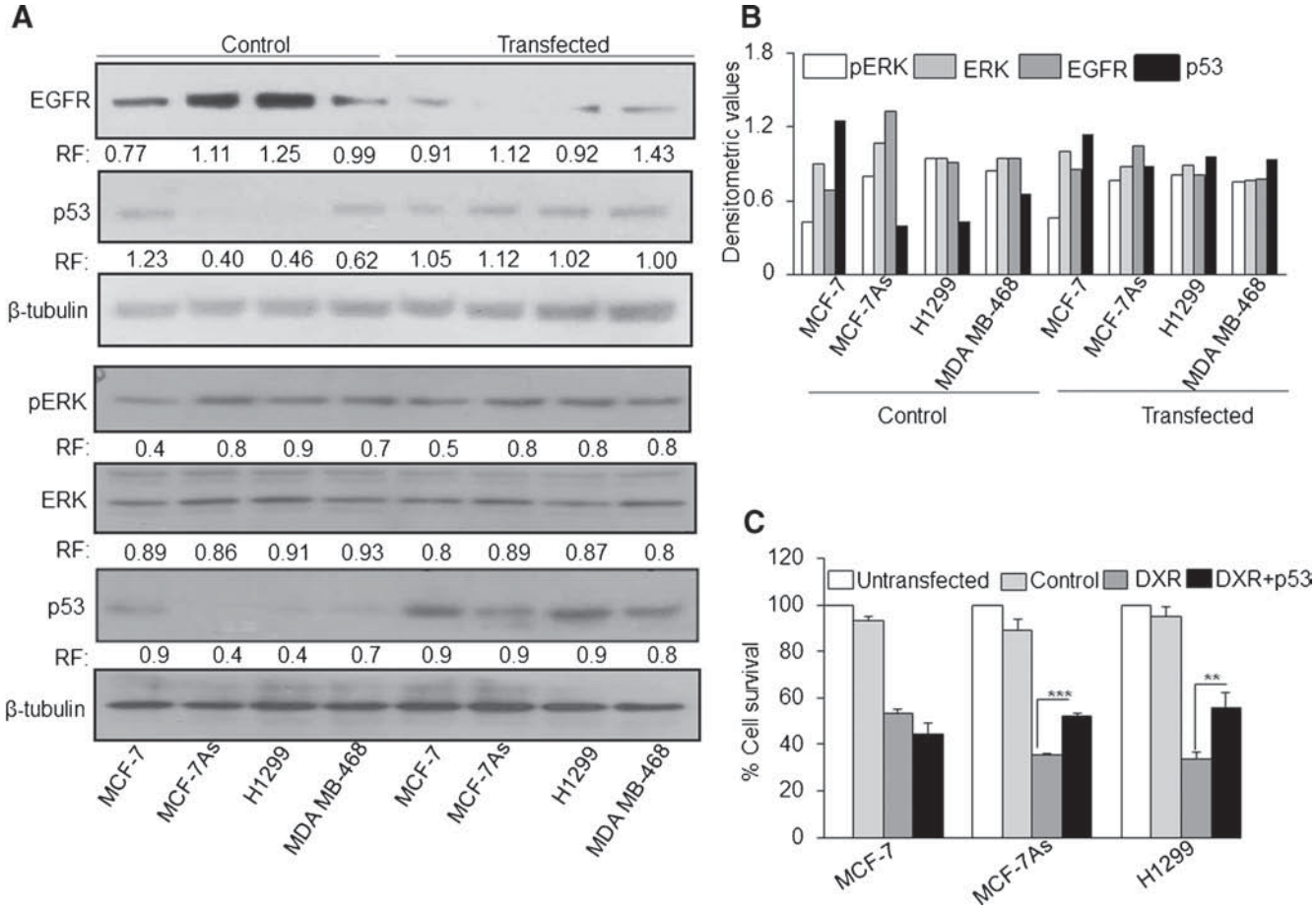

Figure 4. EGFR/p53-independent activation of ERK. (**A**) MCF-7As, H1299 and MDA MB-468 cells were transfected with p53 wild-type plasmid and pEGFP control plasmid. After 16 h, EGFR, pERK, ERK levels were detected in whole cell lysates of transfected cells by western blotting. Relative fold value (RF) calculated by densitometric analysis after normalizing with respective β-tubulin. (**B**) Densitometric analysis representing average values calculated from two independent experiments represented in (**A**). (**C**) MCF-7, MCF-7As and H1299 cells were transfected with wild-type p53 and pEGFP plasmids. After 16 h, cells were treated with DXR; MCF-7 (10 μM), MCF-7As (5 μM), H1299 (2.3 μM) and MDA MB-468 (5 μM) for additional 24 h and 48 h. After drug treatment, cells were allowed to grow for 24 h in drug free medium before performing MTT assay as described in materials and methods section. All the bar graphs represent the mean±SD of an experiment done in triplicate. Statistical comparison was performed by Student's two-tailed unpaired *t*-test. The values of $P<0.05$ were considered statistically significant (*$P<0.05$, **$P<0.01$, ***$P< 0.001$).

3.6 *Inhibition of ERK activation contributes towards DXR sensitivity*

Activated ERK has been correlated with DNA-damage-induced apoptosis (Singh *et al.* 2007; Upadhyay *et al.* 2008). ERK activation can be inhibited by U0126 (an upstream inhibitor of ERK activation) (Marampon *et al.* 2009). Therefore, we probed into the role of inhibition of ERK activation in DXR induced apoptosis. When cells were treated with ERK inhibitor U0126, pERK level was barely detectable, whereas basal ERK remained unchanged (figure 5A). To investigate the involvement of pERK in sensitivity to DXR, cells were exposed to U0126, and subsequently treated with DXR (figure 5B). Significant reduction in cell death was observed in the cells exposed to ERK inhibitor and treated with DXR. These results are also complimented by phase contrast microscopic pictures, which demonstrate the presence of more number of surviving cells (figure 5C).

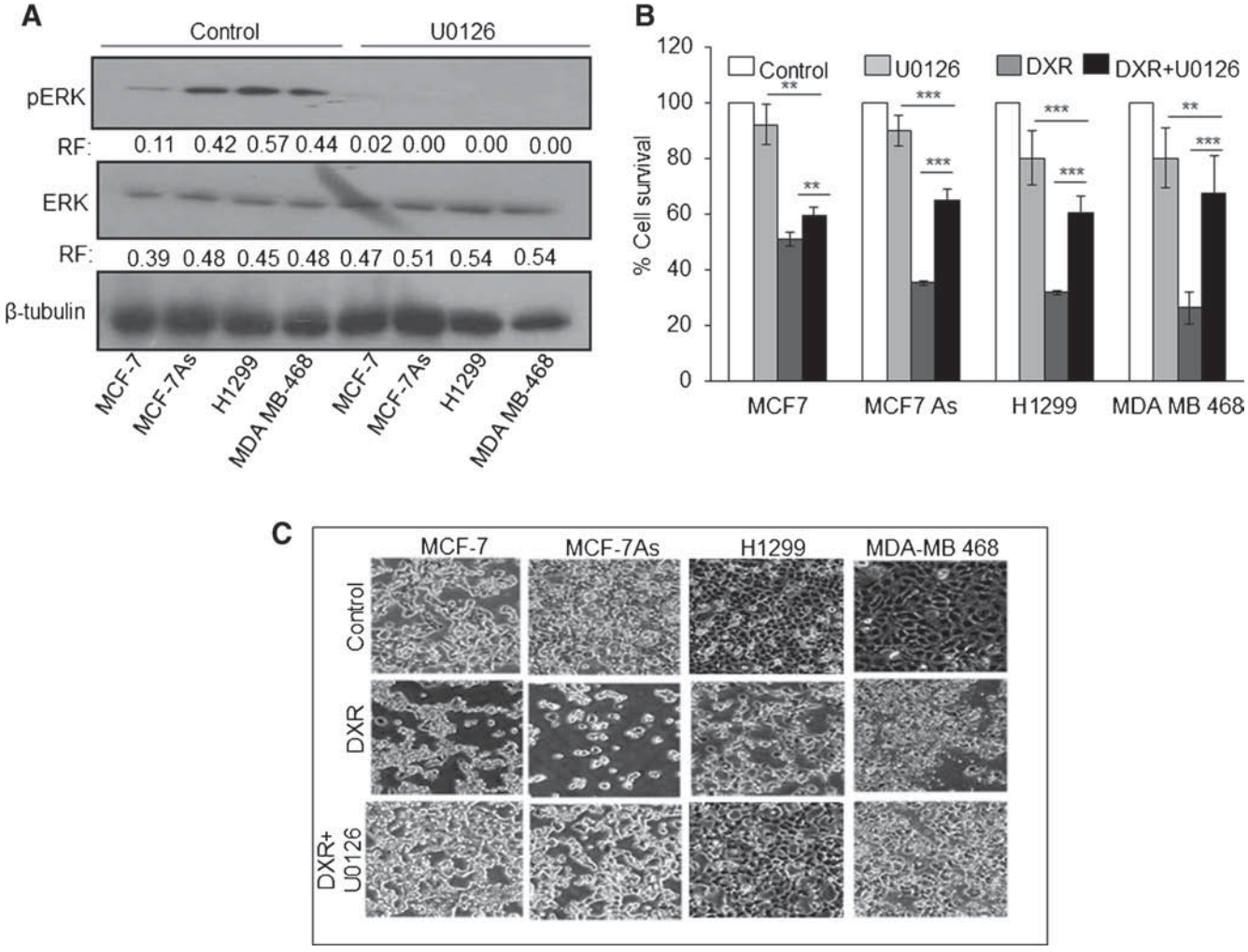

Figure 5. Inhibiting pERK activation promotes survival in DXR-treated cells. (**A**) MCF-7As, H1299 and MDA MB-468 cells were treated with U0126 (20 μM) and whole cell lysates were processed for Western blotting for detection of pERK protein. Relative fold value (RF) calculated by densitometric analysis after normalizing with respective β-tubulin. The experiment was repeated twice and data represents western blot from one of the experiment. (**B**) MCF-7, MCF-7As, H1299 and MDA MB-468 cells were pre-treated with U0126 (20 μM) for 1 h followed by DXR; MCF-7 (10 μM), MCF-7As (5 μM), H1299 (2.3 μM) and MDA MB-468 (5 μM) treatment for 24 h in the presence or absence of U0126. All the bar graphs represent the mean±SD of an experiment done in triplicate. Statistical comparison was performed by Student's two-tailed unpaired *t*-test. The values of $P<0.05$ were considered statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$). (**C**) Photomicrographs of cells MCF-7, MCF-7As, H1299 and MDA MB-468 cells pre-treated with U0126 (20 μM) for 1 h followed by DXR; MCF-7 (10 μM), MCF-7As (5 μM), H1299 (2.3 μM) and MDA MB-468 (5 μM) treatment for 24 h in the presence or absence of U0126 (magnification, ×400; scale bars, 100 μm).

3.7 *Inhibition of EGFR and ERK together reduces sensitivity to DXR*

To evaluate the role of EGFR and ERK in sensitivity, cells were exposed to AG1478, U0126 or both together followed by treatment with DXR and survival was assessed by MTT assay. It was observed that percentage of surviving cells following DXR treatment in p53 non-functional cell lines was significantly enhanced in the presence of either AG1478 or U0126 or both together as compared with percentage survival in p53 functional cells treated similarly (figure 6A). Also, increased survival of p53 non-functional cells was observed upon exposure to U0126 than AG1478, indicating EGFR dependent and independent pathways are contributing to ERK induced sensitivity towards DXR. These observations are in agreement with results obtained in long-term colony formation assay, under similar treatment conditions (figure 6B and supplementary figure III).

Schematic representation of proposed model of ERK promoted DXR-induced cell death is depicted in figure 7.

4. Discussion

Over the past few decades, although major advancements in therapies for cancer have taken place, still cancer remains a leading cause of mortality worldwide (Maruthampu *et al.* 2016). A number of studies have documented the role of p53 mutations towards the therapeutic outcome for various cancers (Licitra *et al.* 2004; Wansom *et al.* 2010; Mollgard et al. 2011; Fernández *et al.* 2012). However, the p53 downstream signalling pathways involved in sensitivity towards chemotherapeutic drugs are yet to be completely understood.

In the present study we have explored the relationship between p53 status and sensitivity of cancer cells to doxorubicin. We employed p53 wild-type cells MCF-7 and p53 non-functional cells MCF-7As, H1299 and MDA MB-468. These cells were treated with MMC, DXR, 5-FU and Carb. Sensitivity towards 5-FU and Carb was not dependent on p53 status, whereas in case of MMC, p53-null cells appear to be more sensitive than wild-type but in p53-mutated cells survival is more than that of wild-type. Since only DXR effect was p53 dependent, we further explored its mechanism of action. This observation is complimented by occurrence of nuclear disintegration indicative of apoptosis in

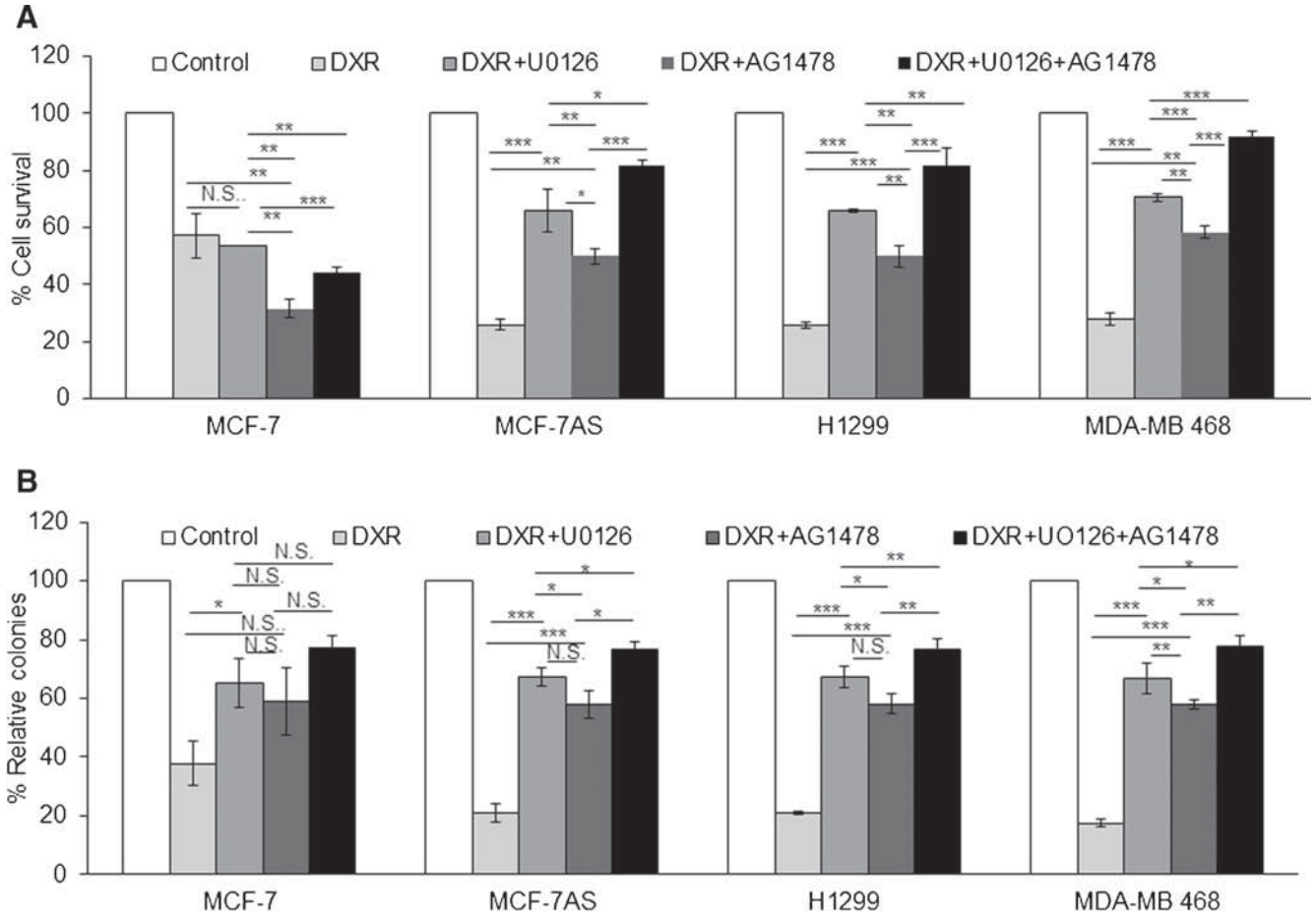

Figure 6. Inhibition of EGFR and activation of ERK leads to enhanced survival under DXR treatment. (**A**) MCF-7, MCF-7As, H1299 and MDA MB-468 cells were pre-treated with U0126 (20 μM) and/or AG1478 (10 μM) for 1 h and then treated with DXR treatment for 24 h. Cells were allowed to grow in drug free medium for additional 24 h then MTT assay was performed. All the bar graphs represent the mean ±SD of an experiment done in triplicate (*P<0.05, **P<0.001, ***P<0.001). (**B**) MCF-7, MCF-7As, H1299 and MDA MB-468 cells were pre-treated with U0126 (20 μM) and/or AG1478 (10 μM) for 1 h and then treated with DXR treatment for 24 h. Cells were allowed to grow in drug free medium for 21 days and colonies were visualized with crystal violet staining. Densitometric analysis was performed and relative colonies were plotted as bar graph. All the bar graphs represent the mean±SD of an experiment done in triplicate (*P<0.05, **P<0.001, ***P<0.001). Original photograph is included as supplementary figure III. Statistical comparison was performed by Student's two-tailed unpaired *t*-test. The values of P<0.05 were considered statistically significant (*P<0.05, **P<0.01, ***P<0.001).

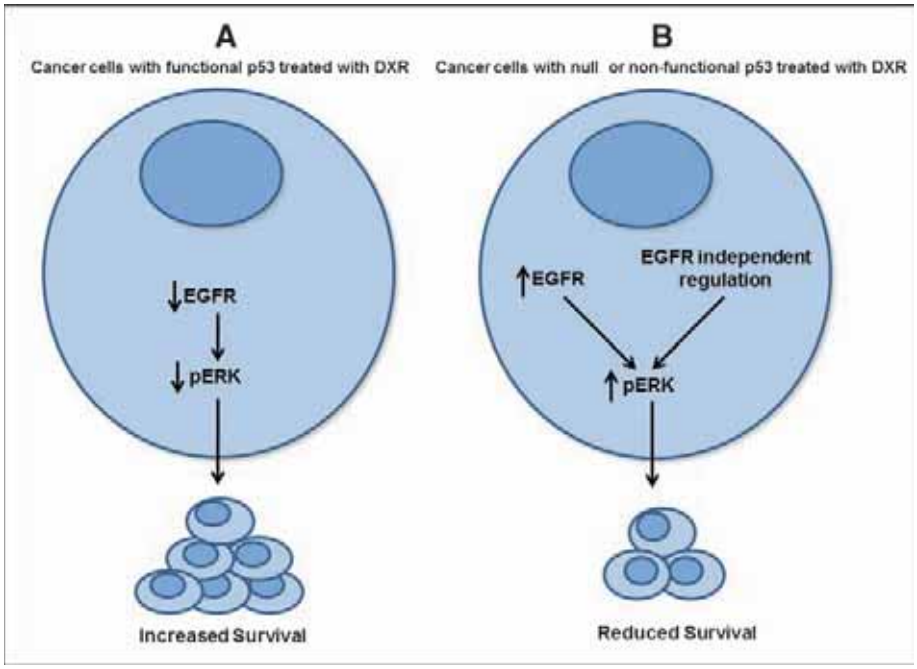

Figure 7. Schematic representation. (**A**) Functional p53 decreases EGFR level, causing a decrease in pERK level resulting in decreased sensitivity towards doxorubicin. (**B**) Activation of ERK either by EGFR or independent of EGFR, decreases survival by increasing sensitivity to DXR in p53 non-functional cells.

DXR treated MCF-7As, H1299 and MDA MB-468 cells. This finding is consistent with the earlier reports documenting enhanced cytotoxicity in cells with p53-null phenotype (Lehmann-Che *et al.* 2010; Fernández *et al.* 2012).

It has been reported that colorectal cancer cells having mutant p53 are more sensitive towards cetuximab therapy because of increased EGFR level (Oden-Gangloff *et al.* 2009). This suggests an inter-relationship between p53 and EGFR, which warrants investigation. We observed that cancer cells with non-functional p53 have increased level of EGFR. Expression of wild-type p53 in these cells reduces EGFR protein level indicating that in p53 non-functional cells there is a constitutive level of EGFR, which is kept under check by wild-type p53. Inhibition of EGFR by AG1478 reduces EGFR level. Treatment of cells with DXR in the presence of AG1478 promotes cell survival suggesting that EGFR plays a role in sensitization of p53 non-functional cells to DXR. Also, an increase in the levels of EGFR achieved by treatment of MCF-7 cells with EGF ligand increases its sensitivity to DXR. However, in MCF-7As cells EGF treatment does not cause any change in the EGFR level. These findings collectively indicate that p53 regulates EGFR, which is an important determinant for sensitivity of cancer cells to DXR. The above-stated observations are in agreement with that reported earlier (Huang *et al.* 2011).

Downstream target of EGFR is ERK and its phosphorylated form is linked to DNA-damage-induced apoptosis (Wang *et al.* 2000; Singh *et al.* 2007; Rieber and Rieber 2009). High level of pERK has been detected in malignant tumours and decrease in pERK level has been shown to reduce tumour formation (Hoshino *et al.* 2001). The relationship between pERK and sensitivity of the cells toward drugs is not well understood. It is evident from our finding that inhibition of EGFR decreases pERK level while the basal ERK level remains unchanged. U0126 significantly reduce pERK level in all the cells. Inhibition of EGFR promotes cell survival in MCF-7As, H1299 and MDA MB-468 cells but not in MCF-7 cells. Although inhibition of EGFR does not change percentage cell survival in MCF-7 cells, inhibition of ERK increases survival of DXR treated cells. This finding is supported by observation that MCF-7 cells have low level of EGFR. On the other hand, in MCF-7 cells the survival increased in the presence of U0126 suggesting sensitivity of cells towards DXR exhibits dependency on ERK activation. Inhibition of EGFR and pERK increases survival in p53 non-functional cells. However, in MCF-7 cells the presence of both inhibitors together does not alter sensitivity to DXR.

Another well-known target of EGFR is AKT and thus we attempted at investigating role of AKT in sensitivity towards DXR in p53 functional and p53 non-functional cell lines. We

observed that pAKT levels change in response to EGFR inhibition in p53 wild-type cells but not in cells having non-functional p53 (supplementary figure IV A). Additionally, we observed that inhibition of AKT by LY294002 increases survival of cells with functional p53 while no significant effect was observed in cells with non-functional p53 (supplementary figure IV B). These results suggest that pAKT affects sensitivity towards DXR in only p53 wild-type cells while in p53 non-functional cells AKT does not affect the sensitivity towards DXR.

Further we demonstrate that inhibition of constitutively activated ERK increases survival in all the cells treated with DXR. Surprisingly, the presence of wild-type p53 in cells with non-functional p53 does not alter the pERK level. However, an increased survival of cells in which p53 is not functional is observed in the presence of wild-type p53. These results indicate that ERK-mediated cell death is independent of p53 status in cancer cells. Also, high levels of pERK in p53 non-functional cells might be due to ERBB2-dependent signals (Kim *et al.* 2008). However, despite of EGFR dependency, p53 status seems to be a crucial factor in determining the sensitivity of the cells to DXR. This finding is consistent with reports where it has been shown that activation of ERK following DNA damage leads to cell cycle arrest causing apoptosis in a p53 independent manner (Tang *et al.* 2002; Wei *et al.* 2011).

Activation of EGFR by EGF has been shown to enhance the cytotoxic effect of cisplatin (Jen-Chung Ko *et al.* 2008; Ahsan *et al.* 2010). It is also reported that abrogation of EGFR by its antisense decreases cisplatin induced apoptosis in MDA MB-468 cells (Dixit *et al.* 1997). High level of EGFR expression has also been correlated with increased sensitivity to cisplatin in cervical cancer cells (Donato *et al.* 2000). All these findings propose a role of EGFR in sensitivity towards chemotherapeutic agents. To delineate the role of EGFR, we exposed MCF-7 cells to EGF and treated with DXR. Elevated levels of EGFR by EGF enhances cell death induced by DXR.

To conclude, we propose that p53 non-functionality causes increased sensitivity of cells towards DXR. This is because of increase in EGFR protein and pERK levels. However, pERK-mediated cell death is not directly dependent on p53 status in cancer cells. Therefore, cells with p53 non-functionality and elevated EGFR are more sensitive to DXR.

Acknowledgements

The authors thank Dr SC Mande, Director, NCCS, Pune, and Dr GC Mishra, former Director, NCCS, Pune, for their support and encouragement in carrying out this work. RK and SS thank the University Grants Commission (UGC), RRC and SC thank the Council for Scientific and Industrial Research (CSIR), AKA thanks the Indian Council of Medical Research (ICMR), India, for research fellowships. This work was supported in part by intramural funding from National Centre for Cell Science (NCCS), Department of Biotechnology (DBT), India. The funding agencies had no involvement in study design, data collection, interpretation and analysis, decision to publish or writing of the manuscript.

Work described in this paper is a part of thesis submitted by RK in 2010 to University of Pune, Pune.

References

Ahsan A, Hiniker SM, Ramanand SG, *et al*. 2010 Role of epidermal growth factor receptor degradation in cisplatin-induced cytotoxicity in head and neck cancer. *Cancer Res.* **70** 2862–2869

Busse D, Doughty RS, Ramsey TT, *et al*. 2000 Reversible G(1) arrest induced by inhibition of the epidermal growth factor receptor tyrosine kinaserequires upregulation of p27(KIP1) independent of MAPK activity. *J. Biol. Chem.* **275** 6987–6995

Chhipa RR and Bhat MK 2007 Bystander killing of breast cancer MCF-7 cells by MDA-MB-231 cells exposed to 5-fluorouracil is mediated via Fas. *J. Cell. Biochem.* **101** 68–79

Chhipa RR, Kumari R, Upadhyay AK, *et al*. 2007 Abrogation of p53 by its antisense in MCF-7 breast carcinoma cells increases cyclin D1 via activation of Akt and promotion of cell proliferation. *Exp. Cell Res.* **313** 3945–3958

Coradini D, Biganzoli E, Ardoino, *et al*. 2015 p53 status identifies triple-negative breast cancer patients who do not respond to adjuvant chemotherapy. *Breast* **3** 294–297

Cui T, Chen Y, Yang L, *et al*. 2012 The p53 target gene desmocollin 3 acts as a novel tumor suppressor through inhibiting EGFR/ERK pathway in human lung cancer. *Carcinogenesis* **33** 2326–2333

Dixit M, Yang JL, Poirer MC, *et al*. 1997 Abrogation of cisplatin-induced programmed cell death in human breast cancer cells by epidermal growth factor antisense RNA. *J. Natl. Cancer Inst.* **89** 365–373

Donato NJ, Perez M, Kang H, *et al*. 2000 EGF receptor and p21WAF1 expression are reciprocally altered as ME-180 cervical carcinoma cells progress from high to low cisplatin sensitivity. *Clin. Cancer Res.* **1** 193–202

Dong P, Xu Z, Jia N, *et al.* 2009 Elevated expression of p53 gain-of-function mutation R175H in endometrial cancer cells can increase the invasive phenotypes by activation of the EGFR/PI3K/AKT pathway. *Mol. Cancer.* **8** 103

El-Deiry WS 2003 The role of p53 in chemosensitivity and radiosensitivity. *Oncogene* **22** 7486–7495

Fernández CL, Oakman C, Falagan LP, *et al*. 2012 Prognostic and predictive value of TP53 mutations in node-positive breast cancer patients treated with anthracycline- or anthracycline/taxane-based adjuvant therapy: results from the BIG 02-98 phase III trial. *Breast Cancer Res.* **14** R70

Garcia R, Franklin RA and McCubrey JA 2006 Cell death of MCF-7 human breast cancer cells induced by EGFR activation in the absence of othergrowth factors. *Cell Cycle* **16** 1840–1846

Hoshino R, Tanimura S, Watanabe K, *et al.* 2001 Blockade of the extracellular signal-regulated kinase pathway induces marked G1 cell cycle arrest and apoptosis in tumor cells in which the pathway is constitutively activated: up-regulation of p27(Kip1). *J. Biol. Chem.* **276** 2686–2692

Huang S, Benavente S, Armstrong EA, *et al*. 2011 p53 modulates acquired resistance to EGFR inhibitors and radiation. *Cancer Res.* **71** 7071–7079

Kim KK, Lee JJ, Yang Y, *et al*. 2008 Macrophage inhibitory cytokine-1 activates AKT and ERK-1/2 via the transactivation of ErbB2 in human breast and gastric cancer cells. *Carcinogenesis* **29** 704–712

Ko JC, Ciou SC, Cheng CM, *et al*. 2008 Involvement of Rad51 in cytotoxicity induced by epidermal growth factor receptor inhibitor (gefitinib, IressaR) and chemotherapeutic agents in human lung cancer cells. *Carcinogenesis* **29** 1448–1458

Lachat Y, Diserens AC, Nozaki M, *et al*. 2004 INK4a/Arf is required for suppression of EGFR/DeltaEGFR(2-7)- dependent ERK activation in mouse astrocytes and glioma. *Oncogene* **23** 6854–6863

Lehmann-Che J, André F, Desmedt C, *et al*. 2010 Cyclophosphamide dose intensification may circumvent anthracycline resistance of p53 mutant breast cancers. *Oncologist* **15** 246–252

Licitra L, Suardi S, Bossi P, *et al*. 2004 Prediction of TP53 status for primary cisplatin, fluorouracil, and leucovorin chemotherapy in ethmoid sinus intestinal-type adenocarcinoma. *J. Clin. Oncol.* **22** 4901–4906

Marampon F, Bossi G, Ciccarelli C, *et al*. 2009 MEK/ERK inhibitor U0126 affects in vitro and in vivo growth of embryonal rhabdomyosarcoma. *Mol. Cancer Ther.* **8** 543–551

Maruthampu M, Watkins J, Noor AM, *et al.* 2016 Economic downturns, universal health coverage, and cancer mortality in high-income and middle-income countries 1990-2010: a longitudinal analysis. *Lancet* **388** 684–695

Moerkens M, Zhang Y, Wester L, *et al.* 2014 Epidermal growth factor receptor signalling in human breast cancer cells operates parallel to estrogenreceptor α signalling and results in tamoxifen insensitive proliferation. *BMC Cancer* **14** 383

Mollgard L, Saft L, Treppendahl MB, *et al*. 2011 Clinical effect of increasing doses of lenalidomide in high-risk myelodysplastic syndrome and acute myeloid leukemia with chromosome 5 abnormalities. *Haematologica* **96** 963–971

Oden-Gangloff A, Di Fiore F, Bibeau F, *et al.* 2009 TP53 mutations predict disease control in metastatic colorectal cancer treated with cetuximab-based chemotherapy. *Br. J. Cancer.* **100** 1330–1335

Patricia AJ, Muller PAJ and Vousden KH 2014 Mutant p53 in cancer: new functions and therapeutic opportunities. *Cancer Cell* **25** 304–317

Rieber M and Rieber MS 2009 DN-R175H p53 mutation is more effective than p53 interference in inducing epithelial disorganization and activation of proliferation signals in human carcinoma cells: role of E-cadherin. *Int. J. Cancer* **125** 1604–1612

Singh S, Upadhyay AK, Ajay AK, *et al*. 2007 p53 regulates ERK activation in carboplatin induced apoptosis in cervical carcinoma: a novel target of p53 in apoptosis. *FEBS Lett.* **581** 289–295

Tang D, Wu D, Hirao A, *et al*. 2002 ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. *J. Biol. Chem.* **277** 12710–12717

Upadhyay AK, Ajay AK, Singh S, *et al*. 2008 Cell cycle regulatory protein 5 (Cdk5) is a novel downstream target of ERK in carboplatin induced death of breast cancer cells. *Curr. Cancer Drug Targets* **8** 741–752

Ventura A, Krisch DG, McLaughlin ME, *et al*. 2007 Restoration of p53 function leads to tumour regression in vivo. *Nature* **445** 661–665

Wang X, Martindale JL and Holbrook NJ 2000 Requirement for ERK activation in cisplatin-induced apoptosis. *J. Biol. Chem.* **275** 39435–39443

Wansom D, Light E, Worden F, *et al*. 2010 Correlation of cellular immunity with human papillomavirus 16 status and outcome in patients with advanced oropharyngeal cancer. *Arch. Otolaryngol. Head Neck Surg.* **136** 1267–1273

Wei F, Yan J and Tang D 2011 Extracellular signal-regulated kinases modulate DNA damage response - a contributing factor to using MEK inhibitors in cancer therapy. *Curr. Med. Chem.* **18** 5476–5482

MS received 15 June 2016; accepted 12 January 2017

Corresponding editor: Sorab N Dalal