EGFR Nuclear Translocation Modulates DNA Repair following Cisplatin and Ionizing Radiation Treatment

Gianmaria Liccardi, John A. Hartley, and Daniel Hochhauser

Abstract

Epidermal growth factor receptor (EGFR) overexpression is associated with resistance to chemotherapy and radiotherapy. It modulates DNA repair after radiation-induced damage through association with the catalytic subunit of DNA protein kinase (DNA-PKcs). We investigated the role of EGFR nuclear import and its association with DNA-PKcs on DNA repair after exposure to cisplatin or ionizing radiation (IR). The model system was based on EGFR-null murine NIH3T3 fibroblasts in which EGFR expression was restored with isoforms that were wild-type (wt), derived from human cancers (L858R, EGFRvIII), or mutated in the nuclear localization signal (NLS) sequence. In cells expressing wtEGFR or EGFRvIII, there was complete unhooking of cisplatin-induced interstrand cross-links and repair of IR-induced strand breaks. In contrast, cells expressing L858R or NLS mutations showed reduced unhooking of interstrand cross-links and repair of strand breaks. Immunoprecipitation showed wtEGFR and EGFRvIII binding to DNA-PKcs, increasing 2-fold 18 hours after cisplatin therapy. Confocal microscopy and proximity ligation assay showed that this interaction in the cytoplasm and nucleus was associated with increased DNA protein kinase complex (DNA-PK) activity. Cells expressing the EGFR L858R mutation, which has constitutive kinase activity, exhibited reduced DNA repair without nuclear localization. EGFR-NLS mutants showed impaired nuclear localization and DNA-PKcs association with reduced DNA repair and DNA-PK kinase activity. In summary, EGFR nuclear localization was required for modulation of cisplatin and IR-induced repair of DNA damage. EGFR–DNA-PKcs binding was induced by cisplatin or IR but not by EGFR nuclear translocation per se. Our findings show that EGFR subcellular distribution can modulate DNA repair kinetics, with implications for design of EGFR-targeted combinational therapies. Cancer Res; 71(3); 1103–14. ©2011 AACR.

Introduction

The epidermal growth factor receptor (EGFR) promotes the activation of survival signaling pathways including RAS/MAPK (mitogen-activated protein kinase), PI3K (phosphoinositide 3-kinase)/AKT, and JAK (Janus activated kinase)/STAT (1, 2). Increased EGFR activation and overexpression is strongly associated with tumorigenesis and cancer progression (3). EGFR is an important target for cancer therapies including antibodies disrupting ligand–receptor interactions such as cetuximab (4, 5) and small molecules inhibiting EGFR kinase activity including gefitinib (6, 7) and erlotinib (8). There has been extensive investigation on the mechanisms by which EGFR inhibition modulates the activity of chemotherapy and radiation (3). Combinations of the monoclonal antibody (mAb) cetuximab with cisplatin or radiation have been useful clinically in the treatment of head and neck and colon cancer (9, 10). In contrast, despite effects in vitro (11), only small benefits have been obtained combining small molecules inhibiting EGFR with conventional treatment. Several studies have shown the association of EGFR with the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), a central component of the nonhomologous end-joining (NHEJ) pathway involved in the repair of DNA strand breaks (12, 13).

Recently, it has been reported that a fraction of intracellular EGFR is located within the nucleus where it may activate transcription of genes, including cyclin D1, iNOS, c-myc, and COX-2, that are associated with cell proliferation and the nitric oxide pathway (14–17). Evidence for the expression and activity of nuclear EGFR has been found using a variety of techniques including fractional immunoblotting, confocal microscopy, electron microscopy, reporter assays, and chromatin immunoprecipitation. Nuclear EGFR has been shown to correlate with worse prognosis in a variety of malignancies including breast, head and neck, and ovarian cancer (16, 18). The intracellular localization of EGFR may therefore have profound effects on response both to chemotherapy and to novel therapies inhibiting the EGFR pathway.

EGFR cellular distribution is dictated by several regulatory motifs within the juxtamembrane (JX) domain (19). Two
basolateral signals control EGFR re-sorting to the transmembrane (20), whereas the lysosomal signal (accessible following EGFR activation) determines EGFR degradation (21). The nuclear localization signal (NLS) sequence, comprising 13 amino acids 645 to 657 (RRRHIVRKKLLRR; ref. 22), has a dual role. It allows nuclear translocation via sequence recognition and binding to importin β (23) and mediates EGFR allosteric conformational change and dimer stabilization, which are indispensable for the receptor activation (24, 25).

Studies on the somatically acquired, constitutively active EGFR mutant L858R, found in certain non–small cell lung cancers, have shown impaired nuclear localization and DNA-PKcs binding (26). This suggests that EGFR activation and nuclear translocation are related and that nuclear localization may modulate DNA repair.

Nuclear translocation of EGFR following ionizing radiation (IR) has been shown to result in increased repair of DNA strand breaks (12). The effects of nuclear translocation on repair of chemotherapy-induced DNA damage are less clear. We therefore investigated the significance of nuclear localization for the repair of cisplatin- and IR-induced DNA damage, using EGFR constructs with mutations in the NLS and mutations found in human cancers (EGFRvIII, L858R). Cells expressing EGFR with mutations that impair nuclear transport showed reduced repair of DNA strand breaks following IR and reduced unhooking of interstrand cross-links following treatment with cisplatin, as compared with cells expressing wild-type (wt) EGFR. Immunoprecipitation experiments confirmed association of EGFR with DNA-PKcs following treatment with cisplatin or IR. Confocal microscopy demonstrated that cells with mutations in the NLS failed to translocate to the nucleus following IR and cisplatin treatment. These findings confirm the importance of nuclear translocation of EGFR in mediating effects on DNA repair and emphasize the significance of subcellular EGFR expression in determining responses to therapy.

Materials and Methods

Materials

Cisplatin (DBL 1 mg/mL) was obtained from Mayne Pharma PLC. Epidermal growth factor (EGF) was obtained from Sigma-Aldrich.

Irradiation condition

Cells were plated at a concentration of 1 × 105/mL. Following 48 hours transfection, cells were serum starved for 24 hours and irradiated with a dose of 4 Gy with the A.G.O. HS 321kV X-ray system.

Cell lines and culture conditions

NIH3T3 mouse fibroblast cell lines (obtained from CR-UK London Research Institute) were grown in Dulbecco’s minimal essential medium (Autogen Bioclear). Transfected NIH3T3 cells were grown in the same medium containing G418-selective agent (Sigma-Aldrich) at a concentration of 1 mg/mL. All cells were supplemented with 10% fetal calf serum and 1% glutamine and incubated at 37°C in 5% CO2.

Plasmids and site-directed mutagenesis

The plasmid DNA was the pUSEampa vector (Upstate Cell Signaling Solutions).

The wtEGFR and the L858R constructs were kindly provided by Dr. Daphne Bell and Matthew Meyerson from the MGH Cancer Centre, Harvard Medical School, Boston, MA.

The NLS mutant constructed used the wtEGFR and the L858R plasmids as a template. The 2 designed and SDS-purified mutagenic primers (forward: 5’-CTCTTCTATGG-CAGCCGGCCACATCCTTGGAAGCCAGCTGGGCGCC-TGCTGCAGG-3’; and reverse: 5’-CTCTTGACGACGGCCGCAC-GGTGGCCCTCGCAACGATGTGGGCCGCTGCCATGAAGA- GG-3’) were utilized according to the site-directed mutagenesis XL kit protocol (Stratagene) to change the EGFR NLS sequence 645-RRRHIVRKKLLRR-657 into 645-AAAHIVAKA TLAA-657.

EGFRvIII was kindly provided by Prof. William Gullick from the Department of Biosciences University of Kent, Canterbury, UK.

EGFR M1, M12, KMT, and ANLS encoding point mutations of the EGFR NLS sequence (M1: AAAHIVRKKLR; M12: AAAHIVAAATLRR), the deletion of the NLS sequence (ΔNLS), and a mutation within the kinase domain (KMT: K821A) were kindly obtained from Prof. M.C. Hung (MD Anderson Cancer Center, Houston, TX).

Plasmid transfection

Cells were plated at 1 × 105/mL and following 2 hours, cells were transfected according to the GeneJuice transfection reagent protocol (Novagen EMD Bioscience). Cells were then treated 48 hours following transfection, or serum starved for 24 hours and then treated.

Alkaline single-cell gel electrophoresis (comet) assay

Measurement of DNA interstrand cross-links was done as previously described (27).

Repair of DNA strand break in cells irradiated with a dose of 15 Gy was measured using the comet assay as previously described (28).

Data were presented as a percentage of tail moment, that is, as a percentage of the amount of strand breaks resulting immediately following IR treatment.

Statistical analysis

The 2-way ANOVA, Bonferroni’s posttests, and Student’s t test were used for calculating the significance of the differences in repair and DNA-PK activity. All the cell lines were considered individually and compared with the wtEGFR-expressing cell line. Statistical values of P < 0.01 were considered significant.

Immunoprecipitation

Stably expressing NIH3T3 cell lines were plated at 2 × 105/mL and left overnight before treatment. Cells were then incubated in growing medium or treated with 50 μmol/L cisplatin for 1 hour in serum-free media and then left in drug-free medium for 18 hours, or serum starved for 24 to 36 hours and treated with 100 ng/mL EGF or treated with 4-Gy IR
and incubated 20 minutes in serum-free media. Approximately 5 x 10^6 cells were lysed on ice in 500 µL of CellLyticM Cell lysis reagent (Sigma) supplemented with protease and phosphatase inhibitor (Roche) and Benzonase (Merck) according to manufacturer’s protocol. A 1.5 mg sample of protein was incubated with 2 µg of anti-EGFR antibody (clone R19/48; Invitrogen) and left rotating at 4°C for 2.5 hours. Immunoprecipitation was done as previously described (29).

**Western blotting**

Western blotting was done as previously described (27). Proteins were probed using anti-EGFR (Cell Signaling; 1:1,000), anti-DNA-PKcs (AbCam; 1:400), anti-PY20 (Santa Cruz; 1:1,000). Finally, the primary antibody was probed with horse-anti-DNA-PKcs (AbCam; 1:400), anti-PY20 (Santa Cruz; 1:1,000), and anti-EGFR antibodies (Cell Signaling; 1:1,000), respectively. Immunoprecipitation was carried out until the primary antibody incubation. Cy3 signal amplification was utilized for the assay. Cells were examined by confocal microscopy (objective: ×40; Leica TCS SP2). Nuclear slice images were acquired by sequential scanning by using the LAS AF Lite program.

**DNA-PK functional assay**

DNA-PK activity was detected using the Promega Signa-TECT DNA-PK assay system, according to the manufacturer’s protocol. The enzymatic activity of DNA-PK was analyzed by scintillation counting and expressed as a percentage change of control DNA-PK activity as measured in untreated cells.

**Proximity ligation assay**

Proximity ligation was done according to the manufacturer’s protocol, using the Duolink Detection Kit (Cambridge BioScience Ltd.). NIH3T3 cells were grown on 13-mm glass cover slips (VWR) and treated with cisplatin or IR as detailed earlier. Immunofluorescence staining protocol was carried out until the primary antibody incubation. Cy3 signal amplification was utilized for the assay. Cells were examined with a confocal microscope (objective: ×40; Leica TCS SP2).

**Results**

**EGFR nuclear translocation modulates DNA repair**

The synergistic effects of both cisplatin and IR with EGFR inhibition have been well described (12, 23). However, the role and consequences of EGFR nuclear translocation and kinase activation in the repair of drug-induced DNA interstrand cross-links, or radiation-induced DNA strand breaks, have not been fully examined. To probe the role of EGFR in modulating DNA repair, the EGFR-negative cell line NIH3T3 was transfected with each of 10 plasmids [wtEGFR, NLS123, L858R, LNLs123, EGFRvIII, M1, M12, KMT, ΔNLS, and vector control (VC)], and therapy-induced DNA damage and its repair were assessed. The resulting transfectants express either wtEGFR or mutations within the NLS sequence (NLS123, LNLs123, M1, M12, and ΔNLS), kinase domain (L858R, KMT), or extracellular domain (EGFRvIII) of EGFR (Fig. 1A). Their expression was confirmed over a period of 72 hours following transfection (Supplementary Data S1). Transfected cells were incubated with 50 µmol/L cisplatin for 1 hour or treated with 15 Gy IR. Formation and repair of cisplatin-induced interstrand cross-links, critical cytotoxic lesions produced following drug treatment, were measured over a 48-hour period by a modification of the comet assay as previously described (ref. 27; Fig. 1B). Repair of IR-induced strand breaks was measured over a 4-hour period (Fig. 1C).

There was no alteration in the formation of the peak of DNA interstrand cross-links by cisplatin in cells expressing any of the constructs (Supplementary Fig. S2 and Supplementary Table S1). However, cells expressing mutations of the NLS sequence (NLS123, LNLs123) and of the kinase domain (L858R, KMT, ΔNLS) clearly showed reduction in repair (unhooking) of interstrand cross-links (Fig. 1B). Unhooking of cisplatin-induced interstrand cross-links was greater than 95% by 36 hours and complete by 48 hours in cells expressing wtEGFR and EGFRvIII. In contrast, cells expressing mutations NLS123 and LNLs123 showed only 26% ± 4.07% and 19.3% ± 4.8% unhooking at 48 hours, respectively. Intermediate levels of unhooking were observed for the L858R (46.57% ± 2.13%), KMT (54.95% ± 2.56%), and ΔNLS (37.21% ± 9.72%) mutants, whereas M1- and M12-expressing cell lines showed 84.09% ± 4.87% and 97.41% ± 2.73% unhooking of interstrand cross-links, respectively, at 48 hours following cisplatin treatment. Statistical analysis showed significance (P < 0.01) at the 48-hour time points and/or at earlier time points among the different mutants (Table 2A Supplementary Data).

The effect of the EGFR constructs on repair of DNA strand breaks induced by IR was also investigated. Repair of IR-induced DNA strand breaks is shown as the percentage of the IR-induced tail moment calculated from the comet assay data (Fig. 1C). Decrease of tail moment was 100% in both wtEGFR- and EGFRvIII-expressing cell lines at 4 hours following treatment, indicating complete repair of strand breaks. Significant differences (P < 0.001) in repair kinetics between wtEGFR and the mutant EGFR-expressing cell lines were found at 30 minutes following IR (Table 2B Supplementary Data). At 4 hours, cells expressing NLS123 and LNLs123 showed significant delay in repair of strand breaks with 22.48% ± 3.72% and 24.94% ± 1.45% unrepaired strand breaks, respectively. Intermediate levels of repair were observed for cells expressing L858R (12.33 ± 1.00%), KMT (18.86 ± 3.45%), ΔNLS (17.38 ± 5.06%), M1 (8.51 ± 1.12%), and M12 (9.28 ± 2.26%) plasmids.

**wtEGFR and EGFRvIII associate with DNA-PKcs following treatment with IR or cisplatin**

Previous studies have shown association of EGFR and DNA-PKcs following IR treatment (12, 30, 31). However, this...
association and its significance have not been described following cisplatin treatment. NIH3T3 cells were transfected with wtEGFR and treated with 50 μmol/L cisplatin for 1 hour. Cells were then collected at various time points up to 24 hours following treatment, protein extracts were prepared, immunoprecipitated using an anti-DNA-PKcs mAb, and blotted with an anti-EGFR antibody (Fig. 2A). There was a time-dependent association of EGFR and DNA-PKcs, resulting in a 2.7-fold increase at 18 hours following the cisplatin treatment.

To determine the levels of EGFR–DNA-PKcs association and whether it is induced by cisplatin or IR, we compared the levels of this association following cisplatin, IR, and EGF treatment. Experiments were carried out using extracts from cells expressing wtEGFR, NLS123, L858R, LNLS123, EGFRvIII, M1, M12, KMT, ΔNLS, and VC were treated with (B) 50 μmol/L cisplatin alone and (C) 15-Gy IR. Interstrand cross-link formation is represented as a percentage decrease of the peak of cross-link, and strand breaks are shown as a percentage of tail moment.
antibody, and blotted using an anti-DNA-PKcs antibody. Western blot analysis showed that in cells expressing wtEGFR and EGFRvIII, there is association with DNA-PKcs following treatment with IR or cisplatin. However, cells expressing NLS123, L858R, and LNLS123 showed no interaction between EGFR and DNA-PKcs. The immunoprecipitated samples were also blotted with a pan-phosphotyrosine antibody to determine the activity of EGFR. Cells expressing wtEGFR showed maximal activation of the receptor following EGF treatment. Intermediate levels of activation were detected following IR or cisplatin treatment, L858R-, LNLS123-, and EGFRvIII-expressing cells showed a constitutive activation of the receptor, whereas NLS123 showed no receptor activation. Therefore, the EGFR-DNA-PKcs binding is triggered by cisplatin or IR treatment and not by the EGFR nuclear translocation per se.

**DNA-PKcs and EGFR localize in the same cellular compartments following IR or cisplatin treatment**

Having established an association between EGFR and DNA-PKcs following cisplatin or IR treatment, we investigated their cellular localization by confocal microscopy following IR and cisplatin treatment. Cell transiently transfected with wtEGFR and EGFRvIII showed clear EGFR nuclear expression following cisplatin (Fig. 3) or IR treatment (Fig. 4). In contrast, cells transiently transfected with NLS123, L858R, LNLS123, KMT, and ΔNLS showed a lack of EGFR nuclear accumulation. M1- and M12-transfected cells showed only reduced EGFR nuclear expression following IR or cisplatin treatment. Next, stably transfected cells were utilized to investigate this pattern. Cells expressing wtEGFR and EGFRvIII showed nuclear expression of both EGFR and DNA-PKcs following cisplatin (Fig. 5) or IR
treatment (Fig. 6). In contrast, L858R-expressing cells showed impaired EGFR nuclear localization following cisplatin or IR treatment. Expression of EGFR and DNA-PKcs was exclusively cytosolic in NLS123- and LNLS123-expressing cell lines following cisplatin (Fig. 5) or IR treatment (Fig. 6). Therefore, the NLS123 mutation inhibits EGFR nuclear localization and also indirectly inhibits DNA-PKcs subcellular localization following IR or cisplatin treatment. Nuclear translocation of EGFR was verified via cellular fractionation following cisplatin and IR treatment (data not shown).

**EGFR and DNA-PKcs association following cisplatin or IR treatment**

Previous studies have shown binding between EGFR and DNA-PKcs in the nucleus following EGFR nuclear translocation (23, 32). To investigate the colocalization of EGFR and DNA-PKcs following treatment with cisplatin or IR, we conducted a Duolink proximity assay. This assay allows visualization of the interaction between 2 proteins in fixed cells. Each interaction is represented via a single red fluorescent dot (Fig. 7A). In cells expressing vector and NLS123 constructs, no interaction was detectable. In contrast, cells expressing wtEGFR and EGFRvIII showed subcellular interaction between EGFR and DNA-PKcs following IR or cisplatin treatment.

**EGFR modulation of DNA-PK activity**

We previously showed that the association of EGFR and DNA-PKcs resulted in stimulation of DNA-PKcs activity (31). The experiments detailed earlier showed that nuclear translocation of EGFR is required for the association with DNA-PKcs. To investigate whether this association resulted in an alteration in enzyme activity, we investigated the effects of expression of different EGFR constructs on DNA-PK kinase activity. As compared with individual untreated control, cells expressing wtEGFR showed a 27.5% ± 7.22% increase in DNA-PK activity following IR treatment (4 Gy) and a 37.52% ± 4.01% increase following 50 μmol/L cisplatin treatment for 1 hour (Fig. 7B). In cells expressing EGFRvIII, there was a 32.42% ± 16.58% increase in DNA-PK activity following IR treatment and a 26.6% ± 8.49% increase following cisplatin treatment. In contrast, no significant change in DNA-PK activity compared with controls was found in L858R- and LNLS123-expressing cells following IR (−5.29% ± 8.27% and 2.72% ± 7.06%) or cisplatin treatment (9.04% ± 3.46% and 2.25% ± 6.07%). Results are shown in
Table 2C Supplementary Data. Only cells expressing NLS123 showed a clear decrease in DNA-PK kinase activity compared with control following IR (−36.95% ± 8.08%) or cisplatin treatment (−43.30% ± 5.82%). Therefore, nuclear localization is required for EGFR-induced stimulation of DNA-PK activity. EGF treatment did not induce a significant change in DNA-PK kinase activity (P > 0.05) in NLS123-, LNLS123-, and EGFRvIII-expressing cell lines; only L858R showed borderline significant differences (P < 0.05) compared with the wtEGFR-expressing cells.

Discussion

Inhibitors of EGFR play a major role in cancer therapeutics. However, the activity of these agents as monotherapies is low, and it is important to investigate regimens with optimal combinations by using chemotherapy and radiation (3). There is evidence of the effects of EGFR inhibition on DNA repair following irradiation. In this study, we show the importance of nuclear EGFR in modulating the repair of DNA damage following cisplatin chemotherapy or radiation.
Expression of EGFR in the nucleus is well established, but the implications on the effects of therapy are not clear. According to recent reports, EGFR nuclear translocation requires receptor dimerization and activation, as, following internalization, mature and active EGFR may become a poor substrate for lysosomal degradation (33, 34). This allows either indirect sorting of the receptor through the Golgi or direct sorting through the endoplasmic reticulum. Subsequent association with Sec61 results in retrotranslocation to the cytosol where EGFR is stabilized following association with HSP70 (33). Binding of importin β, mediated through the NLS sequence, translocates the receptor to the nucleus (22, 33). Inactive receptors (or those without an active conformation) are usually sent back to the plasma membrane via the recognition of a basolateral signal in the JX domain (19).

The interaction of EGFR with DNA-PKcs has been shown in several studies (12). The DNA-PK complex plays a key role in NHEJ, the major method of repair of DNA strand breaks following IR treatment. Interaction of EGFR with DNA-PKcs has been shown to contribute to the repair of DNA strand breaks. Inhibition of EGFR, by cetuximab or gefitinib, inhibits repair of IR-induced DNA strand breaks and impairs EGFR–DNA-PKcs interaction (4, 11).

It is well known that interstrand cross-links contribute significantly to cisplatin cytotoxicity and that unhooking of interstrand cross-links may be used to determine clinical sensitivity (35). In previous studies, we showed that the unhooking of cisplatin DNA interstrand cross-links was inhibited by gefitinib and that this is mediated through the DNA-PK pathway (31). This pathway has been shown to have relevance in the repair of cisplatin-induced DNA damage (36).

In this study, we show that EGFR nuclear expression in transfected EGFR-null cells modulates repair of DNA damage through the DNA-PK pathway. Cells expressing EGFR constructs with mutated NLS sequence are inhibited in their ability to unhook DNA interstrand cross-links (35). Abrogation of nuclear expression of EGFR results in significant delay in repair of interstrand cross-link in these cells. This correlates with reduced association of EGFR with DNA-PKcs. Cells expressing constructs that do not translocate to the nucleus

Figure 5. EGFR and DNA-PKcs cellular localization following cisplatin treatment. NIH3T3 cells stably expressing wtEGFR, NL123, L858R, LNLS123, EGFRvIII, and VC were treated with 50 μmol/L cisplatin for 1 hour in serum-free media and then fixed with 4% PFA 18 hours following treatment. Cells were stained with goat anti-rabbit Alexa Fluor 647 (EGFR), goat anti-mouse Alexa Fluor 488 (DNA-PKcs), and DAPI (nucleus).
showed increased sensitivity to cisplatin (Supplementary Fig. S3). Previous studies have shown that EGFR nuclear translocation correlated with repair of IR-induced strand breaks.

It has been shown previously that the association between DNA-PKcs and EGFR peaks at 20 minutes following IR treatment (37), but association following cisplatin treatment has not been described. Here, we show that binding peaks at 18 hours following cisplatin treatment. The different timing following cisplatin and IR treatment likely reflects different types of DNA lesions and repair mechanisms. There was reduced repair of DNA IR-induced strand breaks in cells expressing EGFR constructs that do not translocate to the nucleus. Repair of DNA strand breaks following IR treatment has been shown to be modulated by EGFR (37) and the less marked effects of impaired EGFR nuclear translocation as compared with the repair of cisplatin-induced interstrand cross-links may be secondary to the activation of other DNA repair pathways (12).

In this study, the nuclear translocation of EGFR was shown by confocal microscopy. Interestingly, these experiments suggest that there is colocalization of EGFR and DNA-PKcs both within the nucleus and the cytoplasm. Although the primary location of DNA-PKcs is in the nucleus in the formation of complexes on damaged DNA, cytoplasmic expression of DNA-PKcs has been shown in several studies (38–40). The results of the Proximity ligation assay show physical proximity of DNA-PKcs and EGFR following DNA damage by cisplatin or IR treatment in cells with intact nuclear localization. This was not shown in cells expressing EGFR constructs deficient in nuclear localization.

There is contradictory evidence regarding nuclear expression of EGFRvIII. Although EGFRvIII and STAT3 colocalization within the nucleus was shown in some studies (17, 41), other studies have reported a lack of nuclear expression in glioma models (42). Cells expressing EGFRvIII show elevated activation of DNA-PKcs and enhancement of DNA strand breaks repair. In this study, we show that EGFRvIII and
wtEGFR undergo nuclear translocation and binding with DNA-PKcs following cisplatin or IR treatment.

Although cells expressing kinase-dead EGFR showed a lack of nuclear translocation, expression of the L858R mutant also resulted in impaired nuclear expression despite constitutive kinase activity. This resulted in a reduction in DNA strand breaks repair that is consistent with the observation that non-small cell lung cancer lines expressing L858R show increased sensitivity to IR (43) and reduced nuclear expression (26). Moreover, the difference in repair between cells expressing L858R (kinase active but with impaired nuclear localization) and M1or M12 (kinase active and expressed in the nucleus) suggests that it is the impaired nuclear EGFR accumulation (which is a consequence of the lack of allosteric activation) and not kinase activity per se that determines the reduced DNA repair in these models.

Figure 7. A, EGFR-DNA-PKcs complex cellular localization. Stable NIH3T3 cells expressing wtEGFR, NLS123, EGFRvIII, and VC were treated with 50 μmol/L cisplatin for 1 hour in serum-free media and then fixed with 4% PFA 18 hours following treatment or 4-Gy IR and then fixed with 4% PFA 20 minutes following irradiation. Cells were then immunoblocked with anti-rabbit EGFR and anti-mouse DNA-PKcs. Interacting complexes were then visualized via the Duolink proximity assay. Each red spot represents a single interaction. B, EGFR modulation of DNA-PK kinase activity. Stable NIH3T3 cells expressing wtEGFR, NLS123, L858R, LNLS123, and EGFRvIII were treated with 50 μmol/L cisplatin for 1 hour or 4-Gy IR or 100 ng/mL EGF in serum-free media. Eighteen hours following the treatment with cisplatin, 20 minutes following the treatment with IR, and at 1 hour following EGF incubation, samples were prepared for the DNA-PK kinase assay. The graph shows the percentage change in DNA-PK activity following each treatment compared with untreated control. Stars, statistical significance.
The impaired kinase activity shown by the NLS123 mutant supports the previously described allosteric mode of activation of EGFR and the importance played by the third cluster of arginines (646-RR-647) within the NLS sequence in adopting an α-helical conformation that is indispensable for EGFR activation (23, 34). The impairment of other EGFR functions, as a result of mutations in the NLS sequence, has not been excluded. Interestingly, the LNS123 mutant shows kinase activation despite bearing the same NLS mutation that renders the NLS123 kinase dead. This suggests that the L858R mutation, which has been shown to thermodynamically stabilize EGFR (44, 45), allows receptor activation that does not require the allosteric conformational change. Further work will be required to investigate whether the L858R inhibition of nuclear translocation is due to a structurally hidden NLS sequence.

EGFR inhibition by gefitinib has been shown to suppress DNA repair following treatment with radiation and cisplatin (31). Similarly, in this study, the kinase-dead mutant KMT (mutation K721A) shows no nuclear localization, suggesting that the targeting of the EGFR kinase domain interferes with nuclear translocation and consequently with repair. Maximal effect of gefitinib on the inhibition of interstrand cross-links was observed only in EGFR constructs translocating to the nucleus (Supplementary Fig. S4). A recent study showed that cisplatin resistance and DNA repair were dependent on nuclear translocation (23). Here, we have shown that a variety of EGFR mutants deficient in nuclear expression show impaired repair and that nuclear accumulation is a major determinant of repair of cisplatin-induced interstrand cross-links. In addition, we recently showed that the expression of HER2 modulated repair of cisplatin-induced interstrand cross-links and that this also requires nuclear expression (27). Other factors, including ubiquitination of EGFR induced by cisplatin in head and neck cancer cells, may contribute to the interaction between cisplatin and the EGFR pathway in therapy (46).

There has been extensive study on the interaction of the EGFR and DNA-PK pathways. Following the initial observation that cetuximab treatment inhibits EGFR–DNA-PKcs interaction (47), the role of this interaction in the modulation of DNA repair has been confirmed. Cells expressing specific EGFR mutations found in human cancer such as the L858R in non-small cell lung cancer have been found to show sensitivity to IR. This includes delayed DNA repair kinetics, defective IR-induced arrest in DNA synthesis, and increased apoptosis (26). Here, we show that inhibition of EGFR nuclear translocation alters DNA-PKcs cellular distribution.

Stimulation of DNA-PK kinase activity was associated with nuclear expression and binding. There are likely other factors apart from EGFR–DNA-PKcs binding that may influence the effect of EGFR on DNA repair. EGFr induces nuclear translocation of EGFR, but this is not associated with EGFR–DNA-PKcs interaction. Receptor kinase activation may influence, indirectly, DNA-PK possibly by the activation of the AKT pathway, which has been shown to be a kinase of DNA-PKcs (48, 49).

These results suggest that nuclear expression of EGFR plays a significant role in response to cisplatin and radiation. The intracellular localization of EGFR may play a critical part in response to therapies combining inhibitors of the EGFR pathway with chemotherapy or radiation. Understanding of the mechanisms by which nuclear expression modulates therapeutic effects of these modalities will optimize design of clinical studies in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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