Tumour Response to Gefitinib Is Associated with EGF- and Gefitinib- but not Radiation-modulated EGFR Expression

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Abstract. *Aim: This study was conducted to explore the relationship between different treatment-modulated EGFR expression and gefitinib sensitivity. Materials and Methods: Gefitinib-sensitive (A431) and -resistant (A375, MALME-3M, and SK-MEL 5) tumour cell lines were treated with epidermal growth factor (EGF), gefitinib or radiation in vitro, and EGFR expression levels were measured by using ELISA. Results: EGF, and gefitinib treatment resulted in significantly higher levels of total and/or phosphorylated EGFR in sensitive than in resistant tumours and this was associated with gefitinib IC_{50}. In contrast, radiation-modulated EGFR expression, both total and phosphorylated, did not correlate with the efficacy of gefitinib. Stimulation of proliferation by EGF was significantly stronger in A431 than in the other three lines, indicating sensitive tumours were more EGFR-dependent than resistant tumours for cell proliferation. Conclusion: These findings imply a potential role of EGF- and gefitinib-modulated EGFR expression in predicting gefitinib sensitivity.*

Gefitinib (ZD 1839; Iressa™) is an anilinoquinazoline that inhibits the epidermal growth factor receptor (EGFR) tyrosine kinase activity by binding the receptor at ATP site. Antitumour activity of gefitinib has been shown in many preclinical studies and clinical trials (1, 2). Nevertheless, resistance to gefitinib has been seen in clinical trials where objective response rates only reached 10%-20% in patients with non-small cell lung cancer (NSCLC) (3, 4). In recent years, enormous efforts have been made to identify biomarkers that are capable of predicting the efficacy of gefitinib and other EGFR tyrosine kinase inhibitors (TKIs). EGFR as the targeted biomarker of gefitinib has undergone intensive investigations. Unfortunately, there is insufficient evidence for the use of basal EGFR expression in predicting tumour response to gefitinib or other EGFR-TKIs (5). Wide screening of other biomarkers has suggested a potential role for *EGFR* gene copy number and *EGFR* mutation (6) in predicting gefitinib sensitivity, but these findings are controversial (5).

It is known that EGFR expression can be modulated by various chemical (7), biological (8) or physical (9) factors. More importantly, Van Schaeybroeck *et al*. demonstrated a correlation between cisplatin- or taxol-induced EGFR phosphorylation and the efficacy of gefitinib combined with either of the two chemotherapeutics in NSCLC cells (10). Ono *et al*. found that gefitinib sensitivity is linked to the extent of EGF-induced down-regulation of cell surface EGFR (11). These findings imply the feasibility of modulating EGFR expression by different treatments and the possibility of predicting gefitinib effectiveness by treatment-modulated EGFR expression. The relationship between modulated EGFR expression and gefitinib sensitivity is yet to be elaborated, however. In this context, it is interesting to investigate what sorts of treatments would be able to modulate EGFR expression, to what extent modulated EGFR expression would differ from basal EGFR expression, and the usefulness of a link between modulated EGFR expression and gefitinib efficacy. Such information would provide insights into the role of treatment-modulated EGFR expression to predict tumour response to gefitinib.

The purpose of this study was to investigate the relationship between treatment-modulated EGFR expression and gefitinib sensitivity by using an *in vitro* tumour model. One gefitinib-sensitive cell line (A431 human epidermoid carcinoma) (12) and three gefitinib-resistant lines (A375, MALME-3M and SK-MEL 5 human melanoma) were treated with EGFR-related factors (EGF as EGFR agonist and gefitinib as EGFR antagonist) or an EGFR non-related factor (radiation), and correlation between EGFR responsiveness to treatment and antitumour activity of gefitinib were analysed. The proliferative effect of EGF on

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the tumour cells was also assessed. These data demonstrated that gefitinib sensitivity was associated with EGF- and gefitinib- but not radiation-modulated EGFR expression, which was determined by the tumour's dependence on the EGFR system for proliferation and survival.

Materials and Methods

Cell culture. The A431 cells (ATCC, Rockville, MD, USA) were cultured in DMEM with 4 mM L-glutamine, and A375 (ATCC), MALME-3M and SK-MEL 5 cells (National Cancer Institute at Frederick, USA) in RPMI-1640 medium with 2 mM L-glutamine. All media were supplemented with 10% GIBCO foetal bovine serum (Invitrogen Australia, Mount Waverley, VIC, Australia), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Sigma-Aldrich, Castle Hills, NSW, Australia). Cells were incubated at 37˚C in a humidified incubator with 5% CO_2-95% air supply. In all experiments, cells were seeded in 96-well microplates at a concentration of 1,000 to 6,000 cells/200 μl/well, to ensure exponential growth of cells throughout the whole experiment. The media were refreshed every 2-3 days unless stated otherwise. The cell passage number was less than ten for each experiment.

Antitumour effects of gefitinib. Gefitinib [Parling (Shanghai) PharmaTech, Baoshan, Shanghai, P.R. China] was first dissolved in dimethylsulfoxide (DMSO) and then diluted in culture media to make up a range of concentrations (0, 0.1, 0.3, 1, 3, 10, and 30 μM) which equally contained 1% DMSO (v/v). At approximately 48 h after cell seeding, the cells were exposed to one of the drug concentrations for 48 h. The position of cell lines and drug concentrations in each microplate was randomised. Cell survival was determined by using the crystal violet cell staining method and materials provided in the FACE™ EGFR ELISA kits (Active Motif, Carlsbad, CA, USA). In brief, at the end of drug treatment, cells were fixed with neutral buffered 10% formalin solution (100 μl/well) and stored at 4˚C overnight or longer. On the day of assay, the microplates were washed with wash buffer and then PBS. Cells were stained *via* incubation with crystal violet solution (100 μl/well) at room temperature (RT) for 30 min. After removing the crystal violet solution and washing three times with PBS, the cells were incubated with 1% SDS solution (100 μl/well) on a shaker at RT for 1 h. Absorbance was read on a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 595 nm. The IC_{50} for gefitinib was calculated from the sigmoidal dose-response curve in which the concentration that inhibited 50% cell growth was determined.

Cell treatments. (a) *EGF*. At approximately 72 h post cell seeding, the culture media were replaced with serum-free media. Twenty-four hours later, the media were changed into recombinant human EGF (Invitrogen Australia) solutions in serum-free media (0 or 100 ng/ml) and incubated with the cells for 5 min. The procedures for baseline groups were identical to those for the EGF groups, except that normal culture media were in use throughout the whole experiment.

(b) *Gefitinib*. After seeding in microplates for approximately 48 h, cells were incubated with gefitinib solutions (0 or 10 μM) for 48 h.

(c) *Radiation*. Approximately 24 h post cell seeding, cells received a dose of radiation (0 or 8 Gy at a dose rate of 0.34 Gy/min) using a ^{60}Co irradiator, and then incubated as usual for 72 h. For all experimental groups, after the aforementioned treatments, the media or drug solutions were removed and the cells were fixed with neutral buffered 10% formalin (100 μl/well) and stored at 4˚C overnight or longer for EGFR assay (see EGFR assay below) or cell survival assay (see *Antitumour effects of gefitinib* above).

EGFR assay. Total EGFR (t-EGFR) and phosphorylated EGFR (p-EGFR, represented by p-Y845 and p-Y992) expression levels were determined by using the FACE™ EGFR ELISA kits, according to the manufacturer's instructions. In brief, after removing the formalin solution, microplates were washed with wash buffer or PBS before and after each manipulation. At RT, cells were incubated with quenching buffer for 20 min first and then with antibody blocking buffer for 1 h. After adding antibody against t-EGFR, p-Y845, and p-Y992, the microplates were sealed with sealing tape and Parafilm and stored at 4˚C. The following day, at RT, the samples were first incubated with HRP-conjugated secondary antibody for 1 h and then with developing buffer for 2.5 min, finally by the addition of stop buffer. Absorbance was read on a VersaMax microplate reader at 450 nm. After reading, cell density was determined by using the crystal violet staining method described above, in which the absorbance was read at 595 nm. The measured EGFR expression (OD_{450} nm) was corrected for cell density by using the equation: Corrected EGFR=OD_{450} nm/OD_{595} nm.

EGF effects on cell proliferation. Approximately 24 h post cell seeding, the normal culture media were replaced with different concentrations of EGF in serum-free media (0, 1, 3 and 30 ng/ml). After 72 h incubation, the EGF solutions were removed and cell proliferation was determined by using the crystal violet staining method as described above.

Statistical analysis. Differences among the four cell lines were determined by using *post hoc* Dunnett's test, following a significant effect in one-way ANOVA. Gefitinib effect and EGFR expression level were regarded correlative, provided that the two sets of data presented a same or opposite trend in difference between sensitive and resistant cell lines and the difference had reached a statistically significant level. The criterion for statistical significance was set at $p<0.05$. Data were expressed as mean±standard error of the mean. All statistical analyses were conducted with the SigmaStat 2.03 software package (SPSS Inc., Chicago, IL, USA).

Results

Antitumour effects of gefitinib. The dose–response curves for gefitinib displayed a clear dose- and cell line-dependence of the tumour responses. Compared to the melanoma cell lines, A431 line was much more susceptible to gefitinib at lower to medium concentrations (0.1-10 μM), albeit the maximum concentration effect (30 μM) was similar among all cell lines where the cell survival rates ranged from 1.3±0.7 to 5.1±0.6% relative to Control (Figure 1A). The IC_{50}s for gefitinib were 0.3±0.1, 14.6±0.4, 17.8±0.6 and 13.3±1.2 μM for A431, A375, MALME-3M and SK-MEL 5, respectively (Figure 1B), where the value for A431 was significantly lower than those for the three melanoma lines (all $p<0.05$). According to the conventional criteria for gefitinib sensitivity (13), A431 was highly sensitive to the drug ($IC_{50}<1$ μM) while the three melanoma lines were resistant ($IC_{50}>10$ μM).

EGFR expression. Under baseline conditions, EGFR expression levels were variable among the four tumour cell lines. Compared to the A431 line, the t-EGFR levels of A375 and SK-MEL 5 were significantly lower (both $p<0.05$), the p-Y845 level of MALME-3M was significantly higher ($p<0.05$), while the p-Y992 levels of all three melanoma lines were not significantly different (Figure 2A). Five-minute stimulation with EGF (100 ng/ml) did not cause significant changes in t-EGFR expression among the four cell lines. In contrast, the same treatment resulted in a distinct difference in p-EGFR responsiveness, in which the p-Y845 and p-Y992 levels of A431 were significantly higher than the melanoma lines (all $p<0.05$) (Figure 2B). After incubation with gefitinib (10 μM) for 48 h, the expression levels of t-EGFR, p-Y845 and p-Y992 in A431 cells were unanimously and significantly higher than those in the three melanoma lines (all $p<0.05$) (Figure 2C). On the contrary, irradiation (8 Gy) of the cells failed to produce a superiority for A431 unanimously over the melanoma lines in t-EGFR and p-EGFR expression, in spite of the significantly lower levels of p-Y845 and p-Y992 in the MALME-3M line (Figure 2D).

Correlation between gefitinib effect and basal or modulated EGFR expression. Given that the gefitinib IC_{50} of A431 was significantly and unanimously lower than the melanoma lines (Figure 1B), EGFR expressions which displayed a significant and corresponding difference between the two cohorts were deemed correlative to gefitinib sensitivity. Under baseline conditions, neither t-EGFR nor p-EGFR expression of the three melanoma lines was unanimously higher or lower than the A431 line. Therefore, the basal EGFR expression did not seem to be associated with the effectiveness of gefitinib. The same was for EGF-modulated t-EGFR expression, in which no significant difference was seen among the four tumour lines. Interestingly, EGF-modulated p-Y845 and p-Y992 expression in A431 was superior to every melanoma line and sustained a positive correlation between these two EGFR measurements and efficacy of gefitinib. This indicates that the higher the p-EGFR levels induced by EGF, the more sensitive to gefitinib the tumours will be. In a similar pattern, gefitinib treatment resulted in significantly higher levels of t-EGFR, p-Y845 and p-Y992 in A431 compared to each of the melanoma lines, supporting a positive correlation between gefitinib-modulated t-EGFR/p-EGFR expression and tumour sensitivity to the drug. On the other hand, there was no significant and unanimous difference between A431 and the melanoma lines in radiation-modulated t-EGFR, p-Y845 and p-Y992 expression, thus ruling out a correlation of gefitinib sensitivity with these modulated EGFR expressions.

EGF effects on cell proliferation. As shown in Figure 3, EGF exerted opposite influences on cell proliferation between the A431 and the melanoma lines. In a dose-dependent manner,

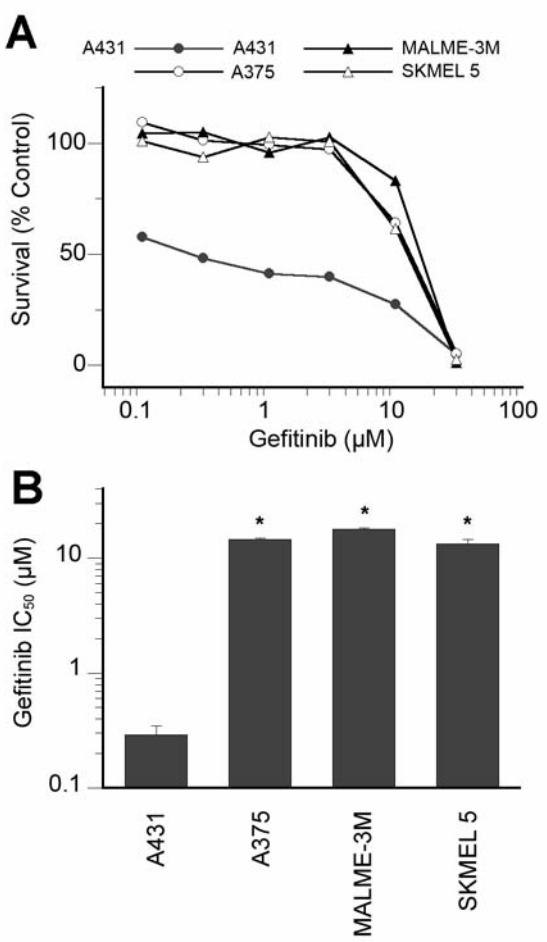

Figure 1. *Antitumour effects of gefitinib in A431, A375, MALME-3M and SK-MEL 5 tumour cell lines. A: Dose–response curves. B: IC_{50}s for gefitinib. Tumour cells were exposed to different concentrations of gefitinib (0, 0.1, 0.3, 1, 3, 10 and 30 μM) for 48 h, and cell survival was determined by using the crystal violet staining method. Each symbol or column plus error bar respectively represents group mean or group mean±SEM (N=10 from 5 experiments). *p<0.05, compared to the A431 group (post hoc Dunnett's test).*

EGF significantly increased cell growth in the former and markedly reduced cell proliferation in the latter (all $p<0.05$). Compared to the A431, cell proliferation levels were significantly lower in all melanoma lines at the two higher EGF doses of 3 and 30 ng/ml (all $p<0.05$) and was so in the A375 only at the lowest dose of 1 ng/ml ($p<0.05$). The data suggest that the A431 is much more dependent on the EGFR system for cell proliferation than the melanoma lines. The EGF proliferative effect was closely associated with the antitumour effect of gefitinib (Figure 1B), with EGF-modulated p-Y845 and p-Y992 expression (Figure 2B), and with gefitinib-modulated t-EGFR, p-Y845 and p-Y992 expression (Figure 2C).

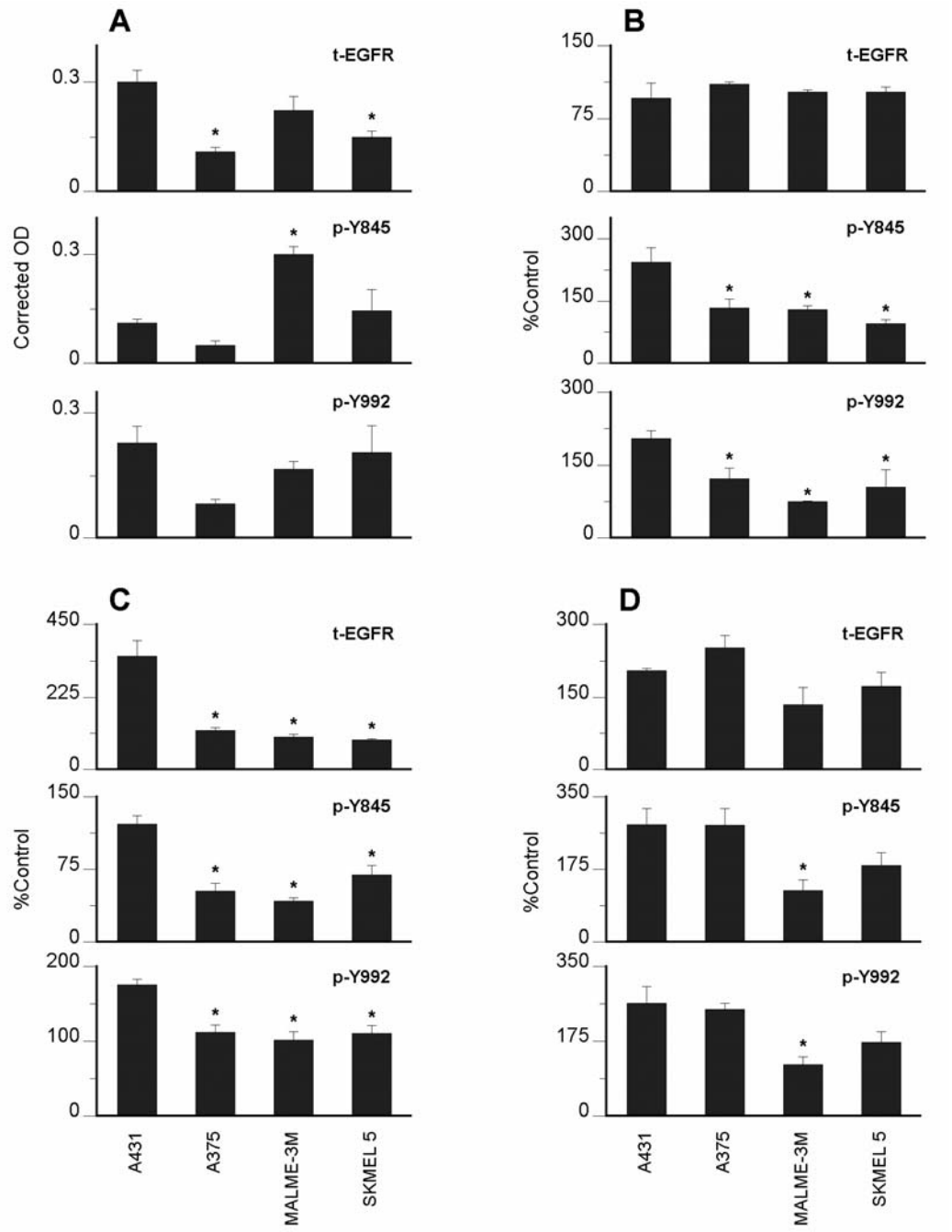

Figure 2. *Total EGFR (t-EGFR) and phosphorylated EGFR (p-Y845 and p-Y992) expression in A431, A375, MALME-3M and SK-MEL 5 tumour cells: (A) baseline, (B) modulated by EGF (100 ng/ml), (C) modulated by gefitinib (10 μM), (D) modulated by radiation (8 Gy). See Materials and Methods for procedure details. Each column and error bar represents mean±SEM (N=4 from 2 experiments). *p<0.05 compared to the A431 group (post hoc Dunnett's test).*

Discussion

Consistent with previous reports (14, 15), the present study demonstrated gefitinib sensitivity in A431 cells and gefitinib resistance in the three melanoma lines (Figure 1). The A431 cells were positive for EGFR expression, as known in the literature (12, 14). For the melanoma cells, variation in the basal EGFR expression (Figure 2A) is reminiscent of a study by de Wit *et al*. (16) that displayed heterogeneity in EGFR expression among six human melanoma cell lines. It was

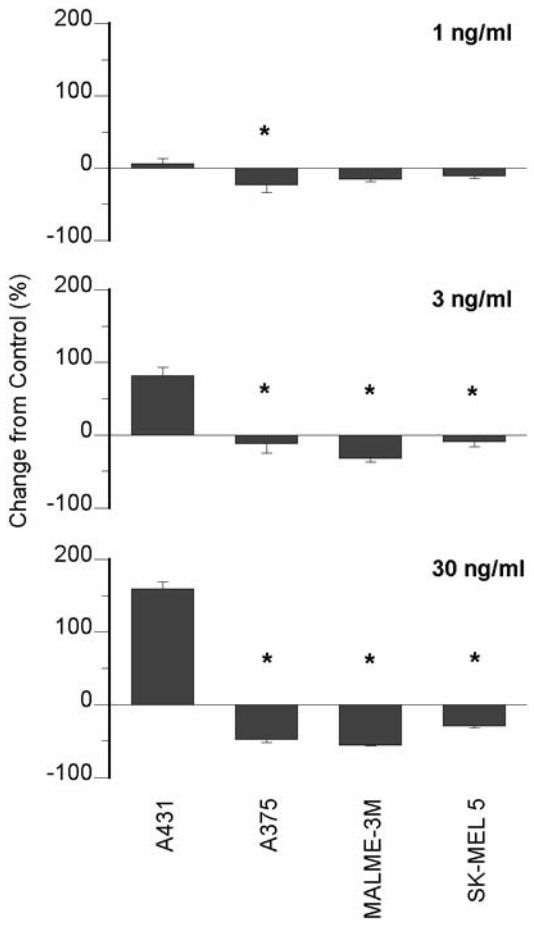

Figure 3. *Effects of EGF on proliferation of A431, A375, MALME-3M and SK-MEL 5 tumour cells. Serum-starved tumour cells were incubated with EGF (0, 1, 3 and 30 ng/ml) for 72 h, and cell proliferation was determined by using the crystal violet staining method. Each column and error bar represents the mean±SEM (N=8 from 2 experiments). *p<0.05, compared to the A431 group (post hoc Dunnett's test).*

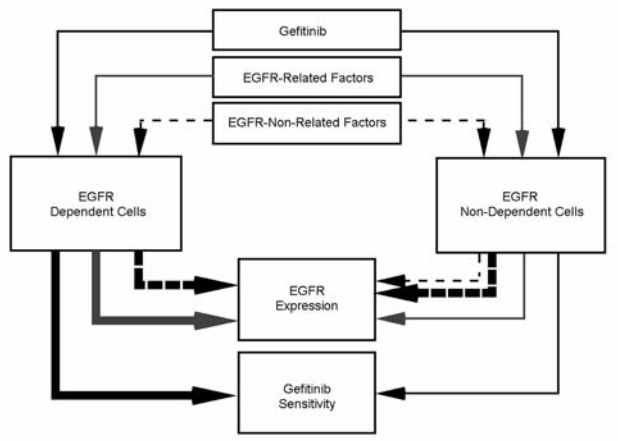

Figure 4. *Hypothesis of how treatment-modulated EGFR expression correlates to gefitinib sensitivity. From this study, tumour cells were be divided into two categories, EGFR-dependent (e.g. the A431 line) and EGFR-non-dependent (e.g. the melanoma lines), upon their dependence on the EGFR system for proliferation. The former was more sensitive to the antitumour effects of gefitinib than the latter. In parallel, when treated with EGFR-related factors (e.g. EGF and gefitinib), t-EGFR and/or p-EGFR responsiveness of the EGFR-dependent cells was stronger than the non-dependent cells. In contrast, when treated with EGFR non-related factors (e.g. radiation), the EGFR responsiveness was overlapped between these two different categories of cells. Therefore, it might infer that dependence on the EGFR system for cell proliferation or survival determines the correlative relationship between treatment-modulated EGFR expression and gefitinib effectiveness.*

previously demonstrated that short-time (10 min to 1 h) EGF stimulation of A431 cells causes an increase in p-EGFR expression but no significant change in t-EGFR expression (7). Similar EGFR responsiveness in A431 cells was observed in the present study when EGF stimulation was applied for 5 min. The necessity of a longer interval for synthesis of new EGFR protein might explain the ineffectiveness of rapid EGF stimulation on t-EGFR expression. The effect of EGF on melanoma EGFR expression was tested for the first time in the present study and no significant change was detected in either t-EGFR or p-EGFR (Figure 2B).

Discrepancies in EGFR responsiveness to gefitinib have been seen in the literature. In a phase II trial on head and neck squamous cell carcinoma (HNSCC), Cohen *et al*. reported that 50% patients were EGFR-positive prior to gefitinib treatment, while they were 100% positive after 7-week gefitinib treatment (oral 500 mg/d) (17). By incubating HNSCC cells with gefitinib (1 and 10 μM) for 4 h, Pernas *et al*. failed to demonstrate any significant changes in t-EGFR and p-EGFR expression (18). After 24 h exposure of NSCLC cells to gefitinib (1 μM), Helfrich *et al*. observed a significant decrease in p-EGFR expression in gefitinib-sensitive but not in -resistant cell lines, in addition to no significant t-EGFR changes in all cell lines (19). In contrast, data of the present study showed that gefitinib treatment (10 μM for 48 h) resulted in significantly higher levels of t-EGFR and p-EGFR in the sensitive cell line (A431) than in the drug-resistant melanoma lines (Figure 2C). To verify the results, in a separate experiment a range of gefitinib concentrations (0.1-10 μM) were applied to cells, and the differences between A431 and the melanoma lines were reproduced in a dose-dependent style (data not shown). It is important to notice the variances in methodology among the studies mentioned above, such as cell lines, gefitinib dosage, time course, tissue processing procedures, EGFR detecting technique *etc*. Differences in these experimental parameters may contribute to the disparities in gefitinib-induced EGFR responses. Moreover, data of the present study showed that 8 Gy of irradiation caused significant increases in t-EGFR and p-EGFR expression in A431 and A375 cells and slight

but insignificant increases in MALME-3M and SK-MEL 5 cells (Figure 2D). These results extend the previous findings by Schmidt-Ullrich *et al*. (20) and Kraehn *et al*. (21).

The most interesting finding of the present study was on the relationship between treatment-modulated EGFR expression and gefitinib sensitivity. These two variables correlated with each other in a treatment agent-dependent style. EGF is an endogenous EGFR-binding ligand with high affinity to the EGFR (22), and gefitinib is a selective inhibitor of the EGFR tyrosine kinase (2). Both are EGFR-related agents. EGF stimulation induced significantly higher levels of p-Y845 and p-Y992 in the gefitinib-sensitive line, A431, than in the melanoma lines that were gefitinib-resistant (Figure 2B). Similarly, gefitinib treatment increased the expression levels of t-EGFR, p-Y845 and p-Y992 in A431 cells, which were significantly higher than their counterparts in the melanoma lines (Figure 2C). These modulated EGFR responses were closely associated with the effectiveness of gefitinib, *i.e*. the stronger the EGFR responsiveness, the more sensitive to the drug the cells are. On the other hand, as a physical agent without direct action on EGFR, radiation caused significant up-regulation of t-EGFR and p-Y845 and p-Y992 in A431 cells and one of the melanoma lines (A375) without significant changes in the other two melanoma lines (MALME-3M and SK-MEL 5) (Figure 2D). Therefore, radiation-modulated EGFR expression was not associated with gefitinib sensitivity. Besides, the baseline t-EGFR, p-Y845 and p-Y992 levels of A431 did not unanimously and significantly differ from the melanoma lines (Figure 2A) and thus failed to correlate with gefitinib efficacy among the four cell lines. This is in agreement with findings in previous studies (5, 11).

The positive correlation between EGF-, and gefitinib-modulated EGFR expression and gefitinib sensitivity implies potential of the EGFR responsiveness as a predictive factor of tumour sensitivity to the EGFR-TKI. At present, there is still a lack of clinically validated biomarkers predicting the effectiveness of gefitinib (5). *EGFR* gene copy number and mutation have been suggestive of potential predictive biomarkers (6), but detection of these biomarkers involves invasive specimen collection and labour-intensive laboratory operation. *In vivo* t-EGFR and p-EGFR are detectable by PET imaging with radiolabelled molecules (23, 24) which is a non-invasive and convenient tumour monitoring technique. Therefore, EGF- or gefitinib-modulated EGFR expression is believed to be superior to *EGFR* gene copy and mutation as a predictive factor of gefitinib sensitivity. In particular, EGF-induced changes in p-EGFR expression take place in minutes and do not involve alteration of tumour mass. In addition, oral and intravenous administration of EGF is reportedly safe and effective in the clinical treatment of gastrointestinal ulcers (25, 26). The potential of EGF-modulated p-EGFR expression in predicting gefitinib effectiveness is worthy of further investigation.

The fact, that gefitinib sensitivity was associated with EGFR responsiveness to EGFR-related agents (*i.e*. EGF and gefitinib) but not to an EGFR-non-related agent (*i.e*. radiation), supposes EGFR dependence may be an important mechanism that determines the correlative relationship between gefitinib action and EGFR response. To verify this assumption, this study investigated the effect of EGF on cell proliferation in the four cell lines. The results showed that EGF increased proliferation of gefitinib-sensitive cells (A431) and inhibited growth of gefitinib-resistant cells (the three melanoma lines) (Figure 3), indicating the former more depends on the EGFR system for proliferation than the latter. EGFR is known to play an important role in mediation of cell proliferation and survival in tumours (27). However, this role of EGFR could be played down due to other possible mechanisms such as gene mutations that interfere with drug binding, oncogenic pathways driven by other receptor tyrosine kinases, or EGFR-independent activity of downstream signalling molecules (28). Previous studies have proposed that the weight of dependence on the EGFR system for cell survival determines tumour sensitivity to gefitinib (11, 29). By providing new evidence, the present study substantiated this notion. It is thus rational to hypothesise that dependence on EGFR for cell proliferation and/or survival is a common mechanism that links treatment-modulated EGFR expression to gefitinib sensitivity. The hypothesis is illustrated in Figure 4.

In conclusion, the results of this study revealed a positive correlation between gefitinib sensitivity and EGF-, and gefitinib-modulated EGFR expression, suggestive of a potential role of the modulated EGFR expression in predicting gefitinib effectiveness. These data also demonstrated that the proliferative effect of EGF was correlated with both gefitinib sensitivity and EGF-, and gefitinib-modulated EGFR expression. This indicates that the relationship between gefitinib effect and treatment-modulated EGFR expression is determined by a tumour's dependence on the EGFR system for cell proliferation and survival.

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