Prediction of Synergistic Antitumour Effect of Gefitinib and Radiation *In Vitro*

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Abstract. *Aim: This study investigated the potential of a series of biomarkers in predicting the interaction of gefitinib and radiation in tumour treatment. Materials and Methods: In vitro assays were performed on human skin cancer and melanoma cell lines. The antitumour effect was measured by using the MTT assay. Total and phosphorylated epidermal growth factor receptor (EGFR and pEGFR) levels were determined by cell-based ELISA. Results: Gefitinib and radiation interacted to inhibit tumour cell proliferation in a cell line-dependent manner. Synergism dominated the interaction (76%), followed by additive effect (20%) and a few instances of antagonism (4%). Correlation analyses revealed a significant correlation between the median combination index (CI) and gefitinib IC_{50}, radiation ID_{50}, gefitinib- or EGF-modulated EGFR and/or pEGFR expression (all $p \leq 0.05$). Conclusion: A potential role of gefitinib efficacy, radiation efficacy and gefitinib- or EGF-modulated EGFR and/or pEGFR expression in the prediction of interaction between gefitinib and radiation is supported.*

The combination of molecular blockade of epidermal growth factor receptor (EGFR) signalling and radiation therapy is an attractive anticancer strategy currently under intensive preclinical and clinical investigations. In *in vitro* studies, gefitinib enhanced the cytotoxic effects of radiation in different tumour types, *e.g.* skin (1), thyroid (2), colon, ovarian, non-small cell lung and breast cancer (3). In parallel, a synergistic or additive effect between gefitinib and radiation was demonstrated in animal tumour models with epidermoid carcinoma (1), colorectal cancer (4) and glioma (5) xenografts. Several early-stage clinical trials have reported promising activity and/or tolerable toxicity of combination therapy with gefitinib and radiation in patients with various types of tumour (6, 7).

In the clinic, predicting efficacy is a major challenge in the treatment of cancer with gefitinib, either alone or combined with other therapeutic agents, because of the large individual difference in patient response to these treatments. In recent years, significant research efforts have been directed to the identification of biomarkers for the effectiveness of gefitinib in combination therapy. Van Schaeybroeck *et al.* showed that the nature of interaction between gefitinib and oxaliplatin was determined by the level of basal and oxaliplatin-modulated phosphorylated EGFR (pEGFR) in colorectal cancer cells (8). In phase I/II or II studies on patients with advanced colorectal cancer, Zampino *et al.* found that baseline EGFR serum level was associated with best objective response to gefitinib-FOLFOX6 (folinic acid, 5-fluorouracil and oxaliplatin) followed by gefitinib alone (9), Ogina *et al.* revealed a negative correlation between p21 and p53 expression and patient response rate to combination therapy with gefitinib (10), while Cascinu *et al.* failed to link EGFR expression, *EGFR* gene amplification and nuclear factor-kappa B (NFκB) activation to the efficacy of gefitinib-FOLFOX4 treatment (11). Nevertheless, little is known about the predictability of tumour response to combination therapy with gefitinib and radiation thus far.

Previously, we have demonstrated a close association between gefitinib effectiveness and EGF-, gefitinib- but not radiation-modulated EGFR expression in human skin cancer and melanoma cells (12). It is likely that the efficacy of a therapeutic agent, represented by IC_{50} or ID_{50}, may play a role in combination therapy with that agent. Analyses of the relationship between these biomarkers and the combined effects of gefitinib and radiation may lead to the discovery of potential predictors for interactive effects of the two agents. In this study, using human skin squamous cancer and melanoma cell lines, the efficacy of interaction between gefitinib and radiation was investigated and then correlations between the combination therapy and the aforementioned biomarkers were analysed.

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Materials and Methods

Cell culture. A431 human skin squamous carcinoma cells (American Type Culture Collection, Rockville, MD, USA) were grown in DMEM medium with 4 mM L-glutamine. Human melanoma cell lines (M14, MALME-3M, SK-MEL 2, SK-MEL 5, SK-MEL 28 and UACC 257) (National Cancer Institute, Frederick, MD, USA) were cultured in RPMI-1640 medium with 2 mM L-glutamine. All the 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 atmosphere with 5% CO_2-95% air supply. In all the experiments, the cells were seeded in 96-well microplates at a density of 1,000 to 6,000 cells/200 μl/well, to ensure exponential growth of cells throughout the whole experiment.

Gefitinib and radiation treatments. Gefitinib alone: gefitinib (Parling (Shanghai) PharmaTech, Baoshan, Shanghai, China) was first dissolved in DMSO and then diluted in culture media to contain 1% DMSO (v/v) at each concentration level. At 48 h post-plating, the cells were incubated with gefitinib (0, 0.1, 0.3, 1, 3, 10 or 30 μM) for 48 h. Radiation alone: at 24 h post-plating, the cells were irradiated with gamma ray (0, 1, 2, 4 or 8 Gy at a dose rate of 0.34 Gy/min) by using a ^{60}Co irradiator and allowed to grow for 72 h. Gefitinib and radiation combination: at 24 h post-plating, the cells were irradiated with one of the graded radiodoses (0, 2, 4 or 8 Gy). Twenty-four hours later, the cells were incubated with gefitinib (0, 0.3, 1, 3 or 10 μM) for 48 h. At the end of each experiment, cell survival assay and/or EGFR assay were performed as described below.

EGF stimulation. After 24 h deprivation of serum, the cells were incubated with 100 ng/ml of EGF (Invitrogen Australia) for 5 min. Then the cells were fixed with 10% neutral buffered formalin and stored at 4˚C overnight or longer before EGFR assay (see below).

Cell survival assay. As described previously (12), cell survival was determined using an MTT-Based *In Vitro* Toxicology Assay Kit (Sigma-Aldrich), according to the manufacturer's instructions. The IC_{50} for gefitinib and ID_{50} for radiation were determined from the sigmoidal dose-response curves produced by each agent, in which the concentration or dose that inhibited 50% cell growth was identified. For those cell lines whose maximum inhibitory rate by irradiation did not reach 50%, the ID_{50} was predicted from the linear best fitting curve. The combined effects of gefitinib and radiation, indicated by combination index (CI), were analysed by Chou's median-effect method (13) using CompuSyn software (ComboSyn Inc., Paramus, NJ, USA). The criterion of data conformity to the median-effect principle was set at r≥0.90, where r is the linear correlation coefficient of the median-effect plot.

EGFR assay. EGFR and pEGFR (Tyr845(Y845) and Tyr992 (Y992)) were measured by using a FACE™ EGFR ELISA Kit (Active Motif, Carlsbad, CA, USA). As described previously (12), the assay was performed in accordance with the instructions of the manufacturer.

Statistical analyses. The relationship between median CI and various biomarkers (*i.e.* gefitinib IC_{50}, radiation ID_{50}, gefitinib-, radiation- or EGF-modulated EGFR/pEGFR expression) was analysed by Pearson product-moment correlation or Spearman rank order correlation, whichever was appropriate. The criterion for statistical significance was set at *p*≤0.05. All the statistical analyses were performed using the SigmaStat 2.03 software package (SPSS Inc., Chicago, IL, USA).

Results

Antitumour effects of gefitinib and radiation. Figure 1A shows dose–response curves for gefitinib and radiation treatments. Both agents, whether alone or in combination, produced a dose- and cell line-dependent, inhibitory effect on proliferation in all the cell lines. The optimal antitumour effects (*i.e.* minimum survival fraction) were 0.29-0.80, 0.41-0.87 and 0.18-0.65 for gefitinib alone, radiation alone and gefitinib combined with radiation, respectively. The data revealed considerably large variations in tumour responses. Figure 1B illustrates the degree of interaction between gefitinib and radiation by plotting the CI values against combined treatments with the two agents. Among the 12 gefitinib and radiation dose combinations, synergism (CI<0.9) was shown in 12/12 (100%) in the A431 cells, in contrast to a range of scores from 7/12 (58%) to 10/12 (83%) in the melanoma lines. An additive effect (CI=0.9-1.1) was seen in all the melanoma lines and ranged from 1/12 (8%) to 5/12 (42%). Antagonism (CI>1.1) only occurred in two melanoma lines, SK-MEL 2 and UACC 257 with a rate of 2/12 (17%) and 1/12 (8%), respectively. The synergistic effect in the A431 cells was in agreement with previous findings in the literature (1, 14), validating the experimental settings in this study.

Figure 2 depicts the median effects of gefitinib and radiation in single and combination therapy. The median CI value of the A431 cells (0.38) was lower than those of the melanoma lines (0.68-0.83) (Figure 2A). So were the IC_{50}s for gefitinib, where the value for the A431 cells was 0.3 μM, much lower than those of the melanoma lines (5.0-15.4 μM) (Figure 2B). Although the gap between the A431 cells and melanoma lines was narrower in ID_{50}s for radiation, the A431 cells still had a lower ID_{50} (6.9 Gy) compared to the melanoma lines (9.2-26.2 Gy) (Figure 2C).

EGFR and pEGFR expression. The levels of treatment-modulated EGFR/pEGFR are shown in Figure 3. Incubation with gefitinib 10 μM for 48 h resulted in unanimously higher levels of EGFR, Y845 and Y992 in the A431 cells than in any of the melanoma lines (Figure 3A). In contrast, the A431-dominated pattern was not seen in EGFR/pEGFR responses caused by radiation (Figure 3B). Stimulation with EGF for 5 min caused diverse responses, in which the responses in EGFR were low and irregular while the A431-dominated pattern appeared in the Y845 and Y992 response to EGF (Figure 3C).
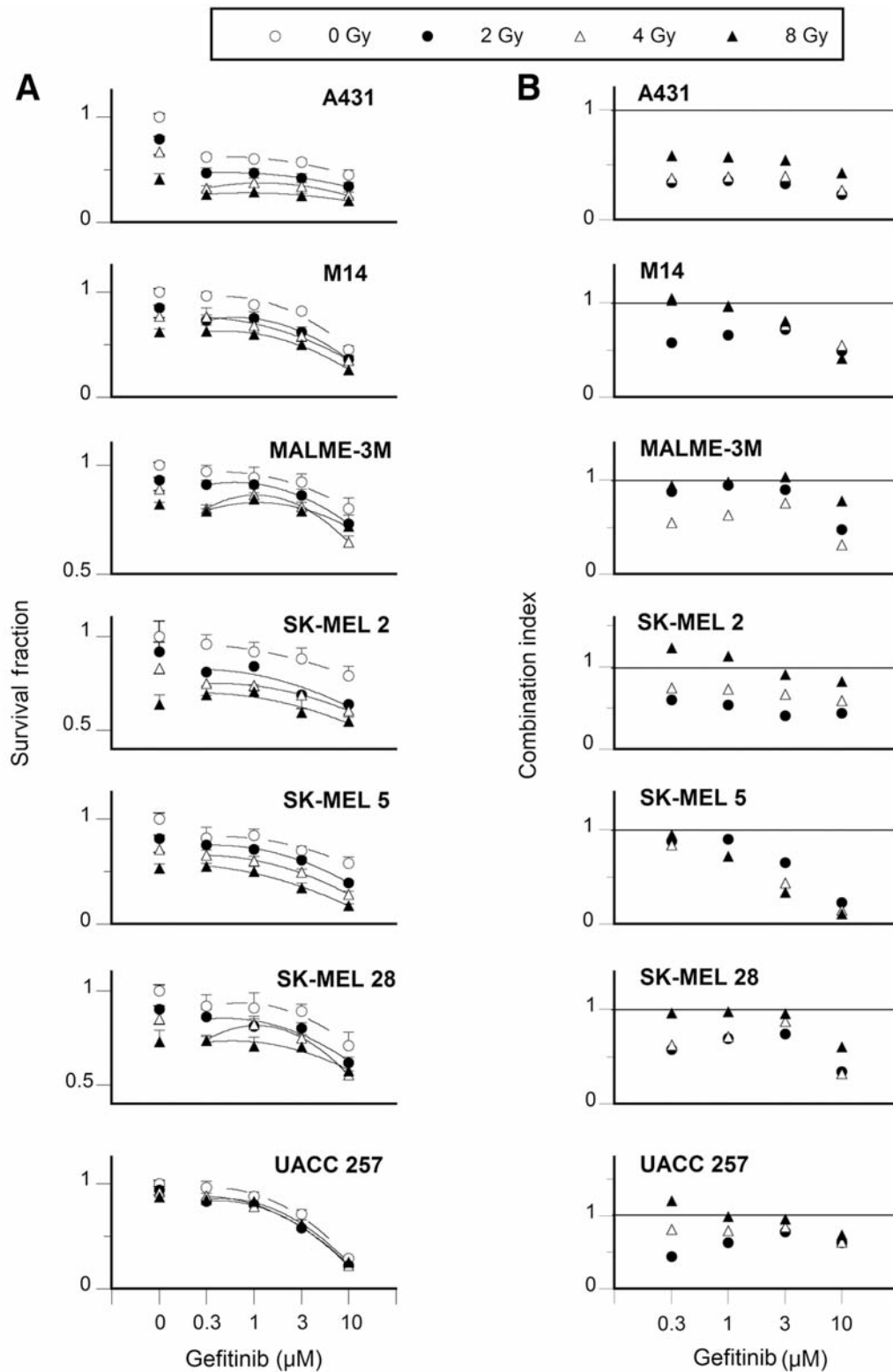
Figure 1. *Antitumour effect of gefitinib and radiation combination in human skin cancer and melanoma cell lines. A: Dose–response curves. MTT assay of cell survival after irradiation followed 24 h later by gefitinib treatment for 48 h. The curves are polynomial (order 2) fitting curves. Mean±SEM for 6 replicates from 3 experiments. B: Combination index (CI) (for the corresponding group mean shown in Figure 1A) calculated by Chou's median-effect method. CI<0.9, CI=0.9-1.1 and CI>1.1 denote synergism, additive effect and antagonism, respectively (13).*

Table I. *Correlation between median CI and gefitinib (Gef.) IC$_{50}$, radiation (Rad.) ID$_{50}$, Gef.-, Rad.- and EGF-modulated EGFR/pEGFR (Y845 and Y992) expression in human skin cancer and melanoma cells.*

	Gef. IC$_{50}$	Rad. ID$_{50}$	Gef.-Modulated			Rad.-Modulated			EGF-Modulated		
			EGFR	Y845	Y992	EGFR	Y845	Y992	EGFR	Y845	Y992
r	0.767	0.964	–0.963	–0.799	–0.920	0.429	–0.223	–0.590	0.321	–0.748	–0.916
p-Value	**0.04**	**0.00**	**0.00**	**0.03**	**0.00**	0.30	0.63	0.16	0.44	**0.05**	**0.00**
N	7	7	7	7	7	7	7	7	7	7	7
Method	Pearson	Spearman	Pearson	Pearson	Pearson	Spearman	Pearson	Pearson	Spearman	Pearson	Pearson

Pearson: Pearson product-moment correlation; Spearman: Spearman's rank order correlation; bold figure: statistically significant (*p*≤0.05).

Correlation between median CI and various biomarkers. In an attempt to identify potential predictors for gefitinib and radiation interaction, the correlative relationship between median CI and several biomarkers was analysed. The results are summarised in Table I. A significant positive correlation was revealed between median CI and gefitinib IC$_{50}$ or radiation ID$_{50}$ (both *p*<0.05). Moreover, gefitinib-modulated EGFR, Y845 and Y992 were significantly and negatively correlated with median CI (all *p*<0.05), as were EGF-modulated Y845 and Y992 (both *p*≤0.05). On the other hand, no significant correlation was established for median CI against radiation-modulated EGFR/pEGFR and EGF-modulated EGFR (all *p*>0.05).

Discussion

By using Chou's median-effect method, this study revealed three types of interaction between gefitinib and radiation, *i.e.* synergism, additive effect and antagonism, in skin cancer and melanoma cells (Figure 1B). As a whole, synergism dominated the interactions (76%), followed by additive effect (20%) with few instances of antagonism (4%). The optimal CI values were 0.23 for the A431 skin cancer line and 0.11-0.44 for the six melanoma lines, indicating that median (CI=0.3-0.7) to strong (CI=0.1-0.3) synergism (13) was achievable by combination of gefitinib with radiation. The results imply an advantage of combining the two therapeutic agents in tumour treatment. In the literature, a body of evidence suggests beneficial effects of gefitinib and radiation combination in the treatment of different types of tumour (2, 15), compatible with the findings of this study. However, quantitative analysis of the drug–radiation interaction has rarely been seen in previous studies, and the present new data provides more accurate and quantitative information about the degree of the interaction.

To our best knowledge, this is the first reported study of gefitinib and radiation interaction in melanoma tumours. In the clinic, resistance to antitumour drugs or radiation is the main obstacle to effective treatment of advanced melanoma.
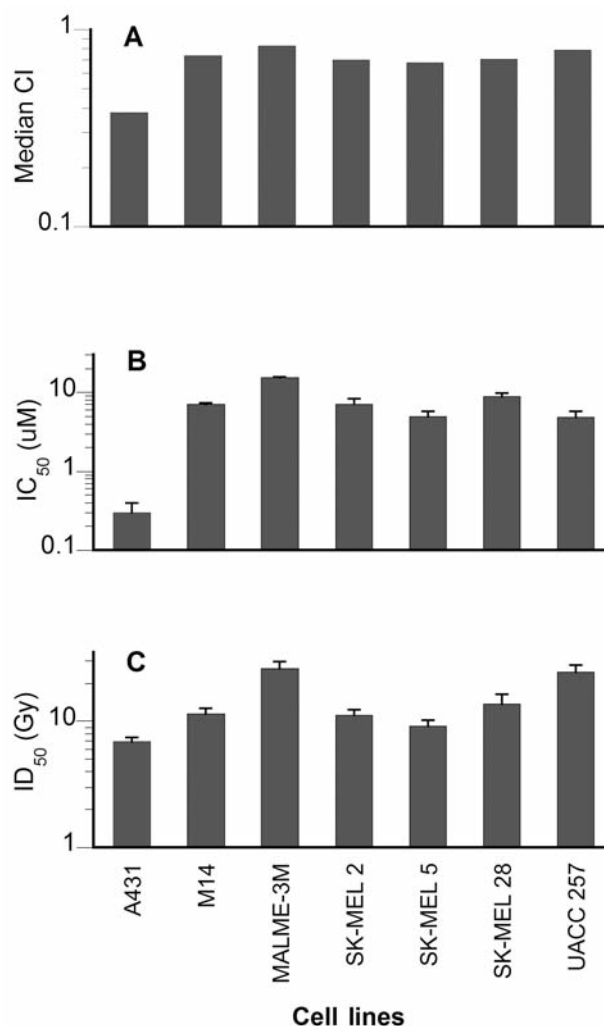

Figure 2. *Median effects of gefitinib and radiation alone or in combination in human skin cancer and melanoma cell lines. A: Median CI values (determined from individual CIs (N=12) presented in Figure 1B) for gefitinib and radiation combination. B: IC$_{50}$ values for gefitinib, calculated from dose–response curves with 0, 0.1, 0.3, 1, 3, 10 and 30 μM. Mean±SEM for 6 replicates from 3 experiments. C: ID$_{50}$ values for radiation, calculated from dose–response curves with 0, 1, 2, 4 and 8 Gy. Mean±SEM for 6 replicates from 3 experiments.*
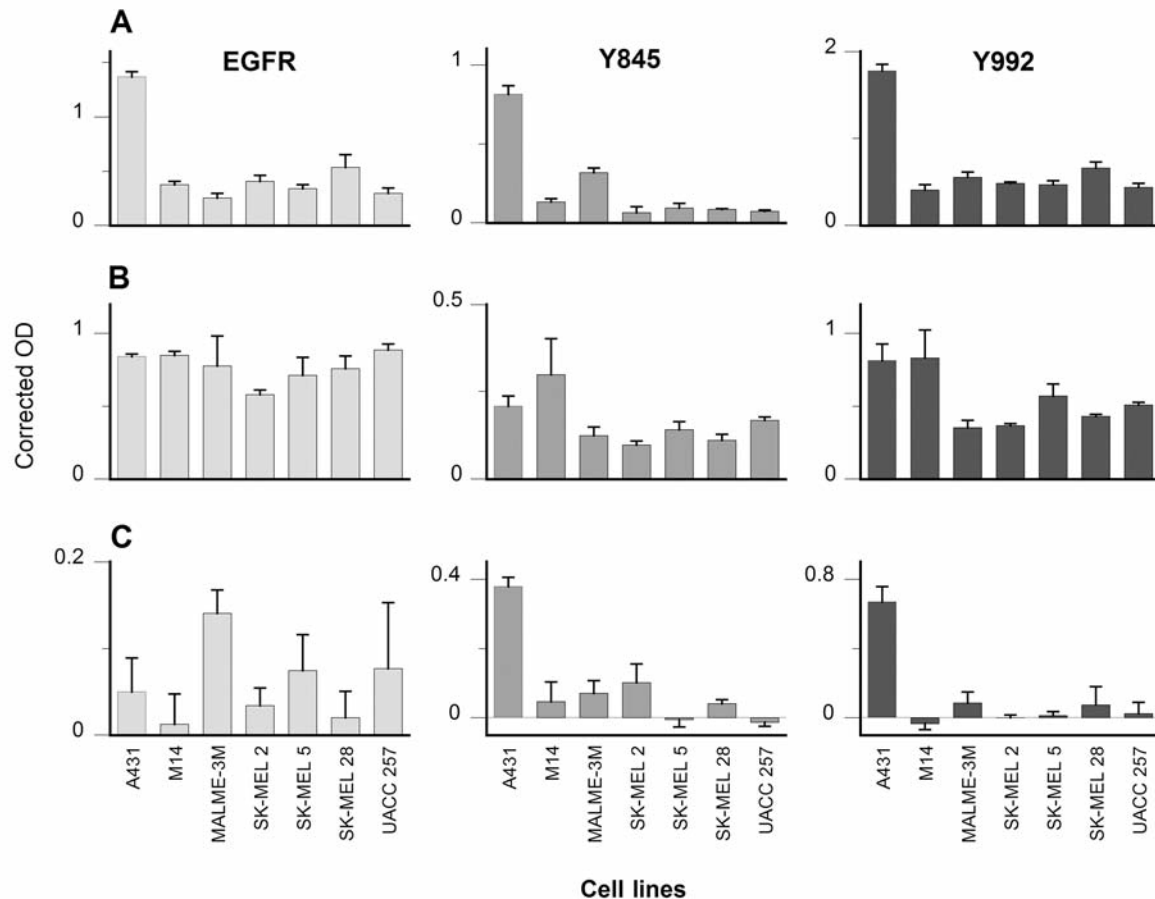
Figure 3. *A: Gefitinib- (10 μM for 48 h), B: radiation- (8 Gy at 0.34 Gy/min), and C: EGF- (100 ng/ml for 5 min) modulated EGFR (left panels) and pEGFR, Y845 (middle panels) and Y992 (right panels) expression in human skin cancer and melanoma cell lines, determined by ELISA, and absorbance data (OD) corrected for cell density. Mean±SEM for 4 replicates from 2 experiments.*

Previously, no survival benefit was found with adjuvant chemotherapy, nonspecific (passive) immunotherapy, radiation therapy, retinoid therapy, vitamin therapy or biologic therapy (16). However, the present study demonstrated that synergism (72%) and additive effect (24%) dominated the interaction between gefitinib and radiation in the melanoma cells, suggesting that combination of the two agents may be a beneficial treatment for melanoma.

Considerable large heterogeneity in tumour response to gefitinib treatment combined with other agents has repeatedly been reported (8, 10). Similarly, in this study, up to a 3.6-fold difference in minimum survival fraction and a 4-fold difference in minimum CI between cell lines was observed, underlining the importance of predicting tumour response in combination therapy with gefitinib and radiation. The data (Table I) supported a significant positive correlation between median CI and gefitinib IC_{50} or radiation ID_{50}, indicating that gefitinib or radiation sensitive tumours would gain more benefit from the combination therapy than the

non-sensitive or resistant ones. Moreover, median CI was significantly and negatively correlated to gefitinib-modulated EGFR/pEGFR and to EGF-modulated pEGFR expression, implying the potential of these biomarkers as predictive factors for the efficacy of gefitinib and radiation combination therapy. On the hand, no significant correlation between median CI and radiation-modulated EGFR/pEGFR was found in this study.

It is known that the cell cycle is an important determinant of radiosensitivity. Cells in G_2/M-phase are most sensitive, whereas those in late S-phase are the most radiation resistant (15). Previous studies showed that gefitinib arrested tumour cells in the G_0/G_1-phase of the cycle and thus led to a decrease of cell number in the S-phase (17). Such cell biological interaction between gefitinib and radiation may explain the high correlation between median CI and gefitinib IC_{50} and radiation ID_{50}. In our preceding study, tumour response to gefitinib alone was associated with gefitinib- and EGF- but not radiation-modulated EGFR and/or pEGFR

expression, which seemed to be underlined by tumour dependence on EGFR for proliferation and survival (12). Interestingly, an identical correlation pattern was shown in the present study. Probably, the EGFR dependence mechanism shown in gefitinib single therapy also contributes to the synergistic effect of gefitinib and radiation.

In summary, gefitinib and radiation are able to produce synergistic antitumour effects in human skin cancer and melanoma cells. The interaction efficacy is significantly correlated with gefitinib IC_{50}, radiation ID_{50}, gefitinib- and EGF-modulated EGFR and/or pEGFR expression. These findings provide a rationale for combination therapy with gefitinib and radiation in the treatment of these tumour types, and warrant further investigation into the potential role of these biomarkers in prediction of the effectiveness of gefitinib and radiation combination.

Acknowledgements

References

1 Solomon B, Hagekyriakou J, Trivett MK, Stacker SA, McArthur GA and Cullinane C: EGFR blockade with ZD1839 ('Iressa') potentiates the antitumor effects of single and multiple fractions of ionizing radiation in human A431 squamous cell carcinoma. Epidermal growth factor receptor. Int J Radiat Oncol Biol Phys *55*: 713-723, 2003.

2 Lopez JP, Wang-Rodriguez J, Chang CY, Sneh G, Yu MA, Pardo FS, Aguilera J and Ongkeko WM: Gefitinib (Iressa) potentiates the effect of ionizing radiation in thyroid cancer cell lines. Laryngoscope *118*: 1372-1376, 2008.

3 Bianco C, Tortora G, Bianco R, Caputo R, Veneziani BM, Damiano V, Troiani T, Fontanini G, Raben D, Pepe S, Bianco AR and Ciardiello F: Enhancement of antitumor activity of ionizing radiation by combined treatment with the selective epidermal growth factor receptor-tyrosine kinase inhibitor ZD1839 (Iressa). Clin Cancer Res *8*: 3250-3258, 2002.

4 Williams KJ, Telfer BA, Stratford IJ and Wedge SR: ZD1839 ('Iressa'), a specific oral epidermal growth factor receptor-tyrosine kinase inhibitor, potentiates radiotherapy in a human colorectal cancer xenograft model. Br J Cancer *86*: 1157-1161, 2002.

5 Geoerger B, Gaspar N, Opolon P, Morizet J, Devanz P, Lecluse Y, Valent A, Lacroix L, Grill J and Vassal G: EGFR tyrosine kinase inhibition radiosensitizes and induces apoptosis in malignant glioma and childhood ependymoma xenografts. Int J Cancer *123*: 209-216, 2008.

6 Joensuu G, Joensuu T, Nokisalmi P, Reddy C, Isola J, Ruutu M, Kouri M, Kupelian PA, Collan J, Pesonen S and Hemminki A: A phase I/II trial of gefitinib given concurrently with radiotherapy in patients with nonmetastatic prostate cancer. Int J Radiat Oncol Biol Phys *78*: 42-49, 2010.

7 Ma S, Xu Y, Deng Q and Yu X: Treatment of brain metastasis from non-small cell lung cancer with whole-brain radiotherapy and gefitinib in a Chinese population. Lung Cancer *65*: 198-203, 2009.

8 Van Schaeybroeck S, Karaiskou-McCaul A, Kelly D, Longley D, Galligan L, Van Cutsem E and Johnston P: Epidermal growth factor receptor activity determines response of colorectal cancer cells to gefitinib alone and in combination with chemotherapy. Clin Cancer Res *11*: 7480-7489, 2005.

9 Zampino MG, Magni E, Santoro L, Zorzino L, Dell'Orto P, Sonzogni A, Fazio N, Monfardini L, Chiappa A, Biffi R and de Braud F: Epidermal growth factor receptor serum (sEGFR) level may predict response in patients with EGFR-positive advanced colorectal cancer treated with gefitinib? Cancer Chemother Pharmacol *63*: 139-148, 2008.

10 Ogino S, Meyerhardt JA, Cantor M, Brahmandam M, Clark JW, Namgyal C, Kawasaki T, Kinsella K, Michelini AL, Enzinger PC, Kulke MH, Ryan DP, Loda M and Fuchs CS: Molecular alterations in tumors and response to combination chemotherapy with gefitinib for advanced colorectal cancer. Clin Cancer Res *11*: 6650-6656, 2005.

11 Cascinu S, Berardi R, Salvagni S, Beretta GD, Catalano V, Pucci F, Sobrero A, Tagliaferri P, Labianca R, Scartozzi M, Crocicchio F, Mari E and Ardizzoni A: A combination of gefitinib and FOLFOX-4 as first-line treatment in advanced colorectal cancer patients. A GISCAD multicentre phase II study including a biological analysis of EGFR overexpression, amplification and NF-kB activation. Br J Cancer *98*: 71-76, 2008.

12 Lin HQ, Katsifis A and Meriaty H: Tumour response to gefitinib is associated with EGF- and gefitinib- but not radiation-modulated EGFR expression. Anticancer Res *30*: 4899-4905, 2010.

13 Chou TC: Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev *58*: 621-681, 2006.

14 Fukutome M, Maebayashi K, Nasu S, Seki K and Mitsuhashi N: Enhancement of radiosensitivity by dual inhibition of the HER family with ZD1839 ('Iressa') and trastuzumab ('Herceptin'). Int J Radiat Oncol Biol Phys *66*: 528-536, 2006.

15 Ochs JS: Rationale and clinical basis for combining gefitinib (IRESSA, ZD1839) with radiation therapy for solid tumors. Int J Radiat Oncol Biol Phys *58*: 941-949, 2004.

16 Swetter SM: Malignant melanoma. eMedicine website. http://emedicine.medscape.com/article/1100753-overview 2010.

17 Hopfner M, Sutter AP, Huether A, Schuppan D, Zeitz M and Scherubl H: Targeting the epidermal growth factor receptor by gefitinib for treatment of hepatocellular carcinoma. J Hepatol *41*: 1008-1016, 2004.