

**ESMO Translational Research Fellowship
(2008 – 2009)**

‘Mechanisms of acquired resistance to anti growth factor receptor agents’

Floriana Morgillo

Final report

Host Institute: AOU Seconda Università degli Studi di Napoli (AOU-SUN), Naples, Italy
Mentor: Prof Fortunato Ciardiello

Different cell-signalling pathways have been successfully targeted in various hematologic and solid malignancies.

Rationale and aim

Non small cell lung cancer (NSCLC) is the major cause of cancer-related death worldwide (1). This is a very heterogeneous disease and this has huge implications for clinical practice. In the past years, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, have represented the first example of molecularly targeted agents developed in the treatment of NSCLC and are, currently, useful tools in the management of patients who progressed on previous chemotherapy and especially for those harbouring EGFR activating mutations (1). Results from phase III clinical showed a great improvement in quality of life and in survival in this subset of patients (2). In spite of these positive aspects, therapy with EGFR TKIs is limited by the emergence of drug resistance. Our group recently demonstrated the importance of the acquisition of a mesenchymal phenotype in an in vitro model of NSCLC cell lines as a mechanism of acquired resistance to anti- EGFR tyrosine kinase inhibitors (3). The epithelial- mesenchymal transition (EMT) is a process characterized by a dramatic remodelling of cell cytoskeleton and by the combined loss of epithelial cell junction proteins such as E-cadherin and the gain of mesenchymal markers such as vimentin. This transition plays a critical role in tumour invasion, metastatic dissemination and the acquisition of resistance to conventional therapies and targeted drugs such as EGFR TKIs. Recent studies suggested the role of EMT as predictive of resistance to erlotinib (4).

Experimental design

To study mechanisms involved in acquired resistance to anti growth factor receptor agents we developed an in vitro panel of acquired resistance by treating over a period of 12 months, the human colorectal cancer cell line (HCT 116) and the human CALU-3 (P-CALU-3) lung adenocarcinoma which were exposed to increasing concentrations of either gefitinib, erlotinib,

vandetanib or sorafenib. This model allowed us to study different pathways implicated in the acquired resistance to different agents targeting growth factor receptors.

The human NSCLC cell line CALU-3 harbours the wild-type EGFR gene. This cancer cell line has been previously characterised by our group for the expression of the four EGF-related growth factor receptors (EGFR, ERBB2, ERBB3 and ERBB4) and of three VEGF receptors (VEGFR-1, VEGFR-2 and VEGFR-3), as well as for the expression of three EGFR ligands (amphiregulin, EGF and TGF α) and of three VEGFR ligands (VEGF-A, VEGF-B and VEGF-C), by using quantitative RT – PCR (1). All tested ligand mRNAs, with the exception of VEGF-C, were expressed in CALU-3 cells. CALU-3 cells also expressed EGFR mRNA; whereas low levels of ERBB2 and ERBB3 mRNAs were measurable. No detectable expression of ERBB4 mRNA was found. Moreover, VEGFR-1 and VEGFR-2 mRNA expression was detected. Expression of EGFR and its specific ligands suggests that in human lung adenocarcinoma CALU-3 cells an EGFR-driven autocrine pathway is relevant for cancer cell proliferation. In fact, CALU-3 cells are growth inhibited by treatment with selective EGFR TKIs, such as gefitinib or erlotinib (1). Furthermore, CALU-3 cancer cells express both VEGF ligands and VEGFRs and are growth inhibited by treatment with anti-angiogenic TKIs (1).

Therefore, CALU-3 cells were selected as a model for exploring the acquired resistance mechanisms to treatment with the EGFR TKIs erlotinib and gefitinib, or with the dual EGFR/VEGFR TKI vandetanib, or with the multi-kinase inhibitor sorafenib.

Results, conclusions and future perspectives

To further characterise the TKI-R CALU-3 cell lines, we examined differential protein expression among parental, sensitive CALU-3 cells and their TKI-R derivatives. As illustrated in Figure 1B, EGF-stimulated activation of the EGFR was efficiently blocked in P- and ERL-R, GEF-R and VAN-R CALU-3 cells, but not in SOR-R CALU-3 cells, as demonstrated by the inhibition of EGFR auto-phosphorylation (P-EGFR). It has been suggested that increased expression and/or activation of other cell membrane growth factor receptors, such as insulin-like growth factor-1 receptor (IGF-1R) and/or MET, could be responsible for the acquired resistance to EGFR-targeted therapies. IGF-1R and MET result activated in all four TKI-R CALU-3 cell lines with increased level of both phosphorylated IGF-1R (P-IGF-1R) and MET (P-MET). Of interest, all four TKI-R CALU-3 cell lines showed also an increased expression of MET protein.

Activation of MAPK and AKT as well as an increase in survivin protein levels were observed in all four TKI-R CALU-3 cell lines as compared with their parental counterpart (Figure 1B). Taken together, these results suggest that in this cancer cell model of acquired resistance to four different TKIs, activation of AKT- and MAPK-driven intracellular signals, which could be activated also by other cell membrane growth factor receptors such as IGF-1R and/or MET, may be responsible for cancer cell growth in the presence of either selective anti-EGFR TKIs, such as gefitinib or erlotinib, or in the presence of broad spectrum TKIs, such as vandetanib or sorafenib.

CALU-3	Erlotinib (IC ₅₀)	Gefitinib (IC ₅₀)	Vandetanib (IC ₅₀)	Sorafenib (IC ₅₀)
P	3 μ M	6 μ M	5 μ M	5 μ M
ERL-R	>25 μ M	>25 μ M	>25 μ M	3 μ M
GEF-R	>25 μ M	>25 μ M	>25 μ M	8 μ M
VAN-R	>25 μ M	>25 μ M	>25 μ M	3 μ M
SOR-R	>25 μ M	>25 μ M	>25 μ M	>25 μ M

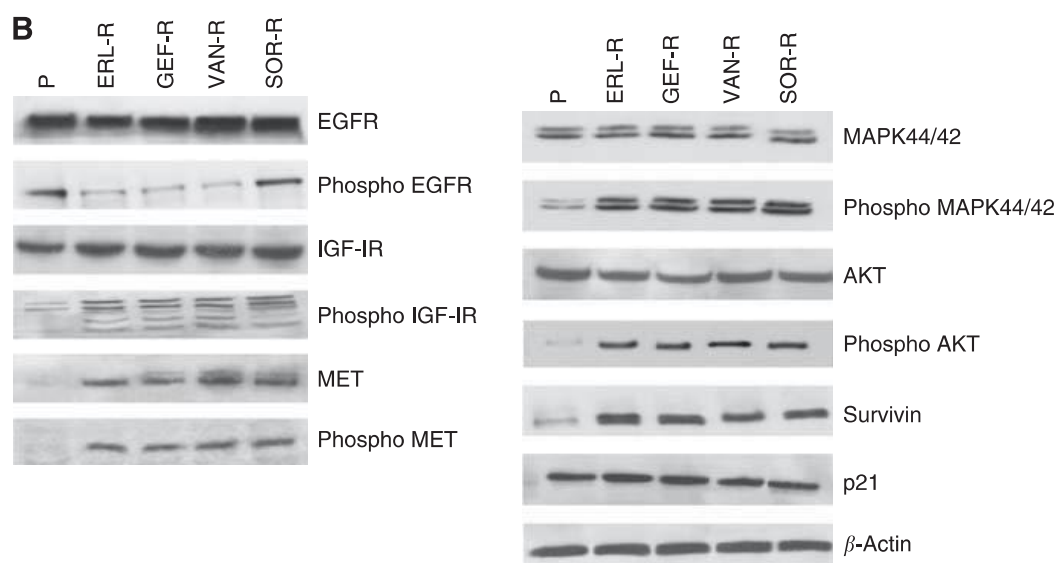
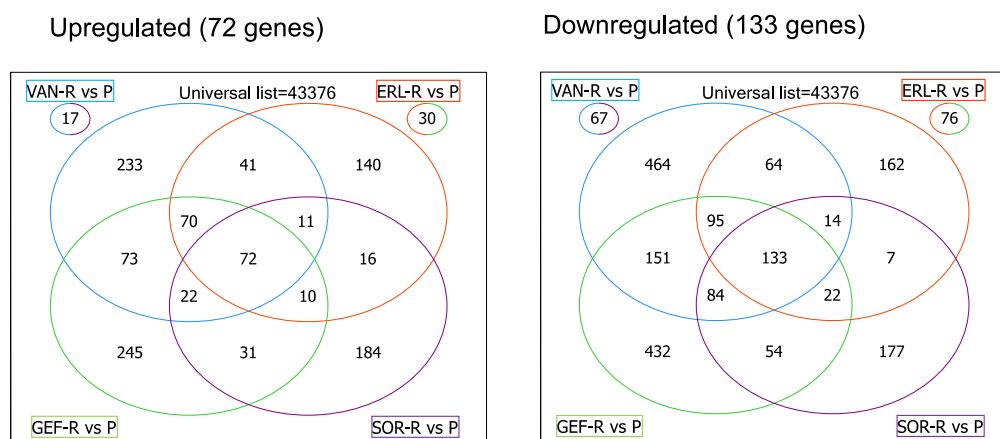


Figure 1 Development and characterisation of TKI-R CALU-3 cancer cells. **(A)** Inhibitory concentration 50 values (IC₅₀) for treatment with erlotinib, gefitinib, vandetanib or sorafenib in parental human lung adenocarcinoma CALU-3 cells (P) and in their TKI-R derivatives (ERL-R, GEF-R, VAN-R and SOR-R). Cell proliferation was measured with the MTT assay. The drug concentrations required to inhibit cell growth by 50% (IC₅₀) were determined by interpolation from the dose-response curves. Results represent the average of three separate experiments each performed in quadruplicate. **(B)** Western blotting analysis of selected growth factor receptors (EGFR, IGF-IR and MET) and of down-stream signalling pathways in parental human lung adenocarcinoma CALU-3 cells (P) and in their TKI-R derivatives (ERL-R, GEF-R, VAN-R and SOR-R). β -Actin was included as a loading control.

Further experiments were conducted to better explore the possible mechanisms of acquired resistance. Basal mRNA gene expression profiles were obtained from P-CALU-3 cells and their four TKI-R CALU-3 derivatives by using Agilent microarrays.

Collectively, the CALU-3-derived cancer cell lines with acquired resistance to four different TKIs shared 133 up regulated and 72 down regulated mRNAs as compared with P-CALU-3 cell gene expression.



Among the down regulated mRNAs, there were epithelial-related genes, such as E-cadherin and amphiregulin. Conversely, among the up regulated genes there were mesenchymal-related genes, including vimentin, caveolin, VE-cadherin, and angiogenesis- related genes, such as VEGFR-1 and HIF-1a. Taken together, these data show the loss of epithelial features and the acquisition of a mesenchymal behaviour, which are consistent with EMT phenotype in all four TKI-R CALU-3 cancer cell lines.

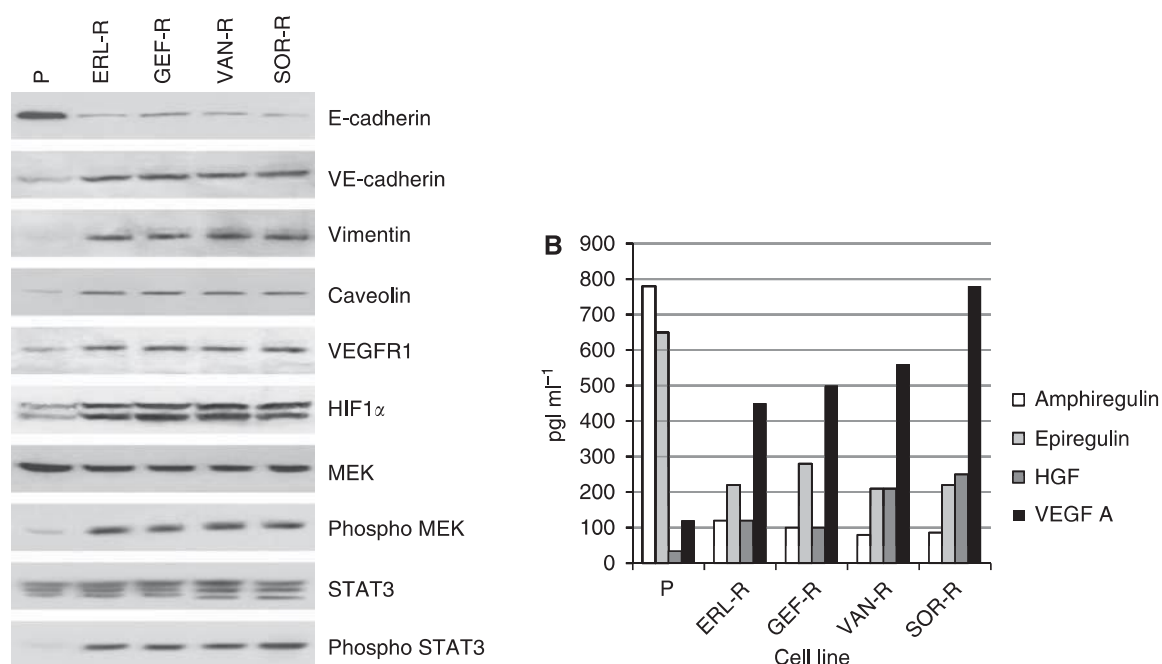


Figure 2 Differential expression of proteins in parental and TKI-R CALU-3 cancer cells. **(A)** Western blotting analysis of the expression of selected epithelial- and mesenchymal-related proteins in parental human lung adenocarcinoma CALU-3 cells (P) and in their TKI-R derivatives (ERL-R, GEF-R, VAN-R and SOR-R). **(B)** Secretion of amphiregulin, epiregulin, HGF and VEGF-A into the conditioned medium, as measured in cell culture media by specific ELISAs.

According to the EMT profile, all four TKI-R CALU-3 cells showed increased invasion, migration and anchorage-independent tumour growth. Treatment with several agents targeting AKT, MET or IGF-1R did not affect TKI-R CALU-3 cell proliferation. In contrast, treatment with MSC19363669B and selumetinib, two selective MEK inhibitors, caused inhibition of cell proliferation, migration and invasion. These results were obtained by in vitro and in vivo experiments.

These data suggest the important role of MEK activation in mediating resistance to anti EGFR/VEGFR targeted therapy, its role in mediating the EMT and the importance of a combined inhibition of cell surface receptors and downstream signalling (2).

The activation of downstream pathways opens several possibilities for combined therapy. Several relevant signalling pathways which are involved in cancer development and progression could be a target for the bortezomib-induced inhibition of proteasome activity. In this respect, bortezomib treatment caused a marked suppression of the PI3K/AKT controlled cell survival pathway in human breast cancer cell lines, suggesting a functional link between activation of the EGFR family of growth factor receptors, PI3K/Akt signalling and proteasome inhibition. Moreover, recent preclinical work from our laboratory and from others has suggested a rationale for the combined use of bortezomib with EGFR inhibitors. Therefore, the inhibition of EGFR-induced activated PI3K/AKT signalling pathway by proteasome inhibition might be used to increase sensitivity and/or to overcome resistance to EGFR inhibitors. In this respect, bortezomib treatment induced a significant inhibition of cancer cell growth and an increase in apoptosis in CALU 3 EGFR inhibitor-resistant cancer cells (GEF-R and ERL-R), suggesting that, in addition to interference with AKT signalling, other mechanisms are involved in the pro-apoptotic effects of bortezomib. Bortezomib treatment activated endoplasmic reticulum (ER) stress-mediated apoptosis, as demonstrated by the induction of GADD153, an ER stress-inducible transcription factor, and of the death receptor DR5, in EGFR inhibitor-resistant cells, but not in parental cells. This effect resulted in the activation of the extrinsic apoptotic pathway, as shown by caspases 8 dependent- PARP and bid cleavage. Bortezomib significantly inhibited the growth of EGFR inhibitor-resistant CALU-3 cells which were established as subcutaneous tumour xenografts in athymic nude mice.

These results suggest that bortezomib treatment could be a useful approach to overcome resistance to anti-EGFR therapies (3).

As compared to parental CALU-3 and HCT116 human cancer cells, TKI-resistant cell lines showed a significant increase in the levels of activated, phosphorylated AKT, MAPK, and of survivin. Considering the role of RAS and RAF as downstream signals of both the EGFR and VEGFR pathways, we treated resistant cells with sorafenib, an inhibitor of C-RAF, B-RAF, c-KIT, FLT-3, RET, VEGFR-2, VEGFR-3, and PDGFR-b. Sorafenib reduced the activation of MEK and MAPK and caused an inhibition of cell proliferation, invasion, migration, anchorage-independent growth in vitro and of tumour growth in vivo of all TKI-resistant CALU-3 and HCT116 cell lines. These data suggest that resistance to EGFR inhibitors is predominantly driven by the RAS/RAF/MAPK pathway and can be overcome by treatment with sorafenib (4).

The clinical activity of the combination of sorafenib and the EGFR inhibitor, erlotinib, has been studied in a multicentre, randomized phase II study in unselected untreated elderly patients with non-small-cell lung cancer (NSCLC). The trial was designed to select the most promising sorafenib-containing combination in previously untreated elderly stage IIIB or IV NSCLC patients, with performance status of zero. Patients were randomly assigned to one of the following

combinations: gemcitabine, 1200 mg/m² days 1 and 8, every 21 days, for a maximum of six cycles, plus sorafenib, 800 mg/day, until disease progression or unacceptable toxicity (arm 1); or erlotinib, 150 mg/day, plus sorafenib, 800 mg/day, until disease progression or unacceptable toxicity (arm 2). Sixty patients were randomly allocated to the study (31 patients in arm 1 and 29 patients in arm 2). After a median follow-up of 15 months, 10 patients [32%, 95% confidence interval (CI) 16% to 49%] in arm 1 and 13 patients (45%, 95% CI 27% to 63%) in arm 2 were alive at 1 year. Median overall survival was 6.6 and 12.6 months in arm 1 and arm 2, respectively. Observed toxic effects were consistent with the expected drug profiles.

The combination of erlotinib and sorafenib was feasible in elderly patients with advanced NSCLC and was associated with a higher 1-year survival rate than the other arm (5).

Recently, we examined the effects of a combined treatment of metformin with a selective EGFR-TKI, gefitinib, on NSCLC cell lines. The combination of metformin with gefitinib induced a strong anti-proliferative and proapoptotic effect in NSCLC cell lines that harboured wild-type LKB1 gene. Treatment with metformin as single agent, however, induced an activation and phosphorylation of mitogen-activated protein kinase (MAPK) through an increased C-RAF/B-RAF heterodimerization. The inhibition of EGFR phosphorylation and of downstream signalling by adding gefitinib to metformin treatment abrogated this phenomenon and induced a strong apoptotic effect in vitro and in vivo. Metformin and gefitinib are synergistic in LKB1 wild-type NSCLC cells. However, further studies are required to investigate better the effect of metformin action on the RAS/RAF/MAPK pathway and the best context in which to use metformin in combination with molecular targeted agents (6-7).

This strong preclinical rationale lead us to initiate a phase II clinical study testing metformin in combination with erlotinib in stage IV NSCLC in second-third line of therapy (METAL Study: METformin in Advanced Lung cancer).

List of publications resulting from the Grant

- 1: Martinelli E, Troiani T, Morgillo F, Rodolico G, Vitagliano D, Morelli MP, Tuccillo C, Vecchione L, Capasso A, Orditura M, De Vita F, Eckhardt SG, Santoro M, Berrino L, Ciardiello F. Synergistic antitumor activity of sorafenib in combination with epidermal growth factor receptor inhibitors in colorectal and lung cancer cells. *Clin Cancer Res*. 2010 Oct 15;16(20):4990-5001.
- 2: Morgillo F, Cascone T, D'Aiuto E, Martinelli E, Troiani T, Saintigny P, De Palma R, Heymach JV, Berrino L, Tuccillo C, Ciardiello F. Antitumour efficacy of MEK inhibitors in human lung cancer cells and their derivatives with acquired resistance to different tyrosine kinase inhibitors. *Br J Cancer*. 2011 Jul 26;105(3):382-92.
- 3: Morgillo F, D'Aiuto E, Troiani T, Martinelli E, Cascone T, De Palma R, Orditura M, De Vita F, Ciardiello F. Antitumor activity of bortezomib in human cancer cells with acquired resistance to anti-epidermal growth factor receptor tyrosine kinase inhibitors. *Lung Cancer*. 2011 Mar;71(3):283-90.
- 4: Morgillo F, Martinelli E, Troiani T, Orditura M, De Vita F, Ciardiello F. Antitumor activity of sorafenib in human cancer cell lines with acquired resistance to EGFR and VEGFR tyrosine kinase inhibitors. *PLoS One*. 2011;6(12):e28841.

5: Gridelli C, Morgillo F, Favaretto A, de Marinis F, Chella A, Cerea G, Mattioli R, Tortora G, Rossi A, Fasano M, Pasello G, Ricciardi S, Maione P, Di Maio M, Ciardiello F. Sorafenib in combination with erlotinib or with gemcitabine in elderly patients with advanced non-small-cell lung cancer: a randomized phase II study. *Ann Oncol.* 2011 Jul;22(7):1528-34.

6: Morgillo F, Sasso FC, Della Corte CM, Vitagliano D, D'Aiuto E, Troiani T, Martinelli E, De Vita F, Orditura M, De Palma R, Ciardiello F. Synergistic effects of metformin treatment in combination with gefitinib, a selective EGFR tyrosine kinase inhibitor, in LKB1 wild-type NSCLC cell lines. *Clin Cancer Res.* 2013 Jul 1;19(13):3508-19.

7: Morgillo F, Sasso FC, Della Corte CM, Festino L, Manzo A, Martinelli E, Troiani T, Capuano A, Ciardiello F. Metformin in lung cancer: rationale for a combination therapy. *Expert Opin Investig Drugs.* 2013 Nov;22(11):1401-9

Selection of courses and workshops attended during the fellowship

ESMO 2008 Congress, Stockholm

ESMO 2009 Congress, Berlin

ESMO 2010 Congress, Milan

Acknowledgments

A special thank you to the ESMO Fellowship Programme and to all patients and their families who contributed to the work.

References

1.7 Ciardiello F, *NEJM.* 2008.

2.7 Rosell R, et al. *Lancet Onc.*2012. 3.7Morgillo F, et al. *BJC* 2011

4.7 Yauch RL, et al. *Can Res* 2005 5.7Maitah MY, et al. *PLoS One.* 2011 6.7Sequist LV, et al. *Sci. Transl. Med.* 2001 7.7Morgillo F, *DRU*, 2005

8.7 Morgillo F, et al. *Can Res.* 2006 9.7Morgillo F, et al. *Clin Cancer Res.* 2007 10.7Martinelli E, et al. *Clin Can Res...*