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# Combination of Gefitinib and Pemetrexed prevents the acquisition of TKI-resistance in NSCLC cell lines carrying *EGFR* activating mutation

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**ABSTRACT**

**Introduction:** Development of resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) is a clinical issue in *EGFR* mutated non-small cell lung cancer (NSCLC) patients. The aim of this study was to investigate the potential of combining gefitinib and pemetrexed in preventing the acquisition of EGFR-TKI-resistance in NSCLC cell lines harboring EGFR exon 19 deletion.

**Methods:** The effect of different combinatorial schedules of gefitinib and pemetrexed on cell proliferation, cell cycle, apoptosis and acquisition of gefitinib-resistance in PC9 and HCC827 NSCLC cell lines and in PC9 xenograft models was investigated.

**Results:** A simultaneous treatment with gefitinib and pemetrexed enhanced cell growth inhibition and cell death and prevented the appearance of gefitinib-resistance mediated by T790M mutation or epithelial to mesenchymal transition (EMT) in PC9 and HCC827, respectively. In PC9 cells and in PC9 xenografts the combination of gefitinib and pemetrexed, with different schedules, prevented gefitinib-resistance only when pemetrexed was the first treatment, given alone or together with gefitinib. Conversely, when gefitinib alone was firstly administered and pemetrexed sequentially alternated, a negative interaction was observed and no prevention of gefitinib-resistance was documented. The mechanisms of resistance developed *in vivo* included T790M mutation and EMT. The induction of EMT was a feature of tumors treated with gefitinib when given before pemetrexed, while T790M was recorded only in tumors treated with gefitinib alone.

**Conclusions:** The combination of gefitinib and pemetrexed is effective in preventing gefitinib-resistance; the application of intermittent treatments requires that gefitinib is not administered before pemetrexed.

**Key Words:** NSCLC, EGFR, gefitinib, pemetrexed, TKI-resistance

## INTRODUCTION

Non-small cell lung cancer (NSCLC) is the leading cause of cancer death in the world. Targeted drugs such as epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) tyrosine kinase inhibitors (TKIs) have deeply changed the management of advanced EGFR mutated and ALK-positive NSCLC patients in the last decade.[1]

It is well known that in patients harbouring *EGFR* gene activating mutations, disease progression occurs after a median of 10-14 months from the beginning of TKIs therapy.[2] Various mechanisms of resistance have been identified.[3] The acquisition of the *EGFR*-T790M secondary mutation is responsible for half of the cases of acquired resistance to EGFR-TKIs;[4-6] *MET* amplification, allowing cell survival by persistent AKT signalling activation, has been described in 5 to 15% of cases.[7-9] *PIK3CA* mutations and transformation to small cell lung cancer have also been implicated as mechanisms of resistance to EGFR-TKIs.[6] Furthermore, recent studies reported that epithelial-mesenchymal transition (EMT), a process in which cells lose their epithelial features and acquire a mesenchymal-fibroblastoid phenotype enhancing their motility and invasion capability, might also play a role in the development of resistance to EGFR-TKIs in NSCLC.[10, 11]

An important unresolved issue is to assess whether the population of *EGFR*-mutant patients may benefit from the combination of EGFR-TKI with systemic chemotherapy. Randomized phase III clinical trials showed no advantage combining EGFR-TKIs with standard first-line chemotherapy for advanced NSCLC, although these trials were conducted in unselected patient population.[12-15]

Recent findings would suggest a potential benefit combining chemotherapy with EGFR-TKI in *EGFR*-mutant patients, in particular with non-concurrent drug administration.[16, 17]

The phase II NEJ005/TCOG0902 clinical trial indicates that the combination of gefitinib and chemotherapy in the *EGFR*-mutated setting, either with concurrent or sequential treatment, shows promising efficacy with predictable toxicities, and concurrent regimen might provide better overall survival.[18] A randomized phase III study comparing gefitinib alone versus gefitinib plus concurrent chemotherapy (NEJ009) is ongoing.[19]

Considering these clinical results, we hypothesize that *EGFR*-mutated patients could benefit from integration of gefitinib and chemotherapy in preventing or retarding the acquisition of EGFR-TKI resistance.

The aim of this study was to explore, both *in vitro* and *in vivo,* the therapeutic potential of gefitinib and pemetrexed combination, and to determine the best schedule of administration in preventing or retarding the acquisition of gefitinib-resistance in NSCLC cell lines harboring a deletion in exon 19 of *EGFR* gene.

## MATERIALS AND METHODS

### Cell Culture

PC9 and HCC827 cells, harboring an in-frame deletion in exon 19 of EGFR gene, were used. Human HCC827 cell line was purchased from American Type Culture Collection (ATCC® CRL-2868™) (Manassas, VA), and the ATCC authenticates the phenotypes of these cell lines on a regular basis (http://www.lgcstandards-atcc.org). PC9 was kindly provided by Dr P. Jänne (Dana-Farber Cancer Institute, Boston MA). Cells were cultured as recommended and maintained under standard cell culture conditions at 37℃ in a water-saturated atmosphere of 5% $CO_2$ in air.

### Drug treatment

Gefitinib (ZD1839/Iressa®) was provided by AstraZeneca (Milan, Italy). Pemetrexed was from inpatient pharmacy of University Hospital of Parma. Gefitinib was dissolved in DMSO (Sigma, ST Louis, MO), while pemetrexed was dissolved in 0.9% sodium chloride injection and diluted in fresh medium before use. Final DMSO concentration in medium never exceeded 0.1% (v/v) and equal amounts of the solvent were added to control cells.

### Analysis of cell proliferation, cell death and cell cycle

Cell number, cell viability and cell death were evaluated as previously described.[20] Distribution of the cells in the cell cycle was determined by propidium iodide (PI) staining and flow cytometry analysis as described elsewhere.[21] The nature of interaction between gefitinib and pemetrexed was calculated using the Bliss additivism model as previously described.[22] A theoretical dose-response curve was calculated for combined inhibition using the equation EBliss= EA + EB - EA * EB, where EA and EB are the percent of inhibition versus control obtained by gefitinib (A) and pemetrexed (B) alone and the E Bliss is the percent of inhibition that would be expected if the combination was exactly additive. If the combination effect is higher than the expected value EBliss the interaction is

synergistic, while if the effect is lower, the interaction is antagonist. Otherwise, the effect is additive and there is no interaction between drugs.

**Colony formation**

Cells were plated in 96-well plates at a density of 5000 cells/well and cultured following the protocols indicated in each experiment. Medium was changed every three days and at the end of the experiment cells were fixed with ice-cold methanol, stained with 0.1% crystal violet (Sigma) and counted. Colonies containing at least 20 cells were scored, and data are given as colony number/96 well plate.

**Cell migration**

The migration assay was carried out using Transwell chamber with 6.5-mm diameter polycarbonate filters (8μm pore size), BD Biosciences, Erembodegem, Belgium) as previously described.[21]

**Western blot analysis**

Procedures for protein extraction, solubilization, and protein analysis by 1-D PAGE are described elsewhere.[20] Antibodies against p-EGFR$^{Tyr1068}$, EGFR, MET, cleaved caspase-3, cleaved caspase-9, Bim, E-cadherin, N-cadherin, Vimentin, SNAIL, SLUG were from Cell Signaling Technology (Beverly, MA); antibody against TS was from Upstate (Lake Placid, NY); antibody against Actin was from Sigma. HRP-conjugated secondary antibodies were from Pierce (Rockford, IL) and chemoluminescence system (Immobilion$^{TM}$ Western Chemiluminescent HRP Substrate) was from Millipore (Temecula, CA). Reagents for electrophoresis and blotting analysis were from BIO-RAD (Hercules, CA).

**Quantitative Real-Time PCR**

Total RNA was isolated using RNeasy mini kit (Qiagen, Venlo, Netherlands). Two μg RNA was retro-transcribed using High Capacity RNA-to-cDNA kit (Applied Biosystems, Forster City, CA), according to the manufacturer's instructions. Primers to specifically amplify vimentin (Hs_VIM_1_SG – cat. no. QT00095795), N-cadherin (Hs_CDH2_1_SG – cat. no.

QT00063196), E-cadherin (Hs_CDH1_1_SG cat. no. QT00080143) were obtained from Qiagen (Venlo, Netherlands). The quantitative real-time PCR was performed in a 20-µl reaction volume containing Fast SYBR® Green master mix (Applied Biosystems). All reactions were performed in triplicate using the StepOne system instrument (Applied Biosystems). Samples were amplified using the following thermal profile: 95℃ for 20 sec, 40 cycles of denaturation at 95℃ for 3 sec followe d by annealing and extension at 60℃ for 30 sec. Amplifications were normalized to HPRT1 (Hs_HPRT1_1_SG cat. no. QT00059066) and PGK1 (Hs_PGK1_1_SG cat. no. QT00013776). The fold change was calculated by the ΔΔCT method and results were plotted as $2^{-\Delta\Delta CT}$.

**T790M evaluation**

Mutational analysis of EGFR was performed using the Therascreen EGFR RGQ PCR Kit (Qiagen) accordingly to the manufacturer's instructions.

**Tumor xenografts**

Two hundred microliters of matrigel (BD Biosciences) and sterile PBS (1:1) containing $5\times10^6$ PC9 cells were subcutaneously injected on the right flank of Balb/c-Nude female mice (Charles River Laboratories, Calco, Italy). The animals were housed in a protected unit for immunodeficient animals with 12-hour light/dark cycles and provided with sterilized food and water *ad libitum*. When tumor volume reached an average size of 150mm$^3$, animals were randomly allocated into five groups (n=8) as indicated in Fig 4A. Gefitinib (10mg/kg in 1% Tween 80) was given once per day, five times per week, by oral gavage. Pemetrexed (100mg/kg in 0.9% NaCl injection) was given intraperitoneally once a day, three times per week. Tumor xenografts were measured as previously described.[23] At the end of the experiments, mice were euthanized by cervical dislocation and tumors weighted and collected for immunohistochemical and analysis.

All experiments involving animals and their care were performed with the approval of the Local Ethical Committee of University of Parma and by the Italian Ministry of Health on

October 2013 (Prot. N. 57/13 25 October 2013), in accordance with the institutional guidelines that are in compliance with national (DL116/92) and international (86/609/CEE) laws and policies.

**Isolation and *in vitro* expansion of neoplastic cells from xenograft tumors.**

Fresh samples were firstly minced with scissors and then put into a 1mg/ml collagenase/dispase solution (Roche, Indianapolis, IN) for 60 minutes in a shaking bath at 37℃. At the end of enzymatic digestion, large frag ments were removed using a nylon filter with pores of 100μm and the cell suspension was centrifuged at 240g for 5 min.

Cells obtained from digestion were suspended in complete culture medium and, after short pre-adherence to remove fibroblasts, cells were seeded for 24h; fresh culture medium was then added to cell culture and changed twice a week.

**Immunohistochemical analysis of tumor xenografts**

To document at tissue level changes in immunophenotype associated with EMT, as previously described,[24] sections were exposed to double immunofluorescent labeling using anti-Cytokeratin7 (CK7) and -vimentin (VIM) antibodies (DAKO, Glostrup, Denmark) followed by TRITC-and FITC-conjugated secondary antibodies (Sigma).

**FISH analysis of human sex chromosomes**

Fluorescence in situ hybridization (FISH) analysis of human sex chromosomes was used to detect the injected human cells within the tumor xenografts according to a methodology previously described[25]. Briefly, following DNA denaturation, a human-specific chromosome fluorescent probe, complementary to the centromeric region of the X chromosome (Cy3-Alpha-Satellite DNA, locus X p11.1-q11.1, Vysis, USA) was applied and sections were post-fixed in formamide buffer. Subsequently, samples were incubated with primary antibody against Vimentin followed by specific FITC-conjugated secondary antibody. Nuclei were counterstained with DAPI, and the analysis was performed at 400× magnification using a fluorescence microscope (Olympus BX60) with Z-stack equipment.

**Statistical analysis**

Statistical analyses were carried out using GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego, CA). Results are expressed as mean values ±standard deviations (SD) for the indicated number of independent measurements. Differences between the mean values recorded for different experimental conditions were evaluated by Student's t-test or by one-way ANOVA followed by Bonferroni's post-test, and P values are indicated where appropriate in the figures and in their legends. P values <0.05 were considered as significant. For *in vivo* studies comparison among groups was made using two-way repeated measures ANOVA followed by Bonferroni's post-test (to adjust for multiple comparisons). Adjusted P values of less than 0.05 were considered significant.

**RESULTS**

**Effects of a short exposure to gefitinib and pemetrexed in NSCLC cell lines carrying EGFR activating mutations**

PC9 and HCC827 cells were highly sensitive to gefitinib, with $IC_{50}$ value of 13±*5* and 8±4nM, respectively. Conversely, a significant difference was observed in pemetrexed sensitivity: $IC_{50}$ values were 50±*5*nM for PC9 cells and 500±*50*nM for HCC827 cells (Fig 1A). Since high levels of thymidylate synthase (TS) expression confer a reduced response to pemetrexed,[26] the reduced sensitivity to pemetrexed of HCC827 compared with PC9 cells may be related to a higher level of TS (Fig 1B).

We then studied the combined effects of gefitinib and pemetrexed using the Bliss interaction model. Comparing the experimental combination points with those expected by the Bliss model, we observed an additive effect in the cell lines tested (Fig1C). We further explored the effect of gefitinib alone or in association with pemetrexed on cell death. The typical apoptotic morphology was observed in cells exposed to gefitinib (50nM), pemetrexed (50nM in PC9 and 500nM in HCC827), or their combination for 72 hours.

Gefitinib induced apoptosis with values of 66% in PC9 and 76% in HCC827; pemetrexed was less effective inducing 25% of cell death in both the cell lines.

The combination of the two drugs further increased cell death up to 87% in PC9 and 96% in HCC827 (Fig 1D) and this effect was additive (Fig 1E).

The therapeutic potential of gefitinib and pemetrexed combination using different schedules of treatment in a 48h time frame experiment was then explored in PC9 cells.

As shown in Fig 2A, higher levels of cell death, as quantified by fluorescence microscopy, were observed with G+P→G, P→G and G+P→G+P schedules; G→P was the least efficient combination revealing an antagonist effect (Fig 2B). Results obtained on cell death were also confirmed by evaluating the sub-G1 region (apoptotic index, %) of the cell cycle profiles (Fig 2C). Induction of cell death was irrespective of the distribution among the different phases of the cell cycle. Indeed, when cells were treated firstly with gefitinib or with gefitinib plus pemetrexed, a G1 phase arrest was observed independently on the subsequent treatment. Otherwise, pemetrexed alone or pemetrexed followed by gefitinib induced S-phase or $G_2$/M arrest, respectively.

Moreover, we observed that expression of TS was down regulated, EGFR was dephosphorylated, the pro-apoptotic BCL-2 family member Bim, required for apoptosis induced by EGFR-TKIs in *EGFR*-mutated cells,[27] was induced and caspases 3 and 9 were activated when gefitinib was given, also as second treatment. In contrast, the schedule G→P maintained high level of TS and EGFR phosphorylation and no induction of pro-apoptotic proteins was observed (Fig 2D).

**Effect of a long exposure to gefitinib and pemetrexed in NSCLC cell lines carrying *EGFR* activating mutations**

When HCC827 and PC9 cells were cultured in the presence of gefitinib for a prolonged period of time (until 1 month), an increasing number of surviving and proliferating cells forming colonies was observed after 10 days of treatment for HCC827 and 20 days for

PC9 cells. On the contrary, we did not observe any colony formation when both the cell lines were simultaneously treated with gefitinib and pemetrexed (Fig 3A).

Fifteen clones for PC9 cells and ten clones for HCC827 cells were isolated and tested in the presence of different gefitinib concentrations and showed an $IC_{50}$ for gefitinib of about 10μM, a value 1000 fold higher than the $IC_{50}$ of the parental cell lines (not shown).

We then evaluated the mechanisms of gefitinib-resistance in the clones isolated after the selection procedures. Thirteen PC9 clones out of fifteen tested, exhibited *EGFR*-T790M secondary mutation as evaluated by Therascreen *EGFR* PCR assay. We performed the EGFR phosphorylation inhibition assay in ten clones and confirmed the maintenance of EGFR activation in all of them (as shown in Fig 3B for one clone). Neither T790M nor *MET* amplification were observed in all the ten HCC827 clones tested. Since they exhibited a different morphology from parental cells, we evaluated whether cell clones underwent EMT. By RT-PCR and Western blot analysis we demonstrated that nine clones out of ten tested had increased expression of mesenchymal markers such as vimentin, N-cadherin and SNAIL and reduction of the epithelial marker E-cadherin (as shown for one clone in Fig 3C and Fig 3D). Moreover, the HCC827 resistant cells displayed increased migratory capability as shown in Fig 3E.

The therapeutic potential of gefitinib and pemetrexed combination in preventing or retarding the appearance of resistance, using different schedules of treatment in long-term experiments (two months) was then explored. In particular, PC9 cells were seeded in microplates of 96 wells and cultured following the protocols indicated in Fig 3F: schedule A (cells continuously treated with a fixed gefitinib concentration), schedule B (cells continuously treated with gefitinib and, every week, a simultaneous treatment with pemetrexed was cyclically performed); schedule C (cells treated with gefitinib intercalated, every week, with pemetrexed); schedule D (cells treated with gefitinib plus pemetrexed

intercalated, every week, with gefitinib alone); schedule E (cells treated with pemetrexed intercalated, every week, with gefitinib).

After 8 weeks, colonies with more than 20 cells were counted and as shown in Fig 3G, the maximum rate of colony formation was recorded following schedule A (cells treated with gefitinib only); a significant number of colonies was present also in schedule C (gefitinib intercalated with pemetrexed), while schedules B, D and E significantly prevented the appearance of resistant cells, with schedule D being the most effective.

We then shifted the colonies to physiologic conditions without drugs for 2 weeks and only cells from schedules A and C were able to proliferate. Conversely, cells from protocols B, D and E did not attach and were considered as abortive cells (data not shown).

**Effect of different combinatory schedules of gefitinib and pemetrexed in the acquisition of gefitinib resistance *in vivo***

Finally, the effect of the different schedules of gefitinib and pemetrexed association in the acquisition of gefitinib resistance was tested *in vivo*.

We performed *in vivo* experiments on PC9 xenografts treated with gefitinib and pemetrexed, mimicking most of the *in vitro* schedules (Fig 4A).

PC9 cells were subcutaneously inoculated into Balb/c-Nude female mice and after tumors had reached an average size of about 150mm$^3$ the animals were randomized into five different groups: vehicle alone (control), gefitinib (1), gefitinib intercalated, every week, with pemetrexed (2), gefitinib plus pemetrexed intercalated, every week, with gefitinib alone (3) and pemetrexed intercalated, every week, with gefitinib (4). Gefitinib was administered at a dosage of 10mg/Kg according to previous studies on PC9-derived xenograft models,[28-30] while the dose of 100 mg/kg pemetrexed was selected on the basis of preliminary experiments documenting its capability to inhibit tumor growth in PC9-derived xenograft tumors (not shown). Tumor growth was monitored for 18 weeks and

during this period, mice showed no signs of toxicity and regularly gained body weight (not shown).

As illustrated in Fig 4B the continuous gefitinib treatment inhibited tumor growth for up to 10 weeks. Subsequently, tumor growth inhibition disappeared and six of eight animals treated with gefitinib alone developed acquired resistance. Conversely, in the group of animals treated with gefitinib plus pemetrexed intercalated, every week, with gefitinib alone (group 3) tumor regression was observed up to week 18 in all treated mice. The treatment with pemetrexed intercalated with gefitinib (group 4) was also effective in inhibiting tumor growth, whereas treatment with gefitinib intercalated with pemetrexed (group 2) was not able to prevent the acquisition of resistance and tumor regrowth (six of eight animals) was observed after 8 weeks of treatment.

We then established three cell lines isolated from tumors regrown from control, gefitinib (group 1) and gefitinib intercalated with pemetrexed (group 2) and termed them PC9C, PC9G and PC9G/P, respectively. All the cell lines maintained the exon 19 deletion mutation in *EGFR*. PC9C cells were highly sensitive to gefitinib as were parental PC9 cells ($IC_{50}$ 25nM). On the contrary, the $IC_{50}$ values of gefitinib in PC9G and PC9G/P were >10μM and >1μM, respectively (Fig 5A). These cell clones were also more resistant to pemetrexed ($IC_{50}$ 1μM) (Fig 5B).

The mechanisms of resistance developed in PC9G and PC9G/P cells were then investigated. PC9G cells contained the T790M mutation as detected by Therascreen *EGFR* PCR assay, while this mutation was not present in PC9G/P cells. To confirm this finding, PC9G and PC9G/P were treated with gefitinib for 24h and Western blot analysis (Fig 5C) showed that EGFR remained phosphorylated in PC9G cells but was completely dephosphorylated in PC9G/P cells. Consistent with morphological changes, PC9G/P cells increased the expression of the mesenchymal markers N-cadherin, vimentin, SLUG and SNAIL and reduced the expression of E-cadherin (Fig 5C).

Therascreen *EGFR* PCR assay was performed in tumor tissues from xenograft mice. Two of six tumors from group1 confirmed the *EGFR*-T790M mutation as observed in PC9G cells. By contrast, all tumors from groups 2, 3 and 4 were negative for EGFR-T790M mutation.

Moreover, tissue analysis of EMT was conducted on tumor samples from experimental groups. A quantitative estimation of the process was performed following double immunofluorescence staining with vimentin and cytokeratin antibodies on sections from xenografts (Fig 6). Thus, tumor areas positive for CK7 only were considered EMT negative, whereas when vimentin was expressed alone or concurrently with CK7 signal, in neoplastic cells, tumor areas were considered EMT positive. In addition, to determine whether vimentin expression belonged to neoplastic cells or to mouse resident interstitial cells surrounding the tumor, FISH analysis of human X chromosome was performed together with Vimentin staining. By this approach, a 4-fold increase ($p<0.001$) in EMT was observed in all resistant tumors from group 2 (EMT positive tumor cells 26±4.8%, n=6) compared to the four tumors in the group 1 without T790M mutation (6.5 ± 2%, n=4) while the fraction of neoplastic cells undergoing EMT was negligible in CTRL (2.8±1 %, n=5), in group 3 (1.8±1.4%, n=2) and in group 4 (1.7±1%, n=3).

These findings indicate that the presence of pemetrexed suppressed the emergence of T790M mutation in resistant tumors, and that the induction of EMT is a prominent feature of tumors treated by gefitinib when given before pemetrexed.

**DISCUSSION**

In this study we demonstrated that the combination of gefitinib with pemetrexed prevented EGFR-TKI-resistance induced by either *EGFR*-T790M mutation or EMT process, in cell lines harboring an in-frame deletion in exon 19 of EGFR gene. Moreover, in PC9 xenograft model, the onset of resistance was prevented only when pemetrexed was the initial treatment either given in combination or in alternate schedule with gefitinib. Conversely,

when gefitinib alone was the first treatment and then alternated with pemetrexed, prevention of gefitinib-resistance was the least effective.

Several preclinical studies investigated the interaction between EGFR-TKIs and pemetrexed. In 2007 Li et al. reported the effect of erlotinib and pemetrexed combination, in 72h experiments, following different schedules of treatment in a panel of NSCLC either sensitive or resistant to erlotinib.[31] They demonstrated schedule-dependent effects with antagonism when erlotinib was administered before pemetrexed in erlotinib-sensitive cells. The antagonism was associated with the G1 blockade of the cell cycle induced by EGFR-TKI, which exerted a protective effect from the cytotoxic activity of pemetrexed. By contrast, erlotinib, gefitinib and BIBW2992 reduced TS expression and activity in NSCLC cells resulting in a synergistic anti-proliferative and pro-apoptotic activity when combined with pemetrexed.[32, 33] Our results on the effect of a short exposure to gefitinib and pemetrexed are consistent with these data. In all the experimental schedules in which gefitinib was maintained or given after pemetrexed the expression of TS was reduced, EGFR was de-phosphorylated and apoptosis induced. Our results further support a negative interaction between gefitinib and pemetrexed when gefitinib was administrated before pemetrexed indicating the maintenance of high level of TS and EGFR phosphorylation as well as the inhibition of apoptosis as possible mechanisms.

The novelty of the present study is related to the effect of different combinatory schedules of gefitinib and pemetrexed in the acquisition of gefitinib resistance in long exposure experiments. The exposure of PC9 and HCC827 cell lines to gefitinib induced the appearance of resistant clones characterized by *EGFR*-T790M mutation and EMT phenotype, respectively. These results are in agreement with many previous reports indicating a prevalence of *EGFR*-T790M in PC9 resistant cells[34-37] and *MET* amplification or EMT without evidence of *MET* amplification in HCC827.[7, 35, 38, 39] Clones selected by exposure to 50nM gefitinib were then able to proliferate normally in the presence of 10µM

gefitinib. Previous reports suggested that *EGFR*-T790M mutation is already present in cis with the activating mutation in a small fraction of PC9 cell population. The frequency has been recently reported as 0.0360% in PC9 cells.[40] When PC9 and HCC827 cells were simultaneously exposed to gefitinib and pemetrexed we did not observe any selection of resistant clones suggesting that combined treatment prevented the appearance of EGFR-TKI resistance. Although it is still unresolved whether the resistance to TKI may derive from a selection of pre-existing resistant cells or from new acquired genetic or epigenetic alterations, our results suggest that the enhancement of cytotoxicity when pemetrexed was added to gefitinib might suppress the small subpopulation of cells harboring T790M mutation or with mesenchymal phenotype.

Interestingly, in PC9 cells the prevention of T790M-mediated gefitinib resistance was also observed in different combinatory schedules of gefitinib and pemetrexed in long exposure experiments (see figure 4 for details). It is noteworthy that the schedule with gefitinib as first treatment and pemetrexed administered subsequently did not prevent the selection of resistant and viable clones accordingly with results obtained in 48h-72h experiments. Similarly, in *in vivo* PC9 xenograft experiments, tumor regrowth was observed when mice were treated either with gefitinib alone or with gefitinib intercalated with pemetrexed. By contrast, pemetrexed (alone or with gefitinib) intercalated with gefitinib resulted in tumor regression up to 5 months in all treated mice.

These preclinical data support very recent promising results from two phase II clinical trials that evaluated the efficacy of gefitinib combined with pemetrexed as first-line therapy in advanced *EGFR* mutated NSCLC patients.[41, 42] In the trial of Yoshimura et al., pemetrexed was administered on day 1 and gefitinib was sequentially administered on days 2-16 every three weeks, obtaining an high response rate (84.6%) and long median progression-free survival (PFS) (18 months), with an acceptable toxicity.[41] Recently a randomized phase II study showed that a similar combination of pemetrexed and gefitinib permits to obtain a

longer median PFS compared with gefitinib alone (15.8 vs 10.9 months; HR 0.68; 95%CI 0.48-096; p=0.029).[42]

An additional potential strategy emerging from our results, that could be further clinically tested, is represented by a continuous gefitinib administration with pemetrexed combined at the start of treatment and subsequently given at regular intervals, as intermittent therapy. In general, intermittent treatments could prolong cancer control and retard resistance appearance by enhancing cell cytotoxicity and reducing the selection pressure.

By analyzing the resistance mechanisms developed in *in vivo* experiments, we demonstrated that EGFR-T790M mutation emerged only in tumors treated with gefitinib alone, while induction of EMT was a prominent feature of tumors treated with gefitinib intercalated with pemetrexed. EMT occurred, although to a lesser extent, also in gefitinib-treated resistant tumors negative for T790M.

In agreement with our data, recent findings[40] indicate that cells from a heterogeneous population are differently selected by particular treatment regimens and a significant prevalence of T790M alleles in parental cells may not necessarily predict the emergence of EGFR TKI resistance associated with T790M.

In conclusion, the combination of gefitinib and pemetrexed is effective in preventing gefitinib-resistance; when intermittent treatment is proposed, gefitinib should not be administered before pemetrexed. These results justify further clinical trials with EGFR-TKI combined with pemetrexed.

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## Figure legends

### Figure 1

**Interaction between gefitinib and pemetrexed on the growth inhibition dose–response curves and apoptosis induction.** *A*, $IC_{50}$ values of gefitinib and pemetrexed in PC9 and HCC827. *B*, Thymidylate Synthase (TS) protein expression in PC9 and HCC827 cells evaluated by Western blot analysis. Representative blot of three independent experiments is shown. *C*, effect of combined treatment on growth inhibition versus theoretical Bliss additivity curve. In the combined treatment, pemetrexed was 25nM in PC9 and 250nM in HCC827. Cells were treated with the drugs for 72 hours and then cell growth assessed by MTT assay. Data are expressed as percent of inhibition versus control cells and represent means ±SD of three separate experiments. *D*, cells were treated with the drugs (gefitinib 50nM; pemetrexed 50nM in PC9 and 500nM in HCC827) for 72 hours and then cell death was quantified by fluorescence microscopy on Hoechst 33342 and propidium iodide-stained cells. Data, expressed as percent values, are means ±SD of three independent experiments (**p<0.01, ***p<0.001 vs ctr; ##p<0.01, ###p<0.001 vs G+P; one-way ANOVA followed by Bonferroni's post-test). *E,* effect of combined treatment (pemetrexed 50nM/gefitinib 1-100nM) on cell death versus theoretical Bliss additivity curve in PC9 cells. Cells were treated with the drugs for 72 hours and then cell death was evaluated. Data are expressed as percent of cell death versus control cells and are means ±SD of three separate experiments.

### Figure 2

**Therapeutic potential of combining gefitinib and pemetrexed using different schedules of administration.** PC9 cells were treated with 50nM gefitinib and/or 50nM pemetrexed based on the following schedules: (G→G) 48h to gefitinib; (G→G+P) 24h to gefitinib and then 24h to the combination gefitinib/pemetrexed; (G→P) 24h to gefitinib and

then 24h to pemetrexed; (G+P→G) 24h to gefitinib and pemetrexed and then 24h to gefitinib; (P→G) 24h to pemetrexed and then 24h to gefitinib; (G+P→G+P) 48h to gefitinib and pemetrexed; (P→P) 48h to pemetrexed. At the end of the treatment period: *A*, cell death was quantified by fluorescence microscopy analysis on Hoechst 33342 and propidium iodide-stained cells. Data are expressed as percent values, *bars* SD (*<0.05, **p<0.01, ***p<0.001 vs ctr; #p<0.05, ###p<0.001 vs G→G; one-way ANOVA followed by Bonferroni's post-test); *B*, cell growth was assessed using MTT assay, curve of growth-inhibitory effects of gefitinib and G→P curve versus theoretical Bliss additivity curve are reported. Data are expressed as percent of inhibition of cell proliferation versus control cells (*<0.05, ***p<0.001 vs G→P (Bliss); Student's t-test); *C*, cells were stained with propidium iodide and analyzed by flow cytometry for cell-cycle-phase distribution. Percentages ±SD are reported in the table (*<0.05, **p<0.01, ***p<0.001 vs ctr, ##p<0.01, ###p<0.001 vs G→G; one-way ANOVA followed by Bonferroni's post-test). *D*, cells were lysed and Western blot analysis was performed on lysate proteins using monoclonal antibodies directed to the indicated proteins. Results are representative of three independent experiments.

**Figure 3**

**Effect of different schedules of gefitinib and pemetrexed association in the acquisition of gefitinib resistance *in vitro.*** *A*, HCC827 and PC9 cells were treated with gefitinib (50nM) in the absence or presence of pemetrexed (500nM and 50nM respectively) and the ability to form colonies was assessed after 30 days by crystal violet assay. A representative image of colony formation is shown; *B*, p-EGFR and total EGFR were detected by Western blotting in PC9 and PC9 resistant cells exposed for 2h to 0.1 to 10µM gefitinib and stimulated with EGF 50ng/ml for 5 min.; *C,* comparison of N-cadherin, E-cadherin and vimentin mRNAs by quantitative RT-PCR in HCC827 gefitinib-resistant versus HCC827 parental cells. The fold change was calculated using the $2^{-\Delta\Delta CT}$ method

relative to HCC827 cell line used as control; *D*, HCC827 and HCC827 resistant cells were lysed and Western blot analysis was performed on lysate proteins using monoclonal antibodies directed to the indicated proteins; *E,* migration assay was performed on HCC827 and HCC827 gefitinib-resistant cells, columns are means ±SD of 10 fields counted (**p<0.01). Results in B, C, D, E are confirmed also in other gefitinib-resistant clones.

Following the schedules indicated in *F,* PC9 cells were treated with 50nM gefitinib and 50nM pemetrexed and after 8 weeks colony number was assessed as described in Material and Methods *(G)*. Columns are means ±SD of five independent experiments (***p<0.001 vs A; #p<0.05, ##p<0.01 vs C; one-way ANOVA followed by Bonferroni's post-test).

**Figure 4**

**Effect of different schedules of gefitinib and pemetrexed association in the acquisition of gefitinib resistance *in vivo*.** The schedules indicated in *A* were tested in a NSCLC xenograft model. PC9 cells were subcutaneously inoculated into Balb/c-Nude female mice and after tumors had reached an average size of about 150 $mm^3$ the animals were randomized into five different groups (*B*). Gefitinib was administered at a dosage of 10 mg/Kg (orally, five times per week) and pemetrexed at a dosage of 100 mg/kg (i.p., three times per week). Tumor sizes were measured two-three times per week and data are expressed as volume ±SEM (n=8 mice per group), (*p<0.05, **p< 0.01, ***p< 0.001 vs group 1; #p<0.05, ##p<0.01, p<0.001 vs group 2; two-way repeated measures ANOVA followed by Bonferroni's post test). Inset: representative images of dissected xenograft tumors.

**Figure 5**

**Characterization of cell lines derived from regrown tumors.** PC9C, PC9G and PC9 G/P, isolated respectively from regrown tumors from control, group 1 and 2, were treated

with increased concentrations of gefitinib (*A*) or pemetrexed (*B*) for 72h and then cell number was assessed by MTT assay. Data are expressed as percent of inhibition of cell proliferation versus control cells and are means ±SD of three separate experiments. *C*, PC9G and PC9G/P cells were exposed to 1µM gefitinib for 24h and Western blot analysis was performed on lysate proteins by using monoclonal antibodies directed to the indicated proteins. Results are representative of three independent experiments.

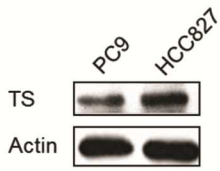
**Figure 6**

**Evaluation of EMT in neoplastic xenograft tissues.** Left panels document the double immunofluorescence detection of CK7 (red fluorescence) and VIM (green fluorescence) in sections of tumor xenografts from control (CTRL), gefitinib (GEF; group1) and gefitinib intercalated with pemetrexed (GEF/PEM; group 2) treated mice. FISH analysis of human X chromosome (red dots) together with VIM immunostaining (green) in corresponding experimental samples is shown in right panels. Nuclei are recognized by the blue fluorescence of DAPI. Asterisks indicate examples of $CK7^{pos}$ (left panels) or hXChromosome carrying (right panels) neoplastic cells labeled by $VIM^{pos}$. Arrowheads point to mouse resident $VIM^{pos}$ interstitial cells. Multiple hXchromosome signals in mitotic (arrow) and polyploid nuclei of neoplastic cells are apparent. Scale bars= 20µm.

A

| | PC9 | HCC827 |
|---|---|---|
| gefitinib, nM | 13±5 | 8±4 |
| pemetrexed, nM | 50±5 | 500±50 |

B

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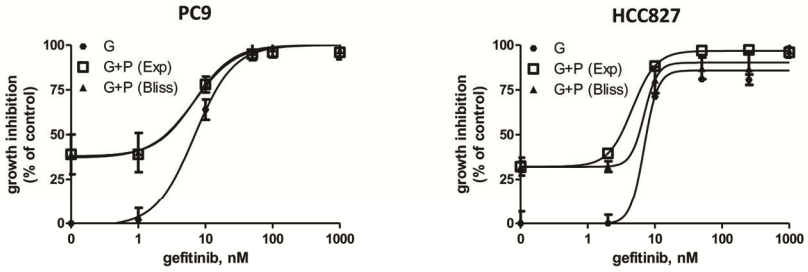


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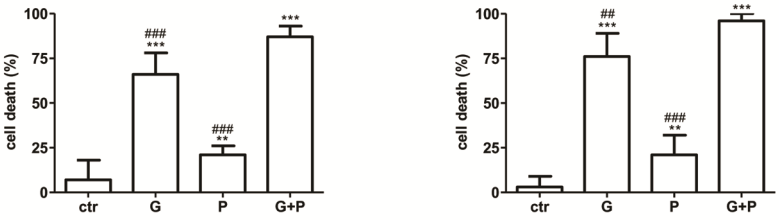


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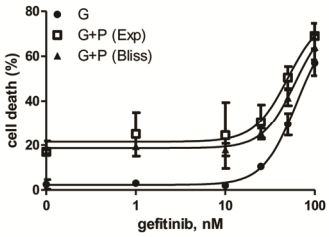
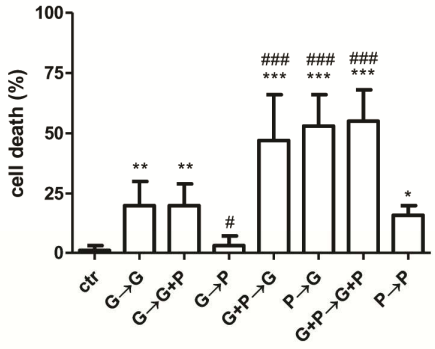


A

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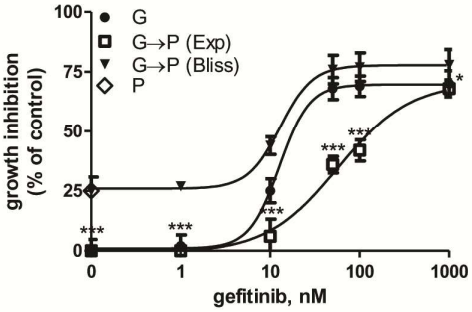
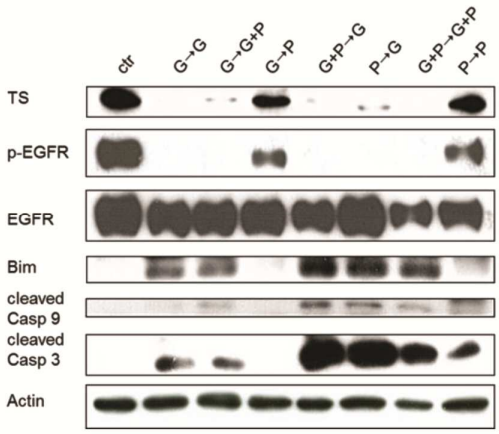
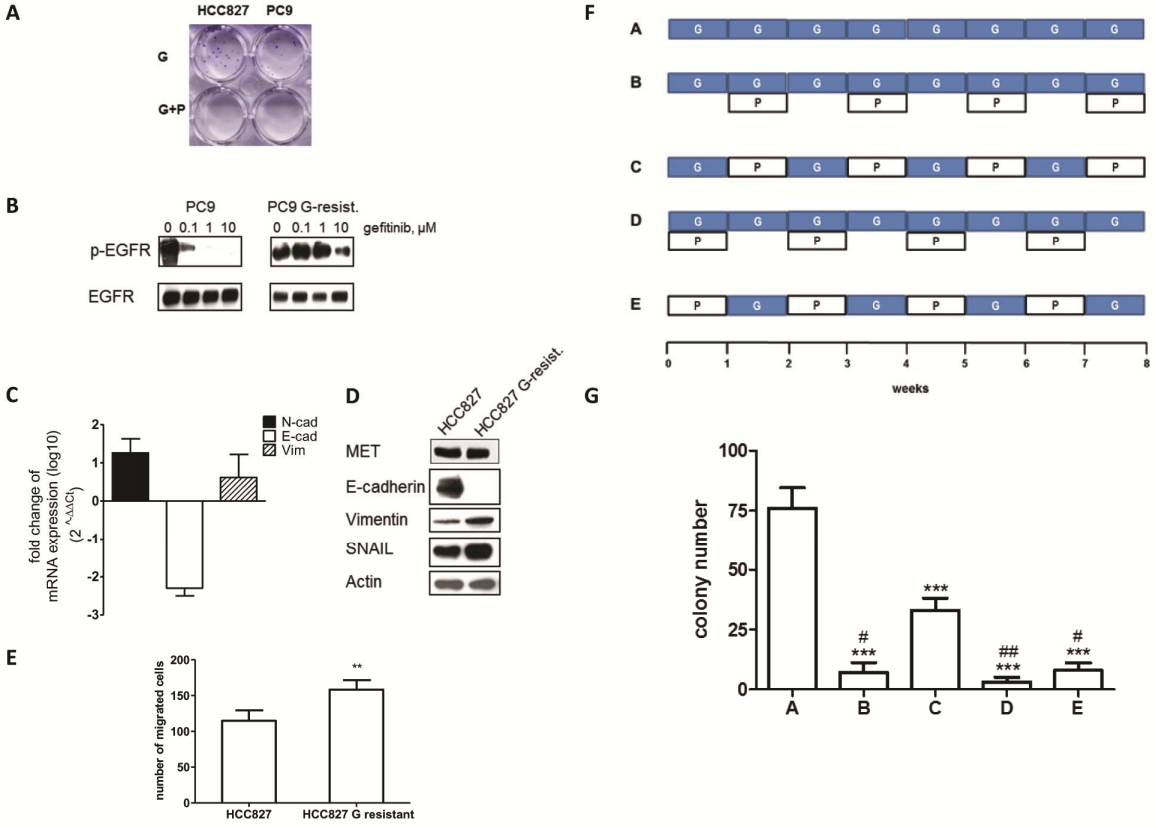


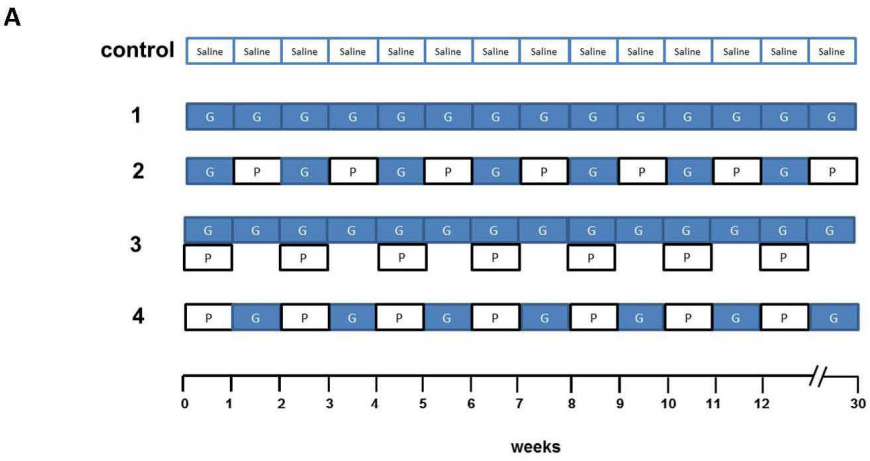

C

| Treatment | $G_1$ phase (%) | S phase (%) | $G_2$/M (%) | Apoptotic index (%) |
|---|---|---|---|---|
| control | 65.3±1.5 | 23.3±0.6 | 11.3±1.2 | 1.2±0.6 |
| G→G | 81.7±1.5 | 5.3±1.2 | 13.0±1.7 | 21.5±2.0* |
| G→G+P | 78.0±1.7 | 10.3±1.2 | 11.7±0.6 | 26.5±2.2** |
| G→P | 81.0±3.0 | 12.3±2.5 | 6.7±0.6 | 0.8±0.5# |
| G+P→G | 83.0±2.7 | 7.7±0.6 | 9.3±3.0 | 51.2±3.1***### |
| P→G | 30.5±0.7 | 34.0±1.4 | 35.5±0.7 | 49.2±8.3***## |
| G+P→G+P | 83.7±2.5 | 9.3±0.6 | 7.0±2.0 | 43.1±.03***## |
| P→P | 42.0±2.8 | 52.0±1.4 | 6.0±1.4 | 13.7±2.7* |

D



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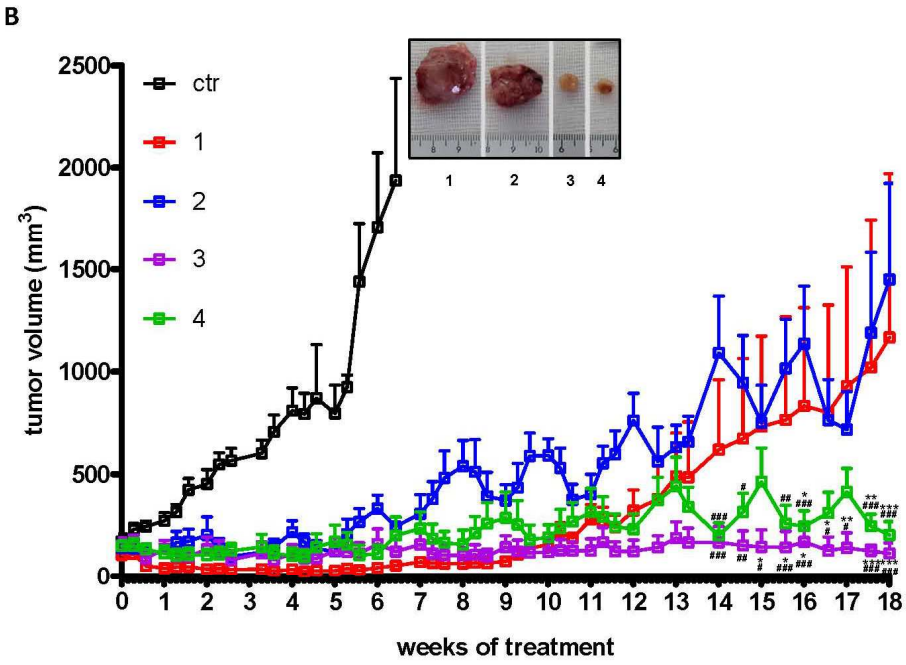
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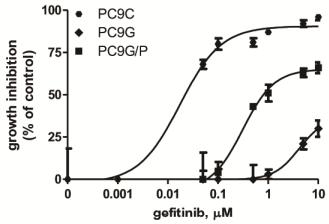


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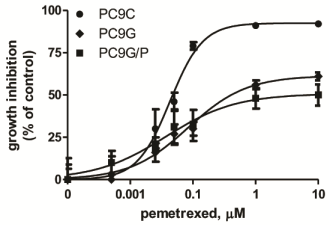


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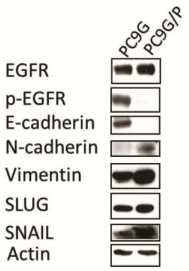

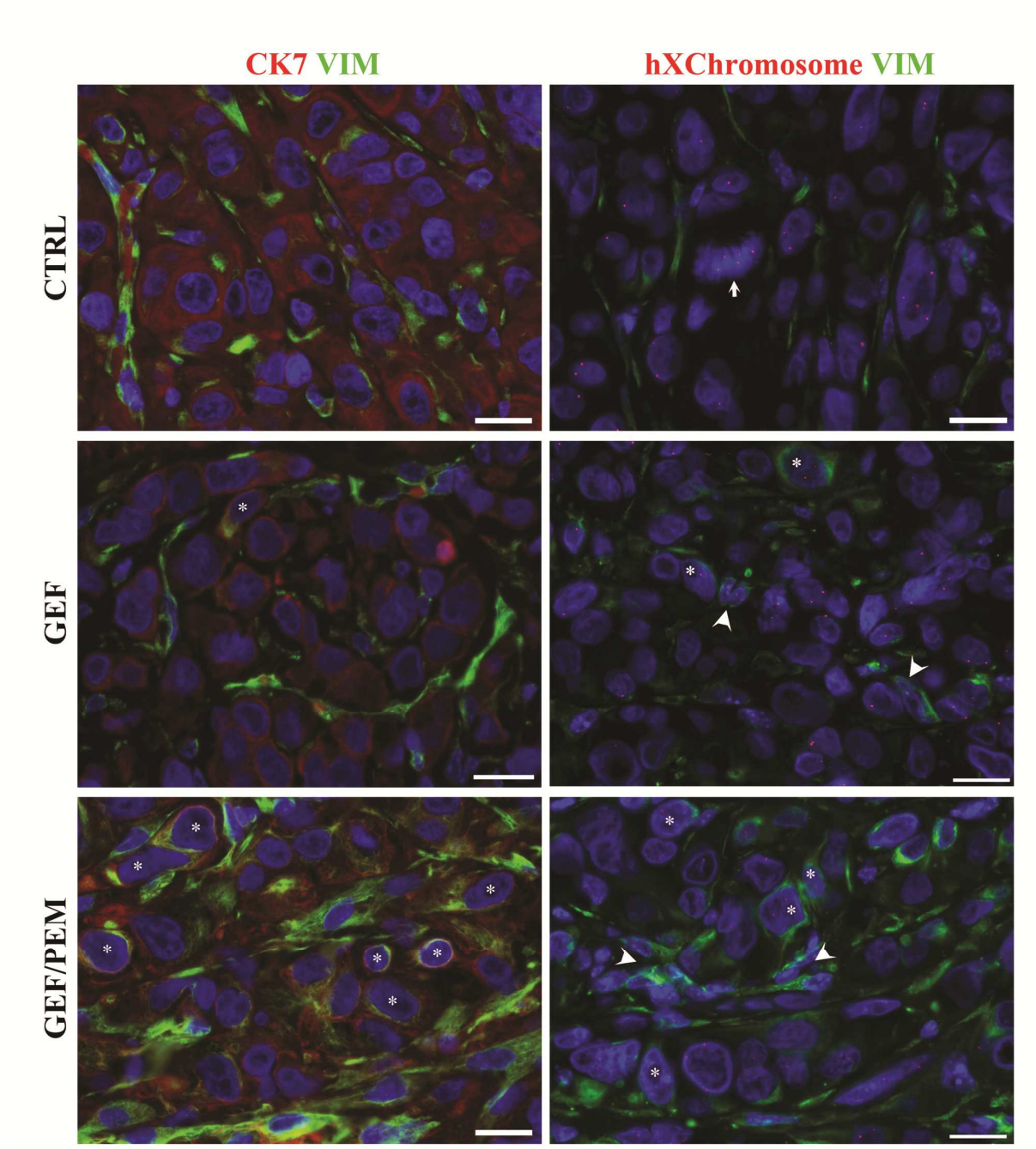
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