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ORIGINAL ARTICLE

In vitro antiproliferative effect of trastuzumab (Herceptin®) combined with cetuximab (Erbitux®) in a model of human non-small cell lung cancer expressing EGFR and HER2

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Abstract Lung cancer is the leading cause of cancer death. For this reason, new therapies are needed for the treatment of this devastating disease. In this study, we investigated the effects of combining cetuximab and the trastuzumab on the growth of a model of human non-small cell lung carcinoma cell line (A549). The results were compared with those obtained from a human lung squamous carcinoma cell line (NCI-H226). Both cell lines were treated with cetuximab and trastuzumab, alone or in combination, at various concentrations, for 24, 48 and 72 h. Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. EGFR and HER-2 mRNA expression was detected by reverse transcription polymerase chain reaction, and the gene amplification status of receptors was evaluated by fluorescence in situ hybridisation. The colorimetric proliferation assay showed that trastuzumab combined with cetuximab significantly inhibited A549 cells at a dose of 40 μg/ml after 72 h of treatment ($p < 0.05$), while no time-dose dependent inhibition was observed in NCI-H226 cells. The combined treatment influenced both levels of EGFR and HER-2 mRNA in A549 cells and only EGFR mRNA levels in NCI-H226 cells. Fluorescence in situ hybridisation showed that both cell lines were aneuploid for the two genes with equally increased EGFR and CEN7 signals, as well as HER-2 and CEN17 signals, indicating a condition of polysomy without amplification. The preliminary results of this study encourage further investigations to elucidate the downstream events involved and to understand how these mechanisms influence non-small cell lung cancers growth.

Keywords Lung cancer · Cetuximab · Trastuzumab · EGFR · HER2

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Introduction

Lung cancer is one of the most common malignant tumours worldwide [1]. The two major subtypes are small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [2]. For early-stage or locally advanced lung cancer, surgery is the most effective treatment and combined chemotherapy is the standard adjuvant approach. However, over 60 % of all NSCLC patients already have advanced or metastatic tumours at the time of diagnosis and are often not suitable for surgery. The prognosis is poor, with a 5-year survival rate of only 14 % [3]. For this reason, the development of novel therapeutic approaches for lung cancer treatment is needed.

The expression of epidermal growth factor (EGF) family receptors (EGFR, HER2, HER3 and HER4) is frequently observed in lung cancer [4]. These receptors regulate signalling pathways involved in the proliferation, invasion, migration, survival, adhesion, and differentiation of cancer cells [5]. High levels of EGFR and/or HER2 receptors are associated with disease progression and poor prognosis [6, 7]. These receptors are activated by dimerisation after

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binding to ligands (EGF/TGF-α, heparin-binding EGF, epiregulin, betacellulin and amphiregulin). HER2 is the preferred heterodimeric partner for all members of the EGFR family [8]. Heterodimers containing HER2 show, in fact, greater affinity for ligands [9] to generate more prolonged signals and to enhance the biological effects of EGFR ligands, such as EGF [10].

Recently, the EGFR and HER2 proteins have been investigated as attractive targets for novel anticancer strategies [11], including monoclonal antibodies and small molecule inhibitors of the intracellular tyrosine kinase activity. Small molecules, such as gefitinib and erlotinib, have progressed to large-scale studies, but randomised clinical trials have shown limited success. Gefitinib was effective only in a sub-group of NSCLC patients with specific mutations in the EGFR gene [12]. Moreover, the addition of gefitinib to conventional chemotherapy regimens did not improve any outcome variable, as demonstrated by two large randomised studies [13, 14]. Erlotinib is an effective treatment for NSCLC patients and has been registered as a second- and third-line treatment for patients whose tumours are EGFR wild-type [15]. The disappointing results of EGFR tyrosine kinase inhibitors have led to increased interest in monoclonal antibodies against EGFR.

The aim of this study was to determine whether a combined treatment of two humanised monoclonal antibodies, cetuximab and trastuzumab, would show significant inhibition of human NSCLC adenocarcinoma A549 cell proliferation. The results were compared with those obtained from the human NSCLC squamous carcinoma H226 cell line. Cetuximab is a chimeric IgG1 monoclonal antibody (mAb) that blocks ligand binding to EGFR, leading to a decrease in receptor dimerisation, autophosphorylation, and activation of signalling pathways [16]. In combination with chemotherapy, it has been approved by the FDA for the treatment of metastatic colorectal cancer and locally advanced head and neck cancer.

Two randomised phase III trials in NSCLC patients, evaluating cetuximab in addition to first-line chemotherapy, showed a small benefit in terms of overall survival for the experimental treatment, which was considered insufficient by the EMA for marketing approval [17, 18]. However, a subgroup analysis of the FLEX phase III trial recently demonstrated greater survival in patients with high immunohistochemical EGFR expression [19]. Trastuzumab is used for the treatment of HER2-positive breast cancer. It was tested in phase II trials as a single agent and in addition to cytotoxic chemotherapy for patients with NSCLC, but these trials have not produced any convincing results of improved antitumour activity by adding trastuzumab to standard chemotherapy in NSCLC [20, 21].

In this study, the effect of combined treatment was also assessed, with respect to cell growth, mRNA expression levels of EGFR and HER-2 receptors and gene amplification status of EGFR and HER2. Since EGFR and HER2 are, respectively, overexpressed in 40–80 and 25–30 % of NSCLC patients [22], improved therapeutic effects might be obtained against NSCLC cells expressing both receptors. Thus, targeting both EGFR and HER2 using a combination of anti-EGFR and anti-HER2 monoclonal antibodies may become a promising strategy to treat NSCLC.

Materials and methods

Drugs

The humanised mouse anti-human EGFR antibody, cetuximab (5 mg/ml, C225, Erbitux®), was a kind gift from Merck (Darmstadt, Germany), and the humanised mouse anti-human HER2 antibody trastuzumab (150 mg, 4D5, Herceptin®) was purchased from Roche Diagnostics (Pensberg, Germany). Both drugs were dissolved in 10 % dimethyl sulfoxide (DMSO) and stored at −80 °C for in vitro studies.

Cell lines, cell culture and cell viability assay

Human lung cancer cell lines A549 and NCI-H226 were a kind gift from the Department of Bio-Medical Sciences at the University of Catania, Italy. Both cell lines express EGF and HER2 receptors. All cell culture media and supplements were purchased from Life Technologies (Carlsbad, CA 92008, USA) unless otherwise indicated. Dulbecco's Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute Medium (RPMI) 1640 were used as culture media for A549 and NCI-H226 cells, respectively. Both media were supplemented with 10 % foetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were maintained under standard cell culture conditions at 37 °C and 5 % CO_2 in a humid environment. Culture media were refreshed every 3 days. Cells were routinely grown for 5–6 days, and then they were washed with Hank's Buffered Salt Solution (HBSS) and detached from the culture flask by incubating for 3–5 min at 37 °C with 0.05 % of trypsin–EDTA solution. A549 cells in DMEM/10 % FBS and NCI-H226 in RPMI/10 % FBS were plated at a density of 2.5×10^3 cells/well in 96-well culture plates. Twenty-four hours after plating, cetuximab and trastuzumab were added, alone or in combination, to a final concentration of 10, 20 and 40 µg/ml for each drug, and the incubation was continued for 24, 48 and 72 h. In other experiments, 10 ng/ml EGF (Invitrogen Carlsbad, CA), alone or in association with the drugs, was added to cell suspensions or to cells previously plated in 96-well plates.

All incubations were terminated by adding 10 µl of a 0.5 g/ml stock of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Inc., St. Louis, MO) to each well. The reaction was allowed to proceed for 3–4 h at 37 °C. The culture medium of each cell line was removed, and formazan crystals were dissolved by adding 200 µl of DMSO to each well. The intensity of the colour, which is proportional to the number of viable cells, was measured at a wavelength of 540 nm. All values were compared to the corresponding controls. Six wells were assigned to each treatment.

RNA isolation and cDNA synthesis

Cells were plated in 6-well plates (BD Falcon™) at a density of 1.5×10^5 cells/dish in DMEM/10 % FBS or RPMI/10 % FBS. Twenty-four hours after plating, cells were serum-starved for 24 h and subsequently incubated for other 72 h in the absence (CTRL) or presence of a combination of cetuximab and trastuzumab (40 µg/ml each). When the incubation was terminated, DMEM/1 % FBS was aspirated and the cells were washed with 1 ml/well of HBSS. Subsequently, 1 ml/well of clean HBSS was added and cells were scraped with a disposable cell scraper (1.8 cm blade; Corning Costar®, UK). Cells were harvested and total RNA was extracted using the RNeasy Micro kit (Qiagen®, USA). RNA quality assurance and concentration were assessed with a spectrophotometer (NanoDrop Technologies, LLC, Wilmington, DE, USA). Total RNA was treated with DNase I (Life Technologies, Carlsbad, USA) and subsequently reverse transcribed using random hexamers and the SuperScript II reverse transcriptase enzyme (Life Technologies, Carlsbad, USA) according to the manufacturer's instructions.

Quantitative real-time reverse transcription PCR (RT-PCR)

The primers used for PCR are listed in Table 1. Quantitative RT-PCR was performed using the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA). For EGFR and HER-2 mRNA quantification, two pairs of sequence-specific oligonucleotides were designed using the Oligo Perfect™ Designer software (Life Technologies, Carlsbad, CA) based on the sequence of human EGFR variant-1 (accession number: NM_005228.3) and HER-2 variant-1 (accession number: NM_004448.2).

Each PCR reaction was carried out in a final volume of 25 µl using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 1 µM of primers. Finally, 1 µl of diluted cDNA (50 ng/µl) was added to each reaction. Each reaction was performed in triplicate. The conditions used for PCR amplification were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. As a negative control, we performed reactions without cDNA (no template control, NTC). The relative abundance of mRNA of the gene of interest was deducted from the cycle number at which fluorescence increased above the background level (Ct) in the exponential phase of the PCR reaction.

Fluorescence in situ hybridisation (FISH) analysis

FISH was performed using the Histology FISH Accessory kit and the EGFR/CEN-7 FISH Probe Mix or the HER-2 FISH pharmDx kit (Dako Italia S.p.A., Milano, Italy), according to the manufacturer's instructions. Briefly, 2×10^5 cells in phosphate-buffered saline (PBS; Life Technologies, Carlsbad, CA) were placed on slides and air-dried for 24 h. Slides were then dehydrated using ascending grades of alcohol and air-dried. The probe mixture was dropped onto the slides, then denaturation was performed for 5 min at 82 °C and hybridisation overnight at 45 °C. The slides were washed with saline-sodium citrate buffer at 65 °C, dehydrated with ascending grades of alcohol, air-dried and mounted in DAPI-containing fluorescence mounting medium. Evaluation was performed using a fluorescence microscope, scoring 60 non-overlapping interphase nuclei. EGFR or HER-2 were visualised as a red signal with a tetramethylrhodamine isothiocyanate filter, chromosome seven α-centromeric (CEN-7) or chromosome seventeen α-centromeric (CEN-17) as a green signal with a fluorescein isothiocyanate filter and nuclei as a blue signal with a DAPI filter. Enumeration of the number of locus-specific identifier (LSI) EGFR and CEN7 signals and of the number of LSI HER2/neu and CEN17 signals, per nucleus, were done by two independent readers. A cellular

Table 1 Primers used in the PCR

Gene	Forward primer	Reverse primer
EGFR	5′-GGGAGTTGATGACCTTTGGA-3′	5′-TGCACTCAGAGAGCTCAGGA-3′
HER-2	5′-CGAGAGGTGAGGGCAGTTAC-3′	5′-AGCAGAGGTGGGTGTTATGG-3′
GAPDH	5′-TCAAGAAGGTGGTGAAGCCAG-3′	5′-TCTTACTCCTTGGAGGCCAT-3′

The nucleotide sequences of the PCR primers used to assay gene expression by real-time quantitative PCR are shown

specimen was considered amplified for the EGFR and HER-2/neu genes with a ratio of EGFR/CEN7 or HER2/CEN17 $\geq$2.0 and non-amplified with a ratio $\leq$2.0. Cells in which both EGFR/HER-2 and CEN-7/CEN17 were equally elevated (more than two signals per nucleus) were regarded as aneuploid.

Statistical analysis

The statistical significance of differential findings between experimental groups and control groups was determined by one-way ANOVA. All the results are expressed as mean + standard error of the mean (SEM) and were analysed using Fisher's exact test. A p value <0.05 was considered statistically significant.

Results

The effect of cetuximab and/or trastuzumab on cell proliferation

We studied the effects of trastuzumab and cetuximab, alone or in combination, on the growth of A549 and NCI-H226 cell lines, at doses of 10, 20 and 40 μg/ml each for 24, 48 and 72 h. Single agent treatments did not produce inhibition in both cell lines growth (Fig. 1), while a combination of trastuzumab with cetuximab, at a dose of 40 μg/ml each for 72 h, induced significant growth inhibition in A549 cells and no inhibition in NCI-H226 cells, compared with controls (Fig. 2). In order to assess the effect of a natural ligand on tumour cell growth, we used EGF alone or in combination with cetuximab and trastuzumab. The addition of 10 nM exogenous EGF to A549 cells stimulated proliferation, but did not influence the inhibition of growth produced by the two antibodies (Fig. 3). In contrast, the addition of exogenous EGF did not affect H226 cell proliferation (Fig. 4).

These experimental observations suggest that targeting EGFR and HER2 by the combination of trastuzumab and cetuximab increases the in vitro antiproliferative activity of both individual agents and seems to be a potential therapeutic strategy against NSCLC.

Influence of treatment with cetuximab and trastuzumab on EGFR and HER-2 mRNA levels

Messenger RNA levels of EGFR and HER-2 were assessed by quantitative RT-PCR in A549 and NCI-H226 cells under basal conditions and 18, 24, 48 and 72 h after treatment with the two monoclonal antibodies. In A549 cells we observed, after 30 h of treatment, an increase in EGFR mRNA levels (Fig. 5a) and an early increase in HER2 mRNA after only 18 h of treatment (Fig. 5b). NCI-H226 cells expressed very high EGFR mRNA levels after 48 h of treatment, and they maintained high levels after 72 h (Fig. 5c). HER-2 mRNA levels were not significantly increased (Fig. 5d).

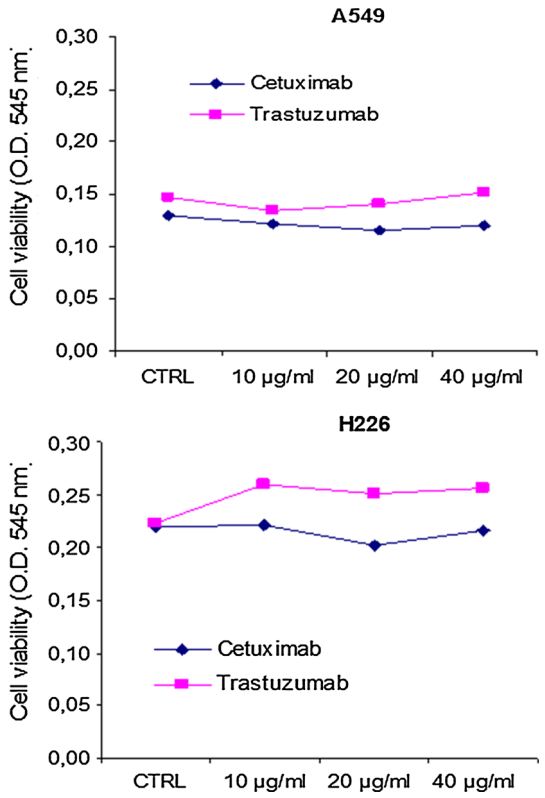

Fig. 1 Effect of 72 h of treatment with cetuximab and trastuzumab alone, at the indicated concentrations, on A549 and NCI-H226 cell growth

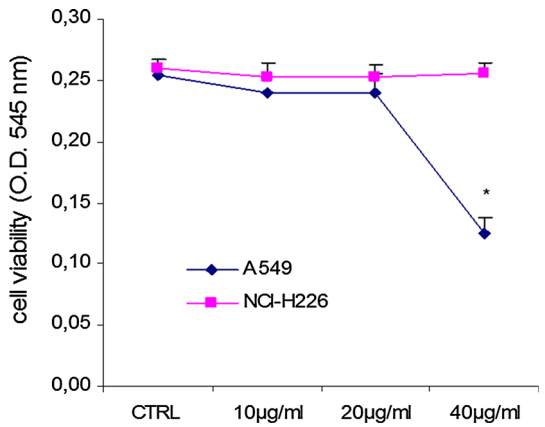

Fig. 2 Effect of 72 h of treatment with combinations of cetuximab and trastuzumab, at the indicated concentrations, on A549 and NCI-H226 cell growth

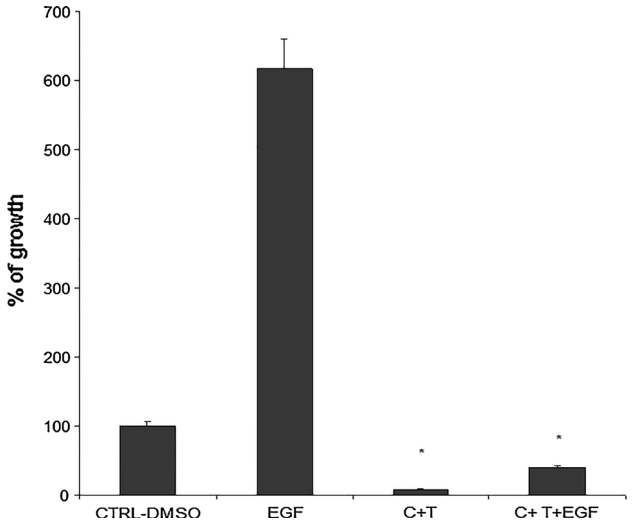

Fig. 3 Effect of EGF, cetuximab and trastuzumab, at the indicated concentrations, on A549 cell growth after 72 h of treatment. Results are expressed as the percentage of control with 100 % representing control cells treated with DMSO alone. CTRL = DMSO; *EGF* epidermal growth factor, *C* cetuximab, *T* trastuzumab

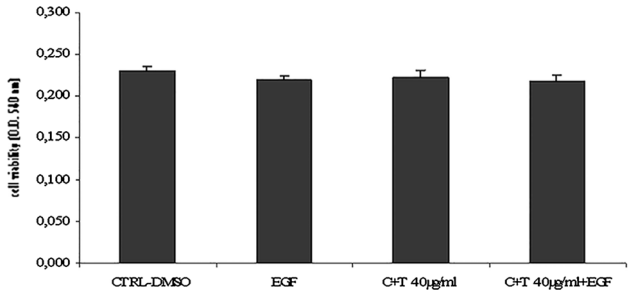

Fig. 4 Effect of EGF, cetuximab and trastuzumab, at the indicated concentrations, on H226 cell growth after 72 h of treatment

EGFR and HER-2 gene amplification

The gene amplification status of EGFR and HER-2 was evaluated by FISH analysis. Both A549 (Fig. 6a, b) and NCI-H226 cells (Fig. 6c, d) were aneuploid for the two genes, with equally increased EGFR and CEN7 signals, as well as HER-2 and CEN17 signals, indicating a condition of polysomy (increased gene copy number) without amplification.

Discussion

Recently, the best comprehension of the molecular biology of lung cancer has led to improvement in the diagnosis and treatment of lung cancer based on the genotype of tumour. It is known that EGF family receptors (EGFR, HER2, HER3 and HER4) are involved in the proliferation, invasion, migration, survival, adhesion, and differentiation of lung cancer cells [5]. A lot of researchers have studied the changes in EGFR and HER2 gene-protein expression and gene amplification. Hirsch et al. first reported that EGFR gene copy number correlates with EGFR protein expression, but not with prognosis [23]. Other researchers reported that EGFR gene amplification and a higher protein expression are significantly associated with better clinical response, disease control rate, time to progression and survival, while EGFR mutations have been associated with a good tyrosine kinase inhibitor (TKI) response in NSCLC [24, 25]. Among lung cancer biomarkers, HER2 is a poorly described target. It is widely known that HER2 protein overexpression or gene amplification is associated with sensitivity to HER2-targeting drugs in breast cancer [26], but the involvement of HER2 in lung cancer has not been much studied. HER2 protein overexpression and gene amplification are present in 6–35 % and in 10–20 %, respectively, of NSCLC [27, 28], while HER2 mutations were identified in approximately 2–4 % of NSCLC [29, 30]. Although HER2 mutations are more important than HER2 protein overexpression and gene amplification, there are few data on the clinical course of patients with HER2-mutated NSCLC.

Nowadays, simultaneous inactivation of EGFR family members represents a strategy for the development of selective therapies against epithelial cancers. Tumours that co-express EGFR and HER2 present a less favourable prognosis than tumours that express either receptor alone [31], indicating co-operation of the two receptors in tumour progression.

Current EGFR- and HER2-based targeted therapies of cancer include the use of monoclonal antibodies and small molecule inhibitors. Cetuximab (Erbitux®) is a monoclonal antibody, which interacts exclusively with domain III of sEGFR, partially occluding the ligand-binding region on this domain and sterically preventing the receptor from adopting the extended conformation required for dimerisation. This suggests that cetuximab disrupts both ligand binding and receptor dimerisation. Trastuzumab (Herceptin®) is another monoclonal antibody, which has been approved for the treatment of breast cancer patients and was shown to prolong the survival of metastatic breast cancer patients with tumours expressing HER2 [32]. Trastuzumab is able to block HER2 activation and its signalling pathway [33].

This study demonstrates a synergistic growth inhibition in A549 human lung cancer cells treated with a combination of the humanised anti-HER-2 antibody trastuzumab and the humanised anti-EGFR antibody cetuximab. Probably, in these cells the two drugs combined together could block different growth factor-driven signal-transduction pathways, resulting in a more significant antitumour effect.

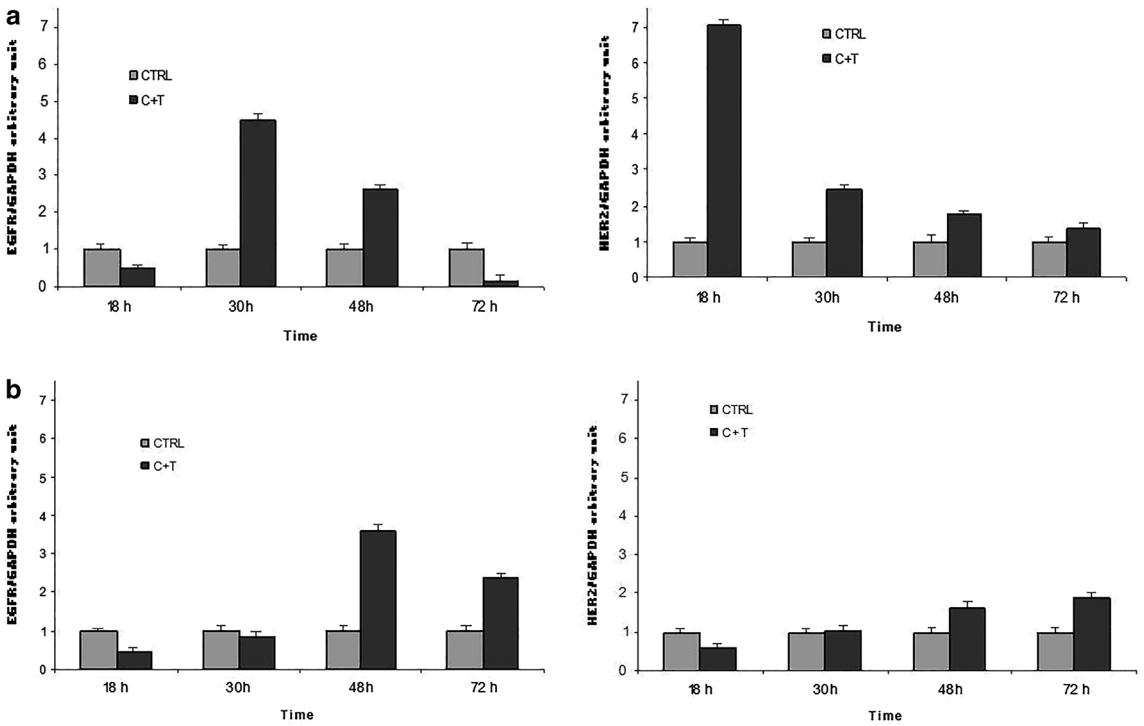

Fig. 5 Effect of combined treatment with cetuximab and trastuzumab on mRNA expression of EGFR and HER2 receptors in A549 cells (**a**) and in NCI-H226 cells (**b**). *Vertical bars* indicate the normalised Ct value ± SEM (Arbitrary Unit)

A similar additive antiproliferative effect of a combination of trastuzumab and cetuximab has been shown in ovarian cancer cells [34] and in oesophageal squamous cell carcinoma with EGFR and HER-2 expression [35]. Moreover, the lack of an increased proliferation of A549 cells, treated with the combination of cetuximab and trastuzumab, after the addition of exogenous EGF, was probably due to the effect of the two drugs. Changes in cell proliferation, expression of EGFR and HER2 mRNA and gene amplification status of EGFR and HER-2 were also evaluated in this study. We analysed mRNA levels of EGF and HER2 receptors of A549 and H226 cells, in order to understand the different behaviour of the two cell lines. In A549 cells, a significant increase in EGFR mRNA expression was observed after 30 h of pharmacological treatment. This could be explained considering the mechanism of action of cetuximab, which interacts exclusively with domain III of sEGFR. The partial occlusion of the ligand-binding region and the steric hindrance of the receptor prohibiting the extended conformation required for dimerisation induce the internalisation and degradation of the antibody/receptor complex, contributing to potent inhibition of EGFR activation [36]. Moreover, we observed that the synthesis of new mRNA, to supply the loss of cell surface receptors, required 30 h of treatment. mRNA levels decreased after 48 and 72 h of treatment towards to normal levels. Similar behaviour was observed for HER2 mRNA levels, in which the internalisation of the receptor, as a consequence of interaction with trastuzumab, led to an increase in receptor expression after 18 h of treatment.

In contrast, NCI-H226 cells showed resistance to the combined treatment. The addition of exogenous EGF did not affect proliferation, probably due to modulation by a TGF-α-mediated autocrine loop [37]. Analyses of the mRNA levels of both receptors showed similar behaviour in relation to that of A549 cells for EGFR. The cetuximab–EGFR interaction and the consequent internalisation and degradation of the receptor drove new receptor synthesis. After 48 h of treatment, a 3.5-fold increase in the mRNA level was observed, with maintenance of high levels after 72 h. The HER2 receptor did not seem to be influenced by the pharmacological treatment; in fact, during the early phases of administration, mRNA levels were unchanged. Only after 72 h of treatment, an increase in these levels was observed, i.e., the level was about doubled. This indicates the possible and late involvement of this receptor following the combined treatment. A possible explanation of this late involvement could be the co-localisation of HER2 with growth factor receptor-bound protein 7 (Grb7), a protein that in humans is encoded by the GRB7 gene [38]. It is normally expressed, at varying levels, in a number of normal tissues, including lung, and it is also

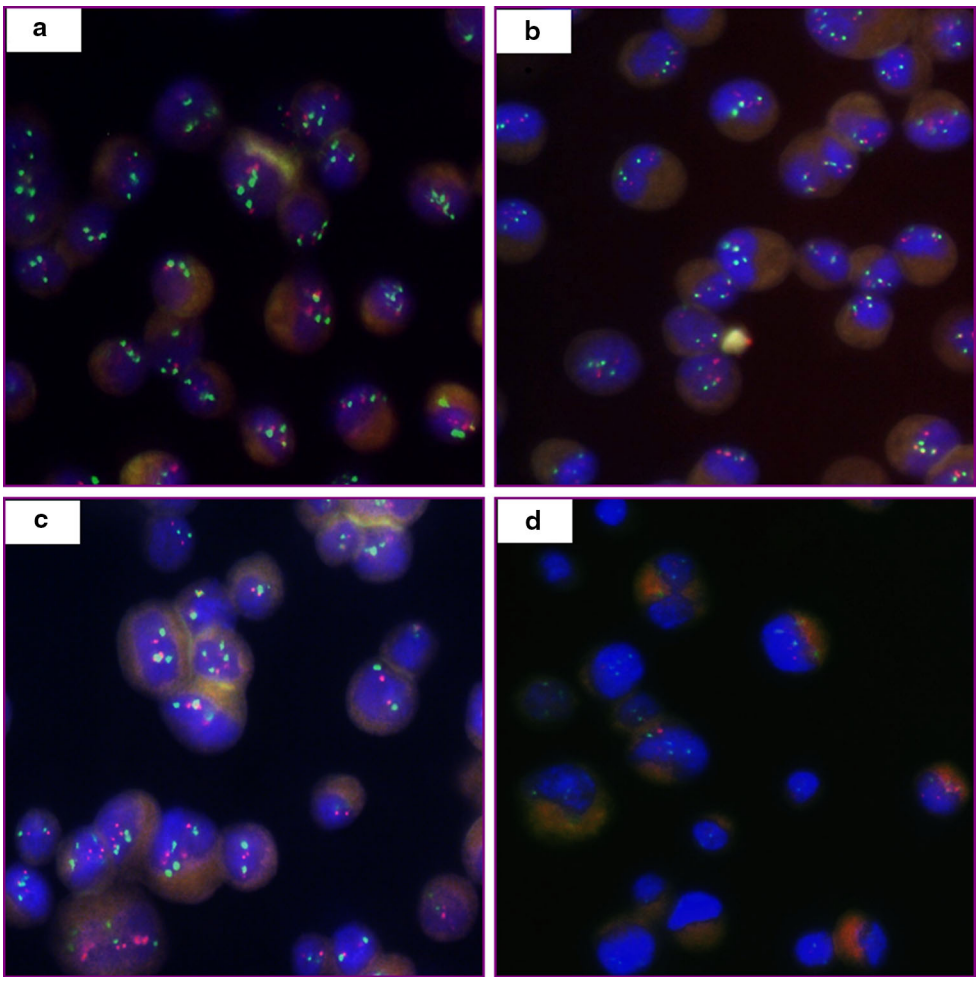

Fig. 6 EGFR and HER2 gene amplification, by DAKO EGFR/CEN 7 and HER2/CEN 17 Dual Colour Probes, in A549 (**a**, **b**) and NCI-H226 (**c**, **d**) lung cancer cells

overexpressed in several malignant tumours and cell lines [39]. Probably, in H226 cells, molecular interactions involving GRB7 could influence HER2 signalling [40].

Several alterations in RNA metabolism were found in these lung cancer cell lines. It is likely that the variable response of A549 and NCI-H226 cells to combined treatment was influenced by RNA metabolism-related molecules involved in the development of this pathology [40]. Moreover, we verified that the two cell lines were aneuploid for the EGFR and HER2 genes, with equally increased EGFR and CEN7 signals, as well as HER-2 and CEN17 signals, indicating a condition of polysomy (increased gene copy number) without amplification. These results suggest that EGFR and HER-2 may interact with each other and lead to effective antitumour activity and encourage us to investigate the downstream events and how these mechanisms influence cell growth and sensitivity in NSCLC cancers expressing EGFR and HER2.

Conflict of interest The authors declare that they have no conflicts of interest.

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