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-(-)Epigallocatechin-3-gallate downregulates EGFR, MMP-2, MMP-9 EMMPRIN and inhibits the invasion of MCF-7 tamoxifen resistant cells

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SYNOPSIS

The activation of Epidermal Growth Factor Receptor (EGFR) signaling pathway is one of the key mechanisms underlying the development of resistance to tamoxifen in breast cancer patients. As (-) Epigallocatechin-3-gallate (EGCG), the most active catechin present in green tea, has been shown to downregulate EGFR, we studied the effects of 10-100 µg/ml EGCG treatment on growth and invasion in a breast carcinoma cell line resistant to tamoxifen (MCF-7Tam) and parental MCF-7. A dose-dependent downregulation of EGFR mRNA expression and protein level occurred after 50 µg/ml EGCG treatment of MCF-7Tam cells. EGFR molecules on the plasma membrane surface of MCF-7Tam cells significantly decreased. EGFR phosphorylation (Tyr992, Tyr1045 and Tyr1068) was higher in MCF-7Tam than MCF-7 and it was reduced by EGCG treatment. ERK and phosphoERK p42/44 were also downregulated by EGCG treatment and in vitro cell growth and invasion decreased. Matrix Metalloproteinases (MMPs) -2 and -9, which are implicated in cell invasion and metastasis and Extracellular Matrix Metalloproteinase Inducer (EMMPRIN), a glycoprotein able to activate MMPs, were significantly reduced after 50 µg/ml EGCG treatment. Coherently, Tissue Inhibitors of Metallo Proteinases (TIMPs) -1 and -2, which downregulate MMPs, increased after EGCG treatment. Altogether the present data demonstrated that EGCG could attenuate the tamoxifen-resistant phenotype of MCF-7Tam cells. EGCG could contrast MCF-7Tam cell growth and in vitro invasion through downregulation of EGFR and other molecules implicated in aggressive biologic behavior. The present data support the hypothesis that EGCG is an interesting molecule to be investigated in tamoxifen-resistant breast carcinoma.

Key words: breast carcinoma, tamoxifen, EGCG, invasion, TIMPs, Receptor Tyrosine Kinases.

Abbreviations: (-)Epigallocatechin-3-gallate (EGCG); Epidermal Growth Factor Receptor (EGFR); MCF-7 breast carcinoma cell line resistant to tamoxifen (MCF-7Tam); Matrix Metalloproteinases (MMPs) -2 and -9; Extracellular Matrix Metalloproteinase Inducer (EMMPRIN); Tissue Inhibitors of Metallo Proteinases (TIMPs) -1 and -2, Extracellular Regulated Kinase (ERK).

INTRODUCTION

The development of breast tumors resistant to anti-estrogens is one of the major obstacles in the therapy of breast carcinomas. The failure of endocrine-targeted therapies can be due to the activation of novel signaling pathways that circumvent the effects of anti-estrogens. Emerging data indicate that altered expression and/or modification of several growth factor receptors and downstream signaling molecules correlate with tamoxifen resistance: in particular Epidermal Growth Factor Receptor (EGFR), human epidermal growth factor receptor type 2 (HER2), and insulin-like growth factor-1 receptor (IGF-1R) signaling pathways are often elevated in non-responsive tumors that exhibit either de-novo or acquired resistance [1-3]. The activity of kinases that function downstream of these receptors such as extracellular-regulated kinase (ERK) 1/2, p38, AKT, and p21-activated kinase-1 (Pak1) is also increased [4]. Altogether these modifications are also associated with the development of more aggressive phenotype. Many of the molecular pathways that are involved in circumventing anti-estrogen-induced blocks in cell proliferation and survival are also implicated in increased migration and metastasis of breast cancer cells. Genotype and phenotype changes occur in the development of the tamoxifen-resistant phenotype. MCF-7 cells selected for tamoxifen resistance showed signaling perturbations associated with the epithelial to mesenchymal transition (EMT): elongated, fibroblast-like phenotype that lacked characteristic cell-cell contacts, enhanced basal motility and an invasive phenotype were associated with increased expression of extracellular matrix proteins, downregulation of E-cadherin, disruption of adherents junctions complexes, accumulation of beta-catenin and NF- κ B in the nucleus and transcription of target genes such as c-Myc and cyclin D1[5,6]. Treatment of these cells with the pharmacological inhibitors gefitinib and AZD0530 inhibited migration and invasion, indicating that the invasive phenotype of these resistant cells was dependent on EGFR and c-Src, respectively [5-7]. Molecules able to down-regulate one or more of these molecular pathways are therefore interesting to the aim of attenuating the malignant phenotype emerging with resistance to tamoxifen.

Many reports demonstrated that EGCG, the most important catechin present in green tea, could downregulate many molecular signaling pathways, including EGFR and HER2 [8-11].

We therefore used EGCG on a MCF-7 tamoxifen resistant cell line developed in our laboratory and we investigated the relation between EGCG and EGFR and molecules implicated in cell motility and invasion.

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MATERIAL AND METHODS

Cell lines

MCF-7 cell line was obtained from American Type Culture Collection (ATCC) (Manassas, Va, USA) and grown in E-MEM supplemented with 10% Fetal Serum (FBS), 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM Na pyruvate (all from Cambrex, Bioland, Mariland, USA). MCF-7Tam cell line was developed growing MCF-7 cells in a-MEM without phenol red, supplemented with 10% charcoal-stripped Foetal Bovine Serum (FBS), 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM Na pyruvate (all from Cambrex, Bioland, Maryland, USA); 10^{-7} M 4-OH-tamoxifen (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium for 1 year before starting the experiments as previously reported [12].

MTT assay

MCF-7 and MCF-7Tam cells were seeded in a 96-well microtiter plate (1×10^4 cells/well) overnight, in E-MEM (MCF-7) or in α -MEM with 10^{-7} M 4-OH-tamoxifen (MCF-7Tam). The day after the cells were incubated with different concentrations of 10-100 μ g/ml EGCG (Sigma-Aldrich, Saint Louis, MO, USA) for 24, 48 and 72 h. Cell growth experiments were conducted in triplicate. The effect of EGCG on cell growth was examined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich, St. Louis, MO, USA) assay. Briefly, 10 μ l of MTT solution (5 mg/ml) was added to each well to make a final volume of 110 μ l and incubated for 3 hr at 37 °C. The supernatant was aspirated, and the MTT-formazan crystals formed by metabolically viable cells were dissolved in 200 μ l of Dimethyl Sulfoxide (DMSO). After 1 hr incubation in DMSO, the absorbance of OD at 570 nm was detected and recorded by means of an Enzyme-Linked Immunoabsorbent Assay (ELISA) reader (Beckton Dickinson, Boston, MD, USA).

Western blot analysis

MCF-7 and MCF-7Tam cells (40000 cell/cm^2) were plated and treated with EGCG (10-100 μ g/ml). The cells were detached and were collected by centrifugation at 300 x g for 10 min. The pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5% Triton X-100, 5 mM Na_3VO_4) and sonicated on ice, in the presence of protease inhibitors. Protein concentration was determined by the method of Lowry. Cell lysates (50 μ g of protein per lane) were size fractionated in 7.5-12% SDS-polyacrylamide gel, prior to transfer to Hybond TM-C Extra membranes (GE Healthcare, UK) by standard protocols. Membranes were blocked for 2 hr with 5 % milk in transfer buffer saline (TBS) (0.2 M Tris-HCl, 0.5 M NaCl, 1% Tween 20, pH 7.6) at room temperature. The membranes were incubated overnight at 4°C with the following primary antibodies: anti-MMP-2, or anti-MMP-9, or anti-TIMP-1, or anti-TIMP-2 (Santa Cruz Biotechnology, USA), or anti-EMMPRIN (Zimed Lab, USA) or anti-EGFR (Lab Vision, USA) or anti-ERK p42/44 (ERK1/2) and anti-phosphoERK p42/44 (Cell Signaling Technology, USA) diluted 1:500, anti-phosphoEGFR Tyr1068, phosphoEGFR Tyr1045 and phosphoEGFR Tyr992 (Cell Signaling Technology, USA) diluted 1:300. Anti-rabbit or anti-mouse peroxidase conjugated antibody were diluted 1:1000 in TBS containing 5% dry milk and used as secondary antibodies. The proteins were detected by luminol (GE Healthcare). Bands were quantified by using a densitometry images analysis software (Image Master VDS, Pharmacia Biotech, Uppsala, Sweden). Actin (Sigma-Aldrich, St Louis, USA) was used as a control.

Toluidine blue staining

MCF-7 and MCF-7Tam cells were grown on coverslip in 3.5 mm Petri dishes and fixed in 4% Formalin (Carlo Erba, Milan, Italy) for 15 min. After washing twice in PBS and once in water, the cells were incubated (10 min.) with a solution 0.5% Toluidine blue in Sodium Tetraborate 1%, prepared and filtered 24 hr before using. The cells were rinsed with bidistilled water several times, air dried and mounted with mounting medium (Diapath, Martinengo, Bergamo, Italy). The samples were visualized with a Nikon (Nikon Italy, Florence, Italy) epi-fluorescence microscope ECLIPSE

E600W equipped with a 100 KV lamp and filters for FITC and DAPI. Pictures were taken by a PanFluor X100 objective with 0.50-1.25 lens aperture, recorded by a Nikon Digital Camera DXM 1200F and elaborated by ATC-2U software.

Immunofluorescence

MCF-7 and MCF-7Tam cells were grown on coverslip in 3.5 mm Petri dishes and fixed in cold methanol (4°C) for 10 min. Mouse monoclonal anti EGFR antibody Ab-12 1:800, (for both extracellular and cytoplasm domain) and Ab-5 1:500 (specific for the extracellular domain) (both from Lab Vision Corporation, Westinghouse, Fremont, CA, USA) were used. Anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Sigma, St Louis, MO, USA) was added 1:800 to the cells for 1 hr in the dark at 37°C. Nuclei were counterstained by 4'-6-Diamidino-2-phenylindole (DAPI, 200 mg/ml) dissolved 1:500 in 1,4 diazabicyclo[2.2.2]octane (DABCO) (both from Sigma-Aldrich, St Louis, MO, USA) and added to the samples. The edges were sealed by rubber cement and kept in the dark. Samples were visualized with a Nikon (Nikon Italy, Florence, Italy) epi-fluorescence microscope ECLIPSE E600W equipped with a 100 KV lamp and filters for FITC and DAPI. Pictures were taken by a PanFluorX60 objective with 0.50-1.25 lens aperture, recorded by a Nikon Digital Camera DXM 1200F and elaborated by ATC-2U software.

Fluorescence Activated Cell Sorting (FACS)

Mouse monoclonal EGFR Ab-5 antibody (Lab Vision Corporation, Westinghouse, Fremont, CA, USA) specific for extra-cellular domain was used to detect and estimate the EGFR molecules present on the plasma membrane of control and EGCG treated MCF-7Tam cells. Cells were plated in 3.5 mm Petri dishes. The antibody was dissolved in sterile PBS (1:800). In vivo incubation (1 hr) was followed by two washings in PBS and fixation in 1% Formalin in PBS for 15 min. Then, the samples were incubated with the secondary antibody (1:800 anti-mouse FITC-conjugated) (Sigma, St Louis, MO, USA) for 30 minutes at 37°C. After washing in PBS the cells were evaluated by a FACSlibur (Becton Dickinson, Franklin Lakes, NJ, USA).

Zymography

MMP-2 and MMP-9 activity of MCF-7Tam and MCF-7 cells was determined by gelatin zymography. Cells were seeded: after 18 hr they were washed with PBS and replaced in serum-free medium (α -MEM, control) or serum-free α -MEM medium with EGCG for 24 hr. The cells were centrifuged (300 x g for 10 min) and the protein concentration determined. Ten μ g proteins per lane were added to sample buffer (1M Tris-HCl pH 6.8, 2% sodium dodecyl sulphate, 10% glycerol) and loaded to 10% SDS-polyacrilamide gel containing 1 mg/ml gelatin (Sigma, St. Louis, MO, USA). After electrophoresis, SDS was removed from gel by washing twice with 2.5% Triton X-100 for 1 hr. After a brief rinse, the gel was incubated at 37°C for 18 hr in buffer, pH 7.6, containing 100 mM Tris-HCl, 10 mM CaCl₂, 20 mM NaCl. The gel was stained with 1% Coomassie Brilliant Blue R250 for 2 hr and then treated with destaining solution (40% methanol, 10% acetic acid, 50% distilled water). The MMPs activity, indicated by clear bands of gelatin digestion on a blue background, were quantified by using a densitometry images analysis software (Image Master VDS, Pharmacia Biotech, Uppsala, Sweden).

Invasion assay

MCF-7Tam and MCF-7 cell invasion was determined using a Boyden chamber (N.T.G., Italy) with polycarbonate membrane, 8.0 μ m pore size (N.T.G., Italy). The filter was coated with 12.5 μ g of Matrigel (Sigma-Aldrich, St. Louis, MO, USA). Control and 24 hr EGCG treated cells were seeded into the upper part of each chamber (4×10^5 cell/well in 800 μ l serum-free α -MEM medium). Chemoattractant (200 μ l MEM with 10% FBS) was added to the lower part. After incubation for 12 hr at 37°C, non-migratory cells on the upper surface of the filter were wiped with a cotton swab while cells migrated on the lower surface of the filter were fixed and stained with Toluidine blue (Sigma-Aldrich, St. Louis, MO, USA). The number of invasive cells on the lower surface of the

membrane was counted in five random fields. The total number of migrating cells under control and EGCG treatment conditions were statically compared.

RNA extraction and RT-PCR

RNA was extracted by guanidinium-phenol-isoamyl alcohol method according to Chomczynski and Sacchi with modifications [13]. RT-PCR reaction was performed using the cMaster RT plus PCR system kit according to the instruction of the supplier (Eppendorf, Hamburg, Germany). The following primers were used: (EGFR) **F**: 5'-CTCACGCAGTTGGGCACTTT-3', **R**: 5'-TCATGGGCAGCTCCTTCAGT-3'; β -actin was used as an internal control: The sequence of the primers were: **F**: 5'-GGCATCGTGATGGACTCCG-3', **R**: 5'-GCTGGAAGGTGGACAGCGA-3'. Annealing temperature was 58°C. The β -actin primers were put together with EGFR primers into the same tube.

Statistic analysis

Experiments were conducted 3 times when not otherwise specified. Data were expressed as mean and standard deviation (\pm SEM). Statistically significant differences in the mean were assessed by ANOVA followed by Bonferroni's multiple comparison test, or two-tail Student's *t*-test, as appropriate, using the GraphPad Prism 5.1 software statistical package. The level for accepted statistical significance was $p < 0.05$.

RESULTS

EGFR expression in MCF-7 and MCF-7Tam cells

MCF-7 cells were grown in a medium deprived of estrogens and phenol red and in presence of 10^{-7} M 4-OH-tamoxifen for one year before starting the experiments, as previously reported [12]. Tamoxifen treatment induced a strong selection of MCF-7 cells: MCF-7 cells sensitive to tamoxifen treatment underwent cell death, whereas a part of MCF-7 cells became resistant and acquired the ability to grow and duplicate in presence of the drug. We named these cells MCF-7Tam cells. Together with the ability to proliferate and grow in presence of tamoxifen, MCF-7Tam cells also displayed an altered morphology reminiscent of an Epithelial Mesenchymal Transition (EMT), as already described [6,7,8]. As shown in Fig.1b, MCF-7 cells grew in monolayer with tight cell-cell contact and they gave rise to roundish colonies. In contrast, MCF-7Tam cells lacked of cell-cell contact. They showed an elongated, fibroblast-like phenotype (Fig.1a) and ruffled membranes observed in actively spreading cells.

EGFR expression was poorly detectable in the cytoplasm of MCF-7 cells by immunofluorescence (Fig.1d), whereas bright EGFR immunostaining was present in both cytoplasm and plasma membrane of MCF-7Tam cells (Fig.1c). With respect to MCF-7, MCF-7Tam cells expressed EGFR to a high extend, as also demonstrated by Western Blot analysis (Fig.1e).

When EGCG was given to MCF-7 and MCF-7Tam cells, a gradual decrease in EGFR was found by Western blot in both groups of samples (Fig.2a and b). FACS analysis of the cell surface-associated EGFR in MCF-7Tam cells showed a dose-dependent decrease, statistically significant after 10 μ g/ml EGCG treatment for 24 hours (Fig.2c). EGCG treatment also suppressed the activity of EGFR signalling pathway leading to cell proliferation, as demonstrated by decreased expression of phosphoEGFR Tyr992 and Tyr1068 (supplementary material, Fig.1). PhosphoEGFR Tyr1045, which is involved in the direct interaction of Grb2-mediated Cbl-induced ubiquitination of EGFR and subsequent endocytosis, showed a modest increase after 10 and 50 μ g/ml EGCG treatment and decreased after 100 μ g/ml EGCG treatment (supplementary material, Fig.1). Loss of EGFR after EGCG treatment found by Western blot and FACS was also mirrored by RT-PCR: dose-dependent downregulation of EGFR transcription occurred in MCF-7Tam cells. EGFR gene transcription decrease was statistically significant after 50 μ g/ml EGCG treatment for 24 hours (Fig.3). In MCF-7 cells, EGFR down-regulation was also detectable, although to a lesser extend than in MCF-7Tam cells (data not shown).

ERK p42/44 activity, viability and invasion in MCF-7 and MCF-7Tam cells

EGFR represents one of the most important signaling pathways able to induce survival and proliferation in the course of treatment with tamoxifen and in many other human cancer. The ERK and PI3K–Akt signaling pathways function downstream of EGFR: the continuous activity of the EGFR is essential for the phosphorylation of the ERK1/2 downstream signaling molecules, which have been shown to be essential for cell proliferation. We investigated whether EGFR signaling pathway was active in both MCF-7Tam and MCF-7 cells by ERK and phosphoERK p42/44 analysis. We also asked whether EGCG treatment was able to downregulate ERK phosphoERK molecules and, consequently, to reduce cell proliferation. ERK p42/44 expression was higher in MCF-7Tam than in MCF-7 cells (Fig.4a) and EGCG treatment decreased ERK p42/44 expression to a similar level in the two cell lines. In contrast, the effect of EGCG treatment was remarkably greater in MCF-7Tam cells than in MCF-7 cells (Fig.4b). In fact, phosphoERK p42/44 expression decreased significantly in MCF-7Tam cells after 10 μ g/ml EGCG treatment for 24 hours, whereas in MCF-7 cells, only 100 μ g/ml EGCG treatment was significantly effective.

Accordingly, loss of EGFR expression and ERK-phosphoERK p42/44 downregulation were associated with reduced cell growth. MCF-7 and MCF-7Tam cells were treated with various concentrations of EGCG for 24, 48 and 72 hr. At the end of the treatment, cells were subjected to MTT assay to determine the cell viability. As shown in Fig.5a and 5b, MCF-7Tam cell treatment with high EGCG concentration (50 and 100 μ g/ml) caused a remarkable cytotoxic effect, which developed

earlier and was greater in MCF-7Tam than in MCF-7 cells. After 50 $\mu\text{g/ml}$ EGCG treatment for 72 hr, MCF-7Tam cell growth was reduced to 60% with respect to control cells and a further decrease in cell growth to 33% was found after 100 $\mu\text{g/ml}$ EGCG treatment for 72 hr. MCF-7 cell growth was inhibited to a lesser extent after EGCG treatment (Fig.5b): in fact, 50 $\mu\text{g/ml}$ EGCG treatment for 72 hr reduced MCF-7 cell growth to 84%. A further inhibitory effect on cell growth was detected after 100 $\mu\text{g/ml}$ EGCG treatment (49%).

Cell invasion assay was performed by incubating MCF-7Tam and MCF-7 cells with EGCG at various concentrations for 24 hr. We observed a dose-dependent reduction of invasive cells at a minimum of 10 $\mu\text{g/ml}$ EGCG treatment. The inhibition of migration was statistically significant at the concentration of 50 $\mu\text{g/ml}$ EGCG, whereas 100 $\mu\text{g/ml}$ EGCG treatment reduced MCF-7Tam cell invasion to more than 50% (Fig.6a and 6b). In contrast, MCF-7 cells did not show a remarkable attitude to invasive growth, presumably due to MCF-7 strong cell-cell contact (Fig. 6c).

MMP-2, MMP-9, TIMP and EMMPRIN expression in MCF-7 and MCF-7Tam cells

We hypothesized that the invasion inhibitory effect of EGCG might be due to the suppression of MMP-2 and MMP-9 activity, two molecules involved in a wide range of proteolytic events, including tumor growth, tumor cell migration and metastasis. MMP-2 and MMP-9 were less expressed in MCF-7 cells than in MCF-7Tam cells (Fig.7a). We analyzed MMP-2 and MMP-9 expression by Western blot in control and EGCG treated MCF-7Tam and MCF-7 cells and we found that EGCG inhibited MMP-2 and MMP-9 expression in a dose-dependent manner in both the cell lines. As shown in Figs. 7a and 7b, the inhibitory effect of EGCG on MMP-2 and -9 expression was statistically significant after 50 $\mu\text{g/ml}$ administration. The zymography analysis of MMP-2 and MMP-9 activity in MCF-7Tam and MCF-7 cells treated with 50 and 100 $\mu\text{g/ml}$ EGCG for 24 hr clearly showed a dose-dependent decreased activity (Fig.7c). MMP-2 and MMP-9 decreased activity was statistically significant after 50 $\mu\text{g/ml}$ EGCG treatment for 24 hr.

MMP activity is controlled by endogenous inhibitors, the Tissue Inhibitors of Metalloproteinases (TIMPs). Changes in TIMP levels directly affect MMP activity since they have the ability to form tight 1:1 complexes with the active MMP enzymes. Particularly, TIMP-1 regulates MMP9 and TIMP-2 regulates MMP2. Therefore we evaluated TIMP-1 and TIMP2 expression after EGCG treatment (Fig.8a). A significant dose dependent TIMPs increase was only detected in MCF-7Tam cells. TIMP-1 and TIMP-2 expression increased in MCF-7Tam cells after 50 $\mu\text{g/ml}$ EGCG treatment and 100 $\mu\text{g/ml}$ EGCG treatment, respectively. In contrast, EMMPRIN, a transmembrane glycoprotein that is highly expressed on the surface of malignant tumor cells and that induces production of MMPs, was highly expressed in MCF-7Tam cells and significantly reduced after EGCG treatment (Fig.9b): MCF-7Tam cells expressed EMMPRIN to a high extent with respect to MCF-7 cells, but after 100 $\mu\text{g/ml}$ EGCG treatment for 24 hr, EMMPRIN was reduced to a level comparable to MCF-7 cells (Fig.9b). In MCF-7 cells, EGCG treatment at the highest dose (100 $\mu\text{g/ml}$) reduced EMMPRIN expression scarcely.

DISCUSSION

The present results represent the first report about the effects of EGCG on a breast carcinoma cell line resistant to tamoxifen, which we developed and we used as a model to better understand the molecular mechanisms underlying the EGCG effects. In the present study, MCF-7Tam cells were established by long-term exposure of estrogen-dependent MCF-7 cells to tamoxifen [12]. In agreement with other reports, MCF-7Tam cells displayed elevated levels of EGFR, increased motility and a fibroblast-like phenotype [6,7]. Previously, we have also demonstrated that pS2 transcription, which is considered a good indicator of active estrogen signaling pathway, was downregulated in our MCF-7Tam cells [12].

The development of tamoxifen resistant tumors is one of the major obstacles in the endocrine therapy of breast carcinoma. Many data support the hypothesis that it occurs via activation of novel signaling pathways that circumvent the effect of anti-estrogens. We found that EGFR signalling pathway was activated to a greater extent in MCF-7Tam than in MCF-7 cells and EGCG was able to downregulate it. Particularly, phosphorylated Tyr992 was undetectable in MCF-7 cells, as already reported [14], whereas it was highly expressed in MCF-7Tam cells, together with increased phosphorylation of Tyr1068: both were reduced by EGCG treatment. Tyr992 phosphorylation results in activation of PLC γ -mediated downstream signaling [15] and phosphorylated Tyr1068 binds Grb2, a crucial molecule to the EGF-induced Ras/mitogen-activated protein kinase signaling pathway, able to induce breast cancer cell motility and invasion [16]. On the basis of these results we considered that the increased sensitivity of MCF-7Tam cell with respect to MCF-7 cell to EGCG treatment could not be only due to the downregulation of the oestrogen receptor signalling pathway that occurred after tamoxifen treatment, but also to the activation of alternative signalling pathways that made MCF-7Tam cells able to survive even in the presence of the drug.

These findings support the hypothesis that in absence of oestrogen receptor signalling pathway, EGFR might be one of the driving molecules for MCF-7Tam cells survival and proliferation and that EGCG was able to downregulate it. Cell proliferation and cell motility were inhibited and the activity of molecules strongly implicated in local invasion and metastasis (MMP-2, MMP-9, EMMPRIN and TIMPS) was modulated.

The effect of EGCG and green tea catechins on EGFR has already been described in many *in vitro* and *in vivo* models [8, 9, 17]. Emerging data indicate that EGCG exerts anticancer effects, at least in part, through inhibition of activation of the specific Receptor Tyrosine Kinases (RTKs) [10]. EGCG seemed to alter the lipid rafts and to inhibit the homo and heterodimerization of EGFR, inactivating the signaling pathway [18]. As a consequence, the EGFR is internalized and degraded and the signaling pathway is interrupted. In keeping with these studies, we also found a significant and dose-dependent loss of EGFR on the plasma membrane after EGCG treatment. Low dose EGCG treatment increased the phosphorylation of Tyr1045, directing EGFR to lysosomal degradation, therefore reducing the quantity of EGFR present on the plasma membrane [19].

The alteration of the lipid rafts may also explain why EGCG can inhibit activation of other membrane-associated RTKs: this ability might play a critical role in the anticancer effects of green tea catechins. In contrast, several studies supported the hypothesis that EGCG can directly inhibit the tyrosine kinase activity of EGFR [20] and there is evidence that EGCG can directly target downstream intracellular signaling molecules. The numerous molecular targets of the green tea catechins have been subject of various review [20, 21] but, taken together, the numerous studies present in the literature suggest that the antitumor effects of EGCG and green tea catechins may be due to binding to numerous and various cell targets. This characteristic is not a limit but an advantage, conferring this molecule potential utility towards different types of cancer [22].

In the present study we also found that EGCG was effective in inhibiting the *in vitro* invasion of MCF-7Tam cells and that this event was related with down-regulation of MMPs and EMMPRIN, molecules implicated in local invasion and metastasis. The relation between down-regulation of

EGFR and MMPs inhibition has been demonstrated in oral squamous cell carcinoma after ZD1839 (Iressa) treatment, which selectively inhibited the EGFR tyrosine kinase activity. Downregulation of EGFR was associated with reduction of MMP-2 and MMP-9 activity [23]. Evidences that linked EGFR and MMP-2 and -9 regulation were provided for thyroid carcinoma [24], glioma [25], head and neck squamous cell carcinomas [26] and ovarian cancer [27]. In association with MMPs and the plasminogen activator receptor (PAR), EGFR concurs to form structures that mediate migration through the basal membrane. We can not exclude that the effect of EGCG on MMPs could also be direct, as suggested by many studies on different human cell lines and animals [28-31].

The decreased in vitro matrigel invasion after EGCG treatment was associated with MMP-2 and -9 reduced expression. As already demonstrated, EGCG inhibits MMPs activity, leading to accumulation of non activated MMP-2 at the cell surface [31]. In the present study, we observed increased expression of both TIMPs, but the effect on TIMP-2 was significant after 50 µg/ml EGCG treatment for 24 hr, whereas the increase of TIMP-1 was significant after 100 µg/ml EGCG treatment. These findings contribute to explain the impaired in vitro invasion after EGCG treatment. Pro-MMP-2 activation needs TIMP-1 and -2 contribution. The active forms of MMPs subsequently activate the remaining pro-MMPs. Inactivation of the physiological function of MMPs, or even pro-MMPs, is accomplished by non-covalent TIMP binding [32]. Beside growth factors, such as Her2/neu, cytokines and hormones, MMP-2 expression is also dependent on EMMPRIN. In the present study we found that EMMPRIN expression was very high in MCF-7Tam with respect to MCF-7 cells, and we found that EGCG could reduce EMMPRIN expression to a level present in MCF-7 cells. Then, the final inhibitory effect on MMP-2 and -9 activity was due to both TIMP-1 and -2 upregulation and EMMPRIN downregulation. Blocking MMP-2 secretion and activation during breast carcinoma development may decrease metastasis: a low level of MMP-2 is linked to a favorable prognosis in patients with hormone receptor-negative tumors, usually associated with high risk [33]. Our results contribute to show that the anticancer effect of EGCG occurred via multiple cellular and molecular mechanisms and they also support the hypothesis that EGCG may be useful in inhibiting and/or preventing metastasis.

In the present study, we have also investigated the effect of EGCG on MCF-7 cells, the parental cell line used to develop the MCF-7Tam cells. EGFR, ERK and phosphoERK p42/44 expression was higher in MCF-7Tam cells than MCF-7 cells, as well as cell migration and EMMPRIN expression. Differences concerning the expression of MMPs and TIMPs were less pronounced in the two cell lines. EGCG treatment demonstrated to be effective in both cell lines, but the most significant effects were detected in MCF-7Tam cell line. On the base of these findings we remark that EGCG was able to attenuate a certain number of cell features acquired during the development of tamoxifen resistance.

So far, no study has been published about the effect of down-regulation of EGFR in a cell line of breast carcinoma resistant to tamoxifen, but a potent cytotoxic effect was observed when green tea and tamoxifen were combined on MCF-7, ZR75, T47D human breast cancer cells in vitro and in vivo [34]. Interestingly, the combination of tamoxifen and EGCG increased the toxicity in MDA-MB-231 cells, a breast carcinoma cell line that lack of estrogen receptor alpha (ER α) and this effect was obtained by downregulating EGFR and mTOR signaling pathways [35,36]. As a complement of the aforementioned results about the effects of EGCG and tamoxifen, the present data support that EGCG can be effective in downregulating the expression of some of the most implicated molecules that confer increased malignancy to tamoxifen resistant breast carcinoma cells. EGCG might be a valuable molecule to associate with tamoxifen even in case of resistant disease. As demonstrated by Zhang and coworkers [37] the combined treatment of squamous cell carcinoma of the head and neck with EGCG and EGFR-tyrosine kinase inhibitor (EGFR-TKI) erlotinib revealed a synergistic antitumor effect. As green tea is cheap, safe and healthy, its use ought to be encouraged and better investigated: EGCG might be a useful molecule in contrasting the drug-resistance development and in attenuating the drug-resistant phenotype.

The effects of EGCG described in the present and many other studies were obtained by treating cells with relatively high EGCG concentrations, which could be hardly reached by a single dose administration in humans. Studies on EGCG bioavailability reported plasma EGCG concentration as high as 4.4 and 7.4 $\mu\text{mol/L}$ or lower: instability of EGCG at the intestinal pH of 8.5, the balance of transporters involved in EGCG influx and efflux and the phase II enzymes involved in its transformation are factors that would contribute to its low bioavailability [38,39]. To overcome the limits of bioavailability of EGCG, or the potentially toxic effects at high doses, Siddiqui and coworkers recently developed a nanoparticle-mediated delivery technique that enhanced bioavailability of EGCG and might limit any unwanted toxicity [40]. Moreover, we can not deny the obvious that the low EGCG concentration following oral administration was demonstrated to be effective to inhibit or delay cancer development in rodents [41]. Finally, we can not exclude that the combinations of various phytochemicals, or phytochemicals and anticancer drugs at low doses, which might exert synergistic anticancer effects, might be more effective and less toxic.

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Legends to figures

Fig.1. MCF-7-Tam and MCF-7 cells showed different morphology and different level of EGFR expression. The cells were cultured on coverlips, fixed in Formalin 4%, stained by Toluidine blue 0.1% and observed by a Nikon optical microscope with a X100 objective. MCF-7Tam cells (a) showed a fibroblast-like morphology whereas MCF-7 cells (b) presented a roundish shape and very tight cell-cell junctions. Bar 30 μ m. Fig.1c and d: immunofluorescence detection of EGFR in MCF-7Tam and MCF-7 cells. The cells, cultured on coverslips, were fixed in cold methanol and incubated with a monoclonal antibody against EGFR followed by an anti-mouse FITC-conjugated antibody. The samples were analyzed by a Nikon optical microscope equipped with a X60 objective. Pictures were taken by using the same exposition time, in order to assure that the different fluorescence intensity was only due to the intrinsic characteristics of the samples. Bar 30 μ m. EGFR expression was scarce in MCF-7 cells (d) and restricted to the cytoplasm. In contrast, MCF-7Tam cells showed bright EGFR staining (c) in both cytoplasm and plasma membrane. Fig.1e: Western blot analysis of MCF-7Tam and MCF-7 cells. Western blot analysis showed a fainter and thinner band in MCF-7 than in MCF-7Tam cells.

Fig.2. Western blot and FACS analysis of EGFR expression in MCF-7Tam and MCF-7 cells treated with EGCG. EGFR protein level was assessed by Western blot in cell lysate of MCF-7Tam and MCF-7 cells treated with 10-100 μ g/ml EGCG for 24 hr (a). Densitometry data are expressed as percentage of treated samples with respect to the control (CTR) (b). EGFR expression in MCF-7Tam cells was assumed as 100% with respect to all the other samples, included untreated MCF-7 cells, in order to compare the different level of EGFR expression in the two cell lines and the corresponding decrease after EGCG treatment. EGCG reduced EGFR in both MCF-7Tam and MCF-7 cells. Bar shows mean with SEM and asterisk indicates significant difference with respect to the control: * p <0.05, ** p <0.01. FACS analysis of EGFR expression in MCF-7Tam cells (c). MCF-7Tam cells were treated with 10-100 μ g/ml EGCG for 24 hr. Mouse monoclonal antibody anti EGFR external domain (1:800) was used on control (CTR) and EGCG treated cells and detected by a secondary anti mouse antibody FITC-conjugated (1:800). FACS analysis detected EGFR dose-dependent decrease. Bar shows mean with SD and asterisk indicates significant difference with respect to the control: * p <0.05, ** p <0.01.

Fig.3. RT-PCR analysis of EGFR expression in MCF-7Tam cells treated with 10-100 μ g/ml EGCG for 24 hr. One-step RT-PCR was performed on RNA extracted from control and treated MCF-7Tam cells. PCR product was loaded onto a 1% agarose gel stained with Ethidium Bromide. Densitometry analysis was reported as β -actin/EGFR ratio of treated on control samples. A dose-dependent decrease of EGFR expression was detectable and it was statistically significant after 50 μ g/ml EGCG treatment for 24 hr. Asterisk indicates significant difference with respect to the control: * p <0.05.

Fig.4. ERK and phosphoERK p42/44 Western blot analysis in MCF-7Tam and MCF-7 cells after EGCG treatment for 24 hr. ERK p42/44 expression in control (CTR) and EGCG treated MCF-7Tam and MCF-7 cells (a). The results are expressed as percentage of treated on control samples. ERK p42/44 and phosphoERK p42/44 expression in MCF-7Tam control cells were assumed as 100% with respect to all the other samples, included untreated MCF-7 cells, in order to compare the different level of expression in the two cell lines and the corresponding relative decrease after EGCG treatment. PhosphoERK p42/44 expression in control (CTR) and EGCG treated MCF-7Tam and MCF-7 cells (b). EGCG treatment was significantly more effective in MCF-7Tam cells than in MCF-7 cells. Bar shows mean with SEM and asterisk indicates significant difference with respect to the control: * p <0.05.

Fig.5. MTT assay in MCF-7Tam and MCF-7 cells treated with 20-100 $\mu\text{g/ml}$ EGCG for 24, 48 and 72 h. Dose-dependent cytotoxic effect after EGCG treatment developed earlier and, at high dose, it was greater in MCF-7Tam than MCF-7 cells. Data are expressed as percentage of treated samples with respect to the control (CTR). Asterisk indicates significant difference with respect to the control: * $p < 0.05$, ** $p < 0.01$.

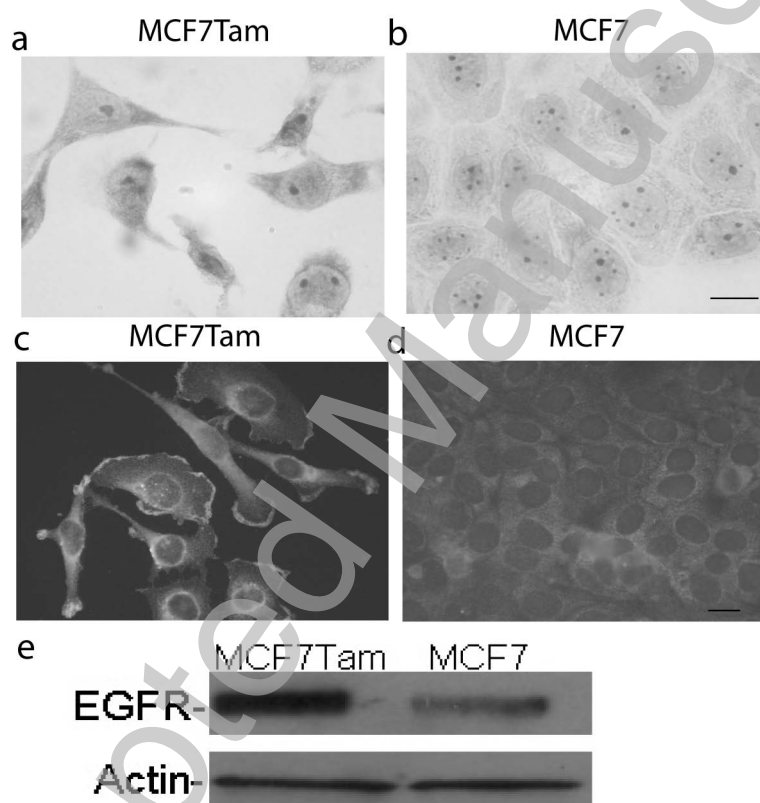
Fig.6. Cell Invasion Assay in MCF-7Tam and MCF-7 cells. Effect of 10-100 $\mu\text{g/ml}$ EGCG treatment on matrigel invasion of MCF-7Tam and MCF-7 cells (a) as detected by Boyden chamber. After 12 hr migration through the matrigel-coated membranes, the cells were fixed, stained, photographed and counted under light microscopy. MCF-7 cells showed a limited attitude to invasion with respect to MCF-7Tam cells. The effect of 100 $\mu\text{g/ml}$ EGCG treatment in MCF-7Tam and MCF-7 is shown (a). Data are expressed as number of cells for field for treatment (b). Each bar represents the mean (\pm SEM) of three independent experiments. Horizontal Bar: 100 μm . * $p < 0.05$, ** $p < 0.01$.

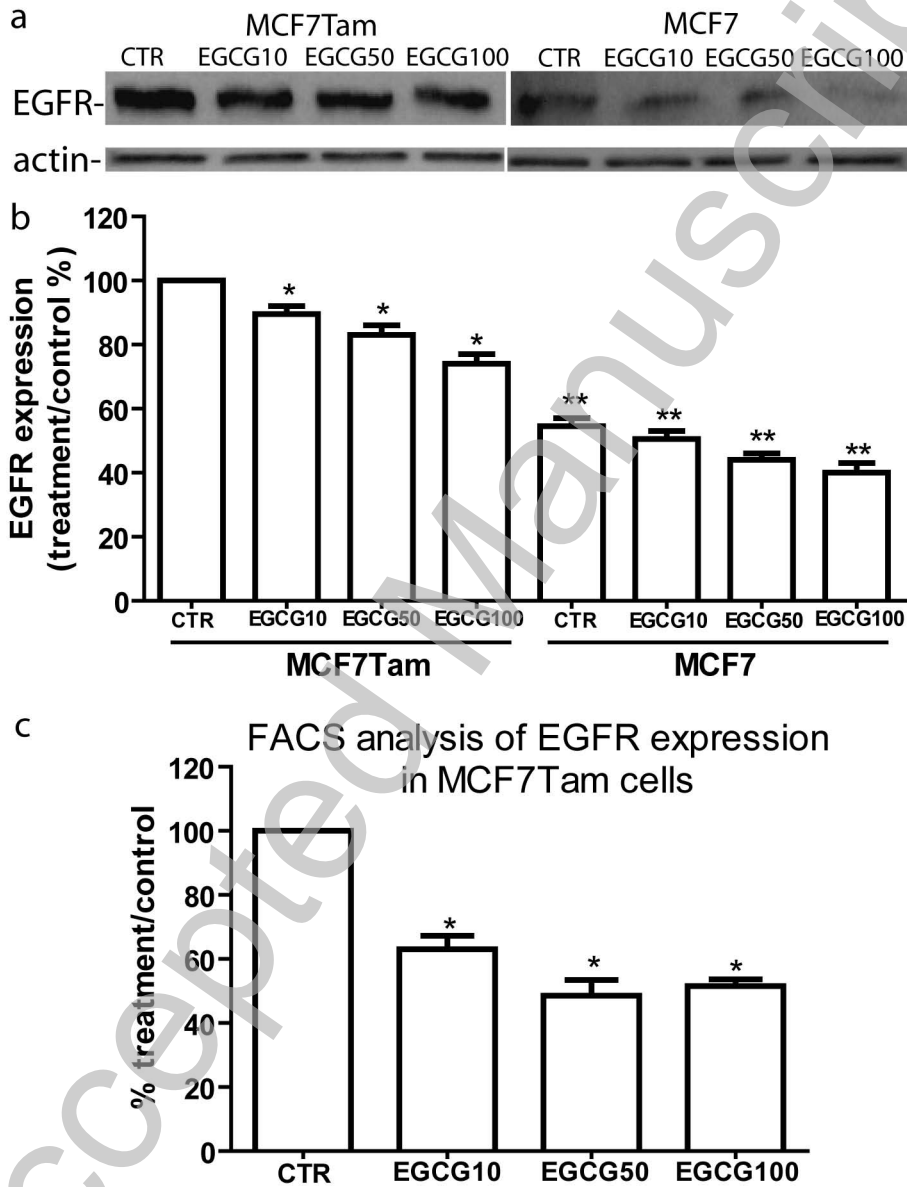
Fig.7. Effect of 10-100 $\mu\text{g/ml}$ EGCG treatment on MMP-9 and MMP-2 protein levels as assessed by Western blot and zymography. Cell lysates of MCF-7Tam and MCF-7 cells were used for Western blot analysis after 10, 50 and 100 $\mu\text{g/ml}$ EGCG treatment. MMPs decrease occurred to a comparable extent into the two cell lines. Densitometry data (b) are expressed as percentage of treated on control samples. MMPs expression in MCF-7Tam control cells was assumed as 100% with respect to all the other samples, included untreated MCF-7 cells, in order to compare the different level of expression in the two cell lines and the corresponding relative decrease after EGCG treatment. Bar shows mean with SEM and asterisk indicates significant difference with respect to the control: * $p < 0.05$, ** $p < 0.01$. The effect of 10-100 $\mu\text{g/ml}$ EGCG treatment for 24 hr on MMP9 and MMP2 activity was evaluated by zymography in MCF7Tam and MCF-7 cells (c). Gelatin zymogram showed the MMP9 (83 kDa) and MMP2 (62 kDa) form in serum free conditioned media. After 50 and 100 $\mu\text{g/ml}$ EGCG treatment the bands were not detectable.

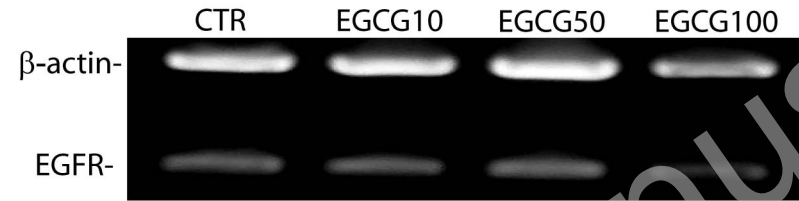
Fig.8 Effect of 10-100 $\mu\text{g/ml}$ EGCG treatment for 24 hr on TIMP-1 and TIMP-2 protein levels, as assessed by Western blot in cell lysate of MCF7Tam and MCF-7 cells (a). TIMPs increase was only statistically significant in MCF-7Tam cells. Densitometry data are expressed as percentage of treated samples with respect to the control (CTR) (b). TIMPs expression in MCF-7Tam control cells was assumed as 100% with respect to all the other samples, included untreated MCF-7 cells, in order to compare the different level of expression in the two cell lines and the corresponding relative decrease after EGCG treatment. Bar shows mean with SEM and asterisk indicates significant difference with respect to control: * $p < 0.05$.

Fig.9 Effect of 10-100 $\mu\text{g/ml}$ EGCG treatment for 24 hr on EMMPRIN protein levels, as assessed by Western blot in MCF-7Tam and MCF-7 cells. MCF-7Tam cells showed a greater amount of EMPPRIN than MCF-7 cells (a). A significant decrease of EMMPRIN expression was found after 24 hr EGCG treatment (b). Densitometry data are expressed as percentage of treated samples with respect to the control (CTR). EMMPRIN expression in MCF-7Tam control cells was assumed as 100% with respect to all the other samples, included untreated MCF-7 cells, in order to compare the different level of expression in the two cell lines and the corresponding relative decrease after EGCG treatment. Bar shows mean with SEM and asterisk indicates significant difference with respect to control: * $p < 0.05$.

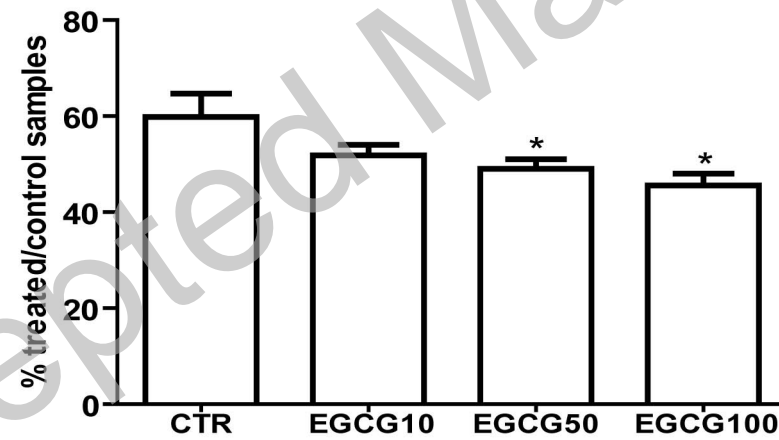
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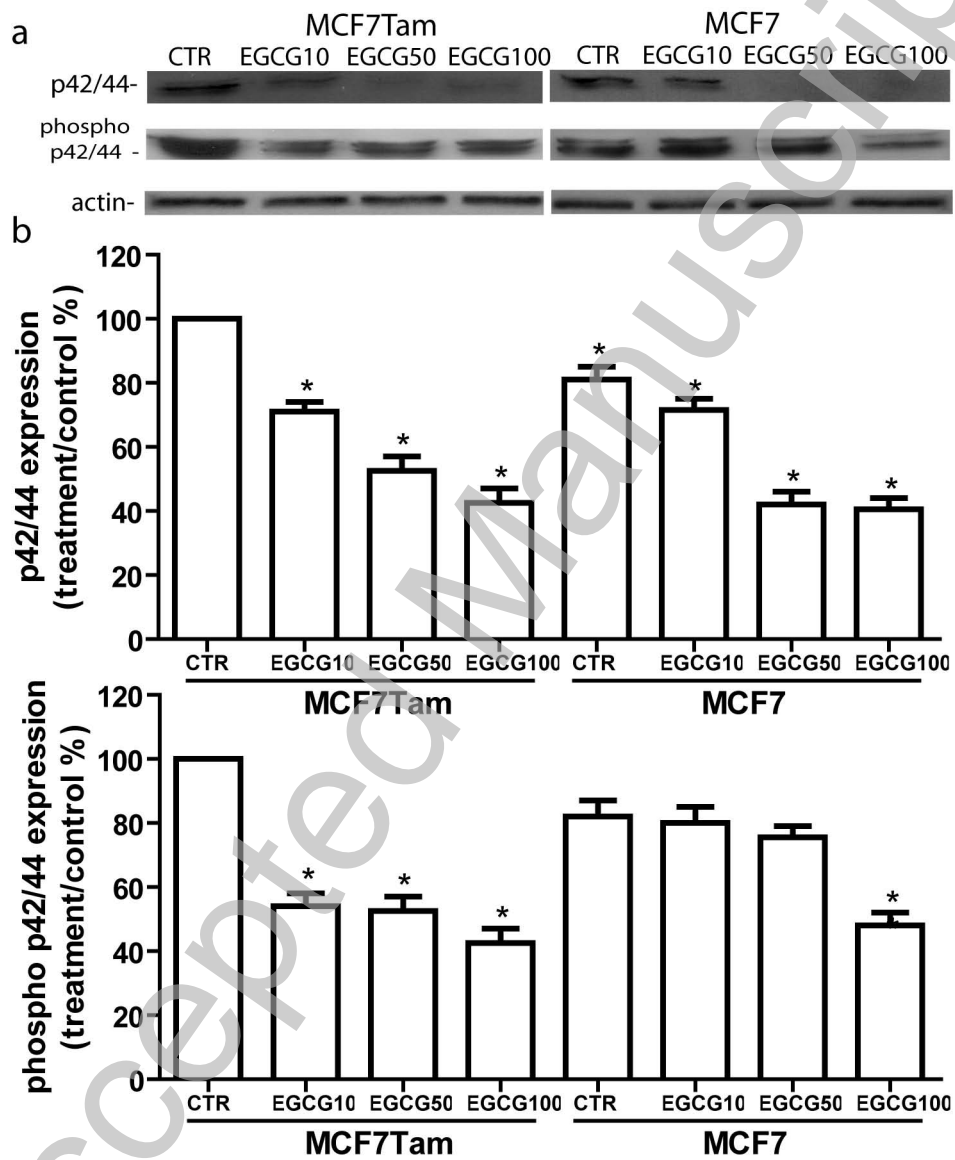


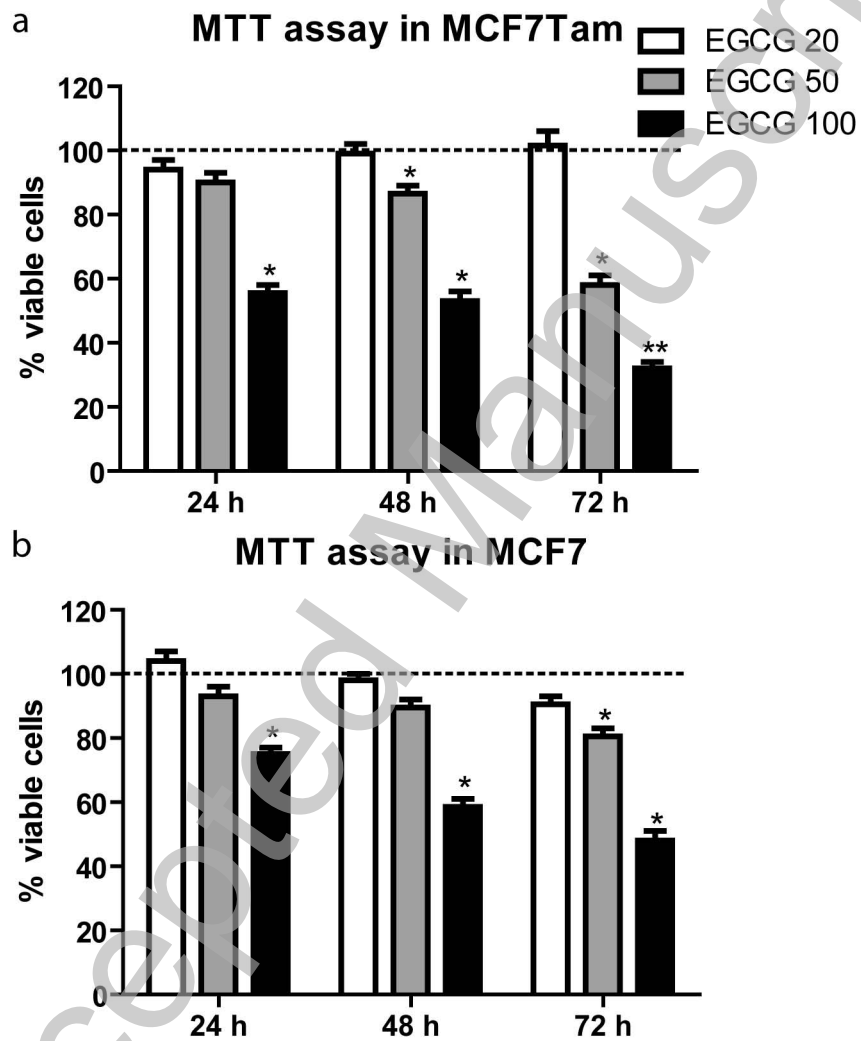


Densitometry analysis of EGFR expression as evaluated by RT-PCR



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