EGF/EGFR signaling axis is a significant regulator of the proteasome expression and activity in colon cancer cells

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ABSTRACT

Colon cancer is the third most common type of cancer worldwide. Epidermal growth factor receptor (EGFR) plays a crucial role in the (patho)physiology of the disease. EGFR controls vital cellular processes, while this action is associated with poor prognosis. In addition, K-Ras mutations are associated with the promotion of the disease and the anti-EGFR resistance. The ubiquitin-proteasome system plays also a very important role in cancer, modulating cell cycle and other cellular processes such as the growth and the survival of cancer cells. Proteasome inhibition affects, in several cases, the action and the protein levels of EGFR. Nevertheless, little is known whether the reversed option is possible. In this study, we, therefore, investigated the impact of epidermal growth factor (EGF)/EGFR signaling axis on gene expression and the proteolytic activity of the proteasome subunits, as well as whether nuclear factor erythroid 2 related factor 2 (Nrf2), an activator of proteasome expression, plays a role in this process. Moreover, we evaluated whether EGF regulates the expression of its own receptor and the proliferation rate of DLD-1 (K-Ras mutated) colon cancer cells. The obtained data showed that, although EGF has no significant effect on the proliferation of DLD-1 colon cancer cells, it significantly upregulates the expression of EGFR as well as the expression and the activity of the proteasome, suggesting that the EGF-mediated proteasome activation could possibly lead to enhanced EGFR degradation leading to autoregulation of EGF–EGFR pathway. Nrf2 activation did not induce proteasome gene expression in DLD-1 colon cancer cells.

INTRODUCTION

Colon cancer is estimated to be one of the most common types of cancer in Europe and USA [1, 2]. The disease is characterized by poor prognosis and short lifetime, especially for patients with the metastatic type. Epidermal growth factor receptor (EGFR) is a cell surface receptor, with vital role for cancer [3], regulating crucial cell functions and the expression of several ECM molecules [4], which are key players in cancer, as recently reported by our research group [5, 6]. EGFR is one of the four members of ErbB family, a subgroup of tyrosine kinase receptors. The activation of the receptor is triggered by specific ligands, mainly by EGF [7]. EGF binding to the receptor causes its dimerization. EGFR has the ability to form both homodimers and heterodimers with other members of ErbB family. The dimerization of the receptor causes cross-phosphorylation of its intracellular part and, therefore, the phosphorylated sites of the receptor act as docking sites for several signaling molecules, containing SH2 or PTB binding sites [8]. The activation of such molecules initiates specific signaling pathways, which in turn activate several transcription factors. EGFR mainly uses four signaling pathways: Ras-Raf-Mek-Erk, PI3K-Akt, Jak-Stat, and PLCγ-PKC. Another very important and well-studied receptor of the ErbB family is also HER2/neu. HER2 is expressed in several types of cancer, commonly in breast and lung cancer [9, 10] and is also associated with cancer progress and development. Although HER2 has no natural ligands, this remains a key player into the ErbB family due to its ability to form stable heterodimers with the other receptors of the group [11]. Relating to its

signaling pathways, HER2 shares almost the same pathways with EGFR. The overexpression rates of EGFR in colon cancer range between 35% and 49% [12–14]. EGFR overexpression has been associated with advanced stages of colon cancer [15]. Due to the significance of the receptor in this particular type of cancer, a variety of studies are strongly focused on EGFR inhibition. Specific tyrosine-kinase inhibitors (TKIs), which can pass through extracellular membrane and bind to the intracellular part of the receptor blocking its cross-phosphorylation, as well as monoclonal antibodies, that bind to the extracellular part of the receptor antagonizing EGF have been used as EGFR inhibitors [16]. In most cases, EGFR inhibition causes antitumor effect in both in vitro and in vivo studies. For example, the TKIs AG1478-induced colon cancer cells apoptosis [17]. In a previous study of our laboratory Gialeli *et al.* [18], it has been demonstrated that the monoclonal antibody Panitumumab against EGFR exerts an anti-proliferative and anti-migratory role in colon cancer cell lines. Moreover, the simultaneous use of the monoclonal antibody IMC-C225 with irinotecan resulted in enhanced anti-tumor effect in vivo [19]. Despite EGFR's main role in colon cancer, HER2/neu also appears to be significant in this particular type of cancer. Due to HER2 heterodimerization with EGFR, a variety of studies in colon cancer examine the effect of the simultaneous inhibition of both receptors. More specifically, the monoclonal antibody against HER2, named trastuzumab, in combination with the specific TKI against EGFR, erlotinib, suppressed growth development both in vivo and in vitro. It is significant that the inhibition of both the receptors enhanced the growth suppression as compared to that caused by pertuzumab alone [20]. Mutations are a common phenomenon in colon cancer. Several signaling molecules downstream of EGFR are often mutated. K-Ras mutations are well-studied cases in colon cancer. K-Ras is a guanosine-5′-triphosphate (GTP)-bounded protein, located downstream of EGFR at the Ras-Raf-Mek-Erk signaling pathway. K-Ras mutation rates in colon cancer accounts for *ca*. 20%–50% [21–23]. The mutation of K-Ras protein has as a result the constitutive activation of the molecule, which in turn, activates other downstream signaling molecules. This may be a mechanism for the resistance to EGFR targeted therapies. For this reason, the monotherapy with anti-EGFR monoclonal antibodies is not recommended for patients with K-Ras mutated status [24–28]. The existence of such mutations makes therapies for colon cancer, based on EGFR inhibition, much more complex. The ubiquitin-proteasome system is the main regulator of the nonlysosomatic degradation of a variety of intracellular proteins, including abnormal, misfolded, denatured, or otherwise damaged proteins. The 20S proteasome is a 700-kDa protease composed of 28 subunits arranged as a barrel-shaped structure of four heptameric rings. The two outside rings contain one copy each of seven different but related α-type subunits. Likewise, both of the inside rings contain one copy each of seven different but related β-subunits where the catalytic sites are located. The proteasome possesses multiple endopeptidase activities including chymotrypsin-like (β5 subunits), trypsin-like (β2 subunits), and caspase-like (β1 subunits) [30]. Proteasome is responsible for the degradation of many intracellular proteins. Through this process, proteasome controls the viable cellular functions and is strongly associated with cell survival and anti-aging [31]. Proteasome promotes cell survival mainly by regulation of specific transcription factor such as NF-kB, p53, and c-Jun, etc. Its ability to promote cell viability makes proteasome a possible target for anticancer therapies. Indeed, in most cases, proteasome inhibition leads to cancer cells apoptosis, both in vivo and in vitro. More specifically, in colon cancer cells, proteasome inhibition by MG-132 suppressed proliferation and inducted apoptosis. In other cases, the combined therapy with proteasome inhibitor and other chemotherapeutic agents enhanced the antitumor effects [32, 33]. Nrf2 is a key transcription factor for cell defense mechanisms that regulates the expression of electrophile and xenobiotic detoxification enzymes and drug efflux pump proteins, which confer cytoprotection against oxidative stress and apoptosis in normal cells. Under the basal conditions, the low constitutive amount of Nrf2 is maintained by the Kelch-like ECH-associated protein1 (Keap1)-mediated ubiquitination and proteasomal degradation system, largely localized in the cytoplasm. When cells are exposed to oxidative stress, electrophiles, or chemopreventive agents, Nrf2 escapes Keap1-mediated repression, escapes proteasomal degradation, translocates to the nucleus, and activates antioxidant responsive element (ARE)-dependent gene expression to maintain cellular redox homeostasis [34]. Interestingly, in some cases, the induction of the proteasome and expression is mediated through an Nrf2-dependent manner [35], indicating that, Nrf2 protective role may be, partly, due to proteasome expression. However, in cancer, the activation of Nrf2 may have different effects. More specifically, Nrf2 promotes cancer cells survival and development [36–38] and, therefore, enhances chemoresistance against anticancer therapies [39, 40]. EGFR is one of the proteins which are subjected to degradation by the proteasome. In several types of cancer, including colon, proteasome inhibition seems to affect the protein levels or the phosphorylation of EGFR [41–43]. Interestingly, several studies demonstrate that the simultaneous EGFR and proteasome inhibition causes enhanced antitumor effects [41, 42, 44], indicating that the combined EGFR/proteasome inhibition could be a possible therapeutic strategy against cancer. However, there are limited data regarding the involvement of EGFR on proteasome subunits expression and activation. For this reason, in this study, we evaluated whether EGF could affect via EGFR or HER2 the gene expression and activity of the catalytic proteasome subunits in DLD-1 colon cancer cells. Moreover, we evaluated, whether the activation of Nrf2 could possibly affect the proteasome expression. According to EGFR significance in colon cancer, we further investigated the effect of EGF on EGFR expression as well as cell proliferation.

MATERIALS AND METHODS

Chemicals and reagents

Dubleco's modified Eagle's medium (DMEM) fetal bovine serum (FBS), L-glutamine, sodium pyruvate, penicillin, streptomycin, gentamycin, and amphotericn B were all obtained from Biochrom AG (Berlin, Germany). The selective inhibitor CP-724,714 against HER2/neu was supplied by Selleck Chemicals. EGF-R's specific ligand EGF, receptor's selective inhibitor AG1478, as well as the fluorogenic substrate SucLLVY-AMC for 20S proteasome chymotrypsin-like subunit β5 (PSMB5) and synthetic Oltipraz were purchased from Sigma Chemical Co (St. Luis, MO, USA). All other chemicals used were of the best commercially available grade.

Cell lines and culture conditions

DLD-1 (K-Ras mutant) cancer cell line was purchased from the American Type Culture Collection (ATCC) and cultured at 37°C in a humidified atmosphere of 5% (v/v) CO_2 and 95% air. Cancer cells were cultured in DMEM, supplemented with 10% FBS, 1.0 mM sodium pyruvate, 2 mM L-glutamine, and a cocktail of antimicrobial agents containing 100 IU/mL penicillin, 100 mg/mL streptomycin, 10 mg/mL gentamycin sulfate, and 2.5 mg/mL amphotericin B. All experiments were conducted in serum-free conditions.

Total RNA isolation and cDNA synthesis

Total RNA isolated after cell lysis with guanidine thiocyanate using NucleoSpin RNA II Isolation System Macherey-Nagal (Germany). All of the total RNA preparations were free of DNA contamination as assessed by real-time polymerase chain reaction (RT-PCR) analysis, and this purification was determined by the absorbance ratios A_{260}/A_{280}. The amount of isolated RNA was quantified by measuring its absorbance at 260 nm (1 A_{260} unit = 40 μg/ml RNA). Total RNA was reverse transcripted with PrimeScript™ first strand cDNA synthesis kit, Takara (Takara Bio Inc, Japan).

End-point and RT-PCR analysis

For end-point PCR analysis, the KAPA Taq ReadyMix DNA Polymerase (KAPABIOSYSTEMS) method was used. Afterward, cDNA sequences were semiquantitive, measured using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as "housekeeping" gene. All amplification products were separated by electrophoresis in a 1.5% agarose gel containing Gelred™ Nucleic Acid Gel Stain (Biotium). Bands were visualized with a ultraviolet (UV) lamp, and gels were photographed with a charge-coupled device (CCD) camera. For semiquantitative analysis, gene expression was determined as the relative fluorescence obtained for each molecule compared with the reference gene (GAPDH). Image analysis was performed using the program UVIpro (UVITec). Real-time PCR was performed in 20 μL mixture consisting of 10 μL KAPA SYBR FAST Master Mix (2×) Universal (KAPABIOSYSTEMS), containing *Taq* DNA polymerase, oligonucleotide primers (0.2 μM each) and 1 μM of template cDNA. The amplification consisted of a two-step procedure denaturation at 95°C for 3 min and 40 cycles with denaturation at 95°C for 3 sec and then annealing/elongation at 60°C for 20 sec using Rotor Gene Q (Giagen, USA). GAPDH was used as an endogenous control. All reactions were performed in triplicates, and a standard curve was always included for each pair of primers for assay validation. In addition, a melting curve analysis was performed for detecting the SYBR Green-based objective aplicon. The relative abundance of mRNA of the gene of interest was deducted from the cycle number at which fluorescence increased above background level (Ct) in the exponential phase of the PCR reaction, after normalization to the Ct of the calibrator. The sequence of primers as well as all their product characteristics and information are shown in Table 1.

Determination of proteasome activity in cell monolayers

Cells were washed twice in phosphate-buffered saline and then lysated with a solution of 1 mM DTT in water for 1 h at 4°C (500 μl /well of a 6-well plate) [45]. Lysates were centrifuged at 14,000 *g* for 30 min at 4°C to remove nonlysated cells, membranes, and nuclei, and protein concentrations were determined in supernatants by the method of Bradford [46], using bovine serum albumin as standard. Then the

Table 1. Sequence of the primers used for end-point and RT-PCR analysis.

Gene	Primer sequence	Annealing temperature ($T_{annealing}$) °C
End-point PCR		
β5 subunit		
Sence	5′-GAG-ATC-AAC-CCA-TAC-CTG-CTA-G-3′	58
Anti-sence	5′-AGT-CAC-CCC-AAG-AAA-CAC-AAG-C-3′	
β1 subunit		
Sence	5′-CAC-CTA-TTC-ACG-ACC-GCA-TT-3′	58
Anti-sence	5′-ACG-GCG-AAT-TTG-GGT-ATC-TG-3′	
β2 subunit		
Sence	5′-ACA-CAG-ACA-TGA-CAA-CCC-AG-3′	58
Anti-sence	5′-AGC-CTG-GTC-CCC-TTC-TTG-T-3′	
Nrf2		
Sence	5′-AAA-CCA-GTG-GAT-CTG-CCA-AC-3′	49
Anti-sence	5′-GAC-CGG-GAA-TAT-CAG-GAA-CA-3′	
GAPDH		
Sence	5′-TCA-AGA-TCA-TCA-GCA-ATG-CCT-CC-3′	60
Anti-sence	5′-AGT-GAG-CTT-CCC-GTT-CAG-C-3′	
RT-PCR		
EGFR		
Sence	5′-ATG-CTC-TAC-AAC-CCC-ACC-AC-3′	60
Ant-sence	5′-GCC-CTT-CGC-ACT-TCT-TAC-AC-3′	
GAPDH		
Sence	5′-AGG-CTG-TTG-TCA-TAC-TTC-TCA-T-3′	60
Ant-sence	5′-GGA-GTC-CAC-TGG-CGT-CTT-3′	

supernatant were assayed for the main chymotrypsin-like activity of 20S proteasome using the fluorogenic substrate N-Succinyl-Leu-Leu-Val-Tyr-AMC (Succ-LLVY-AMC; Sigma-Aldrich, Dorset, UK), as previously described [47]. Cell lysates (50 μl) were incubated with the fluorogenic substrate (dissolved in DMSO) at final concentration of 20 μM in 50 mM HEPES, 20 mM KCl, 5 mM $MgCl_2$, and 1 mM DTT pH 7.8 buffer, in a total volume of 100 μl, for 1 h at 37°C. To determine the specific degradation of Succ-LLVY-AMC by proteasome, MG-132, a proteasome inhibitor, at final concentration 200 μM, was added. The reaction was stopped by the addition of 100 mM sodium acetate buffer pH 4.3 (150 μl). Fluorescence signal for AMC was monitored at 370 nm excitation and 440 nm emission in a TECAN infinite M200 (Austria) fluorometer, using free AMC as a standard. Proteasome activity was expressed as p mole AMC/μg total protein.

Cell proliferation

To determine whether these agents affect the proliferation of DLD-1 colon cancer cell line, the water-soluble tetrazolium salt (WST-1) assay was used. The assay is based on the reduction of WST-1 by viable cells, producing a soluble formazan salt. DLD-1 cells (6×10^3 per well) were plated in 100 μL cell culture medium with 10% FBS in a 96-well plate. Twenty-four hours after plating, cells were washed twice with fresh culture medium serum free. Before addition of EGF (20 ng/mL), cells were incubated with the specific inhibitors of EGFR (AG1478) and HER2 (CP-724,714), alone or in combination for 30 min. After preincubation of inhibitors, EGF was added to culture medium for 24 and 48 h incubation time. Cell proliferation was determined using a microplate reader Infinite 200 (Tecan Austria Gmbtl). The absorbance was measured at a wavelength of 440 nm and a reference wavelength of 650 nm.

Statistical analysis

The statistical analysis of the results assessed with unpaired "*t*-test" or "test ANOVA" Origin 6.0 (Microcal Software Inc., USA). All values are given as mean ± standard deviation of three separate experiments in triplicate.

RESULTS

EGF/EGFR is the main signaling axis for the EGFR gene expression

EGFR is a vital receptor in colon cancer and a significant regulator of gene expression. Therefore, we, first, evaluated the impact of EGF on gene expression of EGFR. For this purpose, two specific TKIs were used: AG1478 against EGFR and CP-724,714 against HER2. Colon cancer cells after starvation in serum-free medium for 24 h were preincubated with the inhibitors (1 μM) for 30 min followed by further incubation with EGF (20 ng/mL) for 24 h. The mRNA levels of EGFR were assessed by RT-PCR analysis as described in section on materials and methods.

As shown in Figure 1, EGF significantly upregulated the expression of EGFR. In addition, the inhibition of EGFR activation by AG1478 abolished the EGF induction by restoring the mRNA levels of the receptor lower to that of the control cells. On the other hand, the inhibition of HER2 activation by CP-724,714 in the presence of EGF elevated the EGFR expression. Moreover, the combined inhibition of both receptors activity strongly decreased the levels of EGFR, reaching the levels obtained when the EGFR inhibitor AG1478 was used alone. The above data highlight that the EGF/EGFR-mediated effect of EGF is the crucial axis for the gene expression of EGFR in DLD-1 colon cancer cells.

The effect of EGF/EGFR on proliferation rate of DLD-1 colon cancer cells

It is known that EGFR regulates several cellular processes in colon cancer such as survival, differentiation, and proliferation. Taking into account that EGF affects EGFR expression, we further examined whether EGF could affect the proliferation of DLD-1 cells. As shown in Figure 2, EGF had no effect on cell proliferation. Moreover, the inhibition of the receptors alone and in combination have no any regulatory effect on the proliferation of DLD-1 cell, following either 24 or 48 h incubation periods (Figure 2). These data may well be in

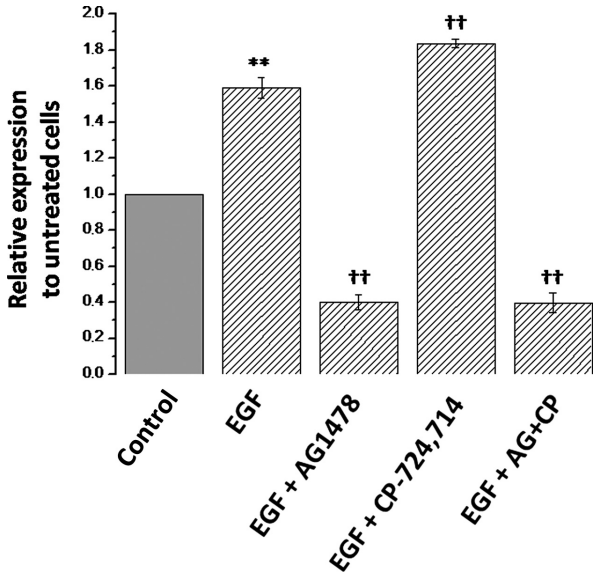

Figure 1. The effect of EGF on gene expression of EGFR in DLD-1 colon cancer cells. The mRNA levels were assessed by RT-PCR analysis. Cancer cells were first preincubated with AG1478 (1 μM) and CP-724,714 (1 μM) for 30 min, followed by the introduction of EGF (20 ng/mL). Total incubation time was 24 h. Results are expressed as relative expression and normalized to untreated cells.
Statistically significant differences are compared with control or EGF-treated cells are symbolized with $^{**}p < 0.01$ or $^{††}p < 0.01$, respectively.

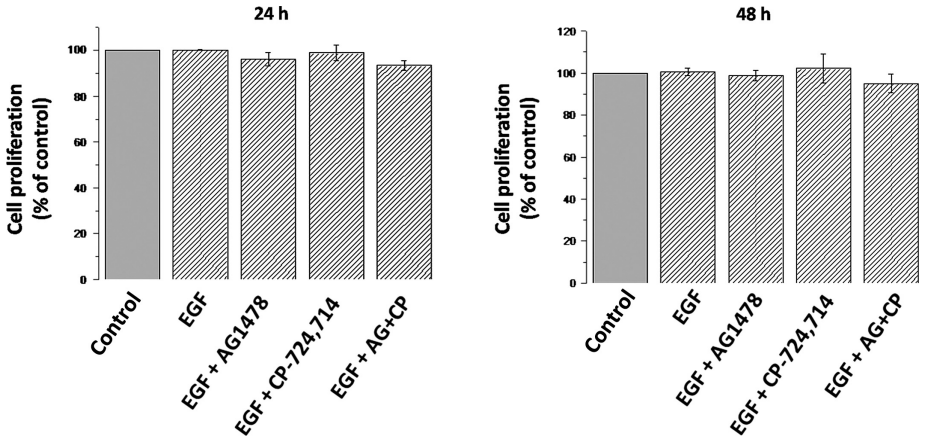

Figure 2. The effect of EGFR and/or HER2 inhibition on DLD-1 proliferation. Cells were incubated with AG1478 (1 μM) and CP-724,714 (1 μM) for 30 min, followed by the introduction of EGF (20 ng/mL). Cell proliferation was evaluated for 24 and 48 h. The results are expressed as mean ± SD of three separate experiments in triplicate.

accordance with the existence of K-Ras mutation. However, the case that the EGF/EGFR signaling axis could affect other intracellular components crucial for cell proliferation and survival could not be excluded; therefore, we are forced to evaluate the EGF effect on proteasome expression and activity.

The effect of EGF/EGFR on proteasome subunits gene expression

Taking into consideration the above data, we investigated the impact of EGF and EGF-mediated, EGFR, and/or HER2, inhibition on gene expression of the catalytic proteasome subunits. The proteasome subunits expression was assessed by end-point PCR, as described in the section on materials and methods.

It is significant that the results obtained for the expression of the three proteasome subunits exhibited a similar pattern. As shown in Figure 3, despite the presence of K-Ras mutation, EGF significantly increases the expression of β5, β1, and β2 proteasome subunits, indicating that the action of EGF overwhelms the effect of K-Ras mutation in order to induce proteasome expression. Following treatment with EGF and blocking either EGFR or both EGFR and HER2 activities by specific TKIs, the EGF-stimulatory effects were significantly reduced. Moreover, blocking of HER2 activity alone does not

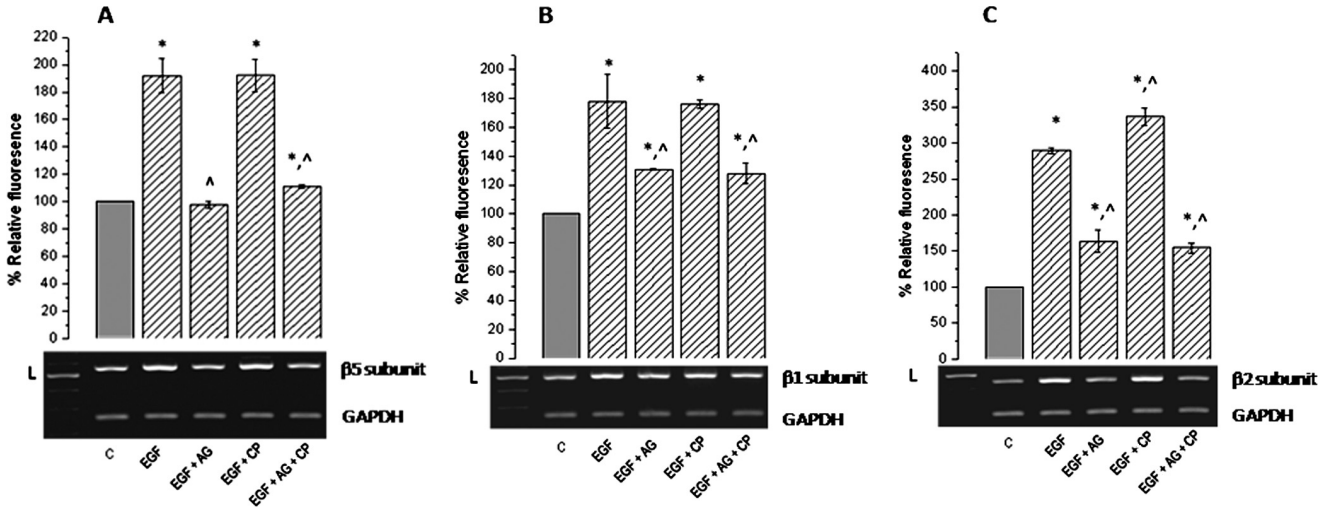

Figure 3. The effect of EGF on gene expression of (A) β5, (B) β1, and (C) β2 catalytic proteasome subunits, respectively, in DLD-1 colon cancer cell line. The mRNA levels are assessed by end-point PCR analysis. Cancer cells are incubated with AG1478 (1 μM) and CP-724,714 (1 μM) for 30min, followed with the introduction of EGF (20 ng/mL). Total incubation time was 24 h. The percentage of relative fluorescence of the gene of interest/the reference gene (GAPDH) is given in all of the diagrams.

Statistically significant differences compared with control or EGF-treated cells are symbolized with *$p < 0.05$ or ^$p < 0.05$, respectively. All treatments are given as abbreviations. C: control cells, EGF: Epidermal growth factor (20 ng/mL) AG: AG-1487 EGFR inhibitor (1 μM), CP: CP-724,714 HER2 inhibitor (1 μM).

suppress the EGF-enhanced expression of proteasome subunits. These data clearly show that, despite the presence of K-Ras mutation, EGF sensitizes its own receptor in order to significantly regulate gene expression of all three proteasome catalytic subunits. Interestingly, the expression patterns of proteasome and EGFR are very similar, indicating a possible correlation between them.

The effect of EGF/EGFR on β5 catalytic proteasome activity

Similarly to gene expression, we evaluated the impact of EGF on the activity of the β5 catalytic proteasome subunit. For that reason, we used the same specific TKIs as described before, and the total incubation time was 48 h. The activity of β5 (chymothrypsin-like) catalytic proteasome subunit was determined using the fluorogenic substrate N-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin, as described in the section of materials and methods.

Interestingly, the activity levels of β5 proteasome subunit (Figure 4) share a similar pattern with the expression levels of the subunit (Figure 3A). Particularly, EGF significantly increases the β5 proteasome subunit activity levels, despite the presence of K-Ras mutation. Moreover, after stimulation with EGF, the inhibition of HER2 activity does not change the EGF-mediated effect, whereas the blocking of EGFR activity or of both EGFR/HER2 abolished the stimulatory effect of EGF. According to the results, it is suggested that EGF significantly regulates the activity of the β5 proteasome subunit via the EGFR, without the involvement of HER2.

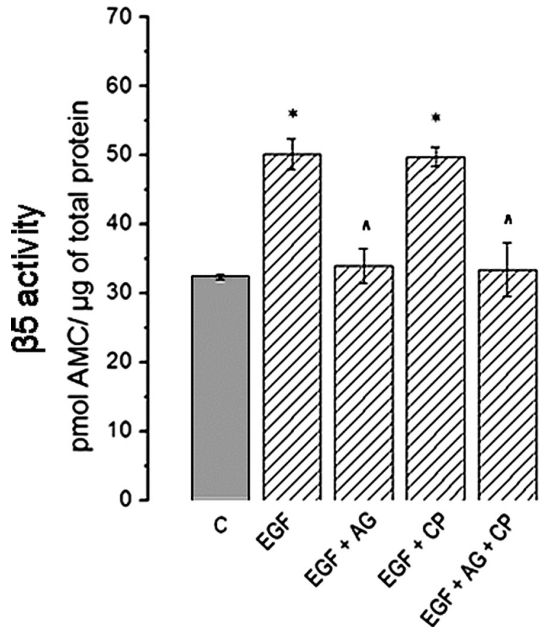

Figure 4. The effect of EGF on the activity of the β5 catalytic proteasome subunit in DLD-1 colon cancer cells. The activity levels were determined using the fluoregenic substrate SucLLVY-AMC. Colon cancer cells were first preincubated with AG1478 (1 μM) and CP-724,714 (1 μM) for 30min, followed with the introduction of EGF (20 ng/mL). Total incubation time was 48 h. In all the diagrams are presented the p mol of hydrolyzed AMC/the mg of total protein.

Statistically significant differences compared with control or EGF-treated cells are symbolized with $*p < 0.05$ or $\wedge p < 0.05$, respectively.

Activation of Nrf2 does not affect the gene expression of proteasome subunits

It is known that EGF induces the activation of Nrf2 [48] and in turn the activation of Nrf2 induces, in some cases, the proteasome expression [35]. We, therefore, examined the impact of the activation of Nrf2 on gene expression of the catalytic proteasome subunits in DLD-1 colon cancer cell lines. For this experiment Oltipraz, a synthetic activator of Nrf2, was used. Colon cancer cells were incubated with Oltipraz (30 μM) for 24 h and then the proteasome subunits gene expression was determined by end-point PCR analysis, as described in the section of materials and methods. As shown in Figure 5, Oltipraz did not exhibit any significant effect on gene expression of all three catalytic proteasome subunits, indicating that in DLD-1 colon cancer cells, the EGF mediated-induced proteasome expression is not depended on the Nrf2 activation.

DISCUSSION

Several in vivo and in vitro studies relate the importance of EGFR in colon cancer development and progression. Through its signaling pathways, EGFR is able to regulate vital cellular processes as proliferation, survival, and metastasis. Moreover, EGFR is responsible for the expression of several molecules which contribute to cancer promotion. The inhibition of EGFR results in anti-tumor effects both in vivo and in vitro. A very common coreceptor for EGFR is HER2. In colon cancer, the combined EGFR/HER2 activity inhibition enhances the anticancer effect, indicating interplay between those two receptors. Moreover, K-Ras is a very important signaling molecule downstream of EGFR and HER2, which is often mutated in colon cancer. The existence of K-Ras mutations induces carcinogenesis and demonstrates resistance against anti-EGFR therapies. The ubiquitin-proteasome is the main regulator of the nonlysosomatic degradation of a variety of intracellular proteins. Several studies highlight the significance of the proteasome in several types of cancer since proteasome inhibition induces apoptosis of cancer cells. Proteasome affects the protein levels of EGFR and its phosporylation/activation in several types of cancer, including colon [49–52]. This indicates a correlation between EGFR and proteasome. In one study, proteasome inhibition affected some signaling molecules downstream of EGFR which in turn affected Nrf2 activation, also implicating this transcription factor in the correlation between EGFR and proteasome [53]. Although it has been demonstrated that proteasome is able to modulate EGFR, it is not clear whether the reverse option is possible.

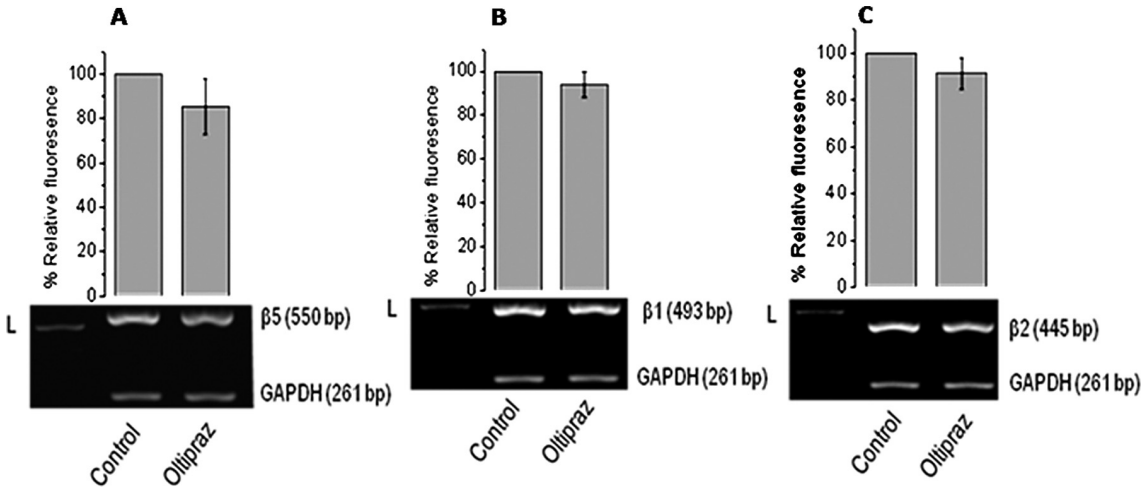

Figure 5. The effect of the activation of Nrf2 on gene expression of (A) β5, (B) β1, and (C) β2 catalytic proteasome subunits in DLD-1 colon cancer cells. The mRNA levels are assessed by end-point PCR analysis. Cancer cells are incubated with Oltipraz (30 μM) for 24 h.

Taking into consideration previous studies showing that EGF appears to regulate the survival time of *Caenorhabditis elegans* by activating proteasome [54] and that EGF increases proteasome expression through activation of EGFR in prostate cancer cells [55], our basic goal was to evaluate the effect of EGF/EGFR and second, HER2 on the expression and the

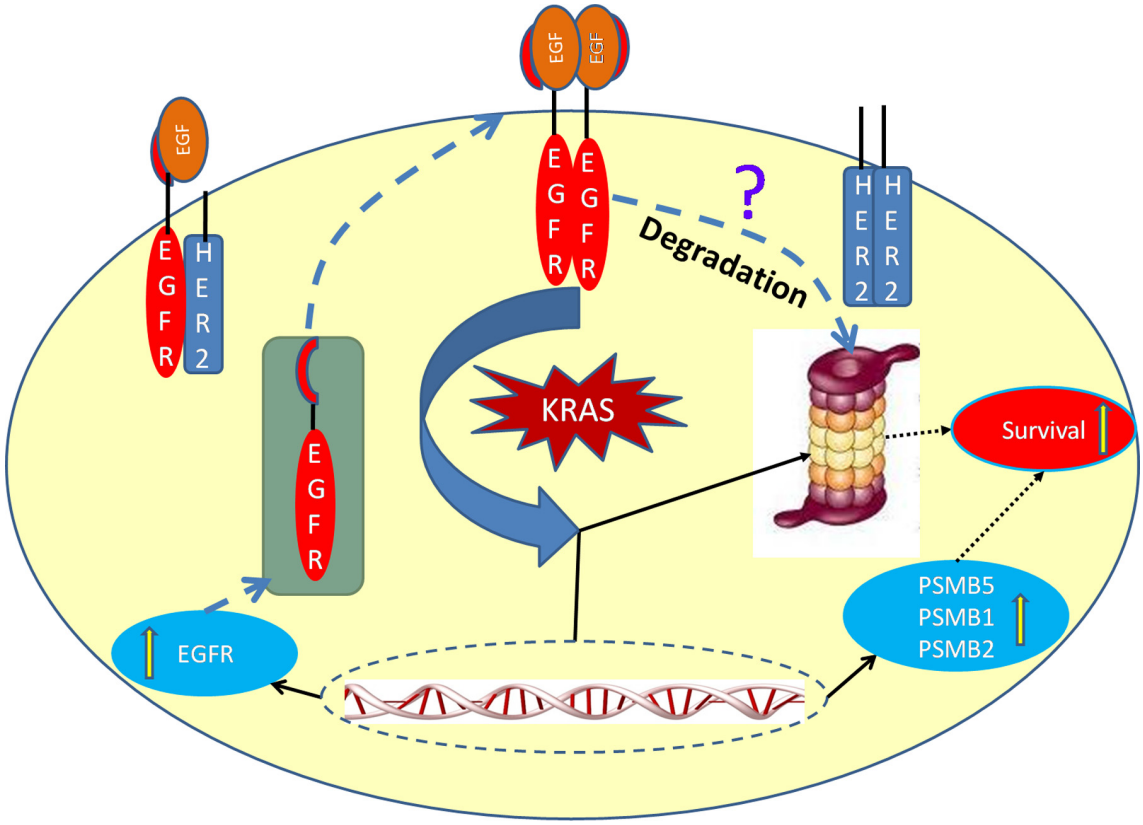

Figure 6. Schematic representation is showing that the EGF/EGFR signaling axis overwhelms the action of K-Ras mutation and significantly enhances proteasome expression and activity possibly contributing to cancer cells survival. Taking into consideration that EGF also increases the expression of EGFR, it is plausible to suggest that the EGF-mediated proteasome activation could possibly lead to enhanced EGFR degradation leading to autoregulation of EGF–EGFR pathway.

activity of the catalytic proteasome subunits. It is worth mentioning that in DLD-1 cancer cells, the introduction of exogenous EGF leads to phosphorylation of EGFR, as demonstrated previously [19, 56]. As shown in Figures 3 and 4, EGF-EGFR signaling axis strongly regulates both the proteasome expression and activity, overwhelming the action of K-Ras mutation. As previously reported [55], EGF increases proteasome expression, almost exclusively through JAK-STAT3 signaling pathway in prostate cancer cells, while the MAPK and PI3K signaling pathways do not actually involve in that process. This could be also a possible scenario in DLD-1 cells and could partly explain the fact that despite the presence of K-Ras mutation, EGF significantly regulates proteasome expression and activity.

It is known that both enhanced proteasome and EGFR activation contribute to increased tumor survival, while proteasome and EGFR inhibition results in apoptosis in several types of cancer, including colon [15, 17, 45–48]. Therefore, it is plausible to suggest that EGF enhances cancer cells survival through the activation of proteasome, whereas the inhibition of EGFR may partly causes apoptosis due to proteasome inhibition. This may explain that the combined EGFR/proteasome inhibition results in enhanced antitumor effect [41, 42, 44]. In addition, the proteasome may regulate the EGF–EGFR pathway by degradation of EGFR as previously reported [57]. According to the significance of EGFR in colon cancer, we evaluated the action of EGF on the expression of its own receptor and the proliferation of DLD-1 cells. Our data suggest that EGF/EGFR signaling axis is the main mediator for the expression of the receptor. Interestingly, the expression patterns of proteasome and EGFR were very similar. On the other hand, although EGFR is a vital receptor for colon cancer cells, the proliferation rates of DLD-1 cells were not affected by EGF/EGFR system, possibly due to existence of K-Ras mutation. It has been reported that Nrf2 activation is modulated by proteasome. However, other studies indicate that the activation of Nrf2 is able to induce proteasome expression [58–60]. In the present study, we report that the EGF-induced proteasome subunits expression is not mediated by Nrf2 activation. In summary, we report that EGF/EGFR signaling axis significantly regulates the expression and the activity of the catalytic proteasome subunits in DLD-1 colon cancer cells, overwhelming the action of K-Ras mutation. This regulation appears to be Nrf2-independent. Moreover, EGF/EGFR signaling axis regulates the EGFR expression in a similar mode that regulates proteasome, indicating an EGFR/proteasome correlation. However, EGF does not affect the proliferation of DLD-1 cells, possibly due to the enhanced proteolytic action of proteasome on EGFR. The conclusions are summarized in Figure 6. Those data provide the basis for more studies, which will declare the pathway(s) that EGFR regulates proteasome and how this could contribute to the development of more effective anticancer approaches targeting on EGFR and proteasome in colon cancer.

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COMPETING INTERESTS

The authors declare no competing interests.

PUBLISHING NOTES