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Original Articles

Overcoming cetuximab resistance in HNSCC: The role of AURKB and DUSP proteins

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ABSTRACT

Unraveling the underlying mechanisms of cetuximab resistance in head and neck squamous cell carcinoma (HNSCC) is of major importance as many tumors remain non-responsive or become resistant. Our microarray results suggest that "resistant" cells still exhibit RAS–MAPK pathway signaling contributing to drug resistance, as witnessed by low expression of *DUSP5* and *DUSP6*, negative regulators of ERK1/2, and increased expression of *AURKB*, a key regulator of mitosis. Therefore, interrupting the RAS–MAPK pathway by an ERK1/2 inhibitor (apigenin) or an AURKB inhibitor (barasertib) might be a new strategy for overcoming cetuximab resistance in HNSCC.

Introduction

Tumors originating from the squamous epithelium of the upper aerodigestive tract, including the lip, oral cavity, pharynx, larynx and paranasal sinuses are classified as head and neck squamous cell carcinoma (HNSCC) and comprise 90% of all head and neck cancers. Despite improvements in therapy for HNSCC patients, the 5-year survival rate is only 50% [1,2]. Moreover, HNSCC has a severe impact on the quality of life of patients and survivors [3]. Personalized medicine using targeted therapies – based on the molecular profile of the tumor – may contribute to the much-needed progress in HNSCC treatment.

Improvements in therapy of patients with HNSCC have evolved from the integration of epidermal growth factor receptor (EGFR) targeted therapeutics into conventional treatment regimens [4]. The promising EGFR targeting agent cetuximab is approved by the Food and Drug Administration and by the European Medicines Agency for the treatment of HNSCC patients. Cetuximab exerts antitumor activity by inhibiting cell proliferation, triggering antibody-dependent cell-mediated cytotoxicity and increasing the cytotoxic effects of chemo- and radiotherapy [5–8]. However, HNSCC tumors display remarkable heterogeneity in drug response, since only 10%–20% of patients are reported to have a favorable response to cetuximab in monotherapy [9]. Nevertheless, in combination with platinum-fluorouracil-based chemotherapy or radiotherapy, better clinical outcome was observed [10,11]. For instance, the addition of cetuximab to platinum-fluorouracil chemotherapy improved overall survival when given as first-line treatment in patients with recurrent or metastatic HNSCC [10]. However, drug response does not correlate unequivocally with EGFR expression. Therefore, the identification of biomarkers predicting drug response to cetuximab or other anti-EGFR biotherapeutics is pressing.

In other cancer types, certain mechanisms of resistance to EGFR-targeting agents, e.g. *KRAS* mutations, have already been identified [12]. However, the low prevalence of *EGFR* and *KRAS* mutations in HNSCC will likely preclude a major role in defining treatment options in these patients [13–15]. Consequently, it is becoming clear that what is relevant in one cancer type may not necessarily apply to other forms of cancer [16]. Thus, further unraveling of the underlying resistance mechanisms will undoubtedly reveal predictive

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Table 1
Schedule of administration of monotherapy.

Drug	Concentration	Incubation time	Vehicle solution
Cetuximab	0–15 nM	168 h	PBS
Panitumumab	0–15 nM	168 h	PBS
Barasertib	0–25 nM	168 h	DMSO
Apigenin	0–40 μM	168 h	DMSO

biomarkers for anti-EGFR therapy, and the design of rational combinations of targeted therapies has the promising potential to eventually overcome therapy resistance.

We conducted this study to explore cetuximab-induced transcriptional responses and diversification of signaling events downstream of the EGFR receptor, in an effort to identify genes that are likely involved in (intrinsic) resistance toward the EGFR targeting monoclonal antibody cetuximab in HNSCC cell lines. We focused on genes with aberrant expression that are related to the RAS–MAPK pathway; aurora kinase B (*AURKB*) and dual-specificity phosphatase (*DUSP*) 5 and 6. Sustained ERK1/2 signaling despite EGFR inhibition was observed in cetuximab resistant cell lines. Consequently, inhibitors of ERK and AURKB were tested for their ability to induce cytotoxicity in cetuximab resistant cell lines. Furthermore, AURKB expression was evaluated in HNSCC patients eligible for cetuximab therapy. Our findings provide new insights into mechanisms of drug resistance toward cetuximab in HNSCC and highlight the development of a rational combination therapeutic strategy.

Materials and methods

Cell lines and culture conditions

The human HNSCC cancer cell lines Cal27 and FaDu were obtained from American Type Culture Collection. SC263 was kindly provided by Prof. Dr. Sandra Nuyts (University Hospital Leuven, Leuven, Belgium) and TR146, LICR-HN1, LICR-HN2 and LICR-HN5 were kindly provided by Prof. Dr. Olivier De Wever (Laboratory of Experimental Cancer Research, Ghent University Hospital, Ghent, Belgium). All HNSCC cell lines were grown as monolayers in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum, 2 mM glutamine and 1% penicillin/streptomycin. All media and supplements were obtained from Life Technologies (Merelbeke, Belgium). Cultures were maintained in exponential growth in a humidified 5% CO_2/95% air atmosphere at 37 °C. Cells were confirmed free of mycoplasma infection through regular testing (MycoAlertTM, Plus Mycoplasma detection kit, Lonza, Verviers, Belgium).

Pharmaceuticals

The reagents, cetuximab (anti-EGFR monoclonal antibody, Merck, Darmstadt, Germany) and panitumumab (anti-EGFR monoclonal antibody, Amgen, Brussels, Belgium), were diluted in sterile PBS. Barasertib (aurora kinase B inhibitor, Selleck Chemicals, Houston, USA) and apigenin (ERK inhibitor, Biaffin GmbH & Co KG, Kassel, Germany) were diluted in DMSO (Merck).

Growth inhibition experiments

Cytotoxicity studies were performed in 48 well plates after determination of the optimal seeding densities for each cell line, to ensure exponential growth for the entire duration of the assay. Cells were counted automatically with a Scepter 2.0 device (Merck Millipore SA/NV, Overijse, Belgium). After an incubation period of 24 hours, cells were treated with the drug of interest in monotherapy (Table 1), or in combination therapy (Table 2). After incubation, cell survival was determined by the sulforhodamine B assay (SRB), as previously described [17]. All experiments were performed in triplicate and each concentration was tested six times within the same experiment.

The IC_{50} value, representing the concentration of a drug that is required for 50% growth inhibition as compared to cells grown in the vehicle-control medium, was calculated using the WinNonlin software (Pharsight, Mountain View, USA), "pharmacodynamic model: inhibitory effect sigmoid Emax model".

EGFRvIII mutation detection

RNA was isolated using the TRIzol-based method (Invitrogen, Merelbeke, Belgium). Concentration and purity were measured by Nanodrop ND-1000 spectrophotometry (Isogen, Sint-Pieters-Leeuw, Belgium). The RNA quality was assessed by capillary electrophoresis with an Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands) using Agilent RNA 6000 Series Nano kits (Agilent Technologies). Next, after treatment with DNase (Invitrogen) following the manufacturer's protocol, isolated RNA was reverse transcribed using SuperScript® II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Primers were designed to flank exon 2 and exon 7 of the *EGFR* cDNA, forward primer 5′-GGAGCAGCGATGCGACCCTC-3′ and reverse primer 5′-ACACTTGCGGACGCCGTCTT-3′. The reaction mixture consisted of 1× PCR buffer, 2% DMSO, 50 μM of each primer, 200 μM dNTPs, 1u Taq DNA polymerase II (DyNAzyme, Finnzymes, Espoo, Finland), 20 ng cDNA and water to a total volume of 25 μl. PCR cycling was performed on a PX2 Thermal Cycler PCR (Thermo Electron Corporation). The PCR was run according to the following conditions: one cycle of 95 °C for 15 minutes and 35 cycles in the following sequence: 94 °C for 1 minute, 68 °C for 1 minute and 72 °C for 1 minute, followed by one cycle of 92 °C for 10 minutes. The results were visualized on a 2% agarose gel. The length of the PCR product was dependent on the mutation status. The wild type sequence produced a fragment length of 987 bp and the mutant form (*EGFRvIII*) generated a fragment of 187 bp. RNA from the U87del cell line (EGFRvIII overexpressing U87, a generous gift from Dr. Furnari) was used as a positive control.

KRAS mutation detection

DNA isolation was performed using the GenEluteTM Blood Genomic DNA kit (Sigma Aldrich, Bornem, Belgium) and was stored at –20 °C. Concentration and purity of the DNA were measured by Nanodrop ND-1000 spectrophotometer (Isogen, Temse, Belgium). The cell lines were screened for mutations in codon 12 and 13 of the *KRAS* gene using high-resolution melting analysis (HRMA) technique, as described previously [18].

HPV infection

The cell lines were screened for the presence of HPV DNA, as described previously [19].

xCELLigence real-time cell analysis (RTCA) and genome-wide gene expression

Prior to microarray experiments, the optimal incubation period at which a clear effect of 15 nM cetuximab treatment on SC263 cell growth could be observed, was determined using the xCELLigence RTCA DP system (Roche Diagnostics GmbH, Mannheim, Germany). Thereafter, cetuximab sensitive and resistant cell lines were treated with 15 nM cetuximab for the predefined incubation period. RNA was isolated according to the TRIzol method, as described above, and used for microarray experiments. In order to maintain minimal experimental variability, all cell lines were grown and treated in identical culture conditions.

For microarray experiments, RNA samples were amplified using the Illumina Totalprep RNA Amplification kit (Ambion, Austin, TX, USA). In short, mRNA fractions were converted to double stranded-cDNA. A subsequent *in vitro* transcription reaction produced cRNA strands with incorporated biotin-UTP nucleotides. 750 ng of the resulting cRNA was hybridized to an Illumina human HT12v4 beadchip (Illumina, San Diego, CA, USA). For every biological state, three independent biological replicates were loaded on a chip. After overnight sample hybridization at 58 °C, subsequent washing steps and sample labeling with a streptavidin-Cy3 dye (Amersham, Buckinghamshire, England), intensity values were read out on an Illumina

Table 2
Administration schedule of combination therapy in cetuximab resistant cell lines; simultaneous (sim) and sequential (seq) treatment schedules.

Drug 1	Concentration	Incubation	Drug 2	Concentration	Incubation	Schedule
Barasertib	10 or 20 nM	24 or 48 h	Cetuximab	15 nM	120 or 144 h	seq
Cetuximab	15 nM	24 or 48 h	Barasertib	10 or 20 nM	120 or 144 h	seq
Cetuximab	15 nM	168 h	Barasertib	10 or 20 nM	168 h	sim
Apigenin	17.5 or 24 μM	24 or 48 h	Cetuximab	15 nM	120 or 144 h	seq
Cetuximab	15 nM	24 or 48 h	Apigenin	17.5 or 24 μM	120 or 144 h	seq
Cetuximab	15 nM	168 h	Apigenin	17.5 or 24 μM	168 h	sim