Erlotinib (Tarceva®) inhibits oral cavity carcinoma and synergizes with cisplatin and ionizing radiation *in vitro*

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Epidermal growth factor (EGF) plays important roles in the growth and development of normal epidermis and numerous malignant human tumors. EGF is a secreted protein and binds to a heterodimeric receptor which possesses an intrinsic tyrosine kinase activity. EGF receptor (EGFR) kinase inhibitor has been shown to specifically inhibit the tumor growth of lung cancer, head and neck cancer, colon cancer and others. The cytotoxic effect of the newly developed tyrosine kinase inhibitor erlotinib or OSI-774 (Tarceva®, OSI Pharmaceuticals) on the oral cavity cancer in cell culture condition was investigated. Tarceva showed a dose-dependent inhibition of the growth of squamous carcinoma of the tongue (SCC-015) in cytotoxic assays. The inhibition of the SCC-015 cells by Tarceva appeared to be within the physiologically achievable concentrations (low µm concentrations). The effect of Tarceva on SCC-15 cells appears to be intra-S phase inhibition as well as an inhibition of G2/M transition of the cell cycle by flow cytometry study. Furthermore, Tarceva can synergistically inhibit the SCC-15 cell growth with cisplatin and radiation. It has also been shown by this group that approximately 56% of the head and neck squamous cell carcinoma tumor specimens express EGFR by tissue microarray study in combination with immunohistochemical staining and automated imaging analyses. These results represent promising preliminary data for further clinical trials of this kind of drug as adjuvant therapy for squamous carcinoma of the oral cavity.

Keywords:
cisplatin, cytotoxicity, EGFR inhibitor, head and neck squamous cell carcinoma, SCC-015 cells, Tarceva

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Head and neck squamous cell carcinoma (HNSCC) accounts for 2–4% of all malignancies in the USA. Chemo- and radiotherapy is the standard treatment option for uncontrollable or metastatic diseases [1]. Increasing understanding of the molecular mechanisms of tumor growth and proliferation makes it possible to introduce new targeted therapy alone or in combination with standard chemotherapy and radiation for patients to achieve better treatment outcomes [1]. Two types of molecular targets have been successfully used for the treatment of a variety of human malignancies, including leukemia, lymphoma and other solid tumors. First, is to use monoclonal antibodies (mAbs) against the cell surface molecules important for cell growth and tumor survival, and such examples are best demonstrated by trastuzumab (Herceptin®, Roche) for breast cancer and rituximab (Rituxan®, Genetech Inc.) for lymphoma. The secon approach is to use small organic compounds to target the intracellular tyrosine kinases important for cell growth and such examples are best illustrated by imatinib (Gleevec®, Novartis) for chronic myelogenous leukemia and gefitinib (Iressa®, AstraZeneca) for non-small cell lung cancer. Many related drugs based on similar principles are at various stages of clinical trials for many types of human malignancies. One of the most important molecular targets, the epidermal growth factor receptor (EGFR), is a transmembrane receptor that plays a key role in epithelial cell growth [2–4]. Binding of ligand, epidermal growth factor (EGF), to EGFR activates a cascade of tyrosine kinase signaling pathways that transduce the proliferating signal from the cell membrane to the nucleus [3,4]. In addition, EGFR-mediated signaling is also associated with other malignant phenotypes, such as tumor cell invasion, metastasis and upregulation of angiogenic factors [2,5]. EGFR overexpression appears to play a vital role in the development of solid tumors, such as HNSCC and lung cancer [6,7]. Down-modulation of EGFR or its activity has been shown to be effective in inhibiting tumor cell proliferation as well as the production of angiogenic factors, thereby suppressing tumor growth [8–10].

Erlotinib or OSI-774 (Tarceva®, OSI Pharmaceuticals) is a synthetic compound that specifically inhibits the tyrosine kinase activity of the EGFR [11]. It competitively binds to the ATP-binding site at the catalytic domain of the EGFR [12]. Similar to Iressa, Tarceva was

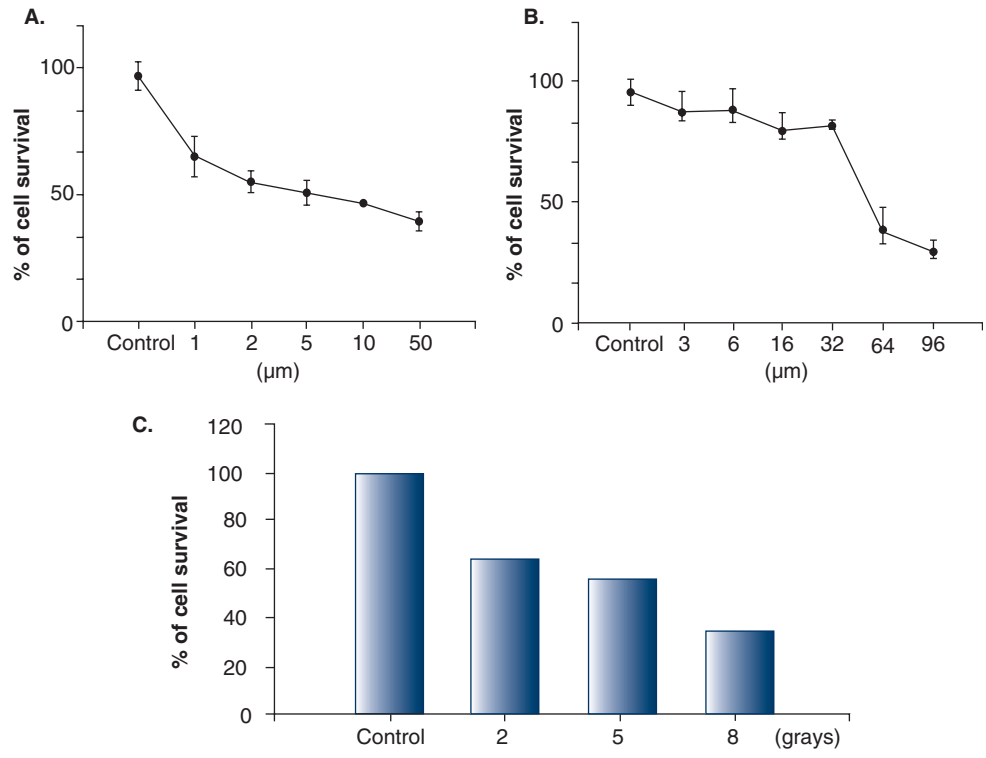
developed to treat non-small cell lung cancer and is currently in the Phase II/III clinical trials for advanced lung cancer [13]. Since EGFR is the key regulator of HNSCC growth and Tarceva was shown to be a promising agent for blockade of EGFR signaling, we were interested in the potential role of Tarceva as either a single agent or an adjuvant therapeutic agent for HNSCC, and sought to investigate the cytotoxic effect of the drug on the growth of HNSCC cell line SCC-015 under cell culture condition. We report here that Tarceva can effectively inhibit the growth of the HNSCC cell line in culture and can synergize with cisplatin and radiation to inhibit the growth of HNSCC. Furthermore, we have also demonstrated that the HNSCC tumor tissues express abundant EGFR protein by tissue microarray and immunohistochemical staining.

Materials & methods

Cell culture & MTT cytotoxicity assays

The human oral cavity squamous carcinoma cell line SCC-015 was obtained from American Type Culture Collection (ATCC). The SCC-015 cell line was maintained at 37°C under 5% CO_2 in monolayer culture in methoxyethoxymethyl (MEM) supplemented with 10% fetal bovine serum (Gibco BRL) and 1% penicillin–streptomycin. All experiments were performed under these conditions. MTT assay is based upon the cleavage of the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide) to purple formazan crystals by metabolically active cells. This group have previously demonstrated that this method is accurate in measuring the number of viable cells in culture [14]. The tested concentration of Tarceva ranged from 1 to 50 µM. The SCC-015 cells were seeded into a 96-well culture plate at a cell density of 2 × 104 and maintained for culture for 24 h before Tarceva was added to the medium for 48 h. After treatment, 10 µl of MTT labeling reagent was added to each well and plates were incubated at 37°C for 4 h. Following MTT incubation, the cultures were solubilized and the spectrophotometric absorbance of the samples detected using a microtiter plate reader. The wavelength to measure absorbance of formazan product is 570 nm, with a reference of

Figure 1. Dose-dependent inhibition of SCC-015 cells in culture.



A. The effect of the increasing concentrations of Tarceva on SCC-015. **B.** The effect of cisplatin on SCC-015 cells. **C.** The effect of γ-radiation on SCC-015 cells.

750 nm. Each concentration of Tarceva was tested in quadruplet wells and each experiment was repeated three times. A representative experiment data was shown.

Flow cytometry for cell cycle study

The SCC-015 cells were cultured in the six-well plates and treated with Tarceva at a concentration of 5 μM for 6 h. The cells were harvested by trypsin and fixed with 70% ethanol. Propidium iodine (PI) was added at a concentration of 10 μg/ml for 30 min before being analyzed using a FACSCaliber instrument (Becton–Dickinson) as previously described. For each sample, 20,000 events were analyzed and the data were plotted with Cell Quest software (Becton–Dickinson).

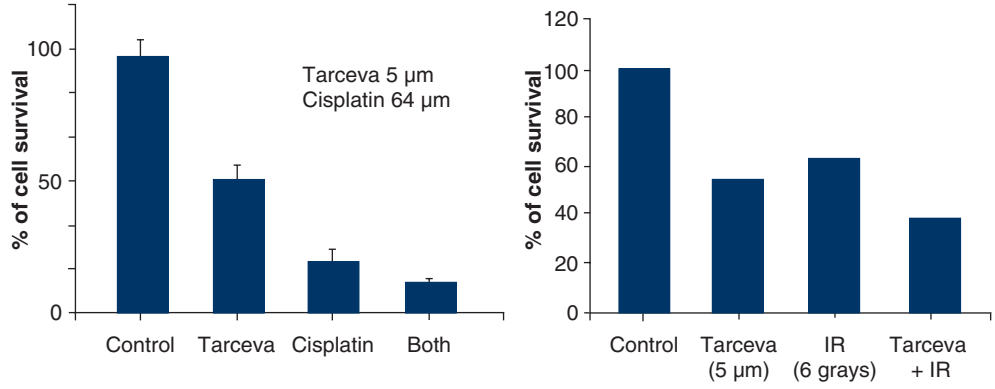
Western blot analysis

The SCC-015 cells were cultured in the six-well plate under the normal condition and treated with Tarceva (1 and 2 μM) or cisplatin (33 or 66 μM) for 24 h. The cells were lysed in the lysis buffer containing 50 μM Tris (pH 7.4), 150 mM NaCl, 0.1% Triton® X-100 (Hach Co.), 0.1% Nonidet P-40, 4 μM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT). The phenylmethylsulfonyl fluoride of 50 μg/mL and the protease inhibitor cocktail containing antipain of 10 μg/mL, leupeptin of 10 μg/mL, pepstatin A of 10 μg/mL and chymostatin of 10 μg/mL were also added to the cell lysates. The protein concentrations of the lysates were determined by the Bradford assay (Bio-Rad) and in total 50 μg of the proteins were separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to the nitrocellulose membrane (Biorad) and incubated with the anticell cycle checkpoint kinase (CHK)2 antibody. CHK2 protein was visualized by horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescence detection (Pierce).

Immunofluorescent microscopy & quantification of IR-induced apoptosis

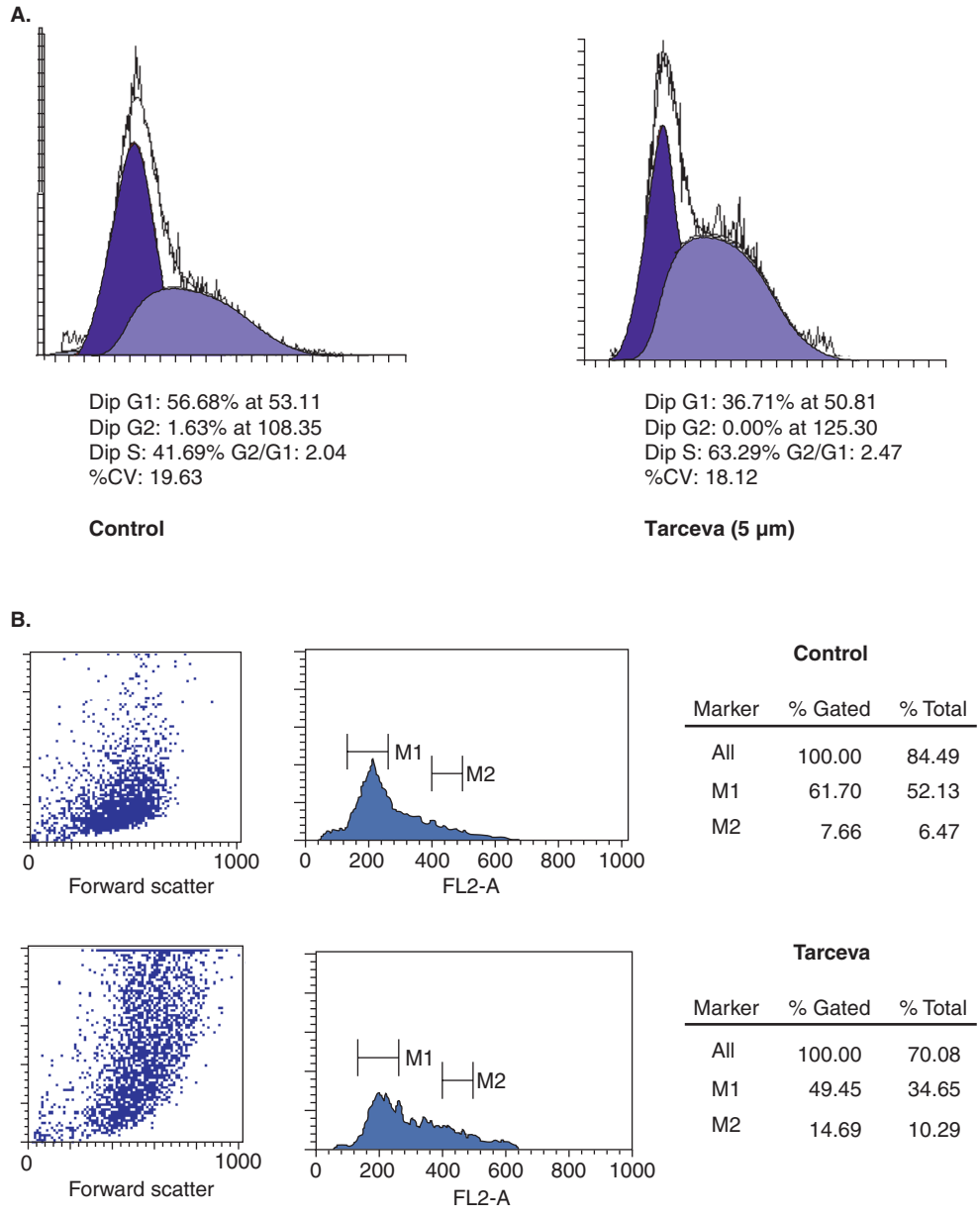
The γ-radiator for ionizing radiation (IR) can only accommodate small 100 mm culture dishes and the 96 cell plates cannot be directly irradiated. Therefore, immunofluorescent microscopy was used to quantify the apoptosis before and after radiation. The immunofluorescent staining procedure was as described [14]. Briefly, the SCC-015 cells were cultured on glass coverslips, treated with or without Tarceva (5 μM) for 48 h, fixed in 4% paraformaldehyde in phosphate buffer solution (PBS) and permeabilized with 0.3% Triton X-100 in PBS. Coverslips were stained with diamidinophenylindole (DAPI) for 30 min. The image was analyzed by a Zeiss fluorescent microscope at the imaging facility of West Virginia University Department of Anatomy. The apoptotic nuclei were counted and represented as percentage of all nuclei per 500 cells. The results were expressed as an average percentage of apoptotic nuclei per 500 cells on three coverslips.

Figure 2. Synergy between Tarceva and cisplatin and Tarceva and α-radiation.



The SCC-015 cells were cultured on glass coverslips and treated with Tarceva alone or with cisplatin or radiation. The cells were fixed, stained with DAPI and visualized under UV microscope. The apoptotic nuclei were counted in a total of 500 cells. The percentage of cells shown was the nonapoptotic cells (survival).
DAPI: Diamidinophenylindole; IR: Ionizing radiation; UV: Ultraviolet.

Figure. 3 Flow cytometry study of Tarceva effects on SCC-015 cells.



The SCC-015 cells were cultured in the six-well plates, treated with Tarceva, harvested by trypsinization, stained with propridium iodine and analyzed by FACS Analyzer. Increased populations at the S phase and the G2 phase were shown.

Tissue microarray, immunohistochemical staining & automated image analysis

Immunohistochemical staining was used to detect the presence of EGFR in the HNSCC tumor specimens. The HNSCC tumor tissue array slides were obtained from Clinomics Biosciences, Inc. (MA, USA). A classic immunohistochemical staining method was used to detect the EGFR expression. The HNSCC tissue microarray slides consisted of 29 HNSCC tumors and eight matched normal control tissues. The slides were sectioned at 5 µM in thickness, deparaffinized in xylene, dehydrated, rehydrated in a series of 100, 90 and 70% ethanol solution and stained with the EGFR antibody (Santa Cruz Biotechnologies) in the

Table 1. Expression of EGFR in human head and neck cancer.

Cancer type	% positive	Intensity	Total scores
Squamous carcinoma (n = 29)	3	3	9 (n = 4) (14%)
	3	2	6 (n = 12) (41%)
	3	1	3 (n = 7) (23%)
	2	1	2 (n = 2) (7%)
	0	0	0 (n = 4) (14%)
Match normal (n = 8)	3	3	9 (n = 0) (0%)
	3	2	6 (n = 4) (50%)
	3	1	3 (n = 2) (25%)
	0	0	0 (n = 2) (25%)

Ventana Benchmark II Autostainer (Ventana Medical International, Inc.).

Automated imaging analyses were performed using the densitometric quantitation by Quantimet Q570 computerized imaging/microscopy system (Leica, IL, USA). The scoring method was essentially as described [15,16]. Briefly, the total areas of the microcore of the tumors or normal control tissues were scanned digitally under the microscope and acquired by the software within the imaging/microscopy system. The stained cells for EGFR were quantified as the following: scores of 0–3 were assigned according to the percentage of the positive tumor cells (0 = 0%, 1 = < 25%, 2 = 25–50%, 3 = > 50%) and the staining intensity of the tumor cells (0 = 0, 1 = 1+, 2 = 3+, 3 = 3+). The two scores were multiplied to give an overall score of 0–9, of which 0 is considered negative, 1–2 weak, 3–6 moderate and 9 strong staining [16]. The imaging analysis has been described and used for Her-2/Neu expression in breast cancer patients and prostate stem cell antigen expression in the prostate cancer patients [15,16].

Results

Tarceva inhibited the growth of SCC-015 in a dose-dependent manner

The SCC-015 cells were cultured under the normal condition. Increasing concentrations of Tarceva in the culture media induced significant growth inhibition of the SCC-015 cells in a dose-dependent manner (**Figure 1A**). Tarceva concentration that inhibited 50% of the growth of SCC-015 cells was around 5 μM (IC_{50}). Cisplatin can also inhibit SCC-015 cell growth in a dose-dependent manner but the IC_{50} was between 32 to 64 μM (**Figure 1B**). Ionizing α-radiation can also induce inhibition of the growth of SCC-015 cells and the inhibitory concentration of 50% (IC_{50}) was approximately 5 gray (**Figure 1C**). These results indicate that Tarceva can effectively inhibit the growth of SCC-015 cells under the cell culture condition.

Tarceva synergized with cisplatin in SCC-015 growth inhibition

Next we investigated whether Tarceva can synergize with either cisplatin or ionizing radiation. IC_{50} concentrations were chosen for both Tarceva and cisplatin. SCC-015 cells were treated with both Tarceva (5 μM) and cisplatin (64 μM) (**Figure 2A**). The combination of Tarceva and cisplatin showed a significantly greater reduction in the survival of the SCC-015 cells than any agent alone. Similarly, a combination of Tarceva and radiation demonstrated a greater decrease in tumor cell survival than the single agent alone. These results indicate that Tarceva can potentiate the cytotoxic effects of cisplatin or α-radiation.

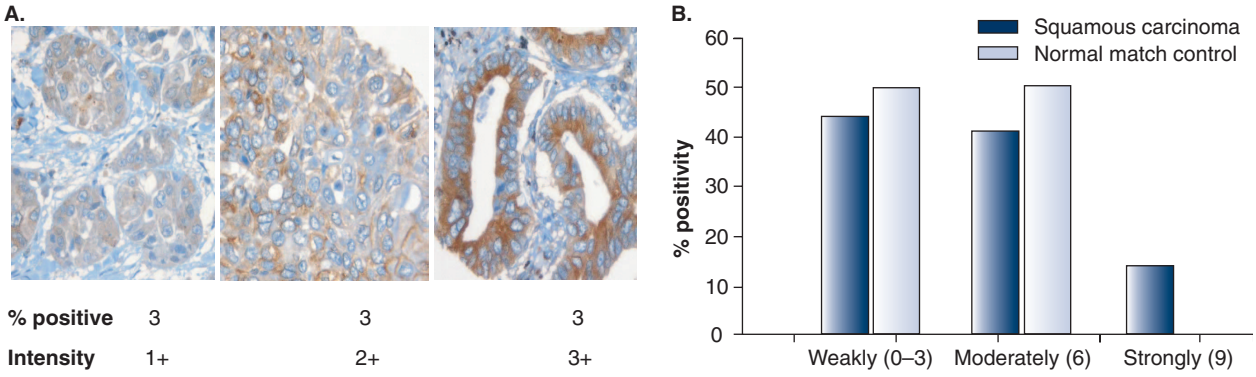
Tarceva inhibited the SCC-015 growth in the S phase

The potential mechanism of Tarceva's effects on SCC-015 cells was investigated. As a specific inhibitor of EGFR, an inhibition of the tumor cell growth was expected. Flow cytometry studies demonstrated that the treatment of SCC-015 cells by Tarceva for 6 h increased accumulation of the tumor cells in both the S and the G2 phase of the cell cycle, suggesting that the effect of Tarceva is at the intra-S phase as well as G2/M transition (**Figure 3A & 3B**). This result is consistent with the role of EGF in DNA synthesis and cell proliferation.

HNSCC tumor tissues expressed more EGFR compared with normal tissues

The expression of EGFR on the human HNSCC tumor tissues was assessed. The conventional immunohistochemical staining method was used in the HNSCC tumor tissue microarray combined with automated imaging analyses. EGFR is a membrane receptor and the nuclear stains were

Figure 4. Immunohistochemical staining profiles on HNSCC tumor tissues.



The tissue microarray slide contains a total of 29 head and neck squamous cell carcinoma tumor tissues and eight matched normal tissues were stained with epidermal growth factor receptor antibody using a conventional immunoperoxidase method. The stained slides were analyzed by the utomated imaging system. The scoring system was used as described. **A.** Representative example of the automated scoring method. **B.** The percentage of positive cases in each category, weakly stained, moderately stained and strongly stained. The table summarized the staining results. The percentage of the positive staining cases was used for the graphic representation.

considered to be nonspecific. As shown in **Table 1**, more than 56% of the human HNSCC expressed EGFR moderately or strongly (total scores 6 or 9), 22% expressed weakly (total score 3) and 2% expressed no detectable level of EGFR (total score < 3). The example of the automated scoring system for the tumors is illustrated in **Figure 4A**. In eight matched normal squamous mucosa tissues, 50% expressed EGFR moderately (total score 6), 25% weakly (total score 3) and 25% had no detectable level (total score < 3). These data indicate that EGFR expression is commonly seen in the HNSCC tumor tissue and EGFR inhibitors, such as Tarceva, may have a role in the treatment of the uncontrollable or metastatic HNSCC.

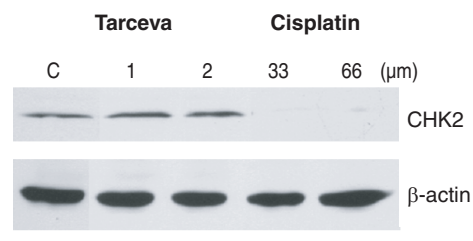
Cisplatin, but not Tarceva, down-regulates CHK2 kinase expression

Since the effect of Tarceva on SCC-015 cells was intra-S phase as well as G2/M transition, we sought to determine if CHK2, a key regulator of the G2/M checkpoint, was affected by Tarceva or cisplatin treatment. As shown in **Figure 5**, there was no change in CHK2 expression in response to Tarceva. However, CHK2 protein expression was completely inhibited to an undetectable level in response to cisplatin. The molecular mechanism of CHK2 inhibition by cisplatin is yet to be established.

Discussion

We have shown that Tarceva, a specific EGFR inhibitor, can inhibit the growth of the HNSCC in cell culture conditions. The IC_{50} of Tarceva appears to be in the low µm range. We have also shown that Tarceva can synergize with cisplatin or α-radiation, two commonly used anticancer modalities. These results are consistent with those described previously [17]. The effect of Tarceva on HNSCC cells appears to be in intra-S phase and G2/M transition of the cell cycle. These results are consistent with the known effect of EGF that stimulates cell growth and DNA synthesis [4]. We have also shown that a high percentage of human HNSCC tumor tissues (56%) express moderate or high levels of EGFR by tissue microarray analyses. The level of EGFR expression in the HNSCC tumor tissues appears to correlate with a poor prognosis and poor survival [7]. These data provide

Figure 5. Immunoblot of CHK2 kinase expression in response to Tarceva and cisplatin.



Western blot was performed as described and the primary antibody was against CHK2 kinase. Control α-actin was used to normalize the amount of proteins in each lane.

an *in vitro* cell culture basis for further clinical investigation of the role of Tarceva as a single agent or in combination with other conventional anticancer options in the treatment of the advanced stage of HNSCC.

We have also found that the effect of the combination of cisplatin with Tarceva on SCC-015 cell death was more significant than any single agent alone. This combined effect appears to be a synergy between the two agents. Such synergistic effect of Iressa with cisplatin was observed when Iressa was used for a variety of tumor cell lines [18]. Interestingly, the synergy between Iressa and the other antitumor agents appeared to be irrelevant to the tumor's EGFR expression status [18]. We have detected the EGFR expression in our SCC-015 cells by immunofluorescent staining (not shown). How Tarceva or Iressa works on the EGFR-negative tumor cells is unclear at the present.

Highlights

- Tarceva®, a specific epidermal growth factor receptor (EGFR) inhibitor designed for non-small cell lung cancer, can inhibit the growth of oral cavity cancer cell line SCC-015 *in vitro*.
- Tarceva inhibits SCC-015 cells in a dose-dependent manner by cytotoxicity study.
- Tarceva inhibits the SCC-015 cell growth in the S phase by flow cytometry study.
- Tarceva can potentiate the effect of cisplatin in cell killing.
- Human head and neck squamous cell carcinoma frequently expresses EGFR by immunohistochemistry.

Conclusion

Tarceva is effective in the inhibition of HNSCC in the *in vitro* cell culture model. These results represent promising preliminary data for further clinical trials of this type of drug as a single agent or adjuvant therapy for the treatment of patients with advanced HNSCC.

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