

The Impact of Timing of EGFR and IGF-1R Inhibition for Sensitizing Head and Neck Cancer to Radiation

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Abstract. *Background:* Targeting the epidermal growth factor receptor (EGFR) improved radiotherapy outcome by 10-15% in head and neck tumors (HNSCC). We tested the therapeutic benefits of co-targeting EGFR and insulin-like growth factor-1 receptor (IGF-1R) to further enhance tumor response to radiation. *Materials and Methods:* Mice bearing FaDu tumor xenografts were treated with ganitumab (previously known as AMG479, an anti-IGF-1R antibody), panitumumab (an anti-EGFR antibody), or both in combination with fractionated doses of radiation. Tumor growth delay and tumor cure/recurrence served as end-points. *Results:* The best tumor growth delay was achieved when ganitumab and panitumumab were given concurrently with radiation. Tumor cure/recurrence studies showed that combining ganitumab, panitumumab and radiation resulted in significantly higher radiocurability rates than use of either of the agents given with radiation. *Conclusion:* These findings provide the rationale for clinical testing of the combination of ganitumab and panitumumab for the treatment of HNSCC.

Recent reports show that cross-talk exists between epidermal growth factor receptor (EGFR), and other EGFR family members, with the insulin-like growth factor receptor (IGF-1R) pathway (1, 2). The EGFR family of receptors and IGF-1R contribute to tumor development and progression through their effects on cell proliferation, inhibition of apoptosis, angiogenesis, anchorage-independent growth and tumor-associated inflammation (2-4). Overexpression of EGFR has

been reported for a number of epithelial malignancies, including squamous cell carcinoma of the head and neck (HNSCC), non-small cell lung cancer (NSCLC), breast cancer, and ovarian cancer (5, 6). Activation of EGFR has an inverse relationship with prognosis in HNSCC (6). An inverse relationship between the expression levels of EGFR and response to radiation was also reported by our group for mouse tumor model systems (7). A series of studies on the radiosensitizing effects of the human-mouse chimeric monoclonal antibody (mAb) cetuximab, an anti-EGFR antibody, established the capacity of EGFR inhibition to enhance tumor response to radiation, both *in vitro* and *in vivo* (7, 8). A key phase III trial showed that adding cetuximab to radiation for the treatment of locally advanced HNSCC (9) improved locoregional control and overall survival by 10-15%, which is similar to the benefit of radiation with concurrent cisplatin, but with no increase in radiation-related toxicity. This trial result led to US Food and Drug Administration approval of cetuximab for use in conjunction with radiation for the treatment of patients with locoregionally advanced HNSCC. However, the need for further improvement and targeting of the IGF-1R pathway has emerged as an appealing approach.

Many neoplasms express high levels of IGF-1R, associated with tumor resistance to therapy making it a potential target (10-12). Over the past decade, several anti-IGF-1R agents have been developed. Among them are anti-IGF-1R antibodies and small-molecule kinase inhibitors (13). Ganitumab, developed by AMGEN Inc. is a monoclonal antibody (IgG1) directed to IGF-1R. Ganitumab binds IGF-1R and inhibits binding of IGF (14). The antibody does not cross-react with the insulin receptor (INSR) and it does not interfere with insulin binding to INSR homodimers *in vitro*. Ganitumab exhibited antitumor activity as a single agent in many tumor models and had at least an additive effect when combined with chemotherapeutic agents (14, 15). Combination of IGF-1R inhibitors with EGFR antagonists resulted in enhanced tumor response to therapy (16). However, there is no report on the radiation-sensitizing

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effects of ganitumab alone or combined with EGFR antagonists. Building on our previous EGFR work, we investigated the effects of targeting IGF-1R by ganitumab alone or with inhibition of EGFR, on tumor radiation response in FaDu (a HNSCC) tumor xenografts grown in mice. We used panitumumab, an anti-EGFR IgG2 monoclonal antibody that has been demonstrated to bind EGFR, prevent binding of EGF to the receptor, and inhibit receptor auto-phosphorylation (17). Pre-clinical studies indicate that panitumumab can improve HNSCC radiation sensitivity by suppressing EGFR activation induced by radiation (18).

Materials and Methods

Tumor cell line. FaDu cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in minimum essential medium (MEM), supplemented with 1% nonessential amino acids, 1% sodium pyruvate, 10% fetal calf serum and 10,000 U/ml of penicillin-streptomycin at 37°C under 95% CO₂. FaDu cell suspensions were prepared from cells propagated as monolayers *in vitro*.

Animals. Nude (Swiss nu-nu/Ncr) male mice were used in these studies. Animals were bred and maintained in our specific pathogen-free facility, approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), and in accordance with current regulations of the United States Department of Agriculture and Department of Health and Human Services. Mice were 3-4 months of age and weighed about 30 g at the start of the experiments. The experimental protocol was approved by, and was in accordance with, institutional guidelines established by the Institutional Animal Care and Use Committee.

Tumor growth delay (TGD) assay. Solitary tumors were generated by inoculating 1×10⁶ FaDu cells into the right hind leg of mice. When tumors grew to ~6 mm diameter (designated as day 0), mice were randomly assigned to treatment groups of 8 mice. Control mice received no treatment. For dose-response study with ganitumab, three dosages (*i.p.* 30, 100, or 300 µg/mouse/dose) were given six times at 3-day intervals from day 0-15. For the optimal timing study, 300 µg/mouse/dose of ganitumab was given three or six times at 3-day intervals. For combination studies, 300 µg/mouse/dose of ganitumab was given six times either concurrently with panitumumab or sequentially and panitumumab was given *i.p.* at 0.5 mg/mouse/dose (six times at 3-day intervals). Local tumor irradiation was delivered at a dose rate of 5 Gy/min using a small animal ¹³⁷Cs irradiator, designed so that the tumor in the legs of unanesthetized mice was centered within a 3-cm diameter field and the animal's body shielded. Twelve fractions of 2 Gy were given as twice daily treatments for a total dose of 24 Gy in 6 days with the first dose delivered 4 days after tumors reached 6 mm; thus, radiation was given from day 4 to 9. In the combined therapy groups, ganitumab or panitumumab was given 6 h before the second fraction of radiation of that day. For sequential administrations of ganitumab and panitumumab, two regimens were followed: (i) ganitumab was given from day 0-15 and panitumumab was given from day 10-25; (ii) panitumumab was given from day 0-15 and ganitumab was given from day 10-25. In both regimens, fractionated doses of radiation were given from day 4-9.

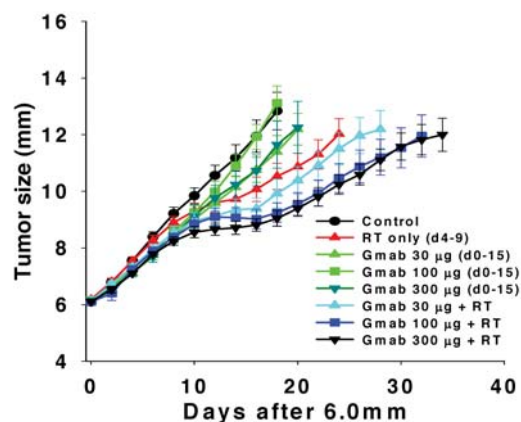


Figure 1. Effect of ganitumab (Gmab) doses (30, 100 or 300 µg/dose, given six times) on the FaDu tumor response to fractionated doses of radiation. Tumor growth delay assay. Mice bearing 6-mm tumors in the right hind leg were exposed to various treatments: ganitumab treatment was initiated on day 0 (6-mm tumor) and given six times at 3-day intervals. Fractionated doses of radiation were initiated 4 days after the first dose of ganitumab in the combination group, or 4 days after tumors reached 6-mm in radiation-only group. RT: Radiation; d: day. Each data point represents the mean size of 8-10 tumors±SE.

In order to obtain tumor growth curves, three mutually orthogonal diameters of tumors were measured 2-3 times/week with a Vernier caliper, mean values were calculated and tumor growth delay (TGD) plots were generated using average tumor diameters. Mice were euthanized when tumors grew to 14 mm diameter. Regression and regrowth of tumors were expressed as the time in days for tumors in the treated groups to grow from 6 mm to 12 mm in diameter minus the time in days for tumors in the control group to reach the same size. This was termed absolute growth delay (AGD). For groups treated with ganitumab or panitumumab and radiation, normalized growth delay (NGD) was determined as the time for tumors in the combined therapy group to grow to 12 mm minus time for tumors in the group treated with drug alone to grow to 12 mm. The radiation enhancement factor (EF) was then determined by dividing the NGD for the group receiving ganitumab or panitumumab plus radiation by the AGD for the group given radiation only.

Tumor cure assay (TCD₅₀). TCD₅₀ is defined as the dose of radiation yielding tumor cure in 50% of animals. TCD₅₀ experiments were performed as described elsewhere, with some modifications (19). Mice bearing 6-mm tumors in the leg were treated with ganitumab, panitumumab or both, given concurrently. Local tumor irradiation was given twice daily for 6 consecutive days. Radiation doses were 2.5 Gy to 8 Gy for control mice receiving local tumor irradiation only (group A) and 1.5 Gy to 7.5 Gy for mice receiving panitumumab (group B), ganitumab (group C), or both (group D) with radiation. Each group had 8 subgroups with different doses of radiation. Tumor recurrence was defined as a tumor regrowing to 7 mm in diameter. Mice were euthanized by CO₂ inhalation when tumors reached a size of 14 mm. Tumor cure was assessed 100-140 days after treatments. For each group, TCD₅₀ values were calculated using the Kaplan Meier method of analysis and data were plotted as the progression-free survival.

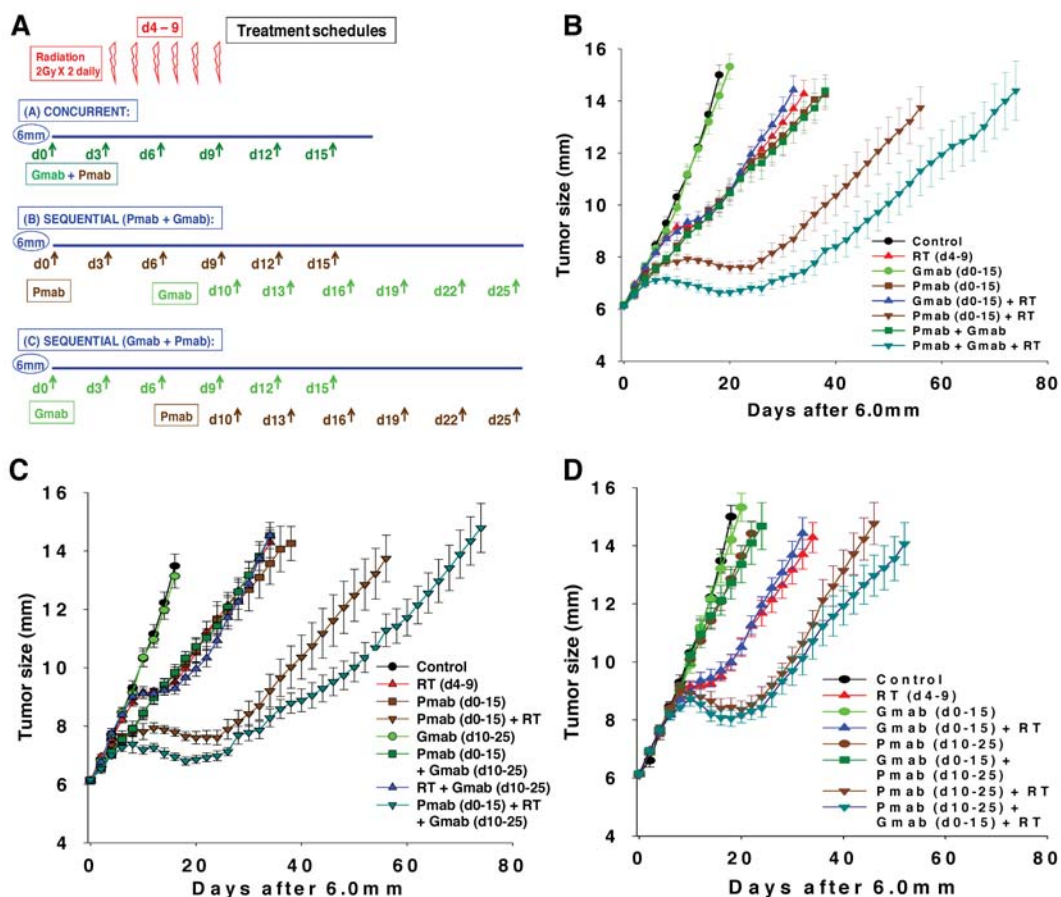


Figure 2. Effect of ganitumab (Gmab) and panitumumab (Pmab), given at three different schedules, on tumor response to fractionated doses of radiation. Tumor growth delay (TGD) assay. A: Diagram showing the three different schedules of drug administration and radiation delivery: concurrent, sequential with panitumumab first and then ganitumab, and sequential with ganitumab first and then panitumumab. B: FaDu tumor growth curves after concurrent administration of ganitumab and panitumumab with radiation. Ganitumab and panitumumab treatments were initiated on day 0 (6-mm tumor) and were given six times, at 3-day intervals (from d0-15). Fractionated doses of radiation were initiated 4 days after the first dose of ganitumab/panitumumab in the combination group or 4 days after tumors reached 6-mm in radiation-only group. C: Sequential administration with panitumumab given first from d0-15 and ganitumab given from d10-25. Fractionated radiation doses were given from d4-9. D: Sequential administration with ganitumab first from d0-15 and panitumumab from d10-25. Fractionated radiation doses were given from d4-9. Day. Each data point represents the mean size of 8-10 tumors \pm SE.

Results

Optimal dose of ganitumab combined with fractionated radiation. Treatment with ganitumab, given as 30, 100, or 300 μ g per mouse per dose, commenced when the tumor diameter reached 6 mm and a total of six doses were administered as 3-day intervals (from day 0 to 15). Fractionated radiation doses were delivered starting 4 days after the first ganitumab dose for 6 consecutive days (from day 4 to 9). Table I shows the time for xenografts to grow from 6 mm to 12 mm in diameter in the control and in various study groups. There were two out of eight mice cured in the group in which mice were treated with 300 μ g plus radiation. Figure 1 (excluding tumor cures) shows TGD

Table I. Effect of ganitumab (Gmab) on the response of FaDu tumor xenografts to radiation.

Treatment	Time in days ^a required to grow from 8-12 mm	No. of cures
Control	18.4 \pm 2.9	
RT (d4-9)	25.7 \pm 2.3	1/8
Gmab 30 μ g (d0-15)	19.8 \pm 1.3	
100 μ g (d0-15)	16.6 \pm 1.0	1/8
300 μ g (d0-15)	28.3 \pm 8.1	
30 μ g + RT	26.6 \pm 2.6	
100 μ g + RT	36.0 \pm 4.4	
300 μ g + RT	33.5 \pm 2.5	2/8

^aMean number of days \pm SE; 8 mice per group.

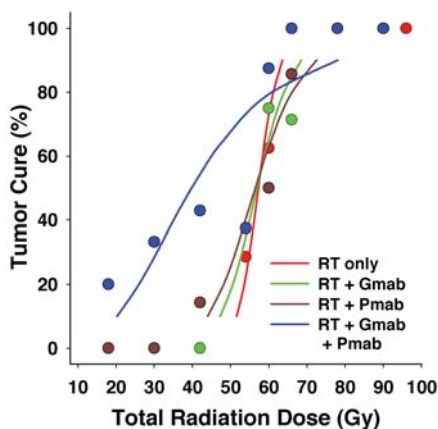


Figure 3. Effect of ganitumab (Gmab) and panitumumab (Pmab) given concurrently on tumor response to fractionated doses of radiation: Tumor cure assay (TCD50). For radiation (RT), only groups the doses ranged from 2.5 Gy to 8 Gy twice daily for 6 days (total radiation dose ranged from 30 to 96 Gy) and for combination therapy groups the radiation doses ranged from 1.5 Gy to 7.5 Gy (total radiation dose ranged from 18 to 90 Gy). The percentage of tumor cure for each radiation dose group of mice was plotted against the total radiation doses given with or without panitumumab or ganitumab. The presented data were obtained 100 days after treatments.

curves after treatments with ganitumab, radiation, or both. Based on these data, 300 µg of ganitumab were used for further studies.

Optimal timing of ganitumab combined with fractionated radiation. Ganitumab (300 µg) was given three times from day 0 to 6 before radiation, or from day 10 to 16 after radiation, or six times from day 0 to 15 delivered before and after radiation. Fractionated radiation was given from day 4 to 9. TGD that was induced by radiation was further enhanced by ganitumab when given either for 6 days or 3 days after radiation and the NGD were 12.78 ± 5.2 and 12.79 ± 2.5 , with two and three tumor cures respectively and two cures in the radiation-only group. However, there were no significant differences in the magnitude of TGD induced by various scheduling of ganitumab and radiation (data not shown).

Antitumor efficacy of triple therapy combining ganitumab and panitumumab with fractionated radiation.

TGD assay: To determine the optimal scheduling of ganitumab and panitumumab administration we used three different treatment regimens. Figure 2A depicts the three treatment schedules tested: (a) both agents were given concurrently for 15 days (panitumumab 1 h before ganitumab on each day), (b) panitumumab was given from day 0 to 15 and ganitumab was given from day 10 to 25, and (c) ganitumab was given from day 0 to 15 and panitumumab

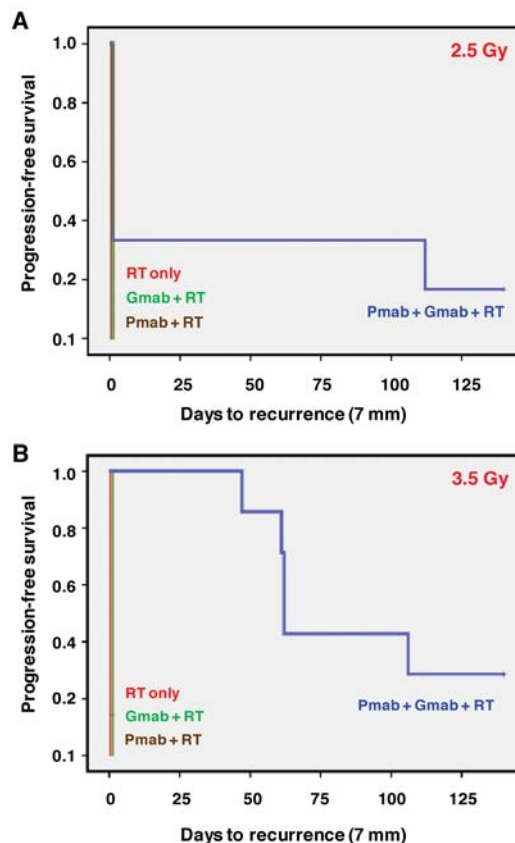


Figure 4. Tumor cure rates and recurrences examined by the Kaplan-Meier method of analysis. The progression-free survival against the number of days to recurrence is plotted. The survival curves were generated after treatments with radiation-only (RT), and in combination with panitumumab (Pmab) or ganitumab (Gmab), or both. A: Survival curves of all four groups treated with 2.5 Gy twice daily for 6 days. B: Survival curves of all four groups treated with 3.5 Gy twice daily for 6 days.

was given from day 10 to 25. In all three regimens, radiation was given from day 4 to 9, 6 h after drug treatment. Figure 2B-D show the tumor growth curves of concurrent and two sequential regimens combined with radiation, plotted with respective controls. Untreated control tumors took 13.7 ± 0.8 days to reach 12 mm. Tumors exposed to radiation alone took 26.0 ± 1.8 days with a growth delay of 12.3 ± 1.8 days. Addition of panitumumab increased the radiation-induced growth delay to 35.9 ± 4.1 days with an EF of 1.7. When ganitumab was added to the treatment regimen the tumor growth was further delayed to 50.7 ± 5.7 days and the EF was 3.0 (Figure 2B). Sequential regimen administration of panitumumab followed by ganitumab augmented tumor growth delay, with an EF of 2.9 (Figure 2C). In contrast, the regimen with ganitumab followed by panitumumab had relatively less effect, with an EF of 2.0 (Figure 2D). Table II summarizes the extent of regrowth delays of various groups

Table II. Effect of ganitumab (Gmab) and panitumumab (Pmab) on the response of FaDu tumor xenografts to radiation.

Treatment	Time in days ^a required to grow from 8-12 mm	Absolute growth ^b delay	Normalized ^c growth delay	Enhancement ^d factors	No. of cures
Control	13.7±0.6				
RT (d4-9)	26.0±1.8	12.3±1.8			
Gmab (d0-15)	14.1±0.8	0.4±0.8			
Pmab (d0-15)	28.3±2.4	14.6±2.4			1/8
RT + Gmab (d0-15)	25.1±1.2	11.5±1.2	11.1±1.2	0.9	1/8
RT + Pmab (d0-15)	49.6±4.1	35.9±4.1	21.4±4.1	1.7	
Gmab + Pmab (d0-15)	27.8±2.7	14.1±2.7			1/8
RT + Gmab + Pmab (d0-15)	64.4±5.7	50.7±5.7	36.6±5.7	3.0	
Gmab (d10-25)	14.2±0.7	-0.2±0.7			
Pmab (d0-15) + Gmab (d10-25)	26.1±1.5	12.4±1.5			
RT + Gmab (d10-25)	26.9±0.7	13.3±0.7	13.5±0.7	1.1	
RT + Pmab (d0-15) + Gmab (d15-25)	61.8±3.7	48.1±3.7	35.7±3.7	2.9	2/8
Pmab (d10-25)	16.0±1.0	2.3±1.0			
Gmab (d0-15) + Pmab (d10-25)	16.7±1.9	3.0±1.9			
RT + Pmab (d10-25)	37.2±1.9	23.5±1.9	21.2±1.9	1.7	1/8
RT + Gmab (d0-15) + Pmab (d15-25)	41.6±3.6	27.9±3.6	24.9±3.6	2.0	

^aMean number of days±SE; 8 mice per group. ^bAbsolute tumor growth delay (AGD) is the days required for tumors to grow from 8 to 12 mm in diameter minus the time in days control tumors required to grow from 8-12 mm. ^cNormalized tumor growth delay (NGD) is defined as the time in days required for tumors to reach 12mm in mice treated with combination of Gmab, Pmab or both and RT, minus the time in days required for tumors to reach 12 mm in mice treated with Gmab, Pmab or both alone. ^dEnhancement factors obtained by dividing NGD in mice treated with Gmab, Pmab or both plus RT by the AGD in mice treated with RT alone.

with the EFs for each combination group. Concurrent administration of these agents as well as panitumumab application before ganitumab and radiation, resulted in enhancing the radiation effect by a factor of 3.0 and 2.9, respectively.

TCD₅₀ assay: To test the effects of ganitumab, panitumumab and their combination given concurrently, in increasing the rate of radiation-induced tumor cures, a TCD₅₀ assay was performed. For radiation-only (group A) the doses per fraction ranged from 2.5 Gy to 8 Gy twice daily for 6 days and for combination therapy (groups B, C, D), the radiation doses per fraction ranged from 1.5 Gy to 7.5 Gy. Percentage tumor cures were plotted against total radiation doses given with or without ganitumab or panitumumab. Figure 3 presents the TCD₅₀ assay data obtained 100 days after treatments. The TCD₅₀ was 57.3 Gy for radiation-only, 56.9 Gy for radiation plus ganitumab, 56.5 Gy for radiation plus panitumumab, and 39.8 Gy for radiation plus ganitumab and panitumumab. It is important to note that the radiation dose-response curves of triple therapy groups shifted to the left substantially at lower doses of radiation, implying that the combination of these agents resulted in better outcome at lower doses compared to higher doses per fraction (*p*-values <0.05). Additionally, tumor cure rates and recurrences were examined by the Kaplan-Meier method of analysis. Figure 4A and B show the curves for treatment of 12 fractions of 2.5 Gy and 3.5 Gy,

respectively. Results showed that combination of ganitumab, panitumumab and radiation resulted in significantly fewer recurrences than either of the agents given with radiation.

Discussion

In previous pre-clinical studies, the combination of radiation and panitumumab demonstrated a favorable interaction in upper aerodigestive tract tumor models, both *in vitro* and *in vivo* (18). A recently completed phase Ib trial (20) in patients with advanced NSCLC reported that addition of panitumumab to motesanib (a multireceptor kinase antagonist) and/or chemotherapeutic agents (carboplatin or paclitaxel) with intensity-modulated radiotherapy, showed encouraging results, warranting further clinical investigation for other tumor types. Early-phase clinical data suggest that antiIGF-1R monoclonal antibodies may have therapeutic benefit in several malignancies (21). IGF-1R signaling is known to mediate resistance to radiation as well as to anti-EGFR therapy (22, 23), perhaps by radiation-induced acceleration of tumor cell repopulation. Inhibition of IGF-1R has been shown to enhance radiosensitivity in several types of human tumor cell lines in pre-clinical models (23-25). Iwasa *et al.* (24) showed that CP-751,871, a fully human monoclonal antibody specific for IGF-1R, blocked radiation-induced IGF-1R activation and consequently sensitized tumor cells to radiation by inhibiting

DNA repair and promoting apoptosis. Cellular stress induced by radiation triggers the activation of IGF-1R and EGFR signaling, promoting cell survival (26, 27). Blocking both IGF-1R and EGFR pathways potentially enhances the radiation-induced tumor cell killing. To our knowledge, the present study is the first to show the effect of triple therapy with an anti-EGFR antibody, with anti-IGF-1R and radiation, for the treatment of HNSCC in a tumor model.

EGFR and IGF-1R interact on multiple levels, either through direct association between the two receptors, by mediating the availability of each other's ligand, or indirectly *via* common interaction partners such as G-protein-coupled receptors or downstream signaling molecules (2, 28-30). Resistance to the EGFR inhibitor is associated with up-regulation of IGF-1R levels; conversely, IGF-1R overexpression is found to correlate with decreased efficacy of EGFR targeting, suggesting the importance of IGF-1R signaling in resistance to EGFR inhibitors (22, 31). Chakravarti *et al.* (22) showed that IGF-1R was up-regulated in a glioma cell line resistant to AG1478 (an EGFR tyrosine kinase inhibitor) and co-targeting the IGF-1R kinase activity with AG1024 greatly enhanced both spontaneous and radiation-induced apoptosis *in vitro*. These studies establish the existence of a strong interaction between EGFR and IGF-1R pathways.

Pre-clinical studies have shown that IGF-1R could be used as a successful co-target with anti-EGFR therapy. Treatment with cetuximab (an anti-EGFR antibody) in combination with the IGF-1R antibody, IMC-A12, resulted in greater inhibition of pancreatic carcinoma xenograft growth than treatment with either agent alone (28). The recombinant human IgG-like bispecific antibody, Di-diabody, which blocks both IGF-1R and EGFR receptors simultaneously, has been shown to lead to enhanced anti-tumor activity (32).

Lammering *et al.* (33) reported that exposure of tumor cells to ionizing radiation in the therapeutic dose range 1-5 Gy, resulted in immediate activation of EGFR, and that repeated radiation exposure of 2 Gy led to increased EGFR expression. Radiation-induced EGFR activation contributes, at least in part, to the mechanism of accelerated proliferation (34). Inhibition of the EGFR pathway by panitumumab potently enhanced tumor response to radiation. However, in tumors that were exposed to panitumumab and radiation, activation of IGF-1R may occur as a mechanism of tumor cells to escape the anti-EGFR and radiation therapies. Hence, inhibition of IGF-1R by administration of ganitumab in addition to panitumumab markedly increased anti-tumor efficacy by inhibiting an alternative pathway for repopulation.

This study showed that when combined with radiation, co-targeting both EGFR and IGF-1R pathways yielded better therapeutic effects than inhibition of either pathway alone. However, the magnitude of effects depended significantly on the sequence of signaling blockade. Marked increases in tumor response, measured by regrowth delay assay, were

obtained when ganitumab was given concurrently with panitumumab or after panitumumab. Tumor cure data showed that with lower doses per fraction, close to those used in the clinic, the combinations of both ganitumab and panitumumab substantially improved tumor cure rates but the effects were minimal with higher doses per fraction (Figure 3). The mechanisms underlying such differential effect as a function of radiation doses per fraction have yet to be elucidated. Nonetheless, these pre-clinical data show the therapeutic potential of the regimens and warrant further clinical investigations, testing for combinations of ganitumab and panitumumab with radiation in cancer therapy.

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