Novel *EGFR* Ectodomain Mutations and Resistance to anti-EGFR and Radiation Therapy in H&N Cancer Stem Cells

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Purpose: EGFR-targeted monoclonal antibodies (mAbs) provide clinical benefit in some patients with H&N squamous cell carcinoma (HNSCC), but others progress with minimal response. Missense mutations in the EGFR ectodomain (ECD) can be acquired under mAb therapy by mimicking the effect of large deletions on receptor untethering and activation. Little is known about the contribution of EGFR ECD mutations to EGFR activation and anti-EGFR response in HNSCC. **Methods:** We selected patient-derived HNSCC cells (UM-SCC-1) for resistance to mAb Cetuximab (CTX) by repeated, stepwise exposure to mimic what may occur clinically and identified two concurrent EGFR ECD mutations (UM-SCC-1R). We examined the competence of the mutants to bind EGF ligand or CTX. We assessed the potential impact of the mutations through visual analysis of space-filling models of the native sidechains in the original structures vs. their respective side-chain mutations. We performed CRISPR in combination with site-directed mutagenesis to test for the effect of the mutants on ligand-independent EGFR activation and sorting. We determined the effects on receptor internalization, endocytosis, downstream signaling, and radiation sensitivity. **Results:** UM-SCC-1R cells carried two non-synonymous missense mutations (G33S and N56K) mapping to domain I in or near the EGF binding pocket of the EGFR ECD. Structural modeling predicted that these mutants restrict the adoption of a tethered, inactive EGFR conformation while not permitting association of EGFR with the EGF ligand or CTX. Binding studies confirmed that the mutant, untethered receptor displayed a reduced affinity for both EGF and CTX but demonstrated sustained activation and presence at the cell surface with diminished internalization and sorting for endosomal degradation. Single and double-mutant models demonstrated that the G33S mutant is dominant over the N56K mutant in its effect on EGFR activation and EGF binding. CTX-resistant UM-SCC-1R cells demonstrated cross-resistance to mAb Panitumuab but, paradoxically, remained sensitive to the reversible receptor tyrosine kinase inhibitor Erlotinib. **Conclusions:** HNSCC cells can select for EGFR ECD mutations under EGFR mAb exposure that converge to trap the receptor in an open, constitutively activated state. These mutants impede the receptor's competence to bind mAbs and EGF ligand and alter its endosomal trafficking, possibly explaining certain cases of clinical mAb and radiation resistance.