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ROLE OF GRB2-ASSOCIATED BINDER 1 (GAB1) IN EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)-INDUCED SIGNALING IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Abstract

The Epidermal Growth Factor Receptor (EGFR) plays an important role in the pathogenesis of head and neck squamous cell carcinoma (HNSCC). Despite the high expression of EGFR in HNSCC, EGFR inhibitors have only limited success as monotherapy. The Grb2-associated binder (GAB) family of adaptor proteins acts as docking/scaffolding molecules downstream of tyrosine kinase receptors. We hypothesized that GAB1 may amplify EGFR-induced signaling in HNSCCs and therefore could play a role in the reduced sensitivity of HNSCC to EGFR inhibitors. We used representative human HNSCC cell lines overexpressing wild type EGFR, and expressing GAB1 but not GAB2. We demonstrated that baseline Akt and MAPK signaling were reduced in HNSCC cells in which GAB1 expression was reduced. Furthermore, the maximal EGF-induced activation of the Akt and MAPK pathway was reduced and delayed, and the duration of the EGF-induced activation of these pathways was reduced in cells with GAB1 knock-down. In agreement with this, HNSCC cells in which GAB1 levels were reduced showed an increased sensitivity to the EGFR inhibitor gefitinib. Our work demonstrates that GAB1 plays an important role as part of the mechanism of by which EGFR induces induced activation of the MAPK and AKT pathway. Our results identify GAB1 as an amplifier of the EGFR-initiated signaling, which may also interfere with EGFR degradation. These findings support the emerging notion that reducing GAB1 function may sensitize HNSCC to EGFR inhibitors, hence representing a new therapeutic target HNSCC treatment in combination with EGFR targeting agents.

Introduction

Head and neck squamous cell carcinoma (HNSCC) arises in the oral cavity, oropharynx, larynx or hypopharynx and is the sixth leading cancer by incidence worldwide ¹. It is likely that up to 600.000 cases will arise this year worldwide and that only 40-50% of patients with HNSCC will survive beyond 5 years. The most important risk factors so far identified are tobacco use and alcohol consumption, which act in a synergistic fashion ^{2, 3}. A subgroup of HNSCCs, particularly those of the oropharynx, is associated with the infection with high-risk types of human papillomavirus (HPV) ^{4, 5}. The prognosis for patients with HNSCC is largely determined by the stage at presentation, which is dependent on the extent of the tumor, as well as the presence of lymph node metastases and distant metastases ⁶. Early stage tumors are often treated with surgery or radiotherapy and have a favorable prognosis. While the mainstays of treatment for advanced tumors are surgery combined with postoperative radiotherapy, in the past decade, the role of organ-preservation protocols, with combined chemoradiation and surgery for salvage, has increased.

The limited information available on the molecular drivers of HNSCC pathogenesis^{1, 7}, together with the genetic and biological heterogeneity of the disease has hampered the development of new more targeted therapeutic strategies. One exception, however, is the introduction of cetuximab, a monoclonal antibody directed to the epidermal growth factor receptor (EGFR), which has been shown to improve overall survival in patients with HNSCC not amenable for curative treatment when used in combination with chemotherapy⁸. It is also approved for use in combination with radiation in previously untreated patients⁹. Zalutumumab, another human monoclonal antibody targeting EGFR, has been shown to improve the progression free survival in recurrent or metastatic HNSCC after failure of platinum-based chemotherapy, compared to best supportive care¹⁰.

The EGFR protein was found to be overexpressed in 38%-47% of HNSCC, while mRNA for EGFR is found to be elevated in up to 90% of HNSCC lesions¹¹. Moreover, EGFR levels increase in advanced-stage tumors and in poorly differentiated tumors, and elevated levels of EGFR in HNSCC often correlate with poor prognosis¹². The overexpression of EGFR is a result of increased mRNA synthesis, as well as decreased down regulation of the EGFR protein. Factors for increased EGFR mRNA synthesis include dysregulated p53, single nucleotide polymorphisms (SNP) in the EGFR promoter region, and CA dinucleotide repeat in intron 1 of the EGFR gene, with a less prominent frequency of EGFR gene amplification¹³⁻¹⁶. In contrast to the numerous reports documenting the presence of activating mutations of EGFR in lung cancer, EGFR mutations are very rare in HNSCC¹⁷. An important mechanism for EGFR activation in HNSCC is instead the autocrine or paracrine activation by EGFR ligands that are often expressed by the tumor cells, or that accumulate in the tumor microenvironment. Despite the overexpression of EGFR in HNSCC and the important role in HNSCC pathogenesis, EGFR inhibitors have only limited success as monotherapy in this malignancy.

In this regard, it is possible that the large overexpression of EGFR may render EGFR inhibitors clinically ineffective, as even a limited fraction of active EGFR may be sufficient to promote tumoral growth at the tissue levels of EGFR inhibitors safely achievable in the clinic. Alternatively, we hypothesized that HNSCC may harbor additional molecular alterations amplifying EGFR signaling, thus enabling the growth promoting function of EGFR even in the presence of low levels of active receptor after pharmacological inhibition. To address this possibility, we began exploring the expression of possible EGFR signal amplifiers in HNSCC.

Among them, GAB1 is part of a family of docking proteins, which also include GAB2 and GAB3, that are important signaling proteins employed by a plethora of transmembrane receptors^{18, 19}. These proteins encompass an N-terminal PH domain, which mediates their interaction with specific membrane lipids and contain a large number of tyrosine residues and proline-rich regions that allow their interaction with signaling proteins encompassing SH2 and SH3 domains and consequently the formation of multimolecular signaling complexes.

GAB1 was originally isolated as a Grb2-binding protein from a human glial tumor expression library¹⁹. It was also identified independently as a Met-receptor interacting protein in a yeast-two hybrid screen and as the major tyrosine phosphorylated protein in cells transformed by the Tpr-Met oncogene (constitutively active version of Met). GAB1, in contrast to GAB2 and GAB3, contains a Met binding domain (MBD), which is sufficient for the interaction of this protein to the Met receptor and hence has been often associated with Met signaling in growth promotion and cell scattering and pro-migratory activity²⁰. Indeed, the MBD site of GAB1 is distinct from the Grb2-binding sites, which are necessary for the binding the EGF receptor²¹. Thus, the distinct biological functions of the EGF and Met

receptors might be explained, at least in part, to the different mechanisms of GAB1 recruitment. Here, we observed that GAB1 is highly expressed in HNSCC cells, and this prompted us to explore whether GAB1, a well known docking/scaffolding molecule, may serve as a possible amplifier of EGFR-induced signaling in HNSCC.

Materials and Methods

Cell culture and reagents

HNSCC cell lines HN4, HN6, HN12, HN13 and HN31 were obtained from Dr. J.F. Ensley (Wayne State University, Detroit, MI) and described previously ²². The human HNSCC cell lines CAL27 and HEP-2 were purchased from the American Type Culture Collection (Manassas, VA). HaCaT cells were derived from normal skin adjacent to a melanoma, and these cells often served as a control, non-tumorigenic epithelial cell line. NOK-SI is an spontaneously immortalized normal human oral epithelial cell line ²³. All cell lines were grown and maintained in DMEM and 10% fetal bovine serum, penicillin, and streptomycin at 37 °C in the presence of 5% CO₂. HNSCC cell lines were grown to 60% to 70% confluence prior to treatment or stimulation. For in vitro studies, cells were treated with gefitinib (LC laboratories), rapamycin, LY294002 or Wortmannin, all purchased from Calbiochem (San Diego, CA), at the indicated concentrations and time points. Prior to stimulation with EGF, cells were serum starved for 16 h. Serum-starved cells were stimulated with EGF 100 ng/mL. Cells were then harvested, lysed, and proteins subjected to Western blot analysis.

Transfection of siRNA

Transfection of siRNAs (Qiagen) at the indicated final concentrations was performed using HiPerFect (Qiagen) according to the manufacturer's recommendation. At least two targeting sequences were used for each knockdown experiment.

Cell lysis and Immunoblotting

Cells were rinsed twice in PBS, lysed with protein lysis buffer (62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 1% beta mercaptoethanol) scraped, immediately transferred to microcentrifuge tubes and sonicated for 20 seconds. Protein yield was quantified using the detergent compatible protein assay kit (Bio-Rad, Hercules, CA). Equivalent amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Equivalent loading was confirmed by staining membranes with Ponceau-S. The membranes were blocked for 1 hour in blocking buffer (5% nonfat dry milk in 0.1% Tween 20-TBS), which was then replaced by the primary antibody diluted in blocking buffer at the concentration described above, and incubated overnight at 4°C. The membranes were then washed thrice in washing buffer (0.1% Tween 20-TBS). Primary antibody was detected using horseradish peroxidase-linked goat anti-mouse or goat anti-rabbit IgG antibody at 1:5,000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized with ECL plus chemiluminescent substrate.

Antibodies

Rabbit monoclonal anti-phospho-threonine 308-Akt (pT308-Akt), and rabbit polyclonal antiserum against MET, phospho-Tyr1234/1235-MET, Grb2, GAB1, phospho-Tyr627-GAB1, S6, phospho-S6 (p-S6), Akt, phospho-tyrosine 1173-EGFR (pY1173-EGFR) and phospho-ERK1/2 (p-ERK1/2) were purchased from Cell Signaling Technology, Beverly, MA. Rabbit polyclonal antiserum against GAB2(H200), ERK1/2(MK1), EGFR(E-8), and tubulin(H-300) were obtained from Santa Cruz. As described above, all antibodies were used for Western blot dilution of 1:1,000.

[³H]Thymidine Incorporation Assay

HN13, HeLa and HN6 cells were grown in 24-well plates and transfected with GAB1 siRNA and labeled with 1 μ Ci/ml [³H]-thymidine for 4 h. Cells were washed twice with ice-cold PBS and three times with ice-cold 10% (w/v) trichloroacetic acid. After resuspension in 0.3 M NaOH, the radioactivity present in the trichloroacetic acid-insoluble material was determined by liquid scintillation counting.

Results

GAB1 and GAB2 expression in HNSCC cell lines

In order to select a suitable HNSCC cell line for our studies, we determined the expression of GAB1, GAB2 and EGFR in various HNSCC cell lines. Most HNSCC cell lines express both GAB1 and GAB2, which makes it difficult to study the effects of the downregulation of GAB1, since GAB2 could partially compensate for the lack of GAB1. Hence, to examine specifically the role of GAB1 in EGFR-dependent signaling we selected the HN13 cell line, derived from human oral squamous cell carcinoma, as a model, since this cell line expresses GAB1 at high levels, while GAB2 expression is almost undetectable (Figure 1). The HeLa cell line was used as a negative control, since this cervical cancer-derived cell line expresses low levels of GAB1 and EGFR, but high levels of GAB2. In further experiments we also used the HN6 cell line, in which EGFR is persistently activated primarily due to the concomitant overexpression of EGFR and its ligand, TGF- α ²⁴.

GAB1 is phosphorylated upon EGF stimulation

c-MET, the tyrosine kinase receptor for hepatocyte growth factor (HGF), is overexpressed in a variety of tumors in which it plays a central role in malignant transformation ²⁵. In HNSCC, c-MET expression has been reported, and it is known that GAB1 is a direct interaction partner of c-Met, as GAB1 exhibits a c-MET binding site (MBS) ¹⁹. This 13 amino acid MBS allows GAB1 to directly associate with the c-MET receptor. The MBS is unique for GAB1 and is not present in the other mammalian GAB family members. In this regard, the HN13 cell line expresses both EGFR and c-MET, and thus allows to determine if GAB1 phosphorylation is induced upon activation of EGFR or c-MET through stimulation with the corresponding ligands, or whether EGFR promotes GAB1 phosphorylation through a cross talk with c-Met, as proposed in other cancer types ¹¹. EGF stimulation induced strong phosphorylation of the EGFR and GAB1, with a subtle increase in the phosphorylation of c-MET (Figure 2). On the other hand, HGF stimulation of HN13 resulted in strong phosphorylation of the c-MET receptor and to a lesser extent also to phosphorylation of EGFR and GAB1 (Figure 2). Quantification of the Western blot results showed that in this cell line, GAB1 phosphorylation is significantly higher upon EGF/EGFR stimulation compared to HGF/c-MET stimulation. In agreement with this, siRNA mediated knockdown of the c-MET receptor did not influence GAB1 phosphorylation upon addition of EGF, although it abolished the phosphorylation of GAB1 in response to HGF (Figure 2), as expected. Phosphorylation of MAPK was also not affected by knockdown of c-MET, which suggests that c-MET is not required for the baseline MAPK signaling of this cell line. These data revealed that GAB1 can be phosphorylated downstream of activated EGFR in HNSCC, and that GAB1 phosphorylation by EGFR is independent of c-MET activity.

EGF/EGFR induced GAB1 phosphorylation is inhibited by gefitinib treatment

We next analyzed if GAB1 phosphorylation responds to the EGFR inhibitor gefitinib. We tested this in the HN6 cell line, which has a persistently active EGFR kinase, and in the HN13 cell line stimulated by EGF. As already shown in Figure 2, phosphorylation of GAB1 was associated with activation of EGFR. While EGFR was persistently phosphorylated in

HN6 cells, both EGFR and GAB1 phosphorylation were increased even further upon EGF stimulation, and both basal and EGF-stimulated EGFR as well as GAB-1 phosphorylation were abolished by gefitinib treatment (Figure 3A). Similarly, EGF induced the phosphorylation of EGFR in HN13 cells, caused an increase in the phosphorylation of GAB1, which could be completely inhibited by gefitinib (Figure 3A). We also tested the effect of gefitinib on the proliferation of the HN6 and HN13 cell lines, with HeLa (a cell line with very low EGFR expression) as a control. In agreement with the Western blot data, HN6 responded very well to gefitinib, with an IC_{50} value below 300 nM (Figure 3B). HN13 responded intermediate to gefitinib (IC_{50} below 10 μ M). The cell line HeLa did not respond to gefitinib (IC_{50} above 10 μ M).

GAB1 is required for EGFR signaling and cell proliferation

To explore role of GAB1 in EGFR signaling in the EGFR-dependent HNSCC cell lines HN13 and HN6, we decreased GAB1 expression using siRNA transfection. Only 5 nM of GAB1 siRNA was needed to selectively knockdown GAB1 levels 72 h after transfection in both HN13 and the HN6 cell lines. Knockdown of GAB1 had a clear effect on total EGFR expression levels in HN13 cells, as well as on baseline Akt and MAPK phosphorylation (Figure 4A). In HN6, however, GAB1 knock down did not affect EGFR expression levels, and had a lower impact on MAPK and Akt activation, albeit both were reduced at higher concentrations of GAB1 siRNAs. In addition, [3 H]-thymidine incorporation assays showed that, with decrease of GAB1 levels, the proliferation rate of the cells also decreased in HN13, HN6, but not in HeLa cells, that served as a control. These data suggest that GAB1 might be required for the stability of EGFR protein and for its signaling output, particularly in HNSCC cells that do not exhibit a persistently active EGFR, as in most HNSCC cases.

To analyze the contribution of GAB1 to EGFR signaling in more detail, we determined the effect of GAB1 knockdown on a time dependent activation of EGFR upon EGF stimulation. The HN13 cell line shows phosphorylation of EGFR as early as one minute after stimulation with EGF. At this time point, the phosphorylation of EGFR was clearly decreased when GAB1 is absent. Similarly, phosphorylation of MAPK, Akt, and the Akt-mTOR downstream target, S6, was clearly reduced after GAB1 knock down (Figure 5A). One hour after stimulation, EGFR remains still highly phosphorylated in cells transfected with control siRNA, while EGFR, MAPK, Akt, and S6 phosphorylation already decreases at nearly basal conditions at this time point in cells transfected with GAB1 siRNA. These data show that loss of GAB1 results in a shorter maximal activation of EGFR activity and signaling, and suggests that GAB1 acts as an amplifier of EGFR signaling.

Downregulation of GAB1, using siRNA, increases gefitinib sensitivity of HN13 and HN6 cells

As in most HNSCC lesions, HN13 cells overexpress a non-oncogenically active EGFR, and as a consequence, the IC_{50} is rather high when treating these cells with gefitinib. Indeed, this cell line is resistant to the concentrations of gefitinib achievable experimentally *in vivo* as well as in cancer patients in clinical trials 26, 27. Western blot analysis showed that the phosphorylation levels of GAB1, Akt and MAPK significantly decrease using gefitinib concentrations between 10 and 30 μ M. remarkably, however, the baseline of phosphorylation of MAPK and Akt decreased after knocking down GAB1, and cells become significantly more sensitive to gefitinib (figure 6A). As judged by the proliferation assays, the IC_{50} of gefitinib was displaced nearly one log unit to the right in this representative HNSCC cells (Figure 6B). In HN6, which expresses a persistently active EGFR, these cells are already gefitinib sensitive, and yet knocking down GAB1 results in a reduction of the basal proliferation rate of these cells, and the gefitinib sensitivity was increased even further. HeLa served as a negative control, as these cells expresses only very low levels of EGFR,

and hence its proliferation is driven by alternative mechanisms. Accordingly, proliferation assay showed that this cell line is gefitinib resistant even after knock down of Gab1.

Discussion

Worldwide more than half a million patients are diagnosed with squamous cell carcinoma of the head and neck each year ¹. Patients who present with an early stage I or II disease are often treated with either radiation or surgery and have an excellent prognosis ⁶. However, these patients are still at high risk for recurrence and second primary tumors. Most HNSCC patients, however, present with locally advanced, stage III or IV disease, which requires a combination of chemotherapy, radiation or surgery. The curation rate of these tumors is very low, with a high risk of recurrence and metastasis. The median survival of patients with metastasized HNSCC is only 11 months and a 1-year survival rate of 20% to 40% ⁶. The limited information available on the molecular carcinogenesis of HNSCC and the genetic heterogeneity of the disease has hampered the development of new therapeutic strategies.

Based on gene expression profiles, Chung *et al.* identified four subgroups of HNSCC with different prognoses. One particular subgroup with an EGFR associated expression profile exhibited a relatively poor prognosis ²⁸. Hence, one would predict that the subset of HNSCCs with an EGFR associated expression profile would benefit from EGFR targeted therapy. However, multivariate analysis of the EXTREME trial however showed that neither EGFR expression level, nor EGFR gene copy number appeared to be predictive of survival or disease progression benefits after treatment with cetuximab in combination with cisplatin based chemotherapy ²⁹.

Only a very small proportion of EGFR-dependent HNSCCs have an activating EGFR mutation, with less than 1% of the Caucasian and 7% of the Asian population. Of the 7% of the Asian, only a small subgroup of EGFR mutations seemed to be activating and therefore oncogenic ^{30, 31}. Amplification is an alternative method by which EGFR can be oncogenically activated. Although the frequency of amplification at 7p11.2, which correlated with EGFR overexpression both at the RNA and protein level, varies between studies, it is generally 38-47% ^{32, 33}. Hence, these or alternative mechanisms may help explain the intrinsic resistance of HNSCC to EGFR inhibitors in the clinical setting. In this study, we propose that at least in a subset of EGFR-dependent HNSCCs the adaptor protein GAB1 plays an important role in the amplification of the EGFR-dependent signaling.

GAB1 is an adaptor protein, which can bind to PIP₃ enriched cell membrane regions through its PH-domain ¹⁹⁻²¹. In our *in vitro* model, the HN6 cell line expresses a persistently activated EGFR even after EGF deprivation due to over expression of the EGFR ligand TGF- α ^{24,34}. In this case, thymidine incorporation assays demonstrate that these HNSCC cells are very sensitive to gefitinib, thus supporting their EGFR-dependence for cell growth. In this case, GAB1 appears to amplify EGFR-dependent downstream signaling, since the baseline proliferation rate of HN6 cells decreased after GAB1 downregulation. However, we did not see any changes in the sensitivity of HN6 to gefitinib after GAB1 expression was reduced, thus raising the question of whether GAB1 plays a biological relevant role in EGFR signaling in HNSCC. However, as discussed above, most HNSCC patients do not respond to gefitinib as a single agent, similar to lung cancer patients lacking activating mutations in EGFR. Thus, our results suggest that in few HNSCC cases in which EGFR is persistently active either by the acquisition of activating mutations or by the local generation of autocrine loops, cells may become addicted to, and hence dependent on, EGFR for tumor cell growth, and consequently these particular tumors may be highly sensitive to EGFR inhibitors, such as gefitinib. In this case, we can speculate that HNSCC cells exhibiting persistently active EGFR may require both GAB1-dependent and independent signaling for

growth, and hence low doses of gefitinib may decrease cell proliferation even when GAB1 is expressed.

However, most HNSCC cases are not sensitive to gefitinib, which was nicely reflected in our *in vitro* model of HN13 cell line, which is derived from a human HNSCC²². In these cells the receptor is not constitutively activated and the antiproliferative response of these cells to gefitinib is rather low, with a high IC₅₀ that cannot be accomplished in clinic, due to grade III-IV side effects. In this case, we can postulate that a signal amplification system may exist to enable a low level of active EGFR after gefitinib inhibition to sustain tumoral growth. We now show that GAB1 endows EGFR with the ability to stimulate Akt and MAPK even after blocking this receptor partially with gefitinib at concentrations achievable in the clinic. Using our *in vitro* model, we can postulate that GAB1 amplifies the EGFR-dependent downstream signaling, achieving a maximal phosphorylation levels of different downstream proteins faster, and for a more prolonged time. Our results show that inhibiting GAB1 also decreases MAPK and Akt baseline signaling levels. Most importantly, we show that when we remove GAB1 in these cells and thus remove the ‘amplification step’, the tumor cells become much more sensitive to gefitinib, shifting the IC₅₀ to a clinical relevant concentration.

Another interesting finding is the decrease of total EGFR levels after knocking down GAB1. It is known that Cbl proteins are negative regulators of EGFR signaling³⁵. Cbl proteins all contain an amino-terminal tyrosine kinase binding (TKB) domain, a ring finger domain and a region of proline-rich sequences in their carboxyl-terminus. Cbl proteins are recruited to the activated EGFR either by the direct binding of their TKB domain to the phosphotyrosine residue at position 1045 in the EGFR, or by an indirect mechanism mediated by Grb2, as the SH3 domains of Grb2 bind the proline-rich region of Cbl proteins and the SH2 domains of Grb2 binds the phosphorylated EGFR. The RING finger domain of the Cbl proteins allows them to function as ubiquitin ligases and thus target the EGFR signaling complex for internalization and subsequent degradation in the lysosome³⁶. We can postulate that GAB1 competes with Cbl for binding to EGFR, and therefore after knocking down GAB1 EGFR becomes more sensitive to Cbl-mediated internalization and degradation, which ultimately reduces EGFR function. Aligned with this possibility, in preliminary studies we have observed that after GAB1 knock down, the internalization and degradation of EGFR after EGF stimulation occurs at earlier time points, potentially decreasing the duration of downstream signaling, a possibility that warrants further investigation.

In addition to competing for Cbl binding, GAB1 can facilitate the formation of high order molecular complexes. The association of GAB1 with EGFR is thought to occur predominantly via Grb2, resulting in tyrosine phosphorylation of GAB1 on several sites. Phosphorylation of Tyr472 regulates its binding to p85 PI3 kinase, phosphorylation at Tyr307, Tyr373 and Tyr407 modulates its association to PLC γ , while phosphorylation of Tyr627 and Tyr659 is required for Gab1 binding to and activation of the protein tyrosine phosphatase SHP2¹⁹⁻²¹. GAB1 mediated recruitment of p85 leads to PI3K activation and the production of phosphatidylinositol(3,4,5)-triphosphate (PIP3) in the plasma membrane. This GAB1-PI3K interaction generates positive feedback in PI3K stimulation: the PH domain of GAB1 binds PIP3 and this leads to a further recruitment of GAB1 to the membrane^{37, 38}. These properties of GAB1 together may help explain its role as an EGFR signal amplifier, and thus exposing GAB1 as a new target for therapies that may sensitize EGFR to small molecule inhibitors. Indeed, we believe that our work may now support the future development and evaluation of EGFR signaling inhibitors that may sensitize to EGFR blocking agents. As a proof of principle, it would be interesting to evaluate whether inhibiting this major docking protein, GAB1, has a broad impact on EGFR and of c-MET-

induced tumors the *in vivo*, considering that both tyrosine kinase receptor pathways converge in GAB1 to activate proliferative pathways, and the emerging evidence that this signal redundancy might play a role in resistance of some HNSCCs to EGFR inhibitors and even to cisplatin- and radio-resistance in HNSCC cell lines 39, 40. Overall, by inhibiting GAB1, one would expect to be able to overcome the c-Met/EGFR potential signaling redundancy-as well as EGFR signal amplification.

Alternatively, potential GAB1 inhibiting strategies could be combined with inhibitors of the PI3K-Akt-mTOR pathway, which is overactive in most HNSCCs ⁴¹. Regarding this possibility, preclinical data have defined mTOR complex 1 and 2 (TORC1 and TORC2) as suitable targets in HNSCC, as shown based on the significant antiproliferative and antiangiogenic effect of the mTOR inhibitor, rapamycin, in HNSCC xenografts and oral specific experimental cancer models ⁴². In particular, although further *in vivo* experimentation may be required to extend our *in vitro* data, we can postulate that in those HNSCCs overexpressing a non-oncogenic EGFR, inhibition of the docking protein GAB1 may increase the sensitivity to both EGFR and mTOR inhibitors, thus leading to a more robust antitumoral effect.

References

1. Leemans CR, Braakhuis BJM, Brakenhoff RH. The molecular biology of head and neck cancer. *Nature Reviews Cancer*. 2011; 11:9–21.
2. Blot WJ. Alcohol and cancer. *Cancer Res*. 1992; 52:2119s–23s. [PubMed: 1544150]
3. Zheng TZ, Boyle P, Hu HF, Duan J, Jiang PJ, Ma DQ, Shui LP, Niu SR, MacMahon B. Tobacco smoking, alcohol consumption, and risk of oral cancer: a case-control study in Beijing, People's Republic of China. *Cancer Causes Control*. 1990; 1:173–9. [PubMed: 2102288]
4. Fakhry C, Gillison ML. Clinical implications of Human Papillomavirus in Head and Neck Cancers. *Journal of Clinical Oncology*. 2006; 24:2606–11. [PubMed: 16763272]
5. Syrjanen S. The role of human papillomavirus infection in head and neck cancer. *Annals of Oncology*. 2010; 21:243–5.
6. Specenier PM, Vermorken JB. Recurrent head and neck cancer: current treatment and future prospects. *Expert Rev Anticancer Ther*. 2008; 8:375–91. [PubMed: 18366286]
7. Haddad RI, Shin DM. Recent Advances in Head and Neck Cancer. *New England Journal of Medicine*. 2008; 359:1143–54. [PubMed: 18784104]
8. Vermorken JB, Mesia R, Schueler A, Amellal N, Hitt R. Platinum-Based chemotherapy plus cetuximab in head and neck cancer. *New England Journal of Medicine*. 2008; 359:1116–27. [PubMed: 18784101]
9. Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, Jones CU, Rowinsky EK, Ang KK. Radiotherapy plus Cetuximab for Squamous Cell Carcinoma of the Head and Neck. *New England Journal of Medicine*. 2006; 354:567–78. [PubMed: 16467544]
10. Machiels J-P, Subramanian S, Ruzsa A, Repassy G, Lifirenko I, Flygare A, Sorensen P, Nielsen T, Lisby S, Clement PMJ. Zalutumumab plus best supportive care versus best supportive care in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck after failure of platinum-based chemotherapy: an open-label phase 3 trial. *Lancet Oncology*. 2011; 2045
11. Kalyankrishna S, Grandis JR. Epidermal growth factor receptor biology in head and neck cancer. *Journal of Clinical Oncology*. 2006; 24:2666–72. [PubMed: 16763281]
12. Psyrris A, Yu Z, Weinberger PM. Quantitative determination of nuclear and cytoplasmic epidermal growth factor receptor expression in oropharyngeal squamous cell cancer by using automated quantitative analysis. *Clinical Cancer Research*. 2005; 11:5856–62. [PubMed: 16115926]
13. Nagatsuka H, Ishiwari Y, Tsujigiwa H. Quantification of epidermal growth factor receptor gene amplification by competitive polymerase chain reaction in pre-malignant and malignant oral epithelial lesions. *Oral oncology*. 2001; 37:599–604. [PubMed: 11564582]

14. Grandis JR, Zeng Q, Drenning SD. Normalization of EGFR mRNA levels following restoration of wild-type p53 in a head and neck squamous cell carcinoma cell line. *International Journal of Cancer*. 1998; 13:375–8.
15. Amador ML, Oppenheimer D, Perea S. An epidermal growth factor receptor intron 1 polymorphism mediates response to epidermal growth factor receptor inhibitors. *Cancer Research*. 2004; 64:9139–43. [PubMed: 15604284]
16. Etienne-Grimaldi MC, Pereira S, Magne N. Analysis of the dinucleotide repeat polymorphism in the epidermal growth factor receptor (EGFR) gene in head and neck cancer patients. *Annals of Oncology*. 2005; 16:934–41. [PubMed: 15829495]
17. Lee JW, Soung YH, Kim SY. Somatic mutations of EGFR gene in squamous cell carcinoma of the head and neck. *Clinical Cancer Research*. 2005; 11:2879–82. [PubMed: 15837736]
18. Liu Y, Rohrschneider LR. The gift of Gab. *FEBS letters*. 2002; 515:1–7. [PubMed: 11943184]
19. Nishida K, Hirano T. The role of Gab family scaffolding adapter proteins in the signal transduction of cytokine and growth factor receptors. *Cancer Science*. 2003; 94:1029–33. [PubMed: 14662016]
20. Bolanos-Garcia VM. MET meet adaptors: functional and structural implications in downstream signaling mediated by the Met receptor. *Molecular and Cellular Biochemistry*. 2005; 276:149–57. [PubMed: 16132696]
21. Wohrle FU, Daly RJ, Brummer T. Function, regulation and pathological roles of Gab/DOS docking proteins. *Cell Communication and Signaling*. 2009; 7:1–28. [PubMed: 19187548]
22. Yeudall WA, Jakus J, Ensley JF, Robbins KC. Functional characterization of p53 molecules expressed in human squamous cell carcinomas of the head and neck. *Molecular Carcinogenesis*. 1997; 18:89–96. [PubMed: 9049184]
23. Castilho RM, Squarize CH, Leelahavanichkul K, Zheng Y, Bugge T, Gutkind JS. Rac1 is required for epithelial stem cell function during dermal and oral mucosal wound healing but not for tissue homeostasis in mice. *PlosOne*. 2010; 5:1–12.
24. Cardinali M, Pietraszkiewicz H, Ensley JF, Robbins KC. Tyrosine phosphorylation as a marker for aberrantly regulated growth-promoting pathways in cell lines derived from head and neck malignancies. *International Journal of Cancer*. 1995; 6:98–103.
25. Lesco E, Majka J. The biological role of HGF-MET axis in tumor growth and development of metastasis. *Front. Biosci.* 2008; 13:1271–80. [PubMed: 17981628]
26. Cohen EEW, Kane MA, List MA, Brockstein BE, Dekker A, Vokes EE. Phase II trial of Gefitinib 250 mg daily in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck. *Clinical Cancer Research*. 2005; 11:8418–24. [PubMed: 16322304]
27. Kirby AM, A'Hern RP, D'Ambrosio C, Tanay M, Nutting CM, Harrington KJ. Gefitinib (ZD1839, Iressa) as palliative treatment in recurrent or metastatic head and neck cancer. *British Journal of Cancer*. 2006; 94:631–6. [PubMed: 16495923]
28. Chung CH, Parker JS, Karaca G, Yarbrough WG, Perou CM. Molecular classification of head and neck squamous cell carcinomas using patterns of gene expression. *Cancer Cell*. 2004; 5:489–500. [PubMed: 15144956]
29. Licitra L, Mesia R, Rivera F, Senger S, Stroh C, Vermorken JB. Evaluation of EGFR copy number as a predictive biomarker for the efficacy of cetuximab in combination with chemotherapy in the first-line treatment of recurrent and/or metastatic squamous cell carcinoma: EXTREME study. *Annals of Oncology*. 2011; 22:1078–87. [PubMed: 21048039]
30. Lee JW. Somatic mutations of EGFR gene in squamous cell carcinoma of the head and neck. *Clinical Cancer Research*. 2005; 11:2879–82. [PubMed: 15837736]
31. Loeffler-Ragg J. Low incidence of mutations in EGFR kinase domain in Caucasian patients with head and neck squamous cell cancer. *European Journal of Cancer*. 2006; 42:109–11. [PubMed: 16324836]
32. Bei R, Budillon A, Masuelli L. Frequent overexpression of multiple ErbB receptors by head and neck squamous cell carcinoma contrasts with rare antibody immunity in patients. *Journal of Pathology*. 2004; 204:317–25. [PubMed: 15476268]
33. Ongkeko WM, Altuna X, Weisman RA. Expression of protein tyrosine kinases in head and neck squamous cell carcinomas. *American Journal of Clinical Pathology*. 2005; 124:71–6. [PubMed: 15923166]

34. Grandis JR, Tweardy DJ. TGF- α and EGFR in head and neck cancer. *Journal of Cell Biochemistry*. 1993; 17F:188–91.
35. Mosesson Y, Mills GB, Yarden Y. Derailed endocytosis: an emerging feature of cancer. *Nature Reviews Cancer*. 2008; 8:835–50.
36. Pennock S, Wang Z. A tale of two Cbls: interplay of c-Cbl and Cbl-b in Epidermal growth factor receptor downregulation. *Molecular and Cellular Biology*. 2008; 28:3020–37. [PubMed: 18316398]
37. Rodrigues GA, Falasca M, Zhang Z, Schlessinger J. A novel positive feedback loop mediated by the docking protein Gab1 and phosphatidylinositol 3-kinase in epidermal growth factor receptor signaling. *Molecular and Cellular Biology*. 2000; 20:1448–59. [PubMed: 10648629]
38. Mattoon DR, Lamothe B, Lax I, Schlessinger J. The docking protein Gab1 is the primary mediator of EGF-stimulated activation of the PI-3K/Akt cell survival pathway. *BMC Biology*. 2004; 2:24. [PubMed: 15550174]
39. Zimmermann M, Ozsahin M. The epidermal growth factor receptor (EGFR) in head and neck cancer: its role and treatment implications. *Radiat. Oncol*. 2006; 1:11. AZ. [PubMed: 16722544]
40. Sano D, Valdecanas DR, Myers JN. Vandetanib restores head and neck squamous cell carcinoma cells' sensitivity to cisplatin and radiation in vivo and in vitro. *Clinical Cancer Research*. 2011; 17:1815–27. FM. [PubMed: 21350000]
41. Molinolo AA, Squarize CH, Castilho RM, Gutkind JS. Dysregulated molecular networks in head and neck carcinogenesis. *Oral oncology*. 2009; 45:324–34. PA. [PubMed: 18805044]
42. Amornphimoltham P, Patel V, Gutkind JS. Mammalian target of rapamycin, a molecular target in squamous cell carcinomas of the head and neck. *Cancer Res*. 2005; 65:9953–61. [PubMed: 16267020]

Figure 1. Expression of GAB1, GAB2 and EGFR in different HNSCC cell lines
 NOK-SI: immortalized human oral keratinocytes; HaCaT: immortalized human keratinocytes, derived from normal skin. HeLa cells express low abundance of EGFR and GAB1. This cell line was used as a negative control in further experiments. HN6 is a HNSCC cell line with a persistently activated EGFR. This cell line was used as a positive control in further experiments. HN13, a human-derived HNSCC cell line that expresses both wild type EGFR and GAB1, but no GAB2, was used to investigate the role of GAB1 in EGFR-dependent signaling. Blots on the left have been overexposed to detect GAB1 expression in HeLa cells.

Figure 2. c-MET-independent EGF-induced GAB1 phosphorylation

A. HN13 cells were transiently transfected with control siRNA (10 nM), c-MET siRNA #1 (10 nM) and c-MET siRNA#2 (10 nM) respectively. Control siRNA does not influence protein levels; both c-MET siRNAs decrease c-MET protein levels selectively. HGF (20 ng/ml) stimulation increases c-MET phosphorylation after HN13 transfection with control siRNA, but not EGFR phosphorylation. EGF (100 ng/ml) promotes the phosphorylation of EGFR and to a lesser extent MET. Both EGF and HGF trigger the phosphorylation of GAB1. Phosphorylated GAB1 has a higher molecular mobility compared to the non-phosphorylated protein (gel shift). After knocking down c-MET, EGF still induces the phosphorylation of EGFR, GAB1 and MAPK, suggesting that the activation by EGF of GAB1 is EGFR-dependent and not c-MET-dependent. Representative gels from three experiments. B. Semiquantitative GAB1 phosphorylation index (p-GAB1/GAB1).

Figure 3. Gefitinib limits the activation of GAB1 by EGFR in HNSCC cells
A. Response to EGF (100ng/ml; 5 min) stimulation and gefitinib (30 μM; 5 min) inhibition in HN13 and HN6 HNSCC cell lines. GAB1 is persistently phosphorylated in HN6 cells, which exhibit an activated EGFR. Upon EGF stimulation there is an increase in EGFR, GAB1 and MAPK phosphorylation. EGFR activation is completely abolished by gefitinib at baseline and after EGF stimulation. In HN13 cells EGFR is not activated at baseline. EGF induces EGFR, GAB1 and MAPK phosphorylation, which is also completely inhibited by gefitinib. B. Thymidine incorporation assay of HN13, HN6 and HeLa cells in response to gefitinib (dose response curve).

Figure 4. Biochemical and biological relevance of GAB1 in HNSCC cells

A. Biochemical relevance. Dose response curve siRNA GAB1 vs control siRNA: Effect on downstream signaling in HN13 and HN6 cells. The knockdown of GAB1 in HN13 cells reduces baseline activation of MAPK and of AKT. There is also a decrease of total EGFR levels. In HN6 cells, which express a persistently activated EGFR, the decrease in baseline phosphorylation of AKT and MAPK is less remarkable as compared to HN13. **B. Biological relevance.** a. Effect of knocking down GAB1 on proliferation rate of HN13 cells, using thymidine incorporation assay. In HN13 cells the knockdown of GAB1 induces a significant reduction in proliferation rate. b. This is also shown in HN6 cells, but not in HeLa cells that hence served as a control.

Figure 5. GAB1 acts as an EGFR-signal amplifier in HNSCC cells

Knocking down GAB1 (10 nM GAB1 siRNA vs 10 nM control siRNA) reduces baseline phosphorylation levels of MAPK and AKT and also decreases the level of maximal phosphorylation of EGFR, MAPK and AKT. The duration of maximal phosphorylation of EGFR, MAPK and AKT is also shortened.

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Figure 6. Biological relevance of GAB1 in HNSCC cell lines

A. Effect of gefitinib on downstream signaling after knocking down GAB1 (10nM GAB1 siRNA vs 10nM control siRNA) in HN13 cells. The baseline levels of phosphorylated EGFR, MAPK and AKT are again decreased. The decrease in phosphorylated EGFR occurs at low concentrations of Gefitinib (1μM) after knocking down GAB1 efficiently, compared to cells transfected with control siRNA. The effect of gefitinib on MAPK, AKT, and S6 phosphorylation levels is less remarkable, since the baseline phosphorylation is very low. B. Effect of Gefitinib on proliferation rate using thymidine incorporation assay after knocking down GAB1 (10 nM GAB1 siRNA vs 10 nM control siRNA) in HN13, HN6 and HeLa cells. HN6 cells are very sensitive to gefitinib, since they express activated EGFR. HeLa is gefitinib insensitive. Knocking down GAB1 does not increase gefitinib sensitivity, since these cells express only low levels of GAB1 (data not shown). In HN13 cells, which express a wild type EGFR and high levels of GAB1, knock down of GAB1 increases gefitinib sensitivity dramatically.