The MET Receptor Tyrosine Kinase Is a Potential Novel Therapeutic Target for Head and Neck Squamous Cell Carcinoma

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Abstract

Recurrent/metastatic head and neck cancer remains a devastating disease with insufficient treatment options. We investigated the MET receptor tyrosine kinase as a novel target for the treatment of head and neck squamous cell carcinoma (HNSCC). MET/phosphorylated MET and HGF expression was analyzed in 121 tissues (HNSCC/normal) by immunohistochemistry, and in 20 HNNSC cell lines by immunoblotting. The effects of MET inhibition using small interfering RNA/two small-molecule inhibitors (SU11274/PF-2341066) on signaling, migration, viability, and angiogenesis were determined. The complete MET gene was sequenced in 66 head and neck cancer tissue samples and eight cell lines. MET gene copy number was determined in 14 cell lines and 23 tumor tissues. Drug combinations of SU11274 with cisplatin or erlotinib were determined in 14 cell lines and 23 tumor tissues. MET gene copy number was present in >10 copies in 3 of 23 (13%) tumor tissues. A greater-than-additive inhibition of cell growth was observed when combining a MET inhibitor with cisplatin or erlotinib and synergy may be mediated via erbB3/AKT signaling. MET is functionally important in HNSCC with prominent overexpression, increased gene copy number, and mutations. MET inhibition abrogated MET functions, including proliferation, migration/motility, and angiogenesis. MET is a promising, novel target for HNSCC and combination approaches with cisplatin or EGFR inhibitors should be explored. [Cancer Res 2009;69(7):3021–31]

Introduction

Head and neck cancer (HNC) is the sixth most common cancer worldwide, with an annual incidence of >640,000 cases worldwide (47,560 cases in the United States; ref. 1). More than 90% of head and neck cancers are of squamous histology (HNSCC). Thirty-five percent to 45% of head and neck cancer patients ultimately die from their disease. Little progress has been made in the treatment for metastatic/recurrent HNC during the past two decades, with the singular exception of cetuximab, an epidermal growth factor receptor (EGFR) antibody, which improves median survival by 2 months when added to standard chemotherapy (2). Overall survival remains poor (median 6–10 months).

To improve HNSCC treatment, relevant molecular targets need to be identified. Receptor tyrosine kinases (RTK) seem to play a pivotal role in the pathogenesis of HNC, with prior research focusing on EGFR. Despite EGFR overexpression in >90% of tumors, EGFR inhibition has only yielded low response rates of 4.3–13% in clinical practice (3–4). Multiple lines of evidence indicate that RTK pathway redundancies/cooperation are common in RTK-driven malignancies and may account for resistance (3–5). We studied the MET RTK and also explored EGFR/MET crosstalk based on reports of cooperation in other diseases (6–9).

MET, located on chromosome 7q31, encodes several functional domains, including the semaphorin (SEMA) domain (ligand-binding), juxtamembrane (JM) domain (regulatory), and the receptor tyrosine kinase (TK) domain (10, 11). The sole ligand for MET is hepatocyte growth factor (HGF, scatter factor), which is produced by stromal and sometimes tumor cells (10, 11). HGF binding activates MET via intracellular phosphorylation initiating RAS-RAF-ERK, and phosphatidylinositol 3-kinase-AKT-mTOR signaling as well as several other pathways. In vivo, HGF/MET signaling leads to increased cell growth, cell motility, invasion/metastasis, angiogenesis, wound healing, and tissue regeneration (10, 11). Studies show that HGF/MET signaling increases motility, epithelial cell dispersion, endothelial cell migration, and chemotaxis. Furthermore, MET overexpression and activation has transforming properties for normal cells (10, 11).

MET is overexpressed in a number of solid tumors, and expression correlates with an aggressive phenotype and poor prognosis (10, 11). Previously, we had shown that in lung cancer, MET mutations can occur in the JM domain and the SEMA domain, and not the TK domain (12). The precise function of most mutations is not yet fully understood. MET mutations have been described for HNC, especially in lymph node metastases (relative frequency of up to 25% in some reports; ref. 13) and are located in the TK domain similar to TK domain mutations found for renal cell carcinomas (11), suggesting an important role for MET in HNC (13). However, mutations in the SEMA and JM domains have not been previously investigated for HNSCC. Further highlighting the...
importance of MET is the observation of MET amplification in several solid tumors, including subgroups of lung and gastric cancers (6, 7, 14, 15).

In our study, we used a large cohort of HNC and normal mucosa tissues as well as cell lines to identify prominent MET expression, increased gene copy number, and mutations in the TK/JM/SEMA domains. Furthermore, we show that MET inhibition alone and in combination with cisplatin or an EGFR inhibitor is a promising target for head and neck cancer.

Materials and Methods

Tumor tissue arrays/immunohistochemistry. Tissue microarrays of 97 HNC tissues and 24 normal mucosa samples were built (IRB: 9890). Immunohistochemistry was performed for MET (C-12, 1:100), p-MET (pY1003, Invitrogen, 1:25), HGF (H145, Santa Cruz, 1:50), human CD31 (JC70A, DAKO, 1:40), and Ki67 (RM-9106-S, NeoMarkers/Labvision, 1:300) as previously described (12, 15, 16). Appropriate negative controls were prepared. Immunohistochemistry results from tumor and adjacent normal tissue were compared semiquantitatively by a senior pathologist (grading: 0 = negative, 1+ = low, 2+ = strong, 3+ = very strong expression; ref. 12).

Reagents and antibodies. Antibodies used for immunoblotting were MET (3D4, Invitrogen/Zymed, C-12, Santa Cruz Biotechnology), phosphorylation site–specific MET pY1003, and pY1230/4/5 (Biosource/Invitrogen, 1:1000), (Anti-actin/CD44/ERCC1/Ron-α antibodies (H-170, Santa Cruz Biotechnology), pTyr (4G10, Upstate/Millipore), and insulin-like growth factor-I receptor (IGF-IR; Cell Signaling; dilution 1:1000) as previously described (12, 15–17).

The following drugs were purchased: SU11274/IGF-IR inhibitor (Calbiochem), cisplatin (Sigma Aldrich), and erlotinib HCL (ACC). PF-2341066 was kindly provided by Pfizer.

Cell lines and culture. Cell lines were obtained from the American Type Culture Collection (SCC9/15/25/68/Cal27/Fadu), Dr. Ralph Weichselbaum (Department of Radiation Oncology, University of Chicago, Chicago, IL; SQ20B, SQ93, SCC3/51/294/151), Dr. Gary Clayman (M.D. Anderson Cancer Center, Houston, TX; 1483, the Ludwig Institute for Cancer Research (London, United Kingdom; HN5), Dr. David Raben (University of Colorado Health Sciences Center, Aurora, CO; MSK921), and Dr. Mark Lingen (Department of Radiology, University of Chicago, IL; OSCC3, SCC17B;28/58) and maintained in DMEM/F12 or RPMI medium and penicillin/streptomycin (Cellgro) with 10% fetal bovine serum (FBS; Gemini Bioproducts). HaCaT is a spontaneously transformed human keratinocyte cell line. HNX was derived from HN5 after prolonged subculture showing overexpressed (2+/3+) MET and 66% (>2) HNSCC tumors (July 2008). Please note that the MET transcript MET-001 (ENST00000318493) was used for identifying genetic changes (e.g., R898C) and MET-002 (ENST0000397752) for identifying phosphorylation sites (e.g., Y1230), which is consistent with common practice (12, 16).

Real-time PCR. Quantitative real-time PCR for gene copy number measurement was done as previously described (15) using ABI StepOnePlus (Applied Biosystems) and iQ-SYBR green (Bio-Rad Laboratories). Reactions were done in triplicates under standard thermocycling conditions (one cycle 95°C × 12 min, 45 cycles 95°C × 20 s, 60°C × 1 min). The mean threshold cycle number was used.

Fluorescence in situ hybridization. Fluorescence in situ hybridization (FISH) analysis was done using two different BAC probes: RP11-433C10, localized to 7p11.2 (full-length EGFR gene), and RP11-163C9, localized to 7q31.2 (MET gene). Two-color FISH was done using RP11-144B2 (red) together with RP11-163C9 (green). The procedure was done as previously described (14), analyzing at least 10 metaphase cells.

Human papillomavirus testing. Human papillomavirus (HPV) testing was performed in cell lines evaluating for p16 expression (JC-8, Santa Cruz Biotechnology) and by PCR using L1 PGM09/11 primers (21), followed by sequencing. HPV-positive results were confirmed using the Digene HPV test (Qiagen).

In vivo Matrigel plug nude mouse xenograft modeling. Tumor cells were mixed with Matrigel (BD Biosciences) and injected s.c. into the flanks of nude mice (5 × 10⁶ cells/flank) following Institutional Animal Care and Use Committee–approved protocols. The animals were monitored for 2 wk and subsequently sacrificed. Tissues were fixed in 10% formalin and paraffin embedded.

Statistical analyses. Data are expressed as mean ± SE. Statistical significance was tested with Graphpad Prism5. For comparison between two groups, Student’s t test or the χ² test was used. For comparing between >2 groups, one-way ANOVA was used. For evaluation of correlation, Spearman’s test was used.

Results

MET/HGF are expressed in HNSCC tissues and cell lines. MET immunohistochemistry was done on 121 cores (97 cancers/22 normal mucosa) as well as in phosphorylated MET (86 cancers/22 normal mucosa). Eighty-five percent (n = 84) of HNSCC tumors overexpressed (2+/3+) MET and 66% (n = 57) overexpressed (2+/3+) activated phosphorylated MET compared with adjacent normal mucosa (Fig. 1A and B). Normal mucosa also expressed MET (21% 1+, 21% 2+), albeit staining was weaker and primarily limited to the basal layer of the mucosa (Fig. 1A; 23% 1+/2+ for phosphorylated MET). No cases of 3+ expression were seen for normal mucosa. MET localized primarily to the membrane and the cytoplasm.

Immunoblot analysis confirmed strong MET expression in 16 of 20 HNC cell lines [excluding HNX (derived from HN5) and HaCaT (transformed keratinocytes)]; however, SCC17B and SCC151 expressed low levels of MET, which were outside the dynamic range (Fig. 1C). SQ20B and SCC294 had low to moderate MET expression. OSCC3, a HPV-positive cell line [p16+; PCR positive (HPV18), Digenet high-risk HPV positive], showed strong MET expression. EGFR,

The cells were imaged on an Olympus IX81 inverted microscope and magnified with IPLab software (Analytical). Images at ×100 magnification were saved every 5 min and processed as mpeg movies (Sonic DVD). Cell movement/morphologic changes were processed with ImageJ (NIH, Photoshop (Adobe), and MetaMorph (Universal Imaging Corporation/Molecular Devices). The positions of the cell nuclei were tracked, and distance/speed was calculated over 21 h.

Mutational analysis. Genomic DNA from 53 HNSCC tissues from formalin-fixed paraffin-embedded tissues was obtained from the University of Chicago Head and Neck Cancer tissue bank (IRB: 9890). Genomic MET reference sequences were obtained from position chr7:116,099,682–116,225,676 from Ensembl (release 50, July 2008). Please note that the MET transcript MET-001 (ENST00000318493) was used for identifying genetic changes (e.g., R898C) and MET-002 (ENST0000397752) for identifying phosphorylation sites (e.g., Y1230), which is consistent with common practice (12, 16).

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IGF-IR, RON, and ERCC1 expression were prominent in several cell lines. There was no statistical correlation with MET expression. Analysis of MET gene expression using the publicly available Oncomine database and data by Ginos and colleagues showed increased MET gene expression in 41 HNSCC compared with 13 normal controls (Supplementary Fig. S1).

HGF expression was evaluated in 68 HNC tumors by immunohistochemistry. The tumors showed strong (3+; 21%), moderate (2+; 24%), and weak (1+; 41%) HGF expression. Fifteen percent of the tumors were HGF negative.

MET-specific small-molecule inhibitors or siRNAs inhibit MET signaling. Using small-molecule MET inhibitors SU11274 (for cell lines, DMSO soluble, Figs. 2 and 3), PF-2341066 (water soluble, clinical candidate, Fig. 4; see Supplementary Table S1), and MET siRNA (Fig. 3B), MET activation/expression were suppressed. Figure 2A shows immunoblotting results for phosphotyrosine, whereas Fig. 2B shows results for phosphorylated MET and downstream signaling effects in six HNSCC cell lines. Serum-starved cells lines were pretreated with 0, 2, or 5 μmol/L of the MET inhibitor SU11274 followed by treatment with HGF for 8 minutes. In cell lines SCC15, SCC28, and to a lesser degree SCC9 and SCC61, HGF stimulation led to a strong p-Tyr signal, which was suppressed with SU11274 MET inhibitor treatment. SCC17B overall had low p-Tyr expression, suggestive of either a less RTK-driven phenotype (5) or a more ligand-dependent phenotype. Despite low MET expression, external HGF stimulation and SU11274 pretreatment showed typical signaling effects of the HGF/MET axis.

Phosphorylated MET expression was weak at baseline in most starved cells. Following HGF stimulation in all cell lines, a strong phosphorylated MET response is observed that can be suppressed in a dose-dependent fashion (Fig. 2A and B). Downstream signaling for phosphorylated AKT was also increased with HGF and decreased by MET inhibition in cell lines SCC15, SQ20B, SCC28, and to a lesser degree in SCC61 (Fig. 2B). Phosphorylated ERK was only mildly affected by MET inhibition with SU11274.

MET inhibition decreases viability in HNSCC. MET gene silencing with MET-specific siRNA was used to validate effects of MET inhibition in HNSCC. MET-specific siRNA duplexes were transiently transfected into SCC61 and SQ20B cells (Fig. 3A), and
protein expression was decreased by >80% 72 hours after transfection.

siRNA down-regulation of MET protein expression in SCC61 and SQ20B cells resulted in inhibition of the serum-stimulated cell growth and viability by >62%/55% as determined by MTS assays (Fig. 3B). We used SU11274 to test for its inhibitory effects on seven HNSCC cell lines (Fig. 3C). MET inhibition was effective with IC50 values varying between 1 and 8 μmol/L: SCC61 (IC50 1 μmol/L), SCC35 (IC50 3 μmol/L), and SCC9 (IC50 3.8 μmol/L) were the most sensitive lines followed by HN31 (IC50 5 μmol/L) and MSK921/S SCC28 (IC50 5.4 μmol/L), SQ-20B, which has lower MET expression and strong EGFR expression (EGFR amplification), showed an elevated IC50 of 8 μmol/L (extrapolated from Fig. 3C). Generally, a 50% to 90% decrease in cell viability compared with control cells was observed.

Furthermore, MET inhibition with SU11274 (3.5 μmol/L) led to suppression of cell motility and migration. Figure 3D shows a graphical depiction of distances covered by individual cells (SCC61) over a period of 21 hours. SU11274-treated cells covered significantly shorter distances (P = 0.0001) than untreated control cells. This effect is consistent during the entire 21-h observation period.

MET inhibition in vivo. To study MET inhibition effects on angiogenesis, water-soluble PF-2341066 was used in vivo (Supplementary Table S1). PF-2341066 inhibited HGF-dependent MET phosphorylation in a dose-dependent manner at concentrations of 10 to 100 nmol/L in HNC cell lines SCC61 and SCC35 in vitro (Fig. 4A) and also in a soft-agar colony formation assay (OSCC3; Fig. 4B); no large colonies formed. Comparable results in colony formation assays were observed for SCC61 and SCC35 (data not shown).

Effects on angiogenesis were investigated with an in vivo Matrigel xenograft tumor model of OSCC3 and SCC35 treated with PF-2341066 (25 mg/kg/d) versus control-treated cells (n = 3 in each group). Figure 4 (C and D) shows abundant tumor growth in a vehicle control–treated mouse, compared with minimal residual tumor nests in the PF-2341066 group. Staining for the proliferation marker Ki67 shows >80% to 90% suppression of proliferation in PF-2341066–treated animals. Finally, staining of endothelial cells in blood vessels with CD31 shows extensive tumor vessels between tumor nests in control-treated animal versus marked angiogenesis suppression in PF-2341066–treated animals, consistent with prior reports using a related MET inhibitor in vivo (23).

SU11274 can synergize with erlotinib and cisplatin. Figure 5 shows four examples of dual treatment with MET inhibitor SU11274 in combination with commonly used agents—cisplatin or erlotinib.

SCC35 and SCC61, which required doses of >10 μmol/L to approach IC50 toxicity (SCC35 >10 μmol/L cisplatin; SCC61 16 μmol/L cisplatin), were synergistically inhibited by combined treatment with SU11274/cisplatin (SCC35 IC50 1.3/1.3 μmol/L, and SCC61 IC50 1/2 μmol/L). Based on the median effect model by Chou (20), the isobologram graph shows combinatorial index (CI) values below 1 for the ED50 and ED75 (values <1 indicate synergy).

For combination with erlotinib, HN5 and SCC35 were chosen. Cells were treated with either agent alone or with a combination of both at equimolar doses. Both single agents showed efficacy, decreasing viability. The combination, however, was consistently
A. in SCC61 and SQ20B, MET-specific siRNA (100 μmol/L) led to a significant decrease in MET protein expression, whereas control siRNA did not suppress MET expression.

B. SCC61 and SQ20B cells 72 h after transfection with MET-specific siRNA and control siRNA were analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and showed significant decreases in viability compared with control (72%/55%).

C. SU11274 treatment led to a dose-dependent decrease in cell viability compared with untreated control (DMSO solvent). In decreasing order of sensitivity (IC50), the following HNSCC cell lines responded to increasing SU11274 concentrations: SCC61, SCC35, SCC9, HN31, MSK921, SCC28 and SQ20B. D. migration was significantly decreased after treatment with SU11274 over a tracking period of 21 h. Colored lines, individual cell movement over 21 h. Cells treated with SU11274 moved significantly shorter distances (38%; P < 0.0001).
significantly superior to either agent alone. The isobologram shows that at ED25 (CI = 0.73/0.35), ED50 (CI = 0.32/0.36), and ED75 (CI = 0.21/0.36), there was synergistic activity between erlotinib and SU11274 (CI < 1). Evaluation of downstream signaling in Fig. 5C indicated that activation of erbB3 and subsequently AKT are synergistically inhibited. MET mutations in HNSCC tumor tissues and cell lines. The entire MET coding region (schema in Supplementary Fig. S2) was sequenced in 66 HNSCC and 8 cell lines. Three mutations in the ligand-binding SEMA domain (T230M/E168D in the tumor tissue; N375S in the SCC25 cell line) and two mutations in four tumor samples in the transmembrane or JM domain (R988C, 3xT1010I; Table 1A; Supplementary Fig. S2) were identified (previously reported in other tumor types; refs. 12, 17). Furthermore, two mutations in the TK domain (T1275I, V1333I) were identified, which have not been described previously. No classic Y1230C/Y1235D mutations were identified. All mutations were heterozygous. The rate of TK domain mutations was 3% (2 of 66) and the rate of non-TK domain mutations was 9% (6 of 66). Overall, mutations occurred in 12% of tumors analyzed (8 of 66). There was no apparent correlation with smoking status or anatomic site, although the sample number was too small to allow sufficient statistical power.

MET gene copy number. We analyzed a panel of nine HNSCC cell lines by FISH and followed this up with qPCR due to the ready availability of DNA from HNSCC tumor tissues. Repetition of cell lines previously analyzed by FISH now using qPCR was done (Table 1B). FISH analysis showed three cell lines with >4 copies, although qPCR copy number was lower (2.79 and 1.91). Generally, qPCR showed similar or lower copy numbers compared with FISH analysis. We subsequently analyzed 23 HNSCC tumor tissues from patients by qPCR (Table 1C): 3 of 23 (13%) tumors showed gene copy numbers of >10 with one sample showing a copy number of 22.1 and two samples 10.50/10.33 respectively. Furthermore, 15 of 23 (65%) HNSCC tumors showed copy numbers of 4 to 10. There was no apparent correlation with smoking status or anatomic site, although small sample numbers in subgroups do not allow for proper assessment.
MET SNPs in HNSCC tumor tissues and cell lines. In addition to mutations, multiple SNPs in the MET gene were identified as heterozygous (A48A in 2, S178S in 4, Q648Q in 5, I706I in 1, K1250K in 1, and D1304D/A1357A/P1382P occurred together in 22 samples) and homozygous (Q648Q in 2, D1304D/A1357A/P1382P occurred together in 9 samples; Table 1D).

Discussion

In this study, we show that MET is a novel target for HNSCC showing prominent overexpression, mutations, and increased gene copy number. We show the effectiveness of MET inhibition on cell signaling, viability, migration, and angiogenesis. Our data provide a strong rationale to use MET inhibition in translational and clinical studies in HNC and suggest studying the integration with established treatments.

MET is activated in HNSCC patient samples and the presence of phosphorylated MET (66%) closely correlated with overall expression (79%). This is consistent with literature reports for HNC (70–90% expression; refs. 24–29) and is comparable with NSCLC (12).

Our study helps to explain the prominent MET overexpression demonstrating increased copy numbers in a subset of tumors. Although karyotypic analysis is still considered the gold standard, Bean and colleagues confirmed the usefulness of qPCR when compared with array CGH analysis (7). Although no MET-amplified HNC cell lines were identified, MET amplification has previously been reported in gastric carcinoma (14) and NSCLC (6, 7) and correlates with sensitivity to MET small-molecule inhibitors (6, 14). This may be relevant for predicting HNC sensitivity to MET inhibitors in future studies.

Gene array data was also consistent in showing overexpression in HNC (Supplementary Fig. S1); furthermore, Ginos and colleagues reported a link to an increased rate of locoregional HNC recurrence (22).

Normal mucosa weakly expressed MET in the basal mucosa layer (Fig. 1A), possibly linked to mucosa turnover/proliferation or field cancerization. Reports by Chen and colleagues and Ohnishi and colleagues suggest a role of MET in HNSCC dysplastic lesions (24, 30).

The expression pattern was both cytoplasmic and membranous, closely resembling data in lung cancer (31); the relative cellular localization seems to be tissue specific and the functional implications are still being elucidated (31).

Similar to prior reports, we confirm elevated HGF expression in 59% of HNSCC [45% strong expression (2+/3+); 15% weak expression (1+)] including the adjacent stroma, suggesting autocrine and/or paracrine signaling loops, which have been described in other tumor types (gliomas, pancreatic and liver carcinomas; refs. 31–34). This may be another possible predictor of response and e.g. recent reports for the EGFR ligand amphiregulin suggest a correlation with sensitivity to EGFR treatments (35).

The correlation MET/HGF expression/amplification status with treatment outcomes is a high priority for future studies. Preclinically, Akervall and colleagues reported higher MET expression based on gene array analysis in cisplatin-resistant HNSCC cell lines compared with sensitive ones (36) and Aebersold and colleagues reported that MET expression correlated with radioresistance (37, 38). Several studies describe increased MET/HGF expression in more invasive HNSCC (24, 25, 27, 39) as well as metastatic spread (28, 29, 40). Finally, the role of epithelial to mesenchymal transition...
**Table 1.** MET sequencing and gene copy number analysis in HNSCC

### A

<table>
<thead>
<tr>
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### B

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### C

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<td>Increased copy number</td>
</tr>
<tr>
<td>16. FOM</td>
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<td>4.96</td>
<td>0.18</td>
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<tr>
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<td>Yes</td>
<td>4.63</td>
<td>0.24</td>
<td>Increased copy number</td>
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<td>18. Pharynx</td>
<td>Yes</td>
<td>4.51</td>
<td>1.86</td>
<td>Increased copy number</td>
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<td>19. Tonsil</td>
<td>No</td>
<td>3.92</td>
<td>0.54</td>
<td>Increased copy number</td>
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<tr>
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<td>Yes</td>
<td>3.72</td>
<td>0.28</td>
<td>Increased copy number</td>
</tr>
<tr>
<td>21. Larynx</td>
<td>Yes</td>
<td>3.10</td>
<td>0.25</td>
<td>Increased copy number</td>
</tr>
<tr>
<td>22. Tongue</td>
<td>No</td>
<td>2.93</td>
<td>0.59</td>
<td>Increased copy number</td>
</tr>
<tr>
<td>23. Larynx</td>
<td>Yes</td>
<td>2.52</td>
<td>0.65</td>
<td>Increased copy number</td>
</tr>
</tbody>
</table>

Summary >10 Copies: 3/23 = 13.0% 
4–10 Copies: 15/23 = 65.2%

(Continued on the following page)
inhibitors: PF-2341066 used here for in vivo or somatically. Domain mutations/variants may be found in either germline DNA inhibitors (44). In contrast to TK domain mutations, SEMA and JM contribute to MET activation and may alter sensitivity to MET suggesting that both SEMA and JM domain mutations/variants can and may have transforming properties (17). Preliminary reports implicated in increased motility and invasiveness in SCLC (12, 17) reported in lung cancer. The JM domain changes have been mutations/variants. Such mutations/variants have previously been required as seen for the EGFR T790M mutation in NSCLC (43). A role of TK domain mutations in HNSCC remains to be determined cancers (germline or somatic mutations; ref. 42). The functional importance is well established in certain papillary renal cell carcinomas (14), and kinase domain mutations (45) and potentially EGFR selection pressure. Our data for HNSCC is currently in absence of prior EGFR TKI selection pressure. Our data for HNSCC in the United States (48), most HNSCC are sensitive to EGFR inhibitors and overexpression is abundant (4). Recent evidence in NSCLC suggests a common signaling pathway via HER3/erbB3 (5–9). Specifically Engelman and colleagues (6) implicated erbB3 signaling as the mediator of amplified MET “overtaking” mutant-EGFR signaling in a NSCLC model of acquired gefitinib resistance. On the other hand, the recent study by Tang and colleagues (9) suggested a central role for erbB3 in mediating the efficacy of dual MET/EGFR inhibition against T790M-EGFR–mediated resistance in the other hand, the recent study by Tang and colleagues (9) suggested a central role for erbB3 in mediating the efficacy of dual MET/EGFR inhibition against T790M-EGFR–mediated resistance in the absence of prior EGFR TKI selection pressure. Our data for HNSCC now also suggest a similar role of MET/erbB3 in the absence of EGFR selection pressure.

Table 1. MET sequencing and gene copy number analysis in HNSCC (Cont’d)

<table>
<thead>
<tr>
<th>SNP</th>
<th>n</th>
<th>Zygosity</th>
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<tr>
<td>T1010I</td>
<td>66</td>
<td>Hetero</td>
</tr>
<tr>
<td>A1357A</td>
<td>31</td>
<td>Hetero/9 homo</td>
</tr>
<tr>
<td>F1340D</td>
<td>31</td>
<td>Hetero/9 homo</td>
</tr>
<tr>
<td>A48A</td>
<td>2</td>
<td>Hetero</td>
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<tr>
<td>S176S</td>
<td>4</td>
<td>Hetero</td>
</tr>
<tr>
<td>Q648Q</td>
<td>7</td>
<td>5 Hetero/2 homo</td>
</tr>
<tr>
<td>I706I</td>
<td>1</td>
<td>Hetero</td>
</tr>
<tr>
<td>K1250K</td>
<td>1</td>
<td>Hetero</td>
</tr>
<tr>
<td>A1357A*</td>
<td>31</td>
<td>22 Hetero/9 homo</td>
</tr>
<tr>
<td>P1382P*</td>
<td>31</td>
<td>22 Hetero/9 homo</td>
</tr>
</tbody>
</table>

NOTE: A. Table of MET mutations/variants. T1010I JM mutations have been reported to increase MET-related functions (signaling, tumorigenicity, motility, ref. 12). Somatic MET TK domain mutations have been described in HNSCC as gain-of-function mutations (13). The schema of MET gene depicting mutations is provided in Supplementary Fig. S2. Fourteen cell lines (B) and 23 HNSCC tumor tissues (C) were analyzed by FISH and/or qPCR. Increased gene copy number was identified in three tumor samples with >10 copies and 15 tumor samples with 4 to 10 copies (qPCR). Comparison of FISH and qPCR results showed reasonable correlation, with qPCR underestimating copy number. All tissue samples were analyzed by qPCR. D. In addition to mutaions, sequencing of 66 HNSCC tumor tissues identified eight SNPs. Three SNPs clustered together (indicated by *) and were present in 31 samples (47.0%). Other SNPs were repeated in seven, four, and two separate tumor samples.

has also been implicated with poor prognosis for HNSCC (41) and MET is a known driver of epithelial to mesenchymal transition (11). We report for the first time the identification of novel MET alterations in the SEMA, JM, and TK domains in human HNSCC. The precise function is part of ongoing studies (13, 17). Previously, DiRenzo and colleagues and Aebersold and colleagues described TK domain mutations in HNSCC in Y1230C and Y1235D in up to 10.9% of tumors (13, 38). In these studies, MET mutations primarily occurred in lymph node metastases and could only be detected with a higher sensitivity method (single-strand conformational polymorphism). In a subsequent study, however, Morello and colleagues did not identify any MET mutations in HNSCC (26). We used standard PCR amplification and sequencing technology and identified two novel TK domain mutations in one lymph node metastasis and one primary tumor (T1275I and V1333I ). We did not detect Y1230C and Y1235D mutations. TK domain mutations are reported to be somatic mutations in HNSCC and their functional importance is well established in certain papillary renal cell cancers (germline or somatic mutations; ref. 42). The functional role of TK domain mutations in HNSCC remains to be determined and high-sensitivity mutation screening/sequencing may be required as seen for the EGFR T790M mutation in NSCLC (43).

We identify for the first time in HNSCC SEMA and JM domain mutations/variants. Such mutations/variants have previously been reported in lung cancer. The JM domain changes have been implicated in increased motility and invasiveness in SCLC (12, 17) and may have transforming properties (17). Preliminary reports suggest that both SEMA and JM domain mutations/variants can contribute to MET activation and may alter sensitivity to MET inhibitors (44). In contrast to TK domain mutations, SEMA and JM domain mutations/variants may be found in either germline DNA or somatically. MET inhibition can readily be achieved with small-molecule TK inhibitors: PF-2341066 used here for in vivo studies is currently in phase I clinical testing. SU11274 is a poorly water-soluble earlier inhibitor developed by Sugen/Pfizer and clinical development was not pursued.

Various parameters have been suggested as predictors of response to MET kinase inhibitors (31), including strong expression as seen, for example, in NSCLC (12), gene amplification as seen for gastric carcinomas (14), and kinase domain mutations (45) and potentially ligand expression as reported for amphiregulin/EGFR (46). Unlike NSCLC and colon cancer, K-Ras mutations are not commonly observed in HNSCC. Our data suggest that generally higher MET expression levels correlate with increased sensitivity to MET inhibition but are not sufficient to explain the remaining substantial variation in IC50 values. Additional factors modulate responsiveness and future studies may include correlation with increased gene copy number, HGF expression, use of parallel RTK signaling cascades (Fig. 1C), and potentially gene mutation status (e.g., PTEN).

AKT activation and ERK activation are oftentimes separate events, with AKT being more prominently involved in cell survival and ERK in proliferation (47). Although sometimes regulated together (i.e., EGFR TK domain mutated NSCLC; ref. 47), it seems that, for most, HNSCC regulation is separate (Fig. 2B). It is possible that concurrent inhibition of the pathways leading to ERK activation will increase therapeutic benefit.

Despite the lack of EGFR mutations in HNSCC in the United States (48), most HNSCC are sensitive to EGFR inhibitors and overexpression is abundant (4). Recent evidence in NSCLC suggests a common signaling pathway via HER3/erbB3 (5–9). Specifically Engelman and colleagues (6) implicated erbB3 signaling as the mediator of amplified MET “overtaking” mutant-EGFR signaling in a NSCLC in vitro model of acquired gefitinib resistance. On the other hand, the recent study by Tang and colleagues (9) suggested a central role for erbB3 in mediating the efficiency of dual MET/EGFR inhibition against T790M-EGFR–mediated resistance in the absence of prior EGFR TKI selection pressure. Our data for HNSCC now also suggest a similar role of MET/erbB3 in the absence of EGFR selection pressure.
Given the broad use of EGFR inhibition in HNSCC patients and the limited single-agent response rate (3), ways to increase efficacy with dual-kinase or multikinase inhibition are pivotal.

Gene copy numbers seemed to be higher in tumor tissue samples compared with cell lines; most notably, we did not identify any amplified cell lines. Although the reasons for this are unclear (possible selection pressure, bias of cell line/tumor choice), such tumors with higher gene copy numbers may be more sensitive to MET inhibition.

Our data also suggest exploring MET inhibition in combination with cisplatin. Interestingly, Akervall and colleagues when comparing cisplatin-sensitive and cisplatin-resistant HNSCC cell lines by gene microarray techniques identified MET overexpression in resistant lines (36). Henceforth, MET may be involved in mediating cisplatin resistance or could be a general poor prognostic marker. Further studies are indicated.

The proangiogenic properties of the MET/HGF axis are well established and MET signaling can initiate vascular endothelial growth factor production, a critical angiogenic switch via Shc (49). We provide the first evidence of antiangiogenic effects of MET inhibition in HNSCC in vivo using PF-2341066 in a Matrigel plug model. A caveat is that murine HGF does not sufficiently activate human MET (50); therefore, the use of a human HGF transgenic model. A caveat is that murine HGF does not sufficiently activate in vivo inhibition in HNSCC by gene microarray techniques identified MET overexpression in tumor tissues and cell lines. Furthermore, we describe evidence of amplification and the presence of novel TK, SEMA, and JM domain mutations. The consistent effects of MET inhibition validate this target further and synergy with cisplatin and erlotinib is therapeutically relevant. Further mechanistic studies into the role of MET-mutated/amplified HNC are indicated and will allow us to better use MET-specific drugs for selected patient groups.

**Disclosure of Potential Conflicts of Interest**

E.E.W. Cohen and R. Salgia received a major commercial research grant from Pfizer; E.E.W. Cohen, R. Salgia, and E. Vokes are consultants for Pfizer. The other authors disclosed no potential conflicts of interest.

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**References**

The Role of MET in Head and Neck Cancer


The MET Receptor Tyrosine Kinase Is a Potential Novel Therapeutic Target for Head and Neck Squamous Cell Carcinoma


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