HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2012 December 07.

Published in final edited form as:

Oncogene. 2012 June 7; 31(23): 2888–2898. doi:10.1038/onc.2011.460.

A positive feedback loop between HER2 and ADAM12 in human head and neck cancer cells increases migration and invasion

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Abstract

Increased activation of epidermal growth factor receptor (EGFR) family members such as HER2/Erbb2 can result in more aggressive disease, resistance to chemotherapy and reduced survival of head and neck squamous cell carcinoma (HNSCC) patients. In order to identify mechanisms through which these receptor tyrosine kinases accelerate tumor progression, the regulation of metalloprotease expression by EGFR family members was investigated in 11 SCC cell lines. HER2 expression was significantly correlated with ADAM12 (A Disintegrin And Metalloprotease 12) expression in these cell lines and was co-expressed in human head and neck cancers. Inhibition of HER2 or EGFR decreased ADAM12 transcripts while HER2 transfection up-regulated ADAM12 expression. To understand the molecular mechanisms underlying HER2 regulation of ADAM12, we investigated the signaling pathways directing ADAM12 production in SCC cells. Inhibition of phosphatidyl inositol-3-kinase (PI3K) or mammalian Target of Rapamycin (mTOR) decreased ADAM12 transcripts in HER2-expressing SCC cells, while transfection with AKT increased ADAM12 mRNA. Experiments utilizing ADAM12 transfection or siRNA targeting of ADAM12 revealed that the protease increased both the migration and invasiveness of oral SCC cells. Surprisingly, ADAM12 also increased HER2 message, protein levels, and activity through an Ets1-dependent mechanism. Collectively, these results reveal a novel positive activation loop between ADAM12 and HER2 that may contribute to HNSCC progression.

Keywords

HER2; ADAM12; head and neck cancer; oral cancer; migration; invasion

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Conflict of interest: Dr Hansen's work has been funded by the NIH, the Nebraska Cancer and Smoking-Related Diseases Research Program, and The Dobleman Institute for Head and Neck Cancer Research. The other authors declare no conflict of interest.

Supplementary information is available at http://www.nature.com/onc/index.html.

INTRODUCTION

Head and neck cancer is the sixth most common cancer worldwide with more than 35,000 new cases annually in the U.S. alone (Pisani et al. 2002;Jemal et al. 2009). About ninety percent of these cancers are squamous cell carcinomas (SCCs), and they usually present as locally advanced stage III or IV disease and often metastasize even when identified early (Ragin et al. 2007). Despite extensive research and treatment advances, there has been little improvement in patient 5-year survival rates, which are currently 20–40% for those with locoregionally advanced disease (Chin et al. 2006). One promising treatment option is epidermal growth factor receptor (EGFR) targeted therapeutics (Chin et al. 2006), although little investigation of EGFR's mechanisms or biological functions in oral cancer has been undertaken.

Aberrant expression of EGFR and its dimerization partner HER2 are indicators of poor prognosis in head and neck squamous cell carcinoma (HNSCC). EGFR, over-expressed in 80–90% of HNSCC, is an early event in HNSCC that is associated with more aggressive disease, resistance to chemotherapy and poorer survival (Forastiere et al. 2001;Ang et al. 2002;Kong et al. 2006;Ettl et al. 2008;Ibrahim et al. 1997;Hanawa et al. 2006). The frequency of HER2 over-expression varies between 6% and over 80% depending on tumor type and is associated with shorter disease-free and overall survival (Brunner et al. 2010;Sato-Kuwabara et al. 2009). For these reasons, EGFR and HER2 have been considered appealing targets for cancer therapy. Agents targeting EGFR have been used in a number of clinical trials and are now approved for HNSCC treatment, revealing increased response rates and increased overall survival when combined with standard cytotoxic therapy (reviewed in (Moon et al. 2010)). Intriguingly, the activation status of HER2 but not EGFR predicts resistance to the EGFR inhibitor gefitinib in HNSCC (Erjala et al. 2006), suggesting that interactions between family members are important for unknown reasons.

EGFR family members can contribute to SCC invasion and progression by up-regulating matrix metalloproteinases (MMPs) that digest extracellular matrix (ECM), process growth factors, and activate cell adhesion molecules (reviewed in (Hudson et al. 2009)). Tumor cells frequently over-express MMPs allowing for degradation of the basement membrane and invasion of the surrounding the tissue. In recent years, another family of proteases, the ADAMs (A Disintegrin And Metalloproteinases), have been described and subsequently found to be increased in various human cancers (Carl-McGrath et al. 2005;Rocks et al. 2006;Lendeckel et al. 2005;Kodama et al. 2004;Kveiborg et al. 2005). Among the ADAM family members increased in cancer, ADAM12/Meltrin α is expressed at low levels in most normal adult tissues, and is over-expressed in a large proportion of some human carcinomas, including HNSCC (Carl-McGrath et al. 2005;Mino et al. 2009;Kornberg et al. 2005;Markowski et al. 2009;Roepman et al. 2005)[,26]. In oral cancers, ADAM12 up-regulation correlates with HNSCC development and progression to metastasis (Kornberg et al. 2005;Markowski et al. 2009;Roepman et al. 2005) although no investigation of its mechanisms of action in head and neck cancer have been previously reported.

ADAM12 is a multifunctional protein with a metalloprotease domain, disintegrin-like region, cysteine-rich domain, transmembrane domain, a prodomain that remains associated

with the mature form of the protein, and a cytoplasmic tail that can signal through phosphotidyl inositol-3-kinase (PI3K) and other pathways (reviewed in (Jacobsen and Wewer 2009)). In humans there are two ADAM12 proteins produced from alternative splicing: ADAM12L, the long form that includes all the domains mentioned, and ADAM12S, the secreted form that lacks the cytoplasmic tail and transmembrane domain but has a unique 33 amino acid tail of unknown function (Jacobsen and Wewer 2009).

Multiple functions for ADAM12 have been proposed. The protease domain of ADAM12 can cleave extracellular matrix substrates including collagen IV, fibronectin and gelatin (Roy et al. 2004). ADAM12 also causes pathological auto-stimulation of tumor cell growth through proteolytic shedding of EGFR ligands including HB-EGF (heparin-binding EGF) (reviewed in (Kveiborg et al. 2008)). Other ADAM12 domains appear to have a number of different functions as well. For example, the disintegrin domain of ADAM12L associates with β1 integrins at the plasma membrane to facilitate cell adhesion (Thodeti et al. 2005;Kawaguchi et al. 2003). Whether ADAM12 influences HNSCC progression through these or other mechanisms, and the biological significance of its interactions with receptor tyrosine kinases are currently unknown.

The present study was undertaken to evaluate whether EGFR family members modulate metalloproteinase expression in HNSCC. A correlation between HER2 and ADAM12 expression was discovered in HNSCC cell lines and in human head and neck cancers. Further experiments revealed a positive feedback loop between HER2 and ADAM12 that increases HNSCC migration and invasion. This research reveals a novel mechanism through which HER2 and ADAM12 may contribute to the progression of HNSCC and other cancers.

RESULTS

Increased HER2 expression was associated with ADAM12 expression in human HNSCC cell lines

Eleven human HNSCC cell lines and the control keratinocyte cell line HaCaT were used in an initial screen to identify potential associations between metalloproteinases and HER family receptors for further study. Immunoblotting of these lines revealed that most HNSCC cell lines had elevated HER2 compared to the control cells, while EGFR and HER3 were not increased in any of the SCC cells (Fig. 1A–B). Correlations between HER receptor levels and protease expression were assessed following measurement of transcripts for seven different MMPs and five different ADAMs (complete list of proteases provided in the Methods section) using real-time RT-PCR. HER2 levels were significantly correlated with ADAM12 transcripts (Fig. 1C), and were inversely correlated with MMP14 ($R = -0.649$, $P = 0.023$, not shown). No significant correlations between EGFR or HER3 and any of proteases were identified. In order to further investigate the relationship between HER2 and ADAM12, the expression of both the prototypic transmembrane form, ADAM12L, and the smaller secreted form, ADAM12S, was determined using primers specific for these splice variants. Expression of ADAM12S transcripts was significantly correlated with HER2 in the HNSCC cells while the association between HER2 and ADAM12L did not reach significance (Fig. 1D,E). Immunoblotting for ADAM12 revealed ADAM12 protein in all the cell lines (Fig. 1A), with a band at approximately 97 kDa as expected for this antibody

(ProteinTech Group, Chicago, IL). Immunohistochemical analysis of human head and neck cancers revealed that all SCC had detectable HER2, one third had detectable ADAM12, and all tumors that expressed ADAM12 were also immuno-positive for both EGFR and HER2 (Fig. 1F). These data suggested that investigation of a relationship between ADAM12 and HER2 warranted further study.

HER2 and ADAM12 expression were increased in oral SCC cells derived from a recurrence compared to cells from the primary tumor from the same site

The expression of ADAM12 and HER2 was evaluated in a primary tumor and from a recurrence at the same site. Cell line UM-SCC 74A is derived from a tongue tumor, and UM-SCC 74B is from a tumor recurrence one year after the initial biopsy (Worsham et al. 1999). HER2 protein was detected in the recurrent tumor cell line UM-SCC 74B but not in the primary tumor line UM-SCC 74A (Supplemental Fig. 1). The message for HER2 was detected in both of these cell lines, although it was significantly increased in the UM-SCC 74B cells (Fig. 2A). EGFR protein was also elevated in UM-SCC 74B cells compared to UM-SCC 74A cells, while HER3 was not detectable in either line (Figs. 1A–B and Supplemental Fig. 1). ADAM12L and ADAM12S transcripts were elevated in the cells from the recurrence when compared to the primary tumor (Fig 2B). Immunofluorescence and immunoblotting confirmed increased ADAM12 protein in the cell line from the recurrent tumor compared to the original tumor cell line (Fig 2C and Fig. 1A). ADAM12 immunolocalization in the recurrent tumor cell line was particularly intense in perinuclear regions (Fig. 2C arrows).

HER2 up-regulated ADAM12 expression

To test the hypothesis that HER2 signaling upregulates ADAM12, the low HER2-expressing cell line UM-SCC 74A was transfected with a HER2 mammalian expression construct. Immunoblotting and real-time RT-PCR analyses confirmed increased HER2 following transfection of UM-SCC 74A (Fig. 3A,B). HER2 transfection significantly increased transcripts for both ADAM12L and ADAM12S in the UM-SCC 74A cells (Fig. 3A). AG825, a potent and relatively specific inhibitor of HER2 (Madson et al. 2006), reduced HER2 activity in UM-SCC 74B cells, as detected by HER2 phosphorylation on immunoblot (Fig. 3C). Inhibition of HER2 also reduced ADAM12L and ADAM12S transcripts in a dose-dependent manner, such that ADAM12 transcripts in the high-expressing UM-SCC 74B cell line after treatment with the highest dose were similar to the levels in untreated low-expressing cells (Fig. 3D). Collectively, these experiments demonstrated that HER2 induces both ADAM12L and ADAM12S expression in oral SCC cells. Because EGFR and HER2 can heterodimerize to transduce signals, we also investigated whether EGFR influences ADAM12. EGFR activity was inhibited in UM-SCC 74B cells using AG1478, as shown in Fig. 3E, lanes 5–8. Inhibition of EGFR also reduced ADAM12 transcripts in UM-SCC 74B cells (Fig. 3F), similarly to inhibition of HER2.

HER2 up-regulated ADAM12 expression through both PI3K and JNK pathways

To elucidate the mechanisms by which HER2 regulated ADAM12 expression, intracellular signaling pathways downstream from HER2 were examined. Phosphatidyl inositol-3-kinase (PI3K) signaling, as indicated by AKT phosphorylation, was elevated in the high ADAM12

UM+SCC 74B cells when compared to low ADAM12 UM-SCC 74A cells (Fig. 4A). Inhibition of PI3K with either LY294002 or wortmannin, confirmed by immunoblotting for phosphorylated AKT (Fig. 4A), reduced ADAM12L and ADAM12S transcripts in high ADAM12-expressing UM-SCC 74B cells by 45% and 88%, respectively, to levels similar to that of UM-SCC 74A (Fig. 4B). Inhibition of the downstream mTOR (mammalian Target Of Rapamycin) with rapamycin also significantly reduced ADAM12 transcripts (Fig. 4C). Conversely, transfection of the low HER2- and low ADAM12-expressing line UM-SCC 74A with a mammalian expression construct for AKT increased ADAM12S and ADAM12L transcripts by more than four-fold (Fig. 4D).

To eliminate the possibility that the effects of PI3K pathway modulation on ADAM12 were the result of altered HER2 transcription (Ou et al. 2008), UM-SCC 74A cells transfected with a HER2 expression vector, in which HER2 expression was driven by a cytomegalovirus promoter, were also treated with the PI3K inhibitor LY294002. The inhibitor did not affect HER2 transcripts but still reduced ADAM12 transcripts (Fig. 4E, F), consistent with PI3K modulating ADAM12 transcripts independently of any effect on HER2 expression.

Examination of other pathways downstream from HER2 revealed lesser effects on ADAM12 transcripts. Activation of ERK1/2, JNK and p38 kinase was not increased in UM-SCC 74B cells compared to UM-SCC 74A cells (Supplemental Figure 2A–C). MEK, JNK, and P38 kinase inhibitors effectively targeted these pathways (Supplemental Fig. 2A–C) and reduced ADAM12 transcripts in SCC74B cells (Supplemental Fig. 2D), although to a lesser extent than blockade of PI3K signaling. Among these inhibitors, inhibition of JNK was the most effective at reducing ADAM12 transcripts, with an approximately 29% and 79% reduction in ADAM12L and ADAM12S transcripts, respectively. In contrast, inhibition of PKC with either rottlerin or GF109203X had no effect on ADAM12 mRNA (Supplemental Figure 2D). Thus, HER2 regulates ADAM12 mRNA through multiple mechanisms, consistent with previous publications showing multiple mechanisms of ADAM12 regulation in other cell types (Le et al. 2003;Le et al. 2005).

ADAM12 up-regulated HER2 expression through Ets1

In order to determine whether ADAM12 can modulate the expression and activity of the orphan receptor HER2 in HNSCC cells, we examined the influence of ADAM12 on receptor expression and activity. Transfection of ADAM12L increased HER2 message by more than two-fold in the low HER2-expressing cell line while transfection with an ADAM12S construct increased HER2 message by 50% (Fig. 5A–B). HER2 protein, measured using immunofluorescence, and HER2 activity, measured by immunoblotting for phospho-HER2, were also increased by ADAM12L transfection (Fig. 5C). Conversely, siRNA knockdown of ADAM12 (Fig. 5D) reduced HER2 message by more than 40% (Fig. 5E). Modulation of ADAM12 had no significant effects on EGFR expression (Fig. 5B,E).

To identify mechanisms through which ADAM12 regulates HER2 transcription, a number of transcription factors were examined in the low and high ADAM12-expressing cell lines. Ets1 transcripts (Fig. 5F) and protein levels (Fig. 5G) were elevated in the high ADAM12-expressing line UM-SCC 74B when compared to the low ADAM12-expressing UM-SCC

74A. Transfection of cells with ADAM12L or ADAM12S up-regulated Ets1 transcripts (Fig. 5H), while knockdown of Ets1 reduced HER2 transcripts by 60% (Fig. 5I). From these data, we conclude that ADAM12 regulation of Ets1 is one mechanism through which this protease up-regulated HER2 expression.

ADAM12 increased tumor cell migration and invasion

We hypothesized that up-regulation of ADAM12 facilitates tumor progression by increasing tumor cell migration and invasion. Consistent with this hypothesis, migration of high ADAM12-expressing UM-SCC 74B cells was 67% faster than in the low ADAM12-expressing UM-SCC 74A cells in a scratch assay (Fig. 6A). In addition, knockdown of ADAM12 in UM-SCC 74B significantly decreased cell migration (Fig. 6B). Cell invasion through a Matrigel-coated membrane in a Fluoroblok assay was 50% higher in UM-SCC 74B cells when compared to UM-SCC 74A cells (Fig. 6C, black bar compared to white bar) and transfection of ADAM12S or ADAM12L increased the invasiveness of UM-SCC 74A cells by 89% and 113%, respectively (Fig. 6C, gray bars compared to white bar). These data demonstrate that up-regulation of ADAM12 increased both the migration and invasiveness of HNSCC cells.

DISCUSSION

The present study identified a correlation between ADAM12 and HER2 expression in HNSCC. Further investigation revealed a positive feedback loop between HER2 and ADAM12, which increased the migration and invasiveness of HNSCC cells. In addition, EGFR also positively regulated ADAM12 expression. HER2 induced ADAM12 expression, which in turn up-regulated and activated HER2. Thus, there is a positive feedback loop between ADAM12 and HER2 that may play a role in HNSCC progression. Thus, our results suggest that up-regulation of ADAM12 expression may be one mechanism through which EGFR or HER2 over-expression results in more aggressive disease.

Understanding the regulation of ADAM12 expression by HER2 in oral SCC tumors may facilitate the development of additional therapeutic interventions for the treatment of tumors. In this regard we focused on the analysis of HER2-dependent signaling pathways including MAP kinases and PI3K/AKT/mTOR pathways in primary and recurrent SCC tumors. Consistent with other reports demonstrating multiple pathways regulate ADAM12 expression (Le et al. 2003;Le et al. 2005), inhibition of each of these kinases decreased ADAM12 transcription to some extent. Inhibition of PI3K, however, was most effective at reducing ADAM12 expression. Because feedback loops exist whereby inhibition of PI3K can downregulate HER2 transcription (Ou et al. 2008), additional experiments were performed to isolate the influence of PI3K inhibition on ADAM12 independent from any effect on HER2. Transfection of HER2 followed by inhibition of PI3K reduced ADAM12 expression to constitutive levels.

ADAM12 has two splice variants, the soluble, short form (ADAM12S) and the long form (ADAM12L) (Jacobsen and Wewer 2009). HER2 up-regulated the expression of both transcripts. Both ADAM12L and ADAM12S have a prodomain, a metalloprotease domain that encodes a viable enzyme, disintegrin and cysteine-rich domains involved in cell

adhesion, and EGF-like repeats. They are unique in that the long form also contains a transmembrane region and cytoplasmic tail, while the short form instead has a unique 33 amino acid sequence (reviewed in (Jacobsen and Wewer 2009)). Although both ADAM12 splice variants are frequently cytosolic, ADAM12S is more likely to be secreted while ADAM12L can be inserted into the cytoplasmic membrane (reviewed in (Jacobsen and Wewer 2009)). Surprisingly, ADAM12L and ADAM12S both induced HER2 expression, although ADAM12L was more effective at increasing HER2 expression.

We found that ADAM12 up-regulated HER2 through modulation of the transcription factor Ets1. Ets family regulation of HER2 has previously been reported (Xing et al. 2000;Chang et al. 1997;Scott et al. 2000), although further examination of the mechanisms through which ADAM12 regulation of Ets1 altered receptor expression is required. Ets1 over-expression has been reported in invasive cancers of the oral cavity (Pande et al. 1999) and elsewhere (reviewed in (Seth and Watson 2005)). Ets1 is a biological marker of invasive potential and lymph node metastasis in oral SCC and correlates with tumor stage, lymph node involvement, distant organ metastasis and reduced disease-free survival (Pande et al. 2001;Arora et al. 2005). One report suggests that smokeless tobacco extract increases expression of Ets1 and alters expression of cell cycle regulators in oral leukoplakia (Rohatgi et al. 2005). Ets1 expression correlates with intratumoral microvessel density, VEGF and stromyelysin expression in oral precancerous lesions and SCCs (Arora et al. 2005). Ets1 is an effector of EMT downstream of Snail and TGF-β1 that increases invasiveness of SCC (Shirakihara et al. 2007). Thus, Ets1 may be an important effector of ADAM12-stimulated HNSCC progression through up-regulation of HER2 and other mechanisms, a hypothesis warranting further study.

Neither splice variant of ADAM12 had an effect on EGFR transcripts. Both splice variants have proteolytic activity, with distinct substrates identified for each. While ADAM12S cleaves insulin growth factor binding protein-3 and -5, ADAM12L can shed pro-heparin binding epidermal growth factor, resulting in activation of EGFR (reviewed in (Jacobsen and Wewer 2009)). Thus, increased ADAM12L expression may lead to increased EGFR activity by release of EGFR ligands. The cytoplasmic tail unique to the long form of ADAM12 is involved in both localization of the protease at the cell surface as well as signaling through Src homology 3 (SH3)- and SH2-containing proteins, including Src family members and PI3K p85, suggesting additional and diverse possibilities for ADAM12L signaling (reviewed in (Jacobsen and Wewer 2009)). Whether ADAM12L and ADAM12S have distinct roles in HNSCC development and progression, through the activation of EGFR or other mechanisms, remains to be elucidated.

Recently, gene expression analysis has demonstrated elevated ADAM12 in HNSCC tissues correlating with metastasis (Kornberg et al. 2005;Roepman et al. 2005). Our data demonstrated co-expression of ADAM12, HER2 and EGFR in some HNSCC and that increased ADAM12 in HNSCC cells results in increased HER2 signaling. The frequency of EGFR, HER2 and ADAM12 co-expression in HNSCC in vivo and the prognostic significance of their co-expression, remain to be determined. Increased expression of HER2 has been reported with a poor clinical prognosis in breast cancer patients who have shorter disease-free and overall survival (Slamon et al. 1987) suggesting that HER2 expression may

be linked to changes in down-stream gene expression leading to increased metastatic ability. HER2 over-expression in some HNSCC is associated with poor clinical prognosis in which patients have shorter disease-free and overall survival (Brunner et al. 2010;Sato-Kuwabara et al. 2009). In addition, EGFR as well as HER2 up-regulated the expression of ADAM12. Since these receptors can heterodimerize to transduce signals, their individual effects may be due, in part or in whole, to altered EGFR/HER2 dimer-activated signaling. In addition to its effects on HER2 expression in our studies, ADAM12L acts as a sheddase for HB-EGF (reviewed in (Jacobsen and Wewer 2009)), and thus may increase EGFR and HER2 activity through an additional mechanism as well. Since EGFR is an important therapeutic target for head and neck cancer therapy, ADAM12 may be a potentially important target for HNSCC treatment because of its ability to alter both HER2 and EGFR signaling. Somewhat surprisingly, given the evidence of a strong positive correlation between EGFR and MMP9 in cancers including HNSCC (O-Charoenrat et al. 2000), we found no such correlation in our screen of eleven HNSCC cell lines. Although we can only speculate about the causes for this discrepancy, it may have resulted from the low variation in EGFR protein levels among the HNSCC lines that were used.

In summary, our work demonstrated a positive feedback loop between HER2 and ADAM12 that increases HNSCC cell migration and invasion. Since an inhibitor of ADAM12 is currently in clinical trials (reviewed in (Jacobsen and Wewer 2009)), this research suggests that disruption of ADAM12, independently or in conjunction with EGFR/HER2 inhibition, may be a useful target for prevention of HNSCC progression. Further experiments are necessary to elucidate the mechanisms through which ADAM12 acts as well as the biological significance of the events demonstrated here.

MATERIALS AND METHODS

Cell culture

Eleven human HNSCC cell lines (UM-SCC 1, UM-SCC 10A, UM-SCC 11B, UM-SCC 12, UM-SCC 13, UM-SCC 17B, UM-SCC 23, UM-SCC 74A, UM-SCC 74B, and UM-SCC 81B) were utilized (Squire et al. 2002;Grenman et al. 1991;Akervall et al. 2004). The cell lines were previously characterized and genotyped as described in (Brenner et al., 2010;Squire et al. 2002;Grenman et al. 1991;Akervall et al. 2004). The cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 1% penicillin (10,000 units/ml)-streptomycin (10,000 μg/ml)(Invitrogen) and 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA) at 37° C and 5% CO_2. At approximately 80% confluence, this medium was replaced with serum-free DMEM for 24 h prior to sample collection. Cells were also treated with UO126 (10 μM, Calbiochem San Diego, CA), 10 μM SB202190 (Calbiochem. San Diego, CA), 10 μM LY294002 (Calbiochem), 20 μM JNK II inhibitor (SP600125, Calbiochem), 0.5–1.0 μM wortmannin (Calbiochem), AG825 (AG Scientific, San Diego CA), AG1478 (AG Scientific), rapamycin (AG Scientific), 10 μM rottlerin (Calbiochem), 10 μM GF109203X (Calbiochem), or 10 μM SU6656 (Calbiochem) in serum-free DMEM for 24 h. Cells were transfected at 60–80% confluence using Lipofectamine/Plus reagents (Life Technologies, Inc., Carlsbad, CA) according to the manufacturer's protocol with mammalian expression constructs for HER2

(Liu et al. 2001), Akt (plasmid 10841 myrAkt delta4-129, Addgene, Cambridge, MA), Erk1 (plasmid 14440 pcDNA-3-T7-ERK1, Addgene), p38 kinase (Plasmid 12658, pMT3P38 Addgene), ADAM12L (Thodeti et al. 2005) and ADAM12S (Thodeti et al. 2005). Transfection efficiency for each experiment was determined by transfection with an Enhanced Green Fluorescent Protein (GFP)(Clontech, Mountain View, CA). Supplemental Fig. 3A shows representative GFP fluorescence and the mean transfection efficiency of 34%. For siRNA experiments, cells were transfected with siRNA targeting ADAM12 (Santa Cruz Biotechnology, Santa Cruz, CA), Ets1 (Santa Cruz Biotechnology), or with a negative control siRNA (Santa Cruz Biotechnology) using siQuest transfection reagents (Mirus Bio, Madison, WI) according to the manufacturer's instructions. Transfection with Cy3-tagged siRNA was used as a transfection control (Mirus Bio). As shown in Supplemental Fig. 3B, the mean transfection efficiency was 91%. Cells were visualized using a Nikon microscope with 10× and 40× objectives and phase and fluorescence microscopy.

Immunoblotting

Cells were lysed in buffer containing 25 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 10% glycerol, 1% Triton-X-100, 1 mmol/L EDTA, Complete Protease Inhibitor Cocktail (Roche, Manneheim, Germany), 1 mmol/L Na_3VO_4, 1.5 μmol/L EGTA, and 10 mmol/L NaF. Protein was assessed by the Bradford assay (Bio-Rad laboratories, Herculues, CA) using bovine albumin as the standard. Immunoblotting was performed using antibodies recognizing HER2 (Cell Signaling Technology, Inc, Danvers, MA), phospho-HER2 (Cell Signaling Technology, Inc.), EGFR (Santa Cruz Biotech Inc.), phospho-EGFR (Santa Cruz Biotech Inc), HER2 (Cell Signaling Technology, Inc.) β-actin (Sigma Chemical Co, St Louis, MO), phospho-AKT (Cell Signaling Technology, Inc.), phospho-JNK (Cell Signaling Technology, Inc.), ADAM12 (ProteinTech Group, Inc. Chicago, IL) and p-44/42 MAPK (ERK1/2) (Cell Signaling Technology, Inc.) using standard techniques as we have previously published (El Abaseri et al. 2006;Madson et al. 2009). The evenness of loading and transfer was confirmed by Ponceau S (Sigma Chemical Co.) staining and by actin immunoblotting. Immunoreactive proteins were visualized with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) and enhanced chemiluminescence (Thermo Scientific, Rockford, IL). Densitometry was performed using a Chemidoc XRS Molecular Imager (Bio-Rad Laboratories, Inc., Hercules, CA).

Immunostaining

Immunofluorescence and immunohistochemistry were performed using primary antibodies recognizing ADAM12 (Santa Cruz Biotechnology or ProteinTech, Inc.) or HER2 (Cell Signaling) together with negative controls where the primary antibody was omitted, Alexafluor 488-conjugated secondary antibodies (Invitrogen), and 4',6- diamidino-2-phenylindole diactate (DAPI)(Invitrogen). Immunohistochemistry of formalin-fixed sections was performed using a horseradish peroxidase-conjugated secondary antibody and diaminobenzidine substrate with hematoxylin counterstaining. Photomicroscopy was performed using a Nikon microscope and cooled camera with Magnifier software (Boyce Scientific, Gray Summit, MO). Scoring of the immunohistochemistry experiments was performed using a semi-quantitative, four-grade (0, 1+ 2+, and 3+) method with the

investigator blinded as to the identity of the samples. Cases in which fewer than 10% of the tumor cells were only weakly stained were judged as negative.

RNA extraction and real-time RT-PCR

Total RNA was isolated from cells using an RNeasy Mini Kit (Qiagen Sciences, Maryland, MD) according to the manufacturer's instructions. cDNA was prepared with the First Strand cDNA Synthesis Kit (Invitrogen) using oligo dT primers (Invitrogen). Primers included forward (5'- AGC CGC GAG CAC CCA AGT- 3') and reverse (5'- TTG GTG GGC AGG TAG GTG AGT T- 3') primers for HER2, forward (5'-AGC TAT GTC TTA GAA CCA ATG AAA AGT G-3') and reverse (5'-CCC CGG ACG CTT TTC AG-3') primers recognizing both ADAM12 splice variants, forward (5'- CAC CAT TGA AAA ACT AAG GTG TGT G -3') and reverse (5'- GAG CCT GAC AGG GTT GGA AG-3') primers specific for the ADAM12L variant, forward (5'- CTG GGC ACC TCC CTT CTG T -3') and reverse (5'- TGC TTC TGC TTG CCG GA -3') primers specific for the ADAM12S variant, and forward (5'-GAA ACC TGC CAA GTA TGA TGA C-3') and reverse (5'-ACC TGG TCC TCA GTG TAG C-3') primers for GAPDH. MMP2, MMP3, MMP7, MMP9, MMP12, MMP13, MMP14, ADAM9, ADAM10, ADAM 17, and ADAM19 were also assessed by real-time RT-PCR in the 11 SCC cell lines and HaCaT controls (see Supplemental Table 1 for primer sequences). Real-time RT-PCR was performed in triplicate using SYBR Green Universal MasterMix (PE-Applied Biosystems Carlsbad, CA) with a first step of 10 min at 95° C, followed by 40 cycles of amplification (95° C for 3 sec and 60° C for 30 seconds) using the 7500 Fast Real-Time PCR System (Perkin-Elmer, Foster City, CA). Fold expression relative to controls was determined after normalizing to GAPDH expression.

Migration and invasion assays

Scratch assays to assess cell migration were performed using confluent dishes following plating of 325,000 cells/well in a 6-well dish. Some cells were transfected at 80% confluence and the assay performed 24 h later after the cells reached confluence. A wound was created across the confluent dish using a pipette tip, cells were washed with phosphate-buffered saline and then incubated in serum-free DMEM containing 10 μM mitomycin C (Sigma) to prevent cell proliferation. Images were acquired in at least six marked locations at multiple time points using a Nikon phase contrast microscope (Boyce Scientific) with a 10× objective and cell migration assessed using Image J software (NIH Freeware, Bethesda, MD). A stage micrometer was used to convert pixels in the digital images to microns and the area migrated in each field expressed as microns squared. Experiments were performed twice to confirm reproducible results and data from one experiment are shown. The Fluoroblok (Becton-Dickinson, Franklin Lakes, NJ) invasion assays were performed according to the manufacturer's instructions. Cells were transfected at 80% confluence 24 h before staining with 10 μg/ml $DilC_{12}$ (Becton-Dickinson) in medium with serum for 1 h and then were replated. Fluoroblok assays were performed in triplicate using 50,000 cells plated in each upper well in serum-free DMEM with the bottom chamber containing DMEM with 10% serum to allow measurement of invasion through a matrigel-coated membrane. Fluorescence at 549/565 um (Excitation/Emission) was measured using an EnSpire 2300 Multilabel Reader (Perkin Elmer, Waltham, MA). Experiments were performed three times and representative data from one experiment are shown.

Statistical analysis

Analysis of the eleven SCC cell lines in the initial screen for correlations between receptor and protease expression is given as P values for a test of significance of Pearson's Correlation Coefficient (R). In other experiments, statistical analyses for comparison of two groups was performed using a Student's t-test, where significance was considered $p \leq 0.05$. For statistical analysis of data collected at multiple time points, two-way ANOVA was used, where $p \leq 0.05$.

Supplementary Material

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ACKNOWLEDGMENTS

The cell lines used in this study were gifts from Drs. Greg Oakley (University of Nebraska, Lincoln) and Thomas Carey (University of Michigan). We also thank Drs. U. Wewer and C. Frohlich (University of Copenhagen, Denmark) for the gifts of ADAM12 plasmids. This research was supported by the National Institutes of Health (1RO1ES015585), the State of Nebraska LB595, and The Dobleman Head and Neck Cancer Institute. This investigation was conducted in a facility constructed with support from Research Facilities Improvement Program Grant Numbers 1CO6RR17417-01 and G20RR024001 from the National Center for Research Resources, National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Research Resources or the National Institutes of Health.

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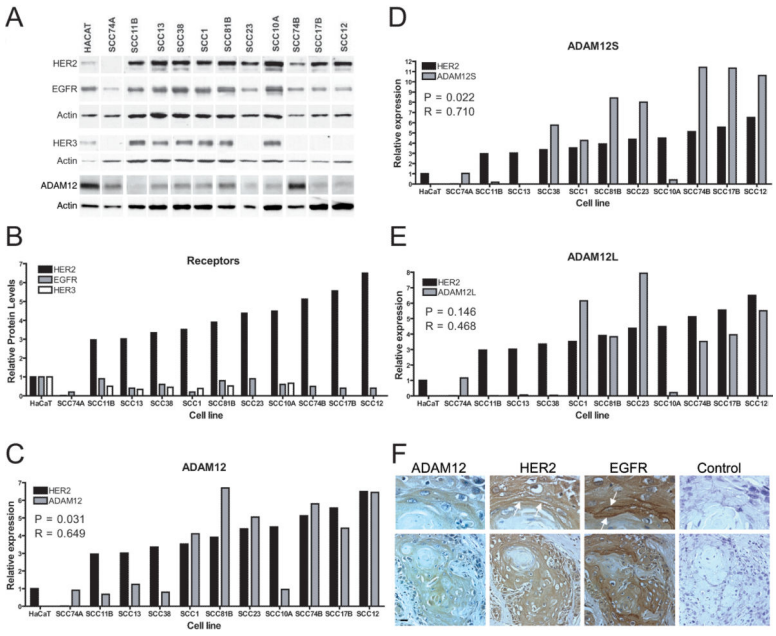

Figure 1. HER2 levels correlated with ADAM12 expression in head and neck cancer cell lines

A) Immunoblotting for EGFR family members and ADAM12 in HNSCC cell lines and HaCaT control cells. The blots have been cropped and the lanes reordered so their display is consistent with the ordering of the cell lines in Figure 1B. B) Quantification of receptor levels from immunoblots of HNSCC and HaCaT control cell lines. C–E) HER2 protein levels obtained by quantification of immunoblotting and quantification of ADAM12 (primers that recognize both ADAM12L and ADAM12S), ADAM12L, and ADAM12S transcripts following real-time RT-PCR. Values shown (B–E) are the mean for at least two replicates for immunoblotting or are the mean of at least four replicates for real-time RT-PCR. P values shown are for a test of significance of Pearson's Correlation Coefficient (R) (C–E). F) Immunohistochemistry for ADAM12, HER2 and EGFR in a human HNSCC or a negative control lacking primary antibody (far right). Arrows indicate membrane localization of receptors. Scale bar indicates 10 μm in the lower panels. Upper panels are expanded views of the lower panels.

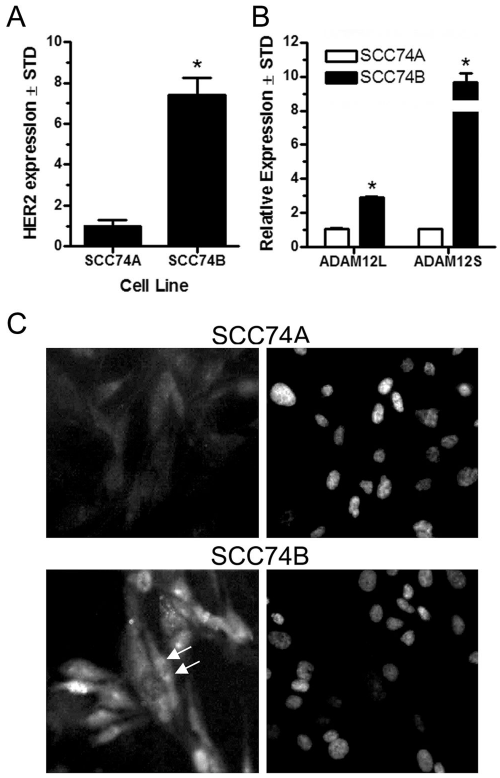

Figure 2. HER2 and ADAM12 were increased in a recurrent tumor cell line compared to cells from the original primary tumor from the same site

A–B) Real-time RT-PCR for HER2 (A), ADAM12L (B), and ADAM12S (B) in UM-SCC 74A and UM-SCC 74B cells. *Indicates statistical significance when compared to UM-SCC 74A using a Student's t-test, where $P \leq 0.05$. C) Immunofluorescence for ADAM12 in UM-SCC 74A and UM-SCC 74B cells. Panels on left show ADAM12 and on right show DAPI. Arrows indicate perinuclear ADAM12 localization.

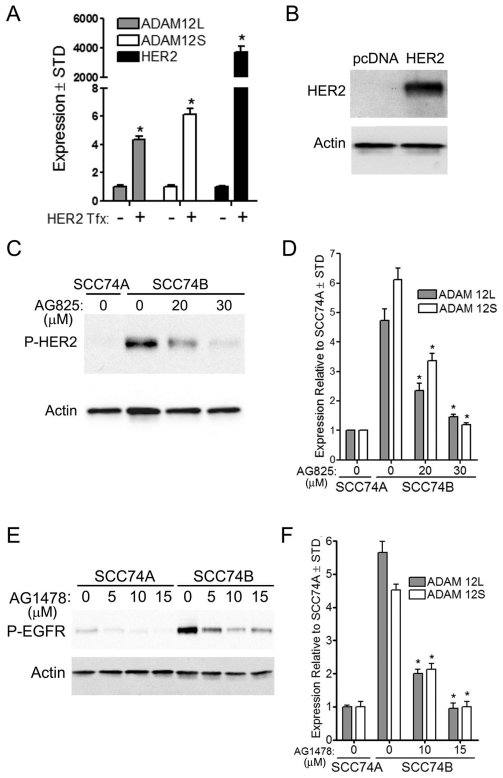

Figure 3. HER2 and EGFR up-regulated ADAM12 in HNSCC cells

A–B) The low ADAM12 expressing cell line UM-SCC 74A was transfected with HER2 or with the empty vector pcDNA. Real-time RT-PCR was performed using primers for ADAM12L, ADAM12S, or HER2 (A). HER2 protein was assessed using immunoblotting (B). C–D) The high ADAM12 expressing cell line UM-SCC 74B and the low ADAM12 expressing cell line UM-SCC 74A (as a negative control) were treated with the vehicle DMSO or with the HER2 inhibitor AG825 at the indicated concentrations for 24 h. Phosphorylation of HER2 (P-HER2) on immunoblot (C) and ADAM12L and ADAM12S transcripts following real-time RT-PCR (D) are shown. E–F) UM-SCC 74A and UM-SCC 74B cells were treated with the EGFR inhibitor AG1478 or with the vehicle DMSO alone. Immunoblotting for phospho-EGFR (P-EGFR)(E) and real-time RT-PCR for ADAM12L and ADAM12S (F) are shown. *Indicates a significant difference compared to the empty vector (A) or vehicle-treated UM-SCC 74B (D,F) controls using a Student's t-test, where $p \leq 0.05$.

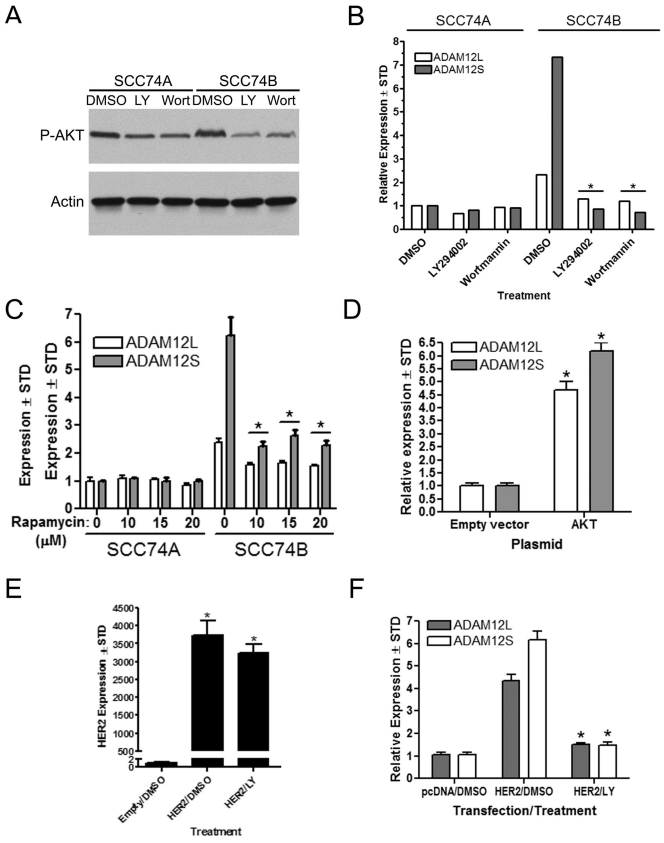

Figure 4. PI3K/AKT signaling modulated ADAM12 levels

A–C) HNSCC cells were treated with the PI3K inhibitors LY294002 (LY) or wortmannin (Wort), with the mTOR inhibitor rapamycin, or with the vehicle DMSO. Immunoblotting for phospho-AKT (P-AKT) is shown 24 h after inhibitor treatment (A). Real-time RT-PCR for ADAM12S and ADAM12L transcripts 24 h after inhibitor treatment is shown (B,C). D) UM-SCC 74A cells were transfected with an AKT vector 24 h prior to analysis of ADAM12L and ADAM12S via real-time RT-PCR. E,F) UM-SCC 74A cells were transfected with HER2 and then treated with LY294002 (LY) for 24 h before real-time RT-PCR for HER2 (E) and ADAM12 (F). *Indicates a significant difference compared to the vehicle (B,C,F) or empty vector (D,E) controls using a Student's t-test, where $p \leq 0.05$.

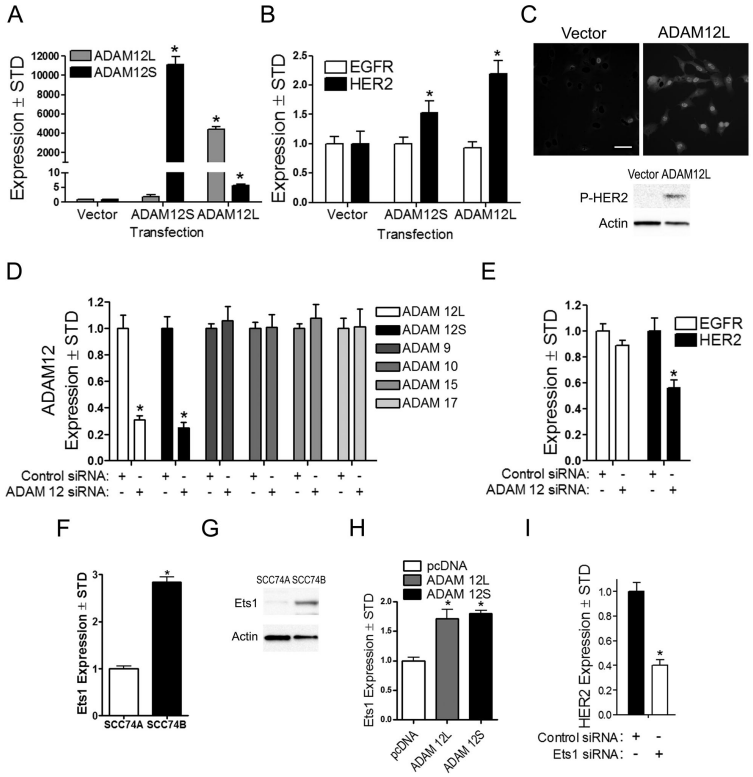

Figure 5. ADAM12 induced HER2 expression through Ets1 up-regulation

A–B) Low ADAM12 expressing cell line UM-SCC 74A was transfected with ADAM12L, ADAM12S or an empty vector control and real-time RT-PCR for the indicated genes performed. C) UM-SCC 74A cells were transfected with the vector pcDNA or with ADAM12L, as indicated above the panels, and immunofluorescence for HER2 (top) or immunoblotting for phospho-HER2 (P-HER2, bottom) performed. Bar indicates 10 μm. D–E) High ADAM12 expressing UM-SCC 74B cells were transfected with ADAM12-targeting or negative control siRNA and real-time RT-PCR performed for the indicated genes. F–G) Real-time RT-PCR (F) and immunoblotting (G) for Ets1 shown in UM-SCC 74A and UM-SCC 74B cells. H) Real-time for Ets1 24 h after UMSCC74A cells were transfected with ADAM12L or ADAM12S. I) Real-time RT-PCR for HER2 performed 24 h after Ets1 or control siRNA transfection in UM-SCC74B cells. *Indicates a significant difference compared to UM-SCC 74A (F), empty vector (A,B,H), or siRNA controls (D,E,I) using a Student's t-test, where $p \leq 0.05$.

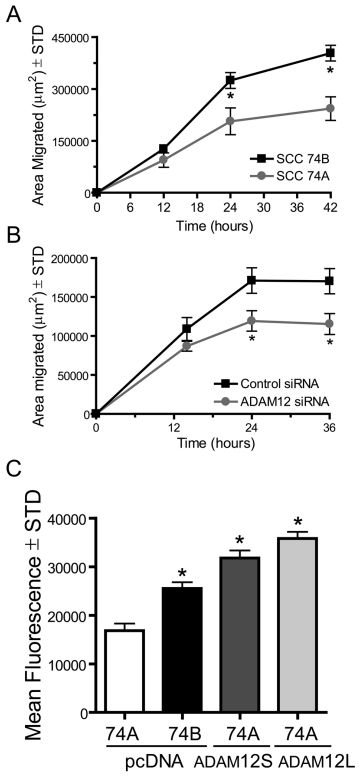

Figure 6. ADAM12 increased HNSCC cell migration and invasion

A–B) A scratch assay was performed in UM-SCC 74A and UM-SCC 74B cells (A) or beginning 24 h after transfection of UM-SCC 74B cells with control or ADAM12-targeted siRNA (B). C) UM-SCC 74A (74A) or UM-SCC 74B (74B) cells were transfected with the empty pcDNA vector or with ADAM12S or ADAM12L and Fluoroblok assays were used to quantify the effect of modulation of ADAM12 on invasion through matrigel. Experiments are representative of at least two performed (A–C). *Indicates significantly different from UM-SCC 74A (A) or from the control siRNA treated (B) using two-way ANOVA or from control transfected UM-SCC 74A using a Student's t-test (C), where $p \leq 0.05$.