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The HGF Receptor c-Met Is Overexpressed in Esophageal Adenocarcinoma[1]

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Abstract

The hepatocyte growth factor (HGF) receptor, Met, has established oncogenic properties; however, its expression and function in esophageal adenocarcinoma (EA) remain poorly understood. We aimed to determine the expression and potential alterations in Met expression in EA. Met expression was investigated in surgical specimens of EA, Barrett's esophagus (BE), and normal esophagus (NE) using immunohistochemistry (IHC) and quantitative reverse transcriptase polymerase chain reaction. Met expression, phosphorylation, and the effect of COX-2 inhibition on expression were examined in EA cell lines. IHC demonstrated intense Met immunoreactivity in all (100%) EA and dysplastic BE specimens. In contrast, minimal immunostaining was observed in BE without dysplasia or NE specimens. Met mRNA and protein levels were increased in three EA cell lines, and Met protein was phosphorylated in the absence of serum. Sequence analysis found the kinase domain of *c-met* to be wild type in all three EA cell lines. HGF mRNA expression was identified in two EA cell lines. In COX-2–overexpressing cells, COX-2 inhibition decreased Met expression. Met is consistently overexpressed in EA surgical specimens and in three EA cell lines. Met dysregulation occurs early in Barrett's dysplasia to adenocarcinoma sequence. Future study of Met inhibition as a potential biologic therapy for EA is warranted.

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Introduction

Esophageal adenocarcinoma (EA) is a highly aggressive malignancy with propensity for early local invasion and systemic metastases. The incidence of EA is increasing rapidly and, currently, EA represents the most common histologic type of esophageal cancer in the United States [1,2]. Despite advances in the diagnosis and treatment of the disease, the overall 5-year survival remains approximately 14% [3]. Molecular events responsible for the development of EA that contribute to such a malignant phenotype are poorly understood. Thus, elucidation of these molecular alterations is important to facilitate the identification of key molecular targets and diagnostic markers.

Met is a transmembrane tyrosine kinase receptor encoded by the c-Met proto-oncogene ($p190^{MET}$) and is expressed in most epithelial and endothelial cells. The receptor is phosphorylated upon binding with high affinity to the ligand hepatocyte growth factor (HGF), also known as scatter factor [4–7]. HGF is produced mostly by mesenchymal cells, acting as a paracrine factor on epithelial tissues [8]. Met receptor activation has mitogenic, motogenic, and angiogenic cellular effects, and has been implicated in tumor development and progression [5,9–11]. Met also appears to have an important role in the healing of gastric and esophageal ulcers through interactions with cyclooxygenase-2 (COX-2) enzymes. Inhibition of COX-2 has been shown to downregulate Met expression in gastric ulcer healing and, importantly, to reduce the frequency of malignant progression of Barrett's esophagus (BE) [12–14].

The intracellular events and pathways involved following Met activation are complex and only partially understood. The signaling cascade is known to be associated with phosphatidyl-3-kinase, Ras-MAP kinase, STAT, PLC-γ, and other proteins related to established cell proliferation and antiapoptotic pathways [15]. Activation of the Met/HGF pathway can result in cancer cell proliferation, resistance to apoptosis, and increased production of serine proteases, promoting tumor growth, cell motility, and invasion [8,16]. In addition, Met has also been shown to directly inhibit apoptosis through sequestration of the Fas receptor in murine hepatocellular carcinoma [17].

The overexpression of Met and/or its ligands has been shown to contribute to the development and progression of different malignancies including lung, colorectal, gastric, breast, prostate, thyroid, pancreas, and oral squamous cell

Abbreviations: HGF, hepatocyte growth factor; EA, esophageal adenocarcinoma; COX-2, cyclooxygenase-2; IHC, immunohistochemistry; NE, normal esophagus; QRT-PCR, quantitative reverse transcriptase polymerase chain reaction; BE, Barrett's esophagus; LGD, low-grade dysplasia; HGD, high-grade dysplasia

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carcinomas [18–22]. Studies have demonstrated the overexpression of Met and HGF in esophageal squamous cell carcinoma [22–24], but only one study has [25] partially evaluated the expression patterns of the Met receptor and HGF in EA.

In this study, we characterized for the first time the expression patterns of Met in human EA tissue samples. We also examined three EA cell lines for *in vitro* studies of Met expression and function. We demonstrate that Met is markedly and frequently overexpressed in EA, and this overexpression is an early event found at stages of dysplasia in premalignant BE.

Materials and Methods

Surgical Tissue Specimens

All human tissues were obtained through protocols approved by the University of Pittsburgh Institutional Review Board. All archived EA specimens from a 10-year time period were identified and retrieved. Patients who had received preoperative radiation therapy were excluded. Specimens were also excluded following histologic review if the tumor sample did not represent at least 50% tumor cellularity, or if the esophageal squamous epithelium specimen contained any malignant cells. The resulting study group represented matched specimens from 26 patients.

For experiments requiring fresh tissues, specimens of patients undergoing esophagectomy at the University of Pittsburgh were prospectively collected as part of an institutional esophageal cancer risk registry. Samples of fresh tissues corresponding to esophageal tumor, normal distant esophagus, and areas of BE (when available) were obtained at the time of surgery, immediately frozen in liquid nitrogen, and stored at −80°C until analyzed. We randomly selected 10 matched cancer tissue specimens and normal esophageal specimens for our analysis.

In Vitro Cell Culture

Three human EA cell lines (FLO-1, SEG-1, and BIC-1) were used for *in vitro* studies. An SV-40 immortalized squamous epithelial cell line (HET-1A) served as a control. Cell lines were maintained in Dulbecco's modified essential medium (DMEM; Gibco-BRL, Rockville, MD) containing 10% FBS and 1% penicillin/streptomycin. For experiments evaluating the phosphorylation status of Met, treatment groups included standard culture conditions, serum starvation for 24 hours, and serum starvation prior to a 30-minute treatment with recombinant HGF at a concentration of 50 ng/ml (R&D Systems, Minneapolis, MN). SEG-1 (COX-2–expressing) and BIC-1 (COX-2–negative) cells were incubated in the presence or absence of a selective COX-2 inhibitor (NS-398 at 10–100 μg) for 18 hours.

RNA Extraction and Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA extraction was performed on EA cell lines and esophageal tissue sections (10 × 10 μm sections) using an RNAeasy kit (QIAGEN, Valencia, CA). All samples were DNAse-treated. Primers and probes were designed using the Primer Express software (Applied Biosystems, Foster City, CA) to flank an intronic sequence. RT reactions were performed in the iCycler PCR instrument (Bio-Rad, Hercules, CA) using specific RT primers for HGF, Met, and an endogenous control gene [either β-glucurosidase (β-Gus) or 18*S* ribosomal RNA (18*S*)]. All RT reactions were performed in duplicates with RNA inputs of 1 and 250 ng per sample, using Superscript II reverse transcriptase as described previously [26]. Five microliters of the first-strand cDNA was amplified as a separate step in a 96-well plate on the TaqMan ABI 7700 instrument (Applied Biosystems) using specific Met, β-Gus, and/or 18*S* primer sets and a florescent-labeled oligonucleotide for signal detection (Table 1). The conditions for the PCR reactions were 95°C for 12 minutes followed by 40 cycles of denaturation (95°C for 30 seconds) and annealing/extension (60°C for 1 minute) steps. Nonreverse transcription controls (no-RT enzyme) were performed for each RNA sample and no-template controls were included on each PCR plate. Amplification plots were generated and the C_t value (cycle number at which fluorescence reaches threshold) recorded. A calibrator RNA sample was included in each batch of RT-PCR to adjust for interplate variability, and relative expression of Met and HGF was calculated using the ΔC_T approach as described previously [27]. In the COX-2 inhibition study, β-Gus was found to be regulated by NS-398 and the Met levels were therefore normalized to those of 18*S* instead.

Immunohistochemistry (IHC) in Tissue Specimens

Five-micron sections of formalin-fixed esophageal specimens were analyzed for Met protein expression by IHC. Rabbit anti–Met polyclonal antibody (SC-161; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at a dilution of 1:100 for IHC staining. Biotinylated anti–rabbit IgG was used as the secondary antibody and then stained with the Fuchsin Substrate System (DAKO, Carpinteria, CA). Antibody specificity was confirmed by primary antibody delete controls. A specimen of hepatocellular carcinoma metastatic

Table 1. Primer and Probe Sequences.

Gene	Primer	Sequence
Met	Forward	5′-CTGCCTGCAATCTACAAGGT-3′
	Reverse	5′-ATGGTCAGCCTTGTCCCTC-3′
	Probe	5′-/56-FAM/TTTCCCAAATAGTGCA CCCCTTGAAGG-3′
HGF	Forward	5′-CCCTATTTCTCGTTGTGAAGGT-3′
	Reverse	5′-TGTTTCGTTTTGGCACAAGA-3′
	Probe	5′-/56-FAM/CGGGATGGTCTAAATTGAC TATTGTAGGTGTG-3′
β-Gus	Forward	5′-CTCATTTGGAATTTTGCCGATT-3′
	Reverse	5′-CCGAGTGAAGATCCCCTTTTTA-3′
	Probe	5′-/56-FAM/TGAACAGTCACCGACG AGAGTGCTGG-3′
18S	Forward	5′-CCCTGTAATTGGAATGAGTCCAC-3′
	Reverse	5′-GCTGGAATTACCGCGGCT-3′
	Probe	5′-/56-FAM/TGCTGGCACCAGACTTG CCCCTC-3′

to the lung, which provided intense but cancer cell–specific staining, served as a positive control. IHC slides were reviewed by a pathologist experienced in esophageal malignancies. A scoring system based on the percentage of tumor cells staining per slide was created. The scoring system had a range of 0 (no staining) to 3 (100% of tumor cells staining for Met). Reported values are the average score per sample (multiple blinded slides for each sample were independently reviewed). For grading BE samples, the intensity was evaluated for a representative area for metaplasia, low-grade dysplasia (LGD), and high-grade dysplasia (HGD), and graded from 0 to 3.

Met Immunoblotting

Protein extraction was performed from total cell lysates as previously described [28]. Proteins were resolved on 8% polyacrylamide gels. Following protein transfer to nitrocellulose membranes, Met immunoreactivity was examined using anti–Met polyclonal rabbit antibody (1:10,000 dilution of SC-161; Santa Cruz Biotechnology, Inc.) or an anti–c-Met phospho-specific antibody (BioSource International, Camarillo, CA), and a peroxidase-labeled secondary antibody. Enhanced chemiluminescence (Pierce, Rockford, IL) was used to detect immunoreactive proteins.

Subcellular Localization of Met, Determination of Phosphorylation Status, and Fas–Met Association Experiments: IP and Laser Scanning Confocal Microscopy

Cells were plated on uncoated glass cover slips, allowed to adhere for 48 hours, and subsequently fixed in 2% paraformaldehyde. After blocking nonspecific antibody binding with a 1:20 dilution of rabbit serum, cells were stained with rabbit polyclonal anti–Met antibodies (1:2500 dilution of SC-161; Santa Cruz Biotechnology, Inc.) and detected with appropriate fluorescent label–tagged secondary antibodies (Jackson Immunolaboratories, West Grove, PA). Specimens treated by omitting the primary antibody served as controls for nonspecific staining. For experiments examining potential Fas–Met interactions, anti-Fas antibodies (1:5000 dilution of clone CH-11; New England Biolaboratories, Waltham, MA) were included in the primary labeling step. Staining was assessed and images were obtained by laser scanning confocal microscopy (Leica Microsystems, Inc., Bannockburn, IL). Immunofluorescence staining was performed as above using two unique phospho-Met–specific antibodies independently (Tyr 1349 and Tyr 1234/1235; Cell Signaling Technology, Beverly, MA).

For immunoprecipitation of Met, protein lysis buffer (20 mM Tris–Cl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton-X, 2.5 mM Na-pyrophosphate, and 1 mM glycerol phosphate) with protease and phosphatase inhibitors was added directly to cell cultures and incubated on ice for 15 minutes. Cell lysates were incubated for 1 hour at 4°C with protein A beads. Following removal of the beads by centrifugation, anti-Met antibody (clone 25H2; Cell Signaling Technology) was added and the lysates incubated overnight at 4°C, following which another aliquot of protein A beads was added to each tube and mixed for 4 hours at 4°C. Following five washes, precipitated protein/protein A beads were pelleted and resuspended in 20 μl of 2 × sample buffer and the proteins resolved on 8% polyacrylamide gel. Total lysate from the SEG-1 cell line was included as a positive control. Following membrane transfer, rabbit anti-Met (SC-161; Santa Cruz Biotechnology, Inc.) was used to detect the precipitated Met proteins. Coprecipitation of Fas was evaluated using rabbit anti-Fas antibodies (Apo-1-3 clone; Kamiya, Seattle, WA). Peroxidase-labeled secondary antibodies and enhanced chemiluminescence (Pierce) were used for detection.

c-met Sequence Analysis

As previously described [28], total RNA was isolated from SEG-1, BIC-1, FLO-1, and HET-1A cell lines and reverse-transcribed. PCR amplification of the tyrosine kinase catalytic domain (bases 3311–4311, accession no. NM 000245) of Met mRNA from the RT product was performed using the forward primer 5′-AGTGCAGTATCCTCTGACAGAC-3′ and the reverse primer 5′-GTCCATGTGAACGCTACTTATG-3′. Amplification products were purified using the Promega Wizard DNA purification kit (Promega, Madison, WI) and sequenced using the same primers by automated sequencing at the DNA sequencing facility at the University of Pittsburgh. A nested set of sequencing primers (5′-ATTCACTGTGCTGTGAAATCC-3′ and 5′-GCGCATTTCGGCTTTAGGGTG-3′) was necessary to obtain high-quality sequence data.

Statistical Analysis

The IHC scores determined for multiple slides from each specimen were averaged. The comparison of matched tissue specimens [EA and normal esophagus (NE) from each patient] from 26 patients was tested with the signed rank test. To examine the hypothesis that Met expression reflects the progression of BE to EA, the average IHC scores of patients with BE were compared to the scores of EA patients with the Jonckheere-Terpstra test. This test is sensitive to differences between groups in which the difference reflects the upward or downward trend among the groups. Specific comparison of the IHC scores between two independent groups was conducted with the Wilcoxon test.

Results

Overexpression of Met mRNA and Protein in EA Specimens

We analyzed surgical specimens from 26 patients undergoing esophagectomy for EA. IHC staining to detect Met protein expression in matched samples of EA and NE revealed consistent and intense anti-Met immunostaining specific to the cancer cells (Figure 1*D*) compared to nearly absent staining in NE samples and stromal tissues (Figure 1*A*). Met protein appeared to localize to the cell surface as well as cytoplasm of the malignant cells, although this assessment must be taken in the context of the resolution limitations of light microscopy. NE tissue had faint IHC staining in a heterogeneous pattern. The semiquantitative

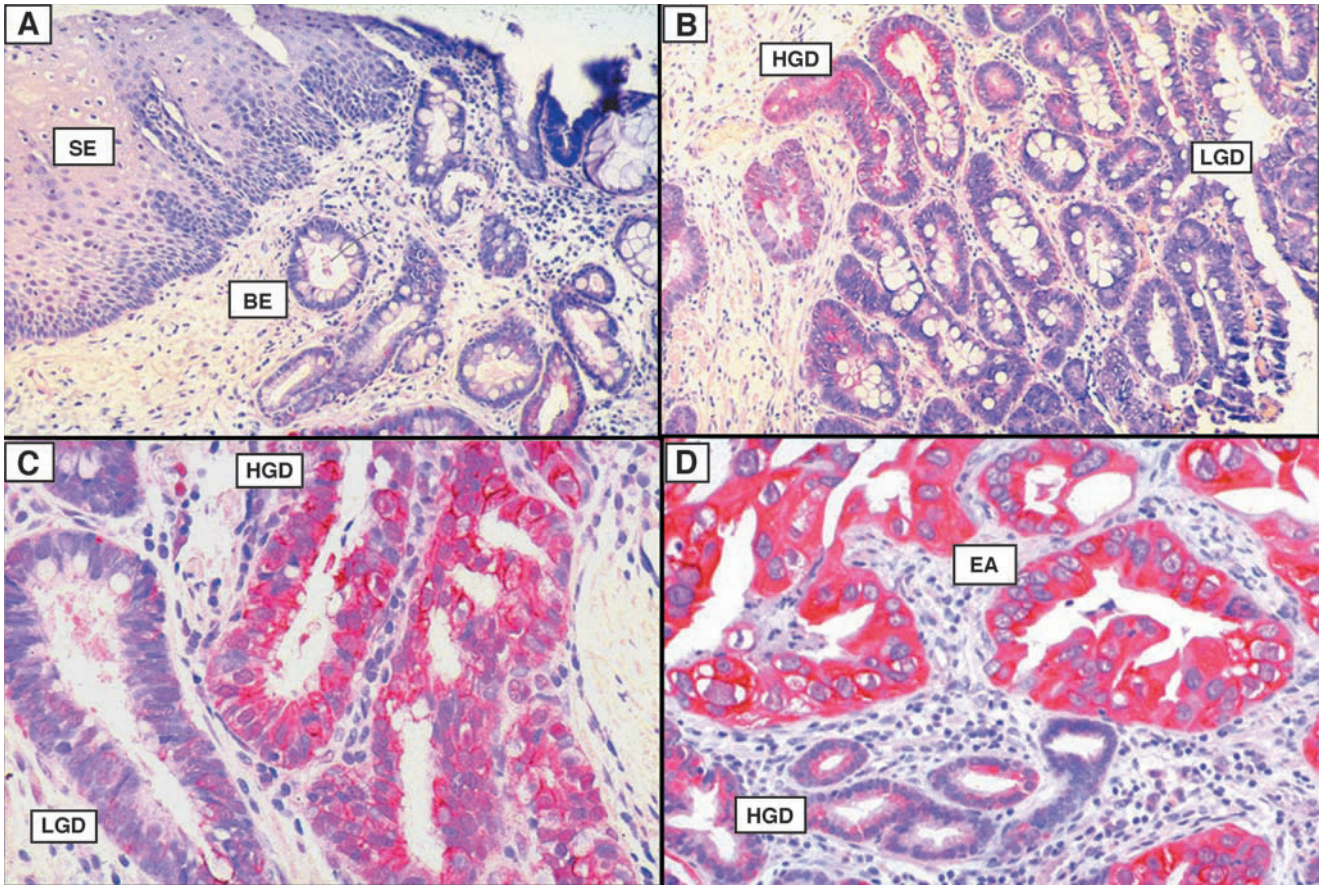

Figure 1. *Met IHC in esophageal surgical specimens. Met-specific staining appears red. (A) Met staining in specimen of NE with Barrett's intestinal metaplasia. Note the lack of staining in the normal squamous epithelium (SE) and even in BE (original magnification, × 100). (B) Met staining in specimen of BE with dysplasia. Met staining is minimal in areas of LGD, but the staining becomes intense in areas of Barrett's with HGD identified by nuclear atypia, and the loss of cellular organization and goblet cells (original magnification, × 100). (C) High-power view of Met staining in a specimen of BE with dysplasia (original magnification, × 400). There is minimal Met staining in the proliferative zone of areas of LGD, but diffuse intense staining in areas of HGD. (D) Met staining in specimen of EA (original magnification, × 400). An area of HGD is also present within this field.*

grading of IHC staining demonstrated a median IHC score of 2.49 on EA specimens. In comparison, the median IHC score for normal control tissue was 0, with 69% of specimens scoring zero in every cross section examined. The IHC scores were significantly higher in EA when compared to matched NE samples, confirming that Met protein is overexpressed in human EA specimens ($P < .0001$, Wilcoxon signed rank test) (Figure 2*A*).

Met Overexpression Occurs Early in Barrett's Metaplasia–Dysplasia–Adenocarcinoma Sequence

To determine if Met overexpression occurs at an early stage in the progression from BE to EA, IHC was performed in eight tissue specimens of BE with varying degrees of dysplasia. Intense Met staining was observed throughout regions of HGD, but this staining was more heterogeneous when compared to the staining seen in EA specimens (Figure 1, *A*–*C*). Met IHC of specimens with dysplasia demonstrated a direct correlation between the intensity of Met staining and the degree of dysplasia. Met staining was minimal in areas of BE (Figure 1, *A* and *B*), but Met staining was stronger in areas of BE with LGD and HGD (Figure 1, *B* and *C*). There was a statistically significant increase in Met IHC staining as the degree of dysplasia progressed from BE to EA ($P < .0002$, Jonckheere-Terpstra test) (Figure 2*B*). Met IHC scores were significantly higher in BE with LGD when compared to BE without dysplasia, suggesting that Met overexpression is present early in the metaplasia–dysplasia–adenocarcinoma sequence ($P = .0242$, Wilcoxon test). Neither HGD nor EA was significantly greater than LGD. These findings of a correlation of Met overexpression with the development of cellular alterations characteristic of dysplasia in BE suggest that Met overexpression can occur as an early event in EA tumorigenesis.

To investigate the mechanism of Met overexpression in EA, we prospectively collected 10 surgical specimens for the evaluation of Met mRNA expression levels. Two of these samples were subsequently excluded due to inadequate percentage tumor representation. Quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) analysis of EA specimens and matching normal esophageal tissues demonstrated Met overexpression in five of eight EA samples examined relative to control squamous epithelium (Figure 3). Met mRNA was two-fold higher in EA

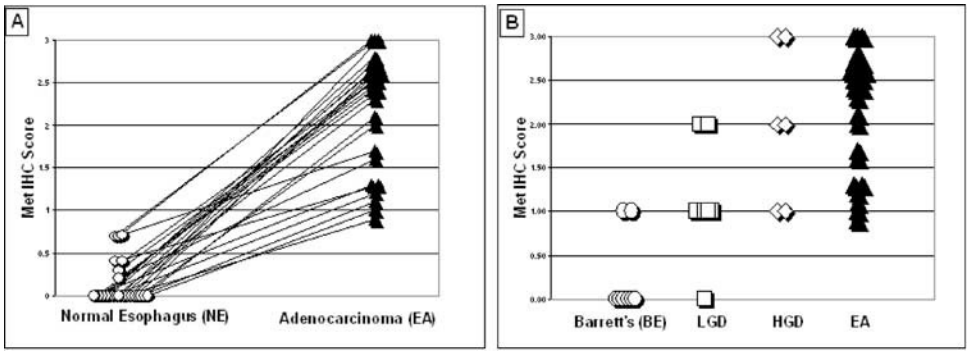

Figure 2. *Anti-Met IHC scores in EA and BE with and without dysplasia. (A) Met (IHC) scores for matched EA and NE tissue samples. The IHC was interpreted by an expert pathologist and graded using a standard score based on the staining intensity and percentage of field stained with a score ranging from 0 to 3. IHC scores were significantly higher in EA when compared to matched NE samples (P < .0001, Wilcoxon signed rank test). (B) Met IHC score for BE, LGD, HGD, and EA. There was a statistically significant increase in Met IHC staining as the degree of dysplasia progressed to adenocarcinoma (P < .0001, Jonckheere-Terpstra test). Met IHC scores were significantly higher in BE with LGD when compared to Barrett's metaplasia, suggesting that Met overexpression is present early in the metaplasia–dysplasia–adenocarcinoma sequence (P = .0242, Wilcoxon exact test).*

(mean expression 0.38) when compared to NE samples (mean expression 0.20) (*P* = .004, Mann-Whitney *U* test) (Figure 3). Taken together, our data suggest that Met protein overexpression in a subset of EA surgical specimens is due to increased Met mRNA. Posttranscriptional mechanisms in other EA tumors are likely responsible for this overexpression.

Met Is Overexpressed at the mRNA and Protein Levels in EA Cell Lines

We next aimed to verify that three EA cell lines were appropriate *in vitro* models of Met overexpression in EA. Met mRNA expression in three EA cell lines (SEG-1, BIC-1, and FLO-1) was analyzed. The SV-40 immortalized squamous esophageal cell line (HET-1A) served as a control. Met mRNA expression in FLO-1, SEG-1, and BIC-1 EA cell lines was on the order of 10,000-fold higher than the control cell line HET-1A (Figure 4*A*) as determined by QRT-PCR. Met mRNA was not detectable in our no-template and no-RT controls, excluding the possibility of sample contamination or genomic DNA amplification. These results demonstrated that Met mRNA is abundantly expressed in all three EA cell lines.

We also examined a potential coexpression of the ligand HGF by these cells. RNA analysis demonstrated HGF (Met ligand) expression in BIC-1 and SEG-1 cells, but not in the FLO-1 or HET-1A cell line (Figure 4*A*). These findings suggest, but do not confirm, that Met may be activated in these two EA cell lines through an autocrine loop.

To verify the presence of mature, full-length Met protein expression in the cell lines, immunoblot analysis of total cellular protein lysates with anti-Met antibodies was performed. These results demonstrated that all three EA cell lines, but not the control squamous epithelial cell line HET-1A, expressed Met protein in its mature 140-kDa form and in its 170-kDa precursor (Figure 4*B*). Of note, Met expression in FLO-1 was predominantly in its mature 140-kDa form. Taken together, these data demonstrate that Met expression in the EA cell lines appears consistent with that observed in the surgical specimens, and these cell lines are an adequate model for *in vitro* experiments to further evaluate Met expression and function in EA.

The Three EA Cell Lines Exhibit Constitutive Met Phosphorylation in the Absence of HGF Stimulation That Is Increased Following HGF Stimulation

Our next objective was to evaluate the phosphorylation status of Met in these cell lines (Figure 5). Immunofluorescence using anti–phospho-Met–specific antibodies demonstrated staining for phosphorylated Met localized to the cell surface of all three EA cell lines that had been serum-starved. Treatment of the SEG-1 and BIC-1 cell lines with recombinant HGF did not result in increases in phospho-Met–specific staining (data not shown) or characteristic morphologic changes (scatter effect). Immunodetection of phosphorylated Met was also observed in serum-starved FLO-1 cells that do not express HGF mRNA. In this cell line, treatment with HGF did result in subtle morphologic changes (scattering) and an appreciable increase in staining for phosphorylated Met (Figure 5*A*). The phosphorylation status

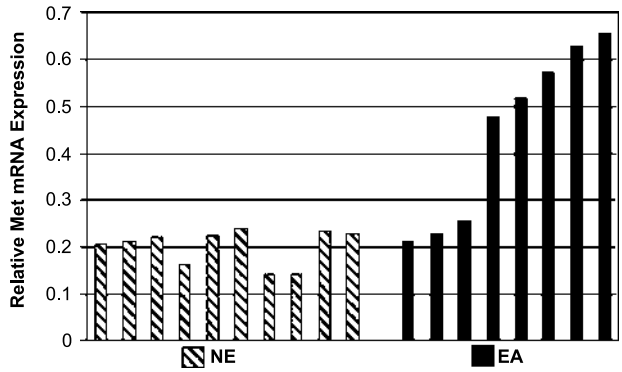

Figure 3. *Met mRNA expression in specimens of EA and NE. Met mRNA expression was analyzed in 10 control esophageal samples and in eight EA samples. Met mRNA was significantly higher in EA (mean expression 0.3768) when compared to matching control NE (mean expression 0.20097) (P = .004, Mann-Whitney U test).*

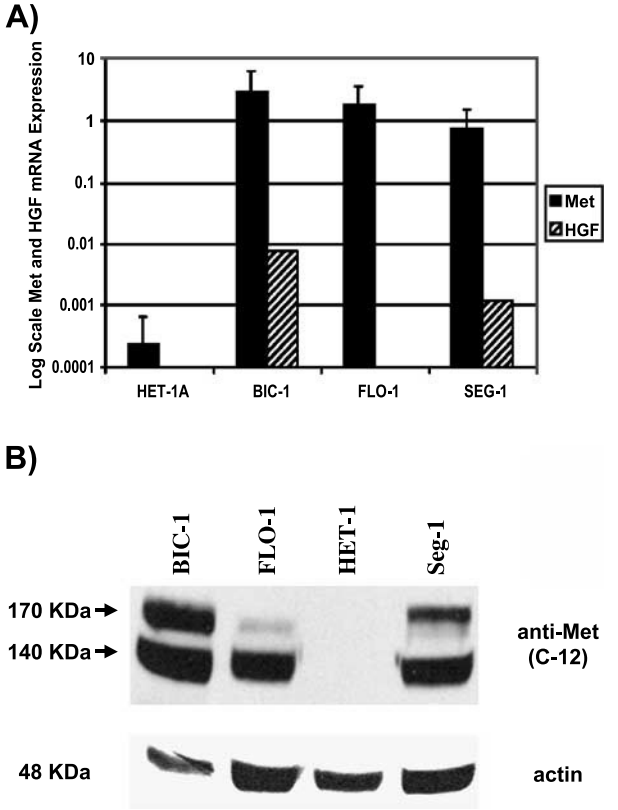

Figure 4. *Met mRNA expression in EA and NE cell lines. (A) Met and HGF mRNA relative expression (log scale) in HET-1A cells, an immortalized human squamous epithelial cell line serving as a control, and three human EA cell lines (BIC-1, FLO-1, and SEG-1) as determined by QRT-PCR. The mRNA expression is relative to the control endogenous gene β-Gus to adjust for mRNA integrity and quantity. (B) Western blot analysis for Met protein expression in EA cell lines (BIC-1, FLO-1, and SEG-1) and the control epithelial cell line HET-1A. Marked overexpression of Met protein (140 kDa mature form and 170 kDa Met precursor) was evident in all three EA cell lines, but not in the control. α-Actin was used as loading control.*

of Met was also evaluated by Western blot analysis using a phosphorylated specific [pYpYpY$^{1230/1234/1235}$] anti–c-Met polyclonal antibody (BioSource International). Phosphorylated Met was detected in FLO-1, BIC-1, and SEG-1 cells following 24 hours of serum starvation, but not in the A549 lung adenocarcinoma cell line serving as a control (Figure 5*B*). HGF treatment (50–100 ng/ml) resulted in phosphorylation of Met in the A549 cell line and increased phosphorylation of Met in the three EA cell lines.

Thus, we concluded that Met is phosphorylated in the absence of serum or exogenous HGF in all three EA cell lines and this activation can be further stimulated by the addition of ligand. Taken together, these data suggest that Met is autophosphorylated in SEG-1 and BIC-1 cells, and this may occur through HGF expression resulting in autocrine activation of the receptor. Ligand-independent phosphorylation of Met occurs in the FLO-1 cell line.

A number of mutations of the *c-met* locus, most involving the kinase domain of the protein, have been shown to increase the activity of the receptor by mechanisms that are not well clarified [16,29]. We hypothesized that the FLO-1 cell line might contain an activating mutation that could explain the above findings. DNA sequence analysis of the kinase domain of Met in SEG-1, BIC-1, and FLO-1 was performed to identify potential activating mutations in the Met coding sequence. However, sequence analysis of cDNA generated from the SEG-1, FLO-1, and BIC-1 cell lines did not reveal any mutations compared to the wild-type sequence. Thus, the mechanism of Met autophosphorylation in FLO-1 cells remains unclear.

Met Protein Expression Localizes to the Cell Membrane and Is Not Associated With Fas Receptor

To determine if the Met receptor localizes to the cell surface in EA, we analyzed Met protein localization in the EA cell lines by immunofluorescent laser scanning confocal microscopy. These studies demonstrated that, as expected, Met protein localizes predominantly on the cell membrane with a smaller fraction of protein observed in a perinuclear reticular pattern suggestive of localization to the ER or endosomes (Figure 6*A*). Because others have reported that Met shares a protein–protein interaction with the death receptor Fas (CD95) in murine hepatocellular carcinoma [30], we sought to determine if this association could be responsible for the lack of cell surface expression of Fas and resistance to Fas-mediated apoptosis in EA that we have previously reported [28]. However, dual-labeled immunofluorescence to Fas and Met did not suggest that there was colocalization of the two proteins at the cell surface. In addition, immunoprecipitation experiments failed to demonstrate that Met and Fas proteins were associated (Figure 6*B*). Thus, we were not able to demonstrate Fas–Met interactions either by imaging or immunoprecipitation. In contrast to Fas, Met appears to be effectively trafficked to the cell surface in the EA cell lines examined.

Selective COX-2 Inhibition Downregulates Met Expression in EA Cell Lines

Recent reports have demonstrated that gastric ulcer patients and mouse models of esophageal ulceration suffer from delayed healing when treated with COX-2 inhibitors due to significant downregulation of Met at the ulcer's edge [12]. This prompted us to investigate whether pharmacologic COX-2 inhibition can result in reduction in Met expression in EA. We evaluated Met mRNA and protein expression after COX-2 inhibition in one EA cell line with no COX-2 expression (BIC-1) and one with marked COX-2 expression (SEG-1) [31]. Cells were treated for 48 hours with celebrex [25] or NS-398 (100 μM) at doses that result in selective COX-2 inhibition. Cells were also treated with PMA (100), a known inducer of Met, through activation of protein kinase C as a positive control. We observed a significant dose-dependent reduction (60% maximum decrease compared to control) of Met mRNA in SEG-1 cells but not in the COX-2–negative BIC-1 cells (Figure 7). At the protein level, a marked reduction in expression was observed following treatment with celecoxib and NS-398 in the SEG-1 cell line, but not in the BIC-1 cell line. PMA treatment increased Met protein expression in both cell lines. These data suggest that COX-2 overexpression may contribute to the Met overexpression

in some EA tumors, but Met overexpression can occur independently of COX-2 overexpression (COX-2–negative BIC-1 cells overexpress Met).

Discussion

The HGF/Met tyrosine kinase receptor pathway has been suggested by others as an important contributor to malignant phenotype by increasing cell proliferation, inhibiting apoptosis, and promoting cell migration and invasion and, thus, metastasis [5,8,10,32]. In this study, we demonstrate for the first time that Met is markedly and consistently overexpressed in EA. This overexpression was evident at the protein level in 26 of 26 surgical specimens evaluated by IHC as well as in three different EA cell lines. We have shown that Met protein overexpression can occur through an increased expression of Met mRNA, but our data also suggest that other mechanisms may also lead to this phenotype. Met overexpression seems to be an early event in the development of EA, as we identified Met protein overexpression in regions of dysplasia in Barrett's epithelium. This suggests that dysregulation of the HGF/Met pathway could have a contributing role in the malignant progression of BE to EA. That Met protein is universally overexpressed in EA and that this overexpression is already present in precursor lesion BE suggests that, similar to other tyrosine kinases, the Met receptor may prove to be an attractive target for chemoprevention or chemotherapy against EA. Based on our findings, future studies focusing on inhibition of this pathway with anti-Met–directed therapy in EA are clearly warranted.

We also report our characterization of Met expression in three EA cell lines and *in vitro* studies aimed at eliciting potential mechanisms that could be responsible for Met alterations in EA. We have shown that Met is constitutively phosphorylated in all three cell lines, and discovered that two of these cell lines express HGF mRNA, suggesting that the Met receptor may be activated through an autocrine loop in EA. We initially suspected that Met phosphorylation in the absence of serum or exogenous HGF in the FLO-1 cell line, combined with the finding that the majority of Met protein was cleaved to the mature form in this cell line, was due to an activating mutation of the Met sequence in FLO-1 as had been reported in other tumor cell lines [29]. Sequence analysis, however, did not identify a mutation. The absence of an activating mutation in kinase domain of these three cell lines does not exclude the possibility that mutations are present elsewhere in the full coding sequence of the *c-Met* gene in the FLO-1 cell line or in some cases of EA. Another alternative is that ligand-independent activation of Met may occur in the FLO-1 cell line, as others have suggested that overexpression of the receptor can result in heterodimerization and autoactivation of the receptor kinase [32]. Finally, our immunoblot data showing that the majority of Met proteins in the FLO-1 cell line is cleaved to the mature form of

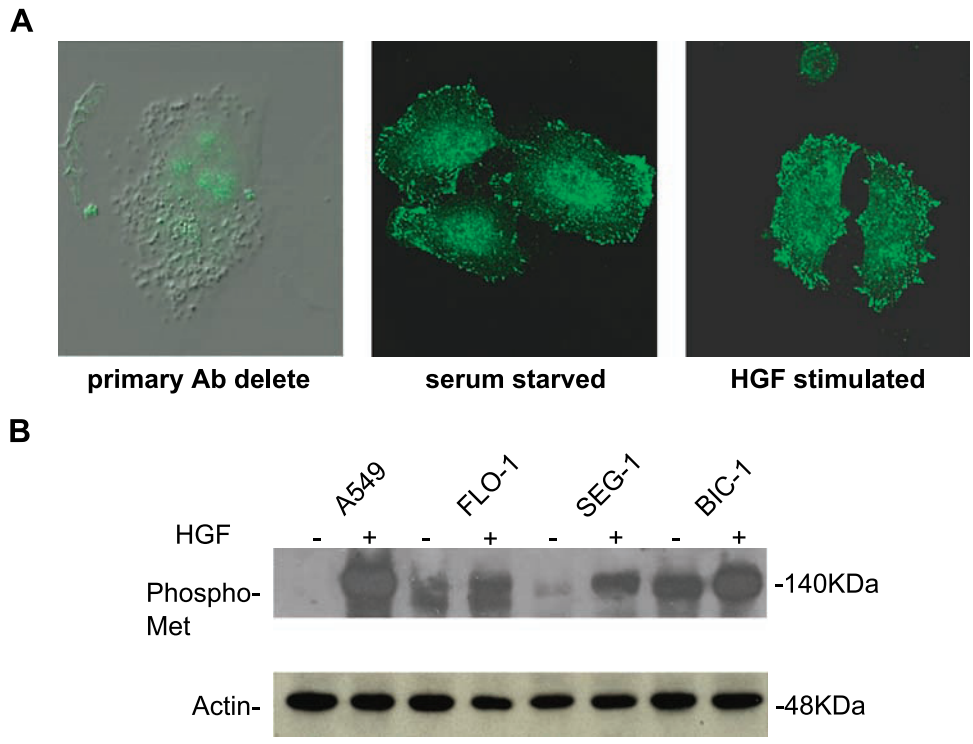

Figure 5. *Constitutive phosphorylation of the Met receptor in SEG-1, FLO-1, and BIC-1 cells. (A) Laser scanning confocal microscopy of FLO-1 cells serum-starved for 24 hours, or serum-starved prior to treatment with 50 nM HGF. Cells were stained with anti–phospho-Met–specific antibodies. Cell surface phospho-Met is detected in the serum-starved FLO-1 cells. Treatment with HGF resulted in increased intensity of phospho-Met staining on the cell surface in the FLO-1 cell line and subtle morphologic changes (scattering). Original magnification, × 600. (B) Immunoblots using a phospho-Met–specific antibody. The A549 lung adenocarcinoma cell line served as a positive control for Met phosphorylation in response to HGF treatment. Phosphorylated Met is observed in all three EA cell lines grown in serum-starved conditions, but not in the A549 cell line. HGF treatment resulted in phosphorylation of Met in the A549 cell line and increased phosphorylation of Met in all three EA cell lines.*

the receptor may suggest that increased proteolytic cleavage of Met to the mature form of the protein could contribute to the observed autophosphorylation.

One limitation of this study is that because we only examined a single population cell culture model, the interactions of Met with other cells and proteins in the tissue stroma could not be examined. We would have liked to examine the effects of Met inhibition and thus have been able to examine Met function in EA in an *in vivo* model where the epithelial–stromal interactions are intact, but a highly specific, small-molecule Met inhibitor has only recently been reported and is currently not generally available for research purposes [33].

A potential explanation for the frequent overexpression of Met in EA may be the role of HGF/Met pathway in the healing process of esophageal and gastric ulcerations [12]. It is well established that processes that chronically injure the esophageal mucosa (i.e., GERD) are risk factors for the development of dysplasia and, eventually, esophageal cancer. The interaction of Met with prostaglandins has been reported, particularly in tissue inflammation and wound healing [12–14]. In our study, we demonstrated that COX-2 inhibition downregulates the mRNA and protein expression of Met in the COX-2–overexpressing EA cell line SEG-1. This effect was specific because the BIC-1 cell line (COX-2–negative) showed no change in Met mRNA or protein expression with

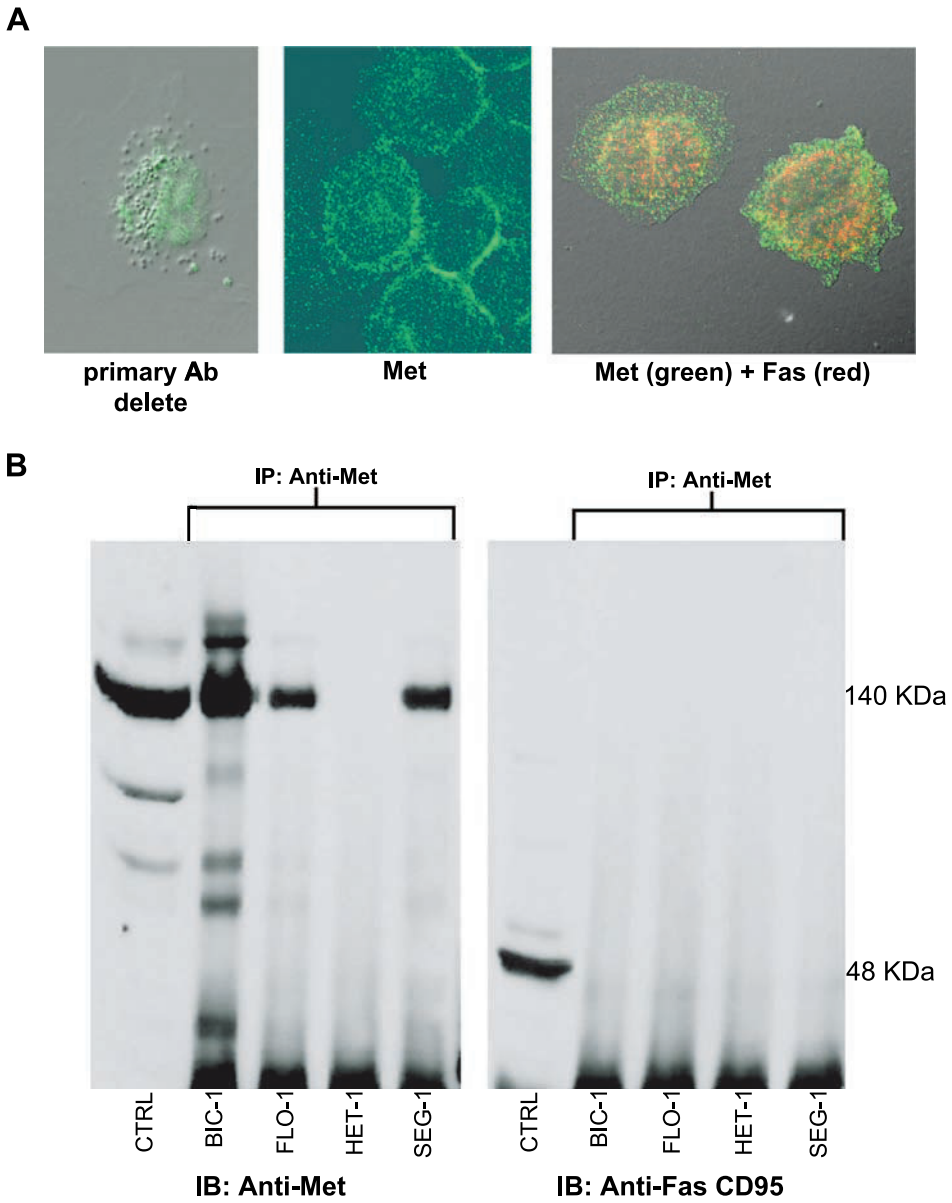

Figure 6. *Cellular localization of Met in EA and lack of colocalization or protein–protein interaction with the death receptor Fas (CD95). (A) Laser scanning confocal microscopy of SEG-1 cells. Perinuclear autofluorescence is visualized in primary antibody delete controls. Met immunofluorescence labeling reveals predominantly cell surface localization of the protein. Dual-labeled immunofluorescence for Fas (red) and Met (green) does not suggest significant colocalization of the two proteins (yellow). Original magnification, × 600. (B) Immunoprecipitation experiment to determine potential Fas–Met interactions in the EA and HET-1A cell lines. Mouse monoclonal Met antibody was used to immunoprecipitate the Met receptor, and associated proteins and immunoblots of the precipitated proteins were performed using anti-Met or anti-Fas rabbit polyclonal AB. Total protein lysate from SEG-1 cell lines (CTRL) was used as a positive control for Met and Fas signals. No protein–protein interaction between Fas and Met was observed.*

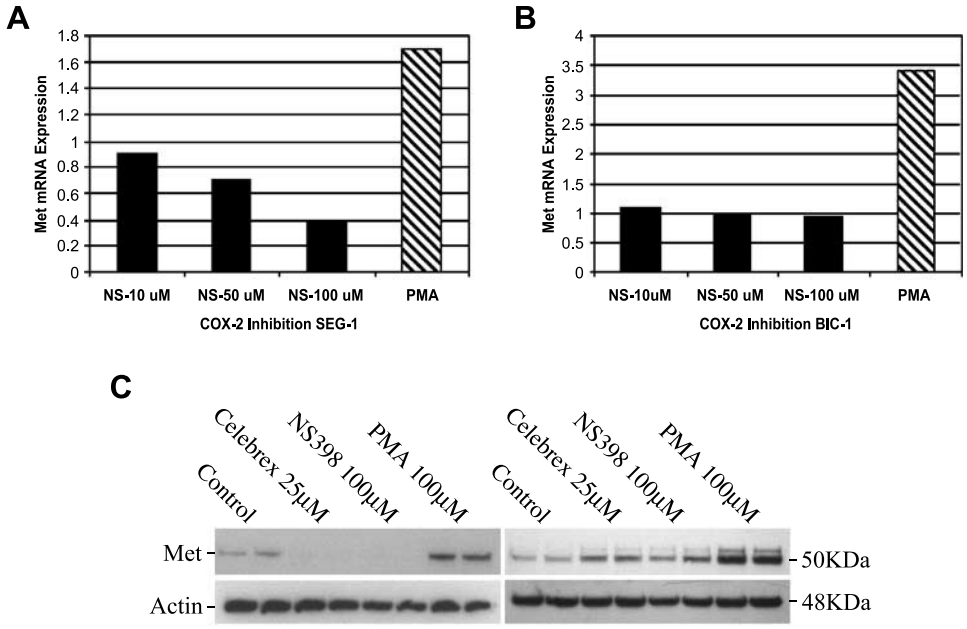

Figure 7. *Met mRNA and protein expression is downregulated by selective COX-2 inhibition in the SEG-1 EA cell line but not in the BIC-1 EA cell line. SEG-1 cells (COX-2–expressing) (A) and BIC-1 cells (COX-2–negative) (B) were treated with increasing doses of COX-2 inhibitors, celecoxib and NS-398. As a known positive control of Met regulation, the protein kinase C inducer PMA was added to the cells. Met mRNA levels were evaluated 48 hours after treatment by QRT-PCR. (A) SEG-1 cells demonstrated a 40% reduction in Met mRNA expression after treatment with 100 μM NS-398. (B) BIC-1 cell line did not demonstrate Met downregulation after COX-2 inhibition; however, this cell line is known to lack COX-2 expression. (C) Met protein expression was found to be significantly downregulated 48 hours following treatment with celecoxib (25 μM) and NS-398 (100 μM) in the SEG-1 cell line but not in the BIC-1 cell line. PMA (100 μM) was found to increase Met expression in both cell lines.*

COX-2 inhibition. Thus, Met overexpression in EA may follow from COX-2 overexpression, but also may occur independently of COX-2 overexpression. Further studies are needed to further clarify the relationship of COX-2 and Met expression in EA. Our results do support the theory that one mechanism through which COX-2 inhibition negatively affects tumor progression is due to this concomitant downregulation of Met.

Finally, Met protein has been shown to interact and sequester Fas receptors at the cellular membrane in murine hepatocellular carcinoma cell lines, causing resistance to Fas-mediated apoptosis [30]. Because our EA cell lines are known to be resistant to Fas-mediated apoptosis [28], we examined whether this mechanism of Fas sequestration was present in the EA cell lines. However, no Fas–Met interaction was demonstrable in our EA cell lines by either immunocoprecipitation of the two receptors or dual-labeled immunofluorescence and laser scanning confocal microscopy. We suspect that this finding is due to the fact that Met is phosphorylated in serum-free conditions in these cell lines, resulting in disassociation of Met and Fas as has been reported by others [34].

Summary

Met expression is altered in EA with marked and consistent overexpression of the proto-oncogene *Met*. This overexpression was observed in BE with HGD and EA. Met is also overexpressed significantly in EA cell lines and Met mRNA expression can be downregulated with COX-2 inhibition, suggesting an interaction in these two pathways in some EAs. Our results suggest that Met would be an attractive target for molecular therapy with tyrosine kinase inhibition. Additional studies of Met overexpression, function, and inhibition in EA are clearly warranted.

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