Regulatory role of transcription factor HBP1 in anticancer efficacy of EGFR inhibitor erlotinib in HNSCC

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1 | INTRODUCTION

Epidermal growth factor receptor (EGFR) is often highly expressed in head and neck squamous cell carcinoma (HNSCC) mainly due to its gene amplification. Abnormal elevation of EGFR expression is closely associated with poor clinical outcomes—high recurrence and low survival rates. Mechanistically, EGFR activation through several transduction pathways, including phosphoinositide 3-kinase (PI3K)/Akt and/or RAS/extracellular signal-regulated kinases (ERK), often leads to a malignant cell phenotype: increased survival, anti-apoptosis, angiogenesis, and metastatic potential. Therefore, drugs such as monoclonal antibodies (eg, cetuximab) and small molecule tyrosine kinase inhibitors (eg, erlotinib and gefitinib) that target EGFR have been developed and clinically used in patients with cancer having elevated EGFR activation.

Erlotinib was approved by the US Food and Drug Administration in 2004 for treatment of patients with locally advanced or metastatic non-small-cell lung cancer after failure of at least one prior chemotherapy regimen. Since then, the therapeutic efficacy of erlotinib has been widely investigated experimentally and clinically in a variety of cancers with deregulated EGFR activation, including HNSCC. A phase II study on the efficacy and safety of erlotinib in patients with advanced recurrent and/or metastatic HNSCC showed that erlotinib was well tolerated in the heavily pretreated population and produced prolonged disease stabilization.

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Mechanistically, erlotinib might inhibit EGFR-mediated signaling pathway through inhibition of the signaling transducer p-Akt, p-ERK, p-STAT3, and/or pSrc, and/or the induction of NADPH oxidase 4-mediated oxidative stress, and/or p21, and/or p27. However, acquired resistance or mutations of the EGFR downstream effectors may contribute to the observed limited clinical outcome. Therefore, understanding the molecular mechanisms of the EGFR signaling in certain cancer phenotypes may help doctors choose and improve the therapeutic efficacy of the antitumor drugs that target EGFR and/or its closely related signaling molecules.

High-mobility group box-containing protein 1 (HBP1) is a transcription factor that regulates several cellular physiologies, including cell proliferation, differentiation, and senescence. Known HBP1 targeted genes include N-myc, c-myc, cyclin D1, MIF, p16 (INK4A), p47phox, and DNA methyltransferase 1 (DNMT1). Ectopic expression of HBP1 results in growth arrest, apoptosis, or differentiation, whereas HBP1 knockdown hastens cell growth, migration, invasion, and xenografted tumor growth in various cancer cell lines. Recently, low-expression HB1 mutants have been isolated from myeloid leukemia and solid tumors such as breast cancer that makes HB1 a tumor suppressor gene. Two major cellular signal transducers, p38 MAPK and RAS, have been shown to regulate HB1 activation and thus cell cycle arrest in the G1 phase and premature senescence, respectively. Previously, we demonstrated that N-acetylcysteine (NAC) exerts its antitumor effect by suppressing the activation of EGFR and Akt and inducing HB1 expression in EGFR-overexpressing oral cancer cells. These data suggest a connection between HB1 and EGFR signaling; however, whether HB1 is a downstream effector of EGFR cannot be asserted from these data.

In the present study, we aimed to establish the role of HB1 as a key downstream effector of EGFR signaling and to further identify its role in erlotinib-mediated growth suppression in EGFR-overexpressing oral cancer cells. Our data suggest that HB1 is an important biomarker of erlotinib action in HNSCC.

## 2. MATERIALS AND METHODS

### 2.1 Reagents, antibodies, and densitometric analysis

All chemicals were purchased from Sigma (St. Louis, Missouri), and antibodies were from Cell Signaling Technology (Beverly, Massachusetts), respectively, unless specified otherwise. Polyvinyliden fluoride membranes and enhanced chemiluminescence detection reagents were from Perkin Elmer Life Sciences, Inc. (Waltham, Massachusetts). The Dual-light system was from Applied Biosystems (Foster City, California). Antibodies for Akt, p-Akt, cyclin D, and HB1 were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Anti-α-tubulin was from Abcam (Cambridge, Massachusetts). EGFR inhibitors AG1478 and erlotinib (Tarceva, OSI Pharmaceuticals, Melville, New York) were purchased from Biochrom AG (Berlin, Germany) and Lumteq (Hsinchu, Taiwan), respectively. The small interfering RNA (siRNA) molecule specific for EGFR was obtained from Santa Cruz Biotechnology (Santa Cruz, California), and two HB1-specific siRNA molecules were purchased from Invitrogen (Carlsbad, California) and Santa Cruz Biotechnology (Santa Cruz, California). Densitometric analyses of Western blots were performed with the ImageJ software 1.8.0 (National Institutes of Health) and normalized to the intensity of the loading control, either α-tubulin or β-actin.

### 2.2 Plasmids

The expressing plasmid pGL3-p27Luc was purchased from Addgene (Cambridge, Massachusetts; Addgene database plasmid 23047). The HB1-expressing plasmid pcDNA3-HB1-Flag was kindly provided by Dr. Xiaowei Zhang (Peking University, China).

### 2.3 Cell culture and treatment

The HSC-3 human oral squamous carcinoma cells were kind gifts from Dr. Hsin-Ling Yang at the China Medical University (Taichung, Taiwan). HSC-3 and FaDu cells were maintained in Dulbecco's modified Eagle medium (DMEM)/F-12 and DMEM and supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic, respectively. Cell viability was measured by the Trypan Blue method. All cell culture reagents were from Invitrogen unless otherwise indicated.

### 2.4 Cell cycle analysis

Cells were seeded onto six-well plates and treated with or without erlotinib for the indicated times, and then trypsinized, washed with phosphate-buffered saline, and fixed in cold 70% ethanol at −20°C. The fixed cells were collected and stained in a solution containing 0.5 mL of 4 μg/mL of PI, 0.5 mg/mL of RNase, and 1% Triton X-100 for 30 minutes at 4°C. The DNA content of these cells was analyzed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey).
2.5 | Tissue sample preparation

As previously described,\textsuperscript{1,29} a total of 35 oral squamous cell carcinoma (OSCC) specimens were acquired from a tissue bank at the China Medical University Hospital (Taichung, Taiwan). RNA specimens from nine normal oral epithelial counterparts were used as reference samples. All tumor specimens were divided into two groups, noninvasive (pN = 0, no metastasis) and invasive (pN > 0, lymph node metastasis).

2.6 | Reverse transcription-polymerase chain reaction and real-time PCR

Total RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. RT-PCR was performed using the SuperScript III One-Step RT-PCR System with the Platinum Taq DNA Polymerase Kit (Invitrogen). The following primers were used: human HBP1, 5′-ATCATCTCCTGTACACATCATAGC-3′ (F) and 5′-CATAGAAAGGGTGGTCCAGCTTAC-3′ (R); 18S, 5′-GTCTGTGATGCCCTTAGATG-3′ (F) and 5′-AGCTTATGACCCGCACTTAC-3′ (R). HBP1 primer sequences for real-time PCR analysis of oral tumor specimens were 5′-GAACCAATTCAGGCTCACA-3′ (F) and 5′-TCAAGACTCAATGCTATCAGTATC-3′ (R).

2.7 | Matrigel invasion assay

Matrigel inserts for 24-well chambers were obtained from BD Biosciences (Bedford, MA) and operated according to the manufacturer’s protocol.\textsuperscript{30} Cell suspensions (5 × 10\textsuperscript{4} cells) were seeded to the upper chamber and 10% FBS-containing medium served as the chemoattractant and was added to the lower chamber. After 24 hours in a 37°C, 5% CO\textsubscript{2} incubator, the noninvading cells from the upper chamber were removed using cotton swabs, whereas the invading cells on the lower surface were fixed with 100% methanol, stained (Giesma in 20% ethanol), and counted. The invading cells were photographed and counted in five, randomly selected microscopic fields (×200 magnification). Error bars represent the variation of the cell numbers between the selected fields.

2.8 | Statistical analysis

Data are expressed as mean ± SD or mean ± SEM from at least three independent experiments. Statistical significance was analyzed using Student’s t test. Results were considered significantly different at $P < 0.05$.

3 | RESULTS

3.1 | HBP1 expression is sensitive to EGFR activity

EGFR is often highly expressed in HNSCC.\textsuperscript{4,31} Previously, we demonstrated that NAC may exert its anticancer effect through collateral downregulation of EGFR and Akt activation and upregulation of HBP1.\textsuperscript{26} Furthermore, we showed that HBP1 is a direct target of the Akt downstream effector FOXO1.\textsuperscript{32} These findings suggest a role of HBP1 in the EGFR signaling pathway. Therefore, in the current study, we tried to examine if HBP1 is an important downstream effector that modulates EGFR action in HNSCC. First, we tested the effect of EGFR level and activity on HBP1 in HSC-3 human tongue squamous cell carcinoma, embedding the highest EGFR protein expression level and activity among the three HNSCC lines tested (Figure 1A). When EGFR expression was suppressed by EGFR-specific siRNA, HBP1 protein levels became upregulated (Figure 1B). In addition, down-regulation of EGFR activation by AG1478,\textsuperscript{33} an EGFR tyrosine kinase inhibitor, also led to increased HBP1 expression in a dose-dependent manner (Figure 1C). It is known that EGFR activation initiates upon ligand binding, which leads to the dimerization and autophosphorylation of the receptors and subsequent activation of the downstream effectors.\textsuperscript{34} With this in mind, it can be postulated that the absence of an EGFR ligand might cause increased HBP1 expression. Indeed, while HSC-3 cells were under serum starvation for 18 hours, EGFR phosphorylation was decreased but HBP1 in both protein and mRNA levels were elevated (Figure 1D,E). The reporter assay from a luciferase gene conjugated with a 2-kb human HBP1 promoter revealed that the serum-free condition also activated the HBP1 promoter (Figure 1F). These results indicated that blockage of EGFR activation led to an increased transcription of HBP1 gene in EGFR-overexpressing HSC-3 cells. Taken together, HBP1 appeared to be a downstream target gene of the EGFR signaling cascade in HSC-3 human oral cancer cells. In short, HBP1 may function as a negative regulator of the EGFR action.

3.2 | Erlotinib may induce HBP1 expression in an Akt-independent fashion

To test the clinical relevance of the EGFR/HBP1 axis, we next investigated the role of HBP1 in the erlotinib-inhibited EGFR signaling in OSCC. As expected, erlotinib treatment potently suppressed the expression of p-EGFR with a concomitant increase of HBP1 levels in both dose- (Figure 2A)
and time-dependent manners (Figure 2B) in HSC-3 cells. The reporter gene assay with a 2-kb human HBP1 promoter further revealed that erlotinib induced the HBP1 promoter activity by 4- and 12-fold after 24 and 48 hours of incubation, respectively, which is consistent with the protein levels (Figure 2C). To further connect the inhibitory effect of erlotinib on cell surface receptor EGFR activity to the induction of nuclear transcription factor HBP1, we examined the phosphorylation of several major mediators of the EGFR signaling pathway, including Akt and mitogen-activated protein kinase (MAPK), ERK and p38, upon erlotinib treatment. Interestingly, erlotinib administration apparently did not suppress Akt phosphorylation in HSC-3 cells, whereas ERK phosphorylation was inhibited, and p38 phosphorylation was induced by erlotinib in a dose-dependent manner (Figure 2D). These data indicate that erlotinib induces HBP1 expression in an Akt-independent manner in HSC-3 cells.

3.3 | HBP1 regulates erlotinib-mediated growth arrest in OSCC cells

Next, we examined the cellular outcome of erlotinib treatment. As shown in Figure 3, erlotinib administration potently inhibited cell growth of three cancer cell lines,
FIGURE 2  Dose- and time-dependent effect of erlotinib on HBP1 expression. A, HSC-3 and FaDu cells reaching 80% confluent density were treated with increasing concentrations (0-30 μM) of erlotinib (E0, E10, and E30) for 24 hours and then cell lysates were subjected to analysis for the expression of p-Y1086-EGFR, EGFR, HBP1, cyclin D1, and α-tubulin by Western blotting. B, The time-dependent effect of erlotinib on EGFR phosphorylation and HBP1 expression was measured by Western blotting in HSC-3 cells treated with 10 μM of erlotinib (ERL). C, The time-dependent effect of erlotinib on HBP1 promoter activity 293T cells were transfected with a 2-kb HBP1 promoter-luciferase construct and a β-galactosidase plasmid for 24 hours, followed by 0 (E0) or 10 μM (E10) of erlotinib treatment for another 24 or 48 hours. Luciferase intensities were measured and normalized to β-galactosidase activities (**, P < .001 as compared with control, E0, erlotinib 0 μM). D, HSC-3 cells reaching 80% confluent density were treated with erlotinib (0, 10, 30 μM; E0, E10, and E30) of for 24 hours and then cell lysates were subjected to analysis for the expression of p-Akt, Akt, p-ERK, ERK, p-p38, p38, and β-actin by Western blotting. EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; HBP1, HMG box-containing protein 1; p-ERK, phosphor-ERK; p-Y1086-EGFR, phosphor tyrosine 1086-EGFR.

FIGURE 3  Effect of erlotinib on cell growth in oral cancer cell lines. A, HSC-3, FaDu, and OECD-1 cells were treated with increasing concentrations of erlotinib (0, 5, 20, 40 μM) for 72 hours and viable cell numbers were counted by the Trypan blue method. B, After 48 hours incubation of 10 μM erlotinib, HSC-3 and FaDu cells were analyzed by flow cytometry for cell cycle distribution (**P < .001 as compared with Control).
HSC-3, OECM-1, and FaDu. Interestingly, among these three cell lines, HSC-3 showed the highest sensitivity to erlotinib (Figure 3A). The flow cytometry analysis of cell cycle distribution further revealed that erlotinib significantly caused cell growth arrest in the G1 phase (Figure 3B) with a concomitant reduction of a positive cell cycle regulator cyclin D1 (Figure 2A) and the induction of a negative cell cycle regulator p27 (Figure 4C). Taken together, these results suggested that HBP1 might be a key downstream mediator in erlotinib-mediated growth inhibition in oral cancer cells. To test this hypothesis, we first used HBP1 siRNA to knockdown its expression under the presence of erlotinib. We found that HBP1 siRNA-mediated knockdown significantly attenuated erlotinib-induced suppression of cell growth (Figure 4A), and G1 arrest (Figure 4B) in HSC-3 cells. In addition, erlotinib-induced p27 expression was also attenuated in the presence of HBP1 siRNA (Figure 4C), suggesting that HBP1 might function upstream of p27 under the erlotinib action. Indeed, transfecting HSC-3 cells with the pcDNA3-HBP1-Flag plasmid to ectopically overexpress HBP1 level led to increased p27 expression in the absence of erlotinib (Figure 4D). This induction of p27 expression became enhanced when cells were cotreated with erlotinib (Figure 4D). Conversely, either erlotinib or HBP1 alone reduced cyclin D1 protein level, and this reduction became more potent in the presence of both (Figure 4D). Furthermore, the overexpression of either p27 or HBP1 resulted in an increased percentage of G1 phase distribution and confirmed the role of both p27 and HBP1 in erlotinib-mediated cell cycle arrest (Figure 4E). Taken as a whole, these results indicate that HBP1 is a crucial mediator in regulating erlotinib-mediated suppression of cyclin D1 and induction of p27 and, ultimately, cell cycle arrest in G1.

**FIGURE 4**  HBP1 regulates erlotinib-mediated cell growth in oral cancer cells. A-C, HSC-3 cells were transfected with either scramble or HBP1-specific siRNA (HBP1 siRNA) for 24 hours and then incubated in the absence (ERL 0) or presence (ERL 10 μM) of erlotinib for the indicated times, followed by hemocytometric counting of viable cell numbers with Trypan blue staining. A, flow cytometric analysis of cell distribution (*P < .05 as compared with HBP1 siRNA (−)), B, or Western blotting analysis for p-Y1086-EGFR, HBP1, p27, and α-tubulin. C-D, HSC-3 cells were transfected with either empty or HBP1-Flag-expressing plasmid for 24 hours and then incubated in the absence (ERL 0) or presence (ERL 10) of 10 μM of erlotinib for another 24 hours, followed by Western blotting analysis for Flag, p-Y1086-EGFR, EGFR, p27, and α-tubulin. E, HsC-3 cell were transfected with empty vector, p27-luciferase, or HBP1-Flag-expressing vector for 24 hours (left panel), followed by flow cytometry analysis of cell cycle distribution (right panel). EGFR, epidermal growth factor receptor; HBP1, HMG box-containing protein 1; p-Y1086-EGFR, phosphor tyrosine 1086-EGFR [Color figure can be viewed at wileyonlinelibrary.com]
3.4 HBP1 mediates the anti-invasion effect of erlotinib, and its expression status predicts invasiveness of oral cancer

In addition to cell growth control, erlotinib administration also suppressed the invasive potential of oral cancer cells. As shown in Figure 5A, erlotinib (10 μM) inhibited cell invasion by 50% as demonstrated by the Matrigel invasion assay. In a previous study, we showed that HBP1 knockdown led to increased cell invasion in HSC-3 cells. Here, HBP1 knockdown potently alleviated the anti-invasion effect of erlotinib (Figure 5A). Together, HBP1 knockdown

**FIGURE 5** Impact of HBP1 expression level on invasiveness in oral cancer. A, HSC-3 cells transfected with either scramble or HBP1-specific siRNA for 24 hours (upper panel) were treated with erlotinib (10 μM) for indicated time, followed by Matrigel invasion assay for invading cells (lower panel). Briefly, 30,000 control or HBP1-knockdown HSC-3 cells were plated in the upper chamber coated with Matrigel in a serum-free medium and allowed to migrate for 48 hours with the addition of a chemoattractant (10% fetal bovine serum-containing medium) in the lower chamber. Cells invaded to the lower chamber were photographed under ×400 magnification and counted in five, randomly selected microscopic fields (×200 magnification). Error bars represent the variation of the cell numbers between the selected fields. Average migrated cell numbers in the HBP1-knockdown group showed a significant difference from the corresponding control (*P < .05). B, Quantitative analysis of HBP1 and EGFR expression levels in oral tumor specimens. Real-time PCR was used to measure HBP1 and EGFR mRNA levels in a subset of oral tumor specimens. Each four-digit number represents an oral tumor sample. The HBP1 mRNA level of “N,” the average from nine adjacent normal tissue specimens, was set as 1. C, HBP1 expression between normal and oral tumor specimens. The mean HBP1 mRNA level of oral tumor specimens was significantly lower than that of the control, normal tissues (Mann-Whitney test, P = .002). Values were expressed as mean ± S.E.M. (*, P < .05). The Kruskal-Wallis test of HBP1 levels among normal, noninvasive, and invasive tumors revealed a significant difference in HBP1 among groups (P = .001). Further analysis of Dunn’s multiple comparison test indicated that the HBP1 level is significantly lower in invasive tumors than in normal tissues (*P < .05). D, Association of the HBP1/EGFR expression status and invasiveness. Tumors with low HBP1 (<0.2-fold of normal) and high EGFR (>3-fold of normal) expression are associated with invasiveness in oral cancer specimens. The 2 × 2 correlation table and Fisher’s exact test were used with a significant two-sided value, P = .04. EGFR, epidermal growth factor receptor; HBP1, HMG box-containing protein 1 [Color figure can be viewed at wileyonlinelibrary.com]
attenuated erlotinib-inhibited proliferation and invasiveness in oral cancer cells with aberrant EGFR expression. Our data further suggest that high EGFR and low HBP1 might be associated with the metastatic potential of oral cancer. To test this hypothesis, we examined the mRNA levels of HBP1 and EGFR from 35 oral tumor specimens and compared them with those of nine normal tissues. Although a great variation between the groups was observed, the mean EGFR mRNA levels of oral tumors were significantly higher than those of the control normal tissues; however, the mean HBP1 mRNA levels of oral tumors were significantly lower than those of the normal tissues (Figure 5B,C). In addition, invasive oral tumors (lymph node metastasis) exhibited significantly lower HBP1 levels than those of normal tissues (Figure 5C). Further analysis indicated a correlation of a low-HBP1 and high-EGFR status with the aggressiveness of oral tumors (Figure 5D). These clinical data suggest that low HBP1 expression is a potential predictor of invasiveness, while a combination of low-HBP1 and high-EGFR status might determine metastatic potential in oral cancer.

4 DISCUSSION

Aberrent EGFR signaling frequently occurs in HNSCC. The use of EGFR-targeting drugs is a conceivable therapeutic strategy for cancer patients with EGFR overexpression; however, their clinical outcome is modest. Deregulation of the phosphatase and tensin homologue (PTEN)/PI3K/AKT pathway may contribute to resistance to the EGFR inhibitors. Here, we demonstrated that the expression of transcription factor HBP1 was enhanced in response to erlotinib, an EGFR tyrosine kinase inhibitor, while HBP1 knockdown significantly attenuated the anticancer action of erlotinib. More importantly, erlotinib mediated induction of p27, a negative regulator of cell cycle program, and was also attenuated upon HBP1 siRNA treatment, whereas ectopic expression of HBP1 additionally enhanced erlotinib-mediated p27 expression (Figure 4D). Our data indicated that in addition to p21, p27, and p53, HBP1 may also be a crucial downstream mediator of EGFR-targeting drugs that negatively regulates tumorigenicity in oral cancer.

Erlotinib (Tarceva), a small tyrosine kinase inhibitor targeting the ATP-binding site of EGFR, has been clinically demonstrated to prolong survival in patients with non-small-cell lung cancer with previous chemotherapy. A phase II trial also indicated that the addition of erlotinib to a first-line combined modality therapy was feasible in improving the overall survival rate in advanced HNSCC. Nevertheless, accumulating evidence indicates that EGFR overexpression or amplification might not be the only factor predicting that patients benefit from EGFR tyrosine kinase inhibitors. Thus, in addition to EGFR, identification of other molecular targets of EGFR-targeting drugs becomes worthy of investigation. Among those, the PI3K/Akt/PTEN pathway, thereby, has drawn a lot of attention and been posited to be crucial in erlotinib sensitivity. However, the HSC-3 human oral cancer cell line we used in the current study was reported to have an intact PTEN level but carry mutations within exon 9 and 20 of PIK3CA gene, encoding a catalytic subunit of PI3K, thereby increasing Akt phosphorylation. Interestingly, erlotinib (10 μM) potently inhibited EGFR phosphorylation but not Akt phosphorylation (Figure 2D) in this cell line, suggesting that sensitivity to EGFR-targeting drugs such as erlotinib appears to be extremely complicated and depends on cellular mutation milieu.

Studies on transcription factor HBP1 have been focused on its downstream target genes, such as c-myc, cyclin D1, DNMT1, MIF, and p47phox; however, the upstream signaling molecules that lead to HBP1 activation are not fully clear. The HBP1 protein has been shown to be a direct target of p38 MAPK phosphorylation, while RAS induction of cell transformation also leads to increased HBP1 expression, resulting in premature senescence. In addition, in a previous study, we showed that HBP1 expression is negatively controlled by Akt and is a direct transcription target of FOXO1. These data indicate that HBP1 could be an important downstream mediator of activated cell surface receptors in response to extracellular stimulation. Here, we provided evidence that HBP1 is a downstream target gene of the EGFR signaling pathway since downregulation of EGFR induced HBP1 expression in HSC-3 human oral cancer cells with high levels of EGFR expression (Figure 1). Therefore, the current study demonstrated a novel role of HBP1 as a transcription target of the Akt-independent EGFR signaling cascade. Although erlotinib action has been extensively focused on the EGFR/PI3K/Akt pathway, we cannot exclude the possibility that other signaling pathways, including ERK, are also involved. Erlotinib-mediated reduction in ERK phosphorylation has been reported in patients with nonmetastatic HNSCC. Nonetheless, HBP1 seems to play a pivotal role in conveying the erlotinib action to downstream effectors such as p27, thereby leading to cell growth arrest. Therefore, additional therapy that targets signaling molecules such as HBP1 in close connection with EGFR may help overcome resistance and improve clinical outcomes of EGFR-targeting drugs.

Low HBP1 status may predict oral cancer malignancy. In the current study, we analyzed one subset of oral cancer specimens, and we found that mean normalized HBP1
mRNA expression in tumors was significantly lower than that of the corresponding normal tissues (Figure 5B,C) (also in figure 6A,B in Chan et al.29), something that is similar to the previous findings in other solid tumors such as breast and prostate tumors.22,25 However, 4 out of 19 (21%) noninvasive specimens and 3 out of 16 (18.7%) invasive specimens exhibited greater HBP1 mRNA levels than 50% of the expression level in the normal tissues (Figure 5B). It is possible that these samples were either HBP1-independent or carrying HBP1 mutants as previously described in breast cancer.25 Nonetheless, the association of low HBP1 expression and oral cancer malignancy was also supported by the results that HBP1 knockdown potently hastened invasiveness (Figure 5A) (also seen in figure 1C in Chan et al.29), and xenografted tumor growth (figure 5 in Lee et al.26). A low HBP1 mRNA level has been found to be associated with a high recurrence of both breast and prostate cancers.25,44 Therefore, accumulated evidence tends to support HBP1 as an important biomarker in the prediction of tumorigenicity and malignancy. In particular, high EGFR/low HBP1 might predict a metastatic potential in oral cancer (Figure 5D).

In conclusion, our data demonstrated that HBP1 expression was sensitive to the EGFR signaling cascade, and, in particular, HBP1 was a key downstream effector of the EGFR signaling pathway in erlotinib-mediated inhibition of cell proliferation and invasion in oral cancer. This study also supports the concept that low HBP1 and high EGFR expression levels may be potential diagnostic and prognostic markers that predict metastatic potential in oral cancer.

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CONFLICT OF INTEREST
The authors declare no potential conflict of interest.

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