

Antitumor Effects of CRM197, A Specific Inhibitor of HB-EGF, in T-Cell Acute Lymphoblastic Leukemia

NAOKO KUNAMI^{1*}, FUSANORI YOTSUMOTO^{2*}, KENJI ISHITSUKA¹,
TATSUYA FUKAMI³, TAKASHI ODAWARA⁴, SADA O MANABE⁴, TOYOKAZU ISHIKAWA⁴,
KAZUO TAMURA¹, MASAHIDE KUROKI² and SHINGO MIYAMOTO³

Departments of ¹Internal Medicine, ²Biochemistry and ³Obstetrics and Gynecology,
Faculty of Medicine, Fukuoka University, Japan;

⁴The Research Foundation for Microbial Diseases, Osaka University, Japan

Abstract. *The therapeutic outcome for T-cell acute lymphoblastic leukemia (T-ALL) remains poor; thus, novel, targeted therapies are urgently needed. Recently, we showed that heparin-binding epidermal growth factor-like growth factor (HB-EGF), a member of the EGF family, is a promising target for the treatment of various types of cancer. The aim of the present study was to investigate whether HB-EGF is a therapeutic target for T-ALL, and to further elucidate the antitumor effects of a specific inhibitor of HB-EGF, cross-reacting material 197 (CRM197). We elucidated the expression of HB-EGF in T-ALL cell lines, and evaluated the effect of CRM197 on these cells alone or in combination with anticancer agent. The expression of EGFR and EGFR ligands was determined by flow cytometry, RT-PCR and real-time quantitative PCR. Induction of apoptosis was assessed by TUNEL assay. HB-EGF was strongly expressed by T-ALL cell lines, and the expression of both HB-EGF and EGFR was enhanced by doxorubicin. CRM197 induced apoptosis, and furthermore, the combination of CRM197 plus doxorubicin enhanced cytotoxicity in a T-ALL cell line. These results suggest that HB-EGF is a promising therapeutic target for T-ALL.*

T-Cell acute lymphoblastic leukemia (T-ALL) is an aggressive disorder of T-lymphocytes (1) and accounts for about 10% to 15% of adult and 25% of childhood ALL cases, respectively (2, 3). The overall survival for patients

with T-ALL has significantly improved over the past decades, mainly owing to advances in therapeutic interventions (4); however, relapse is frequently observed, resulting in a poor clinical outcome (5). Therefore, it is imperative that novel, effective therapies are developed.

We previously reported that heparin-binding epidermal growth factor-like growth factor (HB-EGF), an EGF family ligand, is a target in the treatment of ovarian, breast, and gastric cancer (6-9). HB-EGF is initially synthesized as a transmembrane protein, similar to other members of the EGF family of growth factors (10). The membrane-anchored form of HB-EGF (proHB-EGF) is cleaved at the cell surface by a protease to yield the soluble form of HB-EGF (sHB-EGF) via a mechanism known as ectodomain shedding (11). Inhibition of HB-EGF expression blocks both the mitogen-activated protein kinase (MAPK) and Akt signaling pathways, resulting in suppression of tumor formation. Cross-reacting material 197 (CRM197) is a nontoxic variant of diphtheria toxin isolated from cultures of *Corynebacterium diphtheriae*. CRM197 binds to human HB-EGF and blocks its mitogenic activity by inhibiting EGFR binding (12). A preliminary report showed that CRM197 can be used to treat cancer patients (13). In addition, the ectodomain shedding of HB-EGF induced by anticancer drugs activates antiapoptotic signaling pathways mediated by ERK and Akt via EGFR transactivation, which is thought to be involved in resistance to chemotherapeutics (14). Therefore, CRM197, either alone or in combination with conventional chemotherapy, may potentially be efficacious in the treatment of various types of cancer. A phase 1 study using CRM197 has already begun for patients with advanced ovarian cancer at Fukuoka University.

Several aberrantly activated signaling pathways have been implicated in the progression and drug resistance of T-ALL. Approximately 85% of T-ALL patients show enhanced activation of phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) (15, 16). Up-regulation of the PI3K/Akt/mTOR pathway is induced by

*These Authors contributed equally to this work.

Correspondence to: Kenji Ishitsuka, Department of Internal Medicine, Faculty of Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan. Tel: +81 928011011, Fax: +81 928655656, e-mail: kenjiishitsuka@fukuoka-u.ac.jp

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Notch1 activation which is involved in the molecular pathogenesis of T-ALL (17, 18), leading to HES (hair and enhancer of split)-induced suppression of PTEN (phosphatase and tensin deleted on chromosome 10) function (19, 20). NVP-BEZ235, a novel, dual PI3K/mTOR inhibitor, has apoptotic effects on T-ALL cell lines and synergizes with chemotherapeutic drugs commonly used for the treatment of T-ALL (17). Recently, it was shown that the EGF system plays an important tumorigenic role in both epithelial cancer and hematological malignancies (21, 22).

The objective of this study was to examine HB-EGF expression in T-ALL cells and cell death induced by treatment with either CRM197 alone or in combination with conventional chemotherapeutic agents commonly used to treat this condition.

Materials and Methods

Reagents and antibodies. CRM197 was kindly provided by Professor Eisuke Mekada (Department of Cell Biology, Osaka University, Osaka, Japan). Doxorubicin was obtained from Sigma (St Louis, MO, USA). Recombinant human HB-EGF and a polyclonal antibody against HB-EGF were purchased from R&D Systems (Minneapolis, MN, USA). Fluorescein isothiocyanate-conjugated anti-goat IgG was obtained from Sigma.

Cells and cell culture. The following cell lines were obtained commercially: Human breast cancer cell line, MDA-MB-231; human T-ALL cell line, Jurkat E6-1 and MOLT4F cells (American Type Culture Collection, Manassas, VA, USA). All cell lines were maintained in RPMI-1640 supplemented with 100 U/ml penicillin G, 100 mg/ml streptomycin, and 10% fetal bovine serum (ICN Biomedical, Irvine, CA, USA). The cell lines were treated with CRM197 (10 µg/ml), doxorubicin (5-25 nM) or both for 72 h.

Reverse transcription-PCR (RT-PCR) and real-time quantitative PCR (qPCR) for EGFR ligands and EGFR. RNA extraction and cDNA synthesis were performed using TRIzol and SuperScript II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA), respectively, according to the manufacturer's protocols. Expression of human *HB-EGF*, amphiregulin (*AR*), transforming growth factor- α (*TGF- α*), *EGFR* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were examined by RT-PCR using KOD plus (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The primers used are shown in Table I. TaqMan qPCR was carried out using oligonucleotide primer pairs and TaqMan probes for *HB-EGF*, *AR*, *TGF- α* , *EGFR* and *GAPDH* as previously described (7).

Flow cytometry. Cells were harvested and incubated with a polyclonal anti-HB-EGF antibody for 30 minutes at 4°C followed by incubation with FITC-conjugated anti-goat IgG antibody for 30 minutes at 4°C. Positive cells were quantified by flow cytometric analysis using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Apoptosis assay. After treatment with doxorubicin (5-25 nM) and CRM197 (10 µg/ml) for 72 hours, TUNEL-positive cells were confirmed to be apoptotic using flow cytometric analysis as

Table I. *Primer sequences for RT-PCR.*

Human EGFR Sense	5'-CAACACCCTGGTCTGGAAGT-3'
Antisense	5'-GCTTTCGGAGATGTTGCTTC-3'
Human HB-EGF Sense	5'-GGCAGATCTGGACCTTTTGA-3'
Antisense	5'-CCCTCTGCAGTCTGAAATC-3'
Human amphiregulin Sense	5'-CGGGAGCCGACTATGACTAC-3'
Antisense	5'-AACAGCAACAGCTGTGAGGA-3'
Human TGF α Sense	5'-TTTAAATGACTGCCAGAT-3'
Antisense	5'-AGCAGTGTATCAGCACACAT-3'
Human GAPDH Sense	5'-ACCCAGAAGACTGTGGATGG-3'
Antisense	5'-TGCTGTAGCCAAATTCGTTG-3'

previously described (7). TUNEL-positive control cells (no treatment) were quantified as 0%.

Statistical analysis. Data obtained from two independent experiments were analyzed using Student's *t*-test. A *p*-value of <0.05 was considered significant.

Results

T-ALL cells express mRNA of HB-EGF and EGFR, and protein of HB-EGF. To confirm that HB-EGF was a possible targeted molecule in T-ALL, the expression of EGF family members in the T-ALL cell lines was examined. *HB-EGF* mRNA expression was increased in Jurkat E6-1, MOLT4F, and MDA-MB-231 cells (Figure 1A), which express HB-EGF to a greater extent than other EGFR ligands (8). Jurkat E6-1 cells also expressed EGFR (Figure 1A). Next, because HB-EGF is initially synthesized as a transmembrane protein, its expression on the cell surface was examined using flow cytometry. HB-EGF expression was confirmed in Jurkat E6-1, MOLT4F, MDA-MB-231 cells (Figure 1B). These results suggest that HB-EGF is a candidate target molecule for T-ALL therapy.

CRM197 reduces HB-EGF expression and induces apoptosis in T-ALL cells. To clarify the *in vitro* antitumor effects of CRM197 on Jurkat E6-1 and MOLT4F cells, we examined induction of apoptosis after incubation with CRM197 for 72 h. The percentage of TUNEL-positive Jurkat E6-1 and MOLT4F cells was 10.8 \pm 3.2% (mean \pm SD) and 8.6 \pm 2.5%, respectively (Figure 2A). Next, we confirmed the changes in HB-EGF mRNA and protein expression in Jurkat E6-1 cells treated with CRM197 for 72 h. CRM197 did not remarkably reduce *HB-EGF* mRNA expression, but significantly reduced HB-EGF protein expression in Jurkat E6-1 cells compared with that in untreated cells (Figure 2B and 2C). These results indicate that CRM197-mediated inhibition of HB-EGF expression induces apoptosis in T-ALL cells.

Doxorubicin increases the expression of HB-EGF and EGFR mRNA in T-ALL cells. Cells treated with doxorubicin were used to investigate the relationship between HB-EGF mRNA

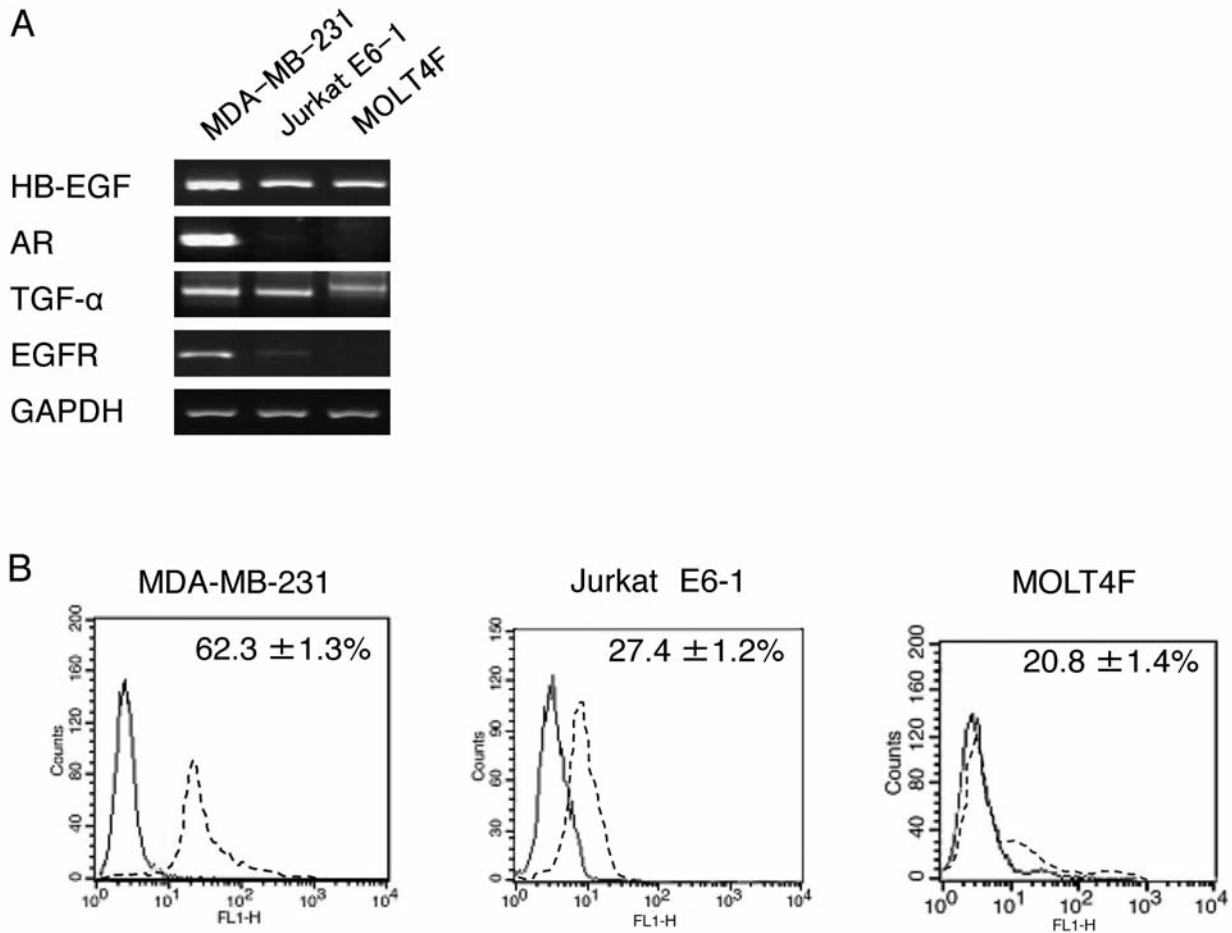


Figure 1. Expression of HB-EGF in T-cell lymphoblastic leukemia cell lines. A: HB-EGF, amphiregulin (AR), TGF- α , EGFR and GAPDH mRNA expression in MDA-MB-231 (breast cancer; positive control), Jurkat E6-1 (T-ALL) and MOLT4F (T-ALL) cells was analyzed by RT-PCR. B: Flow cytometric analysis of HB-EGF (dotted line) on the surface of MDA-MB-231 (positive control), Jurkat E6-1 and MOLT4F cells. The control was labeled with the FITC-conjugated secondary antibody alone (black line). Each histogram is representative of three independent experiments. Data are expressed as the percentage of positive cells (mean \pm SD).

expression and doxorubicin-induced apoptosis. Doxorubicin induced apoptosis in Jurkat E6-1 cells in a dose-dependent manner (Figure 3A). Marked increases in *HB-EGF* and *EGFR* (although not *AR*) mRNA expression were observed at the concentrations of doxorubicin 25 nM (Figure 3B). These results suggest that T-ALL cells can escape from the cytotoxic action of doxorubicin by up-regulating expression of *HB-EGF* and *EGFR*.

CRM197 enhances the antitumor effects of doxorubicin. It was expected that the combination of doxorubicin with CRM197 would be more toxic to T-ALL cells. Therefore, the percentage of apoptotic Jurkat E6-1 cells was examined after treatment with 25 nM doxorubicin and/or 10 μ g/ml CRM197 for 72 hours. The percentage of apoptotic cells after treatment with either CRM197 or doxorubicin alone was 10.8 \pm 3.2% (Figure 2A) and 65.1 \pm 2.5% (Figure 3A),

respectively. However, treatment with doxorubicin plus CRM197 induced apoptosis in 75.6 \pm 2.4% of Jurkat E6-1 cells (Figure 4).

Discussion

In this study, we showed that HB-EGF is the predominant EGFR ligand in T-ALL cell lines, and that suppression of HB-EGF induced significant apoptosis. Doxorubicin, the conventional chemotherapeutic agent used for T-ALL therapy, enhanced HB-EGF expression, thereby probably prevented cell death. Moreover, our results showed that treatment with doxorubicin plus CRM197 induced marked antitumor effects on T-ALL cells *in vitro*.

Doxorubicin resistance is associated with stimulation of the PI3K/Akt pathway (23, 24). The HB-EGF gene is an immediate-early transcriptional target for oncogenic Raf

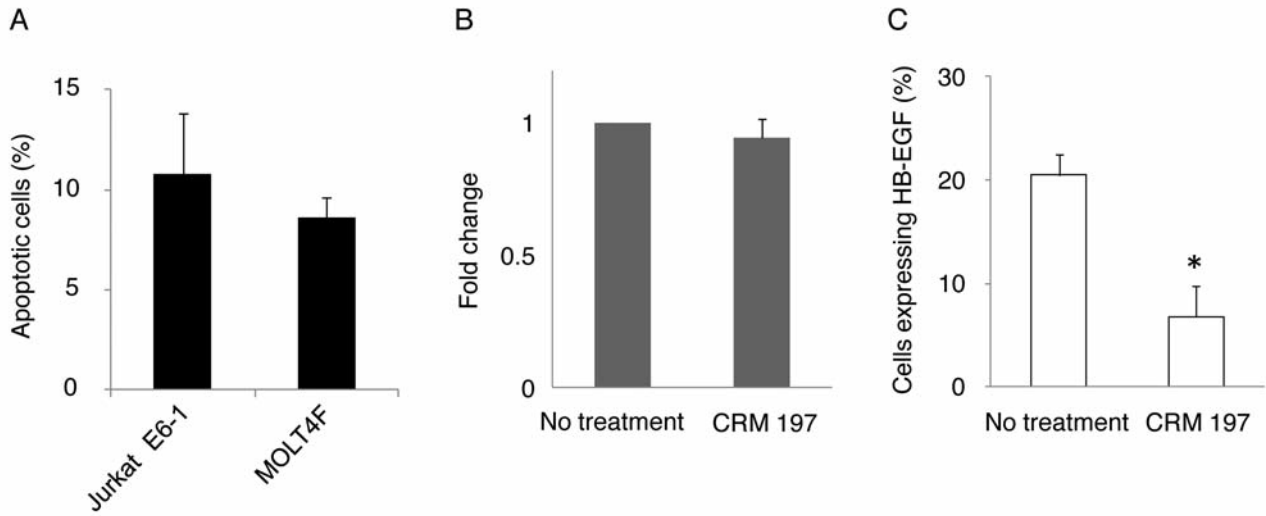


Figure 2. Antitumor effects of CRM197 on T-cell lymphoblastic leukemia. A: Flow cytometric analysis of apoptotic Jurkat E6-1 and MOLT4F cells after incubation with CRM197 (10 µg/ml) for 72 h using TUNEL. Columns represent the mean of three independent experiments and horizontal bars indicate the SD. B: Changes in HB-EGF mRNA expression in Jurkat E6-1 cells treated with CRM197 (10 µg/ml) for 72 h. mRNA levels were analyzed by qPCR. Columns indicate the mean±SD fold-change relative to the values in untreated cells. C: Flow cytometric analysis of HB-EGF on the surface of Jurkat E6-1 cells treated with CRM197 (10 µg/ml) for 72 h. The control was labeled with the FITC-conjugated secondary antibody alone. Columns indicate the mean of three independent experiments and horizontal bars indicate the SD. *p<0.05, versus untreated cells.

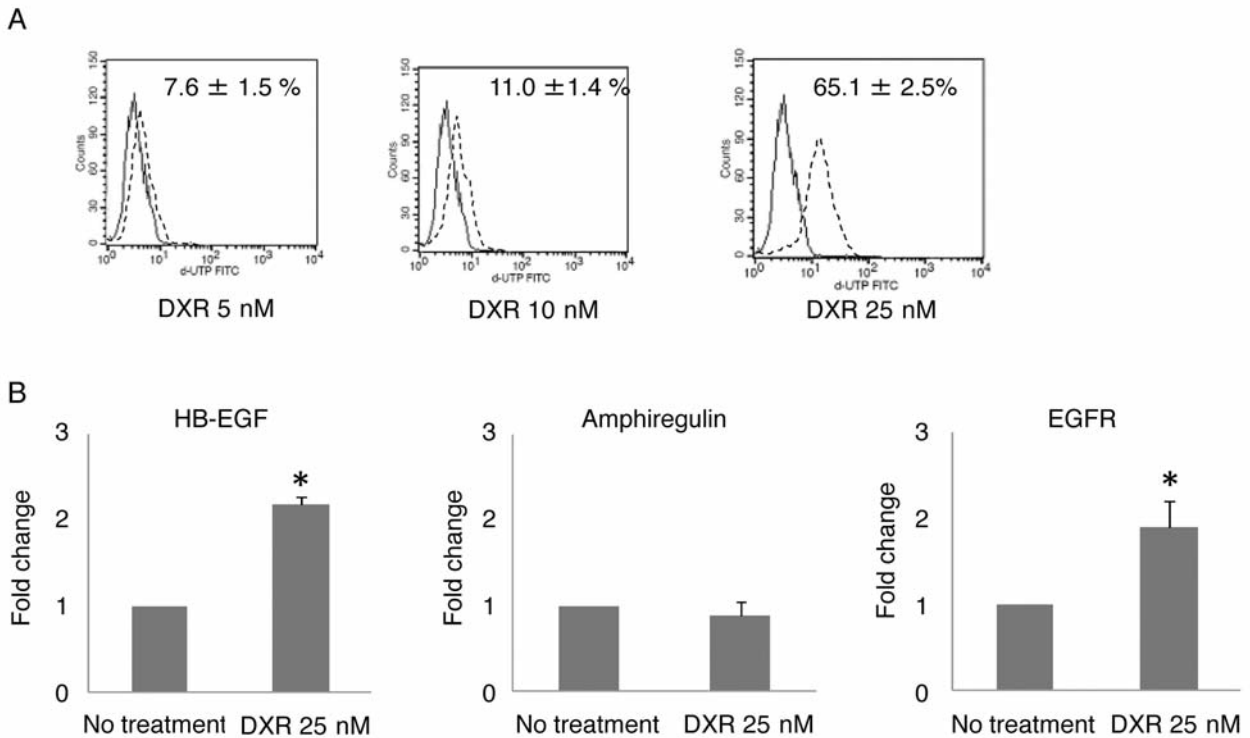


Figure 3. Induction of HB-EGF and EGFR expression by doxorubicin. A: Flow cytometric analysis of apoptotic Jurkat E6-1 cells after incubation with doxorubicin at the indicated concentrations for 72 h using TUNEL. Control (no treatment): solid line; doxorubicin: dotted line. Each histogram is representative of three independent experiments. Data are expressed as the percentage of TUNEL-positive cells (mean±SD). B: Changes in HB-EGF, amphiregulin and EGFR mRNA expression in Jurkat E6-1 cells treated with 25 nM doxorubicin for 72 hours. mRNA levels were analyzed by qPCR. Columns indicate the mean±SD fold-change relative to the values for untreated cells. *p<0.05, versus untreated cells.

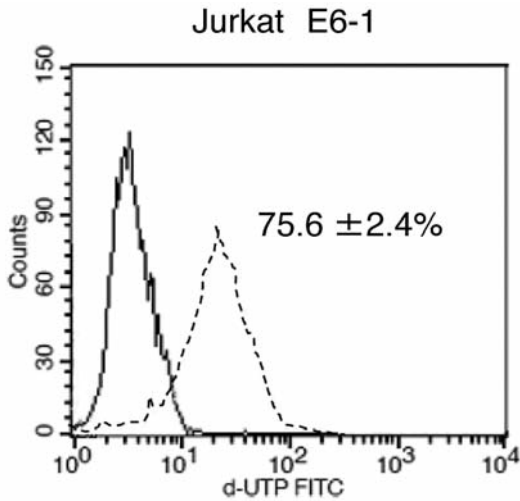


Figure 4. Antitumor effects of CRM197 plus doxorubicin in T-cell lymphoblastic leukemia. Flow cytometric analysis of apoptotic Jurkat E6-1 cells after incubation with CRM197 (10 mg/ml) plus 25 nM doxorubicin for 72 h using TUNEL. Control (no treatment): solid line; doxorubicin (25 nM) plus CRM197: dotted line. Each histogram is representative of three independent experiments. Data are expressed as the percentage of TUNEL-positive cells (mean \pm SD).

kinases, and acts as an early response gene during chemotherapy (25, 26). Increased expression of HB-EGF is regulated by the activation of Akt and ERK kinase (8). Activation of Akt kinase after the addition of doxorubicin may result in enhanced HB-EGF expression in T-ALL cell lines. Moreover, Notch signaling is regulated by ligand-dependent EGFR signaling (27). Combined inhibition of the Notch-EGFR pathways is extremely effective at suppressing tumor growth in mice (28). Therefore, blockade of HB-EGF-mediated signals that drive proliferation and survival may be an important therapeutic strategy for T-ALL. In this study, treatment with CRM197 plus doxorubicin resulted in a marked antitumor effect in T-ALL cells *in vitro*, suggesting a novel combination therapy for T-ALL patients including those that are chemoresistant.

In conclusion, HB-EGF is a critical molecule involved in the pathogenesis of T-ALL and is a reasonable target for T-ALL therapy. The use of CRM197, in combination with conventional chemotherapeutic agents such as doxorubicin, may also lead to an improved prognosis for patients with T-ALL.

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