Activation of HER Family Signaling as a Mechanism of Acquired Resistance to ALK Inhibitors in EML4-ALK–Positive Non–Small Cell Lung Cancer

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Abstract

Purpose: Anaplastic lymphoma kinase (ALK) tyrosine kinase inhibitors (TKI) such as crizotinib show marked efficacy in patients with non–small cell lung cancer positive for the echinoderm microtubule-associated protein–like 4 (EML4)–ALK fusion protein. However, acquired resistance to these agents has already been described in treated patients, and the mechanisms of such resistance remain largely unknown.

Experimental Design: We established lines of EML4-ALK–positive H3122 lung cancer cells that are resistant to the ALK inhibitor TAE684 (H3122/TR cells) and investigated their resistance mechanism with the use of immunoblot analysis, ELISA, reverse transcription and real-time PCR analysis, and an annexin V binding assay. We isolated EML4-ALK–positive lung cancer cells (K-3) from a patient who developed resistance to crizotinib and investigated their characteristics.

Results: The expression of EML4-ALK was reduced at the transcriptional level, whereas phosphorylation of epidermal growth factor receptor (EGFR), HER2, and HER3 was upregulated, in H3122/TR cells compared with those in H3122 cells. This activation of HER family proteins was accompanied by increased secretion of EGF. Treatment with an EGFR-TKI induced apoptosis in H3122/TR cells, but not in H3122 cells. The TAE684-induced inhibition of extracellular signal–regulated kinase (ERK) and STAT3 phosphorylation observed in parental cells was prevented by exposure of these cells to exogenous EGF, resulting in a reduced sensitivity of cell growth to TAE684. K-3 cells also manifested HER family activation accompanied by increased EGF secretion.

Conclusions: EGF-mediated activation of HER family signaling is associated with ALK-TKI resistance in lung cancer positive for EML4-ALK. *Clin Cancer Res; 18(22); 6219–26. ©2012 AACR.*

Introduction

Lung cancer is the leading cause of cancer death worldwide. Fusion of the echinoderm microtubule-associated protein–like 4 gene (*EML4*) with the anaplastic lymphoma kinase gene (*ALK*), which results in the production of a fusion protein (EML4-ALK), occurs in 5% to 10% of cases of non–small cell lung cancer (NSCLC; refs. 1–3). This transforming fusion leads to activation of downstream signaling molecules, and inhibition of ALK signaling has shown marked antitumor effects in NSCLC positive for EML4-ALK in both preclinical and clinical studies (4). Despite their initial response, however, individuals with EML4-ALK–positive NSCLC treated with ALK inhibitors eventually acquire resistance to these drugs (5), and the molecular mechanisms responsible for such resistance remain largely uncharacterized. With the use of an *in vitro* cell model and cells newly derived from a patient with acquired resistance to the ALK inhibitor crizotinib, we have now uncovered a previously unknown mechanism of such resistance.

Materials and Methods

Cell culture and reagents

The H3122 human NSCLC cell line was obtained as previously described (6), and H1299 human NSCLC cells were obtained from American Type Culture Collection. The cells were maintained under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium (Sigma), which contains no biologic ligands including EGF, supplemented with 10% FBS. TAE684, crizotinib, and BIBW2992 were obtained from ShangHai Biochempartner. Paclitaxel was from Sigma, and adriamycin was from Wako. Recombinant

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Translational Relevance

ALK-targeted therapy shows marked clinical efficacy in lung cancer patients with the *EML4-ALK* fusion gene. Such patients eventually develop resistance to ALK tyrosine kinase inhibitors (TKI), however, and the mechanisms of such resistance remain largely unknown. We have now established ALK-TKI–resistant lines of *EML4-ALK*–positive H3122 cells and shown that activation of the epidermal growth factor receptor (EGFR) and the related proteins HER2 and HER3 by autocrine EGF stimulation is associated with such resistance. Furthermore, we established K-3 cells from a specimen of *EML4-ALK*–positive lung cancer obtained from a patient who developed ALK-TKI resistance. These cells also manifested EGF-dependent activation of HER family proteins. Our observations have thus uncovered a previously unknown mechanism of ALK-TKI resistance in *EML4-ALK*–positive lung cancer and they may provide a basis for circumvention of such resistance.

human epidermal growth factor (EGF) was from R&D Systems.

Generation of TAE684-resistant H3122 cells

H3122 cells were initially exposed to 0.01 μmol/L TAE684. They were then isolated by limiting dilution, maintained, and passaged normally with gradually increasing doses of TAE684, up to a maximum of 1 μmol/L. The established resistant cell lines, designated H3122/TR1 and H3122/TR2, were maintained in medium containing the maximal dose of TAE684 (1 μmol/L) to maintain selective pressure for TAE684 resistance.

Growth inhibition assay *in vitro*

Cell viability was assessed with an MTT assay as previously described (7).

Annexin V binding assay

Binding of annexin V to cells was measured with the use of an Annexin-V-FLUOS Staining Kit (Roche), as previously described (7).

Immunoblot analysis

Immunoblot analysis was conducted as previously described (7). Rabbit polyclonal antibodies to phosphorylated human ALK (pY1608), to ALK, to the phosphorylated EGF receptor (pY1068), to phosphorylated HER2 (pY1221), to phosphorylated HER3 (pY1289), to phosphorylated AKT, to AKT, to phosphorylated STAT3, and to STAT3 were obtained from Cell Signaling Technology; those to HER3, to extracellular signal-regulated kinase (ERK), and to phosphorylated ERK were from Santa Cruz Biotechnology; those to HER2 were from Millipore; and those to β-actin were from Sigma. Mouse monoclonal antibodies to the EGF receptor (EGFR) were obtained from Invitrogen.

Reverse transcription and real-time PCR analysis

Total RNA was extracted from cells with the use of an RNeasy Mini Kit (Qiagen) and was subjected to reverse transcription (RT) with the use of a SuperScript Preamplification System (Invitrogen Life Technologies). The resulting cDNA was then subjected to real-time PCR analysis with the use of a One Step SYBR PrimeScript RT-PCR Kit (Takara Bio) and ABI PRISM 7900HT system (Applied Biosystems). The PCR primers (forward and reverse, respectively) included those for EML4-ALK (5′-GTGCAGTGTTTAGCATTCTTGGGG-3′, 5′-T-CTTGCCAGCAAAGCAGTAGTTGG-3′), EGF (5′-TGCAACT-GTGTTGTTGGCTACATC-3′, 5′-TGGTTGACCCCCCATTCTTG-AG-3′), TGF-α (5′-TCAGTTCTGCTTCCATGCAACC-3′, 5′-TT-TCTGAGTGGCAGCAAGCG-3′), and amphiregulin (5′-AGA-GTTGAACAGGTAGTTAAGCCCC-3′, 5′-GTCGAAGTTTCTT-TCGTTCCTCAG-3′). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal standard.

ELISA for ligands

The concentrations of EGF, TGF-α, and amphiregulin in conditioned medium were determined as previously described (8), with the use of a Human Quantikine ELISA Kit (R&D Systems).

Isolation of crizotinib-resistant cells from a clinical specimen

K-3 cells were established at Kinki University Faculty of Medicine from the pleural effusion of a patient with EML4-ALK–positive NSCLC who developed resistance to crizotinib. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. The clinical specimen was obtained from Kinki University Hospital for study with the approval of the Institutional Review Board, and the patient provided written informed consent.

Results

Generation of ALK inhibitor–resistant lines from EML4-ALK–positive H3122 cells

The NSCLC cell line H3122 expresses EML4-ALK and is highly sensitive to the ALK TKI TAE684. We generated TAE684-resistant H3122 cell lines (H3122/TR1 and H3122/TR2) by exposing the parental cells to increasing

Table 1. IC$_{50}$ values of TAE684 and crizotinib for inhibition of the growth of H3122 and H3122/TR cell lines

Cell line	IC50, μmol/L	
	TAE684	Crizotinib
H3122	0.053	0.15
H3122/TR1	1.034	2.26
H3122/TR2	1.100	1.51

NOTE: Data are means of triplicates from representative experiments that were repeated a total of 3 times.

concentrations of TAE684. Short tandem repeat analysis of H3122/TR cells confirmed their H3122 origin (data not shown). The IC_{50} of TAE684 for inhibition of the growth of the parental H3122 cells was 0.053 μmol/L, whereas those for the H3122/TR cell lines as well as for EML4-ALK–negative H1299 cells were all more than 1 μmol/L (Table 1, Fig. 1A). The H3122/TR cells were also resistant to crizotinib, another ALK-targeted TKI, but not to other drugs including paclitaxel and adriamycin (Table 1, Fig. 1A, and data not shown). TAE684 also induced a marked increase in the level of apoptosis in H3122 cells, whereas it had no such effect in H3122/TR cells (Fig. 1B). Collectively, these data suggested that H3122/TR cells had acquired resistance specific to ALK inhibitors.

Activation of alternative signaling pathways in H3122/TR cells

We next investigated the mechanism responsible for resistance to ALK-TKIs in H3122/TR cells. Immunoblot analysis revealed that the abundance of EML4-ALK was reduced in both H3122/TR cell lines compared with that in H3122 cells (Fig. 2A). Quantitative RT-PCR analysis showed that the amount of EML4-ALK mRNA was also reduced markedly in H3122/TR cells relative to that in the parental cells (Supplementary Fig. 1), suggesting that the downregulation of EML4-ALK expression is mediated at the transcriptional level. We did not detect any secondary mutations of ALK in H3122/TR cells (data not shown), with such mutations having previously been shown to give rise to ALK-TKI resistance (5, 9, 10). TAE684 inhibited the phosphorylation of ERK and STAT3 in H3122 cells, but had no such effects in H3122/TR cells (Fig. 2A). Given that signaling via ERK and STAT3 is maintained in H3122/TR cells in the presence of TAE684, we investigated whether alternative upstream pathways are activated in these cells. We found that the level of EGFR phosphorylation was increased in the resistant cells compared with the parental cells, although the abundance of EGFR was decreased in the former cells (Fig. 2B). Phosphorylation of the EGFR-related proteins HER2 and HER3 was also increased in both H3122/TR cell lines (Fig. 2B). To investigate the mechanism responsible for activation of these receptor tyrosine kinases in H3122/TR cells, we examined the expression of ligands for HER family proteins, including EGF, TGF-α, and amphiregulin. Quantitative RT-PCR analysis revealed that the amount of EGF mRNA, but not that of TGF-α or amphiregulin mRNAs, was greatly increased in H3122/TR cells compared with that in parental H3122 cells (Fig. 2C). Consistent with these results, ELISA showed that secretion of EGF, but not that of TGF-α or amphiregulin, was increased in H3122/TR cells (Fig. 2D). We next examined the effect of an EGFR-TKI on apoptosis in the parental and resistant cell lines. Treatment with BIBW2992, an irreversible TKI for EGFR, induced apoptosis in H3122/TR cells, but not in H3122 cells (Fig. 2E). Furthermore, the extent of apoptosis induced by the combination of TAE684 and BIBW2992 in H3122/TR cells
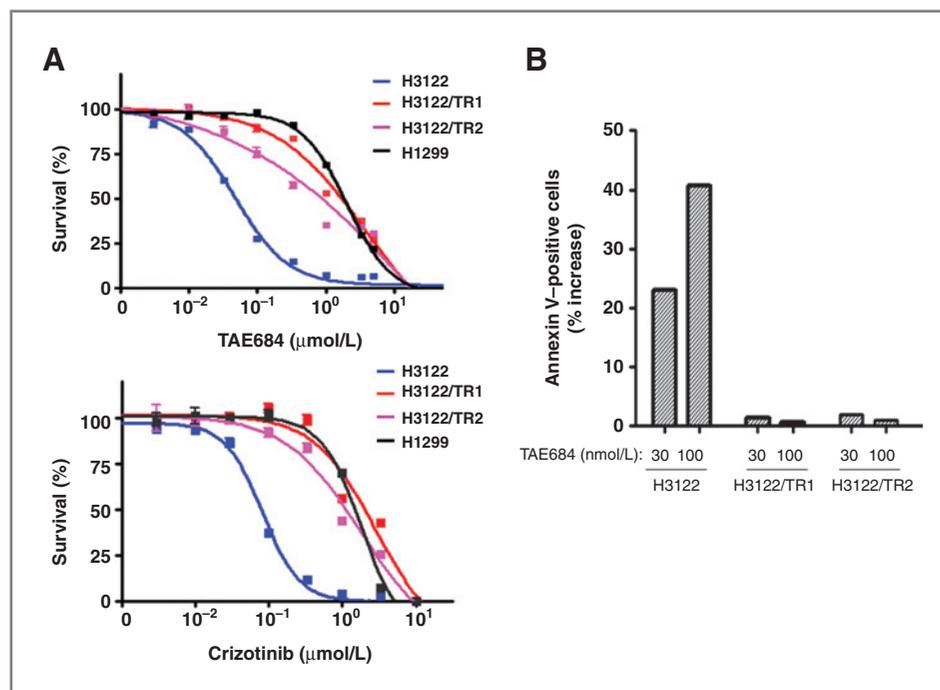
Figure 1. Effects of ALK kinase inhibitors on cell proliferation and apoptosis in H3122, H3122/TR1, H3122/TR2, and H1299 cells. A, cells were cultured for 72 hours in complete medium containing various concentrations of TAE684 (upper panel) or crizotinib (lower panel), after which cell viability was assessed. Data are means of triplicates from representative experiments and are expressed as percentage survival. B, cells were incubated for 72 hours with or without TAE684 (30 or 100 nmol/L), after which the number of apoptotic cells was determined by staining with annexin V and propidium iodide followed by flow cytometry. Data are means of triplicates from representative experiments.
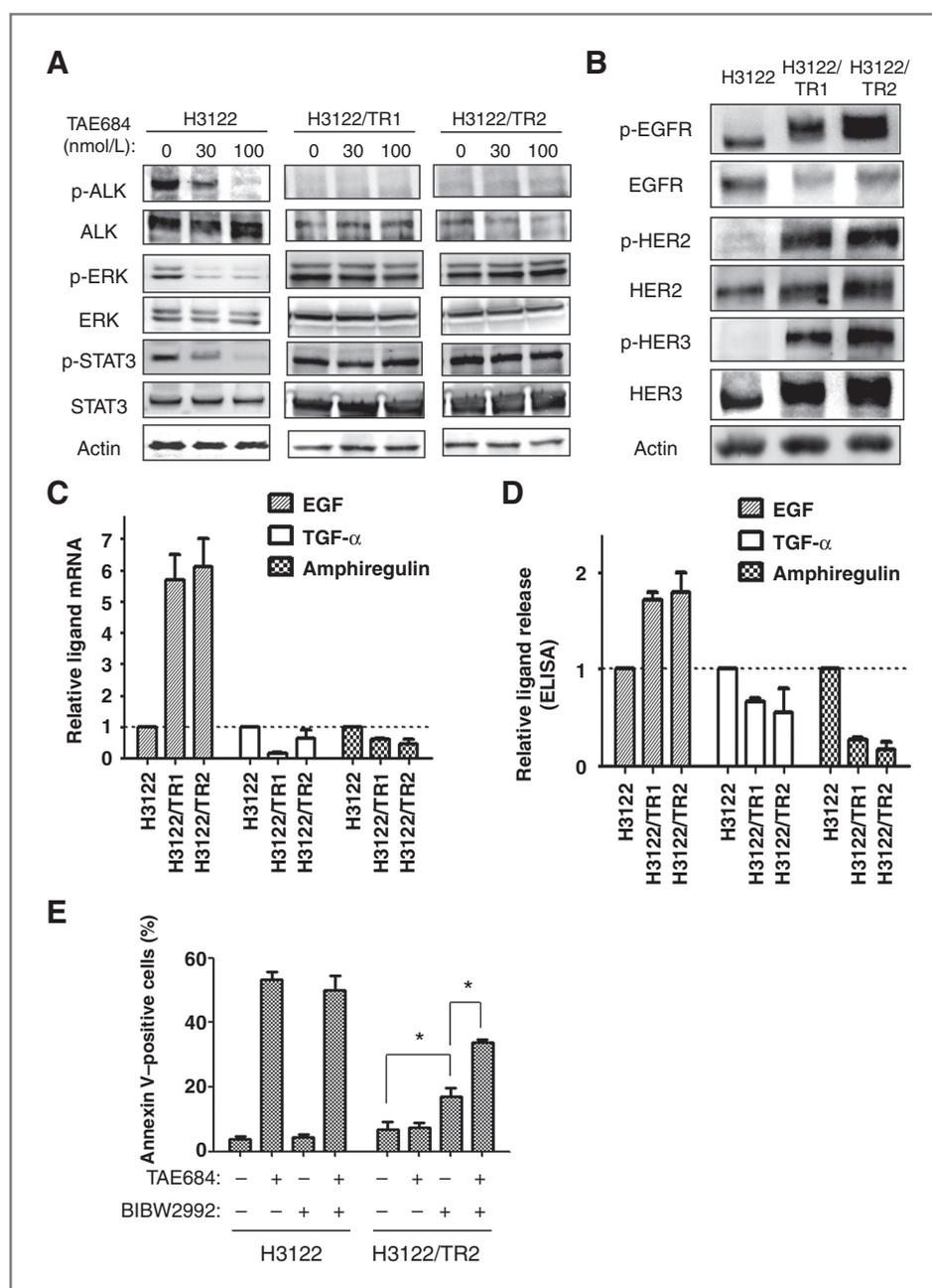
Figure 2. Activation of alternative signaling pathways in H3122/TR cells. A, H3122 or H3122/TR cells were incubated with the indicated concentrations of TAE684 for 6 hours, after which cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of ALK, ERK, or STAT3 as well as with those to β-actin (loading control). B, cells were deprived of serum for 24 hours, lysed, and subjected to immunoblot analysis with antibodies to phosphorylated or total forms of EGFR, HER2, or HER3. C, total RNA extracted from the indicated cell lines was subjected to RT and real-time PCR analysis of EGF, TGF-α, and amphiregulin mRNAs. Data were normalized by the amount of GAPDH mRNA and then expressed relative to the corresponding value for H3122 cells, and they are means ± SE from three independent experiments. D, cells were cultured overnight in medium containing 10% FBS and then incubated for 24 hours in serum-free medium, after which the latter culture supernatants were collected and assayed for EGF, TGF-α, and amphiregulin with an ELISA. Data are expressed relative to the corresponding value for H3122 cells and are means ± SE from three independent experiments. E, cells were incubated for 48 hours in the absence or presence of TAE684 (100 nmol/L) or BIBW2992 (200 nmol/L), as indicated, after which the proportion of apoptotic cells was determined by staining with annexin V and propidium iodide followed by flow cytometry. Data are means ± SE from 3 independent experiments. *, $P < 0.05$ (Student's t test) for the indicated comparisons.

was significantly greater than that induced by BIBW2992 alone (Fig. 2E). Together, these observations thus indicated that EGFR, HER2, and HER3 are activated in H3122/TR cells, that this activation is accompanied by increased secretion of EGF, and that such HER family activation contributes, at least in part, to the survival of H3122/TR cells in the presence of TAE684.

EGF activates HER family signaling and induces TAE684 resistance in parental H3122 cells

To investigate whether EGF secretion plays a role in ALK-TKI resistance, we examined the effect of exogenous EGF on ALK-TKI sensitivity in H3122 cells. We found that exposure to EGF induces resistance to TAE684 in these cells (Fig. 3A), supporting the notion that increased secretion of this ligand can lead to the development of ALK-TKI resistance. TAE684 inhibited EML4-ALK phosphorylation in H3122 cells in the absence or presence of EGF stimulation, whereas it failed to inhibit downstream signaling including the phosphorylation of ERK and STAT3 in the presence of EGF (Fig. 3B). EGF increased the levels of EGFR, HER2, and HER3 phosphorylation in H3122 cells (Fig. 3C). These data suggested that EGF-induced activation of EGFR, HER2, and HER3 might underlie resistance to ALK inhibitors in H3122/TR cells.

Figure 3. Effects of EGF stimulation in H3122 cells. A, cells were treated for 72 hours with various concentrations of TAE684 in the absence or presence of EGF (100 ng/mL), after which cell viability was assessed. Data are means of triplicates from representative experiments and are expressed as percentage survival. B, cells were incubated with or without EGF (100 ng/mL) for 15 min and then in the additional absence or presence of TAE684 (100 nmol/L) for 6 hours. Cell lysates were prepared and subjected to immunoblot analysis with antibodies to the indicated proteins. C, H3122 cells were incubated with or without EGF (100 ng/mL) for 6 hours, after which cell lysates were prepared and subjected to immunoblot analysis.
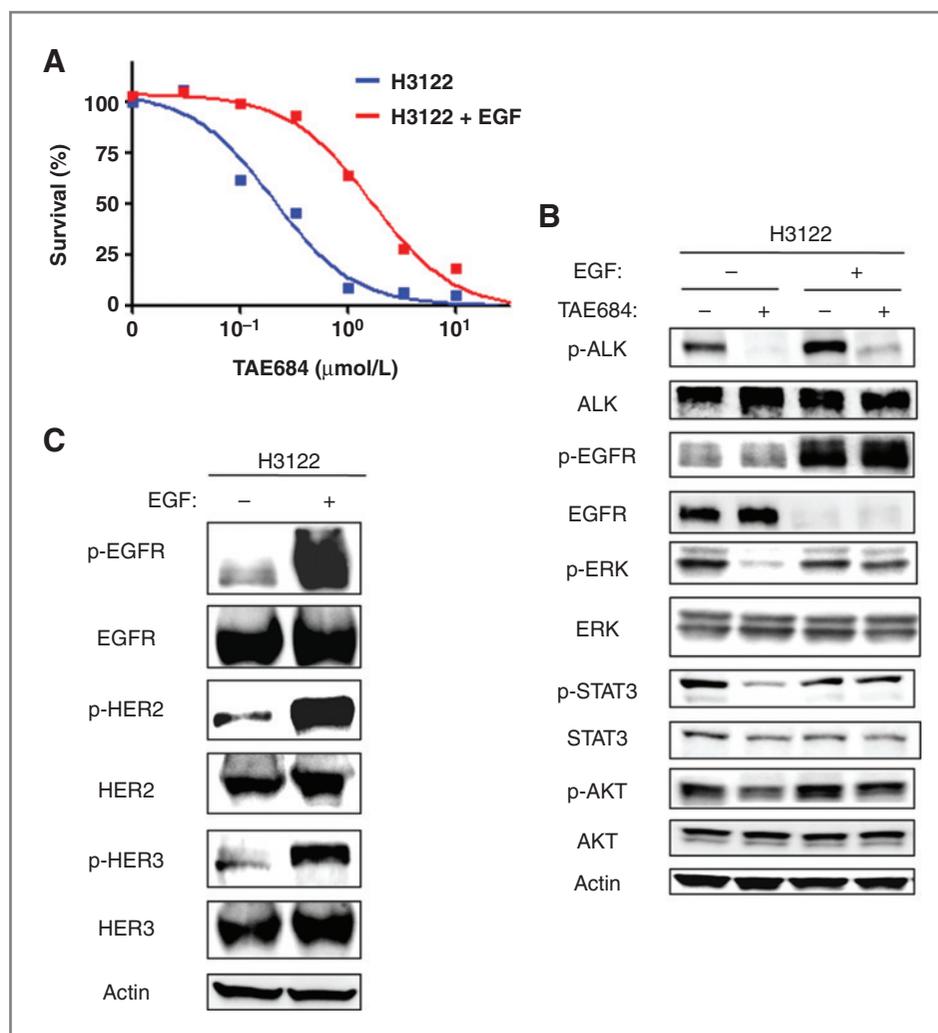
Isolation of drug-sensitive revertants from ALK-TKI–resistant cells

We cultured H3122/TR1 cells continuously in the absence of TAE684 for more than 2 months and thereby obtained H3122/TR revertant (H3122/TR rev) cells that had regained sensitivity to TAE684 (Fig. 4A). RT-PCR analysis and ELISA revealed that both the amount of EGF mRNA and the extent of EGF secretion in H3122/TR rev cells had been restored to levels similar to those in parental H3122 cells (Fig. 4B). This reduced level of EGF expression in H3122/TR rev cells compared with that in H3122/TR1 cells was accompanied by downregulation of EGFR, HER2, and HER3 phosphorylation as well as by upregulation of both the abundance and phosphorylation of EML4-ALK (Fig. 4C). These data suggested that downregulation of EML4-ALK expression and activation of HER family proteins, and the associated shift in signal transduction from the ALK signaling pathway to HER family pathways, are responsible for the acquired resistance to ALK inhibitors in H3122/TR cells.

Establishment of an ALK-TKI–resistant cell line from a clinical specimen

We established a cell line, designated K-3, from the pleural effusion of a patient with EML4-ALK–positive lung cancer that had developed clinical resistance to crizotinib after treatment for 6 months. The K-3 cells were found to be resistant to both crizotinib and TAE684, with IC_{50} values of 3.25 and 5.96 µmol/L, respectively (Fig. 5A). We did not detect any secondary mutations of ALK in K-3 cells. On the other hand, similar to the results obtained with H3122/TR cells, the abundance of EGF mRNA and the extent of EGF secretion were both markedly increased in K-3 cells compared with those in H3122 cells (Fig. 5B). Furthermore, immunoblot analysis showed that the level of EML4-ALK phosphorylation was decreased in K-3 cells, whereas that of EGFR, HER2, and HER3 phosphorylation was increased (Fig. 5C). These results thus showed concordance between an *in vitro* model and clinical experience of ALK-TKI resistance, further supporting the association of such resistance with EGF-dependent HER family activation.
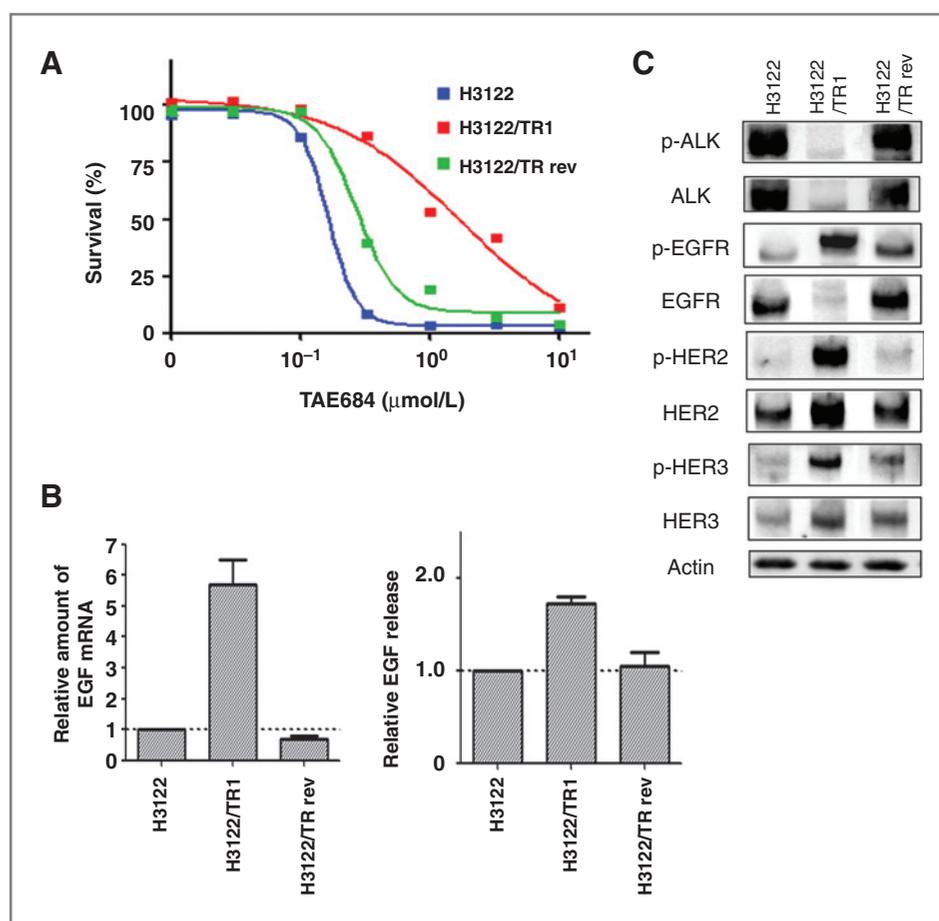
Figure 4. Characterization of H3122/TR revertant cells. A, H3122, H3122/TR1, or H3122/TR rev cells were cultured for 72 hours in complete medium containing various concentrations of TAE684, after which cell viability was assessed. Data are means of triplicates from representative experiments and are expressed as percentage survival. B, total RNA extracted from the indicated cell lines was subjected to RT and real-time PCR analysis of EGF mRNA as in Fig. 2C (left). Culture supernatants were also assayed for EGF by ELISA as in Fig. 2D (right). All data are expressed relative to the corresponding value for H3122 cells and are means ± SE from 3 independent experiments. C, cells were deprived of serum for 24 hours, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to the indicated proteins.

Discussion

EML4-ALK has been identified in 5% to 10% of NSCLC cases, and ALK-TKIs show marked antitumor effects in such tumors (3, 11, 12). However, acquired resistance to ALK inhibitors has already been found to limit the therapeutic potential of these agents (5, 10, 13), with investigation of the underlying mechanisms of such resistance thus being warranted. In the present study, we generated ALK-TKI–resistant (H3122/TR) lines from EML4-ALK–positive H3122 cells by exposing the latter sensitive cells to the ALK inhibitor TAE684, which is a more potent and selective inhibitor of ALK than is crizotinib. The H3122/TR cells were found not to harbor secondary mutations of ALK, which have previously been shown to underlie ALK-TKI resistance (5, 10, 13). Instead, we found that H3122/TR cells manifest increased activation of EGFR, HER2, and HER3 mediated by EGF as well as a reduced level of EML4-ALK activation. Such EGF-induced activation of HER family proteins was associated with sustained downstream signaling in the presence of TAE684, indicative of a shift in survival dependency from the ALK signaling pathway to HER family pathways in the ALK-TKI–resistant cells. Indeed, we found that the combination of an ALK inhibitor and an EGFR inhibitor–induced apoptosis in H3122/TR cells, further supporting the notion that the EGFR signaling pathway contributes to survival in these cells. The clinical relevance of this resistance mechanism was supported by the observation that K-3 cells, which were isolated from a specimen of EML4-ALK–positive NSCLC that developed resistance to the ALK-TKI crizotinib in situ, also manifested EGF-mediated HER family activation. Consistent with our results, amphiregulin-mediated EGFR activation was recently shown to be associated with resistance to ALK inhibitors (9, 14). Collectively, these data suggest that ligand-activated HER family signaling gives rise to ALK-TKI resistance. We and others have shown that ligand-mediated signaling pathway activation accompanied by upregulation of ligand mRNA is associated with resistance to molecularly targeted therapy in several cancer models (9, 15). In the present study, we found that the abundance of EGF mRNA is increased in H3122/TR cells. Given that the *EGF* gene contains consensus binding sequences for many transcription factors, including NF-κB, AP-1, AP-2, AP-3, and SP1 (16), such factors may play a role in the upregulation of EGF expression observed in our study.

In the present study, we found that, in the absence of ALK inhibition, activation of EGFR, HER2, and HER3 in H3122/TR cells becomes downregulated in association with a reversion to TAE684 sensitivity. Consistent with

Figure 5. Characterization of K-3 cells established from a clinical specimen of EML4-ALK–positive NSCLC with acquired resistance to crizotinib. A, K-3 or H3122 cells were cultured for 72 hours in complete medium containing various concentrations of TAE684 or crizotinib, after which cell viability was assessed. Data are means of triplicates from representative experiments and are expressed as percentage survival. B, total RNA extracted from the indicated cells was subjected to RT and real-time PCR analysis of EGF mRNA as in Fig. 2C (left). Culture supernatants were also assayed for EGF by ELISA as in Fig. 2D (right). All data are expressed relative to the corresponding value for H3122 cells, and they are means ± SE from 3 independent experiments. C, cells were deprived of serum for 24 hours, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to the indicated proteins.
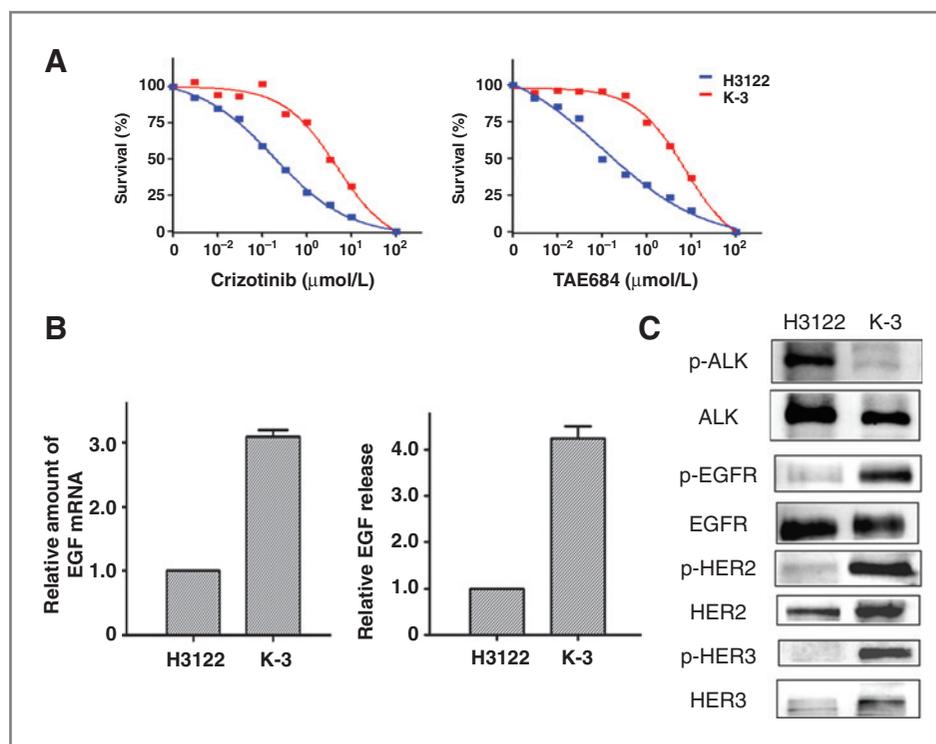
these results, termination of treatment with an EGFR inhibitor after the development of drug resistance in EGFR mutation–positive NSCLC resulted in the loss of the resistance-associated mutation (T790M) and restoration of tumor sensitivity to treatment with EGFR inhibitors in both preclinical and clinical settings (17–19). Our observations now suggest that the development of resistance to ALK inhibitors is potentially reversible. They provide a rationale for temporary cessation of treatment after the development of ALK-TKI resistance in patients with EML4-ALK–positive lung cancer to allow the tumor to regain drug sensitivity. Clinical evaluation of such an approach may thus be warranted.

In conclusion, with the use of *in vitro* cell lines and cells isolated from a patient, we have uncovered a previously unidentified mechanism of acquired ALK-TKI resistance in NSCLC positive for EML4-ALK. Our data add to the complexity of drug resistance mechanisms, further investigation of which is required if they are to be understood and conquered.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Tanizaki, I. Okamoto, T. Okabe, K. Takezawa, K. Nakagawa
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Tanizaki, I. Okamoto, T. Okabe, K. Sakai, K. Tanaka, H. Hayashi, K. Kuwata, H. Yamaguchi, E. Hatashita
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Tanizaki, I. Okamoto, K. Sakai, K. Kuwata, H. Yamaguchi, E. Hatashita, K. Nakagawa
Writing, review, and/or revision of the manuscript: J. Tanizaki, I. Okamoto, H. Kaneda, K. Nakagawa
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Tanizaki, K. Sakai, H. Hayashi, K. Kuwata, H. Yamaguchi, E. Hatashita
Study supervision: I. Okamoto, K. Nakagawa

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