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ORIGINAL ARTICLE

Anti-HER3 monoclonal antibody patritumab sensitizes refractory non-small cell lung cancer to the epidermal growth factor receptor inhibitor erlotinib

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Human epidermal growth factor receptor (HER) 3 is aberrantly overexpressed and correlates with poor prognosis in non-small cell lung cancer (NSCLC). Patritumab is a monoclonal antibody against HER3 that has shown promising results in early-phase clinical trials, but an optimal target population for the drug has yet to be identified. In the present study, we examined whether heregulin, a HER3 ligand that is also overexpressed in a subset of NSCLC, can be used as a biomarker to predict the antitumorigenic efficacy of patritumab and whether the drug can overcome the epidermal growth factor receptor tyrosine kinase inhibitor (EGFR TKI) resistance induced by heregulin. Patritumab sensitivity was associated with heregulin expression, which, when abolished, resulted in the loss of HER3 and AKT activation and growth arrest. Furthermore, heregulin overexpression induced EGFR TKI resistance in NSCLC cells harbouring an activating EGFR mutation, while HER3 and AKT activation was maintained in the presence of erlotinib in heregulin-overexpressing, EGFR-mutant NSCLC cells. Sustained HER3-AKT activation was blocked by combining erlotinib with either anti-HER2 or anti-HER3 antibody. Notably, heregulin was upregulated in tissue samples from an NSCLC patient who had an activating EGFR mutation but was resistant to the TKI gefitinib. These results indicate that patritumab can overcome heregulin-dependent EGFR inhibitor resistance in NSCLC *in vitro* and *in vivo* and suggest that it can be used in combination with EGFR TKIs to treat a subset of heregulin-overexpressing NSCLC patients.

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INTRODUCTION

Members of the human epidermal growth factor receptor (HER) family of proteins—which includes HER1 (also known as epidermal growth factor receptor (EGFR)), HER2, HER3 and HER4—are receptor tyrosine kinases (RTKs)[1–3] that homodimerize or heterodimerize when activated by ligand binding. Aberrant HER signalling, which is caused by overexpression or activating mutations, has a critical role in the oncogenesis of some cancer types.[4,5] *HER3* is occasionally amplified and aberrantly upregulated in cancers such as non-small cell lung cancer (NSCLC),[6,7] and the latter is associated with a poor prognosis.[7] Owing to structural features that limit its intrinsic kinase activity, HER3 cannot be auto-phosphorylated but can be transphosphorylated through heterodimerization or higher-order clustering with other family members, such as HER2.[1–3,8] HER3 is a major mediator of the phosphoinositide 3-kinase/AKT cell survival signalling pathway.[9] The HER3 ligand heregulin is locally secreted and induces HER3 activation through an autocrine mechanism.[10] Alternatively, HER3 can also be activated independently of ligand binding by the dysregulation of other tyrosine kinase receptors.[9,11] For example, in NSCLC cases harbouring an activating EGFR mutation, EGFR can activate HER3 in *trans* through heterodimerization.[9] EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib can prevent HER3 phosphorylation in this subset of NSCLC.

HER3 is a promising oncotherapeutic target.[12] Monoclonal antibodies targeting HER3 have been investigated preclinically and clinically.[13–16] One of these, patritumab, is a novel, fully human monoclonal antibody directed against the extracellular domain of HER3. Early clinical trials designed to evaluate patritumab monotherapy revealed that it was well tolerated and limited tumour growth in some cancers, including NSCLC.[17] Despite these promising results, the drug's mechanism of action is incompletely understood, and a biomarker has not yet been identified for the selection of an optimal target patient subpopulation who can benefit from patritumab treatment.

EGFR is also a drug target in NSCLC, and somatic mutations in the tyrosine kinase domain cause constitutive activation of EGFR in a subset of NSCLC;[18,19] this activity can be blocked by EGFR-TKIs.[20] Although EGFR-TKIs show robust effects on NSCLC with activating EGFR mutations, the cells eventually acquire resistance; in approximately half of such cases, this is attributable to a secondary mutation in the EGFR kinase domain, most frequently a T790M substitution in exon 20.[21,22] In addition, HER3 has a key role in EGFR-TKI resistance.[23,24] Additionally, amplification of the *MET* oncogene can activate HER3, thereby switching on downstream phosphoinositide 3-kinase/AKT survival mechanisms, even in the presence of an EGFR-TKI.[25] Thus targeting HER3 may help prevent the development of cancer cell resistance to EGFR-TKIs.

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To investigate this hypothesis, we evaluated the antitumorigenic efficacy of patritumab in NSCLC as a single agent and in combination with established clinical EGFR inhibitors.

RESULTS

NSCLC cell lines exhibit differential sensitivity to patritumab

To evaluate the efficacy of patritumab *in vitro*, cell proliferation was assessed in 48 NSCLC cell lines with the soft agar colony-formation assay. Cells were treated with patritumab for 7 days at 100 μg/ml, which is comparable to the concentration in the plasma of cancer patients in phase II clinical trials.[13,26] The efficacy of patritumab varied across cell lines, with growth inhibition rates ranging from 0% to 40% (Figure 1a). Although patritumab had a marginal antiproliferative effect on most cell lines, in six of these —QG56, HARA, SW900, H1944, H358 and A549—a growth inhibition rate of >25% was observed (Supplementary Table S1). These lines were defined as patritumab-sensitive, while the other lines were classified as patritumab-refractory. Cell viability was reduced in a dose-dependent manner in HARA, QG56, A549 and SW900 cells treated with patritumab, while in refractory cell lines such as H1437, PC3, H1573 and H520, patritumab did not inhibit cell growth even at the highest concentration (Figure 1b).

Patritumab sensitivity is associated with heregulin expression level in NSCLC

We hypothesized that the differential sensitivity to patritumab was due to molecular differences in the HER3 signalling pathway. Indeed, *heregulin* and *HER3* mRNA expression levels varied across the 48 NSCLC cell lines (Supplementary Figure S1). Cell lines were stratified into three groups ($n=16$ each) based on their *heregulin* mRNA expression level. According to the *HER3* mRNA expression level, cell lines were divided into high and low expression groups ($n=24$ each); growth inhibition rates upon treatment with 100 μg/ml patritumab were compared between groups. Cell lines with high *heregulin* expression were more sensitive to patritumab than those with medium or low *heregulin* expression (16.5% vs 1.1% vs 3.8%; $P<0.002$, Figure 1c), and all six patritumab-sensitive cell lines had high *heregulin* expression. In contrast, there were no differences in the growth inhibition rates between the low and high *HER3* expression groups (7.5% vs 5.2%; $P=0.500$). Notably, A549 and HARA cells had the lowest *HER3* expression among cell lines but were nonetheless sensitive to patritumab due to high *heregulin* expression (Supplementary Figure S1).

Activating EGFR mutations were identified in 10 cell lines (PC9, HCC827, H1650, HCC4006, Ma70, Ma70GR, 11-18, H1975, PC9ZD and HCC827GR5 cells; Supplementary Table S2). Five of these lines (PC9, HCC827, HCC4006, Ma70 and 11-18) were sensitive to EGFR-TKI (data not shown), and all cell lines were refractory to patritumab (Figure 1c, Supplementary Table S1). To verify whether

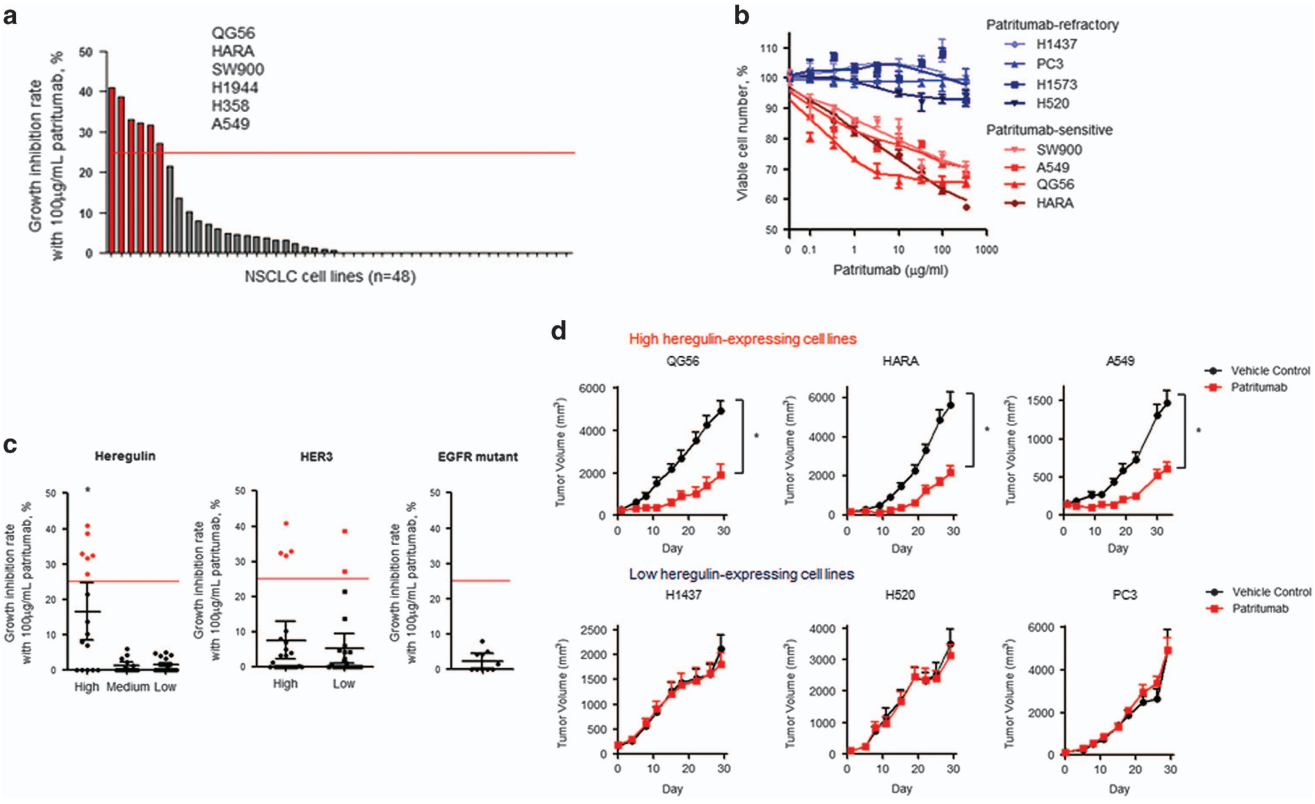

Figure 1. Patritumab sensitivity depends on heregulin expression in NSCLC *in vitro*. (**a**) Patritumab sensitivity was evaluated using a soft agar colony-formation assay. NSCLC cell lines ($n=48$) were treated with 100 μg/ml patritumab, and the growth inhibition rate was calculated relative to untreated control cells. (**b**) Patritumab-sensitive or -refractory cell lines ($n=8$) were treated with the indicated concentrations of patritumab for 3 days; cell viability was evaluated using the CellTiter-Glo viability assay and shown relative to untreated control cells (mean ± s.d. of six independent experiments). (**c**) Relationship between growth inhibition rate at 100 μg/ml patritumab and heregulin and HER3 expression levels among 48 NSCLC cell lines classified as high, medium or low expression ($n=16$ each) for heregulin and high or low expression ($n=24$ each) for HER3. Also shown are the growth inhibition rates for 10 NSCLC cell lines with activating EGFR mutations treated with 100 μg/ml patritumab. Patritumab-sensitive cell lines are highlighted in red. Data represent mean ± s.d. *$P<0.002$, unpaired *t*-test, high vs medium or low heregulin groups. (**d**) In a xenograft NSCLC model, cells expressing high (QG56, HARA and A549) or low (H1437, H520 and PC3) heregulin were treated with patritumab or vehicle. Each treatment group consisted of 10 mice. Data represent mean ± s.e.m. *$P<0.05$, unpaired *t*-test.

our *in vitro* findings could be extended *in vivo*, high (QG56, HARA and A549) and low (H1437, H520 and PC3) heregulin-expressing NSCLC cell lines were implanted subcutaneously in mice, which were then treated with patritumab or vehicle for 4 weeks. Xenograft tumours arising from cells expressing high levels of heregulin were reduced in size by patritumab treatment (Figure 1d), while tumours originating from cells with low heregulin expression continued to grow. Taken together, these results suggest that heregulin contributes to patritumab sensitivity both in NSCLC cell lines and in a tumour model.

Heregulin-dependent NSCLC proliferation *in vitro* is inhibited by patritumab

Depending on the context, heregulin can stimulate or inhibit the proliferation of various cancer cells.[27,28] To evaluate the role of heregulin on NSCLC growth, A549 cells were cultured in medium containing exogenous heregulin at concentrations of 1 or 4 ng/ml, which was replaced every 3 days over 15 days. The growth rate steadily increased from day 8 onwards relative to control cells grown in the absence of heregulin, and this effect was concentration-dependent (Figure 2a). Similar effects were observed in seven other NSCLC cell lines treated with exogenous heregulin at 1 ng/ml for 8 days (Figure 2b).

To determine whether patritumab could inhibit heregulin-induced proliferation, A549 cells were cultured in medium with various concentrations of exogenous heregulin and treated with 100 μg/ml patritumab for 3 days. In these cells, the growth rate steadily increased relative to cells grown in medium without heregulin and in the absence of patritumab (Figure 2c). In contrast, proliferation was inhibited by patritumab, even in the presence of high levels of heregulin.

Finally, to examine whether heregulin knockdown would reduce proliferation in patritumab-sensitive cell lines, we transfected HARA, QG56 and A549 cells (which express high endogenous levels of heregulin) with small interfering RNA (siRNA) against heregulin (siHRG). Compared with siMock cells, siHRG significantly reduced proliferation of these cells, indicating that heregulin overexpression stimulates the growth of NSCLC cells.

Patritumab prevents heregulin-induced HER2/HER3 interaction

To elucidate the molecular mechanisms underlying patritumab sensitivity, the expression of HER3 and the downstream effector AKT was assessed in NSCLC cells expressing high (HARA, A549 and QG56) and low (PC3, H1437 and H520) levels of heregulin following treatment with 100 μg/ml patritumab for 6 h. In cell lines with high heregulin levels, patritumab treatment resulted in the dephosphorylation of HER3 and AKT (Figure 3a), without altering the total HER3 level. In contrast, in cells with low heregulin expression, HER3 phosphorylation was not observed before or after patritumab treatment, and the drug had no effects on AKT phosphorylation in these cells. Patritumab had no effect on the phosphorylation status of ERK in any of the cell lines examined (Supplementary Figure S2). These results suggest that patritumab can abolish heregulin-induced HER3 activation and that cell survival associated with AKT signalling is predominantly driven by HER3 activation in cells that express high levels of heregulin.

Additionally to determine whether HER3-AKT signalling activity depends on heregulin expression, A549 cells were treated with siHRG. Activation of HER3 as well as AKT was prevented by siHRG transfection due to lack of heregulin binding (lane 1 vs lane 3, Supplementary Figure S3). These results suggest that HER3-AKT signalling was activated by heregulin.

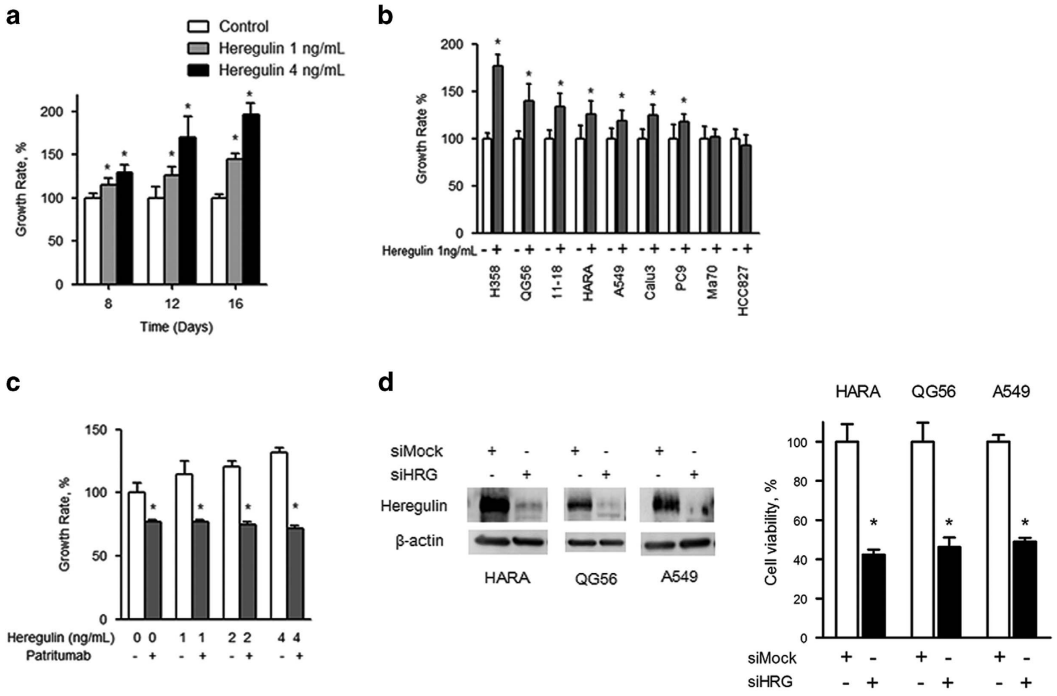

Figure 2. Heregulin stimulates proliferation in NSCLC *in vitro*, which is inhibited by patritumab. (**a**) A549 cells were treated with 1 or 4 ng/ml heregulin or vehicle for 16 days, and the growth rate was determined on days 8, 12 and 16 (mean ± s.d. of four independent experiments) relative to the control on the respective days. Cell viability was evaluated using the CellTiter-Glo viability assay. *$P < 0.05$ vs control, unpaired *t*-test. (**b**) Growth rates were determined for nine NSCLC cell lines treated with 1 ng/ml heregulin or vehicle for 8 days (mean ± s.d. of six independent experiments). *$P < 0.05$ vs control, unpaired *t*-test. (**c**) Growth rate was determined for A549 cells cultured in medium containing 0, 1, 2 or 4 ng/ml heregulin and 100 μg/ml patritumab for 3 days (mean ± s.d. of six independent experiments). *$P < 0.05$ vs untreated cells, unpaired *t*-test. (**d**) Heregulin-overexpressing HARA, QG56 and A549 cells were transfected with siRNA against heregulin (siHRG) or a non-targeting siRNA (siMock); the indicated proteins were detected by western blotting. Cell viability was evaluated using the CellTiter-Glo viability assay and calculated relative to siMock (mean ± s.d. of five independent experiments). *$P < 0.05$ vs siMock, unpaired *t*-test.

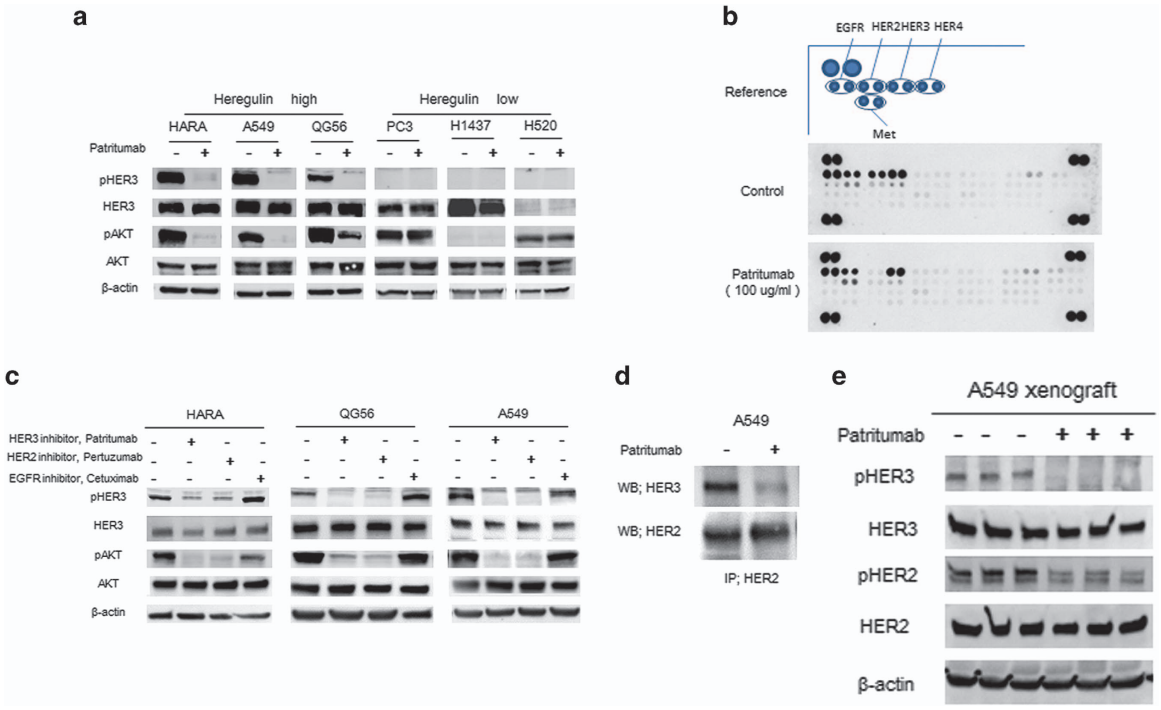

Figure 3. Patritumab blocks heregulin-induced heterodimerization of HER2 and HER3. (**a**) Cells expressing high (HARA, A549, QG56) or low (PC3, H1437, H520) levels of heregulin were treated with 100 μg/ml patritumab for 6 h and then probed for the indicated proteins. (**b**) HARA cells were treated with 100 μg/ml patritumab or vehicle for 6 h, and whole cell lysates were analysed using a phospho-RTK array. (**c**) HARA, QG56 and A549 cells were treated with patritumab, pertuzumab, cetuximab or vehicle for 6 h, and whole cell lysates were probed for the indicated proteins. (**d**) A549 cells were treated with 100 μg/ml patritumab or vehicle for 6 h, and whole cell lysates were immunoprecipitated with an antibody against HER2, which was detected along with HER3 by western blotting. (**e**) A549 cell xenograft mice were treated with vehicle or patritumab for 4 weeks; cell lysates from tumours were probed for the indicated proteins.

Previous reports indicate that HER3 activation occurs preferentially through heterodimerization with EGFR, HER2, HER4 or MET.[1,6,8] To determine whether patritumab inactivates multiple receptors, heregulin-overexpressing HARA cells were treated with 100 μg/ml patritumab or vehicle for 6 h, and the phosphorylation status of EGFR, HER2, HER3, HER4 and MET was evaluated using an RTK array. All of the receptors were phosphorylated in control cells; exposure to patritumab potently inhibited HER3 phosphorylation and moderately inhibited EGFR and HER2 phosphorylation, but it had no effects on MET and HER4 phosphorylation (Figure 3b, Supplementary Figure S4).

We also examined whether EGFR or HER2 inhibition prevents HER3 phosphorylation in heregulin-overexpressing cell lines. To this end, HARA, QG56 and A549 were treated with cetuximab, pertuzumab or patritumab or antibodies against EGFR, HER2 or HER3. Patritumab and pertuzumab, but not cetuximab, blocked the phosphorylation of HER3 and AKT in these cells (Figure 3c), suggesting that HER3 may interact with HER2 in cell lines that express high levels of heregulin. To test this possibility, HER2 was immunoprecipitated from A549 cells and the immune complex was probed for HER3. In untreated control cells, HER3 was detected in complex with HER2 (Figure 3d); however, patritumab-treated cells had a lower level of HER3 than control cells, indicating that the HER3 interaction with HER2 is abolished by patritumab treatment.

To examine whether these findings were relevant *in vivo*, the phosphorylation status of HER2 and HER3 was examined in A549 cells implanted subcutaneously in mice treated with patritumab or vehicle for 4 weeks. Both HER2 and HER3 were phosphorylated in A549 xenografts of untreated animals. However, HER3 was completely and HER2 was partially dephosphorylated in tumours from patritumab-treated mice, (Figure 3e), providing further evidence that HER3 preferentially interacts with HER2.

Heregulin causes EGFR-TKI resistance in NSCLC cells harbouring an activating EGFR mutation

We previously reported that heregulin-dependent resistance to the anti-EGFR antibody cetuximab in colorectal cancer is due to activation of HER2 and HER3 via heterodimerization, as this engenders persistent downstream proliferation signals even in the presence of cetuximab.[29] To determine whether heregulin expression results in EGFR-TKI resistance in NSCLC, the EGFR-TKI-sensitive NSCLC cell lines PC9 and HC827—which harbour an activating EGFR mutation—were stably transfected with heregulin and treated with erlotinib for 3 days (Figure 4a). In contrast to mock-transfected control cells, heregulin overexpression rendered both cell lines refractory to erlotinib. To explore the mechanistic basis for this change, transfected PC9 cells were treated with 1 μM erlotinib for 24 h. Erlotinib inhibited phosphorylation of EGFR in both cell lines (Figure 4b) and reduced HER3 transphosphorylation, as well as phosphorylation of the downstream effectors AKT and ERK in mock-transfected cells. In contrast, in heregulin-overexpressing cells, erlotinib caused HER3 phosphorylation in a time-dependent manner along with an increase in total HER3, despite EGFR inactivation (Supplementary Figure S5). Erlotinib initially prevented AKT and ERK phosphorylation following 3 h of exposure, whereas both proteins were hyperphosphorylated at later time points. These results suggest that HER3 can be activated in a heregulin-dependent manner in heregulin-overexpressing PC9 cells treated with erlotinib and that HER3 activation is amplified due to the increase in total HER3 upon erlotinib treatment. These findings were confirmed *in vivo* in mice implanted with PC9 mock- or heregulin-overexpressing cells and treated with erlotinib daily for 3 weeks; xenograft tumours arising from mock-transfected cells shrank following erlotinib treatment, whereas those overexpressing heregulin continued to grow in the presence of erlotinib (Figure 4c).

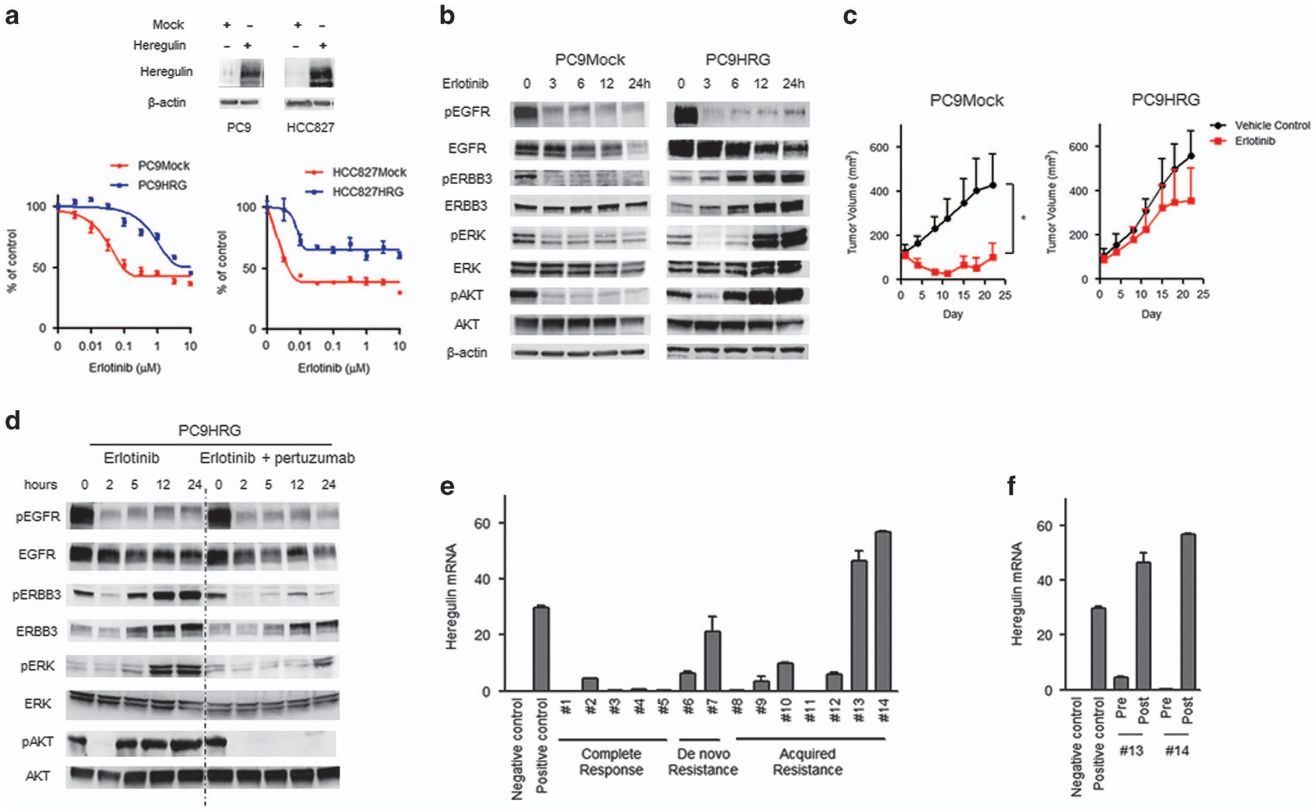

Figure 4. Heregulin induces EGFR TKI resistance in NSCLC cells harbouring activating EGFR mutations. (**a**) EGFR mutant NSCLC PC9 and HCC827 cells were mock-transfected (PC9Mock) or stably transfected with heregulin (PC9HRG) and then treated with the indicated concentrations of erlotinib; cell viability was measured 3 days later using the CellTiter-Glo viability assay, and values are plotted relative to untreated control cells (mean ± s.d., $n = 6$). Whole cell lysates were probed for heregulin expression. (**b**) PC9Mock and PC9HRG cells were treated with 1 μM erlotinib for the indicated times and then probed for the indicated proteins. (**c**) PC9Mock or PC9HRG cells xenograft mice were treated with erlotinib or vehicle for 3 weeks. Each treatment group consisted of eight mice. Data represent mean ± s.e.m. $*P < 0.05$ vs vehicle, unpaired *t*-test. (**d**) PC9HRG cells were treated with either 1 μM erlotinib alone or 1 μM erlotinib combined with 100 μg/ml pertuzumab and then probed for the indicated proteins. (**e**) Tumour samples from 14 NSCLC patients with activating EGFR mutations were analysed for *heregulin* mRNA expression. Five patients achieved complete response, and three patients had primary resistance and six had acquired resistance to EGFR-TKI. *y* Axis, fold changes compared with HCT116 cell line (mean ± s.d. of three independent experiments). (**f**) Tumour samples were obtained from two NSCLC patients prior to and after EGFR-TKI treatment, and *heregulin* mRNA expression was analysed. *y* Axis, fold changes compared with HCT116 cell line (mean ± s.d. of three independent experiments).

To explore the functional mechanisms for this resistance, we treated PC9HRG cells with either erlotinib alone or in combination with the anti-HER2 antibody pertuzumab for 24 h. Erlotinib monotherapy elevated the total HER3 expression level and reactivated HER3 as well as AKT and ERK signalling in PC9HRG cells in a time-dependent manner (Figure 4d, Supplementary Figure S6). In contrast, treatment of erlotinib plus pertuzumab could inhibit re-activation of HER3 as well as AKT and ERK despite the increase in total HER3 expression. These results suggest that HER3 can be reactivated due to HER2/HER3 interaction in PC9HRG cells following erlotinib treatment. In addition, AKT and ERK lie downstream of the HER2/HER3 complex; presumably, AKT is downstream of HER3 and ERK is downstream of HER2.

To assess the clinical relevance of the observed heregulin-induced resistance to EGFR-TKI, *heregulin* mRNA expression was evaluated in clinical samples from 14 NSCLC patients harbouring activating EGFR mutations (Supplementary Table S3). Five specimens were from patients who showed a complete response to gefitinib, and two specimens were from patients resistant to gefitinib who discontinued treatment within a few months because of disease progression. The other seven specimens were from patients who initially responded to gefitinib and then later acquired resistance; tumour samples were obtained by re-biopsy upon disease progression. Heregulin upregulation comparable to the positive control (heregulin-overexpressing PC9 cells) was observed in samples from three NSCLC patients (Figure 4e). One (#7) had an *EGFR* exon 19 deletion and received erlotinib but discontinued treatment after 1 month because of disease progression; two other patients (#13 and #14) showed a partial response but then acquired EGFR-TKI resistance accompanied by an upregulation of heregulin expression, which was negligible prior to EGFR-TKI treatment (Figure 4f). Notably, all three patients were EGFR T790M-negative. DNA sequencing was also performed to investigate genomic amplification of *MET* and *HER2*. None of these genomic alterations were detected in samples #13 and #14,[25,30] and histological examination revealed no evidence of changes such as epithelial-to-mesenchymal transition or small cell lung cancer, which has been observed in some cases of acquired resistance.[22] Heregulin upregulation was not observed in specimens from the five patients who showed a complete response.

Patritumab overcomes erlotinib resistance induced by heregulin in NSCLC

The results described above suggested that patritumab could block HER3 activation in a heregulin-dependent manner in NSCLC. We therefore hypothesized that cells treated with patritumab could overcome erlotinib resistance induced by heregulin. To test this, heregulin-overexpressing PC9 cells were treated with erlotinib or patritumab alone or in combination, and the effect

on proliferation was evaluated. Cells were refractory to erlotinib, with an IC50 $>1\,\mu M$ (Figure 5a). Strikingly, the cells were also resistant to patritumab, despite the high level of heregulin expression. However, a combination of both agents potently inhibited proliferation. Similar effects were observed in heregulin-overexpressing HARA cells (Figure 5b). The combination treatment was also evaluated in a panel of cell lines that were refractory to EGFR-TKIs due to various underlying mechanisms. This included PC9ZD cells with a T790M secondary EGFR mutation and HC827GR5 cells with MET amplification.[25,31] In contrast to the heregulin-overexpressing PC9 cells, these two cell lines were resistant to the erlotinib/patritumab combination, suggesting that patritumab could overcome EGFR-TKI resistance only when it is heregulin-dependent (Supplementary Table S4). Additionally, we examined whether erlotinib/patritumab combination could overcome erlotinib resistance in PC9ZD and HCC827GR5 cells when exposed to the medium containing high concentration of exogenous heregulin. Parent PC9 cells developed the resistance to erlotinib in the presence of exogenous 10 ng/ml heregulin, and this resistance could be overcome by treatment with erlotinib/patritumab combination (Figure 5c). In contrast to the effect observed in parent PC9 cells, erlotinib/patritumab combination therapy could not overcome erlotinib resistance in PC9ZD and HCC827GR5 cells despite the presence of 10 ng/ml heregulin in the medium. These results suggest that erlotinib/patritumab combination might not overcome EGFR-TKI resistance due to a T790M secondary EGFR mutation or MET amplification irrespective of the concomitant presence of heregulin.

To explore the mechanistic basis for the efficacy of patritumab in combination treatments, heregulin-overexpressing PC9 cells were treated with erlotinib or patritumab alone or in combination for 24 h. Treatment with patritumab alone minimally inhibited HER3 phosphorylation, and treatment with erlotinib alone caused re-activation of HER3 as well as AKT and ERK in a time-dependent manner. In contrast, the combination treatment inhibited the re-activation of HER3 as well as AKT and ERK, despite an increase in total HER3 level in PC9HRG cells (Figure 5d, Supplementary Figure S7). These results suggest that co-administration of patritumab can inhibit the heregulin-dependent HER3 activation that occurs following erlotinib treatment. Additionally, we performed an immunoprecipitation assay with anti-HER2 antibody, and the immune complex was probed for HER3 in PC9HRG cells. HER3 was detected in complex with HER2 in cells treated with erlotinib alone, compared with untreated controls cells. However, those complexes were disrupted with patritumab combination therapy (Figure 5e). This in turn provides a potential mechanistic explanation for the antiproliferative effects of patritumab during combination treatment.

The antitumorigenic potential of this drug combination was evaluated in mice bearing heregulin-overexpressing PC9 xenografts. Neither erlotinib nor patritumab alone affected tumour growth, whereas both drugs used in combination potently suppressed tumour growth compared with control mice (Figure 5f). However, after the treatment was discontinued, the tumours began to grow rapidly.

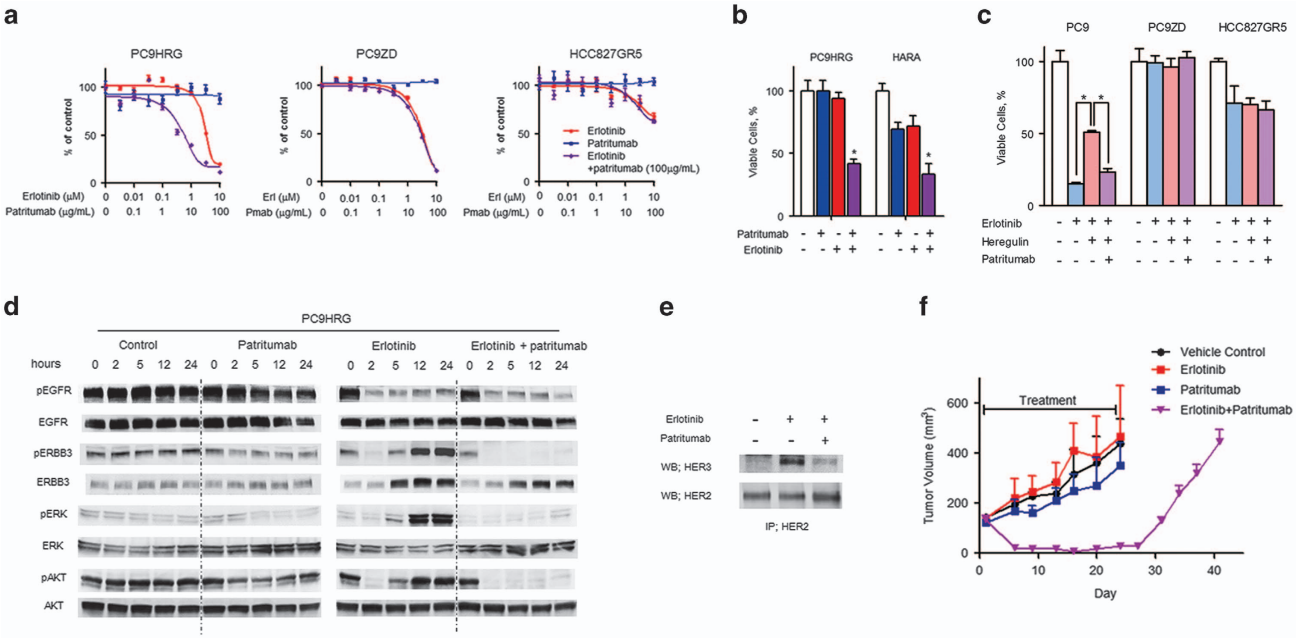

Figure 5. Patritumab overcomes heregulin-induced erlotinib resistance. (**a**) PC9HRG, PC9ZD and HCC827GR5 cells were treated with erlotinib, patritumab or both at the indicated concentrations (in the combination treatment, patritumab concentration was fixed at 100 μg/ml), and cell viability was measured 3 days later using the CellTiter-Glo viability assay. Data represent mean ± s.d. of six independent experiments. (**b**) PC9HRG and HARA cells were treated with patritumab (100 μg/ml), erlotinib (1 μM) or both; cell viability relative to untreated control cells was quantified 3 days later. Data represent mean ± s.d. of six independent experiments. $^*P < 0.05$ vs other treatments, unpaired *t*-test. (**c**) Parent PC9, PC9ZD and HCC827GR5 cells were treated with patritumab (100 μg/ml), erlotinib (1 μM), erlotinib (1 μM) in the presence of 10 ng/ml heregulin or erlotinib (1 μM) combined with patritumab (100 μg/ml) in the presence of 10 ng/ml heregulin. Cell viability relative to untreated control cells was quantified 3 days after treatment. Data represent mean ± s.d. of six independent experiments. $^*P < 0.05$ vs. other treatments, unpaired *t*-test. (**d**) PC9HRG cells were treated with control, 100 μg/ml patritumab alone, 1 μM erlotinib alone or 1 μM erlotinib combined with 100 μg/ml patritumab for the indicated times and then probed for the indicated proteins. (**e**) PC9HRG cells were treated with control, erlotinib (1 μM) alone or erlotinib (1 μM) combined with patritumab (100 μg/ml) for 24 h, and whole cell lysates were immunoprecipitated with an antibody against HER2, which was detected along with HER3 by western blotting. (**f**) PC9HRG cell xenograft mice were treated with vehicle or erlotinib, patritumab or both for 3 weeks. Each treatment group consisted of six mice. Data represent mean ± s.e.m. $^*P < 0.05$, combination therapy vs other treatments, unpaired *t*-test.

DISCUSSION

The anti-HER3 monoclonal antibody patritumab is currently under clinical development and investigations are on-going to identify a biomarker that could be used to select the beneficiary patient subpopulation. Here we showed that *heregulin* expression level correlates with patritumab sensitivity in a preclinical NSCLC model (Figure 1). Mechanistically, this is most likely explained by the patritumab-dependent inhibition of heregulin binding to HER3 and concomitant dephosphorylation of the receptor. Alternatively, patritumab may act as a dimerization inhibitor, in a manner similar to the HER2 antibody pertuzumab or to LJM716, another HER3 monoclonal antibody.[14] Other anti-HER3 antibodies can reportedly inhibit proliferation via inactivation of HER3 in heregulin-overexpressing cancers.[13–16] Furthermore, our data suggest that heregulin expression may predict the efficacy of HER2 inhibitors such as pertuzumab or lapatinib. This is since HER2 plays a key role for HER3 activation in heregulin-overexpressing NSCLC cells. Head and neck cancer cells overexpressing heregulin are sensitive to lapatinib, even in the absence of *HER2* gene amplification, and tissue specimens from head and neck cancer patients have a similar level of *heregulin* mRNA expression as lapatinib-sensitive cell lines.[32]

In contrast to heregulin, sensitivity to patritumab did not correlate with HER3 expression level in the NSCLC cell lines examined. This discrepancy may be explained by the inactivation of HER3 in the absence of ligand binding (Figure 3). Unlike HER3, HER2 is constitutively active without ligand binding, and thus the efficacy of the anti-HER2 antibody trastuzumab depends on the HER2 expression level.[1,12]

Antitumorigenic effects of patritumab monotherapy were not detected in EGFR-mutant NSCLC cell lines. According to a previous study, HER3 is activated independently of ligand binding in EGFR-mutant cell lines.[9,25] HER3 depletion inhibited proliferation in HCC827 NSCLC cells that harbour an activating EGFR mutation.[25] However, in the current study, patritumab did not inhibit proliferation in EGFR-mutant NSCLC cell lines nor did it decrease HER3 phosphorylation or HER3 expression in these cells (Supplementary Figure S8). These results imply that patritumab does not abolish EGFR binding with HER3. Instead, we suggest that the inability of patritumab to internalize and degrade HER3 could limit cell sensitivity to the drug.

Heregulin overexpression induced EGFR-TKI resistance in NSCLC cell lines harbouring activating EGFR mutations, which was also confirmed in clinical specimens (Figure 4). Unlike mock-transfected PC9 cells, those overexpressing heregulin reactivated HER3 as well as AKT and ERK, even in the presence of erlotinib. Moreover, all these heregulin-dependent effects were prevented by treatment with the anti-HER2 antibody, pertuzumab (Figure 4d). These results suggest that the HER2/HER3 complex induces EGFR-TKI resistance in PC9 cells overexpressing heregulin. In addition, total HER3 expression increased in a time-dependent manner in PC9HRG cells treated with erlotinib. This might contribute to re-activation of HER3, although further experimental evidence is required to support this point.

Combination patritumab treatment abrogated heregulin-induced EGFR-TKI resistance (Figure 5). Strikingly, this effect was not found in other EGFR-mutant NSCLC cell lines that have different resistance mechanisms, suggesting that new therapeutic strategies designed to overcome or prevent resistance must be highly targeted.[21,22,25] In an on-going Japanese clinical trial of patritumab combined with erlotinib, 10 of the 12 NSCLC patients with activating EGFR mutations showed prolonged stabilization of tumours that had acquired EGFR-TKI resistance. These are encouraging results compared with the effects of erlotinib alone.[33,34] Thus a combination treatment of patritumab and an EGFR-TKI may improve the clinical outcome in NSCLC cases of acquired resistance.

A limitation of this study is the selection bias in the cell lines used and the *in vitro* approach that did not take into consideration host factors such as circulating heregulin or the tumour microenvironment, which could influence patritumab efficacy. Nonetheless, the latest results of a randomized phase II study of patritumab combined with erlotinib in NSCLC have shown that *heregulin* mRNA expression in tumours is significantly correlated with progression-free survival.[35]

The current study suggests that patritumab can overcome heregulin-induced EGFR inhibitor resistance in NSCLC both *in vitro* and *in vivo*. We recommend that patritumab combination with EGFR TKI should be examined in a clinical setting on a cohort of NSCLC patients with TKI-refractory tumours that have heregulin overexpression and EGFR-activating mutations.

MATERIALS AND METHODS

Cell culture and reagents

The 48 NSCLC cell lines used have been previously characterized[20,25,36,37] (Supplementary Table S1) and obtained from American Type Culture Collection (Manassas, VA, USA) except QG56, PC14, PC1, PC10 (IBL Co., Ltd, Gunma, Japan), HARA (Japan Human Science Foundation, Tokyo, Japan), EBC1, Sq-1 and RERF-LCA1 (Riken Cell Bank, Ibaraki, Japan). Ma70 was established in Kinki University School of Medicine, Osaka, Japan. PC9ZD, Ma70GR and HCC827GR5 are gefitinib-refractory clones of a gefitinib-sensitive EGFR-mutant parent cell line. Cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS). We used cell lines within 6 months of the initial culture. Patritumab was provided by Daiichi-Sankyo Co. Ltd. (Tokyo, Japan). Erlotinib, cetuximab and pertuzumab were obtained from commercial sources.

Patients

Tumour specimens from EGFR TKI-treated patients were obtained from the Kinki University School of Medicine, with approval from the Institutional Review Board and with written informed consent from each patient.

Antibodies and western blotting

Cells were seeded at a density of 1×10^6 cells/plate in Prime Surface 60-mm plates (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) and allowed to grow overnight in medium containing 0.5% FBS before the addition of drugs to the medium. Cells were incubated for various times and then harvested. Western blotting was performed as previously described.[20] Proteins were transferred to nitrocellulose membranes, which were probed with antibodies against phospho-AKT, AKT, phospho-EGFR, EGFR, phospho-HER3 and heregulin (all from Cell Signaling Technology, Danvers, MA, USA) and phospho-ERK 1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Soft agar colony-formation assay

To screen the 48 NSCLC cell lines for patritumab sensitivity, colony formation was assessed using the CytoSelect 96-well cell transformation assay (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's protocol. Briefly, a base agar layer containing 0.6% agar, Dulbecco's modified Eagle's medium and 0.5% FBS was poured into each well of a 96-well plate and was covered with a cell agar layer with the same composition at a density of 5×10^3 cells/well, with patritumab added to a final concentration of 100 μg/ml. After 7 days, the agar was solubilized and the mixture was incubated with 3,(4,5-dimethylthiazol-2-yl)-2,5,-diphenyl-tetrazolium bromide solution, and fluorescence was measured using a 96-well microplate reader at 570 nm. The growth inhibition rate was calculated relative to untreated control cultures.

Cell proliferation and growth inhibition assays

The sensitivity to patritumab, erlotinib and heregulin (HRG1-beta 1 extracellular domain, R&D Systems, Minneapolis, MN, USA) was evaluated using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Quantitative real-time PCR

Total RNA was isolated from cells and paraffin-embedded tissues using the RNeasy Mini Kit and RNeasy FFPE Kit, respectively (both from Qiagen, Valencia, CA, USA), according to the manufacturer's specifications. cDNA was synthesized using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA, USA) and used for real-time PCR to measure heregulin (*NRG1*) and *HER3* expression, with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression used as an internal standard. Expression levels relative to the HCT116 cancer cell line were determined using a standard method with the ABI 7900 HT system SDS software (Applied Biosystems).

siRNA transfection

siRNA against heregulin (siHRG) from the ON-TARGET Plus SMARTpool (no. L-004608-01; Dharmacon, Lafayette, CO, USA) was a mixture of four sets of 21-nucleotide sense and antisense strands. Non-targeting siRNA (siMock) was used as a non-specific control. Cells were plated at 50% confluence in six-well plates and incubated for 24 h in RPMI-1640 medium supplemented with 0.5% FBS and then transfected with siRNAs using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA).

Establishment of a heregulin-overexpressing cell line

A full-length cDNA fragment encoding human heregulin (NRG1, NM_13956) was obtained from Origene (Rockville, MD, USA). The amplified fragment was cloned into the pCR-Blunt II-TOPO vector (Invitrogen) and transferred to the pQCXIH retroviral vector (Clontech Laboratories, Inc., Mountain View, CA, USA). Retroviruses encoding heregulin were used to infect PC9 cells.

DNA sequencing

For multiplex PCR amplification, 10 ng DNA was processed using the Ion AmpliSeq Library Kit and Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies, Carlsbad, CA, USA). The Ion Xpress Barcode Adapters (Life Technologies) were ligated onto the PCR products and purified with Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA). Purified libraries were pooled and sequenced on an Ion Torrent PGM (Life Technologies) using the Ion PGM 200 Sequencing Kit v2 and the Ion 318 vs Chip Kit.

DNA sequencing data was accessed through Torrent Suite v3.4.2 (Life Technologies). Reads were aligned against the hg19 human reference genome, and variants were called using Variant Caller v3.6 (Life Technologies). Raw variant calls were filtered out with the following annotation: homozygous and heterozygous variants, QUAL of < 100, and depth of cover < 19.

Xenograft studies

NSCLC cells were implanted into female athymic nude mice and allowed to grow to at least 150 mm^3 before mice were treated with patritumab (100 μg/mouse), administered twice weekly by intraperitoneal injection, or erlotinib (25 mg/kg), administered daily by oral gavage. Mice were matched for tumour size in each experiment arm and treated without blinded. Studies were performed in accordance with the standards of the Institutional Animal Care and Use Committee under a protocol approved by the Animal Care and Use Committee at Kinki University.

Statistical analyses

Statistical analyses were performed using the StatView v.5.01 software (SAS Institute, Cary, NC, USA). All statistical tests were two-sided, unpaired *t*-tests, and a *P*-value < 0.05 was considered statistically significant. Data were graphically displayed using GraphPad Prism v.5.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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