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HER2 regulates cancer stem-like cell phenotype in *ALK* translocated NSCLC

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Abstract. We have previously shown that cancer stem-like cells (CSLCs) can mediate therapy resistance in *ALK* translocated lung cancers. HER2 has been linked to CSLCs in breast cancers and, therefore, we wanted to assess whether HER2 has a role in CSLCs in *ALK* translocated cancers. *ALK* translocated cell lines, H3122 and H2228, with variable sensitivity to ALK inhibition were used in the study. HER2 overexpression or knockdown was induced by retro- or lentiviral infections and cells were treated with pharmacological agents targeting HER2 and ALK signaling. Furthermore, tumorigenic properties of the cells were assessed *in vitro* using colony and sphere formation assays. In the ALK inhibitor sensitive H3122 cells, HER2 overexpression unaltered the primary response to ALK inhibition, but increased CSLC marker expression and enhanced colony and sphere formation and late AKT and ERK1/2 signaling recovery. In the ALK inhibitor semi-sensitive H2228 cells, *HER2* knockdown reduced basal expression of CSLC markers, modestly increased sensitivity to ALK inhibition in colony and sphere formation assays, and reduced late AKT and ERK1/2 signaling recovery. In addition, HER2 induced cross activation of other ErbB-members of which HER3 followed most closely the CSLC marker expression and neuregulin-1, a HER3 ligand, or pan-ErbB inhibitor afatinib, were able to alter CSLC marker expression and colony formation. The present study suggests that HER2 has an important role in the regulation of the CSLC phenotype in *ALK* translocated lung cancers that is mainly orchestrated by HER2/HER3 heterodimers.

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Abbreviations: ALDH1, aldehyde dehydrogenase 1; ALK, anaplastic lymphoma kinase; CSLC, cancer stem-like cell; EGF, epidermal growth factor; ErbB, erythroblastic leukemia viral oncogene homolog; HER, human epidermal growth factor receptor; NRG1, neuregulin-1; NSCLC, non-small cell lung cancer; RTK, receptor tyrosine kinase; TKI, tyrosine kinase inhibitor

Key words: targeted cancer therapy, resistance, cancer stem-like cells, ALK, HER2

Introduction

Approximately 7% of all non-small cell lung cancers (NSCLCs) contain chromosomal rearrangements of anaplastic lymphoma kinase (*ALK*), resulting in constitutively active ALK. *ALK* rearranged NSCLCs are often highly sensitive to ALK tyrosine kinase inhibitors (ALK TKIs) such as crizotinib. However, the ALK TKI-sensitive *ALK* rearranged NSCLCs will eventually develop targeted therapy resistance. Multiple different mechanisms for ALK TKI resistance have been reported, such as secondary mutations of *ALK*, or mutations in other somatic kinase domains, and activation of alternative signaling pathways through different receptor tyrosine kinases (RTKs), such as EGFR and HER2 (1-3).

Cancer stem-like cells (CSLCs) have often been linked to tumor initiation and therapy resistance. They have shown to be resistant both to the traditional chemo- and radiotherapies, and to targeted therapies, causing tumor relapses (4-8). Many signaling pathways have been described to be essential for CSLCs and potential cancer therapy targets have been discovered based on these pathways. Furthermore, some unspecific agents, like salinomycin and metformin, have been shown to target CSLCs (9,10). Currently, clinical utility of CSLC targeting agents is still unknown. We have previously shown that CSLCs can mediate therapy resistance in *ALK* translocated NSCLC cell lines (H2228 and H3122) and that targeting both ALK and CSLCs results in improved cell killing compared to either alone (6).

ErbB/HER family consists of four members: EGFR, HER2, HER3 and HER4. When activated, ErbB/HER family members form either homo- or heterodimers, which can signal downstream to the PI3K-AKT or Ras-Raf-MEK pathways. EGFR and HER2 receptors are commonly altered in some cancers, like breast cancers and NSCLCs, and act as cancer driving oncogenes. HER3 and HER4 have also shown to be expressed in cancers, but their activating genetic alterations

are uncommon. Co-expression of different HER receptors have been linked to a worse outcome/poor prognosis, especially EGFR and HER2 co-expression (11-14). Furthermore, HER family receptors, especially HER2, have been linked to CSLCs. Overexpression of HER2 has been shown to increase not only the amount of CSLC population in series of breast carcinoma cell lines, but also the tumorigenity in NOD/SCID mice (15-18). Since numerous clinically active HER2 targeting agents, such as trastuzumab, pertuzumab and lapatinib, are approved, characterization of CSLC dependency on HER2 could lead to rapid clinical testing of the agents in the context of CSLC targeting.

In the present study, we investigate the role of HER2 for CSLCs in *ALK* translocated NSCLC cell lines. The results suggest that HER2 plays an important role in the CSLC phenotype.

Materials and methods

Cell lines, inhibitors and growth factors. The cell lines used in the present study included *ALK* translocated NSCLC lines H3122 and H2228, and their counterparts modified to overexpress *GFP* or *HER2* (H3122) or to knock down *GFP* or *HER2* (H2228). The original cell lines were a kind gift from Dr Pasi Jänne (Dana-Farber Cancer Institute, Boston, MA, USA). The cell lines were grown in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA and Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin and streptomycin (Thermo Fisher Scientific). Cells were incubated at 37˚C with 5% CO_2 in the atmosphere. Following inhibitors were used: TAE684 (a gift from Dr Nathanael Gray, Dana-Farber Cancer Institute), crizotinib, afatinib (LC Laboratories, Woburn, MA, USA) and labatinib (Alexis Biochemicals, Lausen, Switzerland). Neuregulin-1 (NRG1) and epidermal growth factor (EGF) were also used (Thermo Fisher Scientific). Inhibitors were diluted in dimethyl sulfoxide (DMSO) and stored at -20˚C while growth factors were diluted in sterile, distilled water and stored at -80˚C.

Lentiviral knockdown and retroviral overexpression. Lentiviral and retroviral vectors were used to achieve overexpression or knockdown of *GFP* and *HER2* in the H3122 and H2228 cell lines. HER2 shRNA vector was purchased from Sigma-Aldrich. GFP shRNA vector and both retroviral vectors used for overexpression were kind gifts from Dr Pasi Jänne (Dana-Farber Cancer Institute). 293T cells were transfected with lenti-/retroviral expression vectors and packaging plasmids using FuGENE 6 reagent (Roche Diagnostics, Mannheim, Germany). Lentiviral supernatants were collected 24 h and retroviral supernatants 48 h after transfection. Both supernatants were filtered through 0.45 μm filter and applied to the target cells in the presence of Polybrene (Sigma-Aldrich). After 48 h of infections, the target cells were selected with puromycin (Sigma-Aldrich) for 72-96 h.

Western blot analysis. The cells were plated on 6-well plates, allowed to attach for 1-2 days and treated with desired drugs for 5 h or 5 days, after which they were lysed with NP-40 lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 1% igepal CA-630, 10 μg/ml aprotinin and 10 μg/ml leupeptin). Protein concentrations were measured with Bio-Rad Protein assay (Bio-Rad Laboratories, Hercules, CA, USA). After equalizing the concentrations Laemmli buffer was added, the samples were boiled and stored at -80˚C.

Equal amounts of protein samples were separated on SDS-PAGE and proteins were transferred to PVDF membranes, after which the membranes were blocked with 5% BSA (1x PBS, 0.1% Tween-20, 0.005% sodium azide) and incubated in primary antibodies overnight at 4˚C. Horseradish peroxidase (HRP)-linked secondary antibody was used, the membranes were developed using chemiluminescence and exposed to radiographic film. The quantification of western blot images was performed with ImageJ software, measuring the intensities (pixel percentages) for each sample in the same membrane with equal, manually selected area.

Following antibodies were used: ALDH1 (BD Transduction Laboratories, Franklin Lakes, NJ, USA), Akt, phospho-Akt (S473), CD44, cleaved PARP, EGFR, phospho-EGFR (Y1068), ERK1/2, phospho-ERK1/2 (T202/Y204), HER2, phospho-HER2 (Y1221/1222), HER3, phospho-HER3 (Y1289), HER4, phospho-HER4 (Y1284), anti-mouse/rabbit HRP-linked secondary antibody (Cell Signaling Technology, Danvers, MA, USA) and β-actin (Sigma-Aldrich). All antibodies were diluted in 5% BSA, and used at 1:1,000, 1:20,000 (β-actin), or 1:3,000 (secondary antibodies) dilutions.

Colony formation assay. Cells (600-1,000) were plated on 24-well plates with duplicates, allowed to attach for 1-2 days and treated with drugs. After 7 days, the drugs were withdrawn and the cells were allowed to recover and form colonies for several weeks. After clear differences were observed in the growth of colonies, the cells were fixed with ice-cold methanol and dyed with crystal violet stain.

Tumor sphere formation assay. The cells were treated with or without ALK TKI for 5 days, after which the cells were plated on 6-well ultra-low attachment plates (Corning Inc., Corning, NY, USA). A total of 5,000 (H3122) or 7,000 (H2228) cells were seeded on each well with or without further ALK TKI treatment. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 media with 20 ng/ml EGF, 20 ng/ml bFGF, 1% B27 supplement and 100 IU/ml penicillin and streptomycin (Thermo Fisher Scientific). The spheres were allowed to grow for 15 days, after which images were taken and the sphere numbers were counted. Magnification (x10) in phase contrast microscope was used when imaging the largest spheres from each well.

Results

HER2 alters expression of CSLC markers. We initially accessed whether two *EML4-ALK* translocated NSCLC cell lines, H3122 and H2228, basally expressed HER2. In the H3122 cells, which are very sensitive to ALK inhibition, basal HER2 expression was undetectable (Fig. 1A). Conversely, the H2228 cells, modestly sensitive to ALK inhibition, showed basal expression of HER2 (Fig. 1A). The overexpression of HER2 with a retroviral expression vector was successful in

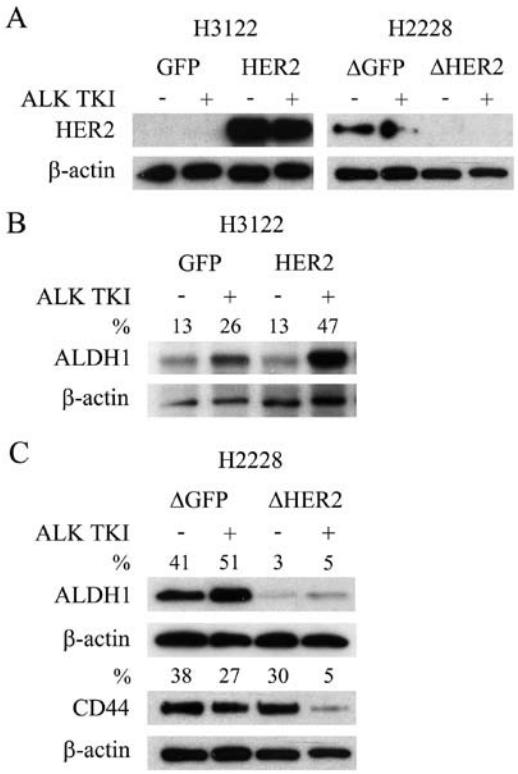

Figure 1. Western blot analysis for cancer stem-like cell markers, ALDH1 and CD44, in *ALK* translocated, GFP or HER2 altered NSCLC lines (H3122, H2228). (A) Validation of HER2 overexpression and knockdown. (B) ALDH1 expression in H3122 with retrovirally induced GFP or HER2 overexpression untreated or treated for 5 days with ALK TKI. (C) ALDH1 and CD44 expression in H2228 cells with lentiviral *GFP* or *HER2* knockdown untreated or treated for 5 days with ALK TKI. The percentages are defined as pixel intensities within equally sized areas for each membrane.

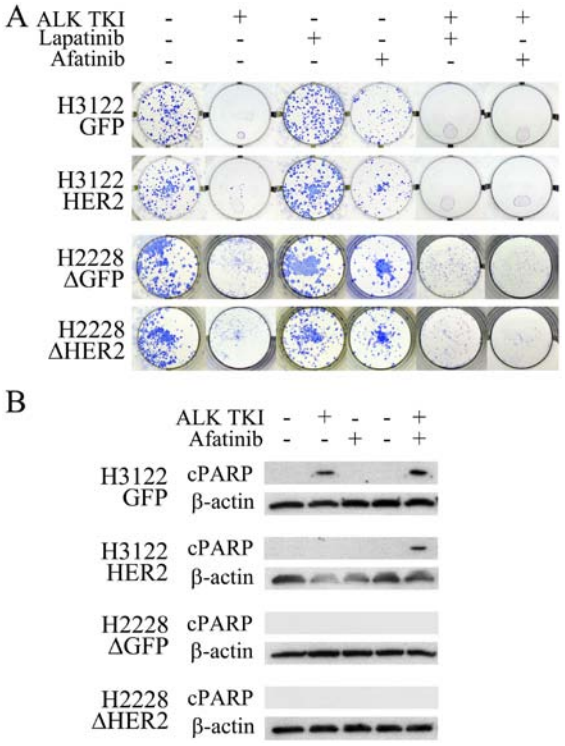

Figure 2. Colony formation and apoptosis of *ALK* translocated cell lines. (A) Colony formation assay in GFP or HER2 altered H3122 and H2228 cells untreated or treated with ALK, HER2 (lapatinib), pan-HER (afatinib) TKIs or their combinations for 7 days, after which the colonies were allowed to regrow without drugs. (B) Western blot analysis for cleaved PARP (cPARP, apoptosis) in GFP or HER2 altered H3122 and H2228 cell lines treated with ALK or HER2 TKIs or their combination.

H3122 cells with a marked increase in the expression level of the protein (Fig. 1A). Furthermore, knockdown of *HER2* with lentiviral shRNA vector induced a complete or near complete downregulation of the protein expression in the H2228 line (Fig. 1A).

We next accessed whether overexpression or knockdown of *HER2* resulted in changes to CSLCs. We have previously shown that CSLCs, indicated by specific markers ALDH1 (H3122) and CD44/ALDH1 (H2228), can mediate the ALK TKI resistance (6). As in our previous studies, ALDH1 expression increased in the H3122 cells in response to ALK inhibition (Fig. 1B), suggesting CSLC mediated resistance. Overexpression of HER2 in H3122 did not change the basal ALDH1 expression, but when the cells were challenged with ALK TKI, this resulted in more pronounced expression of CSLC marker (Fig. 1B). Knockdown of *HER2* in the H2228 cells resulted in basal downregulation of ALDH1, but unaltered expression of CD44 (Fig. 1C), a marker previously linked most strongly to CSLCs in this cell line (6). Moreover, marked CD44 downregulation was only seen in *HER2* knockdown H2228 cells when they were challenged with ALK TKI (Fig. 1C).

Role of HER2 in cytotoxic response to ALK TKI. The HER2 overexpressing H3122 cells or the *HER2* knockdown H2228 cells were next exposed to ALK TKIs to see whether their colony formation and apoptotic responses to ALK inhibition was altered. In the colony formation assay, major cytotoxicity response to ALK inhibition remained unaffected by the HER2 alterations (Fig. 2A). However, the ALK inhibitor treatment in the H3122 cells with HER2 overexpression resulted in increased number of surviving colonies compared to controls (Fig. 2A). Analogously, the ALK inhibitor treated H2228 cells with *HER2* knockdown had a modestly decreased number of surviving colonies (Fig. 2A). When the H3122 and H2228 cells were treated with a single HER2 specific inhibitor lapatinib, no change in cell survival was seen (Fig. 2A). The pan-HER (EGFR, HER2 and HER4) inhibitor afatinib, however, decreased the number of surviving colonies modestly in both tested cell lines (Fig. 2A). There was no difference in single-agent afatinib responses between the HER2 altered and control cells (Fig. 2A). Combination of ALK TKI with either lapatinib or afatinib resulted in total inhibition of colony formation in HER2 overexpressing H3122 cells (Fig. 2A). In H2228 cells, combined inhibition lead to a more pronounced colony inhibition in the *HER2* knockdown cells, afatinib being more potent than lapatinib in this setting (Fig. 2A).

Next, we wanted to assess, whether HER2 alteration would affect apoptotic response in the cell lines using western blot analysis for the apoptotic marker cleaved PARP. In H3122 control cells, cleaved PARP was detected in cells treated with ALK TKI and its combination with afatinib (Fig. 2B). In the H3122 cells overexpressing HER2, cleaved PARP was only detected following a combined treatment

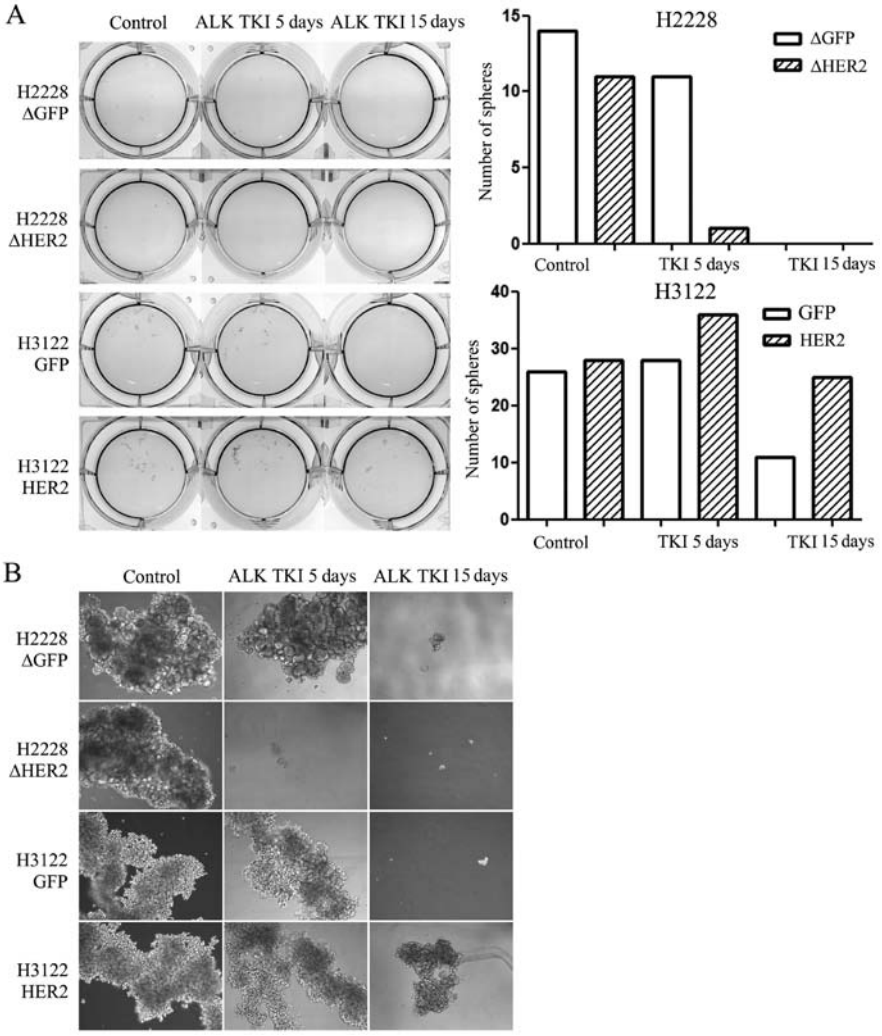

Figure 3. Sphere formation assay. (A) Sphere formation assay in GFP or HER2 altered H3122 and H2228 cell lines untreated or treated with ALK TKI for 5 or 15 days. Number of spheres are shown in the column chart. (B) Representative phase contrast images of the spheres with x10 magnification.

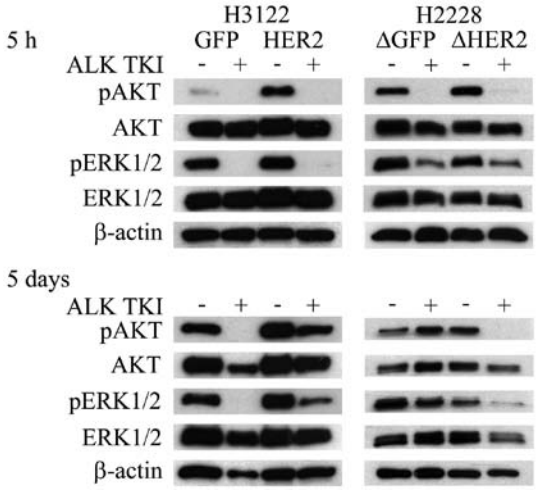

Figure 4. Western blot analysis for receptor downstream signaling proteins AKT and ERK1/2 in GFP or HER2 altered H3122 and H2228 cell lines after 5-h or 5-day treatments with ALK TKIs.

with ALK TKI and afatinib (Fig. 2B). In H2228 cells, analogously to previous study (3), no cleaved PARP signal was seen in control cells, and the *HER2* knockdown did not have an effect on this (Fig. 2B).

Role of HER2 in sphere formation. Sphere formation assay is one of the most used assay to identify CSLCs *in vitro* and therefore, we assessed whether HER2 alteration would modify the sphere formation of the *ALK* translocated H3122 or H2228 cells. We exposed the control and HER2 altered cells to ALK TKI *in vitro* for 5 or 15 days in stem cell enriching sphere formation environment with special media and low-attaching culture plates. In untreated cells, HER2 had no effect in the sphere formation capacity (Fig. 3). In H2228 cells treated with ALK TKI, *HER2* knockdown was able to inhibit sphere formation at 5 days compared to control cells while no surviving spheres where seen at 15 days (Fig. 3). H3122 cells with HER2 overexpression were able to form spheres in the presence of ALK TKI while this capacity was markedly reduced in control cells treated with ALK TKI for 5 or 15 days (Fig. 3).

HER2 is essential to AKT and ERK1/2 downstream signaling in long-term exposure to ALK TKI. PI3K and MAPK pathways are one of the most important downstream signaling pathways controlled by RTKs, such as ALK and HER2 (3,19,20). To

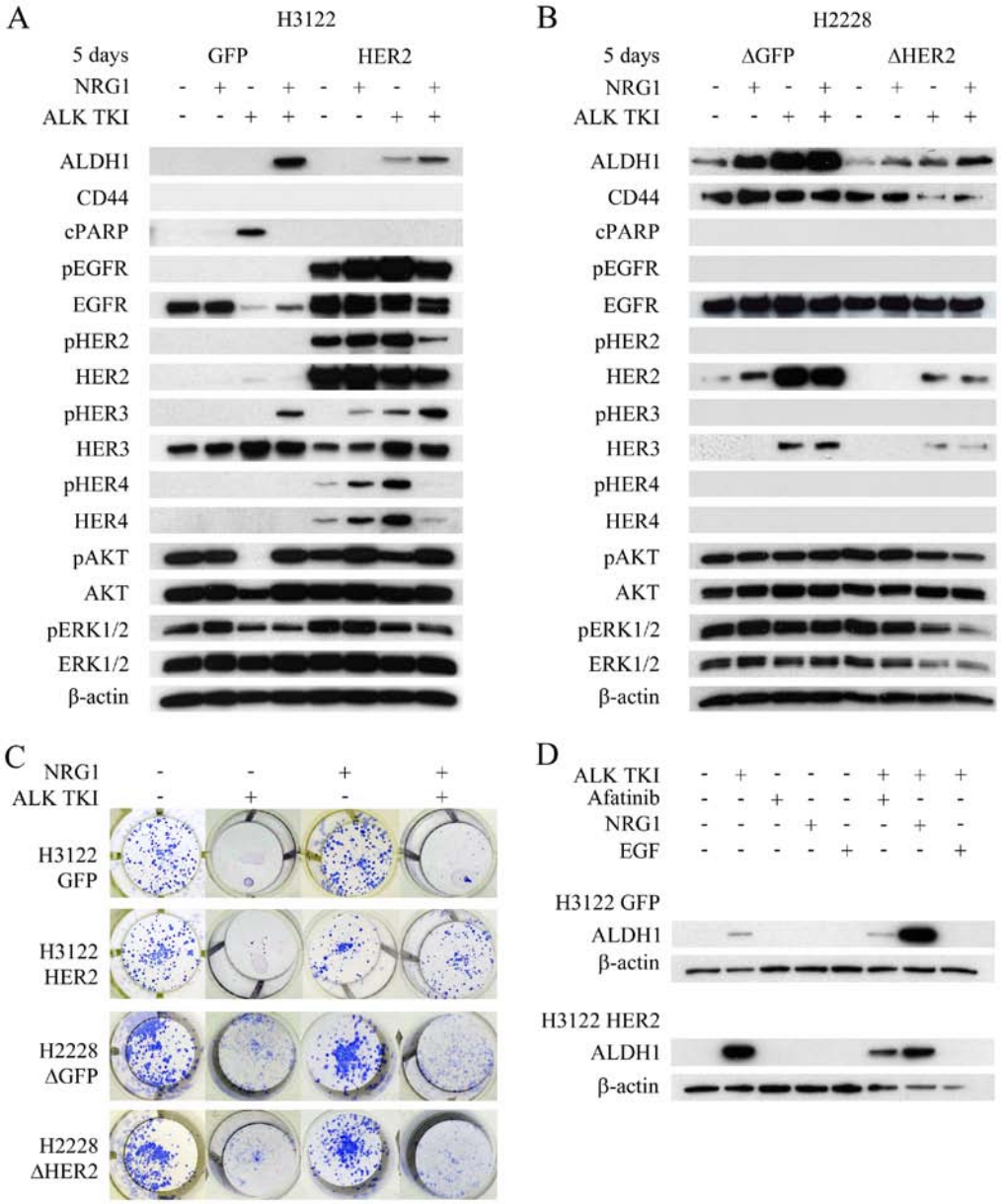

Figure 5. Effects of HER2 alterations and neuregulin-1 (NRG1) on ErbB-family signaling. Western blot analysis for ErbB-family activation, downstream signaling, and PARP cleavage (cPARP, apoptosis) in (A) GFP/HER2 overexpressed cell line H3122, and (B) *GFP/HER2* knockdown cell line H2228 untreated or treated with ALK TKIs and NRG1 for 5 days. (C) Colony formation assay of GFP/HER2 altered H3122 and H2228 cell lines treated with ALK TKI or NRG1 or their combination for 7 days, after which the colonies were allowed to regrow. (D) Expression of cancer stem-like cell marker ALDH1 after a 5-day treatment with ALK TKI, afatinib, NRG1, epidermal growth factor (EGF) or their combinations.

examine the effects of HER2 overexpression and knockdown to initial and long-term downstream signaling, phosphorylation of AKT and ERK1/2 proteins was investigated at 5 h and 5 days after initiation of ALK inhibition. The overexpression of HER2 in H3122 cells did not change the AKT or ERK1/2 responses to short-term (5 h) ALK TKI treatment (Fig. 4). Long-term (5 days) treatment of HER2 overexpressing H3122 cells with ALK TKIs, however, resulted in reformed downstream RTK signaling with major upregulation of both phosphorylated AKT and ERK1/2 (Fig. 4). In H2228 cells, initial response (5 h) to ALK TKIs was similar in the *HER2* knockdown cells (Fig. 4). Similarly to H3122 cells, HER2 altered H2228 cells showed different long-term (5 days) ALK TKI responses in downstream signaling (Fig. 4). In control H2228 cells, initial downregulation of AKT and ERK1/2 phosphorylation was completely recovered at 5 days while the downregulation remained in the *HER2* knockdown cells (Fig. 4).

HER2 orchestrates ErbB-family signaling. Next, we wanted to study whether *HER2* overexpression or knockdown had any effects on other ErbB/HER-family members and their activation, since HER2 forms not only homodimers but also heterodimers with all the other members of the protein family (21). In the H3122 control cells, only EGFR and HER3 were expressed without evidence of their activation by absence of phosphorylated protein counterparts (Fig. 5A). HER2 overexpression increased both expression and phosphorylation of EGFR and HER4 proteins, and phosphorylation of HER3 (Fig. 5A). In H2228 cells, only EGFR and HER2 were basally

expressed and no phosphorylation of the proteins was detected (Fig. 5B). Knockdown of *HER2* resulted in downregulated expression of HER3 (Fig. 5B).

Since HER2 alteration resulted in changes to HER3 and/or HER4 in both cell lines, we accessed whether neuregulin-1 (NRG1), only known joint HER3 and HER4 ligand and linked to tumor initiating cells (22), would modify behavior of the cells. In colony formation assay, NRG1 itself did not alter the number of colonies (Fig. 5C). As previously described, H3122 cells with HER2 overexpression showed increased number of surviving colonies in ALK TKI treated cells compared controls (Fig. 2A). When H3122 cells were treated with NRG1 in combination with ALK TKI, there was marked difference between the HER2 overexpressing and control cells (Fig. 5C). HER2 overexpressing cells showed a marked increase in the number of surviving colonies while the control cells showed only a minor increase (Fig. 5C). In H2228 cells, *HER2* knockdown did not affect the NRG1 response (Fig. 5C).

The cells were further analyzed for cell signaling, apoptotic response and/or CSLC marker expression after the NRG1 exposure. Especially in the H3122 line, NRG1 treatment altered all the analyzed responses (Fig. 5A). In H3122 cells, the most prominent effect of NRG1 was seen in HER3 phosphorylation (Fig. 5A). In control cells, NRG1 was able to induce HER3 phosphorylation in the ALK TKI treated cells, which was accompanied by upregulation of phosphorylated AKT, inhibition of apoptosis (cleaved PARP) and increased expression of CSLC markers (Fig. 5A). In HER2 overexpressing cells, HER3 phosphorylation was already detectable in cells treated with NRG1 alone and the most prominent expression was seen when it was combined to ALK TKI (Fig. 5A). Furthermore, NRG1 and ALK TKI combination induced the strongest expression of CSLC markers (Fig. 5A). In H2228 cells, NRG1 induced increase in ALDH1 expression, most notably in control cells (Fig. 5B). Only minor changes were seen in the CD44 expression, some rescue of ALK TKI induced downregulation of the marker detected in the *HER2* knockdown cells by NRG1 (Fig. 5B). We also assessed the effects of EGF, another HER ligand, mainly activating EGFR, on CSLC marker expression in the H3122 cells with HER2 alterations. However, EGF was unable to stimulate CSLC marker expression alone or in combination with ALK TKI (Fig. 5D).

Discussion

CSLCs have been linked to chemo-, radio- and targeted therapy resistance (4,7,8). Molecular mechanisms behind CSLC phenotype are largely unknown, but some signaling pathways such as wnt/β-catenin, TGF-β and HER2 pathways have been linked to it (23-25). Understanding molecular mechanisms of CSLCs would enable more efficient cancer treatment with combinatory approaches. Early phase clinical trials are testing some agents suggested to target CSLCs but no clear evidence of their effectiveness have been presented.

ALK translocated NSCLC represents a subgroup of disease in which patients are highly sensitive to ALK inhibitors, such as crizotinib. As with other targeted agents, acquired resistance to ALK inhibitors develops ~10-12 months after therapy initiation. Molecular mechanisms of ALK inhibitor resistance includes secondary mutations in tyrosine kinase domain of ALK, activation of by-pass signaling mechanisms and CSLC phenotype (3,6,26). Many previous reports have linked the ErbB-pathway activation mediated by-pass signaling mechanisms to ALK TKI resistance (3,6,27-29). The present study assessed whether ErbB-signaling affects the CSLC acquired in *ALK* translocated NSCLC models. We used two model cell lines, which we have previously shown to be either sensitive or modestly sensitive to ALK inhibition and CSLC phenotype to be related to therapy resistance.

Of the ErbB-pathways, HER2 has been linked most strongly to CSLC phenotype (17). HER2 targeting antibody trastuzumab is approved only for the treatment of *HER2* amplified breast cancer. However, reassessment of studies of *HER2* amplification have identified that some patients without amplification can benefit from adjuvant trastuzumab therapy (30,31). It has been speculated that benefit without *HER2* amplification could relate to CSLC targeting activity of trastuzumab. Our results showed that HER2 expression correlated with CSLC markers and sphere formation in *ALK* translocated models. More precisely, HER2 overexpression resulted more pronounced stem-like cell marker in response to ALK TKI while knockdown of the gene inhibited TKI induced stem-like cell phenotype. These results further highlight the importance of HER2 in CSLC. Our results showed not only the correlation between stem-like cell marker expression and HER2 but also pointed towards functionality of HER2 to stem-like cells assessed by colony or sphere formation. Genomic or pharmacologic alteration of HER2 modifies colony and sphere formation ability of *ALK* translocated models, HER2 correlating with increased capability. Since numerous agents targeting ErbB-signaling components are available in clinic, it would be interesting to test them in context of CSLC targeting as a combinatory approach.

Our experimentation showed that initial cytotoxic response or downstream receptor signaling (occurring in hours) to ALK TKIs was not changed by HER2 alterations. Notably, there was a marked change in the number of surviving colonies and downstream receptor signaling after long-term exposure (days) to ALK TKIs according HER2 status. More precisely, HER2 overexpression was able to markedly reactivate the AKT and ERK signaling after long-term exposure to ALK TKIs compared to controls. Analogously, *HER2* knockdown resulted in less recovery of AKT and ERK signaling after long ALK TKI treatment. More pronounced effects of HER2 alterations were seen in AKT rather than in ERK signaling. Many previous studies have linked AKT-mTOR signaling to CSLC phenotype and targeting this pathway has been shown to inhibit CSLCs (24,32-34). In *ALK* translocated cancers, AKT and ERK signaling is mainly driven by ALK and signaling recovery after long exposures to ALK inhibition is generally unknown. The present study suggests the importance of HER2 in this signaling recovery.

ErbB-family members can form homo- or heterodimers, which signal downstream of AKT and ERK with variable preference (19,21). In *HER2* amplified breast cancer, HER2-HER3 heterodimer is thought to be the most important signaling component, which preferentially signals though AKT-mTOR (35,36). HER3/HER4 ligand NRG1 mainly promotes AKT-mTOR signaling and interestingly, has

previously been linked to tumor initiating cells/CSLCs (22). This study suggests that HER2 orchestrates all other ErbB-family members. Expression of CSLC marker ALDH1 followed most closely HER3. NRG1 and long ALK TKI treatment promoted CSLC marker expression and HER3, which was accompanied by increased colony formation. This suggests that HER2/HER3 heterodimer could play an important role in CSLCs of *ALK* translocated lung cancers.

This study investigated the role of HER2 in CSLCs using *ALK* translocated lung cancer as a model. The results of the study suggest that HER2 has an important role in CSLC phenotype *in vitro* mainly orchestrated by HER2/HER3 heterodimers.

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