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YAP-Dependent AXL Overexpression Mediates Resistance to EGFR Inhibitors in NSCLC

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Abstract

The Yes-associated protein (YAP) is a transcriptional co-activator upregulating genes that promote cell growth and inhibit apoptosis. The main dysregulation of the Hippo pathway in tumors is due to YAP overexpression, promoting epithelial to mesenchymal transition, cell transformation, and increased metastatic ability. Moreover, it has recently been shown that YAP plays a role in sustaining resistance to targeted therapies as well. In our work, we evaluated the role of YAP in acquired resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors in lung cancer. In EGFR-addicted lung cancer cell lines (HCC4006 and HCC827) rendered resistant to several EGFR inhibitors, we observed that resistance was associated to YAP activation. Indeed, YAP silencing impaired the maintenance of resistance, while YAP overexpression decreased the responsiveness to EGFR inhibitors in sensitive parental cells. In our models, we identified the AXL tyrosine kinase receptor as the main YAP downstream effector responsible for sustaining YAP-driven resistance: in fact, AXL expression was YAP dependent, and pharmacological or genetic AXL inhibition restored the sensitivity of resistant cells to the anti-EGFR drugs. Notably, YAP overactivation and AXL overexpression were identified in a lung cancer patient upon acquisition of resistance to EGFR TKIs, highlighting the clinical relevance of our *in vitro* results. The reported data demonstrate that YAP and its downstream target AXL play a crucial role in resistance to EGFR TKIs and suggest that a combined inhibition of EGFR and the YAP/AXL axis could be a good therapeutic option in selected NSCLC patients.

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Introduction

Resistance to targeted therapy is a major issue for cancer treatments. The lesson learned from the clinic reveals that, despite the presence in cancer cells of the genetic lesions predictive of drug response and regardless of an initial response to therapy, at some point, tumors acquire the ability to overcome targeted drug activity and start regrowing. This is the so-called "secondary or acquired resistance." These events are well recapitulated *in vitro*, where cancer cells exposed to a drug for a long period of time become resistant through mechanisms often identical to those observed in patients [1,2]. Indeed, many efforts have been made to create *in vitro* models of resistance to study and possibly bypass tumor resistance and to offer patients efficient second-line treatments designed on the identified mechanisms of resistance.

In this frame, several researchers have rendered lung cancer cells addicted to EGFR resistant to EGFR tyrosine kinase inhibitors (TKIs). Exploiting these *in vitro* models, different mechanisms

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responsible for tumor cell resistance to EGFR TKIs have been identified: the most frequent is a second site mutation on the *EGFR* itself (the T790M mutation) which reduces the affinity of the EGFR ATP binding pocket for the drugs, thus allowing EGFR activation in spite of the presence of EGFR TKIs [3,4]. Other discovered mechanisms involve *MET* [5] and *HER2* [6] gene amplification, *PIK3CA* [7] and *BRAF* [8] mutations, epithelial to mesenchymal transition (EMT) [9], NF-KB [10], and AXL activation [11].

Recently, a role for Yes-associated protein (YAP) in mediating resistance to targeted therapies has been described [12]. The YAP protein, encoded by the *YAP1* gene, is the main mediator of the Hippo pathway [13]. This pathway, originally identified for its role in regulating organ size, is involved in many cellular functions which converge in provoking tumor initiation, progression, and metastasis and in reprogramming cancer cells into cancer stem cells [14–16]. In fact, the YAP pathway is often upregulated in cancer, somehow favoring cell transformation. The activation of the YAP protein upon external stimuli (i.e., low cell density) leads to YAP translocation from the cytoplasm to the nucleus, where it can act, together with TEAD transcription factors, as transcriptional coactivator of several genes, such as CTGF, CCDN1, and AXL, thus promoting cell proliferation and survival programs. Vice versa, when inactive, YAP is phosphorylated and prevalently resides in the cytoplasm, where it elicits less understood functions [17–19].

In this work, EGFR-addicted lung cancer cell lines were rendered resistant to several EGFR TKIs to study the possible involvement of YAP in the acquired resistance to these drugs. Interestingly, many resistant cells displayed increased activation of the YAP pathway compared to the parental, non-resistant cell lines. Moving forward and looking for downstream effector(s) of YAP responsible for resistance onset and maintenance, we demonstrated the causal involvement of the AXL tyrosine kinase receptor in YAP-driven resistance to EGFR TKIs: indeed, AXL was induced in cells with active YAP, and its pharmacological or genetic inhibition was sufficient to restore the sensitivity of resistant cells to the anti-EGFR drugs. The described mechanism is clinically relevant since one of the five examined patients, who had become resistant to EGFR TKIs through a yet unknown mechanism, showed YAP overactivation and AXL overexpression upon acquisition of resistance. The reported data, sustained by this case report, open the possibility of translating the anti-AXL treatment into the clinic.

Material and Methods

Cell Cultures and Compounds

The *EGFR* mutant non–small cell lung adenocarcinoma (NSCLC) cell lines HCC4006 (carrying delE746-A750) and HCC827 (carrying delE746-A750 and *EGFR* amplification) were obtained from ATCC-Sesto San Giovanni, MI, Italy, and cultured in RPMI-1640. The HEK293T cells (Human Embryonic Kidney cells, ATCC) were cultured in ISCOVE. The genetic identity of the cell lines was periodically controlled by short tandem repeat profiling (Cell ID, Promega, Madison, WI).

The *EGFR* mutant NSCLC cell lines were treated with the following EGFR tyrosine-kinase inhibitors: Erlotinib (Tarceva) and Gefitinib (Iressa) from Sequoia Research Products (Pangbourne, United Kingdom) and Afatinib (Gilotrif) and AZD8931 from Selleckchem (Munich, Germany).

The YAP constructs were produced as in reference [20]; AXL targeting shRNAs (#TRCN0000196945 and #TRCN0000195353) were from Sigma Aldrich. GAS6 (#885-GSB) was purchased from R&D Systems (Abingdon, UK).

To generate resistant cell lines, we used a stepwise dose escalation method starting from a drug dose near the IC50 of cell viability and then increasing the dose during a 6-month/1-year period. All the established resistant sublines were maintained in continuous culture with the achieved dose that still allowed cell proliferation. All the assays involving resistant cells were performed in the presence of the TKI to which they have been rendered resistant at the maximum dose reached at the end of the dose-escalation exposure period. The only exception is represented by the Western blot of drug washout, where cells were left untreated.

Quantitative Analysis of mRNAs and gDNA

Total RNAs from cultured cells were extracted using the TRIzol extraction kit (Thermo Fisher, Waltham, MA) according to the manufacturer protocol. Quantitative analysis of mRNAs was performed by reverse transcribing 0.5 μg of total RNA (High Capacity cDNA Reverse Transcription Kit, Thermo Fisher). Genomic DNA was extracted using Wizard SV Genomic DNA purification System (Promega). One microliter of cDNA or 50 ng of gDNA was amplified and analyzed using TaqMan Gene Expression Master Mix (Thermo Fisher). *ACTB* (actin-Hs01060665_g1) and GREB1 (Hs01738470_cn) were used as housekeeping gene for cDNA and gDNA, respectively. qRT-PCR was carried out using ABI PRISM 7900HT. Fold changes were determined by using ΔΔCT method. Taqman probes were as follows: CTGF (Hs01026927_g1), AXL (Hs01064444_m1and Hs1443849_cn), GAS6 (Hs0109035_m1), and MET (hs01277655_cn) (Thermo Fisher). Vimentin and E-cadherin expression was evaluated in SYBER Green. Primers are available from the authors.

Sanger Sequencing

Mutational analysis of *EGFR* exon 6 was performed via PCR amplification of 2 μl of cDNA using AmpliTaq Gold kit (Promega). The following primers were used: forward: CTCCTCTTGCTGCT GGTGGT; reverse: ATCTTGACATGCTGCGGTGT. PCR products were purified using AMPure (Agencourt Bioscience Corp., Beckman Coulter S.p.A, Milan, Italy) according to manufacturer procedures and analyzed on a 3730 DNA Analyzer, ABI capillary electrophoresis system (Thermo Fisher).

Protein Extraction and Western Blot

For Western blot analysis, cells were lysed in LB buffer [2% SDS, 0.5 mol/L Tris-HCl (pH 6.8)]. For stimulation experiments, cells were starved overnight and treated with GAS6 (100 ng/ml) for 10 minutes in the presence of EGFR TKI in resistant cells. Western blots were performed according to standard methods. Primary antibodies were as follows: anti-YAP #4912, anti-phospo YAP#4911, anti-AXL#8661, anti–phospho AXL#5724, anti-AKT#9272, anti–phospho AKT#4060, anti-MAPK#9102, and anti–phospho MAPK#9101, all from Cell Signaling (Leiden, the Netherlands); anti–β-actin #A3854, anti-vinculin #V9131, and anti–β-tubulin #T8328 from Sigma; and anti-TBP#ab818 from Abcam (Cambridge, UK). Peroxidase-labeled antirabbit or antimouse antibodies from Amersham Pharmacia (Milan, Italy) were used as secondary antibodies, and final signal detection was done with enhanced chemiluminescence system (Amersham Pharmacia).

Preparation of Cytosolic and Nuclear Protein Extracts

HCC4006 cells were lysed in 500 μl of Buffer A 10× (100 mM Hepes, 100 mM KCl, 100 mM EDTA, water) supplemented with protease inhibitors, 0.5% NP-40, and 1 mM DTT for 10 minutes on ice. Cells were scraped into a fresh tube, and cell lysates were centrifuged at 13,500 rpm for 15 minutes at 4°C. Supernatants containing the cytosolic fraction were collected and transferred into a separate tube. The pellet containing the nuclei were washed three times with Buffer A and then lysed with 50 μl of Buffer B (20 mM Hepes, 0.4 M NaCl, 1 mM EDTA, 10% glycerol, water) supplemented with protease inhibitors and DTT 1 mM. Tubes containing the nuclei were gently rocked at 4°C for 1 hour and then were centrifuged at 13,500 rpm for 5 minutes. Both the fractions were quantified for the total protein quantity with BCA Protein Assay Kit (Pierce).

Cell Transfection and Transduction

HCC4006 and HCC827 were transfected with siRNAs using Lipofectamine 2000 (Thermo Fisher). Transfection reagents plus siRNAs at final concentration of 20 nM were distributed in each well of a 96-well plate incubated in OptiMEM serum-free media for 20 minutes, and after that, 70 μl of cells (2000 cells/70 μl) in media without antibiotics was added to each well. After 72 hours of growth, cell viability was measured by using the Cell Titer-Glo Luminescent Cell Viability Assay (Promega). SiRNA (Sigma Aldrich) sequences are available from the authors.

Lentiviruses were produced as described in [21]. Cells were transduced with 40 ng/ml of p24.

Cell Viability Assay

For growth curve and cell viability assays, cells were seeded in quadruplicates in 96-well culture plates (2000 cells/well) in the presence of the indicated drugs. After 72 hours of growth, cell viability was measured by using the Cell Titer-Glo Luminescent Cell Viability Assay (Promega).

Immunohistochemistry on Lung Adenocarcinoma Tissue Specimens

Matched biopsies (either bronchial or tru-cut) from nine patients affected by advanced lung adenocarcinomas harboring *EGFR* mutations and treated with EGFR TKIs, obtained before and after the onset of resistance, were analyzed by means of immunohistochemistry. A control group of 10 lung adenocarcinoma cases with matched tissue samples before and after chemotherapy and lacking EGFR mutations was also analyzed. Antibodies against pYAP (#4911 Cell Signaling, diluted 1:50), AXL (Cell Signaling, clone C89E7, diluted 1:50), and CTGF (Santa Cruz, goat polyclonal, diluted 1:100) were employed using an automated platform (Ventana Benchmark). The immunohistochemistry signal was assessed using the H-score which is based on the intensity of the signal and on the percentage of cells stained, and results in a score ranging from 0 to 300.

The immunohistochemical study was conducted according to guidelines and regulations by the Research Ethics Committee of the AOU San Luigi/University of Turin, as explicated by formal approval to M. V. of current projects regulating the use of retrospective solid tumor tissues (see Ethics Committee Approvals no. 167/2015, prot. 17975, 14/11/2015 and no. 204/2016, prot. 20840, 22/12/2016). Before the analysis, the samples have been anonymized by staff members of the Department of Oncology at San Luigi Hospital not involved in the project. No references to the patients can be inferred from the immunohistochemical characterization presented in the work.

Statistics

Results show one representative experiment out of at least three different independent experiments. Comparisons were made using two-tailed Student's *t* test.

Results

The YAP Pathway is Activated in EGFR TKI–Resistant Cells

Since YAP activity has been involved in resistance to B-RAF and MEK targeted therapies [22], we wondered whether it could also be implicated in resistance to EGFR targeted therapies in lung cancer cells. To address this issue, we focused on a panel of lung cancer cell lines (HCC4006, HCC827, and PC9) addicted to EGFR (i.e., dependent on EGFR activity for their growth and survival) that we rendered resistant to four different EGFR TKIs: erlotinib and gefitinib (first-generation TKIs), afatinib and AZD8931 (second-generation TKIs). Cells were treated for several months with increasing concentrations of the different drugs until reaching a dose that was at least five-fold higher than the IC50 (Table 1) and continuously kept in culture with EGFR inhibitors. All the experiments on resistant sublines were performed in the presence of EGFR TKIs if not differently specified. Resistant cells were characterized for the presence of already known mechanisms of resistance and for the activation status of EGFR, MET, AKT, and MAPK (for details, see Supplementary Table 1).

To assess the activity of the Hippo pathway in the resistant cell lines, we screened them for the expression of connective tissue growth factor (CTGF) which is considered one of the major transcriptional targets of YAP [23]. As shown in Figure 1*A* and Supplementary Figure 1*A*, in many resistant cell lines, CTGF expression was significantly higher compared to the parental cells. Interestingly, CTGF increase could be observed also in models of acquired resistance to other TKIs (Supplementary Figure 1*B*), opening the possibility that YAP activation is a shared mechanism of resistance. Since the greatest increase in CTGF expression was seen in HCC4006 cells resistant to the different EGFR TKIs and in HCC827 cells resistant to afatinib, we selected these cell lines for further studies.

According to the literature, upon activation, YAP loses its phosphorylation in serine 127 and translocates into the nucleus, where it activates its transcriptional targets (among them, the already mentioned CTGF) [18]. For this reason, we looked at YAP phosphorylation status and localization to further prove its activation in resistant cells. As shown in Figure 1 *B* and *C*, YAP was less phosphorylated in S127 and showed an increased nuclear localization in resistant cells compared to parental ones, thus indicating a fostering of YAP pathway activation in resistant cells. Moreover, in resistant cells,

Table 1. Characterization of Cells Resistant to EGFR TKIs

Cell Lines		Mechanisms of Resistance	IC50 Resistant Cells	IC50 WT Cells
HCC4006	R400 ERL	EMT phenotype	3000 nM	40 nM
	R40 GEF	EMT phenotype	3000 nM	5 nM
	R6 AFA	EMT phenotype	160 nM	0.3 nM
	R15 AZD	EMT phenotype	320 nM	2 nM
HCC827	R100 ERL	*MET* amplification	2500 nM	7.5 nM
	R100 GEF	*MET* amplification	2500 nM	7.5 nM
	R5 AFA	*MET* amplification	>320 nM	0.3 nM
	R5 AZD	Unknown mechanism	200 nM	0.3 nM
PC9	R100 ERL	*EGFR* T790M mutation	2500 nM	20 nM
	R100 GEF	*EGFR* T790M mutation	2500 nM	20 nM
	R10 AFA	*EGFR* T790M mutation	40 nM	0.2 nM
	R10 AZD	*EGFR* T790M mutation	>320 nM	0.5 nM

The mechanism of resistance was evaluated as follows. In HCC4006 cells, we observed increased expression of vimentin and decreased levels of E-cadherin by qRT-PCR; in HCC827 cells, we observed MET amplification by qRT PCR on gDNA; in PC9 cells, we found the appearance of the EGFR T790M mutation by Sanger sequencing (Supplementary Table 1).

drug washout did not affect YAP activity, suggesting that YAP activation is not an epiphenomenon due to drug exposure but is a stable event, possibly having a functional role in maintaining resistance (Supplementary Figure 2*A*).

YAP Functionally Controls Cell Response to EGFR TKIs

To understand if YAP activation has a functional role in resistance maintenance, we genetically inhibited YAP expression in parental and resistant cells (grown in presence of anti-EGFR drugs) using two different siRNA sequences. As shown in Figure 2*A*, YAP silencing significantly reduced cell viability in resistant cells, while it was ineffective in parental cells.

In the mirror experiment, the overexpression in HCC4006 parental cells of either the WT YAP or an active form of YAP carrying a double mutation in serines 127 and 381 (which renders it unphosphorylable and thus preferentially located in the nucleus; YAP SS) conferred resistance to EGFR TKIs (Figure 2*B* and Supplementary Figure 2*B*). Similar results have been obtained in HCC827 cells as well (Supplementary Figure 2, *C* and *D*).

Altogether, these results show that YAP activation functionally regulates cell sensitivity to EGFR TKIs.

YAP Activation Impairs Drug Response Through Induction of AXL Transcription

Since YAP is able to transcriptionally activate many targets [24], we wondered which of them is critical in sustaining resistance to EGFR TKIs. Among the described YAP targets, we focused our attention on the AXL tyrosine kinase receptor that could, in principle, vicariate the loss of EGFR signal due to TKI treatment [25]. For this reason, we evaluated AXL expression in our resistant and parental cells. As shown, in resistant cells, we observed an increase of AXL expression both at the RNA (Figure 3*A* and Supplementary Figure 3*A*) and at the protein level (Figure 3*B* and Supplementary Figure 3*B*). Notably, no amplification of the AXL gene was observed at the genomic level (Supplementary Figure 3*C*), suggesting that AXL increase might be due to transcriptional activation. As not only AXL protein amount but also its phosphorylation was strongly increased in resistant cells, we evaluated the expression of its ligand, GAS6. GAS6 expression was

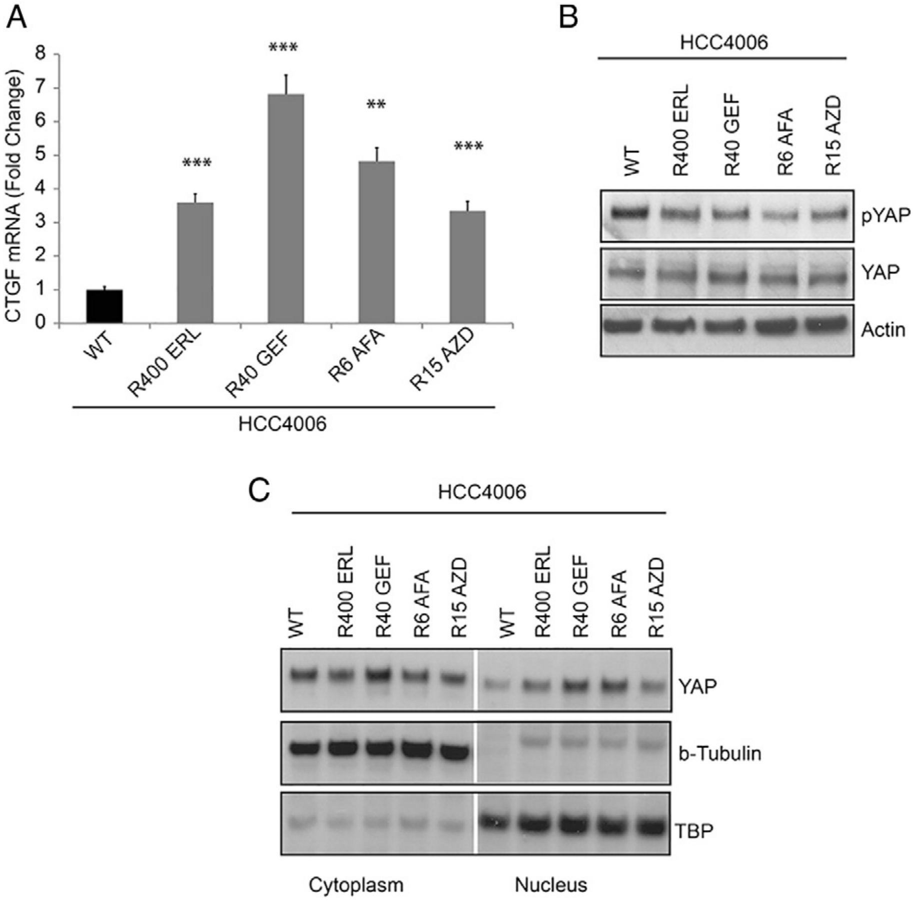

Figure 1. YAP is activated in HCC4006 resistant cells. (A) The expression level of CTGF (one of the major YAP targets) was evaluated by qPCR in HCC4006 resistant cells. Results are expressed as fold change compared to wild-type (wt) cells, considered as 1. CTGF mRNA levels were higher in the resistant cell lines compared to their wt counterpart, testifying to an increased YAP activity in resistant cells. $^{**}P < .01$; $^{***}P < .001$. The error bars represent the SD. (B) Western Blot analysis of total cell lysates demonstrates that all the HCC4006 cells resistant to the different EGFR TKIs showed decreased YAP S127 phosphorylation (that inhibits YAP activity by sequestering the protein in the cytoplasm) compared to parental HCC4006; the amount of total YAP was unaffected. Actin was used as loading control. (C) Nucleus-cytoplasm fractionation: cytoplasms and nuclei of wt and resistant cells were separately lysed, subjected to WB, and probed with the indicated antibodies. As shown, the amount of nuclear YAP was increased in resistant compared to wt cells. TBP (Tata binding protein) and b-tubulin were used as loading controls of the nuclear and cytoplasmic fraction, respectively. ERL = erlotinib; GEF = gefitinib; AFA = afatinib; AZD = AZD8931. R400, R40, R6, R15 = concentrations (nM) of the different drugs to which the cells are resistant.

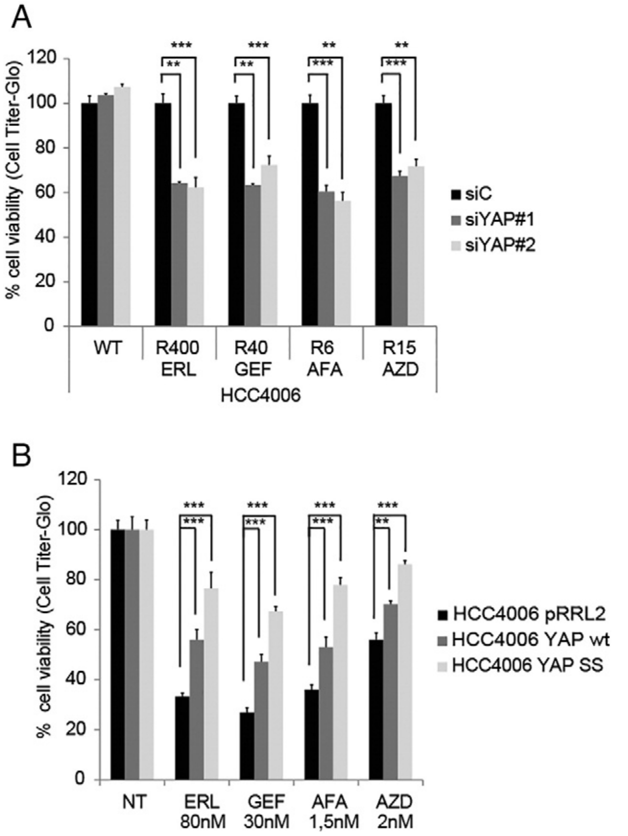

Figure 2. YAP modulation impinges on resistance to EGFR TKIs. (A) Viability assay of wt and resistant cells (in presence of EGFR TKIs) upon YAP silencing with two different siRNA sequences. As shown, viability of resistant cells was significantly affected by YAP silencing. (B) HCC4006 wt cells were transduced with the empty vector (pRRL2), YAP wt, or YAP SS (the constitutively active form of YAP). Cells were then treated with the indicated drugs, and cell viability was assessed 72 hours later. As shown, the overexpression of both YAP wt and SS protected cells from EGFR TKI treatment. The error bars represent the SD. $^{**}P < .01$; $^{***}P < .001$. Drug abbreviations as in Figure 1.

also markedly increased in resistant cells compared to parental ones (Supplementary Figure 3*D*). However, GAS6 expression was not directly controlled by YAP as no modulation of GAS6 expression was observed upon YAP overexpression/inhibition (data not shown).

To prove that increased AXL expression was a consequence of YAP activation, we genetically interfered with YAP expression. A clear reduction of AXL expression was observed upon YAP silencing, both in HCC4006 (Figure 3*C*) and in HCC827 (Supplementary Figure 3*E*) cells. To further strengthen the finding that AXL expression is influenced by YAP activation, we overexpressed either YAP WT or YAP SS in parental HCC4006 and HCC827 cells; as shown in Figure 3*D* and Supplementary Figure 3*F*, in both cells, AXL expression was increased.

AXL Genetic or Pharmacological Blockage Revert EGFR TKI Resistance

The final goal of understanding resistance mechanisms to TKIs is to find a way to overcome resistance, thus offering an effective treatment to patients. With this in mind, we wondered whether AXL could be an actionable target in our system. To address this point, we first undertook a pharmacological approach. HCC4006 resistant cells (maintained in the presence of the EGFR TKI to which they are resistant) were co-treated with increasing doses of TP-0903 (a selective AXL TKI) (Figure 4*A*) or foretinib (a multikinase inhibitor active against many tyrosine kinase receptors, including AXL) (Supplementary Figure 4*A*). As shown in the graphs, both inhibitors significantly decreased cell viability in a dose-dependent manner, the specific TP-0903 inhibitor being more potent than foretinib. As expected, both TP-0903 (Figure 4*B*) and foretinib (Supplementary Figure 4*B*) induced a strong decrease in AXL phosphorylation and in the activation of the downstream transducers MAPK and AKT. Similar results have been obtained in HCC827 resistant cells as well (Supplementary Figure 4*C*). Moreover, AXL basal phosphorylation in HCC4006 resistant cells was further increased by GAS6 stimulation and reverted by foretinib treatment (Supplementary Figure 4*B*).

Finally, to validate the pharmacological data, we carried out a genetic approach, specifically silencing AXL expression with two different shRNA sequences in HCC4006 resistant cells maintained in the presence of EGFR TKIs (Supplementary Figure 5). As shown in Figure 4*C*, upon AXL silencing, HCC4006 reacquired sensitivity to EGFR TKIs at a level near that of wt cells (Supplementary Figure 6).

These data highlight the active role of AXL in mediating resistance to EGFR TKIs in our cellular models and open the possibility of considering AXL an actionable and effective target in EGFR TKI–resistant cells.

Activation of the YAP-AXL Pathway upon Resistance Onset in Human Lung Adenocarcinomas

To validate our *in vitro* data, we analyzed tumor slices obtained from lung cancer patients treated with EGFR TKIs who had become resistant to the treatment (Supplementary Table 2) as compared to a control group of lung cancer patients whose tumors were lacking EGFR mutations and were thus treated with chemotherapy only (Supplementary Table 3). None of the investigated markers was significantly correlated with each other, or different in EGFR mutated versus wt adenocarcinoma samples, or in first biopsies versus samples at tumor progression, as a whole group (data not shown). With regard to the nine patients harboring EGFR mutations and treated with EGFR TKIs, three of them developed the T790M resistance mutation at the time of progression, whereas in the other six patients, the mechanism of resistance was unknown (Supplementary Table 2). Interestingly, in the biopsy of patient #5 obtained upon resistance onset (tissue sample II), we observed, compared to the biopsy at diagnosis (tissue sample I), an important activation of the YAP pathway; indeed, we found decreased pYAP, which is the inactive form of YAP (from H-score 130 in the first biopsy to 30 in the biopsy at tumor progression), and increased CTGF expression (from H-score 30 in the first biopsy to 90 in the biopsy at tumor progression). Concomitantly, AXL expression was significantly increased (AXL score low: 10 in the biopsy at diagnosis, high: 90 in the biopsy at relapse) (Figure 5). The relapsed tumor of this patient was negative for the presence of other known mechanisms of resistance such as *ALK* and *ROS1* translocations; *MET* amplification; and *BRAF*, *PI3K*, *HER2*, and *KRAS* mutations (data not shown). In summary, three out of seven investigated patients developed resistance due to the appearance of the T790M mutation; no other known molecular alteration was identified in the other analyzed resistant cases. YAP-associated AXL activation was found in one out of the six patients not displaying EGFR resistance mutations. This report, although too small to drive conclusions on the real prevalence of AXL-driven resistance, strengthens our *in vitro* data, opening the

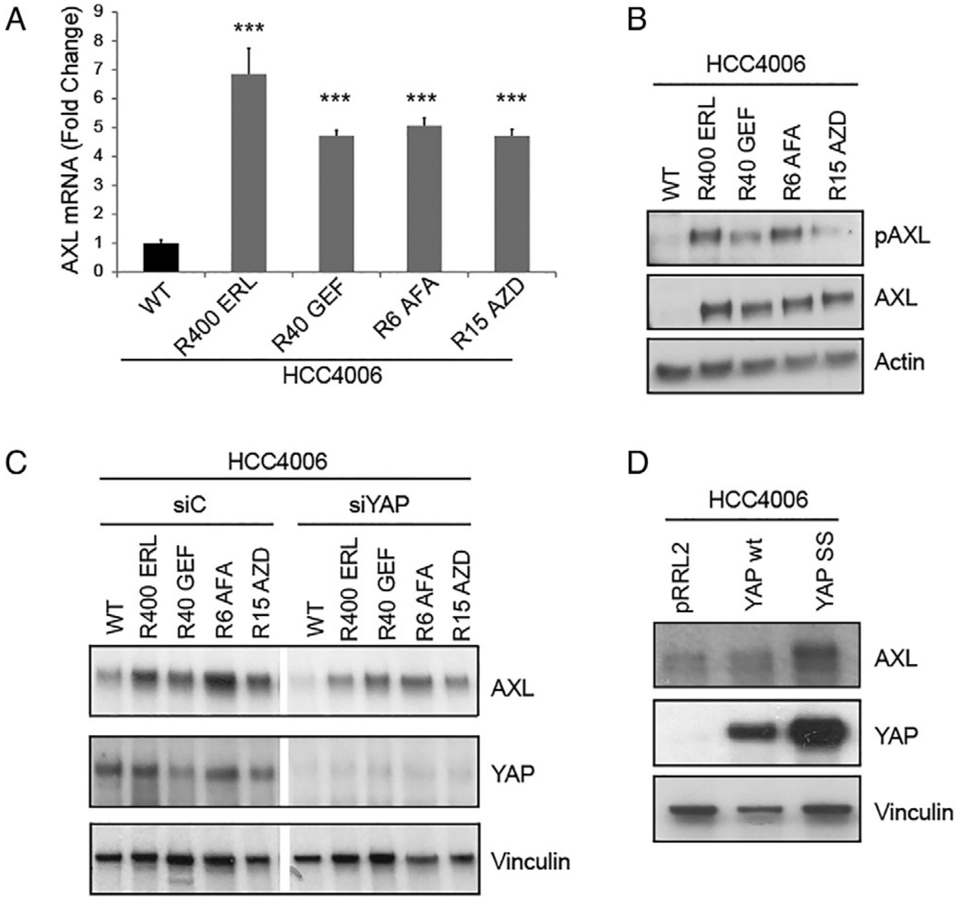

Figure 3. YAP induces AXL expression. (A) The expression level of AXL was evaluated by qPCR in HCC4006 resistant cells and compared to wt cells. Results are expressed as fold change compared to wt cells, considered as 1. AXL mRNA levels were higher in the resistant cell lines compared to the wild-type counterpart. ***$P < .001$. The error bars represent the SD. (B) The expression level of AXL in resistant and parental HCC4006 cells was evaluated by WB. As shown, both AXL expression and activation (phosphorylated AXL, pAXL) were increased in resistant cells. Actin was used as loading control. (C) AXL expression was evaluated by WB upon YAP silencing in HCC4006 cells. As shown, AXL expression was reduced upon YAP silencing. Vinculin was used as loading control. (D) HCC4006 cells were transduced with wt (YAP wt) or active YAP (YAP SS), and the expression of AXL was assessed by WB. As shown, AXL expression was induced upon YAP overexpression. Vinculin was used as loading control. Drug abbreviations as in Figure 1.

possibility of the evaluation of AXL as a pharmacological target in EGFR-resistant patients without other known mechanisms of resistance.

Discussion

In our work, we aimed at evaluating the role of YAP in EGFR-addicted lung cancer cells rendered resistant to first- or second-generation EGFR TKIs. The Hippo pathway effector YAP protein has long been recognized as a critical regulator of organ size and is known to be involved in tumor initiation, progression, and metastasis [14,17]. More recently, some works identified a role for YAP in mediating resistance to targeted therapies [12]. Indeed, Shao and colleagues showed that in a KRAS-driven murine lung cancer model, acquired resistance to KRAS inhibition was due to YAP activation, as both KRAS and YAP converge on the FOS transcription factor and activate EMT [26]. In another work, Lin and collaborators demonstrated that YAP acts as a parallel survival input to sustain resistance to B-RAF and MEK inhibitors and that dual YAP/MEK inhibition is synthetically lethal [22]. The authors found that both YAP and MAPK control the expression of the antiapoptotic protein BCL-xL and that the simultaneous inhibition of both pathways is required to reduce BCL-xL expression to a level sufficient to restore an apoptotic response. Another contribution to understanding the role of YAP in mediating resistance came from the work of Kim et al., who found that resistance to BRAF inhibitors in melanoma cells was due to actin remodeling-induced YAP activation [27]. In fact, inhibition of actin polymerization and actomyosin tension suppressed both YAP activation and resistance to BRAF inhibitors.

A role for YAP in mediating resistance to EGFR inhibition has also been described [28,29]. In line with these evidences, we observed increased YAP activation in all the generated EGFR TKI–resistant cells, testified by decreased phosphorylation on the inhibitory serine 127, enhanced nuclear localization, and augmented expression of its major target CTGF. Interestingly, we detected YAP activation not only in cells resistant to various EGFR TKIs but also in cells resistant to inhibitors directed against other tyrosine kinases such as MET and ROS1. This suggests that YAP activation may represent a more general mechanism to sustain resistance to drugs targeting different tyrosine kinase receptors. Hsu et al. recently reported a role for YAP in mediating resistance to erlotinib in lung cancer cells [29]: they observed increased

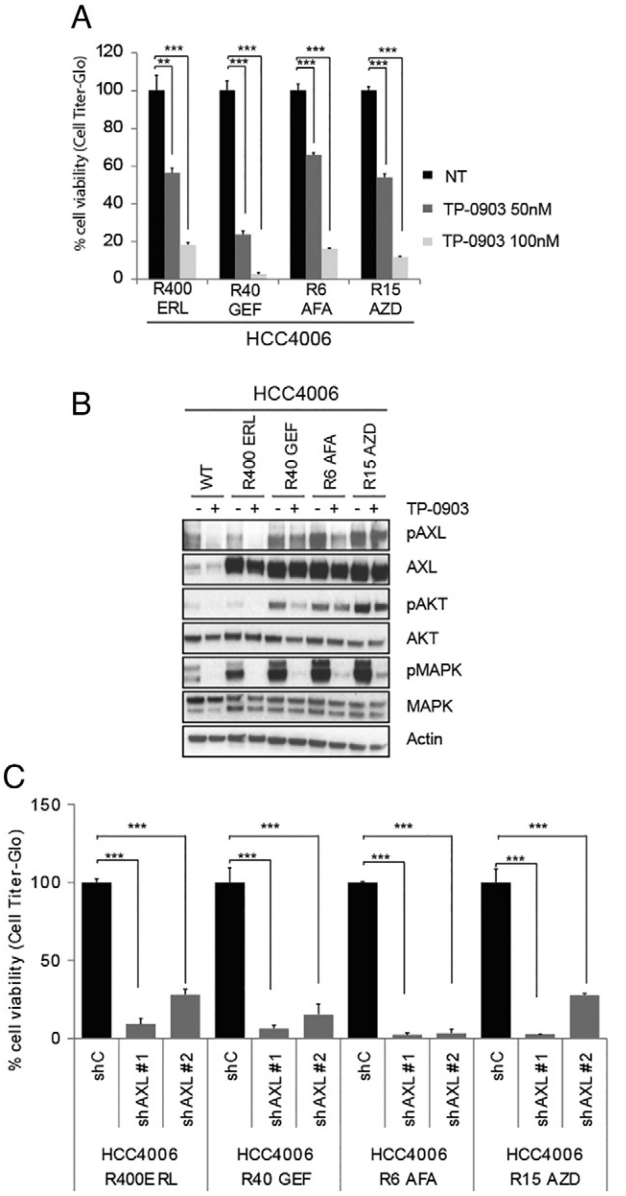

Figure 4. Pharmacological or genetic blockage of AXL impinges on HCC4006 cell viability. (A) HCC4006 resistant cells were maintained in the presence of EGFR TKIs and co-treated with the AXL specific inhibitor TP-0903 for 72 hours, and their viability was assessed. The chart shows that AXL inhibition through TP-0903 reduced cell viability in a dose-dependent manner. The error bars represent the SD. $**P < .01$; $***P < .001$. (B) HCC4006 cells were treated for 2 hours with TP-0903, and the activation of AXL and of its main downstream signal transducers AKT and MAPK was evaluated by WB. As shown, the phosphorylation of AXL (pAXL), AKT (pAKT), and MAPK (pMAPK) was markedly reduced upon TP-0903 treatment. Actin was used as loading control. (C) HCC4006 resistant cells (maintained in the presence of EGFR TKIs) were transduced with two different AXL shRNAs or with a control shRNA (shC). Viability was measured 6 days after seeding. The chart shows that AXL silencing strongly decreased cell viability. The error bars represent the SD. $***P < .001$. Drug abbreviations as in Figure 1.

YAP expression and decreased YAP phosphorylation in HCC827 resistant versus parental cells and YAP-mediated protection to erlotinib treatment in parental cells. It has however to be noted that the reported IC50 (2.48 μM) and the used doses of TKIs were dramatically higher than those reported by others and ourselves ([8,30,31] and Table 1, in the range of low nM). These authors also reported that H1975 erlotinib–resistant cells bearing the second site mutation T790M became more sensitive to erlotinib upon YAP overexpression; the gain in erlotinib efficacy, however, was very poor, and the mechanism through which YAP rendered H1975 cells more sensitive to erlotinib was not addressed.

To prove that YAP activation was not the consequence of resistance onset but rather a critical element in sustaining resistance, we performed experiments of genetic interference or exogenous protein expression in resistant cells, which indeed demonstrated that lowering YAP activity restored sensitivity to the different TKIs, while increasing it impaired response to the drugs. As YAP is a transcriptional coactivator, we reasoned that the observed effect could be due to the transcription of critical effector(s). Among YAP targets, we focused on AXL, a tyrosine kinase that has been identified as a mediator of YAP-dependent oncogenic functions [25] and that has been shown to contribute to resistance to targeted therapies [11]. In fact, Zhang et al. [11] showed that AXL upregulation is sufficient to sustain erlotinib acquired resistance in *EGFR* mutant NSCLC cellular models. In resistant cells, we observed a YAP-dependent AXL increase concomitant with an augmented expression of its ligand GAS6, resulting in autocrine activation of this kinase. Pharmacologic inhibition and genetic interference with AXL expression showed that AXL is critical in mediating YAP-induced resistance to EGFR TKIs, thus representing an actionable target to restore sensibility to targeted therapies. It is worth noting that the described resistance is apparently not due to genetic alterations but is rather sustained by an adaptive mechanism. Interestingly, AXL silencing or pharmacological inhibition is more effective than YAP silencing in reverting cancer cell resistance, suggesting that other mechanisms — in addition to YAP activation — might concur to AXL activation. It has been reported that AXL transcription can be induced by MAPK-AP1 activation [32] and by MZF1 transcriptional activity [33]. In this frame, the possible activation of MAPK or of MZF1 by other cellular stimuli might justify the primary role of AXL in mediating resistance to EGFR TKIs we reported here.

Finally, we demonstrated that the activation of the YAP/AXL axis is present in selected lung cancer patients who become resistant to EGFR inhibitors. It is known that in about 50% of the cases, resistance to EGFR TKIs is due to the appearance of resistance mutations, such as the T790M. Accordingly, in seven lung cancer patients examined, we found that this mutation was present in three biopsies obtained upon resistance onset but not in the corresponding biopsies at diagnosis. In one of the patients that did not show already described genetic alterations supporting resistance, such as new *EGFR* mutations or *ALK/ROS1* translocations or *MET* amplification, we observed YAP activation and AXL overexpression, recapitulating what was found in the *in vitro* generated resistant cells. It is worthwhile to note that the activation of the YAP-AXL pathway in one out of five patients negative for the presence of the T790M mutation might represent a relatively high percentage, in line with other mechanisms of resistance already described, such as *HER2* amplification [6]. Due to the relatively low number of samples analyzed, however, other studies are needed to verify the prevalence of YAP-AXL activation to understand the translation potential of anti-AXL treatments into the clinic.

At present, drugs that specifically inhibit YAP activity are not available. In fact, verteporfin, which was originally described as a specific inhibitor of YAP-TEAD interaction, has been recently shown to exert its activity through selective induction of proteotoxicity rather

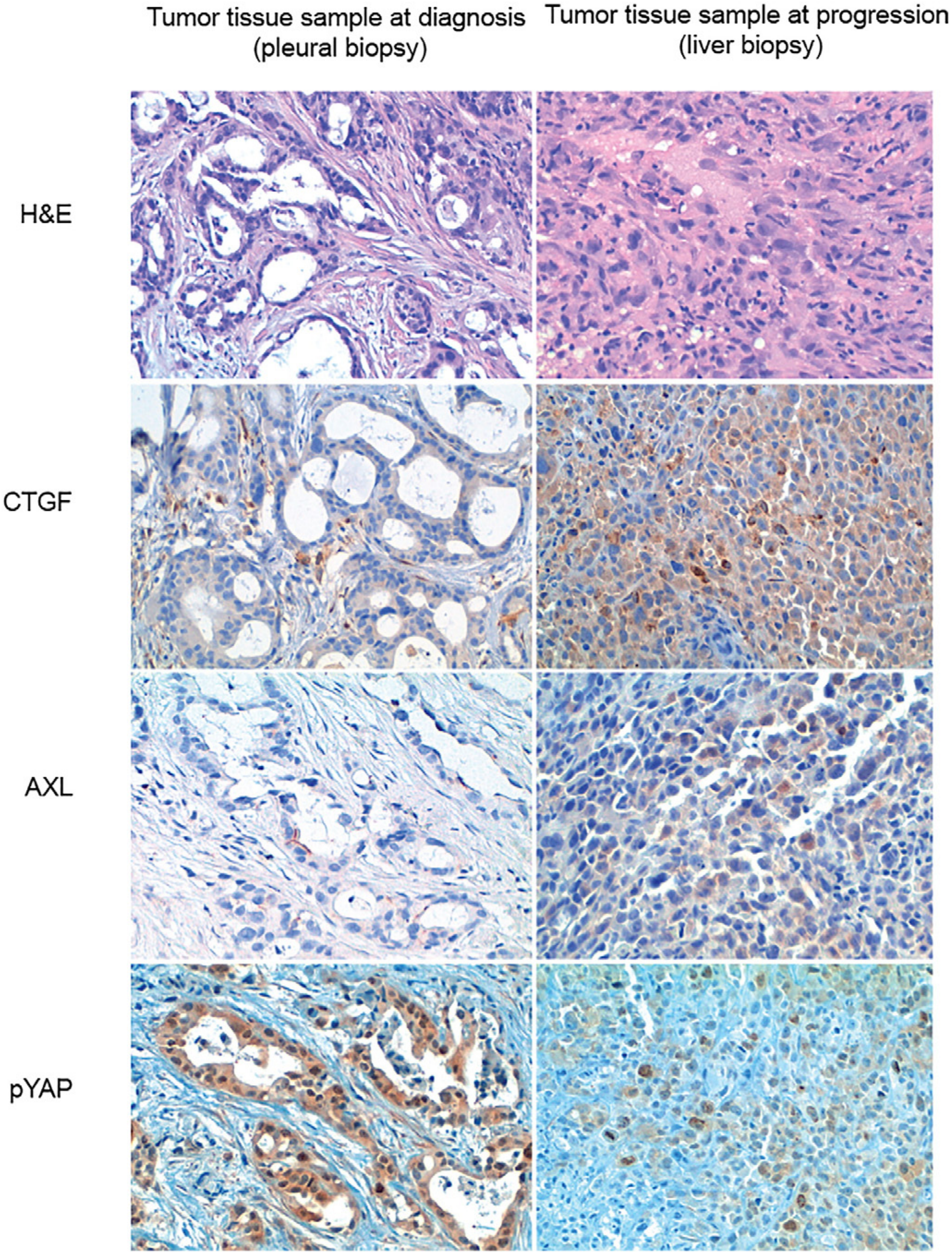

Figure 5. YAP-AXL pathway becomes active upon erlotinib resistance onset in a lung cancer patient. Immunohistochemical evaluation (immunoperoxidase) of CTGF, AXL, and pYAP expression in matched samples from a lung adenocarcinoma patient, at diagnosis (pleural biopsy, histological grade G2; left part), and at the time of progression (liver biopsy, histological grade G3) under TKI inhibitors treatment; original magnification for all figures, 200×.

than through YAP inhibition [34]. However, since, as discussed above, AXL blockage is very effective in bypassing resistance, AXL may represent a more promising actionable target for patients' treatment. A clinical trial testing the effect of cabozantinib (a multikinase inhibitor targeting also AXL) is now recruiting selected patients (NCT01639508), and the first clinical trial of the AXL selective inhibitor TP-0903 is expected to start soon (NCT02729298).

Conclusions

In conclusion, we identified YAP-driven AXL overexpression as a mechanism of resistance to EGFR TKIs in lung cancer cells. Our data add a new mechanism of resistance to EGFR TKIs and could help clinician to select the appropriate therapeutic strategy to overcome resistance to targeted treatments in cancer patients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2017.10.003.

Competing Interest

The authors declare that they have no competing interests.

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Author Contributions

E. G, C. M., and S. G. conceived the study. S. G. and E. G. designed the experiments. E. G., C. M., V. C., E. M., A. P., and S. C. performed experiments. M. V. and G. G. performed the pathological analysis. E. D. L. contributed patients' samples. S. G., E. G., and C. M. wrote the manuscript. All authors revised the manuscript.

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