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ORIGINAL ARTICLE

An activating mutation of *GNB1* is associated with resistance to tyrosine kinase inhibitors in *ETV6-ABL1*-positive leukemia

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Leukemias harboring the *ETV6-ABL1* fusion represent a rare subset of hematological malignancies with unfavorable outcomes. The constitutively active chimeric Etv6-Abl1 tyrosine kinase can be specifically inhibited by tyrosine kinase inhibitors (TKIs). Although TKIs represent an important therapeutic tool, so far, the mechanism underlying the potential TKI resistance in *ETV6-ABL1*-positive malignancies has not been studied in detail. To address this issue, we established a TKI-resistant *ETV6-ABL1*-positive leukemic cell line through long-term exposure to imatinib. *ETV6-ABL1*-dependent mechanisms (including fusion gene/protein mutation, amplification, enhanced expression or phosphorylation) and increased TKI efflux were excluded as potential causes of resistance. We showed that TKI effectively inhibited the Etv6-Abl1 kinase activity in resistant cells, and using short hairpin RNA (shRNA)-mediated silencing, we confirmed that the resistant cells became independent from the *ETV6-ABL1* oncogene. Through analysis of the genomic and proteomic profiles of resistant cells, we identified an acquired mutation in the *GNB1* gene, K89M, as the most likely cause of the resistance. We showed that cells harboring mutated *GNB1* were capable of restoring signaling through the phosphoinositide-3-kinase (PI3K)/Akt/mTOR and mitogen-activated protein kinase (MAPK) pathways, whose activation is inhibited by TKI. This alternative $GNB1^{K89M}$-mediated pro-survival signaling rendered *ETV6-ABL1*-positive leukemic cells resistant to TKI therapy. The mechanism of TKI resistance is independent of the targeted chimeric kinase and thus is potentially relevant not only to *ETV6-ABL1*-positive leukemias but also to a wider spectrum of malignancies treated by kinase inhibitors.

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INTRODUCTION

The *ETV6-ABL1* fusion represents a rare aberration, which can be found throughout a broad range of hematological malignancies. It occurs in approximately 0.2–0.4% of acute lymphoblastic leukemias (ALL) in children and adults; it is found in chronic myeloproliferation and rarely, yet recurrently, in acute myeloid leukemia.[1] To a great extent, *ETV6-ABL1*-positive leukemias resemble *BCR-ABL1*-positive malignancies with respect to disease pathogenesis and unfavorable clinical outcome. Both fusion genes result in the formation of constitutively active non-receptor tyrosine kinases, which largely overlap in their substrate preferences and have similar downstream effects regarding intracellular signaling and cell transforming capacity.[2–7]

Similar to Bcr-Abl1, Etv6-Abl1 kinase can be targeted by selective ATP-competitive tyrosine kinase inhibitors (TKI).[8,9] Currently, TKIs are routinely used in the treatment of *BCR-ABL1*-positive leukemias, and given the reported poor outcome, they represent a promising therapeutic tool also for *ETV6-ABL1*-positive malignancies.[1] However, resistance to TKIs represents a common problem in TKI-treated patients. It occurs in a considerable proportion of patients with *BCR-ABL1*-positive leukemias (both ALL and chronic myeloid leukemia) where it is most frequently caused by mutations in the *BCR-ABL1* kinase domain.[10,11] These mutations may either be acquired during (and potentially induced by) therapy or already pre-exist in a minor leukemia subclone that is selected during therapy. Depending on the type of mutation, cells resistant to the first-line therapy TKI may still be sensitive to another inhibitor.[12] In addition to kinase mutations, genomic amplification and/or enhanced expression of *BCR-ABL1* and enhanced TKI efflux resulting from the over-expression of drug exporters have also been described to cause TKI resistance.[10] Additionally, various *BCR-ABL1*-independent factors may play a role in resistance. Moreover, the resistance may theoretically result from complex or multiple simultaneous mechanisms, and at the molecular level, it still remains incompletely understood in many patients. TKIs have already been used in the treatment of several patients with *ETV6-ABL1*-positive ALL and chronic myeloproliferation.[1,13–26] Although all patients treated with TKIs as first-line therapy responded to treatment and achieved complete remission, half of them developed resistance and suffered disease recurrence.[1] Apart from the T315I mutation of *ETV6-ABL1* kinase, which was described in a single ALL patient,[25] no other mechanisms of TKI resistance have been described in *ETV6-ABL1*-positive malignancies so far.

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RESULTS

Long-term cultivation with imatinib induced multi-TKI resistance in *ETV6-ABL1*-positive leukemic cells

To investigate the potential mechanism of TKI resistance in *ETV6-ABL1*-positive leukemias, we employed a cell line-based model. The *ETV6-ABL1*-positive leukemic cell line ALL-VG was cultivated in medium supplemented with gradually increasing doses of imatinib (the most common first choice TKI in clinical practice). After long-term exposure (16 months) to imatinib, leukemic cells became drug-resistant. We used MTS assays to assess their sensitivity to a wider panel of TKIs and revealed that leukemic cells acquired resistance not only to imatinib but also to more potent TKIs such as nilotinib, dasatinib and ponatinib (Figures 1a). The multi-TKI resistance remained stable even after long-term withdrawal (16 weeks) of imatinib from the growth medium. No apparent differences in proliferation rates between the original (TKI-sensitive) and the established TKI-resistant cell lines were observed. To further study the effect of imatinib on the survival of resistant cells compared to sensitive ones, we treated both cell lines with increasing concentrations of imatinib and assessed viability 3 days post-treatment with an annexin-V/propidium iodide apoptotic assay (Figures 1b). After the first dose of imatinib, there was no clear impact on the cellular viability of both sensitive and resistant cells. The second treatment, however, significantly reduced the survival of sensitive cells at all tested concentrations. In contrast, there were no significant changes in the viability of resistant cells treated with either 0.5 or 5.0 μM imatinib, and an

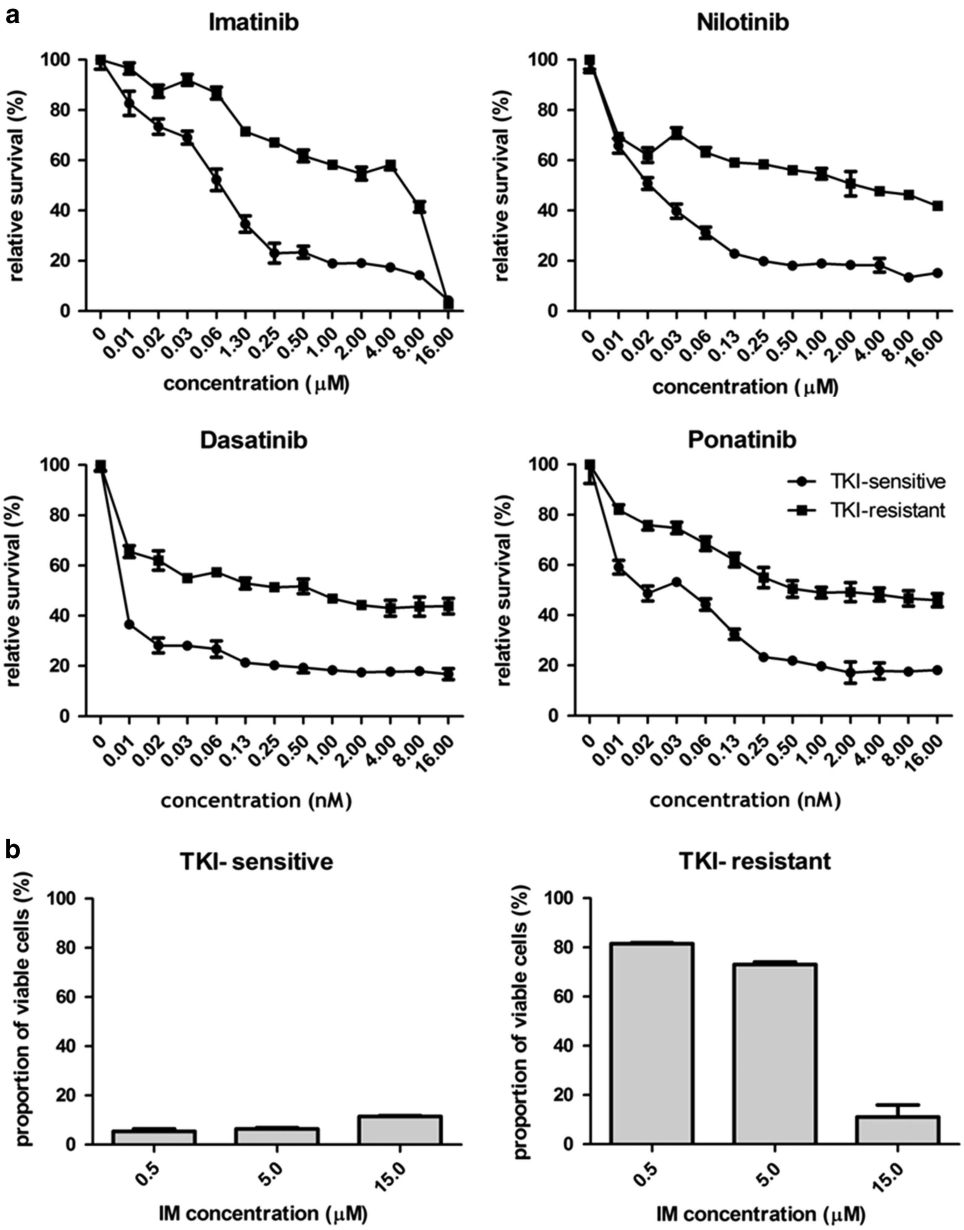

Figure 1. Sensitivity of TKI-sensitive and TKI-resistant cell lines to selected TKIs. Sensitivity to TKIs determined by MTS assay (**a**). Viability of TKI-treated cells normalized to untreated cells is shown on *y* axis. Error bars represent the standard deviation from the mean. Effect of second dose of imatinib (0.5, 5.0 and 15.0 μM) on the viability of TKI-sensitive and TKI-resistant cell lines (**b**). The percentage of viable (Annexin-V-negative, PI-negative) cells is shown on *y* axis. Experiments were performed in triplicates (**a**,**b**).

apparent increase of apoptosis was observed only for the highest drug concentration, 15 μM, which significantly exceeds imatinib serum levels in treated patients[27] and most likely induces off-target cytotoxic effects.

TKI-resistant cells became independent from pro-survival signaling from the Etv6-Abl1 chimeric kinase

To reveal the mechanism of the TKI resistance, we first focused on the most common mechanisms that were previously described in *BCR-ABL1*-positive leukemias. We did not find any mutation within the *ETV6-ABL1* fusion gene (data obtained by whole exome sequencing). Using single-nucleotide-polymorphism (SNP) array and western blot, we excluded *ETV6-ABL1* amplification and an increased expression resulting in increased kinase activity and thus increased autophosphorylation of the encoded chimeric kinase as potential causes for the TKI resistance in our model (Figure 2a).

Next, we studied and compared the impact of imatinib on Etv6-Abl1-triggered signaling in sensitive and resistant cells. It has been previously described that the kinase activation results in autophosphorylation and phosphorylation of downstream substrates including the Crkl adaptor protein, a direct substrate of the chimeric kinase. Analysis by western blot showed that imatinib diminished the phosphorylation of Etv6-Abl1 and Crkl, both in sensitive and in resistant cells (Figures 2a, Supplementary Figure 3). These results demonstrated that imatinib effectively inhibited the kinase activity of Etv6-Abl1 in resistant cells, and therefore, the decreased intracellular drug availability was excluded as a potential mechanism of the TKI resistance. Moreover, these findings strongly implicated the independence of resistant cells from the *ETV6-ABL1* oncogene.

To test this hypothesis, we permanently transduced sensitive and resistant cells with *ETV6-ABL1*-targeting shRNA vectors. While *ETV6-ABL1* knock-down induced proliferation arrest and apoptosis in TKI-sensitive cells, there were no changes in viability or proliferation of TKI-resistant cells, further confirming that the resistant cells became independent from the *ETV6-ABL1* oncogene (Figures 2b, Supplementary Figure 4).

TKI-resistant cells acquired multiple genomic aberrations including the *GNB1* K89M mutation

To further elucidate the molecular basis of the acquired TKI resistance, we compared genome-wide molecular profiles of the sensitive and resistant cell lines using SNP array and whole exome sequencing. Using high-density genome-wide SNP arrays we identified a single acquired copy number alteration in the resistant cell line consisting of a 60 kilobase-long intragenic deletion in the *KDM6A* gene (Supplementary Figure 5), which encodes a lysine-specific histone demethylase. However, subsequent analysis by western blot did not confirm the expression of the predicted aberrant protein from the affected *KDM6A* allele.

Whole exome sequencing revealed a gain of 36 non-synonymous single nucleotide variants (SNV) with variant allele frequency ⩾ 20% in the resistant cell line (Supplementary Table 2).

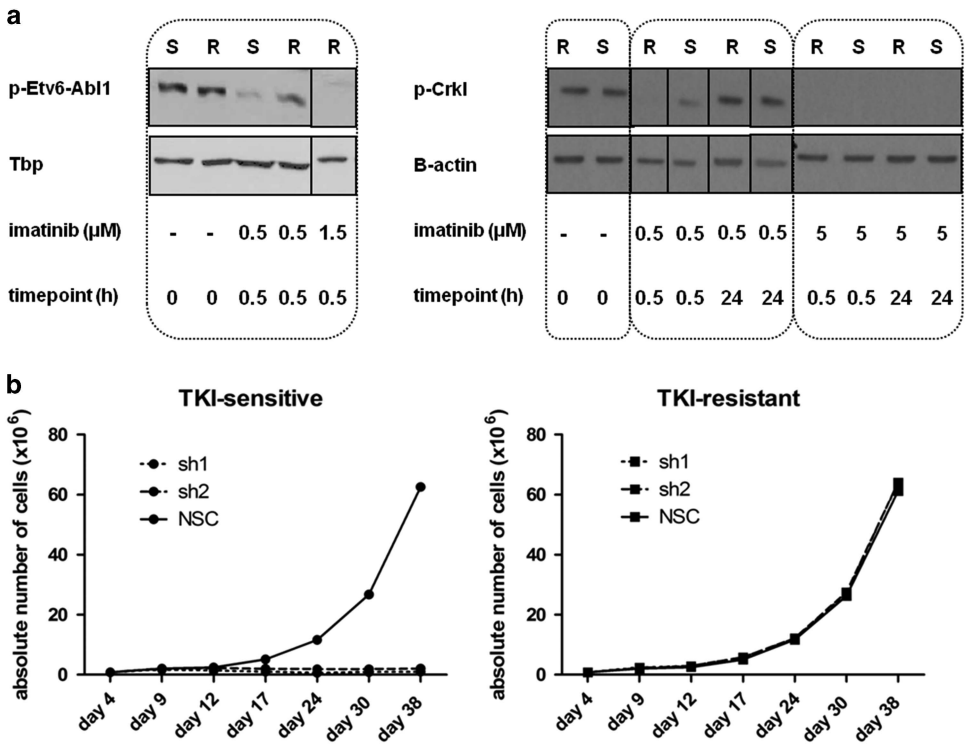

Figure 2. TKI-resistant cells gained independence from the *ETV6-ABL1* oncogene. Effect of imatinib treatment on phosphorylation of Etv6-Abl1 kinase and its downstream target Crkl was analyzed via western blot (**a**). TKI-sensitive (S) and TKI-resistant (R) cells were grown in the absence/presence of 0.5, 1.5 or 5.0 μM imatinib for 30 min and 24 h. Level of phosphorylated (p) Etv6-Abl1 and Crkl was determined in nuclear protein and whole cell lysates, respectively. Tbp and B-actin were used as loading controls. Scans were cut and reorganized to show timepoints followed within the experiment in left to right order. Original scans are shown in Supplementary Figure 3. Effect of shRNA-mediated *ETV6-ABL1* silencing on proliferation of TKI-sensitive and TKI-resistant cells (**b**). Dashed and dotted lines indicate proliferation of cells transduced with *ETV6-ABL1*-targeting and *ETV6*-targeting shRNAs. Cells transduced with negative control shRNA are represented by solid line. Experiments with two different shRNA represent an independent replicate. Results of additional independent experiment with the same two shRNAs are shown in Supplementary Figure 4.

Based on expression in ALL VG cell line and functional annotation of affected genes, we selected seven SNVs (as the most likely resistance-causing lesions) for further verification. Six of seven SNVs were confirmed by Sanger sequencing and were shown to be present at the genomic and transcriptomic level in all tested samples from different passages of resistant cells: *GNB1* K89M, *STAM2* M297L, *AEBP1* Q551H, *KMT2C* I467V, *RREB1* Q399L and *CIITA* G123R.

TKI-resistant cells expressed altered transducin beta chain 1 protein

We performed a differential proteomic analysis of sensitive and resistant cells using two-dimensional electrophoresis in polyacrylamide gel (2-DE). On average we observed 975 protein spots per sample; however, we identified only a single differential spot between sensitive and resistant cells; this spot was present in resistant cells while absent in sensitive cells (Figure 3). Mass spectrometry identified this protein spot as the transducin beta chain 1 (Gβ). This protein is encoded by the *GNB1* gene, which was shown to acquire the K89M mutation in resistant cells (as described above).

TKI-resistant cells were capable of restoring PI3K/Akt/mTOR and MAPK signaling after imatinib treatment

Together with Gα and Gγ, Gβ forms heterotrimeric G proteins that play a pivotal role in cellular signaling downstream of G protein-coupled receptors. In light of our results from genomic and proteomic profiling, we considered a mutation in *GNB1* as the most likely cause of TKI resistance in our model. Moreover, during our study, it was demonstrated that mutations affecting particular codons of *GNB1* (including codon 89, which was mutated in our resistant cell line) alter G protein-mediated signaling and disturb signaling via the phosphoinositide-3-kinase (PI3K)/Akt/mTOR and mitogen-activated protein kinase (MAPK) pathways.[28] Thus, we examined and compared the activity of these pathways after imatinib treatment in sensitive and resistant cells. While treatment with 5 μM imatinib induced prolonged inhibition of the PI3K/Akt/mTOR and MAPK pathways in sensitive cells, resistant cells harboring the *GNB1* mutation were able to restore the signaling 24 h after treatment (Figure 4, Supplementary Figure 6).

$GNB1^{K89M}$ altered PI3K/Akt/mTOR and MAPK signaling and conferred TKI resistance on *ETV6-ABL1*-positive leukemic cells

As described above, additional genetic changes, other than *GNB1* mutation, were present in the resistant cells; to elucidate the capability of $GNB1^{K89M}$, as a single aberration, to induce the resistant phenotype, we introduced the mutated *GNB1* into TKI-sensitive *ETV6-ABL1*-positive cells using a lentiviral vector. In concordance with the previous 2-DE findings in TKI-resistant cell line, both mutated (but not wild type) Gnb1 proteins appeared as distinct differential spots on 2-DE gels due to the mutation-introduced shift in Gnb1 isoelectric point (Supplementary Figure 7).

We studied the impact of $GNB1^{K89M}$ on TKI-sensitivity and kinase signaling and compared it to the effect of $GNB1^{K89E}$, which was previously described to activate kinase signaling.[28] Similar to the imatinib-induced TKI-resistant cell line, the originally sensitive cells became resistant to imatinib, nilotinib, dasatinib and ponatinib when transduced with $GNB1^{K89M}$ or with $GNB1^{K89E}$; in contrast, the sensitive cells transduced with $GNB1^{WT}$ or the empty vector did not became resistant to the aforementioned drugs (Figures 5a). We next studied the viability of transduced cells and their ability to proliferate upon continuous cultivation in the presence of imatinib. In contrast to the cells transduced with $GNB1^{WT}$, which showed significant proliferation arrest and apoptosis upon cultivation with imatinib, cells transduced with $GNB1^{K89M}$ or with $GNB1^{K89E}$ continued to proliferate (Figure 5b) and displayed only a subtle decrease in viability. Furthermore, while the signaling through the PI3K/Akt/mTOR and MAPK pathways was inhibited by imatinib at both early and later time points in cells transduced with $GNB1^{WT}$ or with empty vector, signaling was restored 24 h after treatment in cells transduced with $GNB1^{K89M}$ or with $GNB1^{K89E}$ (Figure 6, Supplementary Figures 8–10), proving that $GNB1^{K89M}$ induces TKI resistance in *ETV6-ABL1*-positive leukemic cells based on alternative activation of pro-survival signaling.

TKI-resistant cells are sensitive to the dual PI3K/mTOR inhibitor

Pro-survival signaling in TKI-resistant cells could be theoretically blocked by inhibitors acting downstream of mutated *GNB1*. Using MTS assay we assessed and compared the sensitivity of TKI-sensitive, TKI-resistant and lentivirally transduced cells to dual PI3K/mTOR inhibitor, BEZ235. All cell lines were comparably sensitive to nanomolar concentrations of BEZ235 (Figure 7).

DISCUSSION

In this study, we used an *ETV6-ABL1*-positive cell line to investigate the resistance to TKIs that may arise as a result of targeted TKI therapy in patients with *ETV6-ABL1*-positive hematological malignancies.

In our cell line, the resistance was induced by long-term culture with a TKI, implying that it was acquired secondarily. Applying complex genomic approaches, we found that resistant cells had gained several genetic aberrations. Taking into account the results from genomic and proteomic profiling, we focused on the K89M mutation of the *GNB1* gene and proved that it is capable of inducing TKI resistance in *ETV6-ABL1*-positive leukemic cells. We showed that $GNB1^{K89M}$ activated the signaling through the PI3K/Akt/mTOR and MAPK pathways when their Etv6-Abl1-mediated activation was blocked by a TKI. Thus, the mutated *GNB1* provided an alternative pro-survival mechanism and conferred independence from the *ETV6-ABL1* oncogene.

The molecular mechanism through which the mutated *GNB1* alters intracellular signaling was recently described by Yoda *et al*.[28] Physiologically, the ligand-induced stimulation of G protein-coupled receptors triggers the dissociation of the G protein heterotrimer into two functional molecules, the Gα subunit and the Gβγ heterodimer, which further mediate signal transduction. The substitution of lysine (K) 89 with a different amino acid (for example, glutamic acid (E) or methionine (M)), alters the interaction surface of the β subunit, which retains the ability to interact with the γ subunit but interacts less efficiently with the α subunit. Consequently, the downstream signaling is disturbed.

Interestingly, immediately after TKI treatment, we observed a comparable inhibition of the intracellular signaling in both sensitive and resistant cells. These data demonstrate that in our TKI-resistant cells the pro-survival pathways were perhaps primarily activated by Etv6-Abl1, and mutated *GNB1* fully exerted its activation potential only when Etv6-Abl1 was 'switched off' by TKI treatment.

Recently, we showed that the prognosis of *ETV6-ABL1*-positive malignancies is poor, especially in adults who may be affected not only by ALL but also by AML or chronic myeloproliferation. Because of the low frequency of this fusion and because it has been identified only after disease progression or even retrospectively in some patients, the number of TKI-treated patients with *ETV6-ABL1*-positive leukemia is insufficient to reliably analyze whether these patients benefit from TKI treatment. Nevertheless, the poor response of the majority of these patients to standard treatment without TKIs and the proven efficacy and safety of respective TKIs in the closely related *BCR-ABL1*-positive malignancies strongly suggest that TKI use in *ETV6-ABL1*-positive

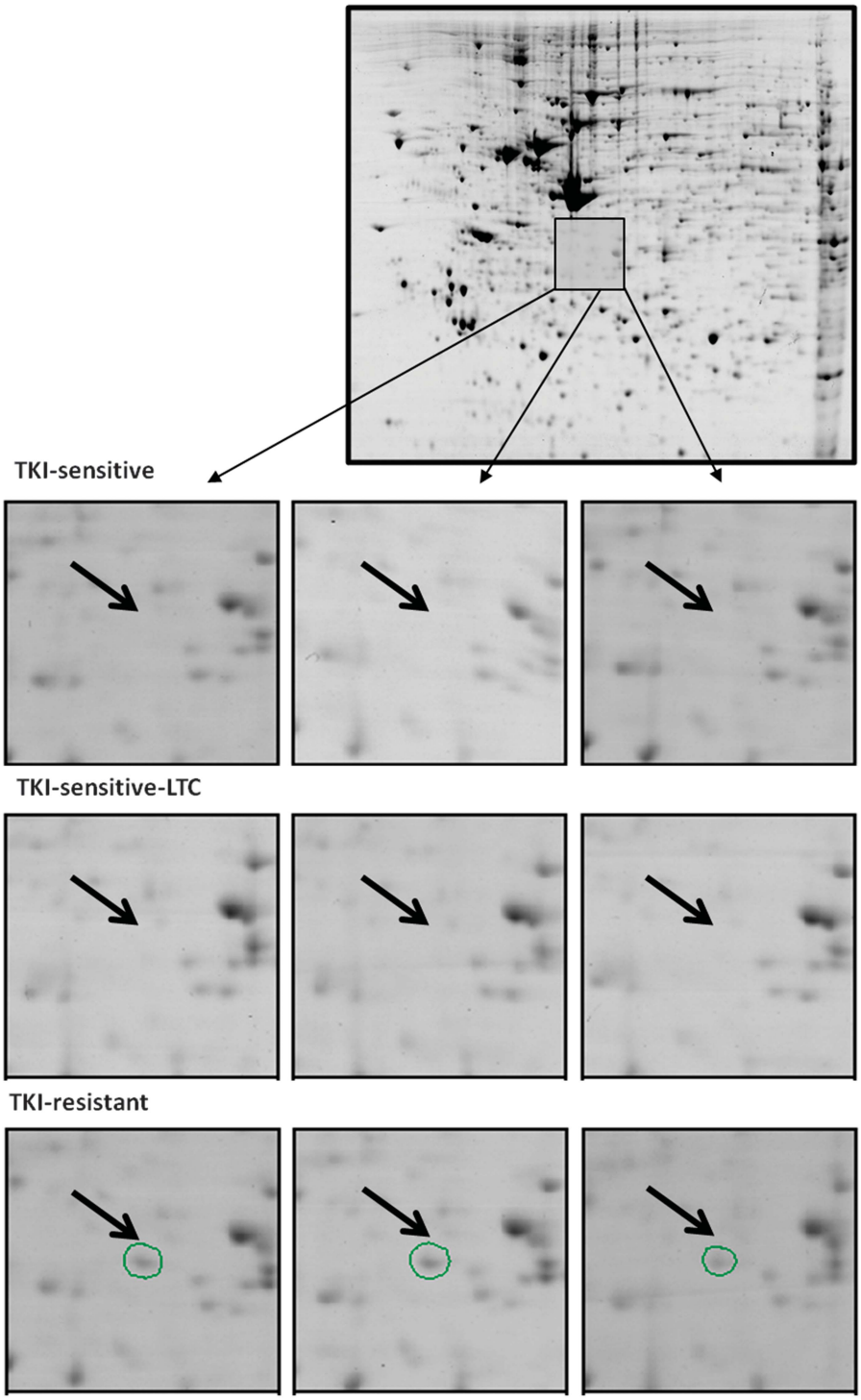

Figure 3. 2-DE based proteomic analysis of TKI-sensitive, TKI-sensitive-LTC and TKI-resistant cell line. A representative 2-DE gel with 975 visualized proteins spots (at the top) with close-ups of the area with a differential GNB1 spot (below). Close-ups of scanned 2-DE gels—black arrow indicates the position of a differential protein spot (identified by mass spectrometry as Gβ), which was detected exclusively in TKI-resistant cells. A representative triplicate of experimental hexaplicate.

leukemias should be considered by treating physicians. Two out of five patients with *ETV6-ABL1*-positive myeloproliferation, treated with TKIs during the chronic stage of the disease, relapsed; both achieved a second complete remission after switching to a different TKI, hence suggesting a kinase domain mutation as a probable cause of the resistance. The assessment of the role of TKI-resistance in ALL recurrence is difficult when TKIs are administered simultaneously with a complex chemotherapeutic regime. However, chimeric kinase mutations were found in the majority of *BCR-ABL1*-positive ALL relapses,[29] thus indicating their

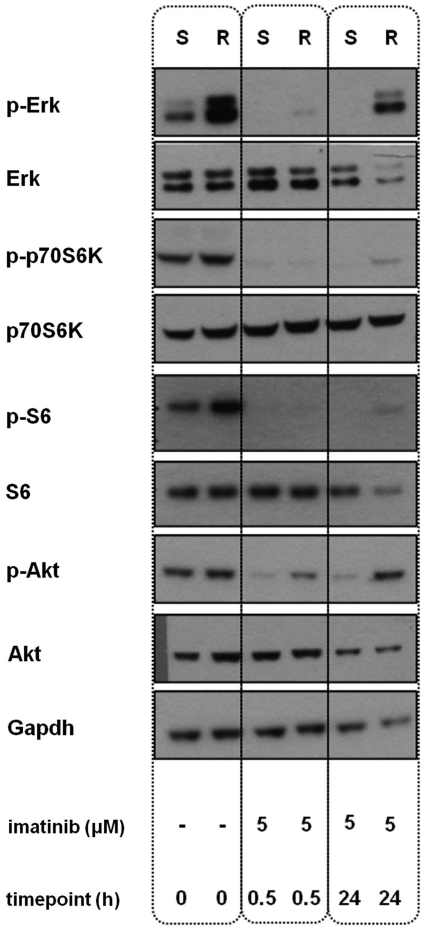

Figure 4. Effect of imatinib treatment on the activation of the PI3K/Akt/mTOR and MAPK pathways in TKI-sensitive and TKI-resistant cells. TKI-sensitive and TKI-resistant *ETV6-ABL1*-positive cells were cultured in the absence/presence of 5.0 μM imatinib for 30 min or 24 h. Levels of total and phosphorylated (p) Erk, p70S6K, S6 and Akt proteins in whole-cell lysates were determined using western blot. Gapdh was used as a loading control. Representative data of three independent experiments. Results of the two additional independent experiments are shown in Supplementary Figure 6.

role in disease progression. Notably, a T315I mutation was recently identified in the relapse of a TKI-treated adult patient with *ETV6-ABL1*-positive ALL,[25] and mutations in fusion genes were described as a mechanism for TKI-resistance in *in vitro* models of *RANBP2-ABL1*-positive and *PAX5-JAK2*-positive ALL.[30]

Although these data suggest that the mutation of fusion kinases is a frequent mechanism in TKI resistance, we did not detect any mutation in *ETV6-ABL1,* even after long-term exposure of our resistant leukemic cell line to therapeutically relevant doses of TKI; instead, cells developed an *ETV6-ABL1*-independent TKI resistance. Importantly, contrary to kinase mutation-mediated TKI resistance, the *GNB1*^{K89M}-mediated TKI resistance described here cannot be overcome by switching to a different *ETV6-ABL1*-targeting TKI; in this case, the downstream pro-survival signaling needs to be inhibited instead.

Activating mutations of *GNB1* and *GNB2* were first identified in a rare subtype of acute leukemia, blastic plasmacytoid dendritic cell neoplasm, and subsequently found in samples from patients with various hematological malignancies (including lymphomas and acute and chronic leukemias of both lymphoid and myeloid origin) and melanoma.[28] Notably, the *GNB1*^{K89E} mutation was found in *BCR-ABL1*-positive ALL with a poor response to TKIs; the *GNB2*^{K87E} mutation was found in *BRAF*-mutated melanoma, and it conferred resistance to a Braf inhibitor in an experimental setting.[28] Moreover, a recently published genomic study identified *GNB1* mutations, albeit with a low frequency (2–3%), in pretreated samples of patients with B-cell precursor ALL.[31] Thus, activating mutations of *GNB1* (and perhaps *GNB2*) might not only be newly acquired during (and potentially induced by) therapy, as we observed in our model, but they might also already be present at the time of disease manifestation before therapy administration.

To conclude, we described here a novel, fusion kinase-independent mechanism of TKI-resistance resulting from an activating mutation of *GNB1* that may be potentially induced by TKI treatment in *ETV6-ABL1*-positive malignancies. Although their frequency still needs to be assessed, activating mutations of *GNB1* (and *GNB2*) may confer both primary and secondary clinically relevant resistance to various kinase inhibitors in a wide spectrum of human cancers.

MATERIALS AND METHODS

Cell lines

The ETV6-ABL1-positive B-cell precursor leukemic cell line ALL-VG[32] was grown in X-VIVO 15 Chemically Defined Serum-free Hematopoietic Cell Medium (Lonza, Basel, Switzerland) with 3 mM L-Glutamine (Lonza) and antibiotics/antimycotics (Thermo Fisher Scientific, Waltham, MA, USA). The established TKI-resistant ALL-VG cell line was cultivated in the same medium supplemented with 1.5 μM imatinib (Glivec; Novartis, Basel, Switzerland). The HEK293T (human embryonic kidney carcinoma) and NIH3T3 (human embryonic fibroblasts) cell lines were grown in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific) with 10% fetal bovine serum (Biowest, Nuaillé, France) and antibiotics/antimycotics.

Establishment of the TKI resistant cell line

The ALL-VG cell line was cultivated in the presence of gradually increasing doses of imatinib (starting from 0.05 μM). After 16 months, the cells became resistant and grew exponentially in the presence of 1.5 μM imatinib (Supplementary Figure 1).

Long-term cultivation of ALL-VG cell line

In parallel to ALL-VG cell line which was exposed to imanitinib to induce TKI resistance (as described above), another ALL-VG cell culture without TKI was continuously grown and passaged for the same time period (16 months). Unlike ALL-VG cells exposed to imatinib, these cells remained TKI-sensitive (Supplementary Figure 2) and are referred as long-term cultured TKI-sensitive cell line (TKI-sensitive-LTC).

Drug sensitivity tests

Cells were seeded in 96-well plates (15 000 cells per well) and exposed to graded concentrations of drugs. After 3 days, cell viability was assessed using a tetrazolium-based MTS assay (CellTiter Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA). For each drug concentration, cell viability was assessed (at least) in triplicate and normalized to the average of control wells, containing no drug. Imatinib, nilotinib and dasatinib were obtained from Novartis; ponatinib was bought from SYNkinase (Parkville, Australia); BEZ235 was bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

shRNA constructs

Two custom-designed shRNAs were used to silence *ETV6-ABL1*: shRNA1 (5′-GAATAGCAGAAGCCCTTCAGCTCAAGAGGCTGAAGGGCTTCTGCTATTC-3′), which targets the *ETV6-ABL1* junction, and shRNA2 (5′-CAGGTGATGTGCTCT ATGAACTCAAGAGGTTCATAGAGCACATCACCTG-3′), which targets exon 4 of *ETV6* (and, thus, it targets *ETV6-ABL1* and potentially also wild-type *ETV6*, which, however, is deleted in the ALL-VG cell line); shRNA (5′- ATC TCGCTTGGGCGAGAGTAATCAAGAGTTACTCTCGCCCAAGCGAGAT-3′), which does not target any human gene, was used as a negative control. All shRNAs were cloned into the pGhU6 lentiviral vector containing the *GFP* gene.

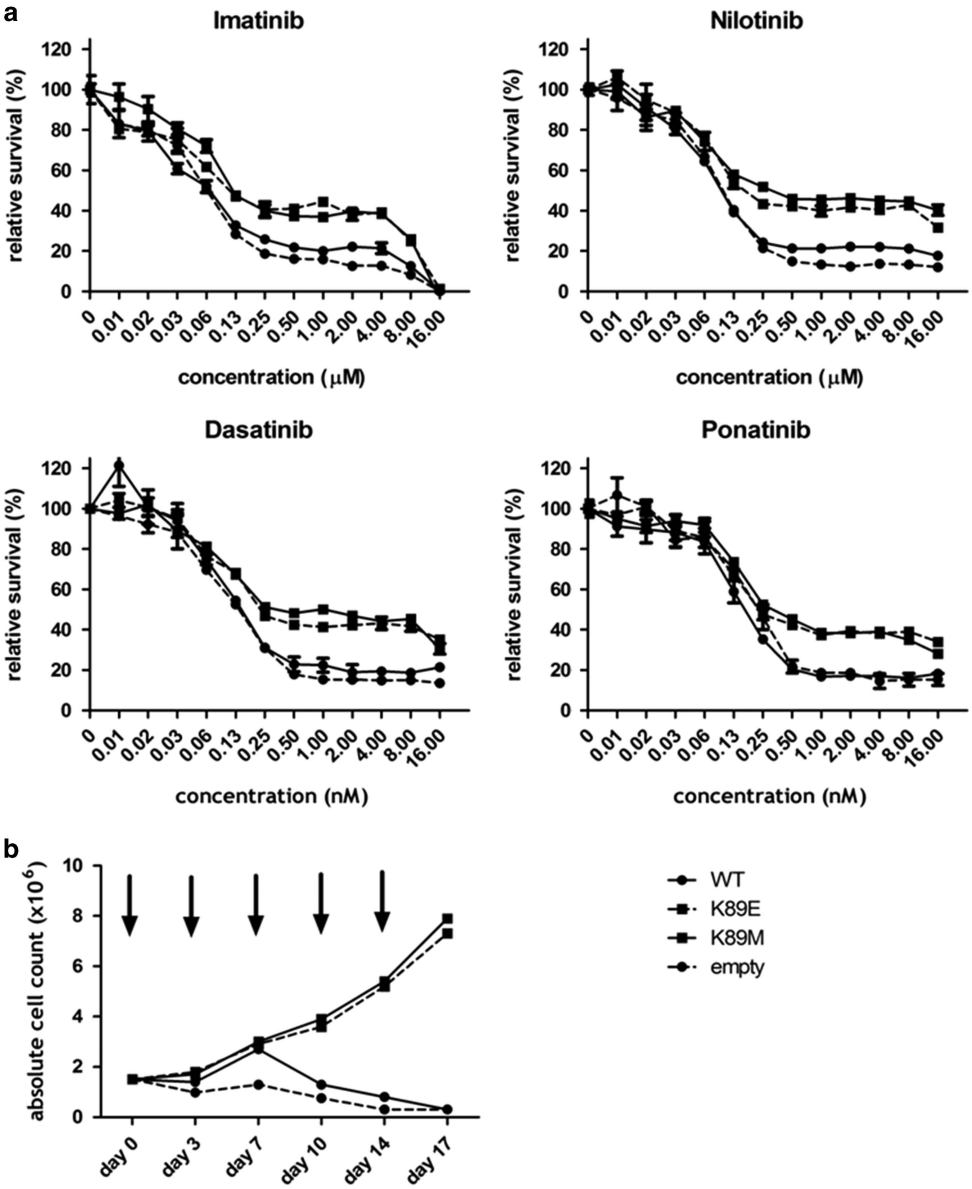

Figure 5. *GNB1*^{K89M}/*GNB1*^{K89E} mutations confer multi-TKI resistance in the TKI-sensitive *ETV6-ABL1*-positive cell line. TKI-sensitive *ETV6-ABL1*-positive cells were transduced with *GNB1*^{wt}/*GNB1*^{K89M}/*GNB1*^{K89E} and empty vector. Sensitivity of transduced cells to TKIs was determined via MTS assay (**a**). Tested concentrations of TKIs are shown on *x* axis. Viability of TKI-treated cells normalized to untreated cells is shown on *y* axis. Experiments were performed in triplicates. Error bars represent the standard deviation from the mean. Proliferation of transduced cells upon repeated treatment with 1.5 μM imatinib (**b**). Black arrows indicate imatinib administration. Absolute number of cells is shown on *y* axis.

GNB1 constructs

The full-length coding sequences of wild-type (*GNB1*^{WT}) and mutant (*GNB1*^{K89M}) *GNB1* were amplified from complementary DNA (cDNA), which was prepared from the resistant cell line, using a PCR Extender System (Thermo Fisher Scientific). The PCR products were cloned into the pWCC19_GFP lentiviral vector containing the *GFP* gene using the InFusion HD Cloning Kit (Clontech, Mountain View, CA, USA), according to the manufacturer's instructions. *GNB1*^{K89E} was generated from *GNB1*^{WT} using the Quick Change Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. All constructs were sequenced using the Sanger method.

Production of lentiviral particles

The lentiviral particles were produced by HEK293T cells. Cells were grown in 15 cm^2 plates to achieve 80–90% confluence and co-transfected with the p-gag-pol (12 μg), p-VSV-G (1.4 μg) and individual *GNB1*/shRNA vectors (15 μg) using Lipofectamine 2000 (85 μl, Thermo Fisher Scientific). The supernatant, containing viral particles, was collected 72 and 96 h after transfection. Viral particles were concentrated using Centricon Plus 70 Centrifugal Filter Units (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. The titer of the produced virus (multiplicity of infection value) was assessed in the NIH3T3 cell line using flow cytometric detection of the *GFP* fluorescence.

Cell transduction

Two-and-a-half million ALL-VG cells were resuspended in 2 ml of growth medium containing lentiviral particles (multiplicity of infection value 20×), centrifuged at 500 *g* for 100 min at room temperature and subsequently incubated for 4 h at 37 °C. Following the incubation, cells were centrifuged, washed in phosphate-buffered saline and resuspended in 2.5 ml fresh

growth medium. Successfully transduced cells were isolated via fluorescence activated cell-sorting 48 h after transduction according to their *GFP*-positivity.

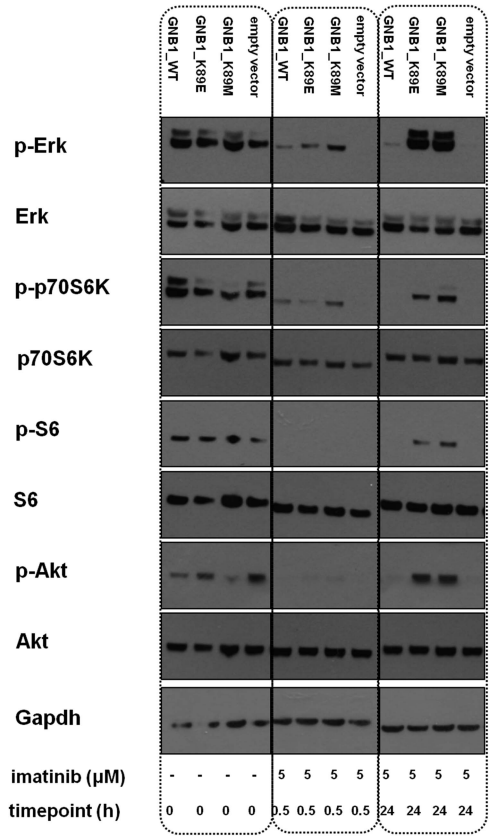

Figure 6. Effect of the *GNB1*^{K89M}/*GNB1*^{K89E} mutations on the activation of the PI3K/Akt/mTOR and MAPK pathways. TKI-sensitive *ETV6-ABL1*-positive cells were transduced with *GNB1*^{wt}/*GNB1*^{K89M}/*GNB1*^{K89E} and empty vector. Transduced cells were treated with 5.0 μM imatinib for 30 min and 24 h. Levels of total and phosphorylated (p) Erk, p70S6K, S6 and Akt proteins in whole-cell lysates were determined using western blot. Gapdh was used as a loading control. Representative data of three independent experiments. Scans were cut and reorganized to show timepoints followed within the experiment in left to right order. Original scans are shown in Supplementary Figure 8. Results of two additional independent experiments are shown in Supplementary Figures 9 and 10.

Western blot analysis

Whole cell protein lysates were prepared using RIPA buffer. Nuclear protein lysates were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) supplemented with Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). Proteins (20–60 μg per well) were resolved by 7% SDS–polyacrylamide gel or Bolt 4–12% Bis-Tris protein gels (Thermo Fisher Scientific) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Non-specific binding was blocked with phosphate-buffered saline containing 5% dry milk for 1 h. The membrane was probed overnight with the chosen primary antibody. Antibodies against phosphorylated (phospho-) Erk T202/Y204 (#4370), phospho-Akt S473 (#4060), phospho-P70S6K T389 (#9234), phospho-S6 S235/236 (#4858), phospho-Abl Y245 (#2861) and phospho-Crkl Y207 (#3181) were purchased from Cell Signaling Technology (Danvers, MA, USA) and used at a 1:1000 dilution. The antibody against Abl was purchased from Abcam (Cambridge, UK) and was used at a 1:200 dilution. Antibodies against GAPDH (1:10 000 dilution, Abcam), B-actin (1:3000 dilution; Sigma-Aldrich, St Louis, MO, USA), Tbp (1:200 dilution, Abcam) and Lamin B (1:200 dilution; Santa Cruz Biotechnology) were used as protein-loading controls. Primary protein-bound antibodies were visualized using appropriate secondary antibodies (Bio-Rad) conjugated with horseradish peroxidase and the Super Signal West Pico or Super Signal West Femto Chemiluminescent Substrate kits (Thermo Fisher Scientific).

Monitoring of proliferation and viability

Cells were counted every 2–3 days using a Cell Countess Automated Counter (Beckman-Coulter, Brea, CA, USA). Trypan blue staining was used to identify dead cells. In selected experiments, the absolute number of cells in a defined volume was determined via flow cytometry using an LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) and AccuCount Spherotech particles (Spherotech Inc., Lake Forest, IL, USA) according to the manufacturer's instructions. Dead cells were identified using the DNA-intercalating fluorochrome DAPI (Thermo Fisher Scientific). Apoptotic cells were identified using Annexin-V-Dyo647 (Exbio, Vestec, Czech Republic) and Propidium Iodide (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The FlowJo software (Tree Star, Ashland, OR, USA) was used to analyze flow cytometry data.

Single nucleotide polymorphism array

Copy number aberrations (CNA) and regions of uniparental disomy (UPD) were identified using HumanOmni Express BeadChip (Illumina, San Diego, CA, USA). DNA labeling and hybridization were performed according to the Infinium HD assay Ultra protocol from Illumina. The GenomeStudio software v2011.1 (Illumina) was used for genotype calling and quality control. Copy number variations and uniparental disomy were called using the copy number variations Partition 2.4.4 algorithm plug-in within the GenomeStudio software. The resulting data (Log R ratio corresponding to copy number and B allele frequency corresponding to SNP genotype) were visually inspected in the Illumina Chromosome Browser and manually

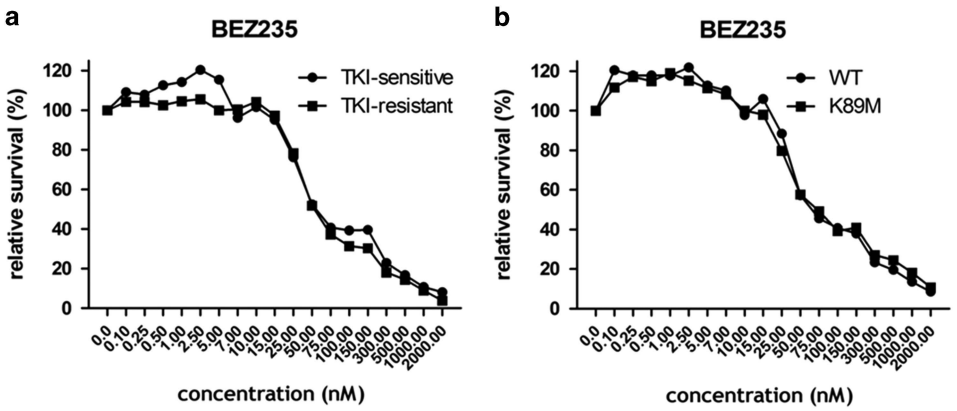

Figure 7. Sensitivity to dual PI3K/mTOR inhibitor BEZ235. Sensitivity of TKI-sensitive and TKI-resistant cells (**a**) and TKI-sensitive cells transduced with *GNB1*^{WT} and *GNB1*^{K89M} (**b**) to BEZ235. Sensitivity was determined by MTS assay. Viability of treated cells normalized to untreated cells is shown on *y* axis. Experiments were performed in triplicates. Error bars represent the standard deviation from the mean.

curated. The deletions corresponding to somatic rearrangements of the immunoglobulin and T-cell receptor gene loci and germline copy number variations/uniparental disomy (present in remission samples) were excluded.

Whole exome sequencing

Whole exome sequencing was performed at ATLAS Biolabs GmBH (Berlin, Germany). The libraries were prepared using the NimbleGen SeqCap EZ Human Exome Library v2.0 Kit (NimbleGen, Madison, WI, USA). Sequencing was performed on a HiSeq2000 (Illumina) instrument. Data were analyzed using an in-house pipeline, as described previously.[33] Briefly, read pairs were aligned to the human genome reference sequence (hg19) using BWA aligner and further processed using Picard tools (http://broadinstitute.github.io/picard/). Variant calling was performed using the VarScan algorithm. Single nucleotide variants and indels were visually inspected using the Integrative Genomic Viewer (IGV, Broad Institute).

Quality control and post-bioinformatic analysis of whole exome sequencing data

Data quality was checked by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Qualimap[34] (see selected data metrics in Supplementary Table 1). To identify mutations newly acquired and specific for TKI-resistant cell line, SNVs and insertions/deletions (indels) were filtered using the following algorithm:

- Variants in non-coding regions and synonymous variants in coding regions were excluded.
- Variants at positions with low sequencing depth in TKI-sensitive cell line were excluded.
- Variants detected in TKI-sensitive and/or TKI-sensitive-long-term-cultured (TKI-sensitive-LTC) cell lines were excluded except of those supported only by single read or those supported by two reads and having allele frequency $\leqslant 2\%$.
- Variants with allele frequency $< 20\%$ in TKI-resistant cell line were excluded (subclonal variants).

SNV verification

Total RNA was extracted using the RNeasy Mini Plus Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad). The target sequences were amplified using in-house designed primers and HotStarTaq Master Mix (Qiagen). The PCR products were sequenced by the Sanger method using a 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were analyzed using the Chromas 2.4 software.

2-dimensional gel electrophoresis (2-DE)

The proteomic profiles were analyzed via 2-DE, which was performed as described previously.[35] Stained gels were scanned using a GS800 Calibrated Densitometer (Bio-Rad). Scans were analyzed using the Progenesis PG220 software (Nonlinear Dynamics). Differential protein spots were digested with trypsin overnight at 37 °C. Extracted peptides were analyzed with a MALDI –TOF Autoflex II mass spectrometer (Bruker Daltonics, Bremen, Germany) using a solid nitrogen laser (337 nm) and the FlexControl software in reflectron mode with positive ion mass spectra detection. Alpha-cyano-4-hydroxycinnamic acid was used as the matrix. The peak lists were generated using the FlexAnalysis software and searched against the Swiss-Prot database using the Mascot software. The following parameters were set as indicated: peptide mass tolerance, 100 ppm; taxonomy, *Homo sapiens*; missed cleavage, 1; fixed modification, cysteine carbamidomethylation; and variable modifications, methionine oxidation and protein N-terminal acetylation. Proteins with a Mascot score over the threshold 56 for $P < 0.05$ calculated for the chosen settings were considered as identified.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

OZ, ED, VK and DK performed experiments; OZ, ED, JStu, VK, DK, HS, JSta, MAJ, JP, JHFF, JT, JZ and MZ designed experiments and analyzed data; MZ designed and led the study; OZ and MZ wrote the manuscript. All authors revised the manuscript and approved the final version.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)