BTK acts as a modulator of the response to imatinib in chronic myeloid leukemia

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Abstract. The use of tyrosine kinase inhibitors, such as imatinib, against the chronic myeloid leukemia (CML)-causing kinase BCR::ABL1 has become the model for successful targeted therapy. Nevertheless, drug resistance remains a clinical problem. Analysis of genome-wide expression and genetic aberrations of an *in vitro* imatinib-resistant CML cell line revealed downregulation of Bruton's tyrosine kinase (*BTK)*, predominantly associated with B cell malignancies, and a novel *BTK* kinase domain variant in imatinib resistance. This raised the question of the role of *BTK* in imatinib-resistant CML. In the present study, *BTK* downregulation and the presence of the *BTK* variant c.1699_1700delinsAG p.(Glu567Arg) were confirmed in imatinib resistance *in vitro*. Similarly, BTK inhibition or small interfering RNA-mediated *BTK* knockdown reduced imatinib susceptibility by 84 and 71%, respectively. *BTK* overexpression was detrimental to CML cells, as proliferation was significantly reduced by 20.5% under imatinib treatment. In addition, *BTK* rescue in imatinib-resistant cells restored imatinib sensitivity. The presence of the *BTK* p.(Glu567Arg) variant increased cell numbers (57%) and proliferation (37%) under imatinib exposure. These data demonstrate that *BTK* is important for the development of imatinib resistance in CML: Its presence increased drug response, while its absence promotes imatinib resistance. Moreover, the *BTK* p.(Glu567Arg) variant abrogates imatinib sensitivity. These findings demonstrate a context-dependent role for *BTK* as an oncogene in B cell malignancies, but as a tumor suppressor in other neoplasms.

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Introduction

The use of tyrosine kinase inhibitors (TKIs), i.e., imatinib, in the hematological malignancy chronic myeloid leukemia (CML) is the most prominent model for successful targeted therapy. This drug targets the disease-causing, constitutively active BCR::ABL1 (breakpoint cluster region/Abelson tyrosine kinase 1) kinase, which results from the reciprocal t(9;22)(q34;q11) translocation forming the so-called Philadelphia chromosome and the *BCR::ABL1* fusion gene (1-3). With a five-year survival rate of 83%, TKI treatment of CML is tremendously successful, however, the handling of CML has become more complex due to the development of TKI resistance (4,5). About half of the TKI-resistant CML patients acquire sequence variants in *BCR::ABL1* that prevent TKI binding and sufficient inhibition of downstream target phosphorylation (6-9). The most prominent pathogenic variant is the so-called gatekeeper mutation p.(Thr315Ile), which impairs the ATP binding pocket of BCR::ABL1 leading to therapy failure for first and second generation TKIs (10-12). In addition, the P-loop mutation p.(Gly250Glu) or the activation loop mutation p.(His396Arg) also lead to treatment failure (13). For the other half of TKI-resistant CML patients, a variety of resistance mechanisms are discussed, e.g., drug-drug interactions via CYP3A4, pharmacogenetic polymorphisms, drug efflux transporter, in particular overexpression of the drug efflux transporters of the ATP binding cassette (ABC) family, epigenetics, microRNA deregulation and activation of alternative signaling pathways, in particular JAK/STAT, MAP kinases or PI3K/Akt signaling (12,14). To analyze drug resistance mechanisms, genome-wide gene expression and exome sequencing data of an *in vitro*-TKI-resistance CML model were performed in a previous study, which identified downregulation of the non-receptor kinase Bruton's Tyrosine Kinase (*BTK)* expression and an acquired novel potentially pathogenic dinucleotide *BTK* variant in imatinib resistance [GSE227347 (15), PRJEB60564 (16)].

First identified in loss-of-function mutations causing X-linked agammaglobulinemia (XLA)/Bruton syndrome, BTK is central for B lymphocyte development (17-19). BTK is known to play a crucial role in Toll-like receptor (TLR), chemokine receptor, as well as B cell receptor (BCR) signaling (20). Besides its essential role in the pathogenesis of XLA, there are also several other malignancies based on BTK dysregulation:

Waldenström's macroglobulinemia, e.g., is an indolent B cell lymphoma caused by myeloid differentiation primary response 88-(MYD88)-dependent BTK activation (21). In chronic lymphocytic leukemia (CLL), a malignant neoplasm resulting in overproduction of mature CD5+ B lymphocytes by a constitutively activated BCR pathway, BTK is an essential component of signal transmission (22). Another malignancy also linked to the BTK-dependent BCR signaling pathway is mantle cell lymphoma (MCL), an aggressive mature B cell non-Hodgkin lymphoma characterized by t(11;14)(q13;q32) translocation, leading to an upregulation of cyclin D1 and BCR activation (23). For all malignancies, in which BTK is a crucial component of pathogenesis, BTK inhibition represents a possible treatment option.

Consequently, BTK became a potential druggable candidate for targeted therapy, leading to the introduction of the selective and FDA-approved BTK inhibitor (BTKi) ibrutinib, which is used as a single agent or combinational treatment in various cancers (17). Similar to other tyrosine kinase inhibitors, relapses and treatment failure can be observed during the treatment with imatinib. This problem, as well as the occurrence of adverse events, led to the development of second generation BTKIs, e.g., acalabrutinib, zanubrutinib and tirabrutinib, with the goal to improve the tolerability by higher selectivity to BTK and to overcome ibrutinib resistance (23-25).

The aim of the current study was to investigate the role of *BTK* and the previously discovered novel dinucleotide *BTK* variant in imatinib resistance in chronic myeloid leukemia cells.

Materials and methods

Reagents and compounds. All chemicals and reagents not indicated differently, were purchased from Sigma-Aldrich or Carl Roth. Imatinib and ibrutinib were obtained from Sigma-Aldrich; Merck KGaA. Imatinib was diluted in 10 mM aqueous stock solutions, ibrutinib in 100 mM stock solutions in DMSO, both stored at -20˚C.

Cell culture. K-562 cells (RRID: CVCL 0004), retrieved from the pleural effusion of a 53-year old woman (26), originate from the German Collection of Microorganisms and Cell Cultures (DSMZ). Cells were cultivated in RPMI-1640 (Thermo Fisher Scientific, Inc.) supplemented with 10% v/v FCS (Bio&Sell), 1% v/v Penicillin/Streptomycin (Carl Roth) and 1% v/v L-Alanyl-L-Glutamin. Imatinib-resistant cell lines were generated as previously described (15). Authenticity of treatment-naïve as well as cell lines resistant against 0.5 μM and 2 μM imatinib was confirmed by short tandem repeats (STR) analysis using the GenePrint 10 system (Promega). *BCR::ABL1* mutations were analyzed as described elsewhere (15). The cell lines showed no *BCR::ABL1* mutations.

Site-directed mutagenesis. Mutagenesis of an *BTK*-encoding plasmid (Gene ID: 2335, ABIN3996197, antibodies-online, Aachen) was performed to insert the *BTK* variants (NM_000061.3,) c.1699G>A p.(Glu567Lys), c.1700A>G p.(GluE567Gly) and c1699_1700delinsAG p.(GluE567Arg) using the primers BTK_G1699A_F 5'-GTCCCCACCGAAAGTCCTGAT-3', BTK_G1699A_R 5'-CACCGGACTGGAAATTTGG-3', BTK_A1700G_F 5'-TCCCCACCGGGAGTCCTGATG-3', BTK_A1700G_R 5'-CCACCGGACTGGAAATTTGG-3', BTK_GA1699-1700AG_F 5'-GTCCCCACCGAGAGTCCTGATG-3' and BTK_GA1699-1700AG_R 5'-CACCGGACTGGAAATTTG-3' obtained from Sigma-Aldrich. 25 ng of template DNA, an annealing temperature of 67˚C and the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) were used according to the manufacturer's protocol. Sequence identification was confirmed using Sanger sequencing.

Isolation of nucleic acids. Total RNA isolation was performed using $1x10^6$ K-562 cells and PeqGOLD TriFast (VWR) according to the manufacturer's recommendation. Cell line DNA was purified using Gentra Puregene Cell Kit (Qiagen) according to the manufacturer's protocol.

Amplicon sequencing (MiSeq, Illumina). Generation of PCR products was performed using MyTaq Polymerase (Bioline), DNA of treatment-naïve, 0.5 μM and 2 μM imatinib-resistant cells, and the primers BTK_Intron_F 5'-TGACACTCTTGTGACCGTGC-3' and BTK_Intron_R 5'-ACAGTAAGCACTCCCCAAGG-3' with annealing at 54˚C. PCR products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, Inc.). After PCR amplicon preparation with the Nextera XT Sequencing Kit (Illumina), Next Generation Sequencing (NGS) SBS technology with Illumina MiSeq was performed as described elsewhere (16,27).

RT-qPCR. According to the manufacturer's protocol, 1 μg total RNA was reversely transcribed using random hexamer primers and the High Capacity cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) for 10 min at 25˚C, 120 min at 37˚C and 5 min at 85˚C. cDNA was stored at -80˚C prior further usage. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) of target genes was performed in triplicates using the TaqMan assays (Thermo Fischer Scientific, Inc.) *HPRT1* (Hs02800695_m1, Chr.X:134460145-134500668), *TBP* (Hs00427620_m1, Chr.6:170554333-170572870) and *GAPDH* (Hs0266705_g1, Chr.12:6534405-653837) as internal controls, as well as *BTK* (Hs00975865_m1, Chr.X:101349447-101390796), with Universal Master Mix II without UNG (Thermo Fisher Scientific, Inc.) on the QuantStudio 7 device (Thermo Fisher Scientific, Inc.) with default cycling conditions. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (28).

Immunoblotting. Whole cell lysates and immunoblotting were performed as described elsewhere (29-31). 20 μg of protein were loaded onto the membranes and blots were probed with the following antibodies, obtained from Santa Cruz, Cell Signaling Technology or LiCOR (Bad Homburg): BTK: Cat# sc-28387, RRID: AB_626770, 1:1,000; p-BTK: Cat# 5082, RRID: AB_10561017, 1:1,000; GAPDH: Cat# sc-47724, RRID: AB_627678, 1:1,000; anti-mouse: Cat# 926-68070, RRID: AB_10956588, all 1:10,000; anti-rabbit: Cat# 926-32211, RRID: AB_621843, all 1:10,000. Primary antibodies were diluted in Intercept/TBS blocking solution (LiCOR), supplemented with 0.2% v/v Tween-20. Secondary antibodies were diluted in TBS, supplemented with 0.1% v/v Tween-20.

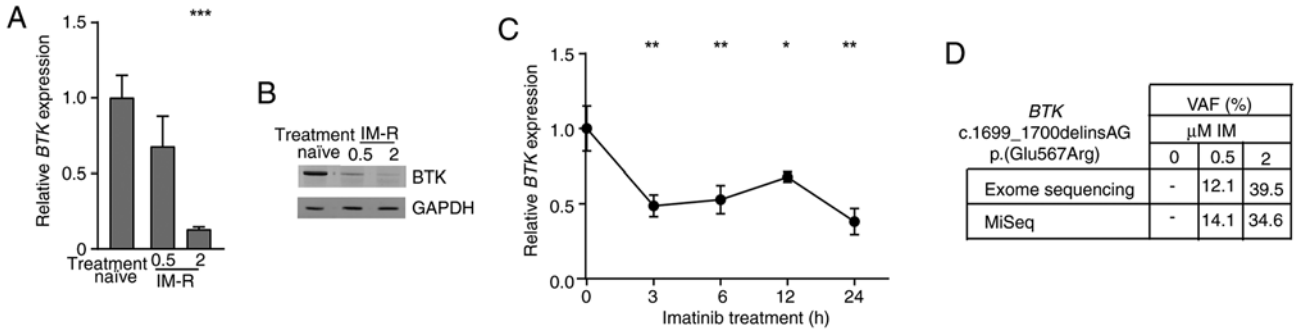

Figure 1. *BTK* downregulation and gain of *BTK* variant p.(Glu567Arg)/E567R in imatinib resistance. (A) *BTK* mRNA expression in imatinib resistance analyzed by RT-qPCR and normalized to *GAPDH*, *TBP* and *HPRT1* as housekeeping genes and treatment-naïve K-562 cells. n=3. (B) Immunoblotting of *BTK* protein levels in imatinib-resistant cells compared with GAPDH. n=3. (C) *BTK* mRNA expression in response to treatment with 2 μM imatinib for 0 to 24 h analyzed by RT-qPCR and normalized to *GAPDH*, *TBP* and *HPRT1* and 0 h. n=3. (D) VAF of the *BTK* dinucleotide variant c.1699_1700delinsAG p.(Glu567Arg)/E567R in imatinib-resistant cell lines (0.5 and 2 μM imatinib) compared with treatment naïve K-562 cells obtained from exome and in-depth sequencing. Error bars indicate standard deviation. *P<0.05, **P<0.01, ***P<0.001 compared with (A) treatment-naïve and (C) 0 h. IM, imatinib; IM-R, imatinib resistance; VAF, Variant allele frequencies; *BTK*, Bruton's tyrosine kinase; RT-qPCR, reverse transcription-quantitative PCR; *HPRT1*, Hypoxanthine-guanine phosphoribosyltransferase.

Plasmid and siRNA transfection. Plasmid and siRNA transfection were performed using the Amaxa nucleofector Kit V and nucleofector 2 b device (Lonza). For plasmid transfection, $2x10^6$ cells were transfected with 10 μg of the respective plasmid. 24 h after transfection, cells were seeded onto cell plates for subsequent experiments. For siRNA transfection, K-562 cells were transfected with 200 nM negative control stealth (1295300) and stealth RNAi Pre-designed *BTK*-siRNA 5'-GGAGUCAGGCUGAGCAACUGCUAAA-3' (HSS101131, both Thermo Fisher Scientific, Inc.). After transfection, cells were seeded onto respective cell culture plates and exposed to 2 μM imatinib for 48 h. Stable transfection was performed exposing the cells to 800 mg/ml G-418 for 4 weeks.

Cellular fitness assays. Cell numbers were obtained by trypan blue staining and performed after 48 h imatinib incubation as described elsewhere (27). WST-1 assay (Sigma-Aldrich; Merck KGaA) was performed after 48 h with $5x10^4$ cells as previously described (29). Proliferation was analyzed using human MKI67 ELISA Kit (MyBioSource) on $1x10^6$ cells according to the manufacturer's recommendation with 50 μg protein/well. Data were analyzed normalizing imatinib-treated to non-treated samples.

Pathogenicity prediction and statistical analysis. Pathogenicity prediction of *BTK* variants was performed using the CADD score [CADD v1.6, (32)]. Statistical analysis was performed using unpaired student's t-test or one-way ANOVA with subsequent Dunnett's test using GraphPad prism software (Version 9.0 for Windows).

Results

Reduction of BTK expression and gain of a BTK variant in imatinib-resistant CML cells. In a previous study, an *in vitro*-TKI resistance model of K-562 CML cells was obtained by stepwise exposure to increasing imatinib concentrations to obtain cells resistant against 0.5 and 2 μM imatinib (15). Analysis of genome-wide expression in this model revealed a *BTK* downregulation in one of the replicate cell lines of imatinib resistance [-3.7-fold, $P=7.08x10^{-7}$, (15)]. To generate insights into the role of *BTK* in the pathogenesis of CML, *BTK* expression in treatment-naïve and imatinib-resistant cell lines was examined on mRNA and protein level. *BTK* was significantly downregulated on mRNA (0.5 μM: -33%; 2 μM: -87%, both P<0.001) and protein level in 2 μM imatinib-resistant cells confirming the BTK downregulation in imatinib resistance (Fig. 1A and B). To investigate, whether *BTK* downregulation occurs in response to imatinib treatment, *BTK* mRNA expression was measured after short-term exposure to imatinib of 3-24 h. Hence, imatinib treatment led to a significant reduction in *BTK* mRNA levels by 33 to 62% (3 h: P=0.002, 6 h: P=0.005, 12 h: P=0.03, 24 h: P=0.001, Fig. 1C).

As previously reported, the novel *BTK* dinucleotide variant [NM_000061.3, c.1699_1700delinsAG p.(Glu567Arg)] was detected in imatinib-resistant cells [PRJEB60564 (16)]. This variant leads to an amino acid exchange from glutamic acid to arginine in the BTK kinase domain, which is likely to have a detrimental effect on protein function according to prediction tools (CADD: 25.2). In depth-sequencing confirmed the presence of this variant in 0.5 (14%) and 2 μM imatinib-resistant cells (34%, considering the K-562 triploidy, Fig. 1D). Overall, our findings show a downregulation of *BTK* expression and a gain of a novel dinucleotide *BTK* variant in imatinib resistance.

Loss of BTK activity or its expression promotes imatinib resistance. Since a putative pathogenic *BTK* dinucleotide variant p.(Glu567Arg) was detected in imatinib resistance, the question arose whether deterioration of the kinase function through inhibition could also influence the imatinib susceptibility of CML cells. Thus, treatment-naïve cells were exposed to the BTK-specific inhibitor ibrutinib and compared to single treatment with imatinib. Treatment with imatinib significantly decreased cell viability of treatment-naïve, but not imatinib-resistant K-562 cells by 73 to 89% (300-2,000 nM: P<0.001), while ibrutinib did not affect the cells (Fig. 2A and B). However, in a combined treatment with low dose (100 nM) imatinib, the presence of ibrutinib dose-dependently increased cell viability by 11 to 37% (ibrutinib (nM): 100: P=0.05, 500:

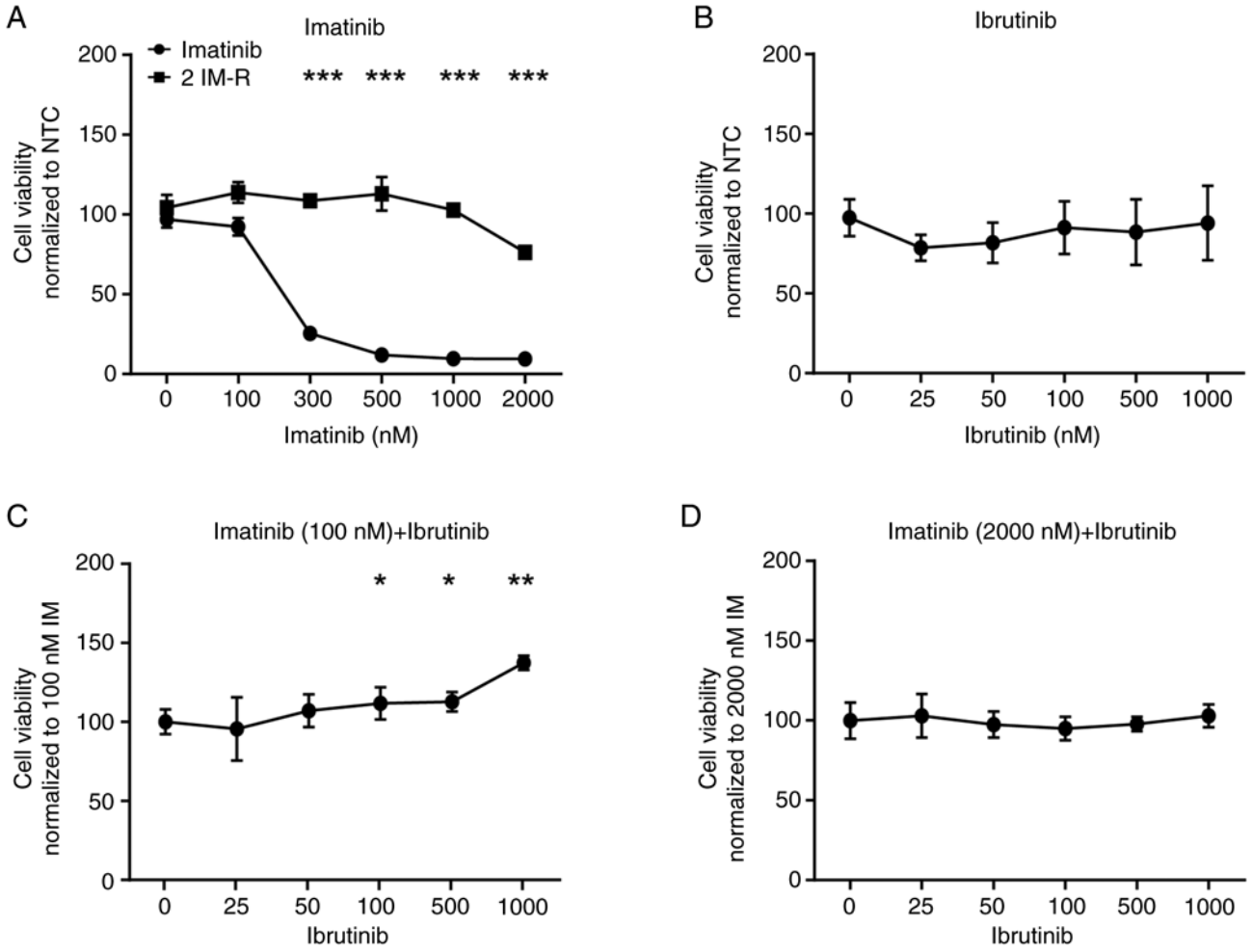

Figure 2. Inhibition of *BTK* by ibrutinib hampers susceptibility to low dose imatinib. Cell viability of K-562 cells after exposure to (A) imatinib (0-2,000 nM), (B) ibrutinib (0-1,000 nM), as well as to (C) 100 or (D) 2,000 nM imatinib, respectively, in a dose-dependent combination with ibrutinib for 48 h. Data were normalized to NTC. n=3. Error bars indicate standard deviation. *P<0.05, **P<0.01, ***P<0.001 compared with the respective cell line at 0 nM. IM, imatinib; IM-R, imatinib resistance; *BTK*, Bruton's tyrosine kinase; NTC, no treatment controls.

P=0.02, 1,000: P=0.002, Fig. 2C), while this was not observed in combination with high dose (2 μM) imatinib (Fig. 2D).

To investigate whether loss of *BTK* expression also affects K-562 cells, a siRNA-mediated *BTK* knockdown was performed in treatment-naïve CML cells. Successful *BTK* downregulation (P<0.001, Fig. 3A) led to a significant increase in cell number (97.2%, P<0.001) and proliferation rate of CML cells compared to negative control-transfected cells (27.5%, P=0.01, Fig. 2B). After subsequent treatment with imatinib, *BTK* knockdown cells also showed increased cell numbers (70.6%, P=0.04), while proliferation was not significantly altered (Fig. 2C). Overall, these findings indicate that both, inhibition and the loss of BTK expression, are beneficial for CML cells and reduce imatinib susceptibility.

BTK overexpression and its rescue reinstate imatinib susceptibility. Vice versa, to analyze whether high *BTK* expression is detrimental for CML cells under imatinib exposure, treatment-naïve cells were stably transfected with a *BTK*-encoding plasmid to induce *BTK* overexpression (Fig. 4A) and challenged with 2 μM imatinib. In response to imatinib, the presence of BTK resulted in a reduction in cell number (-25.9%, P=0.03), proliferation (-20.5%, P=0.04), and cell viability compared to negative control-transfected cells (-25.7%, P<0.001, Fig. 4B). These data indicate that the presence of BTK augments the response to imatinib treatment.

Since *BTK* was significantly downregulated in imatinib-resistant cells, restoration of *BTK* expression in these cells was performed by transfection experiments followed by imatinib exposure. After successful restoration of *BTK* expression, cell number (-28.4%, P=0.002), proliferation (-26.7%, P=0.04) and cell viability (-12.4%, P=0.003) were decreased compared to negative control-transfected cells indicating an improved response to imatinib (Fig. 4C and D). Therefore, our findings demonstrate that the restoration of *BTK* in imatinib-resistant cells reinstates imatinib susceptibility.

Influence of BTK variants on imatinib susceptibility. As the *BTK* variant p.(Glu567Arg) was acquired in imatinib resistance, the question arose whether this mutation affects CML cells and could be involved in the development of resistance. Stable transfection of *BTK* wild-type and the p.(Glu567Arg) variant in treatment-naïve K-562 cells was performed. For comparison, the pathogenic kinase-dead BTK p.(Glu567Lys) (33) and benign p.(Glu567Gly) variants were also transfected. *BTK* mRNA expression was

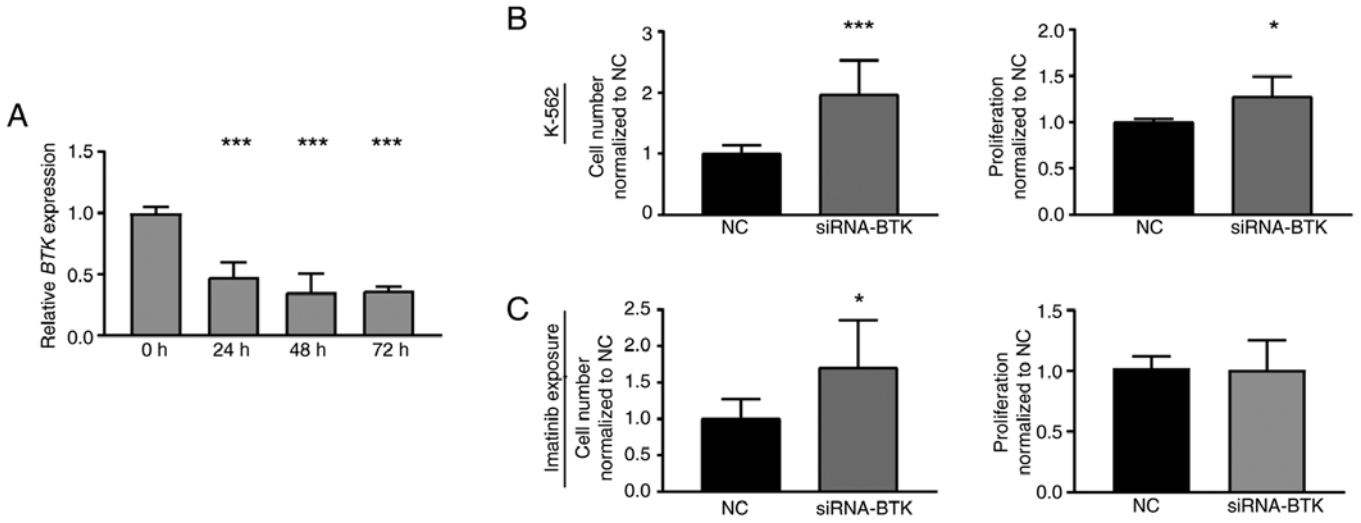

Figure 3. Knockdown of *BTK* is beneficial for CML cells and promotes imatinib resistance. (A) *BTK* mRNA expression 24-72 h after small interfering RNA-mediated *BTK* knockdown measured by RT-qPCR normalized to *GAPDH*, *TBP* and *HPRT1* as housekeeping genes and negative control-transfected cells (NC). n=3. Cellular fitness after *BTK* knockdown of (B) K-562 cells and (C) under exposure to 2 μM imatinib analyzed by cell numbers and proliferation rates. Data were normalized to NC. n=3. Error bars indicate standard deviation. *P<0.05, ***P<0.001 compared with 0 h or NC groups using (A) ANOVA with subsequent Dunnett's test or (B) student's t-tests. IM, imatinib; *BTK*, Bruton's tyrosine kinase; RT-qPCR, reverse transcription-quantitative PCR; *HPRT1*, Hypoxanthine-guanine phosphoribosyltransferase; NC, negative control.

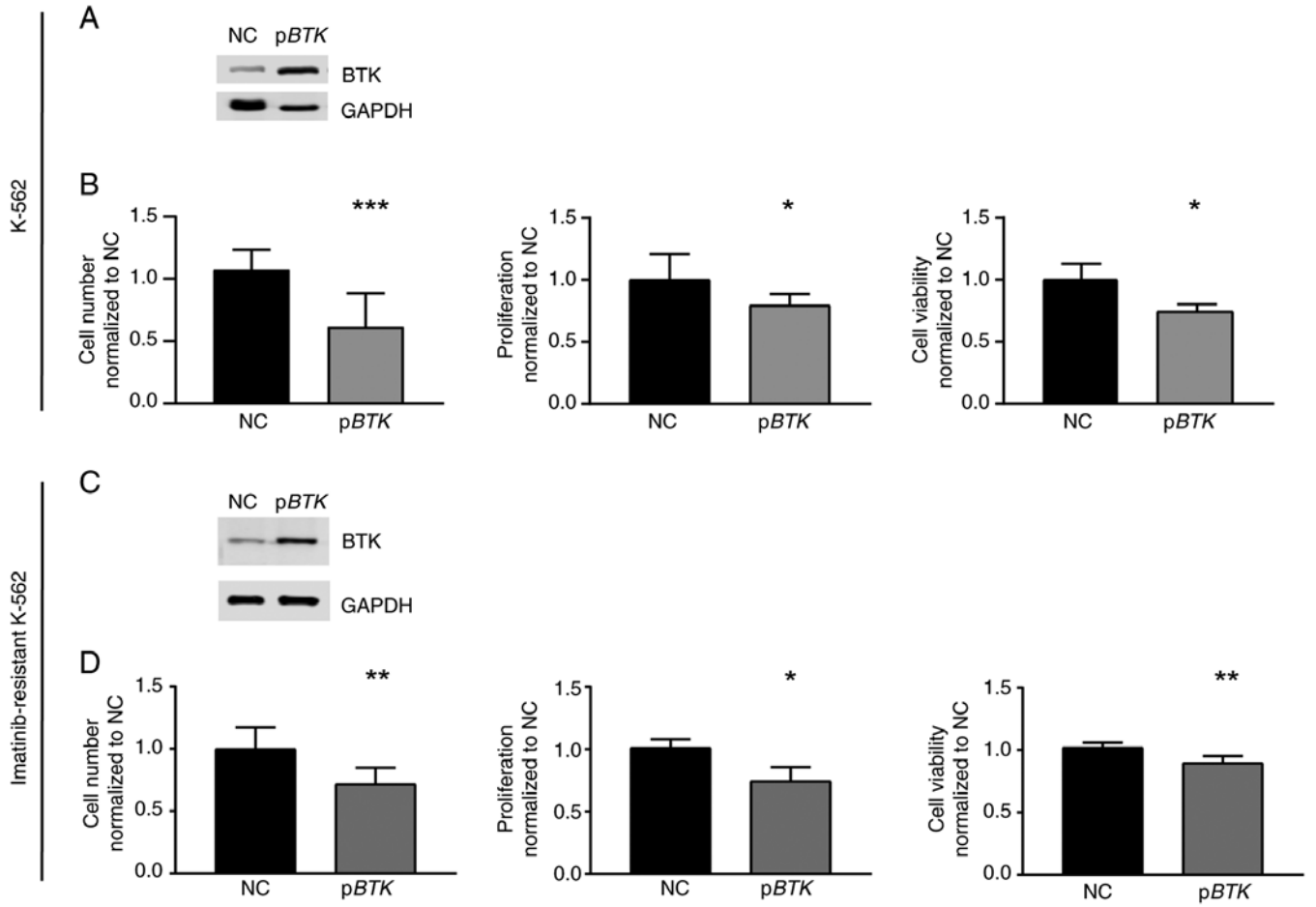

Figure 4. *BTK* modulation alters imatinib susceptibility in treatment naïve and imatinib-resistant CML cells. (A and B) *BTK* overexpression in treatment naïve K-562 cells. (A) *BTK* protein level after stable transfection of a *BTK*-encoding plasmid into K-562 cells analyzed by immunoblotting and compared to GAPDH. (B) Cellular fitness after *BTK* transfection into K-562 cells under exposure to 2 μM imatinib. (C and D) Restoration of *BTK* expression in imatinib-resistant K-562 cells. (C) *BTK* protein level after *BTK* rescue by plasmid transfection of imatinib-resistant cells analyzed by immunoblotting and compared to GAPDH. (D) Cell numbers, proliferation and cell viability after restoration of *BTK* expression after 48 h of imatinib treatment. n=3. Data were normalized to NC-transfected cells. Error bars indicate standard deviation. *P<0.05, **P<0.01, ***P<0.001 compared with NC using student's t-tests. IM, imatinib; NC, negative control; *BTK*, Bruton's tyrosine kinase; p*BTK*, transfection with an *BTK*-encoding plasmid.

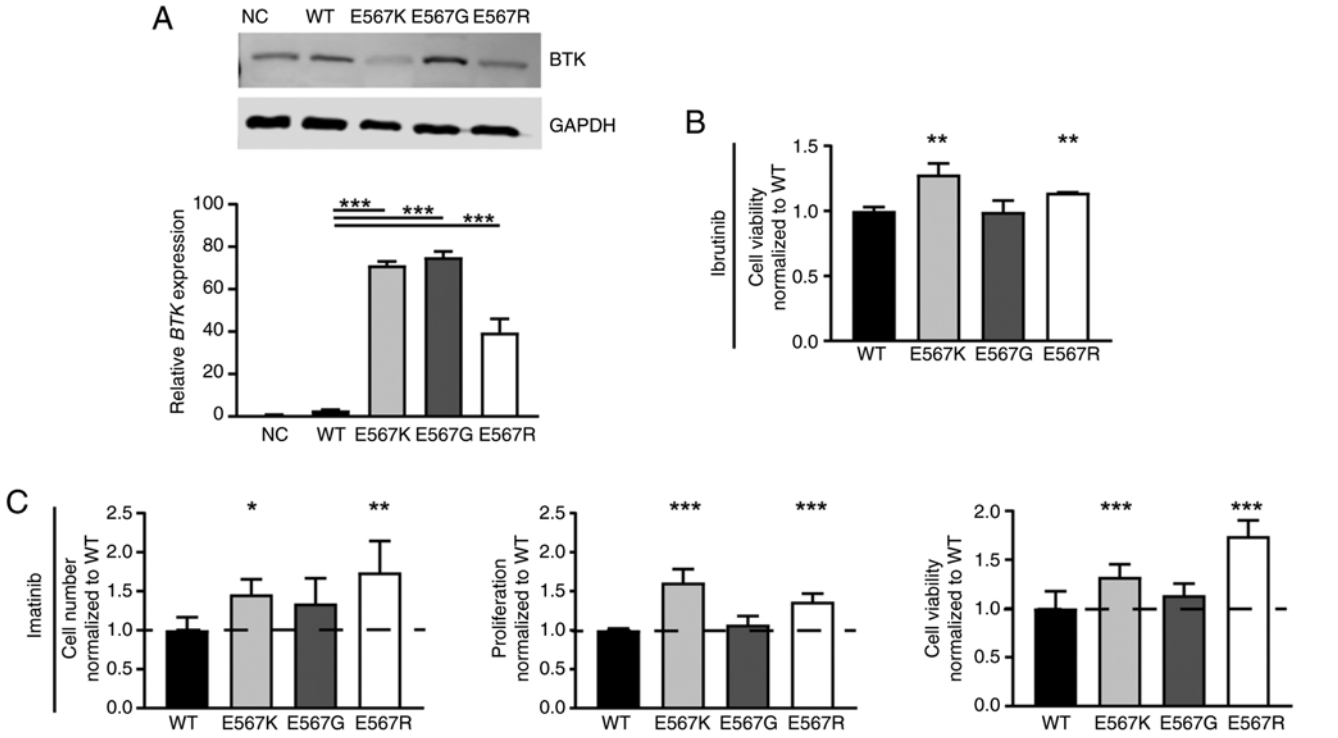

Figure 5. Presence of *BTK* p.(Glu567Lys)/E567K and p.(Glu567Arg)/E567R, but not p.(Glu567Gly)/E567G promotes imatinib resistance. (A) *BTK* mRNA and protein levels after stable transfection of *BTK* WT, the variants p.(Glu567Lys)/E567K, p.(Glu567Gly)/E567G and p.(Glu567Arg)/E567R and the NC into treatment naïve K-562 cells analyzed by immunoblotting compared to GAPDH (top) and by RT-qPCR normalized to *GAPDH*, *TBP* and *HPRT1* (bottom). n=3. Presence of the variants in the stable transfected cell lines was confirmed using Sanger sequencing. (B) Cell fitness after overexpression of *BTK* WT or the variants E567K, E567G or E567R after exposure to 100 nM ibrutinib. n=3. Data were normalized to WT. (C) Cell number, proliferation and cell viability of *BTK* variant cell lines after treatment with 2 μM imatinib compared to WT cells. n=3. Data were normalized to WT. Error bars indicate standard deviation. *P<0.05, **P<0.01, ***P<0.001 compared with WT. WT, wild-type; *BTK*, Bruton's tyrosine kinase; NC, empty vector-control; RT-qPCR, reverse transcription-quantitative PCR; *HPRT1*, Hypoxanthine-guanine phosphoribosyltransferase; NC, negative control.

significantly increased in all stable transfected cell lines indicating successful overexpression (P<0.001, Fig. 5A). On protein level, however, in p.(Glu567Arg) and p.(Glu567Lys)-expressing cells, a reduction in BTK protein level was observed, while p.(Glu567Gly) had the highest BTK levels pointing to decreased protein stability in the presence of lysine or arginine at this residue (Fig. 5A). To investigate if the cells harboring BTK variants are still able to respond to ibrutinib treatment, the cells were exposed to ibrutinib (Figs. 2B, S1). The presence of 100 nM ibrutinib resulted in increased cell viability for p.(Glu567Arg) (22.6%, P=0.008), similarly to p.(Glu567Lys) (45.6%, P=0.006), but not for p.(Glu567Gly) compared to cells overexpressing *BTK* wild-type (Fig. 5B). After exposure to 2 μM imatinib, the presence of the p.(Glu567Arg) variant led to an increase in cell number (57.2%, P=0.003), proliferation (36.5%, P<0.001) and cell viability (60.1%, P<0.001) during imatinib treatment compared to wild-type *BTK* (Fig. 5C). A similar effect was also observed for *BTK* p.(Glu567Lys), as increased cell numbers (35.4%, P=0.04), proliferation (61.2%, P<0.001) and cell viability were observed under imatinib exposure (29.5%, P<0.001, Fig. 5B). The benign variant p.(Glu567Gly), however, did not affect the response to imatinib treatment. These findings show that p.(Glu567Arg), as well as p.(Glu567Lys), result in diminished imatinib susceptibility. Overall, our data show that the acquired *BTK* p.(Glu567Arg) variant appears to be relevant for the development of imatinib resistance.

Discussion

The Bruton's tyrosine kinase (BTK) was initially associated with X-linked agammaglobulinemia (XLA), a primary immunodeficiency disorder characterized by a severe blockade of B cell development in the bone marrow resulting in a lack of B cells and serum antibodies due to (18). Since its discovery, BTK was found to be crucial in oncogenic signaling being important for B cell development and mature B cell function. Accordingly, BTK is deregulated in several B cell malignancies, including CLL, small lymphocytic leukemia (SLL), MCL and follicular lymphoma, as well as in various other tumors, such as pancreatic, lung, breast, ovarian, prostate and colorectal cancer (17,34). In addition, BTK has been shown to be an essential player in chronic graft-vs.-host disease or Waldenström macroglobulinemia (17,35). In the present study, we analyzed the role of *BTK* in the myeloproliferative disease CML. A down-regulation of *BTK* expression, as well as the acquired *BTK* p.(Glu567Arg) variant were detected in imatinib resistance. Subsequent transfection experiments demonstrated that the presence of *BTK* affects the response to imatinib in CML cells. Further, imatinib susceptibility was restored after rescue of *BTK* expression in imatinib-resistant cells. In addition, the *BTK* p.(Glu567Arg) variant seems to be pathogenic to the protein function resulting in a loss-of-function promoting imatinib resistance.

BTK is a 77 kDa protein composed of 659 amino acids and five domains: A plectrin homology (PH), a Tec homology (TH), an SH3 and SH2 domain, and the C-terminal kinase domain (36). The SH2 domain mutation p.(Thr316Ala) and the kinase domain mutations p.(Thr474Ile/Ser), p.(Cys481Ser/Ala/Phe/Gly/Arg/Tyr) and p.(Leu528Trp) with p.(Cys481Ser) are the main *BTK* mutations, associated with BTKI resistance in B cell malignancies (36). Clinical trials of secondary drug resistant CLL have shown the presence of the *BTK* p.(Cys481Ser) mutation in approximately 80% of patients resulting in a loss of drug binding while maintaining kinase activity (36,37). This mutation has also been detected in drug resistant or relapsed MCL and diffuse large B cell lymphoma (DLBCL) leading to a sustained *BTK* signaling and, consequently, to tumor progression (38). In the present study, the *BTK* p.(Glu567Arg) variant, which is located at the C-terminal end of the kinase domain, was acquired in imatinib resistance (16,39). This variant showed similar effects on imatinib susceptibility as the published p.(Glu567Lys) variant, which was shown to suppress BTK-mediated NLRP3 inflammasome activation in brain ischemia (33). Loss-of-function variants at this amino residue, such as the exchange to glutamine p.(Glu567Gln), have previously been associated with XLA (40). The p.Glu567 residue was found to form an ionic bond to Arg641, which stabilizes the kinase structure (41). Therefore, mutations at this residue are likely to affect BTK stability and may also explain the reduction in *BTK* expression in the presence of p.(Glu567Arg) in imatinib resistance observed here. Further, stable transfection of p.(Glu567Arg) was shown to result in increased levels of *BTK* mRNA, but not protein indicating increased protein turnover or reduced translation. Interestingly, both, *BTK* p.(Glu567Arg) and p.(Glu567Lys) variants also resulted in an increased cell fitness in the presence of ibrutinib, which could be a results of decreased binding of ibrutinib to the variant proteins.

BCR::ABL1 TKIs are tremendously successful in CML treatment. However, therapy resistance remains a clinical problem with a need for TKI alternatives for 20-25% of CML patients within five years after therapy onset (4). In B cell malignancies, the TKI ibrutinib also shows significant clinical impact with overall response rates ranging from 90 and 97%, e.g., in CLL (42,43). Ibrutinib is a covalent kinase inhibitor, which irreversibly binds to the BTK cysteine 481 in the catalytic site preventing autophosphorylation at tyrosine 223 and thereby, downstream signaling (39). Interestingly, it was shown that BTK is also a target of the second generation BCR::ABL1 inhibitor dasatinib (44). In the present study, we found that inhibiting BTK by ibrutinib or knocking down its expression prevented CML cells from imatinib resulting in increased cell proliferation rates. These findings contradict a study on primary CML stem cells, where BTK inhibition with ibrutinib was demonstrated as a potential therapy approach in combination with BCR::ABL1 TKI therapy to eradicate leukemic stem cells mediated by inhibition of the Fc gamma receptor IIb (FcγRIIb, CD32B) and a case report from concomitant use of imatinib and ibrutinib in a patient with combined CML and CLL (45,46). However, a study on Ba/F3 cells expressing BCR::ABL1 wild-type or p.(Thr315Ile) mutation found that *BTK* depletion did not affect viability or proliferation indicating that *BTK* is not essential for leukemogenesis (47). Consistent with our findings of a *BTK* downregulation at 0.5 and 2 μM imatinib, concentrations that reflect the observed imatinib plasma levels in patients undergoing therapy (48), decreased *BTK* levels were also shown in imatinib-resistant CML patients (49). In general, BTK appears to be relevant for TKI resistance and CML progression only in cases of a persistent CML, as present in our analyzed K-562 cell line model, but not in the development of the disease. Thus, the BTK downregulation in the imatinib-resistant cell line as presented here, may result in a deregulation of BTK-dependent signaling pathways that are likely involved in the development of imatinib resistance, such as Ras-Map-signaling, to circumvent the imatinib-mediated BCR::ABL1 inhibition. However, the *BTK* deregulation, but also the acquisition of BTK mutations in CML patients should be further analyzed in a clinical study to understand the influence on resistance.

Due to its function as a kinase and its role as an oncogene, it is generally assumed that *BTK* would be upregulated or gain-of-function mutations would be present in drug resistance. Accordingly, in previous studies on CLL and MCL, an upregulated *BTK* expression relative to non-malignant cells have been observed (20,50). However, we found that *BTK* downregulation is associated with CML progression and imatinib resistance. Our results contradict the common assumption that upregulated kinases, such as *BTK*, enhance tumor progression and their downregulation suppresses it. However, this discrepancy is consistent with the description of *BTK* as a context-dependent oncogene or tumor suppressor gene capable to either increase proliferation and cell survival or induce apoptosis and senescence, particularly by affecting p53-signaling (51). Therefore, *BTK* should be considered as a pleiotropic gene with opposing effects in cancer, acting as an oncogene especially in B cell malignancies, but also as a tumor suppressor in other neoplasms.

In conclusion, the present data show that *BTK* is involved in the development of imatinib resistance and progression of chronic myeloid leukemia in an *in vitro*-model. Further, the gain of the *BTK* c.1699_1700delinsAG p.(Glu567Arg) variant in imatinib-resistant cells further highlights the significance of *BTK* in imatinib resistance. These findings demonstrate that *BTK* is not only relevant in B cell malignancies, but may also be involved in other hematopoietic disorders, either as an oncogene or tumor suppressor gene, as well as a factor modulating treatment efficacy. Clinical investigations should confirm whether BTK inhibitors should be considered prior to their usage in imatinib-resistant chronic myeloid leukemia.

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Availability of data and materials

The data generated in the present study may be requested re from the corresponding author. The exome sequencing and gene expression data in the present study may be found in the European Nucleotide Archive (ENA) repository under the accession number (PRJEB60565; https://www.ebi.ac.uk/ena/) and the GEO dataset accession number (GSE227347; https://www.ncbi.nlm.nih.gov/gds).

Authors' contributions

MK and IN conceptualized the study. MK designed the research. LS performed the experiments. LS, MK and IV analyzed the data. LS, IC, MK interpreted the data. LS and MK wrote the original draft. All authors read and approved the final version of the manuscript. MK and LS confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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