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# (−)-Epigallocatechin-3-gallate induces cell apoptosis in chronic myeloid leukaemia by regulating Bcr/Abl-mediated p38-MAPK/JNK and JAK2/STAT3/AKT signalling pathways

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**Summary**

Epigallocatechin-3-gallate (EGCG), a major polyphenolic constituent of green tea, possesses remarkable chemopreventive and therapeutic potential against various types of cancer, including leukaemia. However, the molecular mechanism involved in chronic myeloid leukaemia (CML), especially imatinib-resistant CML cells, is not completely understood. In the present study, we investigated the effect of EGCG on the growth of Bcr/Abl+ CML cell lines, including imatinib-resistant cell lines and primary CML cells. The results revealed that EGCG could inhibit cell growth and induce apoptosis in CML cells. The mechanisms involved inhibition of the Bcr/Abl oncoprotein and regulation of its downstream p38-MAPK/JNK and JAK2/STAT3/AKT pathways. In conclusion, we documented the anti-CML effects of EGCG in imatinib-sensitive and imatinib-resistant Bcr/Abl+ cells, especially T315I-mutated cells.

**KEYWORDS**

Bcr/Abl, chronic myeloid leukaemia, drug resistant, EGCG, T315I mutant

## 1 | INTRODUCTION

Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disease characterized by the Philadelphia chromosome, which generates the Bcr/Abl fusion gene and P210 oncoprotein to produce a constitutively active tyrosine kinase.[1,2] ABL tyrosine kinase inhibitors (TKIs), such as imatinib, nilotinib, dasatinib and ponatinib, can block this kinase activity by occupying the ATP-binding site of Bcr/Abl and inhibit CML cell growth effectively, thus revolutionizing CML treatment.[3] The first generation of TKI imatinib has achieved significant clinical benefit and become the first-line drug for newly diagnosed CML and chronic-phase CML.[4-6] However, many patients develop resistance to imatinib, resulting in remission intervals usually lasting less than 1 year. Primary and secondary resistance is mainly due to the upregulation of Bcr/Abl and point mutations within the Abl kinase domain, which prevents the binding of imatinib to the

ATP-binding site.[7-11] Mutations at over 50 residues that confer varying degrees of imatinib resistance have been reported clinically.[12,13] Although the second- and third-generation ABL inhibitors have proven largely successful in imatinib-resistant CML patients,[14-16] these TKIs (nilotinib, dasatinib and bosutinib) are not capable of inhibiting all the kinase mutants identified in patients, especially the most notably Bcr/Abl T315I mutant. Ponatinib,[17] which has activity against the T315I mutation, has been developed, but its application in CML therapy has been limited by concerns regarding toxicity. Thus, novel therapeutic strategies with high efficacy and low toxicity that target Bcr/Abl, especially Bcr/Abl T315I, are urgently needed for further treatment of CML.[11]

Traditional Chinese medicine and herbal plants have vast histories that facilitate the identification of new compounds in developing therapeutic drugs. Green tea is one of the most widely consumed teas in the world and is reported to have significant benefits to

human health, which is chemically characterized by the presence of high amounts of polyphenolic compounds known as catechins. The most abundant component is epigallocatechin-3-gallate (EGCG), which appears to be the primary active ingredient responsible for the biological effects of green tea.[18,19] EGCG has received much attention in recent years. Previous studies have shown that EGCG exhibits antioxidative, antibacterial, and antitumor effects.[20-23] However, only a few studies have addressed the role of EGCG in malignant haematology, especially leukaemia. It remains unclear whether EGCG regulates the biological functions of CML cells and overcomes drug resistance. In addition, the effect of EGCG on Bcr/Abl activity in imatinib-resistant CML cells has not yet been reported.

Here, we investigated the effect of EGCG on the growth of Bcr/Abl+ CML cell lines, including imatinib-resistant cell lines and primary CML cells. We found that EGCG could inhibit growth and induce apoptosis in these cells. The mechanisms probably involve inhibition of the Bcr/Abl oncoprotein and regulation of its downstream p38-MAPK/JNK and JAK2/STAT3/AKT pathways.

## 2 | RESULTS

### 2.1 | EGCG inhibits the proliferation of CML cell lines and primary CML cells

The K562, K562R, KCL-22, BaF3/p210 and BaF3/p210[T315I] cell lines were treated with EGCG at different concentrations (0, 6.25, 12.5, 25, 50, or 100 μmol/L) for 24 or 48 hours. Cell proliferation was analyzed using the MTT assay. Our results showed that EGCG could
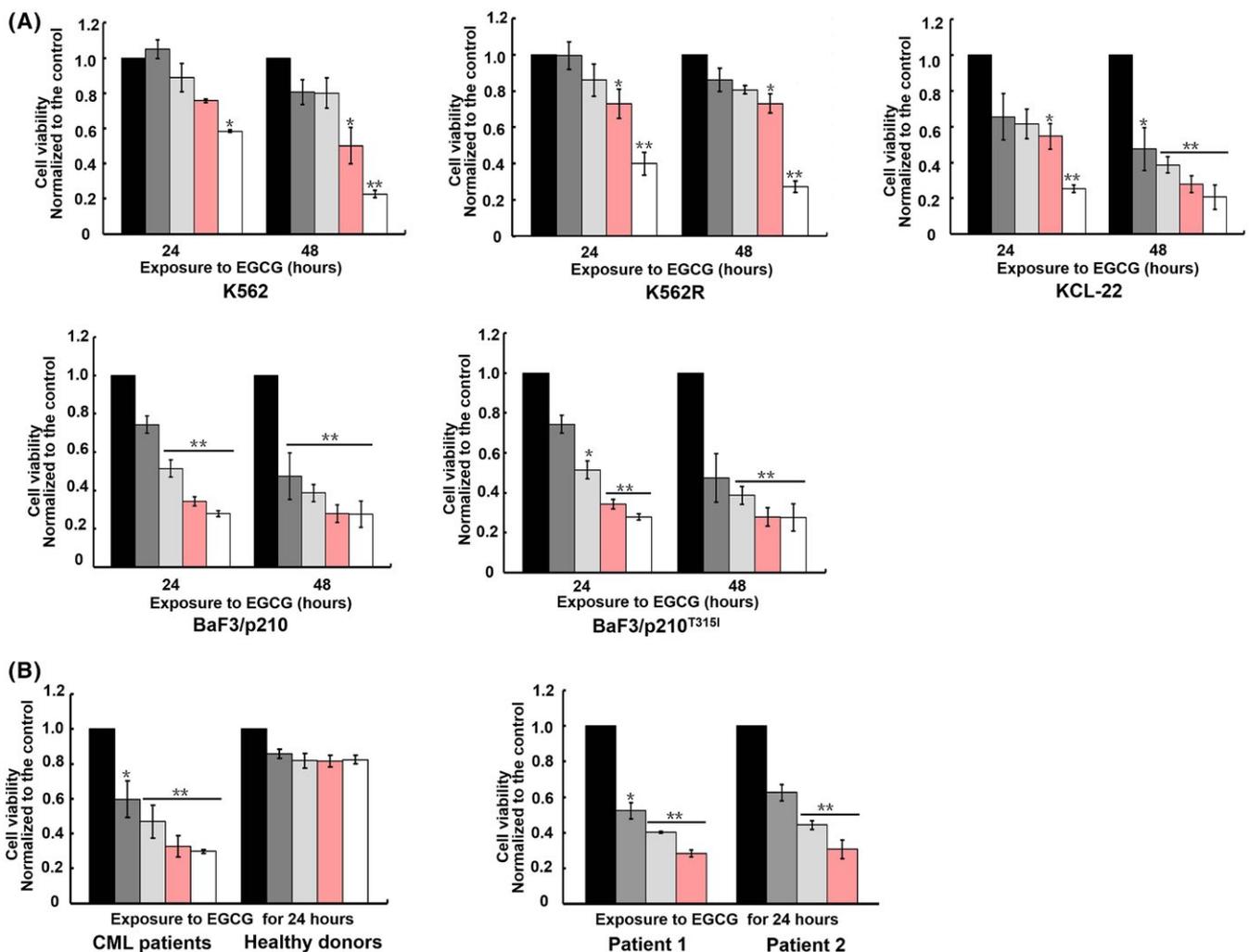


**FIGURE 1** A, Effects of epigallocatechin-3-gallate (EGCG) on the growth inhibition of chronic myeloid leukaemia (CML) cell lines. EGCG reduces the viability of CML cell lines in a dose-dependent manner. After starving the cells with 0.1% FBS, the cells were treated with different concentrations of EGCG for 24 or 48 h, and then, cell viability was measured by MTT assay. (■) 0 μmol/L; (▨) 12.5 μmol/L; (▢) 25 μmol/L; (▨) 50 μmol/L; (□) 100 μmol/L. B, Effects of EGCG on the growth of fresh primary cells (BMMCs) from newly diagnosed CML patient bone marrow (n = 6) or of BMMCs from healthy donors (n = 4) bone marrow (MTT assay). (■) 0 μmol/L; (▨) 6.25 μmol/L; (▢) 12.5 μmol/L; (▨) 25 μmol/L; (□) 50 μmol/L. EGCG reduces the viability of primary CML cells in a dose-dependent manner. In each treatment group, cells treated with medium were the control, and their viability was set as 100%. The values represent the mean ± SD of triplicate cultures. *P < 0.05, **P < 0.01

effectively inhibit the viability of CML cells in a dose-dependent manner with IC50 values of 62.62 µmol/L (K562), 91.12 µmol/L (K562R), 53.76 µmol/L (KCL-22), 12.80 µmol/L (BaF3/p210) and 29.82 µmol/L (BaF3/p210$^{T315I}$) respectively (Figure 1A). We also evaluated the antiproliferation effect of EGCG on primary bone marrow cells from CML patients (n = 6) and from healthy donors (n = 4). Bone marrow mononuclear cells (BMMCs) isolated from newly diagnosed CML patients were inhibited in a dose-dependent manner with an IC50 of 12.4 µmol/L. In contrast, BMMCs isolated from the bone marrow of healthy donors were almost completely resistant to EGCG (IC50: 27.2 mmol/L), indicating the specific toxicity of EGCG towards leukemic cells (Figure 1B). The combination of EGCG and imatinib or nilotinib showed no enhanced growth inhibitory effects on cell lines compared to the EGCG agent alone, which means that there is no synergistic cytotoxicity of EGCG and imatinib or nilotinib towards CML cell lines. Importantly, as shown in Figure 1A, we found that EGCG was able to inhibit the growth of imatinib-resistant K562R and BaF3/p210$^{T315I}$ cells as potently as it inhibited the growth of their respective parental K562 and BaF3/p210 cells, indicating that EGCG, to a certain extent, may play a role in reversing imatinib resistance.

## 2.2 | EGCG reduces the mitochondrial membrane permeability of CML cell lines

The mitochondrial membrane potential (MMP) provides a valuable indicator of cell health and functional status. K562, K562R, BaF3/p210 and BaF3/p210$^{T315I}$ cells were treated with 50 µmol/L EGCG for 24 hours and then stained with JC-1. The cyanine dye JC-1 monomers were taken up into the mitochondrial matrix as a result of changes in the membrane potential, which facilitates discrimination of energized and deenergized mitochondria because the normally red fluorescent dye forms green fluorescent aggregates when concentrated in deenergized mitochondria in response to their lower membrane potential. Flow cytometric analysis showed reduced MMP after EGCG treatment, indicating the irreversible occurrence of early apoptosis (Figure 2A).

## 2.3 | EGCG induces the apoptosis of CML cells through caspase-independent and AIF-mediated cell death pathways

To confirm whether the antileukemia activity of EGCG is associated with apoptosis, externalized phosphatidylserine (PS), an indicator of

apoptosis, as revealed by annexin V-FITC staining, was examined by annexin V-FITC/PI assay. It was observed that EGCG induced apoptosis in CML cells in a dose-dependent manner. After 48 hours of exposure to EGCG, increasing doses resulted in an increased proportion of apoptotic cells in both imatinib-sensitive and imatinib-resistant CML cell lines as well as in primary bone marrow cells from CML patients (Figure 2B). We examined the upstream regulators of mitochondrial membrane permeability. However, EGCG treatment did not alter the level of Bcl-2 protein. To further ascertain whether EGCG induces the mitochondrial-mediated apoptotic pathway, Western blot was performed to assess the expression of caspase-3, caspase-9 and cleaved PARP. The results revealed that the most downstream effectors, caspase-3, caspase-9 and PARP, were clearly not activated, which suggested that EGCG failed to induce a mitochondrial-mediated caspase cascade (Figure 2C).

Apoptosis-inducing factor (AIF) is the major effector of caspase-independent cell death programs. We analyzed the level of AIF in total cellular proteins and in mitochondrial and nuclear fractions from EGCG-treated imatinib-resistant cells by western blot. The specificity of the extracted mitochondrial and nuclear fractions was indicated by the abundance of heat shock protein 60 (HSP60, a mitochondrial protein marker) and histone H3 (a nuclear fraction marker), respectively. As shown in Figure 2D, after 48 hours treatment of K562R and T315I-mutated leukaemia cells with EGCG, AIF clearly increased in the mitochondrial and nuclear fractions compared to that in the control.

Autophagy could trigger apoptosis and/or necrosis. Compared to the control, treatment with EGCG increased the abundance of the autophagy gene (Atg5). Consistently, the amount of LC3 (autophagosomal marker microtubule-associated protein I light chain 3) increased following EGCG treatment (Figure 2E), indicating that autophagy function is increased and may be involved in EGCG-mediated cell apoptosis.

## 2.4 | EGCG suppresses the expression of Bcr/Abl and phospho-Bcr/Abl in CML cells

Since EGCG showed an effect on cell proliferation, we assessed its influence on the Bcr/Abl fusion protein. Our results showed that EGCG decreased the mRNA levels of Bcr/Abl in a dose-dependent manner (Figure 3A). Furthermore, treatment with EGCG reduced the protein expression of Bcr/Abl in primary CML cells and all applied cell lines, including imatinib-resistant K562R and BaF3/p210$^{T315I}$

**FIGURE 2** A, The mitochondrial membrane potential (ΔΨ), which was monitored by JC-1 using flow cytometric detection of green fluorescent aggregates in the FL1 channel, increased significantly in 50 µmol/L epigallocatechin-3-gallate (EGCG)-treated chronic myeloid leukaemia (CML) cells in response to their lower membrane potential. B, EGCG induces apoptosis in imatinib-sensitive and imatinib-resistant CML cell lines and primary CML cells. After starving the cells with 0.1% fetal bovine serum (FBS), the cells were treated with EGCG for 48 h and then analyzed by flow cytometry with annexin V/PI staining. Experiments were repeated, and data from representative experiments are shown. C, Effects of EGCG on caspase-9, caspase-3, cleaved PARP and bcl-2 protein expression (western blot). β-Actin was used as a loading control. D, Mitochondrial/nuclear AIF expression in EGCG-treated K562R and BaF3/p210$^{T315I}$ cells. HSP60 is a mitochondrial protein marker, and histone H3 is a nuclear fraction marker. TP, total cellular protein; Mit, mitochondria protein; Nuc, nuclear extraction. E, Autophagy increased in EGCG-treated K562R and BaF3/p210$^{T315I}$ cells
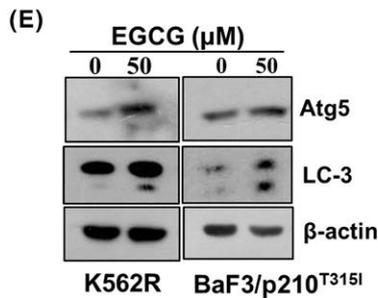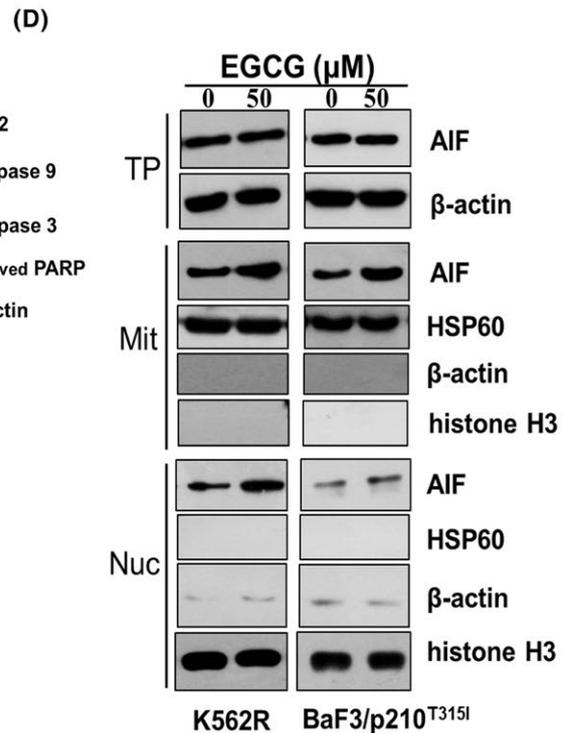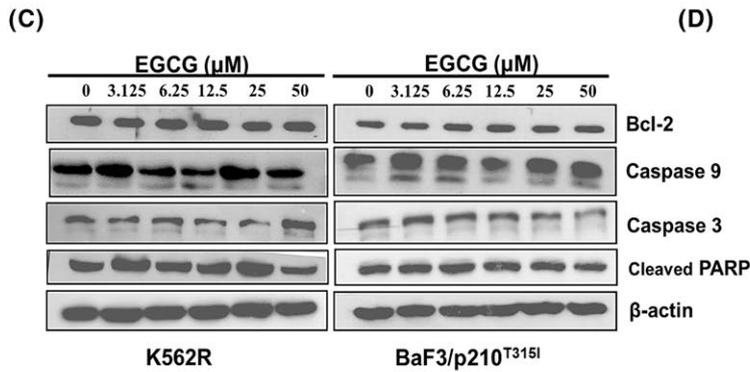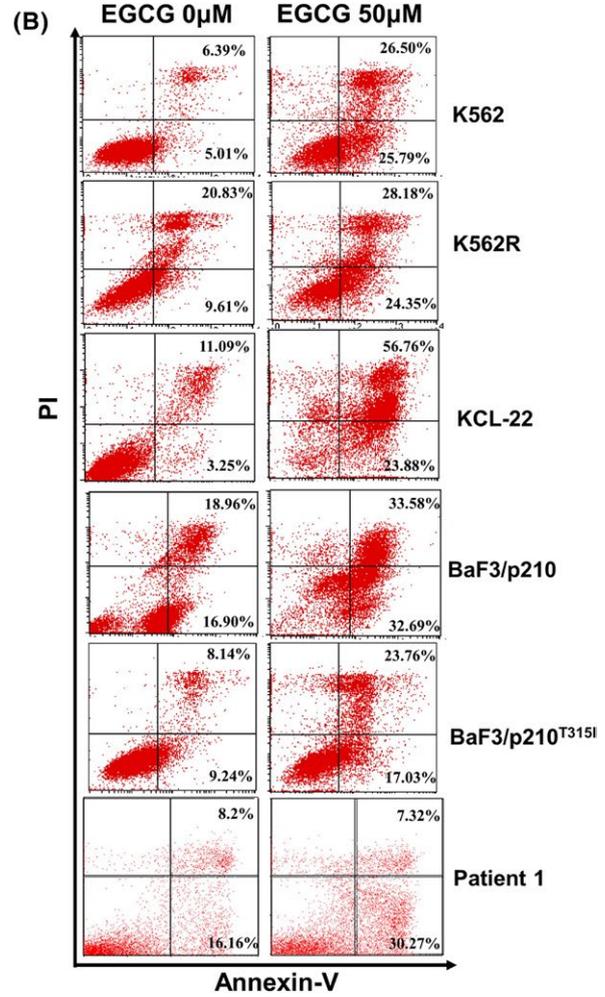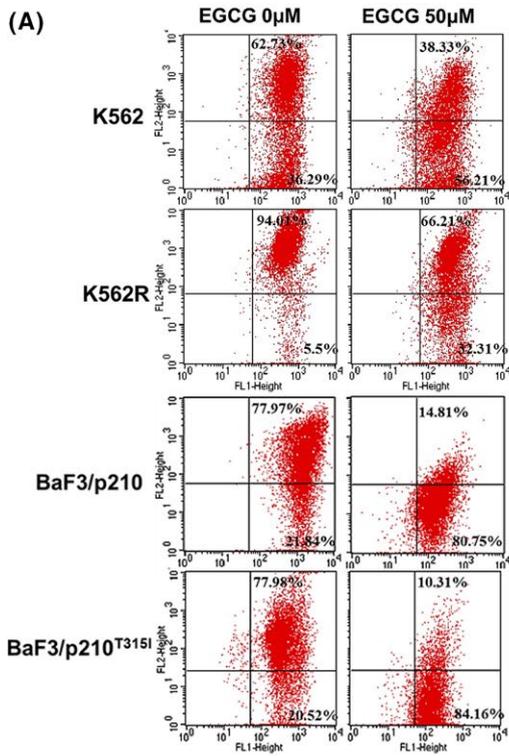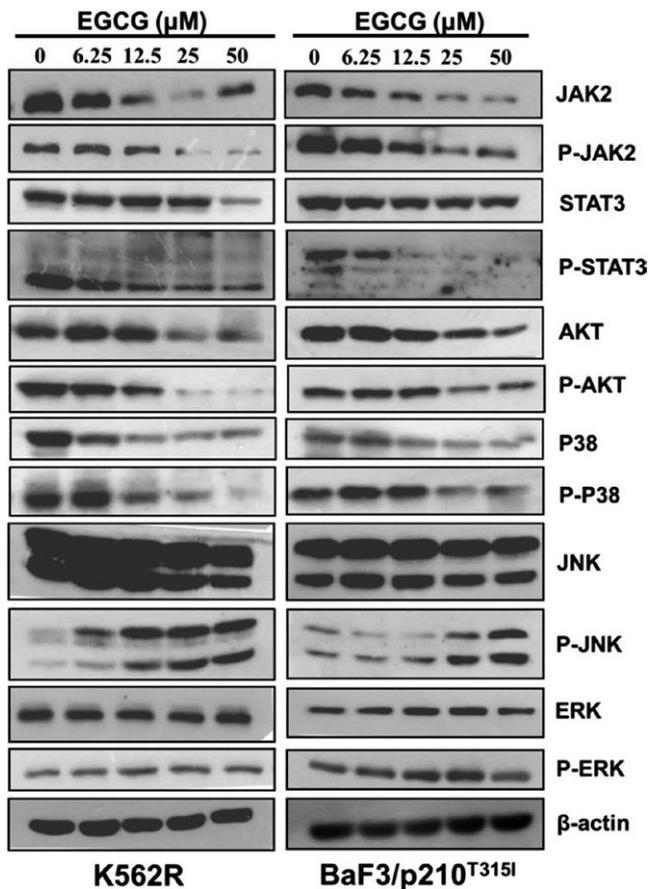
cells in a dose-dependent manner, suggesting that EGCG induced the degradation of the Bcr/Abl protein. The detected autophosphorylation of Bcr/Abl was also inhibited by EGCG, indicating inhibition of ABL kinase activity (Figure 3B). Consequently, we confirmed that EGCG's antileukaemic activity was related to Bcr/Abl expression. In this study, we used imatinib in K562R and nilotinib in BaF3/p210[T315I] cells as a negative control, whereas a third-generation ABL TKI ponatinib was provided as a positive control in BaF3/p210[T315I] cells. Ponatinib has been approved for affecting both unmutated and mutated Bcr/Abl; it is effective against T315I-mutant cells.[24] We observed that EGCG even had a stronger effect on the Bcr/Abl oncoprotein than ponatinib in BaF3/p210 cells harboring T315I mutant.

## 2.5 | EGCG regulates Bcr/Abl downstream JAK2/STAT3/AKT and p38-MAPK/JNK signalling pathways in CML

Bcr/Abl-dependent signal transduction is critical to the vigorous proliferation of CML cells. It has been reported that activation of JAK2 is very important for Bcr/Abl-mediated oncogenicity and may be a therapeutic target. STAT3 is activated by tyrosine kinase JAKs, which are signalling molecules that are located downstream in Bcr/Abl signalling pathways. To assess the effect of EGCG on the JAK2/STAT3/AKT signal network, western blot analysis was completed. As shown in Figure 4, EGCG reduced JAK2 and AKT expression in a dose-dependent manner
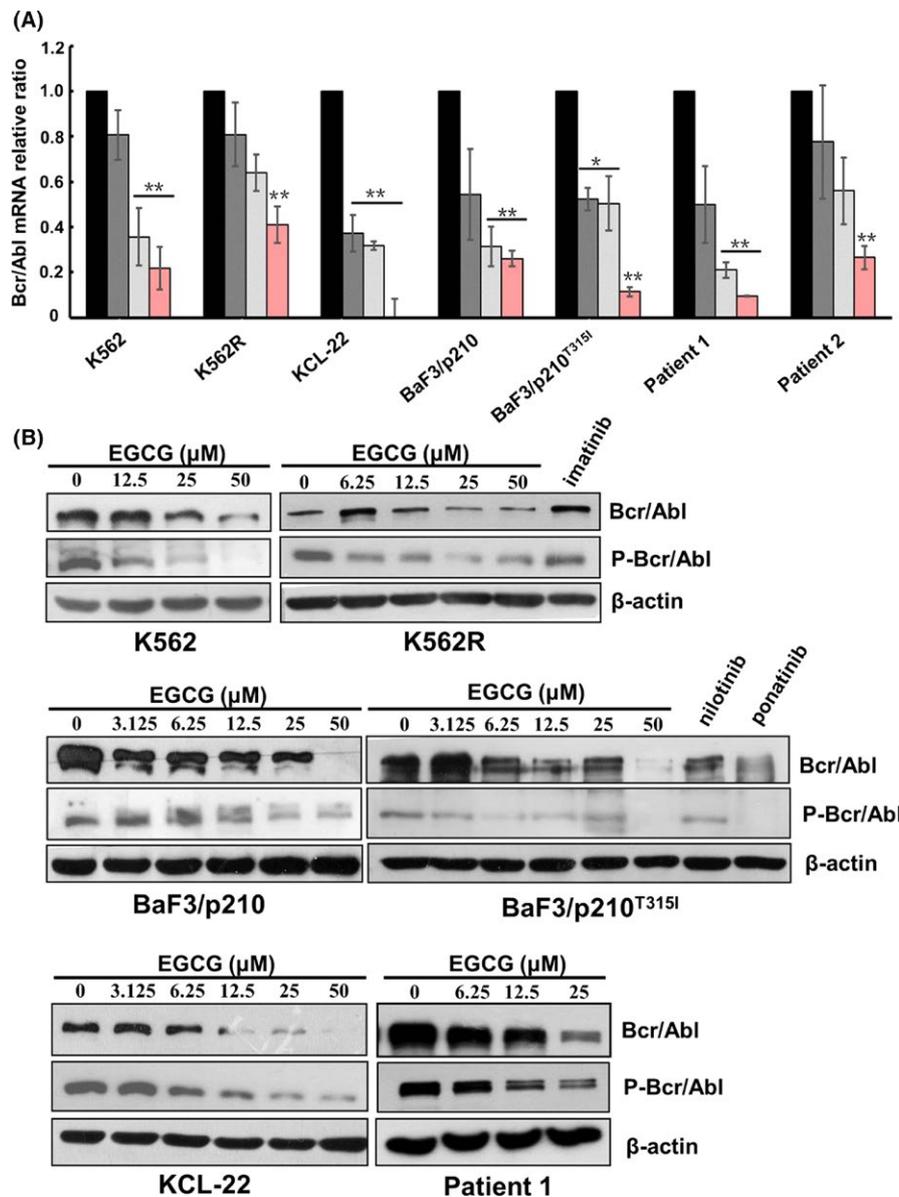


**FIGURE 3** Effect of epigallocatechin-3-gallate (EGCG) on the expression of the Bcr/Abl fusion gene in chronic myeloid leukaemia (CML) cells. The cells were starved in RPMI1640 supplemented with 0.1% FBS. Then, the cells were cultured with different concentrations of EGCG for 48 h. A, Real-time PCR was performed to determine the mRNA level of the Bcr/Abl gene. The relative level was normalized to the value of β-actin. The results are expressed as fold changes compared to the control. The data represent the mean ± SD of three independent experiments performed in triplicate. (■) 0 μmol/L; (■) 6.25 μmol/L; (□) 12.5 μmol/L; (■) 25 μmol/L. B, Western blot was performed to determine the expression of Bcr/Abl, P-Bcr/Abl and β-actin protein. $^*P < 0.05$, $^{**}P < 0.01$

## 3 | DISCUSSION

Epigallocatechin-3-gallate (EGCG), a major component that is derived from green tea polyphenols, is nature's gift molecule endowed with anticancer effects, which has received much attention in recent years. Large amounts of encouraging data from in vitro and animal models have emerged, suggesting that EGCG has the ability to activate cell death and induce apoptosis by perturbing multiple cellular signalling pathways in tumour cells. It has also been documented that the mechanisms of EGCG's effects on cancer cells are very different among cancer cell types. EGCG exerts potent antitumor activity in haematologic malignancies, including several types of leukaemia.[25-31] Jung et al. revealed that EGCG and its derivative EGCG-MP (EGCG-mono-palmitate) suppressed the phosphorylation of Bcr/Abl via SHP-1 or a tyrosine kinase-mediated pathway in a dose- and time-dependent manner in K562 cells.[32] Iwasaki et al. showed that EGCG predominantly caused necrosis-like cell death via a caspase-independent mechanism in K562 and C2F8 cells.[33] However, the exact role of EGCG in chronic myeloid leukaemia, especially in imatinib-resistant CML cells, is less documented.

In the present study, we found that EGCG exhibited potent antileukaemia activities towards Bcr/Abl-positive cells, including CML cell lines and primary bone marrow cells from CML patients. EGCG effectively inhibited the proliferation of K562 cells, imatinib-resistant K562R cells and BaF3 CML cells bearing wild-type or T315I mutant Bcr/Abl (Figure 1). More importantly, we observed that EGCG exhibited strong effects on primary bone marrow cells from newly diagnosed CML patients but did not cause significant normal bone marrow cell death, indicating that EGCG has low or no general cytotoxic effect on normal haematopoiesis (Figure 1B). Although the introduction of first-generation TKI imatinib greatly improved the survival of patients with CML, many patients developed resistance to it. Some of the patients are de novo resistant to imatinib, while others exhibit a good response in the beginning but fail to respond during disease progression.[5,6,8,10] Almost half of the imatinib-resistant patients develop point mutations in the Bcr/Abl gene. Although the second- and third-generation tyrosine kinase inhibitors nilotinib and dasatinib have been proven to be largely successful in overcoming imatinib resistance, the ABL kinase domain mutation T315I-mediated resistance cannot be overcome by currently available clinical drugs. The most important findings from our study are that EGCG could inhibit the growth of both imatinib-resistant K562R cells and BaF3 cells carrying T315I-mutated Bcr/Abl, indicating that EGCG may be a possible candidate to treat imatinib-resistant CML cells and to overcome T315I resistance.

Generally, apoptosis is induced via two pathways, a cell death extrinsic pathway and a mitochondria-dependent intrinsic pathway. Previous reports have concluded that EGCG induced caspase-mediated apoptosis in several solid tumour cells, including chondrosarcoma, gastrointestinal stromal tumour, cholangiocarcinoma, esophageal cancer, pancreatic cancer and prostate cancer.[34-38] EGCG also induced apoptosis in several types of leukaemia. Cornwall et al. tested and showed that EGCG differentially induces



**FIGURE 4** Effects of epigallocatechin-3-gallate (EGCG) on JAK2/STAT3/AKT and p38-MAPK/JNK pathways in imatinib-resistant chronic myeloid leukaemia (CML) cells. Western blot assay was performed to evaluate the levels of JAK2, STAT3, AKT, p38, JNK, ERK and their phosphorylated forms. β-Actin was measured as a control. EGCG downregulated JAK2 and AKT expression in a dose-dependent manner and inhibited the phosphorylation of JAK2, STAT3 and AKT in imatinib-resistant K562R and BaF3/p210[T315I] cells. EGCG could upregulate p-JNK level and inhibit p-P38 expression in a dose-dependent manner, while the p-ERK change was not significant

in CML cells. Importantly, EGCG inhibited the phosphorylation of JAK2 and AKT in all CML cell lines, including imatinib-resistant K562R and BaF3/p210[T315I] cells. The continuous exposure of the cells to EGCG obviously blocked the phosphorylation of STAT3, whereas there was no decrease in STAT3 protein. We also examined three members of the mitogen-activated protein kinase (MAPK) family: P38, JNK and ERK. Our results demonstrated that EGCG was able to significantly reduce the levels of P38 and phospho-P38 in CML cells in a dose-dependent manner. Interestingly, although EGCG had no effect on JNK or ERK proteins, it evidently upregulated the expression of phospho-JNK. Thus, the main effect of EGCG is to abolish p-P38 and activate p-JNK in CML cells. These results, taken together, indicated that proliferation inhibition and apoptosis induction induced by EGCG in CML cells were associated with P38-MAPK/JNK signalling regulation and, in part, could disturb the JAK2/STAT3/AKT pathway, helping to facilitate the growth arrest of Bcr/Abl+ leukaemia cells.

apoptosis in chronic lymphocytic leukaemia (CLL) B- and T-cells, but not in healthy B cells and T cells, in a dose-dependent manner.[30] Lee et al. studied the impact of EGCG on VEGF receptor status and on the viability of CLL B cells. EGCG significantly increased apoptosis in 8 of 10 CLL samples as measured by annexin V/(PI) staining with caspase-3 activation and PARP cleavage.[26] Borutinskaitė et al. demonstrated EGCG's ability to inhibit acute promyelocytic leukaemia (APL) cell proliferation and cause apoptosis.[39] In our research, EGCG induced Bcr/Abl-unmutated, T315I-mutated, and imatinib-resistant CML cell apoptosis. Our data demonstrated increased annexin V-FITC-positive cells and changes in mitochondrial membrane permeability induced by EGCG. However, EGCG did not modulate the caspase-dependent apoptotic pathway in chronic leukaemia cells that were caspase activation and PARP cleavage, the hallmarks of the apoptotic pathway, which are consistent with findings made by Iwasaki et al.[40]

Apoptosis-inducing factor (AIF), belonging to the FAD-dependent oxidoreductase family, is the major effector of caspase-independent cell death programs. AIF is localized to the mitochondrial intermembrane space and has a dual role in controlling cell survival and death. In response to specific death signals, mitochondrial AIF is released into the cytosol and is translocated to the nucleus, where it induces apoptosis in a caspase-independent manner. We analyzed the level of AIF in total cellular proteins and in mitochondrial and nuclear fractions from EGCG-treated imatinib-resistant cells. AIF clearly increased in the mitochondrial and nuclear fractions compared to the control in K562R and BaF3/p210$^{T315I}$ cells. We consider that imatinib-resistant cells undergo AIF-mediated apoptosis with EGCG treatment, which is peculiar and differs from classical apoptosis.

Autophagy, a lysosome-mediated intracellular degradation pathway, could trigger apoptosis and/or necrosis. Autophagy gene (Atg5), an E3 ubiquitin ligase, is required for autophagy function. Our data demonstrated the increased abundance of Atg5 in EGCG-treated leukaemia cells, consistent with the amount of LC3 (Figure 2E), indicating that autophagy function is increased and may be involved in EGCG-mediated cell apoptosis.

The Bcr/Abl fusion gene is a causative oncogene in CML. Accumulating evidence shows that the constitutive tyrosine kinase activity of Bcr/Abl is essential for its leukemogenic activity.[1,2,41] Bcr/Abl could promote the survival, proliferation and adhesion of leukemic cells through the upregulation of downstream pathways.[42,43] Numerous signal transduction pathways activated by Bcr/Abl, such as JAK/STAT, ERK/MAPK and PI3K/AKT pathways, have been implicated.[44-48] To explore the molecular mechanism involved in the EGCG-mediated effect on the response of CML cells, we detected Bcr/Abl and its downstream proteins in imatinib-sensitive and imatinib-resistant cell lines. Our study revealed for the first time that the mRNA and protein expression of Bcr/Abl was inhibited by EGCG in a dose-dependent manner in Bcr/Abl-unmutated, T315I-mutated, and imatinib-resistant CML cells. Bcr/Abl is an oncoprotein that is capable of autophosphorylation. Treatment with EGCG inhibited the active form of Bcr/Abl (p-Bcr/Abl) in all CML cell lines. We even

observed that EGCG had a stronger effect on Bcr/Abl than did ponatinib in BaF3/p210$^{T315I}$ cells.

Janus kinase/signal transducer and activator of transcription (JAK/STAT) is the most common signalling pathway constitutively activated by Bcr/Abl kinase.[49-53] JAKs lead to the recruitment and activation of STAT3, and activated STAT3 promotes tumourigenesis by blocking apoptosis and enhancing proliferation. Jung et al[32] reported that EGCG-MP suppressed the phosphorylation of STAT3 in a concentration-dependent manner in K562 cells. AKT activation has also been identified as a key signalling molecule in Bcr/Abl-mediated leukaemogenesis.[54] AKT remained phosphorylated and activated in CML CD34 + cells even in the presence of Bcr/Abl inhibitors, which contributes to poor survival in CML.[55] The cooperation of STAT3 and AKT signalling pathways is critical during disease progression and the evolution of drug resistance. In our study, we found that EGCG reduced JAK2 expression in a dose-dependent manner in CML cells. Significantly, EGCG can clearly downregulate the expression of phos-JAK2 and phos-STAT3 protein. Therefore, it appears that the inhibition of JAK2 and STAT3 signalling pathways can lead to significant suppression of CML cell growth. In parallel to the inhibition of JAK2 phosphorylation, exposing CML cells to EGCG resulted in a significant decrease in the total and phosphorylated levels of AKT protein, suggesting that the interruption of JAK2/STAT3/AKT axes by EGCG contributes to the growth inhibition and diminished survival of Bcr/Abl+ leukaemia cells.

Among the downstream signalling pathways activated by Bcr/Abl tyrosine kinase, the abnormal activation of the mitogen-activated protein kinase (MAPK) pathway also plays an important role in the occurrence and development of CML. MAPK is involved in the regulation of cell proliferation and differentiation. There are three branches of the MAPK cascade, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAP kinase. ERK mainly responds to growth factors and promotes cell growth. JNK and p38 MAPK have multiple effects, depending on the stimulus and the environment in which activation occurs.[56] p38 can play a dual role as a regulator of cell death in CML through various mechanisms. On one hand, p38 mediates cell survival and inhibits apoptosis in CML cells treated with arsenic trioxide.[57] On the other hand, some reports have suggested that p38 exerts a proapoptotic role in CML cells treated with imatinib and dasatinib.[58,59] It is noteworthy that in our study, the exposure of K562, K562R, BaF3/p210 and BaF3/p210$^{T315I}$ cells to EGCG resulted in diminished phospho-p38 expression and increased phospho-JNK levels.

Taken together, our data indicated that EGCG dramatically inhibited p-JAK2, p-STAT3 and p-AKT expression and upregulated p-JNK levels in Bcr/Abl+ cells, which may be attributed to the crosstalk of different signalling pathways, including proliferation, adhesion, and apoptosis.

In conclusion, we have documented the anti-CML effects of EGCG on imatinib-sensitive and imatinib-resistant Bcr/Abl+ cells, especially in T315I-mutated cells. We delineated that EGCG was able to inhibit CML cell growth without influencing the

caspase-dependent apoptotic pathway. The underlying mechanisms of EGCG's anti-CML activity were involved in the inhibition of JAK2/STAT3/AKT signals and regulation of the p38-MAPK/JNK pathway accompanied by repression of Bcr/Abl. Based on these findings, we believe that EGCG, either alone or in combination with the standard therapy, could be an alternative strategy for the management of CML, which may have great clinical significance in overcoming imatinib resistance. However, further investigations are needed to validate our findings on the mechanisms of EGCG-induced reversal of imatinib resistance in appropriate animal models.

# 4 | MATERIALS AND METHODS

## 4.1 | Reagents and antibodies

EGCG with a purity of up to 95% was purchased from SigmaAldrich (St Louis, MO, USA) and dissolved in sterile distilled water to prepare a stock solution (10 mmol/L). Imatinib mesylate and nilotinib were kindly provided by Novartis Pharma (Basel, Switzerland). A stock solution (10 mmol/L) was prepared in sterile distilled water. Ponatinib (AP24534) was purchased from Selleck (Boston, MA, USA). A stock solution (20 mmol/L) was prepared with DMSO (Sigma, St Louis, MO, USA). All drug reagents were stored in aliquots at −20°C. Antibodies against Bcr/Abl, phospho-Bcr/Abl, p-38, phospho-p38, AKT, phospho-AKT, JAK2, phospho-JAK2, STAT3, phospho-STAT3, ERK, phospho-ERK, JNK, phospho-JNK, caspase-3, caspase-9, Bcl-2, cleaved PARP, histone H3, Atg5 and LC3 were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies for AIF were purchased from Selleck. The HSP60 antibody was purchased from Santa Cruz (Dallas, TX, USA). Anti-β-actin antibody was purchased from SigmaAldrich. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (Sigma) was dissolved in PBS and stored at −20°C.

## 4.2 | Patients

Primary CML cells were harvested from bone marrow samples of six newly diagnosed CML patients, and bone marrow mononuclear cells (BMMCs) were isolated by Ficoll-Paque isolation solution. CML was diagnosed according to clinical and laboratory criteria. All patients provided written informed consent for the collection of samples and subsequent analysis. This study was conducted according to the principles expressed in the Declaration of Helsinki and approved by the Institutional Review Board of the Second Xiangya Hospital, Central South University.

## 4.3 | Cell lines

The parental K562 cell line (Bcr/Abl fusion expression) was maintained in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal calf serum, 2 mmol/L L-glutamine, and penicillin-streptomycin (100 U/mL and 100 U/mL, respectively) at 37°C in a humidified atmosphere of 5% $CO_2$. Imatinib-resistant

K562R cells were kindly provided by Prof. Guangbiao Zhou (Institute of Zoology, Chinese Academy of Science, Beijing, China) and maintained in medium containing 1 mmol/L imatinib, which was cultured in drug-free medium for 2 weeks before all experimental procedures. KCL-22 cells and BaF3 cells stably expressing Bcr/Abl WT (BaF3/p210) and Bcr/Abl T315I mutant (BaF3/p210^T315I) were kindly provided by Prof. Wenli Feng (Department of Clinical Haematology, Key Laboratory of Laboratory Medical Diagnostics Designated by the Ministry of Education, Chongqing Medical University, China) and cultured in a similar medium. Prior to treatment with different concentrations of EGCG, all cells were starved in RPMI-1640 supplemented with 0.1% FBS at 37°C in an atmosphere containing 5% $CO_2$ for 24 hours.

## 4.4 | Cell proliferation assay

Briefly, cells ($5 \times 10^4$ per well) were plated in triplicate wells with 200 µL RPMI 1640 medium supplemented with 10% FBS in 96-well microplates and treated with different concentrations of EGCG (0, 6.25, 12.5, 25, 50, or 100 µmol/L) for 24 or 48 hours. Cell proliferation was evaluated by MTT assay. IC50 values were determined using the nonlinear regression program CalcuSyn (Cambridge, UK). Cell viability was calculated as the percentage of viable cells in the EGCG-treated group versus that in the untreated control.

## 4.5 | Mitochondrial transmembrane potential assay

Cells were seeded in 6-well plates and incubated with 50 µmol/L EGCG for 24 hours. The mitochondrial membrane potential (MMP, ΔΨ) was determined using a JC-1 Apoptosis Detection Kit (KeyGEN BioTECH, Nanjing, China). Briefly, $1 \times 10^6$/mL cells were incubated with JC-1 at 37°C for 15 minutes. After washing with PBS three times, the fluorescence was measured by a FACS Calibur flow cytometer with FL1 and FL2 channels.

## 4.6 | Apoptosis assay

Cells ($1 \times 10^6$/mL) were seeded in 6-well plates and incubated with 50 µmol/L EGCG for 48 hours. Cells undergoing early and late apoptosis were assessed with an Annexin V-FITC/Propidium Iodide (PI) Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) by flow cytometry.

## 4.7 | Real-time PCR assay

Total RNA from EGCG-treated and untreated cells was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), and complementary DNA was synthesized according to a previously published method. Transcribed cDNA was amplified and quantified with real-time quantitative PCR using a SYBR Green qPCR Kit (TaKaRa, Tokyo, Japan). Primers for Bcr/Abl and β-actin were synthesized as previously described.[48,60]

## 4.8 | Protein extraction

After the treatment of cells with EGCG, the total protein, mitochondrial fractions and nuclear fractions were prepared. Mitochondria were isolated using a mitochondria isolation kit (Thermo Fisher Scientific, Waltham, MA, USA). Cells and mitochondria were homogenized on ice in lysis buffer (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1% NP-40, 0.25% Na-deoxycholate, 5 mmol/L EDTA, 1 mmol/L NaF, 25 mmol/L $Na_3VO_4$, 0.1 mmol/L PMSF and 2 mg/mL aprotinin). Then, the contents were incubated on ice for 30 minutes with intermittent mixing, after which they were centrifuged for 15 minutes at 4°C at 14 000 $g$. The supernatant was collected as total protein or the mitochondrial fraction. For collecting the nuclear fraction, the cells were washed with cold phosphate-buffered saline and suspended in lysis buffer (10 mmol/L Hepes (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 2.0 mg/mL leupeptin, 2.0 mg/mL aprotinin, and 0.5 mg/mL benzamidine). The cells were incubated on ice for 15 minutes, after which 10% Nonidet P-40 was added, and the contents were mixed and then centrifuged for 1 minute at 4°C at 14 000 $g$. The supernatant was saved as the "postnuclear fraction". The nuclear pellet was resuspended in ice-cold nuclear extraction buffer (20 mmol/L Hepes (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF, 2.0 mg/mL leupeptin, 2.0 mg/mL aprotinin, and 0.5 mg/mL benzamidine), incubated on ice for 30 minutes with intermittent mixing, and then centrifuged for 5 minutes (14 000 $g$) at 4°C. The supernatant (nuclear extract) was stored at −80°C. The protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific).

## 4.9 | Western blot

Equal amounts of protein were separated by 8% or 10% SDS-PAGE and transferred onto nitrocellulose membranes. After blocking the membranes with 5% fat-free milk, they were probed with various primary antibodies and HRP-conjugated secondary antibodies and visualized with enhanced chemiluminescence detection reagents (Thermo Fisher Scientific, Waltham, MA, USA).

## 4.10 | Statistical analysis

Values are expressed as the means ± SD. Comparisons between two groups were analyzed by two-tailed Student's $t$-test using SPSS 17.0 (Chicago, IL, USA). Statistical significance was defined as a $P$-value <0.05.

## CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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