Abstract. Although the introduction of tyrosine kinase inhibitors greatly improved the survival of patients with chronic myeloid leukemia (CML), drug resistance remains a problem. Thus, mechanism-based novel therapeutic targets warrant exploration. Recently, epidermal growth factor receptor kinase substrate 8 (EPS8), which has been identified as an oncogene and plays an important role in a broad spectrum of solid tumours, was reported to be related to poor prognosis or chemoresistance in acute leukemia patients. However, its role in CML remains unclear. In the present study, using q-RT-PCR, we demonstrated that CML patients expressed a higher level of EPS8 mRNA in bone marrow mononuclear cells than healthy controls. Then, to determine the effect of EPS8 on the biological functions of CML cells, EPS8 expression was knocked down in the human CML cell line K562. Reduced proliferation, increased apoptosis, impaired adhesion and migration were observed in K562 cells after EPS8 silencing. Notably, attenuation of EPS8 increased chemosensitivity both in imatinib-sensitive K562 cells and in the imatinib-resistant murine BCR-ABL+/32D-p210BCR/ABL-T315I cells. Mechanistically, knockdown of EPS8 downregulated p-BCR/ABL and its downstream AKT/mTOR signalling pathway. Finally, knockdown of EPS8 attenuated K562 cell proliferation in BALB/c nude mice. These data indicated that EPS8 regulated the proliferation, apoptosis and chemosensitivity in BCR-ABL positive cells via the BCR-ABL/PI3K/AKT/mTOR pathway.

Introduction

Chronic myeloid leukemia (CML) is a BCR-ABL oncogene-driven malignant disease, characterized by markedly elevated immature myeloid cells in bone marrow and peripheral blood. CML cells progress slowly in the chronic phase (CP) for about one to three years, and then proliferate more rapidly stepping into the accelerated phase (AP) with an increase in blast numbers. The accelerated phase lasts for only several months and eventually converts to acute leukemia in the blast crisis phase (BC) with more aggressive characteristics than de novo acute leukemia. Most CML patients respond well to the tyrosine kinase inhibitor (TKI) imatinib in the chronic phase, however, ~20-30% patients develop resistance to imatinib (1-3). Some of the patients are de novo resistant to imatinib, others exhibit a good response in the beginning, however this response is lost with the progression of this disease. Almost half of the imatinib-resistant patients develop point mutations in the BCR/ABL gene during the course of TKI treatment. Other drug resistance mechanisms include BCR-ABL amplification, additional acquired gene mutation and drug efflux (4,5). Second and third generation tyrosine kinase inhibitors such as dasatinib, ponatinib, are able to overcome imatinib resistance in some patients. However, some mechanisms, for example, BCR/ABL point mutation T315I-mediated resistance cannot be overcome by current available clinical drugs thus highlighting the need for further research on the mechanism of leukemogenesis of CML cells in order to explore novel mechanism-based strategies with high efficacy and low toxicity.

Epidermal growth factor receptor kinase substrate 8 (EPS8) is a cytoplasmic protein that acts as a substrate of receptor and non-receptor tyrosine kinases such as EGFR, FGFR, VEGFR and Src kinase. EPS8 functionally serves as an adaptor protein associating with diverse partner proteins to form complexes that regulate multiple signalling pathways. Physiologically, EPS8 forms a complex with Abi-1 and SOS-1 to regulate the Rac signalling pathway promoting cytoskeletal remodelling. EPS8 also plays a role in membrane flow, pseudopodium...
formation, morphogenesis of microvilli, stereocilia function and length, cellular adhesion and motility (6). Furthermore, EPS8 has been identified as an oncogene, as it enables cellular transformation in vitro and tumour formation in vivo upon overexpression (7). EPS8 has been documented to be highly expressed in a broad spectrum of solid tumours, such as squamous carcinoma, cervical cancer, colon carcinoma, and breast cancer (8-12). However, only a few studies have addressed the role of EPS8 in haematological malignancies. Microarray analysis by Kang et al revealed that a high level of EPS8 predicted a poor prognosis of infant acute lymphoblastic leukemia (ALL) patients with MLL rearrangements (13). In addition, we previously determined that increased expression of EPS8 mRNA in bone marrow was related to a poor response to chemotherapy and a poor prognosis in acute myeloid leukemia (AML) and ALL patients (14,15). However, it remains unclear whether EPS8 is implicated in CML and length, cellular adhesion and motility (6). Furthermore, the G1 phase and reduced adhesion and migration. Notably, silencing EPS8 increased chemosensitivity both in the imatinib sensitive cell line K562 and the resistant cell line 32D-p210 T315I and 32D-p210-WT myeloid precursor cell lines, respectively. These data revealed that EPS8 regulated the cell biology of CML. Targeting EPS8 alone or combined with TKI may be promising therapeutic strategies for refractory and relapsed CML patients.

Materials and methods

Cell lines and human samples. Bone marrow mononuclear cells were collected from patients with CML at the Department of Hematology of Zhujiang Hospital, Southern Medical University from 2013 to 2015. Some of the RNA samples were purchased from Kingmed Diagnostics (Guangzhou, China). In total, 113 cases of CML (male n=60, female n=53) including 32D-p210BCR/ABL-T315I. Mechanistically, knockdown of EPS8 downregulated p-BCR/ABL and its downstream AKT/mTOR signalling pathway. Notably, knockdown of EPS8 attenuated K562 cell proliferation in BALB/c nude mice. Collectively, these data revealed that EPS8 regulated the cell biology of CML. Targeting EPS8 alone or combined with TKI may be promising therapeutic strategies for refractory and relapsed CML patients.

Cell proliferation assay. Cells (1x10⁶ cells/well) were plated in 96-well plates in quadruplicate and cultured in 10% FCS-containing medium with or without the indicated concentrations of drugs. At 0, 20, 44 and 68 h, 10 µl of CCK-8 solution was added to the cells, which were then incubated for an additional 4 h. Then, the OD₄₅₀ values were obtained using a microplate reader.

Apoptosis assay. Cells (5x10⁵) were collected, and 1.24 µl of Annexin V-APC was added to the cells. The cells were incubated at room temperature for 15 min, centrifuged at 1,000 x g for 5 min, and the supernatant was discarded. The cells were then resuspended in 0.5 ml of cold 1X binding buffer. Ten microliters of 7-ADD or propidium iodide (PI) was added then resuspended in 0.5 ml of cold 1X binding buffer. Ten microliters of 7-ADD or propidium iodide (PI) was added to the cells, which were then incubated for an additional 4 h. Then, the OD₄₅₀ values were obtained using a microplate reader.

Cell cycle analysis. Cells (1x10⁶) were collected and washed twice with cold PBS. Then, cold 70% ethanol was added to the cells, which were incubated overnight at 4°C. The following day, the cells were washed once with PBS, and 500 µl of
PBS containing 50 µl/ml PI, 100 µg/ml RNase A, and 0.2% Triton X-100 were added, and the cells were incubated for 30 min at 4°C in the dark. DNA content was detected by flow cytometry.

Adhesion and migration assay. The 96-well plates were coated with fibronectin (30 mg/l) and dried overnight. PBS (20 µl) containing 3% BSA was added to the 96-well plate at 37°C for 2 h, and then removed. Two hundred microliters of cells (0.5x10⁶ cells/ml) suspended in RPMI-1640 and 10% FBS were added to the 96-well plates and incubated for 1.5 h; the plate was then washed gently. The cells that adhered to the bottom of the 96-well plate were observed under a microscope, and the number of adherent cells was assessed using the CCK-8 assay.

The cell migration assay was performed as previously described. Briefly, the cells were suspended in serum-free RPMI-1640 containing 0.1% BSA at a concentration of 1x10⁶ cells/ml. A suspension of 0.1 ml of cells was added to the upper chamber, and the cells were allowed to migrate to the lower chamber, which contained RPMI-1640 and 10% FBS, for 6-8 h at 37°C in a 5% CO₂ incubator. The cells that had migrated to the lower chamber were observed under a microscope. The number of cells in the lower chamber was assessed using the CCK-8 assay.

Chemotherapy drug sensitivity assay. The cells (1x10⁴ cells/100 µl/well) were plated onto 96-well culture plates in quadruplicate in the presence of different concentrations of either daunorubicin or imatinib for the indicated time-points. Cell viability was assayed by adding CCK-8 solution. The percentage of cell viability was evaluated by assessing the absorbance at 450 nm and normalized to the corresponding untreated control.

Western blotting. Total protein lysates were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membranes. The blots were subjected to a standard immunodetection procedure using specific antibodies. The primary antibodies used were as follows: ERK (rabbit, monoclonal, 1:1,000; cat. no. ab196883), p-ERK (rabbit, polyclonal, 1:1,000; cat. no. ab214362) (both from Abcam, Cambridge, UK); STAT5 (rabbit, monoclonal, 1:1,000; cat. no. 94205), p-STAT5 (rabbit, polyclonal, 1:1,000; cat. no. 9459; CST); and GAPDH (mouse, monoclonal, 1:1,000; cat. no. KC-5G4; KangChen Bio-tech).

Statistical analyses. The data are reported as the mean ± standard deviations (SDs). One-way ANOVA followed by the Student-Newman-Keuls' pairwise multiple comparison test were used to compare the EPS8 mRNA expression among CML patients at different phases with healthy donors, as well as differences obtained in the proliferation, apoptosis, cell cycle, adhesion and migration assays. Spearman’s rank correlation coefficient was used to analyze the correlation between EPS8 mRNA expression and clinical characteristics. A two-tailed independent Student’s t-test was used to compare the means in the chemotherapy drug sensitivity assay. Differences were considered significant when P<0.05.

Results

CML patients express a high level of EPS8 mRNA. A published microarray data of 91 cases of CML patients reported by Radich et al (16) revealed an increase in EPS8 mRNA expression of CML patients compared to healthy controls, although the difference was not statistically significant. Furthermore, there was a significant increase in EPS8 expression in the blast crisis phase of CML patients compared to healthy controls. To validate the result of these microarray data, we assessed EPS8 mRNA expression in bone marrow mononuclear cells from 113 cases of CML in CP (50 cases), AP (21 cases), BC (21 cases), CR (21 cases), and 21 healthy controls using quantitative real-time PCR. CML patients in CP, AP and BC exhibited significantly higher expression of EPS8 than those in complete remission and healthy donors (P<0.05). Moreover, there was a tendency for patients in BC to have increased EPS8 expression compared to patients in CP and AP (P=0.05) (Fig. 1A). We further performed correlation analysis between EPS8 expression and clinical features. There was no significant difference in EPS8 expression between the sex (P=0.74), age (P=0.85) and BCR-ABL level (P=0.98) of CML patients, however there was a correlation to blast percentage in bone marrow (P=0.03) (Fig. 1B and C; Table I). Collectively, these data revealed that EPS8 may play a role in CML leukemogenesis.

Knockdown of EPS8 inhibits CML cell proliferation, induces apoptosis and arrests the cell cycle in the G1 phase. To evaluate the effect of EPS8 on the biological function of CML cells, we knocked down the expression of EPS8 in CML cell line K562. EPS8 expression was detected in three human CML...
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cell lines. The K562 and KBM5 cells expressed a higher level of EPS8, while the MEG01 cells exhibited a lower expression (Fig. 2A). Then, the K562 cells were transfected with a lentivirus carrying an EPS8-targeted shRNA vector to knockdown EPS8. As a result, EPS8 expression was attenuated in the K562-shRNA-EPS8 cells that were stably transduced with the lentivirus (Fig. 2B).

To investigate the role of EPS8 in CML cells, we assessed the proliferation, apoptosis and cell cycle after EPS8 silencing. We first examined the proliferation of K562-shRNA-EPS8 cells and determined that knockdown of EPS8 reduced the proliferation of the K562 cells (Fig. 2C). Then, we assessed cell apoptosis and the results revealed that the K562-shRNA-EPS8 cells exhibited enhanced apoptosis compared with their parental cells (Fig. 2D and E). Then, the cell cycle was evaluated and it was determined that more K562-shRNA-EPS8 cells were present in the G1 phase, and fewer cells were present in the S phase compared with the corresponding control cells (Fig. 2F). These data revealed that EPS8 regulated the proliferation, apoptosis and cell cycle in CML cells.

Attenuated EPS8 expression leads to impaired adhesion and migration. EPS8 plays an important role in cytoskeletal remodelling and cellular motility; therefore, we evaluated the effect of EPS8 on adhesion and migration of K562 cells. The K562 cells and their derived cells were plated on fibronectin-coated 96-well plates for 1.5 h and then washed. Less K562-shRNA-EPS8 cells remained on the fibronectin-coated plates compared with the control cells (Fig. 3A and B). Cellular migration was assessed using the Transwell assay, in which the cells were placed into the upper chamber, which contained serum-free medium, and the lower chamber contained medium with 10% FBS. Fewer K562-shRNA-EPS8 cells were observed in the lower chamber compared with the K562-scramble cells demonstrating that the attenuation of EPS8 expression impaired the migration of the K562 cells (Fig. 3C and D).

EPS8 is implicated in the chemosensitivity of CML cells. We then investigated the effect of EPS8 on the chemosensitivity of the K562 cells. Daunorubicin is a widespread used common chemotherapy drug to treat myeloid leukemia. In the present study we determined that the viability of the K562-shRNA-EPS8 cells was significantly reduced in the presence of indicated concentrations of daunorubicin indicating that EPS8 knockdown increases their chemosensitivity (Fig. 4A). However, as an imatinib-sensitive cell line, the K562-shRNA-EPS8 cells did not exhibit further increased sensitivity to imatinib except in the high drug concentration (Fig. 4B).

To investigate whether knockdown of EPS8 can overcome imatinib resistance, we employed a murine imatinib-resistant cell line 32D-p210T325I-BCR/ABL (32D-p210-T315I), which was generated by transfecting myeloid precursor cells with the vector carrying prototype imatinib-resistant BCR/ABL point mutation T315I in CML patients. Compared to the imatinib-sensitive cell line 32D-p210-BCR/ABL (32D-p210-WT) which expressed native BCR/ABL protein p210, 32D-p210-T315I cells expressed a higher level of EPS8 protein (Fig. 4C). To silence EPS8 expression, LV-EPS8-shRNA1 and LV-EPS8-shRNA2 vectors were transfected into 32D-p210-T315I cells, respectively. EPS8 expression was considerably decreased compared with the controls (Fig. 4C). The results revealed that knockdown

Table I. Correlation analysis between EPS8 expression and the clinical features of CML patients.

| Clinical features | EPS8 expression (mean rank) | P-value |
|-------------------|----------------------------|---------|
| Male (n=60)       | 49.83                      | 0.74    |
| Female (n=53)     | 48.99                      |         |
| Age (years)       | 0.019                      | 0.85    |
| p210              | 0.003                      | 0.98    |
| Blast percentage  | 0.246                      | 0.03    |

EPS8, epidermal growth factor receptor kinase substrate 8; CML, chronic myeloid leukemia.

Figure 1. EPS8 mRNA expression is elevated in CML patients. (A) Bone marrow mononuclear cells were collected from 113 cases of CML patients and 21 normal controls. qRT-PCR was performed to assess the expression of EPS8 mRNA. The ratio of EPS8 and 18srRNA was expressed using the 2^-ΔΔCt method. The relative expression of EPS8 mRNA was compared among CP (n=50), AP (n=21), BC (n=21) of CML and healthy controls (n=21). (B and C) Correlation analysis was performed between EPS8 mRNA expression with (B) the BCR-ABL transcript or (C) the blast percentage in bone marrow. *P<0.05 vs. the healthy control; **P<0.01 vs. the healthy control. CML, chronic myeloid leukemia; CP, chronic phase; AP, accelerated phase; BC, blast crisis phase; ns, non-significant.

Figure 2. EPS8 regulates proliferation, apoptosis and cell cycle in CML cells. (A) The K562 and KBM5 cells expressed a higher level of EPS8, while the MEG01 cells exhibited a lower expression. Then, the K562 cells were transfected with a lentivirus carrying an EPS8-targeted shRNA vector to knockdown EPS8. As a result, EPS8 expression was attenuated in the K562-shRNA-EPS8 cells. (B) The cell cycle was evaluated and it was determined that more K562-shRNA-EPS8 cells were present in the G1 phase, and fewer cells were present in the S phase compared with the control cells.

Figure 3. EPS8 regulates adhesion and migration. (A and B) The K562-shRNA-EPS8 cells exhibited impaired adhesion compared with the control cells. (C and D) The K562-shRNA-EPS8 cells exhibited impaired migration compared with the control cells.

Figure 4. EPS8 regulates chemosensitivity in CML cells. (A) Daunorubicin is a widespread used common chemotherapy drug to treat myeloid leukemia. In the present study, the viability of the K562-shRNA-EPS8 cells was significantly reduced in the presence of indicated concentrations of daunorubicin indicating that EPS8 knockdown increases their chemosensitivity. (B) However, as an imatinib-sensitive cell line, the K562-shRNA-EPS8 cells did not exhibit further increased sensitivity to imatinib except in the high drug concentration.

Table II. Correlation analysis between EPS8 expression and the clinical features of CML patients.

| Clinical features (mean rank) | P-value |
|-----------------------------|---------|
| Male (n=60)                 |         |
| Female (n=53)               |         |
| Age (years)                 |         |
| p210                        |         |
| Blast percentage            |         |

EPS8, epidermal growth factor receptor kinase substrate 8; CML, chronic myeloid leukemia.
of EPS8 reduced the cell viability in a dose- and time-dependent manner. Following 24 and 48 h of incubation, cell viability in the EPS8-shRNA1 or EPS8-shRNA2 groups was significantly lower than the scramble group. Therefore, downregulation of EPS8 increased the sensitivity of 32D-p210-T315I cells to imatinib (Fig. 4D and E).
Figure 4. Knockdown of EPS8 increases sensitivity to chemotherapy. K562 cells and their derivatives (1x10^4 cells/100 µl/well) were added to 96-well culture plates and treated with the indicated concentrations of (A) daunorubicin and (B) imatinib for 48 h. Viability was assessed with the CCK-8 assay. (C) EPS8 expression in 32D-p210-WT and 32D-p210-T315I cells were assessed by western blotting. LV-EPS8-shRNA1 and LV-EPS8-shRNA2 vectors were transfected into 32D-p210-T315I cells, respectively. EPS8 expression was revealed in 32D-p210-T315I-shRNA1-EPS8, T315I-shRNA2-EPS8, and the control cell lines. (D and E) 32D-p210-T315I cells and their derived cells (1x10^4 cells/100 µl/well) were added to 96-well culture plates and treated with the indicated concentrations of imatinib for (D) 24 h and (E) 48 h, respectively. Viability was assessed with the CCK-8 assay. Plots are representative of 3 independent experiments. ***P<0.001 vs. the scramble control.

Figure 5. EPS8 knockdown increases imatinib induced apoptosis of 32D-p210-T315I cells. (A) The 32D-p210-T315I cells and their derived cells were stained with Annexin V-APC and 7-AAD, and apoptosis was assessed by flow cytometry. (B) Statistical analysis of the percentage of apoptotic cells in each group. Plots are representative of 3 independent experiments. ***P<0.001 vs. the scramble control.
In accordance with these results, we determined that knockdown of EPS8 notably increased apoptosis in imatinib-treated 32D-p210-T315I cells in a dose-dependent manner. Apoptosis was markedly increased in EPS8-shRNA cells compared with the control groups after imatinib treatment for 24 h (Fig. 5A and B). These results indicated that EPS8 protected imatinib-resistant CML cells against drug-induced apoptosis.

EPS8 regulates apoptosis and proliferation signalling pathways in CML cells. To determine the molecular mechanism through which EPS8 regulates CML cells, key molecules in main signalling pathways involved in leukemogenesis were assessed. Notably, phosphorylated (p)-BCR-ABL was significantly decreased in EPS8-knockdown K562 cells compared with the control group, suggesting that EPS8 regulated BCR-ABL. Furthermore, our results illustrated that p-AKT and phosphorylation of its downstream proteins mTOR, eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and glycogen synthase kinase 3β (GSK3β), as well as p-STAT5 and p-ERK levels were reduced in K562-shRNA-EPS8 cells compared with the control cells. Since Mcl-1 and Bcl-2 function as anti-apoptotic markers in various cancers, we explored whether their expression was affected by EPS8. Indeed, we found that the expression of Mcl-1 and Bcl-2 was suppressed by EPS8 silencing. These data revealed that EPS8 inhibition-induced apoptosis was related with the reduction of anti-apoptotic factors and inhibition of proliferation and anti-apoptotic pathway activity (Fig. 6A).

Given that EPS8 regulated the anti-apoptotic signalling and increased imatinib-induced apoptosis in imatinib-resistant 32D-p210-T315I cells, we next investigated the effect of EPS8 on BCR-ABL expression and its downstream PI3K/Akt signalling pathway in 32D-p210-T315I cells and their derived cells. In accordance with K562-shRNA-EPS8 cells, 32D-p210-T315I-shRNA-EPS8 cells exhibited decreased total BCR-ABL and p-BCR-ABL proteins. Furthermore, EPS8 silencing led to a decrease of p-Akt (T308, S476), p-4EBP1 (T37/46), p-mTOR (S2448) and p-GSK3-β (Fig. 6B). Collectively, these data revealed that EPS8 knockdown interfered with the BCR-ABL/PI3K/AKT/mTOR pathway (Fig. 6C).
Knockdown of EPS8 attenuated the proliferation of K562 cells in vivo. Finally, we determined the efficacy of the inhibition of EPS8 on the proliferation of BCR-ABL positive cells in vivo. Since no EPS8 inhibitor was available, we utilized the shRNA to knockdown EPS8 in K562 cells in a xenograft model. BALB/c nude mice were implanted subcutaneously with 1x10^7 K562-scramble or K562-shRNA-EPS8 cells, and the tumor volume was observed every 3 days. The results revealed that the mice bearing K562-shRNA-EPS8 cells had a significantly smaller tumor volume compared to those bearing the K562-scramble cells (Fig. 7A). Consistently, tumor weight in mice harboring K562-shRNA-EPS8 cells at 24 days after inoculation was significantly lower than that in the control mice (Fig. 7B).

Discussion

EPS8 is a cytoplasmic non-receptor kinase and is well recognized as an oncogene in a variety of solid tumours (6). However, its role in haematological malignant diseases is less documented. A gene expression profiling study of 97 cases of infant ALL identified EPS8, along with six other genes as prognostic factors. The high-EPS8 cohort had an event-free survival of 20%, while the low-EPS8 cohort had a rate of 65% (13). Our previous study revealed similar results. We found that EPS8 expression in AML patients was elevated compared with the control group. Moreover, EPS8 expression was negatively related to the response of AML patients to chemotherapy (15). Furthermore, we addressed the expression of the EPS8 mRNA in 107 cases of ALL patients, and there was a correlation between the expression of EPS8 and MDR1, as well as EPS8 and WT1. A high EPS8/MDR1/WT1 expression profile was associated with a higher risk of relapse. In addition, patients with high expression of EPS8/MDR1/WT1 had a shorter event-free survival (14). Notably, consistent with the reported CML microarray data, we revealed in our present study that our cohort CML patients had a higher expression of EPS8 compared to the controls, indicating that EPS8 plays a role in CML disease. In addition, our data demonstrated that EPS8 mRNA expression was not correlated with BCR-ABL, suggesting that EPS8 may be a BCR-ABL independent factor for CML patients. However, our study did not contain follow-up clinical information, therefore future investigation to address the relationship between EPS8 expression and drug resistance or survival in CML patients is warranted.

To determine the role in CML cells, we knocked down the expression of EPS8 in CML cell line K562 and determined that silencing of EPS8 led to reduced proliferation, increased apoptosis and cell cycle arrest in the G1 phase which was consistent with other studies on EPS8. Xu et al reported that the stable overexpression of EPS8 accelerated proliferation and reduced the serum withdrawal-induced apoptosis of pituitary tumour cells (19). Wang et al found that EPS8 expression was increased in oral squamous carcinoma compared with normal keratinocytes. The NH4 cell line exhibited mildly elevated EPS8 expression and reduced invasion, while the NH12 cell line, which expressed a high level of EPS8, was extremely aggressive. EPS8 overexpression in NH4 cells led to proliferation that was similar to that of NH12 cells (20). Similar results were observed in other cancers. The results in the present study indicated that EPS8 was involved in CML as well.

One of the characteristics of leukemia cells is invasion to other organs. Cytoskeletal proteins have an important role in AML cell migration (21). EPS8 is an important regulator of the cytoskeleton and is reported to be related to the migration and invasion of many solid tumours by regulating MMP9, E-cadherin and N-cadherin (7). EPS8 was demonstrated to upregulate the expression of CXCL5 and CXCL12 via the transcription factor FOXM1 to enhance migration (22). Yap et al demonstrated that EPS8 promoted oral squamous cancer cell migration and invasion by activating Rac in an integrin-dependent manner (23). EPS8, Abi-1 and SOS-1 form a tricomplex and induce Rac-specific guanine nucleotide exchange factor (GEF) activity, which has been revealed to play a critical role in ovarian cancer metastasis (9). In the present study the ability of adhesion and migration was attenuated in K562-shRNA-EPS8 cells compared with the control, which was consistent with previous research indicating that EPS8 may contribute to migration from bone marrow and infiltration to other organs.
Gorsic et al revealed that EPS8 knockdown increased the chemosensitivity of lymphoblastic cell lines, small cell lung cancer cell lines and bladder cancer cell lines to cisplatin (24). Chen et al reported that EPS8 reduced the chemosensitivity of cervical cancer cells to cisplatin and paclitaxel (25). Notably, our results demonstrated that EPS8 regulated the sensitivity of CML cells, including imatinib-sensitive and imatinib-resistant cells, to chemotherapy and tyrosine kinase inhibitors.

To explore the molecular mechanism involved in the EPS8-mediated effect on the response of CML cells to imatinib, we detected BCR-ABL and its downstream proteins in imatinib-sensitive cell line K562 and imatinib-resistant cell line 32D-p210-T315I and their derivatives. EPS8 is a critical signaling molecule and integrates multiple pathways. EPS8 has been reported to regulate PI3K/AKT/mTOR (19,20) and MEK/ERK (8) signalling which control cell proliferation, survival, differentiation and apoptosis. Consistent with the evidence that EPS8 knockdown inactivated Akt and downstream 4EBP1 phosphorylation in colon cancer cells (26), our study provided an extension of this notion, revealing for the first time that the phosphorylation of Akt (S308, S476), GSK3-β (S9), and 4EBP1 (T37/46) were decreased in CML cells after EPS8 silencing. Notably, we determined that the active form of the oncoprotein BCR-ABL was decreased after EPS8 knockdown, highlighting the critical role of EPS8 in BCR-ABL positive cells. The exact underlying mechanism revealing how EPS8 regulates BCR-ABL remains unclear. Although EPS8 is an adaptor protein, it may be less likely that EPS8 directly combines with BCR-ABL to form a complex since recently Reckel et al performed a quantitative comparative proteomic study in p190 or p210 transfected BaF3 cells and EPS8 was not identified as an interactor of BCR-ABL (27). Despite the fact that BCR-ABL is an oncoprotein which is capable of autophosphorylation, proliferation and survival signalling can also regulate its phosphorylation. It is more possible that knockdown of EPS8 leads to attenuation of proliferation and survival signalling, which subsequently decreases the phosphorylation of BCR-ABL. A future study is warranted to test this hypothesis.

In conclusion, our data revealed that EPS8 regulated multiple biological functions such as proliferation, apoptosis, the cell cycle, drug sensitivity of CML cells possibly by mediating the regulation of the BCR-ABL/akt/mTOR signalling pathway. Strategies that target EPS8 in CML patients may help to overcome resistance to tyrosine kinase inhibitors. The EPS8 inhibitor alone or combined with a tyrosine kinase inhibitor may be promising customized strategies to treat refractory and resistant CML patients.

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