Abstract. Ibrutinib, an FDA approved, orally administered BTK inhibitor, has demonstrated high response rates to diffuse large B-cell lymphoma (DLBCL), however, complete responses are infrequent and acquired resistance to BTK inhibition can emerge. The present study investigated the role of the platelet-derived growth factor D (PDGFD) gene and the ibrutinib resistance of DLBCL in relation to epidermal growth factor receptor (EGFR). Bioinformatics was used to screen and analyze differentially expressed genes (DEGs) in complete response (CR), partial response (PR) and stable disease (SD) in DLBCL treatment with ibrutinib, and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to analyze enriched the signaling pathways increasing DEGs. The Search Tool for Interactions of Chemicals database was used to analyze the target genes of ibrutinib. An interaction network of DEGs, disease-related genes and ibrutinib was constructed. The expression of PDGFD in tissues that were resistant or susceptible to DLBCL/ibrutinib was detected via immunohistochemistry (IHC), and the expression of PDGFD in DLBCL/ibrutinib-resistant strains and their parental counterparts were examined via reverse transcription-quantitative PCR and western blot analyses. Subsequently, a drug-resistant cell model of DLBCL/ibrutinib in which PDGFD was silenced was constructed. The apoptosis of the DLBCL/ibrutinib-resistant strains was examined using MTT and flow cytometry assays. EGFR gene expression was then assessed. At the same time, a PDGFD-interfering plasmid and an EGFR overexpression plasmid were transfected into the DLBCL drug-resistant cells (TMDS-ibrutinib, HBL1-ibrutinib) separately or together. MTT was used to measure cell proliferation and changes in the IC_{50} of ibrutinib. A total of 86 DEGs that increased in the CR, PR and SD tissues were screened, and then evaluated with GO and KEGG. The interaction network diagram showed that there was a regulatory relationship between PDGFD and disease-related genes, and that PDGFD could indirectly target the ibrutinib target gene EGFR, indicating that PDGFD could regulate DLBCL via EGFR. IHC results showed high expression of PDGFD in diffuse large B-cell lymphoma tissues with ibrutinib tolerance. PDGFD expression in ibrutinib-resistant DLBCL cells was higher compared with in parental cells. Following interference with PDGFD expression in ibrutinib-resistant DLBCL cells, the IC_{50} value of ibrutinib decreased, the rate of apoptosis increased and EGFR expression decreased. In brief, EGFR overexpression can reverse the resistance of DLBCL to ibrutinib via PDGFD interference, and PDGFD induces the resistance of DLBCL to ibrutinib via EGFR.

Introduction

Diffuse large B-cell lymphoma (DLBCL), a common subtype of non-Hodgkin's lymphoma (NHL), constitutes ~40% of new NHL cases annually in China, according to the World Health Organization Classification (1). As DLBCL is a highly heterogeneous disease, it has varied gene expression and clinical manifestations, requiring different treatment strategies (2). At present, the standard treatment for DLBCL includes rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone (3). Bruton's tyrosine kinase (BTK) plays a key role in B-cell development, proliferation and survival (4). BTK exhibits abnormal expression and mutations in X-linked agammaglobulinemia and diffuse large B-cell lymphoma (5). Ibrutinib, through phosphorylation of phospholipase C γ, inhibits B-cell receptor activation, thereby affecting NF-κB signaling (6). Moreover, ibrutinib, a first-in-class inhibitor of BTK, has become a novel anticancer drug that is widely used as a molecular tool to verify the role of BTK kinase in B-cell tumors (7,8). Despite the promising activity of ibrutinib across DLBCL cases, a large number of patients have shown primary and secondary resistance (9). Primary resistance is characterized by little-to-no response during initial therapy, whereas secondary resistance is characterized by an initial disease response that is subsequently lost (10). Therefore, it
is imperative to study the mechanism of ibrutinib resistance in DLBCL.

Platelet-derived growth factor D (PDGFD) gene belongs to the PDGF family of proteins, is involved in the development and physiological processes of the body, and is also associated with tumorigenesis, fibrosis and atherosclerosis (11,12). An increasing number of studies have shown that PDGFD may play a key role in the occurrence and development of human cancer by regulating cell proliferation, apoptosis, migration, invasion, angiogenesis and metastasis (11,13). The expression of PDGFD has been reported to be upregulated in prostate cancer, lung cancer, kidney cancer, ovarian cancer, brain cancer and pancreatic cancer (11,13-17). In addition, PDGFD has also been reported to exhibit potential carcinogenic activity in prostate cancer (11,14). Wang et al (12) have suggested that the overexpression of PDGFD is closely related to pancreatic cancer occurrence and progression. Xu et al (16) reported that overexpression of PDGFD in renal cell carcinoma SN12-C cells increased cell proliferation and migration in vitro, and increased the coverage of perivascular cells in vivo. Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase belonging to the HER family, and is a key protein in epithelial cell proliferation (18). EGFR is highly expressed in 60-80% of colorectal cancers (19). As an oncogenic factor, EGFR is involved in the processes of numerous cancers, including glioma, colon cancer and pancreatic cancer (20), moreover, high EGFR levels are associated with late-stage disease and poor prognosis (21).

Due to the overexpression and implicated functions of EGFR, EGFR is an effective therapeutic target for various human cancers; EGFR-targeting drugs have been used in the clinic to suppress tumor cell growth and regulate the tumor microenvironment (22-25). At the same time, EGFR is associated with cancer cell resistance to chemotherapeutic agents; for example, in colon cancer, miR-20b reduces colon cancer cell resistance to 5-FU by inhibiting ADAM9/EGFR (26). EGFR is a target gene for ibrutinib, and its abnormal expression leads to drug resistance (27-29). Furthermore, PDGFD could regulate the expression of EGFR (30). Whether PDGFD affects the resistance of DLBCL to ibrutinib through EGFR remains to be elucidated.

Thus, the present study analyzed differentially expressed genes (DEGs) between ibrutinib resistance and sensitivity in DLBCL, and identified that PDGFD was highly expressed in DLBCL with ibrutinib resistance. Then, the effects of PDGFD and EGFR expression on the proliferation, IC$_{50}$, and apoptosis of DLBCL/ibrutinib-resistant cells were evaluated to provide a theoretical basis for alleviating the resistance of DLBCL cells to ibrutinib.

Materials and methods

Data preprocessing and screening of DEGs. The GSE93984 profile (https://www.ncbi.nlm.nih.gov/pubmed/28428442) and its corresponding platform annotation files were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) (31). This dataset consisted of 34 samples. Differential expression of genes in ibrutinib-responsive [complete response (CR; 10 cases) + partial response (PR; 14 cases)] and non-responsive [stable disease (SD; 10 cases)] of DLBCL was tested with cut-off criteria of P<0.05 and fold change (FC)>2. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg/kegg1.html) (32-34) were performed with the Database for Annotation, Visualization and Integrated Discovery (version 6.8; https://david.ncifcrf.gov/) (35,36). Through The Search Tool for Interactions of Chemicals (STITCH) database (version 4.0; http://stitch.embl.de/) (37,38), the genes interacting with ibrutinib were extracted and visualized. The Search Tool for the Retrieval of Interacting Genes (STRING) (version 11.0), which provides information for experimental and predicted interactions, is an online database (39).

Clinical tissue sample collection. Between April 2013 and March 2015, 62 patients who were histologically diagnosed with DLBCL in the Fudan University Shanghai Cancer Center were investigated (Table I). A total of 29 women (46.8%) and 33 men (53.2%) were included. The mean age was 50 years (range, 20-77 years). The inclusion criteria were as follows: Each patient was diagnosed with DLBCL by pathology, received ibrutinib therapy alone and provided informed consent. Cancer tissue samples were then collected from these patients by means of biopsy. DLBCL tissues were defined as showing a PR or CR following ibrutinib treatment, and the resistant DLBCL tissues as showing relapsed/refractory disease. This study was approved by the Research Ethics Committee of Fudan University Shanghai Cancer Center.

Cell culture. The TMd8 and HBL1 cell lines were obtained from the American Type Culture Collection. Cell line authentication was performed by the Cell Check service at IDEXX Laboratories, Inc. Cell lines in the logarithmic growth phase were cultured in RPMI 1640 medium containing 10% FBS (Atlanta Biologicals, Inc.; R&D Systems, Inc.), 1 mM sodium pyruvate and 1% penicillin/streptomycin (Pen/Strep); RPMI 1640 medium, sodium pyruvate and Pen/Strep were obtained from Thermo Fisher Scientific, Inc. Ibrutinib-resistant HBL1 and TMd8 cells were generated via in vitro culture of the parental cell lines for prolonged periods of time with progressively increasing concentrations of ibrutinib (0, 5, 10, 200, 500, 800 and 1,000 nM). These varying concentrations of ibrutinib were added to ibrutinib-resistant HBL1 and TMd8 cells for 24 h prior to the MTI assay. Then, 200 nM ibrutinib was added to ibrutinib-resistant HBL1 and TMd8 cells for 24 h prior to the flow cytometry assay. Lentiviruses Lv-EGFR was obtained from Auragene Bioscience Corporation, Inc.

RNA isolation and quantification. Cells were seeded in 6-well plates (1x10^6/well). Total RNA was extracted using the TaqMan® Fast Cells-to-CT™ kit (Thermo Fisher Scientific, Inc.) and then reverse transcribed into cDNA according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed on a QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Inc.). Amplification was performed in a three-step cycle procedure, 95°C (denaturation) 10 sec, 60°C (annealing) 30 sec, and 72°C (extension) 30 sec, for 40 cycles. The primers were: PDGFD, forward 5'-GAAAGCTACCCAGGAACC-3', reverse 5'-CTGGTGACACACCTGTC-3'; EGFR, forward 5'-CCCTCTGAGCTCTGTAGT-3', reverse 5'-GTTTCC CCTCTGGAGATGC-3'; β-actin, forward 5'-TTGTTACAG
Table I. Clinical tissue information.

| Patient number | Age (years) | Gender | Diagnosis                          | Start date of treatment |
|----------------|-------------|--------|------------------------------------|-------------------------|
| 1              | 56          | Female | Diffuse large B-cell lymphoma      | 2013/4/8                |
| 2              | 50          | Male   | Diffuse large B-cell lymphoma      | 2013/4/12               |
| 3              | 62          | Female | Diffuse large B-cell lymphoma      | 2013/4/19               |
| 4              | 33          | Male   | Diffuse large B-cell lymphoma      | 2013/5/8                |
| 5              | 63          | Female | Diffuse large B-cell lymphoma      | 2013/5/21               |
| 6              | 40          | Male   | Diffuse large B-cell lymphoma      | 2013/5/24               |
| 7              | 77          | Male   | Diffuse large B-cell lymphoma      | 2013/6/7                |
| 8              | 50          | Male   | Diffuse large B-cell lymphoma      | 2013/6/21               |
| 9              | 53          | Male   | Diffuse large B-cell lymphoma      | 2013/6/26               |
| 10             | 57          | Male   | Diffuse large B-cell lymphoma      | 2013/7/12               |
| 11             | 58          | Female | Diffuse large B-cell lymphoma      | 2013/7/26               |
| 12             | 57          | Male   | Diffuse large B-cell lymphoma      | 2013/7/30               |
| 13             | 50          | Male   | Diffuse large B-cell lymphoma      | 2013/8/13               |
| 14             | 49          | Male   | Diffuse large B-cell lymphoma      | 2013/9/3                |
| 15             | 46          | Female | Diffuse large B-cell lymphoma      | 2013/9/3                |
| 16             | 60          | Female | Diffuse large B-cell lymphoma      | 2013/9/17               |
| 17             | 74          | Female | Diffuse large B-cell lymphoma      | 2013/10/9               |
| 18             | 54          | Female | Diffuse large B-cell lymphoma      | 2013/11/5               |
| 19             | 63          | Female | Diffuse large B-cell lymphoma      | 2013/11/26              |
| 20             | 57          | Female | Diffuse large B-cell lymphoma      | 2013/12/2               |
| 21             | 57          | Female | Diffuse large B-cell lymphoma      | 2013/12/10              |
| 22             | 42          | Female | Diffuse large B-cell lymphoma      | 2013/12/10              |
| 23             | 50          | Female | Diffuse large B-cell lymphoma      | 2013/12/16              |
| 24             | 59          | Male   | Diffuse large B-cell lymphoma      | 2013/12/17              |
| 25             | 49          | Male   | Diffuse large B-cell lymphoma      | 2013/12/18              |
| 26             | 51          | Female | Diffuse large B-cell lymphoma      | 2013/12/19              |
| 27             | 45          | Male   | Diffuse large B-cell lymphoma      | 2014/1/3                |
| 28             | 58          | Male   | Diffuse large B-cell lymphoma      | 2014/1/21               |
| 29             | 45          | Male   | Diffuse large B-cell lymphoma      | 2014/1/28               |
| 30             | 64          | Male   | Diffuse large B-cell lymphoma      | 2014/2/21               |
| 31             | 43          | Male   | Diffuse large B-cell lymphoma      | 2014/3/3                |
| 32             | 33          | Female | Diffuse large B-cell lymphoma      | 2014/3/11               |
| 33             | 59          | Female | Diffuse large B-cell lymphoma      | 2014/3/12               |
| 34             | 27          | Male   | Diffuse large B-cell lymphoma      | 2014/3/14               |
| 35             | 46          | Male   | Diffuse large B-cell lymphoma      | 2014/3/18               |
| 36             | 54          | Male   | Diffuse large B-cell lymphoma      | 2014/5/8                |
| 37             | 33          | Male   | Diffuse large B-cell lymphoma      | 2014/5/13               |
| 38             | 50          | Male   | Diffuse large B-cell lymphoma      | 2014/5/19               |
| 39             | 56          | Female | Diffuse large B-cell lymphoma      | 2014/5/19               |
| 40             | 36          | Male   | Diffuse large B-cell lymphoma      | 2014/5/22               |
| 41             | 56          | Female | Diffuse large B-cell lymphoma      | 2014/6/13               |
| 42             | 37          | Male   | Diffuse large B-cell lymphoma      | 2014/6/16               |
| 43             | 40          | Male   | Diffuse large B-cell lymphoma      | 2014/6/23               |
| 44             | 52          | Female | Diffuse large B-cell lymphoma      | 2014/7/4                |
| 45             | 45          | Male   | Diffuse large B-cell lymphoma      | 2014/7/14               |
| 46             | 29          | Male   | Diffuse large B-cell lymphoma      | 2014/7/23               |
| 47             | 68          | Female | Diffuse large B-cell lymphoma      | 2014/8/24               |
| 48             | 43          | Male   | Diffuse large B-cell lymphoma      | 2014/9/28               |
| 49             | 64          | Female | Diffuse large B-cell lymphoma      | 2014/9/4                |
| 50             | 58          | Female | Diffuse large B-cell lymphoma      | 2014/10/5               |
| 51             | 43          | Female | Diffuse large B-cell lymphoma      | 2014/10/6               |
| 52             | 63          | Male   | Diffuse large B-cell lymphoma      | 2014/10/17              |
GAAGTCCCTTGCC-3'; reverse 5'-ATGCTATCACCTCCCCTGTTGTC-3'. mRNA levels were quantified using the 2^(- ΔΔCq) method (40) and normalized to the internal reference gene β-actin. All experiments were repeated three times.

**Construction and identification of stable PDGFD-knockdown cell lines.** The TMD8 and HBL1 ibrutinib-resistant cells in the logarithmic growth phase were seeded in 6-well plates at a density of 3x10^5 cells/well. The PDGF-D shRNA sequence was GGCATCATCAAGCCTTTGC. PDGF short hairpin RNA (shRNA; 20 pmol) and pBTLV-U6-Puro lentiviruses (20 pmol; Auragen Biotechnology Corporation, Inc.) were added to the cells for 24 h in the presence of Polybrene (Santa Cruz Biotechnology, Inc.; 5 µg/ml) after the cells had adhered to the walls of the plates. Following infection for 48 h, cells were observed under a fluorescence microscope (IX70; Olympus Corporation), and uninfected cells were killed with puromycin. The surviving cells were collected, and the PDGF-D protein level was analyzed using western blotting.

**MTT assay.** Cells were seeded into 96-well flat-bottomed tissue culture plates (5-10x10^3/well in 100 µl medium). The cells were cultured for 24-96 h at 37°C under 5% CO_2 before a total of 20 µl MTT (5 mg/ml) was added to cells in the logarithmic growth phase for each group for 4 h. Dimethyl sulfoxide was added to dissolve purple formazan for 10 min. The optical density (OD) value at 490 nm was measured with a microplate reader (Bio-rad laboratories, inc.). The cell survival rate was calculated with a concentration-survival curve.

**Immunohistochemistry (IHC).** Tissue samples from DLBCL and ibrutinib-resistant DLBCL areas were formalin-fixed, dehydrated, cleared in xylene and embedded in paraffin. Sections (5 µm) were deparaffinized, hydrated, and 3% H_2O_2 solution was added for 15 min to remove endogenous catalase and antigen repair. Non-immune normal goat serum was incubated at room temperature for 60 min at 100 µl and then stained with anti-PDGFD in 4°C overnight (1:100, cat. no. ab181845; Abcam). Horseradish peroxidase conjugated secondary antibodies were incubated at room temperature for 30 min (1:1,000, Pv-80000, OriGene Technologies, Inc.), and 3,3-diaminobenzidine substrate was added for the development of immunostaining according to the manufacturer's instructions (Dako; Agilent Technologies, Inc.). Then the slides were counterstained with hematoxylin and eosin at room temperature for 5 min. Positive cells were counted in 10 randomly selected fields with a x40 objective (Olympus CX23; Olympus Corporation).

**Western blotting.** Western blotting was performed as previously described (41). In brief, cells were harvested and lysed with RIPA buffer (cat. no. R0278; Sigma-Aldrich; Merck KGaA) containing 1X protease/phosphatase inhibitor. A BCA protein assay kit (Beyotime) was employed to measure the protein concentrations. Equal amounts (20 µg/well) of protein were separated by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were washed by 1X TBST and blocked by Odyssey Blocking Buffer (cat. no. 927-40000; LI-COR Biosciences) for 1 h at room temperature. Then membranes were incubated with primary antibodies against PDGF-D (1:1,000; cat. no. ab240960; Abcam), EGFR (1:1,000; cat. no. ab52894; Abcam) and GAPDH (1:2,000; cat. no. ab181602; Abcam) overnight at 4°C. Afterwards, membranes were washed and incubated with secondary antibodies for 1 h at room temperature. Signals were measured with a luminescent image analyzer (ImageQuant LAS4000 mini) and GAPDH served as a loading control.

**Flow cytometry.** The ApoDETECT Annexin V-FITC kit (Thermo Fisher Scientific, Inc.) was used to quantify the number of apoptotic cells in the indicated groups. Briefly, 1X binding buffer was used to resuspend cells (5x10^5 cells/ml). Annexin V-FITC was added at room temperature for 10 min in the dark. Then, the cells were resuspended in 190 µl binding buffer containing 10 µl 20 µg/ml propidium iodide (PI) at 4°C for 30 min and analyzed with a flow cytometer (BD Biosciences) using ModFit LT software version 3.0 (Verity Software House, Inc.). The apoptotic rate was calculated as early apoptotic cells + late apoptotic cells.

**Statistical analysis.** Data are presented as the mean ± standard deviation using SPSS 17.0 software (SPSS, Inc.). The statistical significance between multiple experimental groups was analyzed using one-way ANOVA followed by Tukey’s post hoc test. A Student's t-test was used for comparisons between two groups. P<0.05 was considered to indicate a statistically
The use of lentivirus carrying shPdGFd (lv-shPdGFd) was constructed, and the TMd8-ibrutinib and HB11-ibrutinib cell lines were infected. Western blotting was performed to verify the expression of PdGFd in these cell lines (Fig. 3B). The Western blotting results indicated that PdGFd expression was significantly higher in the resistant cell lines compared with the parental cell lines, which was consistent with the qPCR results (Fig. 5B). Compared with that in the parental cell lines, the expression of EGFR mRNA in the drug-resistant cell lines was upregulated (Fig. 5A). In addition, western blotting revealed that the expression of EGFR protein in the drug-resistant cell lines was higher than that in the parental cell lines, which was consistent with the qPCR results (Fig. 5B).

The expression of PdGFd was accompanied by decreased EGFR expression (Fig. 5C). Furthermore, the overexpression of EGFR in the Lv-shPdGFd-infected cells increased the IC50 values of ibrutinib in drug-resistant cell lines. In conclusion, PdGFd may reverse the sensitization of DLBCL to ibrutinib induced by PdGFd interference in the drug-resistant strains. Collectively, the data suggested that PdGFd could induce DLBCL ibrutinib resistance by regulating EGFR expression.

**Discussion**

DLBCL is a subtype of adult non-Hodgkin's lymphoma with significant clinical and biological heterogeneity, including 16 different clinicopathological entities (42). At present, >50% of patients with DLBCL can be cured with R-CHOP regimen; however, ~30-40% of patients still die from drug-resistant or refractory disease (43). Ibrutinib, a targeted inhibitor of BTK, has shown promise in treating B-cell lymphoma (44,45). Ibrutinib can disrupt the tumor microenvironment while directly exerting cytotoxic effects on malignant B-cells (46). Ibrutinib has been shown to inhibit the growth of stomach, breast and colon tumors in mouse models (47,48). Ibrutinib overcomes mesenchymal stem cell (MSC)-mediated drug resistance by inhibiting CXC chemokine receptor 4 expression and inhibits MSC-induced lymphoma cell colony formation (49).

To provide a theoretical basis for the treatment and alleviation of ibrutinib resistance in DLBCL cells, the role of PdGFd in the resistance of DLBCL to ibrutinib was studied. The present study revealed high expression of PdGFd in DLBCL/ibrutinib at the tissue and cellular level. After interfering with PdGFd in TMD8-ibrutinib and HB11-ibrutinib cell lines, it was found that EGFR expression decreased, while the IC50 values of ibrutinib in drug-resistant cell lines increased. In addition, the resistance of TMD8-ibrutinib and HB11-ibrutinib cells to ibrutinib increased, indicating that PdGFd could induce DLBCL ibrutinib resistance. A large number of studies related to ibrutinib resistance in DLBCL have emerged (9,50-52). For example, ibrutinib-resistant tumors were reported to carry mutant myeloid differentiation response 88 (MYD88) and wild-type (WT) CD79A/B, whereas all other genotypic combinations (CD79A/B WT + MYD88 WT, CD79A/B mutant + MYD88 WT and CD79A/B mutant + MYD88 mutant) were responsive to ibrutinib therapy (50-52). In addition, BTK(Cys481Ser) drives ibrutinib resistance via ERK1/2, and protects BTK WT MYD88-mutated Waldenström
Jin et al.: PdGFd induces ibrutinib resistance of dlBcl by activation of EGFR

macroglobulinemia (WM) and activated B-cell DLBCL cells via a paracrine mechanism (9,10,52). These studies are similar to the present study and provide clues to explain new mechanisms of ibrutinib resistance in DLBCL.

PDGFD has been shown to be highly expressed in various cancers (53,54), is associated with the occurrence and development of cancer, and has been implicated in drug resistance to numerous cancer chemotherapeutics (55,56). Zhang et al. (57) that PDGFD overexpression is an independent predictor of platinum chemotherapeutic resistance, and may be a potential biomarker for targeted therapy and poor prognosis. Moreover, PDGFD plays an important role in the epithelial-mesenchymal transition and drug resistance of hepatocellular carcinoma cells (58). The present study for the first time, to the authors' knowledge, reported the expression of PDGFD in DLBCL tissues and cells, with high expression associated with resistance to ibrutinib. In addition, in the TMD8-ibrutinib- and HBl1-ibrutinib-resistant cells that were subjected to PDGFD interference, a decrease in the IC50 of ibrutinib and an increase in the apoptosis rate were observed, indicating enhanced sensitivity of the cells to ibrutinib. These results indicated that PDGFD plays a role in the mechanism of DLBCL cell resistance to ibrutinib.
Bioinformatics analysis suggested that there was an interaction between PdGFd and eGFr. It was speculated that the effect of PdGFd on the drug resistance of DLBCL to ibrutinib may be mediated by eGFr. It was demonstrated that eGFr was overexpressed in drug-resistant cell lines. Other reports have shown that eGFr is overexpressed in cancer cells, and is associated with poor efficacy and a low survival rate (59,60). The effect of interfering with PdGFd on the drug resistance of TMd8-ibrutinib and HB11-ibrutinib cells could be reversed by eGFr overexpression, and eGFr was a target of ibrutinib treatment, indicating that eGFr is a downstream target gene of PDGFD, and that the regulation of eGFr leads to the drug resistance of DLBCL cells to ibrutinib. Accordingly, ibrutinib can effectively block the proliferation and survival of glioma cells mediated by the NF-κB pathway activated by eGFr (61), and promote the chemotherapeutic resistance of glioma cells through Akt-independent activation of the NF-κB pathway (62). However, the relevant experiments investigating PDGFD-regulated signaling in Lv-shPDGFD-treated or non-treated ibrutinib-resistant TMd8 and HB11-1 cell lines were not performed; these will be conducted in future studies. In the pathway analysis, only DEG analysis as a whole was presented; it would be informative to conduct pathway analysis for down- and upregulated
Jin et al.: PdGFd induces ibrutinib resistance of DLBCL by activation of EGFR
genomes separately to further clarify how enriched biological functions may be affected.

The present study was based on in vitro studies of TMD8-ibrutinib and HBL1-ibrutinib, and revealed that PDGF
induced resistance to ibrutinib in DLBCL, potentially via effects on EGFR. The current study is the first, to the best of the authors' knowledge, to evaluate the expression of PDGFd and its effects on drug resistance to ibrutinib. It is concluded that overexpression of PDGFd reduces the sensitivity of DLBCL to ibrutinib by promoting EGFR expression.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JJ and ZT performed experiments and drafted the manuscript; LW, JZ and FL participated in the design of the study. JJ, LW, ZT and JZ performed statistical analysis and data interpretation;
JC and XH designed the study and revised the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of Fudan University Shanghai Cancer Center. Informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Sun J, Yang Q, Lu Z, He M, Gao L, Zhu M, Sun L, Wei L, Li M, Liu C, et al: Distribution of lymphoid neoplasms in China: Analysis of 4,638 cases according to the world health organization classification. Am J Clin Pathol 138: 429-434, 2012.

2. Losso IS and Morgensztern D: Prognostic biomarkers in diffuse large B-cell lymphoma. J Clin Oncol 24: 995-1007, 2006.

3. Kubuschok B, Held G and Pleuendusch M: Management of diffuse large B-cell lymphoma (DLBCL). Cancer Treat Res 165: 271-288, 2015.

4. Herman SE, Gordon AL, Hertlein E, Ramannunni A, Zhang X, Jaglowski S, Flynn J, Jones J, Blum KA, Buggy JJ, et al: Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. Blood 117: 6287-6296, 2011.

5. Tsukada S, Saffran DC, Rawlings DJ, Parolini O, Allen RC, Klisak I, Sparkes RS, Kubagawa H, Mohandas T, Quan S, et al: Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. J Immunol 188: 2936-2947, 2012.

6. Herman SE, Mustafa RZ, Gyamfi JA, Pittaluga S, Chang S, Chang B, Farooqui M and Wiestner A: Ibrutinib inhibits BCR and NF-kB signaling and reduces tumor proliferation in tissue-resident cells of patients with CLL. Blood 123: 3286-3295, 2014.

7. Hendriks RW, Yuvaraj S and KI LP: Targeting Bruton's tyrosine kinase in B cell malignancies. Nat Rev Cancer 14: 219-232, 2014.

8. Wang ML, Rule S, Martin P, Goy A, Auer R, Kahl BS, Jurcak W, Asvani RH, Romaguera JE, Williams ME, et al: Targeting BTK with ibrutinib in relapsed or refractory mantle-cell lymphoma. N Engl J Med 369: 507-516, 2013.

9. Charalambous A, Schwarzbi MA and Wittenz-Harig M: Ibrutinib. Recent Results Cancer Res 212: 133-168, 2018.

10. Zhang SQ, Smith SM, Zhang SY and Lynn Y: Mechanisms of ibrutinib resistance in chronic lymphocytic leukemia and non-Hodgkin lymphoma. Br J Haematol 170: 445-456, 2015.

11. Kong D, Banerjee S, Huang W, Li Y, Wang Z, Kim HR and Sarkar FH: Mammalian target of rapamycin repression by 3'-di-phosphoinositide inversion and angiogenesis in platelet-derived growth factor-D-overexpressing PC3 cells. Cancer Res 68: 1927-1934, 2008.

12. Wang Z, Kong D, Banerjee S, Li Y, Adsay NV, Abbruzzese J and Sarkar FH: Down-Regulation of platelet-derived growth factor-D inhibits cell growth and angiogenesis through inactivation of notch-1 and nuclear factor-kappaB signaling. Cancer Res 67: 11377-11385, 2007.

13. Lokker NA, Sullivan CM, Hollenbach SJ, Israel MA and Giese NA: Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitotic pathways in glioblastoma cells: Evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors. Cancer Res 62: 3729-3735, 2002.

14. Ustach CV, Taube ME, Hurst NJ Jr, Bhagat S, Bonfil RD, Cher ML, Schuger L and Kim HR: A potential oncogenic activity of platelet-derived growth factor D in prostate cancer progression. Cancer Res 64: 1722-1729, 2004.

15. Ustach CV and Kim HR: Platelet-derived growth factor D is activated by urokinase plasminogen activator in prostate carcinoma cells. Mol Cell Biol 25: 6279-6288, 2005.

16. Xu L, Tong C, Cochran D and Jordan J: Blocking platelet-derived growth factor-D/platelet-derived growth factor receptor beta signaling inhibits human renal cell carcinoma progression in an orthotopic mouse model. Cancer Res 65: 5711-5719, 2005.

17. Wang Z, Ahmad A, Li Y, Kong D, Azmi AS, Banerjee S and Sarkar FH: Emerging roles of PDGF-D signaling pathway in tumor development and progression. Biochem Biophys Acta 1806: 122-130, 2010.

18. Yoshida T, Zhang G and Haura EB: Targeting epidermal growth factor receptor: Central signaling kinase in lung cancer. Biochem Pharmacol 80: 613-623, 2010.

19. Cohen RB: Epidermal growth factor receptor as a therapeutic target in colorectal cancer. Clin Colorectal Cancer 2: 246-251, 2003.

20. Hrstustovic G, Lee BJ and Bivona TG: Mechanisms of resistance to EGFR targeted therapies. Cancer Biol Ther 14: 304-314, 2013.

21. Jänne PA, Engelman JA and Johnson BE: Epidermal growth factor receptor mutations in non-small-cell lung cancer: Implications for treatment and tumor biology. J Clin Oncol 23: 3227-3243, 2005.

22. Martinez-Marti A, Navarro A and Felip E: Epidermal growth factor receptor first generation tyrosine-kinase inhibitors. Transl Lung Cancer Res 8 (Suppl 3): S235-S246, 2019.

23. Martin A, Nasser MW, Ravi J, Wani NA, Abbruzzese DK, Zhao H, Oghumu S, Satoskar AR, Shilo K, Carson WE 3rd and Ganju RK: Modulation of the tumor microenvironment and inhibition of EGFR/EGFR pathway: Novel anti-tumor mechanisms of cannabinoids in breast cancer. Mol Oncol 9: 906-919, 2015.

24. Arienti C, Pignatta S and Tesi A: Epidermal growth factor receptor family and its role in gastric cancer. Front Oncol 9: 1308, 2019.

25. Maenling AE, Tur MK, Niebert M, Klockenbring T, Zeppernick F, Wang SQ, Smith SM, Zhang SY and Lynn Wang Y: Ibrutinib inhibits ERBB receptor tyrosine kinases and HER2-amplified breast cancer cell growth. Mol Cancer 15: 2835-2844, 2016.

26. Su Q, Cheng J, Zhang J, Zhang Y, Chen X, Luo S and Xie J: MiR-20b reduces 5-FU resistance by suppressing the ADAM9/EGFR signaling pathway in colon cancer. Oncol Rep 37: 123-130, 2017.

27. Wu H, Wang A, Zhang W, Wang B, Chen C, Wang W, Hu C, Ye Z, Zhao Z, Wang L, et al: Ibrutinib selectively and irreversibly targets EGFR (L858R, Del19) mutant but is moderately resistant to EGFR (T790M) mutant NSCLC cells. Oncotarget 6: 31313-31322, 2015.

28. Wang A, Yan YE, Wu H, Wang W, Hu C, Chen C, Zhao Z, Zhao P, Li X, Wang L, et al: Ibrutinib targets mutant-EGFR kinase with a distinct binding conformation. Oncotarget 7: 69760-69769, 2016.

29. Chen J, Kinoshita T, Sakkuntheng J, Chang BY and Elias L: Ibrutinib inhibits ERBB receptor tyrosine kinases and HER2-amplified breast cancer cell growth. Mol Cancer Ther 15: 2835-2844, 2016.

30. Saito Y, Haendeler J, Hojo Y, Yamamoto K and Berk BC: Receptor heterodimerization: Essential mechanism for platelet-derived growth factor-induced epidermal growth factor receptor transactivation. Mol Cell Biol 21: 6387-6394, 2001.

31. Kuo HP, Ezzell SA, Schweighofer KJ, Cheung LWK, Hsieh S, Apatira M, Sirisawad M, Eckert K, Hsu SJ, Chen CT, et al: Combination of ibrutinib and ABT-199 in diffuse large B-cell lymphoma and follicular lymphoma. Mol Cancer Ther 16: 1246-1256, 2017.

32. Kanehisa M: Toward understanding the origin and evolution of cellular organisms. Protein Sci 28: 1947-1951, 2019.

33. Kanemori and Goto S: KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res 28: 27-30, 2000.

34. Kanemori M, Sato Y, Furumichi M, Morishima K and Tanabe M: New approach for understanding genome variations in KEGG. Nucleic Acids Res 47: D590-D595, 2019.

35. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, et al: Gene ontology: Tool for the unification of biology. The gene ontology consortium. Nat Genet 25: 25-29, 2000.

36. The Cell Ontology Consortium: The gene ontology resource: 20 Years and still GOing strong. Nucleic Acids Res 47: D330-D338, 2019.

37. Szklarczyk D, Santos A, von Mering C, Jensen LJ, Bork P and Kuhn M: STRING 8: Augmenting protein-chemical interaction networks with tissue and affinity data. Nucleic Acids Res 44: D380-D384, 2016.
38. Huang da W, Sherman BT and Lemppicki RA: Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4: 44-57, 2009.

39. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork P, et al. STRING v11: Protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res 47: D607-D613, 2019.

40. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta CT) method. Methods 25: 402-408, 2001.

41. Wu ZH, Tao ZH, Zhang J, Li T, Ni C, Xie J, Zhang JF and Hu XC: MiRNA-21 induces epithelial to mesenchymal transition and gemcitabine resistance via the PTEN/AKT pathway in breast cancer. Tumour Biol 37: 7245-7254, 2016.

42. Sukswai N, Lyapichev K, Khoury JD and Medeiros LJ: Diffuse large B-cell lymphoma variants: An update. Pathology 52: 53-67, 2020.

43. Camicia R, Winkler HC and Hassa PO: Novel drug targets for personalized precision medicine in relapsed/refractory diffuse large B-cell lymphoma: A comprehensive review. Mol Cancer 14: 207, 2015.

44. Cabanillas F and Shah B: Advances in diagnosis and management of diffuse large B-cell lymphoma. Clin Lymphoma Myeloma 17: 783-796, 2017.

45. Maddocks K, Christian B, Jaglowski S, Flynn J, Jones JA, Porcu P, et al. Metformin inhibits metastatic breast cancer progression and improves chemosensitivity by inducing vessel normalization via PDGF-B downregulation. J Exp Clin Cancer Res 38: 235, 2019.

46. Wang Y, Appiah-Kubi K, Wu M, Yao X, Qian H, Wu Y and Chen Y: The platelet-derived growth factors (PDGFs) and their receptors (PDGFRs) are major players in oncogenesis, drug resistance, and attractive oncologic targets in cancer. Growth Factors 34: 64-71, 2016.

47. Zhang M, Liu T, Xia B, Yang C, Hou S, Xie W and Lou G: Platelet-derived growth factor D is a prognostic biomarker and is associated with platinum resistance in epithelial ovarian cancer. Int J Gynecol Cancer 28: 323-331, 2018.

48. Wang X, Li Y, Hou Y, Yang Q, Chen S, Wang X, Wang Z, Yang Y, Chen C, Wang Z and Wu Q: The PDGF-D/miR-106a/twist1 pathway orchestrates epithelial-mesenchymal transition in gemcitabine resistance hepatoma cells. Oncotarget 6: 7000-7010, 2015.

49. Nogi H, Kobayashi T, Suzuki M, Tabei I, Kawase K, Toriumi Y, Fukushima H and Uchida K: EGFr as paradoxical predictor of chemosensitivity and outcome among triple-negative breast cancer. Oncol Rep 21: 413-417, 2009.

50. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, Hernandez-Boussard T, Livasy C, Cowan D, Dressler L, et al.: Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res 10: 5367-5374, 2004.

51. Yue C, Niu M, Shan QQ, Zhou T, Tu Y, Xie P, Hua L, Yu R and Liu X: High expression of bruton's tyrosine kinase (BTK) is required for EGFR-induced NF-kB activation and predicts poor prognosis in human glioma. J Exp Clin Cancer Res 36: 132, 2017.

52. Konig-Behrens E, Tenzer S, Lu X, Lueders C, Funke H, Kugel G, et al.: EGFR signaling activates an mTORC2-NF-kB pathway that promotes chemotheray resistance. Cancer Discov 1: 524-538, 2011.

53. Simonovic M, Doncheva NT, Morris JH, Bork P, et al. STRING v11: Protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res 47: D607-D613, 2019.