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PTBP2 exon 10 inclusion is associated with the progression of CML and it is BCR-ABL1 dependent

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ABSTRACT

Altered or aberrant expression of several splicing factors leads to the progression of different cancers. Though there are several ongoing studies underscoring the role of the splicing regulator polypyrimidine tract binding protein 2 (PTBP2) in neuronal cells, we unveil the role of PTBP2 in chronic myeloid leukemia (CML). Different RNA binding proteins (RBP's) earlier reported in chronic myeloid leukemia blast crisis (CML-BC) cases (n = 28) from Radich Oncomine leukemia dataset, were compared. We observed increased expression of MSI2 followed by PTBP2 in BC cases and increased PTBP2 expression in relapsed cases (n = 10) from the same dataset compared to other RBPs. We also observed increased PTBP2 exon 10 inclusion in KCL22, a granulocytic lineage CML cell line when compared to other CML cell lines of different lineages. As PTBP2 protein expression is associated with PTBP2 exon 10 inclusion, we observed in cell lines and in a set of progressed cases (n = 4) that increased BCR-ABL1 expression potentiates PTBP2 exon 10 inclusion and thus confers the existence of a functional protein. Inhibition of BCR-ABL1 with imatinib not only blocks the inclusion of exon 10 but also deregulates PTBP2 expression in CML cells. Knockdown of PTBP2 in KCL22 cells leads to reduced cell proliferation, increased G2/M cell cycle arrest and increased apoptosis. Taken together our study portrays PTBP2 as a new possible target for CML and progressive inclusion/exclusion of PTBP2 exon 10 might play an important role in CML progression.

1. Introduction

Chronic myeloid leukemia is a clonal hematopoietic stem cell (HSC) disorder, characterized by the presence of BCR-ABL1 oncogene, arising from the fusion of long arms of chromosome 9 and 22 wherein, a part of Abelson (c-*ABL*) gene from chromosome 9 fuses with the Breakpoint Cluster Region gene (*BCR*) of chromosome 22 (Melo, 1996). Although its presence is more than enough for leukemia initiation, the underlying mechanisms that lead to CML progression remains largely unknown. In recent years though the aberrant expression of various RBPs has been implicated in CML disease progression (Perrotti and Neviani, 2007), ours was the first report to show the up-regulation of neuronal-specific splicing regulator PTBP2 in CML progression (Agatheeswaran et al., 2013). PTBP2 expression was initially thought to be restricted to the neuronal cells, but later on, several groups reported its expression in different tissues and recent findings had shown its oncogenic potential in colorectal cancer and osteosarcoma (Ji et al., 2014; Yang et al., 2014; Xu and Hecht, 2011; Nowak et al., 2011). Although there are no reports showing the up-regulation of PTBP2 in CML, thorough literature survey showed PTBP2 as one of the candidate gene likely to be affected by chromosomal aberrations in patients who have progressed to CML myeloid BC and in CML-CP like mice (Makishima et al., 2011; Bolton-Gillespie et al., 2013). In our previous study, we have shown PTBP2 as a direct target of miRNA-223 and up-regulation of PTBP2 in the late chronic phase of CML may contribute to abnormal splicing and stabilization or destabilization of mRNA that may ultimately lead to disease

Abbreviations: CML, chronic myeloid leukemia; PTBP2, polypyrimidine tract binding protein; MSI2, musashi 2; RBP, RNA binding protein; HSC, Hematopoietic stem cells; CP, chronic phase; AP, accelerated phase; BC, blast crisis; PSI, percent splice index; PBMCs, Peripheral blood mononuclear cells; RT-PCR, Reverse transcriptase PCR; RQ-PCR, Real time PCR; Rseq, RNA sequencing

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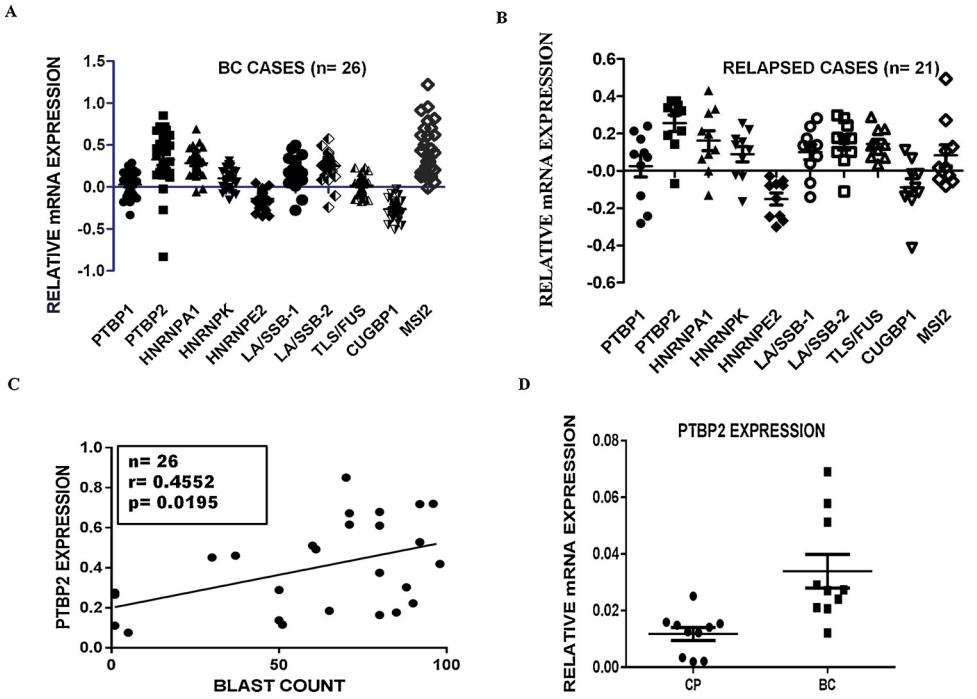

Fig. 1. A. Relative expression of different RNA binding proteins in CML-BC (n = 26) samples from Radich leukemia Oncomine dataset, (PTBP1 vs PTBP2***, PTBP2 vs HNRNPA1 ns, PTBP2 vs HNRNPK**, PTBP2 vs HNRNPE2***, PTBP2 vs LASSB-1 ns, PTBP2 vs LASSB-2 ns, PTBP2 vs TLS/FUS**, PTBP2 vs CUGBP1***, PTBP2 vs MSI2 ns). B. Relative expression of different RNA binding proteins in relapsed (n = 10) samples from Radich leukemia Oncomine dataset, (PTBP1 vs PTBP2**, PTBP2 vs HNRNPA1 ns, PTBP2 vs HNRNPK*, PTBP2 vs HNRNPE2***, PTBP2 vs LASSB-1*, PTBP2 vs LASSB-2 ns, PTBP2 vs TLS/FUS*, PTBP2 vs CUGBP1***, PTBP2 vs MSI2*). (C) Pearson's correlation test depicting a positive correlation between PTBP2 and blast count from Radich Leukemia dataset. (D) RQ-PCR showing PTBP2 expression in our novel set of CP and BC (n = 10) samples. Student's t-test was performed (p = 0.0025).

progression (Agatheeswaran et al., 2013). In this study, we observed that expression of BCR-ABL1 potentiates the inclusion of PTBP2 exon 10 through an unknown mechanism and thus confers the existence of the functional protein.

2. Results

2.1. Increased PTBP2 expression was observed in relapse samples

Comparative analysis of the expression of different RBP's that were reported earlier in CML-BC (n = 26) cases from Radich leukemia Oncomine dataset (GSE4170) showed increased MSI2 expression followed by PTBP2 expression in BC samples when compared to other RBPs (Fig. 1A) which correlates with other earlier studies where MSI2 expression was reported to be high in BC samples whereas none of the studies have shown the expression of different RBP's in relapsed CML cases. However from the same dataset, in a set of relapsed CML cases (n = 10), we observed decreased MSI2 expression compared to PTBP2 expression (Fig. 1B). A positive correlation was observed between PTBP2 and blast count in the same dataset (n = 26) (Fig. 1C). We also observed increased PTBP2 expression in BC compared to CP in our set of patient samples (n = 10) (Fig. 1D).

2.2. PTBP2 exon 10 inclusion increases with increased BCR-ABL1 expression

It has been reported earlier in neuronal and muscle cells, that PTBP2 exon 10 inclusion leads to the production of the functional protein (Rahman et al., 2002; Makeyev et al., 2007; Boutz et al., 2007a,b; Coutinho-Mansfield et al., 2007). RT-PCR (followed by percent splice index (PSI) calculation) was performed to investigate the presence of exon 10 in 32Dcl3 and 32Dcl3 BCR-ABL1 cells and in different CML cell lines (KCL22, K562, KU812, KYO-1 and LAMA 84) using PTBP2 exon 10 flanking primers. Percent splice index (PSI) was calculated as a percentage of an isoform with alternate exon to the total abundance of both the isoforms. Densitometry analysis showed increased inclusion of PTBP2 exon 10 in 32Dcl3 BCR-ABL1 cells and in KCL22 cell lines compared to parental 32Dcl3 cells and other CML cell lines (Fig. 2A). Sequencing of the amplicons by using PTBP2 exon 10 flanking primers further confirmed the presence of exon 10 in 32Dcl3 BCR-ABL1 cells (Supplementary Fig. S1A). Western blot analysis confirmed the presence of functional protein in 32Dcl3 BCR-ABL1, KCL22 and K562 cells, where increased PTBP2 exon 10 inclusion was observed (Fig. 2B).

Comparison of PTBP2 exon 10 inclusion using PTBP2 exon 10 specific primer in a set of normal (n = 5), CP (n = 12) and BC (n = 11) samples by RQ-PCR showed increased PTBP2 exon 10 inclusion in BC samples (Fig. 2C). RQ-PCR in paired CML cases (n = 4) collected longitudinally at different time points showed PTBP2 exon 10 inclusion increases with an increase in BCR-ABL1 expression (Supplementary Fig. S1B) (clinical details provided in Supplementary Table 1). We also observed increased PTBP2 protein expression in one paired sample and a set of BC samples (n = 2) however not in CP samples (n = 3) (Fig. 2D), (clinical details provided in Supplementary Table 2). Increase in PTBP2 exon 10 inclusion was further confirmed by analyzing a publicly available CML Rseq dataset, wherein BC, CP and BCR-ABL1 transduced cord blood cells were used (Supplementary Table 3) (Supporting information reference Jiang et al., 2013). Herein we observed fairly high inclusion of exon 10 in one of the untreated BC sample (BC-02) compared to CP samples and increased PTBP2 exon 10 inclusion in BCR-ABL1 transduced cord blood cells compared to the vector-transduced cells (Supplementary Fig. S2A, B). Thus increased PTBP2 exon 10 inclusion from the above observations supports the expression of PTBP2 in an advanced phase of CML.

2.3. PTBP2 exon 10 inclusion in BCR-ABL1 kinase-dependent

RT-PCR (followed by percent splice index (PSI) calculation) and western blot analysis in imatinib-treated 32Dcl3 BCR-ABL1 (0.2 million cells, 0.5 μM for 24 h) and KCL22 cells (0.5 million cells, 1 μM, 72 h) showed decreased PTBP2 exon 10 inclusion in imatinib-treated cells compared to control cells. (Fig. 3A and B, left and middle panel). Decreased PTBP2 protein expression was observed in imatinib-treated 32Dcl3 BCR-ABL1 and KCL22 cells (Fig. 3A and B, right panel). Imatinib treatment of PBMCs (2 μM) isolated from a CML-AP (19% blast), CML-BC1 (73% blast) and CML-BC2 (84% blast) showed decreased PTBP2 protein expression (Fig. 3C) (clinical details provided in Supplementary Table 4). To further confirm that PTBP2 expression is BCR-ABL1 dependent we treated 32Dcl3 BCR-ABL1 T315I cells, an imatinib-

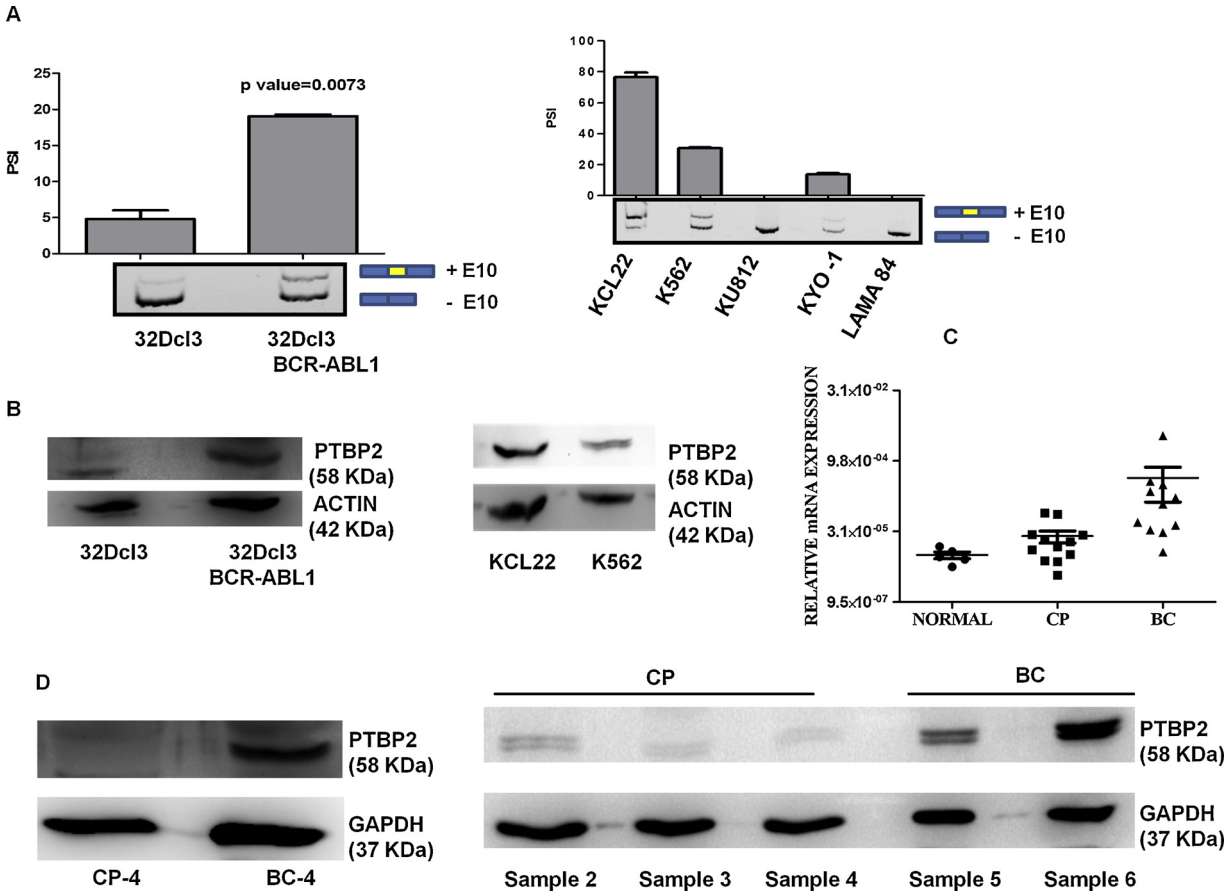

Fig. 2. PTBP2 expression in different cell lines and patient samples. (A) RT-PCR depicting PTBP2 exon 10 inclusion in cell lines. Blue boxes represent constitutive PTBP2 exons 9 and 11 and yellow box represents PTBP2's alternative exon 10. Percent splice index (PSI) was calculated from the densitometry analysis as percentage of isoform with PTBP2 exon 10 to the total abundance of the isoforms. Student's *t*-test was performed ($p = 0.0073$) for comparing two groups. One way ANOVA analysis was performed. Horizontal lines represent mean ± SEM ($n = 5$) ($p < 0.0001$). (B) Western blot depicting PTBP2 expression in cell lines. (C) RQ-PCR depicting PTBP2 expression in normal ($n = 5$), CP ($n = 12$) and BC ($n = 11$) samples. The samples were normalized against the expression of actin gene transcript. (D) Western blot depicting PTBP2 protein expression in one paired case and other CP and BC cases.

resistant cell line with imatinib (0.2 million cells, 3 μM for 24 h). We did not observe any change in BCR-ABL1 and PTBP2 expression which further confirms that PTBP2 expression is mediated by BCR-ABL1 (Fig. 3D).

2.4. Silencing of PTBP2 in KCL22 leads to reduced cell proliferation, increased G2/M cell cycle arrest and increased apoptosis

To examine whether knockdown of PTBP2 has any effect on the growth pattern of CML cells, lentiviral-mediated transduction of KCL22 cells using PTBP2 shRNA was performed. Real-time PCR showed a reduction in PTBP2 expression in PTBP2 transduced cells compared to control cells (Fig. 4A). Western blot showed reduced PTBP2 expression in PTBP2 transduced KCL22 cells compared to control cells (Fig. 4A). Trypan blue staining and counting of stable cells for 96 h at 24 h intervals showed a significant reduction in growth pattern in PTBP2 shRNA treated cells when compared to the control shRNA treated cells (Fig. 4B). We also further investigated whether PTBP2 shRNA transduced KCL22 cells showed any change in the cell cycle pattern. Flow cytometry analysis of propidium iodide stained cells showed an almost 12% increase in G2/M in PTBP2 shRNA transduced KCL22 cells compared to the control cells (Fig. 4C). As increased G2/M arrest is preferentially associated with increased apoptosis, we further investigated whether PTBP2 knockdown induces apoptosis in KCL22 cells. In this regard control shRNA and PTBP2 shRNA transduced KCL22 cells were stained with PE Annexin V-7AAD. A significant increase in the percentage of Annexin V positive and Annexin V-7AAD positive cells in PTBP2 shRNA transduced cells in comparison to control shRNA cells was observed, clearly indicating that knockdown of PTBP2 results in increased apoptosis as well as necrosis of KCL22 cells (Fig. 4D).

3. Discussion

Alternative splicing is a highly regulated and coordinated process that plays a significant role in determining the cellular differentiation and development in all multi-exonic genes leading to the proteomic complexity of eukaryotic organisms. Aberrant splicing occurs as a result of nucleotide change in cis-acting elements such as exonic splicing enhancers or exonic splicing silencers or due to deregulated expression of different trans-acting factors such as splicing factors. Genes involved in apoptosis, metastasis and angiogenesis are among the ones that are found to be aberrantly spliced in cancer (Sveen et al., 2016). Earlier we have shown that decreased miR223 expression leads to the upregulation of PTBP2. We observed that PTBP2 exon 10 inclusion increases with an increase in BCR-ABL1 expression. Analysis of the R-seq data showed very high inclusion of exon 10 in one BC patient (BC-02) who were not treated with either imatinib or hydroxyurea. Follow-up of BCR-ABL1 transduced cord blood cells showed increased inclusion of exon 10 by day 4. Ex vivo treatment of CML cells with imatinib, not only downregulated BCR-ABL1 expression but also decreased the expression of PTBP2. It is already known that apart from imparting their effects on the function of cancer, aberrant splicing events themselves

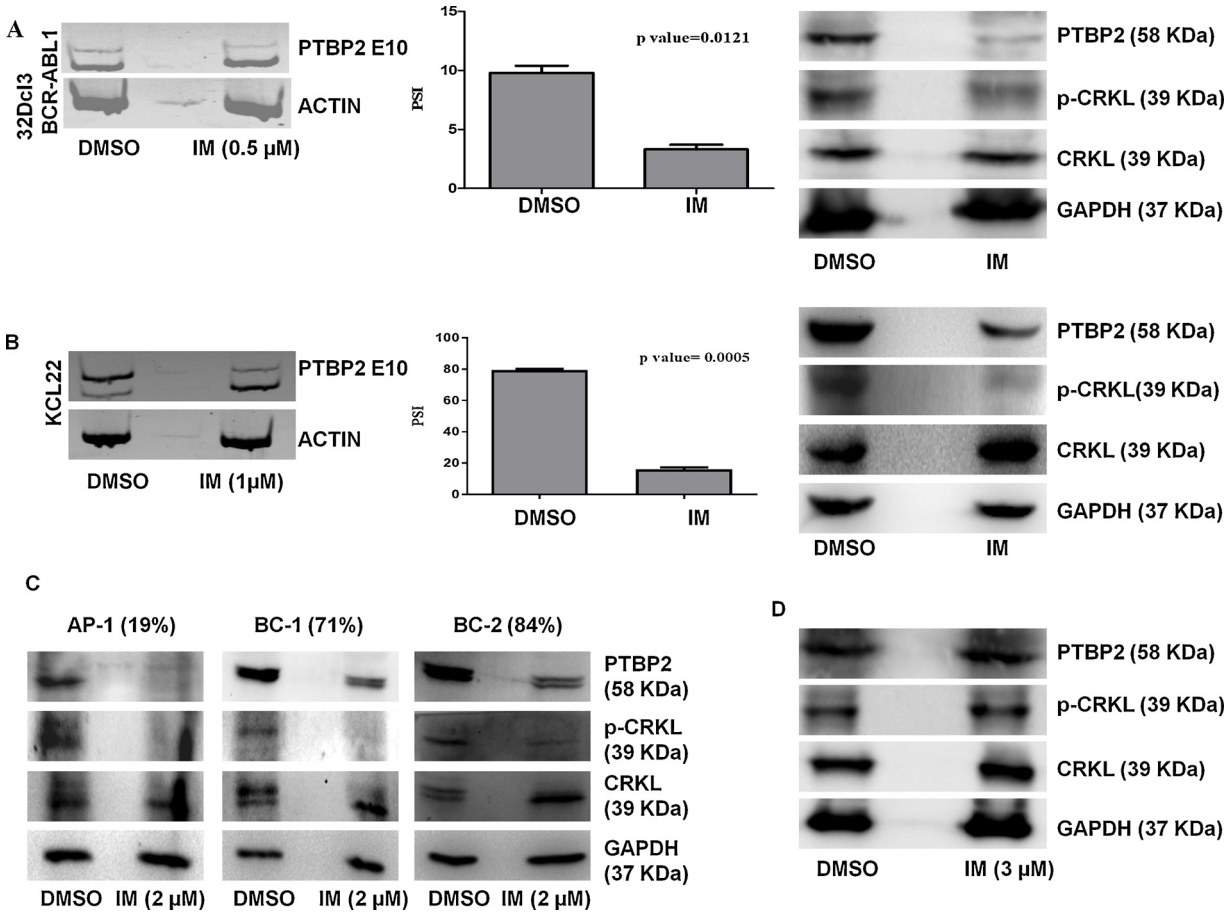

Fig. 3. (A and B) RT-PCR, densitometry analysis and western blot showing PTBP2 expression in imatinib treated 32Dcl3 BCR-ABL1 and KCL22 cell lines. Student's *t*-test was performed ($p = 0.0076$ and 0.0008) respectively. (C and D) Western blot showing PTBP2 expression in imatinib treated patient samples and in 32Dcl3 T315I cell lines.

have the potential to act as cancer biomarkers and therapeutic targets.

PTB knockdown either in neuronal/non-neuronal cell lines leads to increased PTBP2 exon 10 inclusion resulting in induction of PTBP2 protein expression (Spellman et al., 2007) whereas restoration of PTB in mature neuronal cells represses PTBP2 expression via exon 10 splicing changes. These results indicate that PTB plays a crucial role in maintaining the repression of PTBP2 across a wide range of cells.

Silencing of PTBP2 in KCL22 cells showed reduced cell proliferation which is in sync with earlier findings were silencing of PTBP2 along with PTBP1 reduced the proliferation of glioma cells (Cheung et al., 2009). HITs-CLIP data from developing mouse neocortex combined with splicing-sensitive microarray revealed that the significant action of PTBP2 is to inhibit adult specific alternative exons associated with the control of cell fate, proliferation and actin cytoskeleton (Licatalosi et al., 2012). In oral squamous cell carcinoma (OSCC), PTBP1 and PTBP2 impair the autoregulation of SRSF3 by enhancing SRSF3 full-length expression by inhibiting the exon 4 inclusion via interacting with the exonic splicing suppressor which in turn promotes PTBP2 expression (Guo et al., 2015). Ablation of PTBP2 results in germ cell loss due to increased apoptosis of meiotic spermatocytes and postmeiotic arrest of spermatid differentiation (Zagore et al., 2015). Likewise, we observed an arrest in the G2/M phase of the cell cycle when PTBP2 was knocked down in KCL22 cells. Increase in apoptotic and necrotic cell death patterns observed in PTBP2 shRNA treated KCl22 cells compared to the control cells observed in our study supplements previous findings wherein PTBP2-EMxKO pups displayed slower growth and all pups died around weaning age. Staining of EMxKO cortical plate showed increased neuronal cell death, and staining with more specific cell death markers showed evidence of both necrotic and apoptotic cell death (Li et al., 2014). Recently Ptbp2 was found to control a network of genes involved in cell adhesion, migration, and polarity. Ablation of Ptbp2 in germ cells resulted in disorganization of actin filaments suggesting that alternative splicing mediated by Ptbp2 is critical for cellular crosstalk during germ cell development (Hannigan et al., 2017)

As the CML-blast crisis is characterized by differentiation blockade and delayed proliferation of myeloid cells, unraveling the role of splicing factors involved in granulopoiesis might pave clue in understanding the mechanisms leading to disease progression. Evaluation of alternative splicing events, in CML, can be used to identify novel disease markers and drug-sensitive targets to overcome the limitations of small molecule inhibitors that are currently being used for treating patients with CML. Thus unrevealing whether PTBP2 exon 10 inclusion is miRNA dependent as reported in other tissues or miRNA independent will uncover a novel pathway that leads to disease progression. Our study portrays PTBP2 exon 10 as a possible target in CML disease progression, and we speculate that PTBP2-dependent mRNA stabilization and/or splicing may play a decisive role in CML, which can eventually propagate disease progression.

4. Materials and methods

4.1. Patient samples, cell lines, and plasmids

The study was approved by the institutional human ethical committee and the peripheral blood samples were collected from CML patients after obtaining a written informed consent. Peripheral blood

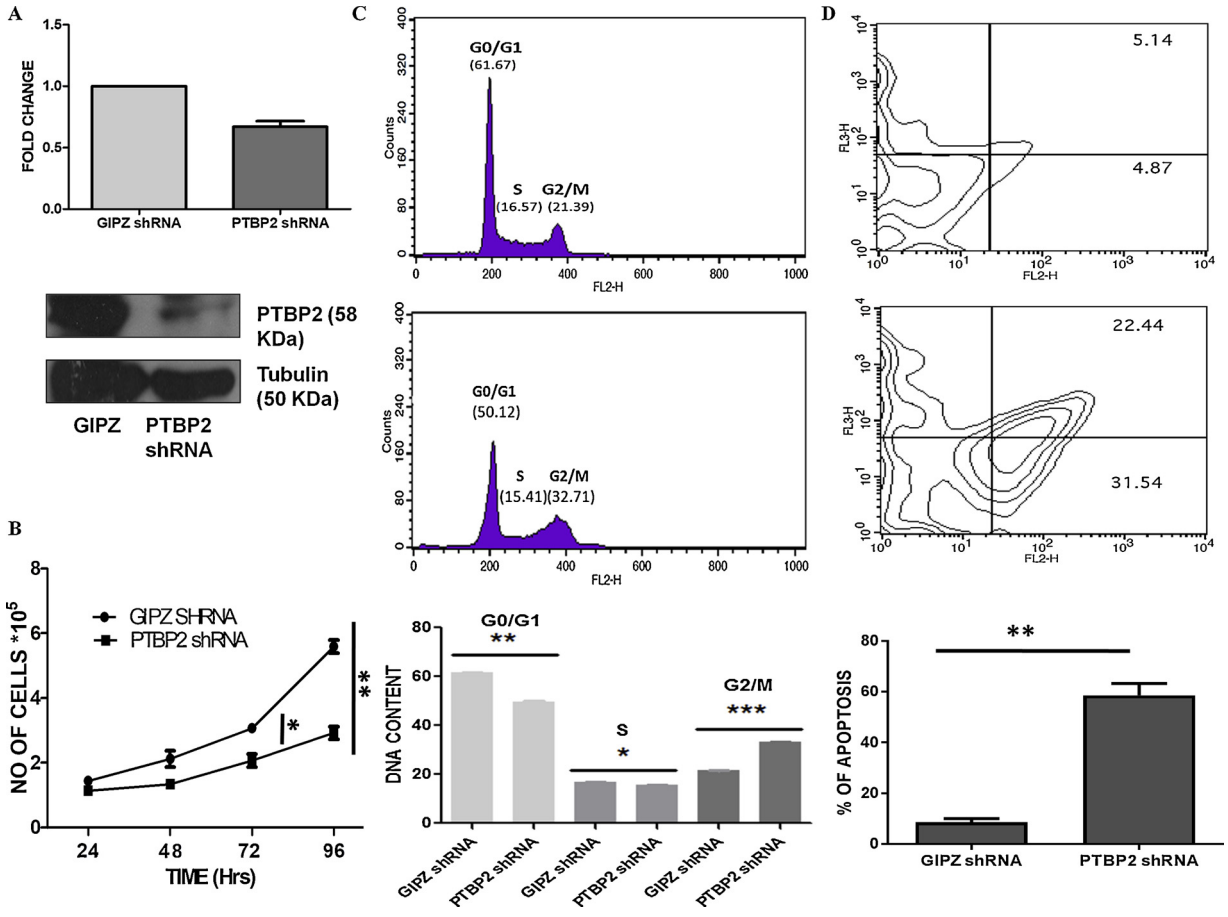

Fig. 4. (A) RQ-PCR and western blot showing PTBP2 expression in shRNA transduced KCL22 cells. (B) Growth curve assay was performed taking 2×10^5 GIPZ shRNA, and PTBP2 shRNA transduced KCL22 cells. The cells were counted using Biorad's automated cell counter and by manual counting using trypan blue exclusion dye for 24–96 h (*p*-value are as follows: 24 and 48 h – not significant, 72 h – 0.0422 and 96 h – 0.0110 respectively). (C) Cell cycle analysis was performed by staining cells using propidium iodide and acquired by flow cytometry. DNA content at each cycle was analyzed. Students test was performed for G0/G1, S and G2/M phase (*p*-value are as follows: 0.0015, 0.0150 and 0.0007 respectively). (D) Annexin V-7AAD apoptosis assay was performed in control and PTBP2 shRNA transduced KCL22 cells. Students test was performed ($p = 0.0092$).

mononuclear cells (PBMCs) were isolated by using Ficoll Paque plus (17-1440-02, GE Healthcare, USA) density gradient centrifugation. Mouse myeloblast cell lines 32Dcl3, BCR-ABL1 transduced 32Dcl3 (32Dcl3 BCR-ABL1), and BCR-ABL1 T315I transduced 32Dcl3 (32Dcl3 T315I), and CML-BC derived cell lines KCL22, K562, LAMA 84 and KYO1 were used for in vitro studies. WEHI-3B supernatant was used as a source of Interleukin-3 to grow 32Dcl3 and its derived cell lines. pSPAX2, pMD2G (lentiviral packaging plasmids) were purchased from Addgene.

4.2. RNA isolation, cDNA preparation and RQ-PCR

RNA isolation, cDNA preparation and real time PCR were performed as mentioned in our previous experiment (Agatheeswaran et al., 2013). The forward and reverse primers used in this study are listed in Supplementary Table S5. For sequencing of PTBP2 exon 10 +/− bands RT-PCR was performed and the PCR products were separated in a 12% polyacrylamide gel (PAGE), and the PCR bands +/− PTBP2 exon 10 was gel excised and eluted using Genelute (Sigma-Aldrich, USA) gel extraction kit according to the manufacture's protocol. For detection of ABL1 kinase domain mutations, RT-PCR was performed for ABL1 kinase domain region and the products were resolved in 1.2% agarose gel and the desired amplicons were purified using Genelute gel extraction kit (Sigma-Aldrich, USA) and sequenced on Applied Biosystems Genetic analyzer 3500 (Life Technologies, USA).

4.3. Mutation detection

Sequence data was analyzed using the chromasliteV 2.01 software (http://www.technelysium.com.au/chromas_lite.html) and was blast searched using NCBI nucleotide Blast, aligned with reference Genbank cDNA sequence (PTBP2 – NM_019550, ABL1 – NM_005157) using EBI's clustalW multiple sequence alignment tool (http://www.ebi.ac.uk/tools/psa/emboss_needle/nucleotide.html).

4.4. Cell culture and immunoblotting

RPMI media with 10% heat-inactivated serum were used for suspension cells. Human PBMCs were cultured in IMDM media with 10% heat-inactivated serum supplemented with IL3 (50 ng/ml) for 48 h followed by G-CSF (50 ng/ml) and imatinib (2 μM/ml) for 48 h. IL3 was added for expansion of cells and G-CSF was added for differentiation of granulocytes. Cytokines (IL3-2503 and G-CSF-2615) were purchased from Stem Cell Technologies (Canada). Imatinib (13139) was purchased from Cayman chemicals. Cells were lysed on ice by Ipegal-40 based lysis buffer (Tris-25 mM, NaCl-50 mM, 1% Ipegal-40). Expression of proteins was checked in patient samples by boiling 1×10^6 PBMC cells with 2X sample buffer. Total protein was resolved by SDS-polyacrylamide (Sigma) gel electrophoresis and was electroblotted to polyvinylidene difluoride membrane (Amersham Biosciences). After blocking the membrane with 3% nonfat milk (Bio-Rad), it was incubated with different primary and secondary antibodies at room

temperature for 1 h to overnight and 40 min respectively. Signals were detected by using chemiDoc (Bio-Rad) after treatment with the enhanced chemiluminescence (GE Healthcare). The membrane was probed with the following antibodies: α-PTBP2 (1:50,000) was kindly provided by Dr. Robert B. Darnell, Rockefeller University, New York and by Dr. Douglas L Black University of California, Los Angeles, α-tubulin (T5168, Sigma Aldrich, WB 1:10,000), α-PTBP1 (8776S, Cell Signalling Technologies, 1:5000), α-pCRKL (Tyr207, 3181L, Cell Signalling Technologies, WB 1:2000), α-CRKL-human (32H4, 3182S, Cell Signalling Technologies, WB 1:5000), α-CRKL-mouse (PA5-28622, Life technologies, WB 1:2000) α-c-ABL (24-11, SC-23, Santa Cruz, WB 1:2000), α-actin (4967S, Cell Signalling Technologies, WB 1:5000), α-GAPDH (2118S, Cell Signalling Technologies, WB 1:5000). The following secondary conjugated antibodies were used α-rabbit HRP (SC2313, Santacruz WB 1:5000) and α-mouse HRP (SC2005, Santacruz WB 1:5000).

4.5. Lentiviral-mediated transduction of KCL22 cells

Lenti virus particles carrying PTBP2 shRNA were produced as reported in our earlier study (Agatheeswaran et al., 2013) and transduced in KCL22 cells. pGIPZ lentiviral empty vector (RHS4351) and PTBP2 shRNA (RHS4430-101126814) were purchased from Thermo Fischer Scientific.

4.6. Cell proliferation, cell cycle, and apoptosis assay

Proliferation was studied in KCL22-GIPZ and KCL22-PTBP2 shRNA stable cells. 2×10^5 cells were cultured in RPMI 1640 media with 10% FBS. The cells were stained with trypan blue and counted every 24 h for 4 days using Biorad's automated cell counter (and manual counting by using trypan blue exclusion principle).

For cell cycle analysis, 1×10^6 KCL22-GIPZ and KCL22-PTBP2 shRNA stable cells were washed with PBS and labeled with propidium iodide solution (20 g/ml PI, 10 g/ml RNaseA, 20% Triton X-100). The cells were washed after 20 minutes before acquisition using FACS Calibur (BD Biosciences).

5×10^5 KCL22-GIPZ and KCL22-PTBP2 shRNA stable cells were grown in RPMI 1640 media with 10% FBS. Apoptosis assay was carried out using the Annexin-V-PE & 7-AAD apoptosis detection kit (BD Biosciences). For Annexin-V assay after labeling with Annexin-V-PE & 7-AAD, the cells were subjected to flow cytometry analysis to detect the externalization of phosphatidylserine (PS) on the cell membrane, a characteristic feature of early apoptosis using FACS Calibur (BD Biosciences).

4.7. Statistics

Student's *t*-test and one way ANOVA with Bonferroni's test were performed for comparing two samples and more than two samples respectively. Continuous data are expressed as mean ± SEM of two independent experiments, each performed in duplicates. Results with a *p*-value of ≤ 0.05 were considered as significant. *p* values represented in figures, ≤ 0.05 = *; ≤ 0.01 = **; ≤ 0.001 = ***. All statistical tests were performed using GraphPad Prism v.5.01 (La Jolla) software.

4.8. Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Author's contribution

SRN, SA and YV did the experiments and wrote the manuscript. SB, GB, NCP provided the samples and analyzed the clinical data. SC designed the study, supervised the work, arranged for the funds and wrote the manuscript. The authors declare no conflict of interest.

Conflict of interest

The authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biocel.2019.01.018.

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