Research Article

Abnormal PTBP1 Expression Sustains the Disease Progression of Multiple Myeloma

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Multiple myeloma (MM) is a hematopoietic malignancy characterized by heterogeneity, which corresponds to alternative splicing (AS) profiles and disadjust gene expression. Bioinformatics analysis of AS factors possibly related to MM progression identified the polypyrimidine tract binding protein (PTBP1) as candidate. The purpose of this study was to confirm the incidence and prognostic value of PTBP1 in MM patients. Several cohorts of 2971 patients presenting newly diagnosed and relapsed MM were enrolled. Correlations between PTBP1 expression and clinicopathological characteristics, proliferative activity, and response to therapy of myeloma cells were analyzed. Moreover, the effect of PTBP1 on the AS pattern of specific aerobic glycolysis-related genes was explored in MM patients. Clinically, PTBP1 expression was present at all stages; it increased with disease progression and poor prognosis, which was even stronger elevated in patients with high tumor burden and drug resistance. Mechanistically, PTBP1 modulated AS of PKM2 and aerobic glycolysis-related genes in MM patients, which play synergistic or additive effects in clinical outcome. PTBP1 may be a novel marker for prognostic prediction and a promising therapeutic target for the development of anti-MM treatments.

1. Introduction

Multiple myeloma (MM) is a plasma cell malignancy and is characterized by hypercalcemia, renal disorder, anemia, and lytic bone lesions, and it is incurable. Although the utilities of novel chemotherapies have obviously conferred survival advantage, MM remains a relapsed or refractory disease [1–3]. Thus continued investigations to identify established markers for risk stratification are still in urgent requirement [4]. To date, several molecular markers have been adopted as a standard staging system (DS/ISS); they are still inadequate in prognostic prediction and providing treatment choices [5]. The appropriate biomarkers that can reduce the probability of recurrence and progression are a clinicopathological priority for MM risk stratification [6, 7].

Adaptation to various stresses is an important characteristic of tumor cells. Recent studies reported how tumor cells regulate gene expression at the level of alternative splicing (AS) to withstand various stresses [8, 9]. AS leads to ligation of exons and excision of introns from the pre-mRNA and is arranged by the spliceosome [10]. When the intron/exon boundaries show high standards of conservation, exons are almost contained in the mRNA, whereas some exons lacking consensus sequences are excluded by alternative regulation [11]. In these cases, exons’ recognitions are regulated by trans-acting splicing factors (SFs). The main members of SFs are serine-arginine proteins and the heterogeneous nuclear ribonucleoproteins, which act antagonistically in AS regulation [10], and the interaction among antagonistic SFs decides whether exon is skipped or included through AS. Therefore, AS increases the coding potential of genomes and represents an evolutionary advantage [12]. However, the changeable regulation adds further opportunities for error, and the defective splicing may contribute to the neoplastic transformation [13–15].

Polypyrimidine tract-binding protein (PTBP1) is a kind of SFs, which participates in variable biological processes [16]. It has been shown that PTBP1 plays important roles in several tumors, such as bladder cancer [17], pancreatic cancer [18], and colon cancer [19]. Accumulating studies
have demonstrated that PTBP1 could modulate the expression of pyruvate kinase M2 isoform (PKM2), which is a vital regulator of glycolysis [16, 19, 20]. For example, Cheng et al. found that PTBP1 knockdown overcomes the resistance to vincristine and oxaliplatin along with the switching of the PKM isoform from PKM2 to PKM1, making for inhibiting glycolysis [19]. However, the role of PTBP1 in MM progression is yet to be elucidated.

In this study, we investigated the impact of PTBP1 expression in MM patients’ survival, as well as the correlation with clinicopathological characteristics, proliferative activity, and response to therapy of myeloma cells. We also explored whether PTBP1 plays a functional role in aerobic glycolysis and influences the prognosis in MM.

2. Methods and Materials

2.1. Data Source and Microarray Analysis. Gene Expression Omnibus ( GEO ) database was carried out to examine the expression of PTBP1 in 2971 MM patients ( GSE5900 [21], GSE2658 [22], GSE24080 [23], GSE31161 [24], GSE38503 [25], GSE9782 [26], GSE19554 [27], and GSE57317 [28]). Data acquisition and normalization methods in the aforementioned databases have been described previously [23, 29]. The gene expression of PTBP1 in plasma cells was determined using the Affymetrix U133Plus2.0 microarray, which was performed as previously described [22].

2.2. Cell Culture and Western Blotting. Human myeloma cell line ( RPMI-8226 ) was cultured in RPMI 1640 medium ( Gibco, USA ) supplemented with 10% heat-inactivated FBS ( Gibco, USA ), penicillin (100 IU/ml), and streptomycin (100 μg/ml) in a humidified incubator at 37°C and 5% CO2. Protein extracts and western blotting were performed as described previously [3, 30]. Primary antibodies include PTBP1 ( Proteintech, USA ), PKM2 ( Zen Bioscience, China ), and GAPDH ( Cell Signaling Technology, USA ). GAPDH was used as a loading control to normalize the protein signal. All western blot experiments were repeated in biological triplicate.

2.3. Statistical Analysis. Various statistical analysis methods were utilized to assess the roles of PTBP1 expression in clinical features and prognosis in MM patients. A Kruskal-Wallis test was used to compare multiple sets of samples. A two-tailed Student t-test was used to compare the mean values of the two groups. The one-way analysis of variance ( ANOVA ) test was used to compare means of more than two groups. The chi-square test was used to compare clinical and pathological features between the PTBP1 high and PTBP1 low groups. Survival curves were plotted according to the Kaplan-Meier method, and the log-rank test was employed to analyze statistical differences between survival curves. The effect of PTBP1 expression on outcome was analyzed using univariate and multivariate Cox regression models. For our analyses, the GraphPad Prism 6 software was employed, and p ≤ 0.05 was considered statistically significant.

3. Results

3.1. PTBP1 Is a High-Risk Myeloma Gene. To evaluate the potential that PTBP1 is important for MM, we examined the expression of PTBP1 in normal plasma ( NP ), smoldering multiple myeloma ( SMM ), monoclonal gammopathy of undetermined significance ( MGUS ), and myeloma cells using GEP datasets. Notably, PTBP1 expression significantly increased from NP, SMM, MGUS, to MM TT2 ( Total Therapy 2 ) and TT3 samples ( * * * p < 0.01, Figure 1(a)). In detail, we found higher PTBP1 expression in the proliferation subgroup ( PR ); the worst subgroup in MM patients ( p < 0.0001, Figure 1(b)). These findings led us to confirm that PTBP1 is a high-risk gene in MM.

3.2. Correlations between PTBP1 Expression and Clinicopathological Characteristics. To confirm the robustness of the PTBP1, we divided the patients into two categories according to their PTBP1 expressions ( low/high expression, using the 50th percentile as cutoffs ) and tested in predicting clinicopathological characteristics distribution. Using 11 clinicopathological characteristics, we found different distributions between the two subgroups in 559 MM patients. Expression levels of β2-microglobulin ( β2-MG ), lactate dehydrogenase ( LDH ), and bone marrow infiltration were significantly increased in the PTBP1 high subgroup compared with the PTBP1 low subgroup by unpaired t-test ( 4.11 ± 0.2308 vs. 5.352 ± 0.3885, p = 0.0062, Figure 2(a)); 166.5 ± 3.627 vs. 177.5 ± 4.22, p = 0.0479, Figure 2(b)); and 43.72 ± 1.642 vs. 49.01 ± 1.534, p = 0.0188, Figure 2(c)). The remaining characteristics were equally distributed between two subgroups ( Table 1 ). Consistent with GSE24080, PTBP1 expression was significantly correlated with low albumin ( 37.79 ± 0.6134 vs. 35.80 ± 0.5878, p = 0.0201, Figure 2(d)) and high β2-MG levels ( 5.227 ± 0.5208 vs. 8.927 ± 1.805, p = 0.0326, Figure 2(e)) in the GSE9782 ( Table 2 ). To validate our findings in Figure 1(b), we also evaluated the correlation of PTBP1 expression and proliferation. PTBP1 expression is positively correlated ( r = 0.3013, p < 0.0001, Figure 2(f)) with myeloma cell proliferation in 246 bortezomib-treated MM patients available at the GSE9782 dataset, using the global gene expression-based proliferation index ( GAPI ) of MM originated by Mayo Clinic as proxy of actual myeloma cell proliferation [31].

3.3. Increased PTBP1 Expression Is Linked to Disease Relapse in MM. The expression of PTBP1 is significantly increased in relapsed MM patients from TT2 and TT3 cohorts compared to baseline patients in the dataset ( 3822 ± 61.71 vs. 4285 ± 127, p = 0.0003; 4291 ± 59.29 vs. 4757 ± 350.8, p = 0.05, Figure 3(a)). Figure 3(b) also confirms this and shows the significantly increased PTBP1 expression in the relapsed group ( 7.101 ± 0.029 vs. 7.254 ± 0.023, p = 0.0002, Figure 3(b)). Furthermore, Figure 3(c) shows that significantly more patients in the PTBP1 low group had a higher bortezomib-treated response rate ( 54.1% vs. 40.1%, p = 0.03). To confirm the correlation between endogenous PTBP1 expression and drug resistance, we used the parental RPMI-8226 (8226) cell line and the RPMI-8226 drug-resistant (8226-DR) cell lines,
which acquired drug resistance by prolonged exposure to low doses of bortezomib. As expected, the expression of PTBP1 was substantially increased for the following three serials at diagnosis, prior to the first (after chemotherapies) and second autologous stem cell transplant, indicating that increased PTBP1 may account for drug resistance and promote cell proliferation \((p = 0.05, \text{Figure } 3(e))\). Consistent with this finding, the TT6 MM patients, who had been treated with more than one cycle of prior therapy excluding autologous hematopoietic stem cell transplant, were divided into two groups based on high and low PTBP1 expression, and the high PTBP1 group had an inferior overall survival (OS) \((p = 0.0195, \text{Figure } 3(f))\).

### 3.4. Higher PTBP1 Expression Predicts Poor Prognosis in MM.

To evaluate the biological outcomes of elevated PTBP1 expression in MM patients, we divided all MM into two groups based on high and low PTBP1 expression. The high PTBP1 expression group had shorter median OS and progression-free survival (PFS) than the low PTBP1 expression group (44 vs. 52 and 39 vs. 45, respectively). As shown in Figure 4, MM patients with strong PTBP1 expression had an inferior OS \((p = 0.0152, \text{Figure } 4(a))\) and PFS \((p = 0.0474, \text{Figure } 4(b))\). Furthermore, Table 3 shows the impact of PTBP1 expression and clinicopathological characteristics on clinical outcomes. Based on the results of univariate Cox proportional hazards regression analysis, \(β2\)-MG, Creatinine (Creat), ALB, and PTBP1 expression \((HR = 1.435, 95\% CI: 1.059–1.943, p = 0.020)\) were included in the multivariable Cox proportional hazards regression analysis which indicated that the PTBP1 expression was still an independent prognostic factor in terms of OS in 559 MM patients \((HR = 1.359, 95\% CI: 1.001–1.845, p = 0.049, \text{Table } 3)\). We also applied the Kaplan-Meier analysis to validated PTBP1 expression in another independent dataset, and the Kaplan-Meier survival analysis suggested that patients in low PTBP1 expression group had better OS and PFS compared with those in high PTBP1 expression group in GSE9782 \((p < 0.0001, \text{Figure } 4(c)); p = 0.0011, \text{Figure } 4(d))\).

### 3.5. PKM2 and Other Key Regulators of Warburg Effect Positively Correlate with PTBP1 Expression and Predict Survival in MM.

Using STRING tools, the protein-protein interaction analysis showed that PTBP1 and PKM interact or coexpress in the Homo sapiens protein interaction network (Figure 5(a)). To confirm the correlation between endogenous PTBP1 and PKM2, we investigated the expression of PTBP1 and PKM2 in 8226 and 8226-DR cell lines by western blotting. As expected, both PTBP1 and PKM2 were upregulated in 8226-DR cells compared to 8226 cells (Figure 3(d)). Given that PTBP1 is involved in PKM2-mediated-myeloma progression, we also investigated the correlation between PTBP1 and PKM2 in MM patients. As shown in Figure 5(b), PKM2 expression was significantly correlated with PTBP1 expression in the GSE2658 with \(r\) value as 0.3666, respectively \((p < 0.0001, \text{Figure } 5(b))\). To further investigate whether PTBP1 and PKM2 have synergistic or additive effects in MM patients' outcome, 351 myeloma patients were divided into 3 subgroups including PTBP1 low/PKM2 low, PTBP1 mid/PKM2 mid, and PTBP1 high/PKM2 high, and survival curve showed that the PTBP1 high/PKM2 high group has the worst outcome in OS \((p < 0.0001, \text{Figure } 5(c))\). These clinical data strongly support findings that PTBP1 interacts with PKM2 and promotes its oncogenic function. Previous studies indicated that PKM2 plays a vital role in aerobic glycolysis [32, 33]. We then investigated whether PTBP1 alters aerobic glycolysis by regulating PKM2 expression. The correlation between PTBP1 and aerobic glycolysis genes was tested in 351 MM patients. The expression of PTBP1 and glycolysis-enhancing genes, such as lactate dehydrogenase A (LDHA), alpha-enzyme (ENO1), and hexokinase 2 (HK2), was significantly positively correlated each other \((r = 0.3329, r = 0.2780, r = 0.3225, p < 0.0001, \text{Figures } 5(d)–5(f))\).
4. Discussion

MM remains incurable despite novel treatments, and plenty of prognostic markers that reflect tumor- or host-related factors have failed to explain thoroughly the heterogeneity in clinical outcomes [34]. Meanwhile, the AS signature of MM is emerging as a detailed marker to distinguish tumor subtypes and accurately stratify patients [35]. The workflow chart is shown in Figure 4(e); we extracted 2971 MM patients’ gene expression microarrays from the GEO database. In GSE24080, we analyzed the association between PTBP1 and clinicopathological characteristics of 559 MM patients.

**Figure 2:** PTBP1 is linked to myeloma progression in MM. (a)–(c) The levels of β2-MG, LDH, and bone marrow infiltration in PTBP1\textsuperscript{high} and PTBP1\textsuperscript{low} subgroups. β2-MG, LDH, and bone marrow infiltration expressed highest in the PTBP1\textsuperscript{high} group, while lowest in the PTBP1\textsuperscript{low} group. (d, e) The levels of ALB and β2-MG in the PTBP1\textsuperscript{high} and PTBP1\textsuperscript{low} subgroups. ALB expressed highest in the PTBP1\textsuperscript{low} group, while lowest in the PTBP1\textsuperscript{high} group. (f) A scatter-plot demonstrating positive correlation of PTBP1 expression and myeloma proliferation in 246 bortezomib-treated MM patients.
In GSE24080, MM patients were treated through TT2 (induction therapy: D(1)-PACE, dexamethasone with or without thalidomide; maintenance: thalidomide) and TT3 (induction therapy: VTD-PACE; maintenance: bortezomib-thalidomide-dexamethasone). In GSE2658, we analyzed the expression of PTBP1 in eight different molecular subgroups. In GSE9782, 264 samples from 264 patients, we analyzed the association between the expression of PTBP1, clinicopathological characteristics, and the global gene expression-based proliferation index. In GSE31161, we analyzed the association between PTBP1 expression and relapse in 937 samples. In GSE83503, 585 samples from 585 cases, we analyzed the expression of PTBP1 and survival in 937 samples. In GSE83503, 585 samples from 585 cases, we analyzed the expression of PTBP1 before and after the first/second transplant in 12 paired MM patients. In GSE57317, 55 samples from 55 cases, we analyzed the association between the expression of PTBP1 and survival in ASCI-treated MM patients. Therefore, our study showed that PTBP1 favors splicing of oncogenic variants and uncovers novel potential prognostic and therapeutic targets for MM patients.

To predict the prognosis of MM patients, the assessment of cellular proliferative activity is regarded with importance [36]. Proliferation status of MM cells had been evaluated by the plasma cell labeling index, Ki-67, or metaphase cytogenetics [37, 38]. More importantly, PTBP1 was significantly higher expressed in the PR subgroup, which is characterized by overexpression of proliferation-related genes and accelerate cell cycles, which was the worst prognosis in comparison to the other molecular subgroups [22, 39]. The degree of bone marrow infiltration by MM cells, as estimated by bone marrow biopsy, is one of the considerable determinants of the MM tumor burden [40]. We performed correlation analysis between PTBP1 expression and bone marrow plasma cell infiltration, as derived from bone marrow biopsies. This significant correlation between bone marrow infiltration and PTBP1 expression raises the possibility that PTBP1 may represent a biomarker that indirectly reflects tumor mass and the level of bone marrow invasion at diagnosis [41]. Similarly, we evaluated BZW2 message levels in 241 bortezomib-treated patients paralleled the myeloma proliferation score, which was scored with the assistance of GPI model constructed by Hose and his associates [42]. Consequently, PTBP1 can facilitate cell proliferation and influences the prognostic impact on MM patients.

Another interesting finding in our study is that PTBP1 expressions appear to correlate in response to bortezomib-based chemotherapy. Bortezomib, which targets the 26S proteasome subunit β5, has induced a high level of positive response rates [43, 44]. However, toxicities associated with global proteasomal inhibition and drug resistance in MM were major concerns, prompting the further development
Figure 3: PTBP1 is linked to disease relapse in MM. (a) The expression of PTBP1 was significantly upregulated in relapsed patients from TT2 and TT3 cohorts in comparison with baseline patients. (b) The expression of PTBP1 was significantly upregulated in relapsed patients from the GSE83503 cohort in comparison with patients without relapse. (c) Bar view presents response rate between PTBP1low MM patients and PTBP1high MM patients treated with bortezomib. (d) Western blots showing the expression of PTBP1, PKM2, and GAPDH in 8226 and 8226-DR cells. (e) The expression of PTBP1 is showed in 12 MM patient samples collected at diagnosis, pre-1st and pre-2nd ASCT. (f) Kaplan-Meier analyses of OS revealed that high PTBP1 expression conferred inferior clinical outcomes in TT6 patients.
of novel target and therapies. In GSE31161, we found a significant increase in the expression of PTBP1 in relapsed MM patients from TT2 and TT3 cohorts in comparison with baseline patients. Furthermore, compared to PTBP1\textsuperscript{high} samples, patients with PTBP1\textsuperscript{low} MM cells were significantly responded to bortezomib evidenced in GSE9782 [26]. Consistent with GEPs derived from patient populations, the protein expression of PTBP1 was substantially increased in 8226-DR cells compared with parental 8226 cells. The above data suggested that myeloma with higher PTBP1 expression represents more aggressive behavior and worse response to chemotherapies.

Aberrant splicing regulation confers alternative advantage to tumor cells by favoring oncogenic splice variants of tumor-related genes [9, 45]. For example, upregulation of PTBP1 in tumor cells affected glycolytic metabolism by...
promoting AS of the PKM2 variant [46], leading to acquisition of drug resistance to chemotherapy [18]. Likewise, by screening PTBP1-interaction targets reported by STRING database, we found that PTBP1 and PKM2 mRNA expression is positively correlated in GSE2658. Additionally, the high expression of the PTBP1 and PKM2 groups showed worst prognosis in various types of MM. The above clinical data forcefully support our findings that PTBP1 upregulates PKM2 expression and promotes its oncogenic function. Because PKM2 is a fundamental enzyme for regulation of aerobic glycolysis in tumor cells, we further determine that PTBP1 expression is positively correlated with aerobic glycolysis genes including LDHA, HK2, and ENO1. Myeloma cells possess increased glycolysis for ATP generation, which is called the Warburg effect [47, 48]. Recently, accelerating studies confirmed that aerobic glycolysis is the hallmark of tumor cells and crucial for proliferation and survival [49, 50]. Despite therapeutic advances, the MM patients eventually relapse and the altered metabolism with increased glycolysis is showed to contribute to drug resistance [48, 51], and increasing research reveals that inhibition of glycolysis restores sensitivity to bortezomib and suppresses tumor growth induced by metabolism [51]. It indicated that targeting glycolysis may be a novel therapeutic strategy to overcome drug resistance.

**Figure 5:** PTBP1 regulates aerobic glycolysis in MM. (a) The protein network was constructed by online software STRING. (b) A scatter-plot showed the correlation between PTBP1 and PKM2. (c) Kaplan-Meier analyses of OS among MM patients with different expression levels of PTBP1 and PKM2. (d–f) Scatter-plots shows the correlation between PTBP1 and glycolysis-enhancing genes, respectively.
5. Conclusions

In conclusion, our results revealed that increased PTBP1 expression was associated with a poor outcome and resistance to chemotherapy in newly diagnosed MM patients. We also characterized PTBP1 as a novel regulator of aerobic glycolysis which contributes to PKM pre-mRNA splicing. Hence, to better individualize the chemotherapy regime, apart from the laboratory markers of prognostic significance, the incorporation of an initial valuation of PTBP1 expression to an individual prognostic profile for MM risk stratification should be considered.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors’ Contributions

All authors contributed to the data analysis and drafting and revising of the article; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work.

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