Aberrant activated APOBEC3B is associated with p53 mutant-driven refractory/relapsed Diffuse Large B-cell Lymphoma

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Research

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Abstract

Background

Although treatment of diffuse large B-cell lymphoma (DLBCL) has progressed considerably in recent years, treatment failure still occurs in about 40% of patients who are refractory/relapse. Recent studies suggest that *TP53* mutation may be an important cause of refractory/relapse in DLBCL, but the cause of *TP53* mutation remains unclear.

Methods

In the present study, the correlation between *TP53* mutation status and APOBEC3A and APOBEC3B expression in DLBCL specimens was searched by detecting the correlation between *TP53* mutation and APOBEC3B expression in combination with database informatics analysis. Further, the relationship between APOBEC3B expression and *TP53* mutation was analyzed by constructing APOBEC3B induced expression DLBCL cell lines. The effects of APOBEC3B-induced *TP53* mutants on DLBCL cell proliferation and drug resistance were also tested.

Results

We identify APOBEC3B as a critical factor that regulates p53-mutant driven drug resistance of DLBCL. APOBEC3B induces *TP53* mutations of DLBCL cells, and its mutation patterns are similar to those in DLBCL patients. Moreover, APOBEC3B-induced p53 mutants promoted growth of DLBCL cells as well as contributed to drug resistance. In human DLBCL, APOBEC3B is aberrantly activated and associated with p53 mutant-mediated refractory/relapsed DLBCL.

Conclusion

These findings yield insights into the mechanism of refractory/relapsed DLBCL induced by p53 mutants and reveal APOBEC3B as a new therapeutic target.

1. Background

Diffuse large B-cell lymphoma (DLBCL), the most frequent subtype of lymphoid malignancy in adulthood, is a heterogeneous disease. Although current standard-of-care immunochemotherapeutic regimen of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) has greatly improved the outcome of DLBCL patients, and durable remission is achieved in 50% of cases, DLBCL remains a significant clinical challenge, with approximately one-third of patients not being cured by this regimen, highlighting a need for more potent and safer therapies against novel targets^{1–3}.

TP53 is an important tumor suppressor which participates in regulation of cell cycle, DNA repair, apoptosis and senescence⁴. *TP53* deletion or mutation is frequent in B-cell malignancies and is associated with a low response rate^{5,6}. Importantly, several hotspot mutations including

283,248,273,175,176 and 213, which are mainly located in DNA binding domain especially the loop-sheet-helix and L3 motifs, are independent negative predictors for DLBCL⁷⁻⁹. However, the mechanism of *TP53* mutation in DLBCL is still not fully understood. Interestingly, most of these mutations, especially the high-risk hotspot mutations, were G/C to A/T mutations¹⁰, which are similar to those induced by apolipoprotein B mRNA editing enzyme catalytic polypeptide-like (APOBEC)3 s family^{11, 12}. These facts prompted us to investigate whether APOBEC3s are responsible for the G/C to A/T mutations in DLBCL.

In the present study, through analysis of *TP53* mutation in specimens from refractory/relapse DLBCL patients, we investigated the relationship between APOBEC3B and *TP53* hotspot mutations in DLBCL. We expect to reveal the mechanism of *TP53* G/C to A/T mutation and then hope to provide some indications for further monitoring and treatment of DLBCL.

2. Materials And Methods

2.1. Patient samples

From November 2016 to July 2018, a total of 61 patients (including 33 females and 28 males), who were diagnosed with de-novo DLBCL by surgical biopsy of the tumor tissues in the Second Affiliated Hospital of Zhejiang University School of Medicine, were included in this study. Diagnosis of DLBCL was made independently by two experienced pathologists according to the 2016 world health organization (WHO) classification. Nineteen patients were relapse or refractory DLBCL according to the criteria defined by Cheson et al¹³. This study was approved by the ethics committee of the hospital in accordance with principles of the Declaration of Helsinki. All the patients provide written informed consent at enrollment.

2.2. Detection of *TP53* G/C to A/T mutation in refractory/relapse DLBCL samples

For all patients, formalin-fixed, paraffin-embedded (FFPE) tumor tissue blocks were collected, and tumor tissue genomic DNA were extracted using QIAamp DNA FFPE Tissue Kit (Qiagen, USA) according to the manufacturer's instructions. Peripheral blood samples were collected as matched normal samples. Total DNAs of peripheral blood mononuclear cells (PBMC) were extracted using QIAamp DNA Blood Mini Kit (Qiagen, USA) according to the manufacturer's instructions. G/C to A/T mutations in *TP53* exon8 were detected by differential DNA denaturation degree PCR base sequencing as described by Burns M.B. et al¹⁴ with some modifications (see supplementary material).

2.3. Cellular localization of APOBEC3s

Immunofluorescence staining of APOBEC3s-HA were described previously¹⁵. APOBEC3s-pEGFP detection were described in supplementary material.

2.4 Online DLBCL database analysis of APOBEC3A and APOBEC3B expression

2.4.1. Oncomine DLBCL database analysis

Because APOBEC3A and APOBEC3B were reported to have the potential activity for inducing G/C to T/A mutation in several human cancers, we analyzed the expression status of APOBEC3A and APOBEC3B in DLBCL using the Oncomine database (<http://www.oncomine.org>) based on existing cancer microarray data sets. APOBEC3A and APOBEC3B expression data of two lymphoma groups, Brune Lymphoma and Compagno Lymphoma, were extracted from GSE12453 and GSE12195 gene chips on GEO database. Then the expression levels of APOBEC3A and APOBEC3B each between DLBCL and different types of normal cell, as well as these two genes in DLBCL, were compared by GraphPad Prism5 software.

2.4.2. GEPIA DLBCL database analysis

Based on <http://gepia.cancer-pku.cn>, expression of APOBEC3A and APOBEC3B in DLBCL on Box Plots were searched via Single Gene Analysis.

2.4.3. GEO DLBCL database analysis

Gene expression profiling data of DLBCL were obtained from the Gene Expression Omnibus (GEO) online database (<https://www.ncbi.nlm.nih.gov/geo/>). The searching results were sorted by number of samples. Expression data of APOBEC3A and APOBEC3B were obtained from these data, and then were compared by GraphPad Prism5 software. Because the expression data are from different sample type including frozen tumor tissue and FFPE tumor tissue. Then expression data of APOBEC3A and APOBEC3B were compared based on different tumor tissue resources in GSE19246, which containing both FFPE and frozen tissue samples, to verify whether sample source could influence the results.

2.5. Detection of APOBEC3B in refractory/relapse DLBCL samples

For all patients, FFPE tumor tissue blocks were collected, and APOBEC3B was detected by immunohistochemical staining method. Anti-APOBEC3B antibody (Cat No. ab191695) was purchased from Abcam USA. DAKO Envision + System (Code K4002, Dako, Denmark) was used for the detection of DAB according the to the manufacturer's instructions.

2.6. Constructions of plasmids and APOBEC3A/APOBEC3B-inducible cell lines

2.6.1. Cell lines

DLBCL cell line Pfeiffer and OCI-LY10 were purchased from ATCC and cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Hek293 and 293T cell lines were purchased from ATCC, and cultured in DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. All cell lines were maintained at humidified, 5% CO₂ atmosphere at 37°C.

2.6.2. APOBEC3A/APOBEC3B-inducible DLBCL cell lines

APOBEC3A/APOBEC3B-flag cDNA was cloned into pLV-Ptight-puro vector (Clontech, Takara Bio, China) to construct inducible APOBEC3A/APOBEC3B-flag/pLV-Ptight-puro plasmid. Pfeiffer, OCI-LY10, 293T and Hek293 were transfected using Polyjet In Vitro DNA Transfection Reagent (SignaGen, USA) according to manufacturer's protocol. The 293T cells were transfected with APOBEC3A/APOBEC3B-flag/pLV-ptight-puro vector as well as PCL and 10A1. Seventy-two hours after transfection, virus supernatants were collected and purified by centrifuge and 0.45 μ m filter. Then the virus supernatant was stored at -80°C for the subsequent experiments. Pfeiffer, OCI-LY10 and Hek293 cells were seeded in 6-well culture plate, 24 hours later the medium was replaced with fresh medium and virus supernatant were added into the medium as well as 8 μ g/ml polybrene (Qiagen, USA). After infection for 12 hours, the medium was replaced with fresh medium. Forty-eight hours later, 3 μ g/ml puromycin (Qiagen, USA) was added to the medium for selection of infected clones. Inducible expression of APOBEC3A/APOBEC3B-flag was confirmed by Western blot using anti-flag antibody (Cat No. M185-3L, MBL, Japan).

After selection by puromycin for 14 days, the remainder cells were seeded at concentration of one cell/well in 96-well plate and checked by Western Blot after cultured with medium containing 4 μ g/ml doxycycline (Sigma, USA). Positive clones were maintained and used in the following steps.

2.7. Detection of APOBEC3A/APOBEC3B-induced G/C to A/T mutations in *TP53* exon8

After 14 days of induction by doxycycline, the APOBEC3A/APOBEC3B-flag-inducible cells as well as empty pLV-pTight-puro vector cells were collected and total DNA were extracted using Qiagen DNA mini kit (Qiagen, USA). The G/C to T/A mutations were detected and analyzed by 3D-PCR based Sanger sequencing method as described above.

2.8. Analysis of proliferation and drug sensitivity of p53-mutants-driven DLBCL cells:

2.8.1 Colony formation assay was performed as previous described¹⁶. Briefly, APOBEC3B-inducible DLBCL cells were induced by 4 μ g/ml doxycycline for 14days, then the doxycycline was removed and cultured for another 24 hours until APOBEC3B-Flag disappeared. APOBEC3B-expression cells and control cells were seeded in 6-well culture plates containing semi-soft agarose at a concentration of 200 cells per well. Then the cells were cultured for another 14 days and colonies were stained by Giemsa and calculated under microscope. Doxorubicin sensitivity was measured by MTT assay as previous described¹⁷.

2.8.2 Construction of p53 mutants (R273C and R282Q) and Doxorubicin sensitivity analysis in DLBCL:

Point mutation of TP53-R273C and R282Q was produced using Hieff Mut™ Site-Directed Mutagenesis Kit (Yeasen, Shanghai, China) with p53-Flag-pLV-Ptight-puro as template, and the primers as follows (mutated base in lower case): R273C-up: 5'-CTTTGAGGTGtGTGTTTGTGCCTGTCCTGGGAGAGACCGGCG-

3'; R273C-dn: 5'-CGCCGGTCTCTCCCAGGACAGGCACAAACACaCACCTCAAAG-3'. R282Q-up: 5'-GGGAGAGACCaGCGCACAGAGGAAGAGAATCTCCGCAAGAAA-3'; R282Q-dn: 5'-TTTCTTGCGGAGATTCTTCTCTGTGCGCtGGTCTCTCCC-3'.

The Ly10-TP53-Mutants was constructed by lentiviral infection which performed according to the standard procedures. Briefly, the 293T cells were co-transfected with viral packaging vectors PCL and 10A1 (Clontech, Takara Bio, China), along with a lentiviral construct expressing vector or the empty vector as control, using Polyjet transfection reagent (SignaGen, Rockville, MD, USA). The transfection medium was replaced after 6 hours with fresh complete DMEM, and 48 hours later the viral supernatants were collected. Then the viral supernatants were added in Ly10 cells and accompany with 5 µg/mL Polybrene. The medium was replaced after 24 hours with fresh RPMI 1640 supplement with 10%FBS, and 72 hours later 2 µg/mL puromycin was added to the infected cells for selection. Doxorubicin sensitivity was measured by MTT assay as previous described¹⁷.

2.9. Statistical Analysis:

The statistical analyses were performed with IBM SPSS Statistics 20. Frequency tables and descriptive statistics (mean, median, minimum and maximum) were used for summarizing characteristics of the patients. Differences between compared groups of patients were assessed by Maximum Likelihood Chi-square test and Fisher exact test in categorical variables and by Mann-Whitney test in continuous variables. PFS was defined as time to disease progression, relapse or death. PFS was estimated using the Kaplan-Meier survival curves method, and compared using the log-rank test. The Kaplan-Meier method was used for univariate survival analysis. Multivariate Cox proportional hazard models were used to evaluate whether *TP53* mutation was independent prognostic factors for progress free survival (PFS). Colony formation data were reported as mean values ± SEM and were analyzed by independent t-test. Doxorubicin IC₅₀ analysis were analyzed by GraphPad Prism 5 (San Diego, California, USA). P values < 0.05 were considered statistically significant.

3. Results

3.1. *TP53* exon8 of refractory/relapse DLBCL samples contain more G/C to A/T mutations

Using 3D-PCR based direct sequencing method, G/C to A/T mutations were detected in R/R DLBCL samples (Fig. 1 and Figure S1), and most of these nucleotide mutations resulted in changes of amino acid in p53 protein (Fig. 2A and Table S1). The distribution of these mutation points was not random, several mutation points, such as R273C, R282W, R282Q, R283C and R290H (Fig. 2B), were more frequent than others. Most of these points were previously reported hotspot mutations including R273C, R282Q, R282W, and R283C. *TP53* exon8 of R/R DLBCL samples contained more G/C to A/T mutations than that of non-R/R DLBCL samples. *TP53* exon8 G/C to A/T mutations were detected in 83.33% of R/R DLBCL patients (15/18), whereas only 23.26% (10/43) of non-R/R patients contained such mutations ($\chi^2 =$

18.93, $P < 0.001$) (Fig. 2C). Hotspot mutations were detected in 14 of 18 R/R DLBCL patients, but only 5 of 43 non-R/R DLBCL patients ($X^2 = 25.89$, $P < 0.001$) (Fig. 2D).

After following-up for 12 months, the complete response (CR) rate of different groups, based on *TP53* exon8 status, were calculated and compared. The overall CR rate for the current cohort was 68.90%, which was similar with that of previous reported^{3,18}. But the CR rates in different groups, based on *TP53* status, were dramatically different. For the *TP53* exon8 wild type group, the CR rate was 81.08% (30/37) (Fig. 3A). The *TP53* exon8 mutation group had the CR rate of 28.57% (8/28) (compared with wild type group, $X^2 = 18.1$, $p < 0.01$, data not shown). Among the *TP53* exon8 mutation group, those who containing the hotspot mutation had the lowest CR rate, which is only 21.05% (4/19)(compared with wild type group, $X^2 = 18.97$, $p < 0.01$, Fig. 3A), however, those who containing non-hotspot mutation had the CR rate of 80% (4/5)(compared with hotspot group, Fisher Exact Test, $p = 0.028$, compared with wild type group, $X^2 = 0$, $p = 1$, Fig. 3A). Thus, the *TP53* wild type group and non-hotspot mutation group could be merged as one wildtype & non-hotspot mutation group, which had a higher CR rate compared with that of *TP53* hotspot mutation group ($X^2 = 23.203$, $p < 0.01$, data not shown). The hotspot mutation group had a median PFS of 6 months (95% CI: 3.245 to 8.755, Fig. 3B). While after follow-up for 12 months, PFS of non-hotspot mutation group and wild type group both were not reached (Fig. 3B). Among these two groups, the patients' characteristics were similar except for gender and B symptoms (Table 1).

Table 1
Patient Clinical Characteristics between different *TP53* Mutation Groups.

Patient Clinical Characteristics between <i>TP53</i> Mutation Groups					
	<i>TP53</i> wildtype & non-hotspot mutation group(42)		<i>TP53</i> hotspot mutation group(19)		
Characteristic	No	%	No	%	p value
Age,years					0.915
≤60	21	50.0	9	47.4	
61 to 70	12	28.6	7	36.8	
71 to 80	6	14.3	2	10.5	
>80	3	7.1	1	5.3	
Median	60.5		61		
Range	18–88		34–83		
Sex					0.036
Male	15	35.7	13	68.4	
Female	27	64.3	6	31.6	
Subtype					0.803
GCB	14	33.3	5	26.3	
non-GCB	28	66.7	14	73.7	
Stage					0.201
I	9	21.4	2	10.5	
II	9	21.4	1	5.3	
III	4	9.5	1	5.3	
IV	19	45.2	15	78.9	
B symptoms					0.029
present	14	33.3	12	63.2	
absent	28	66.7	7	36.8	

The statistical analyses were performed with IBM SPSS Statistics 20. Frequency tables and descriptive statistics (mean, median, minimum and maximum) were used for summarizing characteristics of the patients. Differences between compared groups of patients were assessed by Maximum Likelihood Chi-square test and Fisher exact test in categorical variables and by Mann-Whitney test in continuous variables.

Patient Clinical Characteristics between <i>TP53</i> Mutation Groups				
IPI				0.268
0	4	9.5	1	5.3
1	11	26.2	2	10.5
2	10	23.8	2	10.5
3	7	16.7	7	36.8
4	4	9.5	3	15.8
5	5	11.9	4	21.1
GCB: Germinal center B-like; IPI International Prognostic Index				
The statistical analyses were performed with IBM SPSS Statistics 20. Frequency tables and descriptive statistics (mean, median, minimum and maximum) were used for summarizing characteristics of the patients. Differences between compared groups of patients were assessed by Maximum Likelihood Chi-square test and Fisher exact test in categorical variables and by Mann-Whitney test in continuous variables.				

IPI was the well accepted prognostic factor for DLBCL. In present groups, *TP53* hotspot group contained more IPI > 2 (high-intermediate and high risk) patients compared than *TP53* wildtype & non-hotspot group. So, relationship between *TP53* mutation status and IPI were analyzed. Results showed that in IPI > 2 (high-intermediate and high risk) group, *TP53* mutation status also had a predictive value on PFS (Fig. 3C, $p = 0.04$). While, the PFS of *TP53* wildtype & non-hotspot mutation and IPI > 2 group was similar with that of IPI 0–2 group (Fig. 3D, $p = 0.08$). These results indicated that *TP53* mutation status had a prognostic value that is not currently captured by widely used IPI model.

To assess whether *TP53* mutation was an independent prognostic factor, risk factors were firstly screened by univariate survival analysis using Kaplan-Meier method (Table S2). Then International Prognostic Index (IPI), *TP53* mutation status, lactate dehydrogenase (LDH), stage and gender were analyzed by Multivariate Cox model. Results showed that *TP53* hotspot mutation was the strongest independent factor for PFS (HR 5.146, 95CI 2.134–12.409; $p < 0.0003$), followed by *TP53* mutation (HR 3.616, 95%CI 1.49–8.773; $p = 0.004$).

In summary, these data suggest that *TP53* mutation, especially hotspot mutation, contribute to the poor outcome of DLBCL when treated with R-CHOP.

3.2. APOBEC3B but not APOBEC3A increases in DLBCL compared with normal tissue

APOBEC3s family has seven members including APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3DE, APOBEC3F, APOBEC3G and APOBEC3H. Among these members, APOBEC3A, APOBEC3B, APOBEC3F and APOBEC3G exhibit cytidine deaminase that induces G/C to A/T mutation in single DNA strand.

APOBEC3F and APOBEC3G cause hypermutation of HIV genome, but both of them are localized in cytoplasm (Figure S2). APOBEC3A is localized both in cytoplasm and nucleus, whereas APOBEC3B is predominantly localized in nucleus (Figure S2) and could access to genomic DNA. Previous studies showed that APOBEC3A and APOBEC3B were candidates for host DNA mutation. Based on Oncomine database analysis, Brune lymphoma database revealed that APOBEC3A mRNA level was similar in DLBCL with that of normal tissue (Fig. 4A), whereas APOBEC3B mRNA level was higher in DLBCL than that of normal tissue (Fig. 4B). Using TCGA based GEPIA database, analysis of APOBEC3B and APOBEC3A expression level in DLBCL showed the similar result (data not shown) as that of Brune database. But this result was not confirmed in Compagno lymphoma database (Fig. 4D and 4E). Further analysis showed that expression levels of APOBEC3B were higher than that of APOBEC3A in DLBCL tissues both in Brune lymphoma and Compagno lymphoma database (Fig. 4C and 4F).

Then expression levels of APOBEC3A and APOBEC3B in DLBCL were further compared in GEO database. In GEO database, the results were sorted by number of samples from high to low. And the first 13 data were analyzed. APOBEC3A expression level was higher than that of APOBEC3B in 3 of 13 data including the biggest one (Figure S3A, S3D and S3I). While APOBEC3B expression level was higher than that of APOBEC3A in 10 of 13 data (Figure S3). In the biggest sample number data GSE117556, RNA was extracted from FFPE tumor tissue. So APOBEC3A and APOBEC3B expression levels were analyzed in different sample resources in GSE19246, which contains both frozen tumor tissue and FFPE tumor tissue. The results showed that APOBEC3A expression level was similar between these two kinds of sample resources (Figure S4A). While APOBEC3B expression level in frozen tumor tissue was higher than that of FFPE tumor tissue (Figure S4B). These results indicated that FFPE tumor tissue maybe result in underestimating of APOBEC3B expression. And APOBEC3B expression level was higher than that of APOBEC3A in most of data based on frozen tumor tissues.

Therefore, analysis of online database suggested that APOBEC3B, instead of APOBEC3A, is higher expressed in DLBCL cells.

3.3. APOBEC3B level was higher in R/R DLBCL samples than that of non-R/R ones

Given the fact that APOBEC3B induces G/C to A/T mutation in human cancers such as breast cancer, and the fact that APOBEC3B is up-regulated in DLBCL, we next measured the protein level of APOBEC3B in R/R and non-R/R DLBCL samples using IHC method. Interestingly, we found that APOBEC3B protein levels were higher in R/R DLBCL samples (Fig. 5A-5F) than that of non-R/R ones (Fig. 5G-5K). As expected, APOBEC3B protein was predominantly localized in nucleus. If set the cut-off value of APOBEC3B positive rate at 20%, analysis of APOBEC3B expression level in different *TP53* mutation status groups showed that APOBEC3B protein was higher in *TP53* mutation group (58.33%) than that of *TP53* wild type group (27.03%) ($\chi^2 = 4.657$ $p = 0.038$) (Table S3). More interestingly, APOBEC3B positive rate in *TP53* hotspot mutation group (68.42%) was higher than that of *TP53* wildtype & non-hotspot group (26.19%) ($\chi^2 = 9.776$ $p = 0.004$) (Table S4). These data suggested that over expression of

APOBEC3B is associated with *TP53* mutation, especially hotspot mutation, and refractory/relapsed in DLBCL.

3.4. APOBEC3B could induce G/C to A/T mutation in DLBCL cell lines

To further investigating whether over-expressed APOBEC3B could induce *TP53* mutation in DLBCL, we performed an in vitro assay of *TP53* mutation via inducible APOBEC3B-expression DLBCL cell clones. Using APOBEC3B-flag/pLV-Ptight-puro plasmid, we constructed an APOBEC3B-inducible DLBCL cell clones in *TP53* wild type DLBCL cell lines Pfeiffer and OCI-LY10. APOBEC3B-flag expression was confirmed by western blot after induction by doxycycline. *TP53* exon8 was amplified via 3D-PCR and c-MYC exon2, which is reported less mutated in DLBCL, was also amplified as control. As the denature temperature low-down from 94°C to 87°C, the *TP53* exon8 fragment was detected at 88°C and 87°C in APOBEC3B-inducible cells but not in the control cells (Fig. 6A). While MYC exon2 fragment was not detected at denature temperatures lower than 94°C (Fig. 6B). Also, over expression of APOBEC3A in DLBCL cells could not result in detection of *TP53* exon8 fragment under 89°C via 3D-PCR (Figure S5). Mono-clone sequencing of the *TP53* exon8 PCR products at 87°C indicated that over 20% clones contain more than one G/C to A/T mutation compared with the control group and the 94°C PCR products. The major mutation patterns were TG and GC. The mutation pattern and sites were similar with that of DLBC samples (Fig. 6D). Further analysis showed that there were several known hotspot amino acid mutations including R273C, R282Q, R282W and R283W (Fig. 6D). These data suggested that APOBEC3B could induce *TP53* G/C to A/T mutation, including those hotspot mutations, in DLBCL cells.

3.5. APOBEC3B-induced p53 mutants promoted cell proliferation and caused resistance to doxorubicin

As previous showed that inducible-expression of APOBEC3B could induce p53 mutants, then we analysis the influence of APOBEC3B-induced p53 mutants on proliferation and drug sensitivity of DLBCL cells (Pfeiffer) using previous APOBEC3B-expressed cells. Semi-soft colony formation assay showed that after induced expression of APOBEC3B for 14 days, the APOBEC3B-expression cells generated more colonies that that of control group although APOBEC3B was restored (Fig. 7A and 7B). After induced expression of APOBEC3B for 14 days, APOBEC3B-expression cells showed resistant to doxorubicin, with a 4.65-fold increase of IC₅₀ value as compared with control (6.484ug/ml versus 1.395ug/ml) (Fig. 7C). In vitro doxorubicin sensitivity assay of DLBCL cells (Ly10) expressing p53 mutant also found that Ly10 cells carrying p53 mutants R273C and R282Q had reduced sensitivity to doxorubicin and increased IC₅₀ by 2.28-fold and 2.23-fold, respectively (Fig. 8). These data suggested that APOBEC3B-induced p53 mutants could both promote the proliferation and cause drug resistance in DLBCL cells.

4. Discussion

In this study, we for the first time demonstrate that APOBEC3B is a critical factor that induces p53-mutation and drug resistance of DLBCL. P53 is an important key factor that regulating of cell proliferation, DNA repair, and apoptosis. *TP53* somatic mutations were identified in many types of cancer and were regarded as an important carcinogenesis and drug-resistant mechanism in many cancers¹⁰. Recent studies identified that *TP53* mutation rate is about 20%-30% in DLBCL, with a similar incidence in germinal center B-cell-like (GCB) and activated B-cell-like (ABC) subtypes^{7-9, 19}. While in present study, 3D-PCR-based sequencing method could identify *TP53* exon8 mutation rate at about 35.9% in whole cohort, which is higher than previous data. Even in non-R/R DLBCL cases the *TP53* exon8 mutation rate is 23.26%. This maybe could be explained by selective amplification of DNA fragments containing G/C to A/T mutation²⁰. These results suggest that 3D-PCR could detect the G/C to A/T mutation in a higher sensitivity than that of regular PCR method. But this method also has a shortcoming of missing of other mutation types. While as previous reported that G/C to A/T mutation type is the main mutation type in *TP53* gene, and most of hotspot mutations were G/C to A/T mutation¹⁰, so 3D-PCR also could be used as a valuable method for *TP53* mutation detection.

As an important tumor genetic factor, *TP53* mutation was included as a poor prognostic factor for acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL) in National Comprehensive Cancer Network (NCCN) guidelines as well as for multiple myeloma in Mayo Clinic sSMART system. In recent years, several studies indicated that *TP53* mutation is also a poor prognostic factor for DLBCL⁷⁻⁹. However, this relationship has not been consistent because of some controversial data that failed to demonstrate any correlation between *TP53* mutations and prognosis^{21, 22}. In present study, results showed that *TP53* exon8 G/C to A/T mutation was higher in R/R DLBCL patients than that of non-R/R group. Most of those mutations were missense mutation and caused amino acid change. And the predictive effect of *TP53* exon8 mutation was also studied. Results showed that *TP53* mutation group had a lower CR rate compared with that of wild-type group when treated with standard R-CHOP. Then the prognostic value of *TP53* mutation status was analyzed in different IPI groups, which is the most widely used prognostic factors model. Results showed that *TP53* hotspot mutation also could discern patients with significantly distinct outcomes in IPI > 2 group. On the other side, the group with high risk IPI but wildtype & non-hotspot *TP53* had the similar outcome with those IPI 0–2 group. Also, multivariate Cox model showed that *TP53* hotspot was the strongest prognostic factor for PFS among *TP53* mutation, IPI, LDH, stage and gender. These data indicate that *TP53* hotspot mutation status provide robust prognostic information that is not captured by IPI.

Similar with previous reports, our data also showed that *TP53* mutation could be regarded as a prognostic factor for DLBCL. In present study, only exon8 was sequenced and analyzed, this may underestimate the proportion of *TP53* mutations. *TP53* mutations of other exons also need to be detected to perfect this relationship. But this does not prevent the role of *TP53* mutations in the prognosis of DLBCL.

Among the *TP53* missense mutations, about a third of these mutations are located in six “hotspot” residues that are p.R175, p.G245, p.R248, p.R273, and p.R282²³. Most of the previous reports did not analyze the differential prognostic value of different *TP53* mutation positions in DLBCL. Young KH et al. found that *TP53* mutations in DNA binding domain (DBD), especially in LSH and L3 regions, were associated with worse survival⁸. Xu-Monette et al. reported that special codons including 248, 273, 175, 176, and 213 of the p53 protein had the highest mutation frequency and associated with poor prognosis⁹. These indicated that hotspot mutations maybe more important for p53 activity and were more valuable for prognostic prediction. In present study, we also found that some hotspot mutation rate, such as 273, 282 and 283, were even higher in R/R DLBCL than that of non-R/R DLBCL. Those non-hotspot mutation patients had the similar CR rate and PFS with wild type group. But this need to be further studied because the non-hotspot mutation group was small in present study. These hotspot mutations, instead of non-hotspot mutations, were strongly associated with lower CR rate and shorter PFS in DLBCL. Previously data showed that these hotspot mutations could affect the DNA binding activity of p53 protein and result in failure of regulating of target genes^{24–26}. Some of these hotspot p53 mutants were responsible for gaining of function in carcinogenesis and drug resistance^{27–30}. For example, R273C and R273H mutants were reported to confer a more aggressive phenotype on cancer cells, as well as enhance resistance to DNA damaging drugs³¹. More recently, Boettcher S also found that *TP53* missense mutations, including R273H and R282W, showed a dominant-negative effect, rather than gain of function, and caused drug resistance in AML cells³². Our data found that the hotspot mutations instead of all the mutation sites of *TP53* were associated with worse survival of DLBCL. Which hotspot mutations are more important for prognostic evaluation of DLBCL and how these hotspot mutations affect the role of p53 in tumorigenesis and drug resistance are still unclear.

TP53 mutation could result in resistance to chemotherapy agents such as doxorubicin³² and cisplatin^{33–36}, which are usually used for DLBCL. Although there were some attempts for overcoming of *TP53* mutation but this still needs further study. Lack of understanding of *TP53* gene mutation mechanism is one of the important obstacles to overcome p53 mutant-mediated drug resistance. Among the reported *TP53* mutation types, G/C to A/T is the most common type, especially some hotspot mutation sites. Most of the reported carcinogens, such as PAH (B[a]P), AA, aflatoxin B1, Vinyl chloride and 3-NBA, doesn't induce G/C to A/T mutation¹⁰. Except for the UV radiation was reported could inducing CC to TT mutation in skin cancer³⁷. But the mechanism of the majority G/C to A/T mutation in *TP53* is still unclear. Previously our group and other groups had identified that some APOBEC3s family members could induce G/C to A/T mutation in viral genome including human immunodeficiency virus (HIV)^{38–40}, human T-lymphocytic leukemia virus (HTLV-1)^{41–44}, and hepatitis B virus (HBV)^{15,45–47}. In recent years, APOBEC3B is reportedly one of the most extensive candidate factors for studying G/C to A/T mutations in a variety of human cancers^{14,48–50}. Moreover, some reports proved that APOBEC3s could be up-regulated by interferon^{51,52}, which is an important cytokine involved in inflammation. And chronic inflammation was regarded as a potential factor in lymphoma carcinogenesis. So, we raise the hypothesis that APOBEC3s, especially APOBEC3B, may be responsible for *TP53* G/C to A/T mutation.

Among the seven family members (from APOBEC3A to APOBEC3H), APOBEC3B localized mainly in nucleus, APOBEC3G, APOBEC3F and APOBEC3DE localized mainly in cytoplasm, and APOBEC3A and APOBEC3C localized both sides^{15,47,53}. Based on bio-informatics analysis of APOBEC3s expression in DLBCL, we found that APOBEC3B not APOBEC3A, was up-regulated in DLBCL in several lymphoma database. This suggests that APOBEC3B may be more possibly responsible for *TP53* mutations in DLBCL.

Using inducible expression system, we over express APOBEC3B in DLBCL cells, and found that G/C to A/T mutation could be induced in *TP53* exon8. More importantly, these in-vitro mutation patterns were as the same as that of in-vivo R/R DLBCL samples. Those hotspot mutants were also could be detected in APOBEC3B-induced p53 mutants as the similar pattern of that of DLBCL samples. While over expression of APOBEC3A could not induces G/C to A/T mutation in *TP53*. Also, c-MYC, which is less mutated in DLBCL^{54–56}, was not mutated after APOBEC3B expression in DLBCL cells. These results indicated that APOBEC3B, instead of APOBEC3A, may responsible for the *TP53* G/C to A/T mutation in DLBCL. APOBEC3B could selectively induce G/C to A/T mutation in special genes through an unknown mechanism in DLBCL. Further study found that these APOBEC3B-induced mutants could improve the proliferation of DLBCL cells as well as cause resistant to doxorubicin, one of the main drugs of CHOP. This drug resistance maybe could be explained by previous work by Li J³¹ and Boettcher S³². These results indicated that APOBEC3B-induced p53 mutants maybe responsible for the refractory and resistant of DLBCL. As APOBEC3B target different sequence in different cells, how APOBEC3B selective the targeting sequence and which factors were involved in this process needed to be further investigated. Recently, p53 protein was reported could regulate the expression of APOBEC3B⁵⁷, and APOBEC3B expression also increases APOBEC signature mutation in p53-defective cells⁵⁸. So, the detailed crosstalk between p53 and APOBEC3B, during carcinogenesis and drug resistance, is still needed to be further investigated.

5. Conclusion

In this study, we for the first time demonstrate that APOBEC3B is a critical factor that induces *TP53* mutation and leads to drug resistance in DLBCL. As a DNA mutator, APOBEC3B may serve as a potential target to reduce the rate of *TP53* mutation and improve the prognosis of DLBCL. *TP53* mutants especially those hotspot mutants had the potential value for prognostic evaluation of DLBCL in the era of R-CHOP. We also provide a more sensitive method for *TP53* mutation detection in tumor tissue DNA, and this may be helpful for further study of *TP53* mutation not only in DLBCL but also in other cancer types.

6. Abbreviations

DLBCL: Diffuse large B-cell lymphoma; R-CHOP: rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone; WHO: world health organization; FFPE: formalin-fixed, paraffin-embedded; PBMC: peripheral blood mononuclear cells; APOBEC: apolipoprotein B mRNA editing enzyme catalytic

polypeptide-like; GEO: Gene Expression Omnibus; PFS: progress free survival; IPI: International Prognostic Index; LDH: lactate dehydrogenase; GCB: germinal center B-cell-like; ABC: activated B-cell-like; AML: acute myeloid leukemia; CLL: chronic lymphocytic leukemia; NCCN: National Comprehensive Cancer Network; HIV: human immunodeficiency virus; HTLV: human T-lymphocytic leukemia virus; HBV: hepatitis B virus.

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of the hospital in accordance with principles of the Declaration of Helsinki. All the patients provide written informed consent at enrollment.

Consent for publication

All authors reached an agreement to publish the study in this journal.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author's Contributions:

XZ and RX planned the experiments. XZ, ZW, QY, XZ, YL and TY performed the experiments. JL and XL contributed to the analysis of pathological data. YX, LH, XX, WW, GX and XZ collected the clinical data. QL, YF and AM contributed to the collection of patient material. XZ, ZW and XZ contribute to the analysis of the data. XZ, RX, XZ and XY contributed to writing of the manuscript.

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Figures

		780	*	800	*	820	*	840	
TP53	:	GGAAGACTCCAGTGGTAATCTACTGGGACGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTGGGAGAGACCGGC	:	847					
CZQ-1	:	-----	:	67					
CZQ-2	:	-----	:	67					
CZQ-3	:	-----	:	67					
CZQ-4	:	-----	:	67					
LAG-1	:	-----	:	67					
LAG-2	:	-----	:	67					
LAG-3	:	-----	:	67					
LZQ-1	:	-----	:	67					
LZQ-2	:	-----	:	67					
KMY-1	:	-----	:	67					
KMY-2	:	-----	:	67					
MYZ-1	:	-----	:	67					
MYZ-2	:	-----	:	67					
MYZ-3	:	-----	:	67					
GXL-1	:	-----	:	67					
GXL-2	:	-----	:	67					
XJX-1	:	-----	:	67					
XJX-2	:	-----	:	67					
XJX-3	:	-----	:	67					
XY-1	:	-----	:	67					
YHL-1	:	-----	:	67					
YHL-2	:	-----	:	67					
YHL-3	:	-----	:	67					
WCN-2	:	-----	:	67					
WCN-3	:	-----	:	67					
ZFJ-4	:	-----	:	67					
ZMF-1	:	-----	:	67					
ZMF-2	:	-----	:	67					
ZMF-3	:	-----	:	67					
ZMF-4	:	-----	:	67					
ZMF-5	:	-----	:	67					

		860	*	880	*	900	*	920	
TP53	:	GCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGAGCTGCCCCCAGGGAGCCTAAGCGAGCACTG	:	924					
CZQ-1	:	-----	:	138					
CZQ-2	:	-----	:	138					
CZQ-3	:	-----	:	138					
CZQ-4	:	-----	:	138					
LAG-1	:	-----	:	138					
LAG-2	:	-----	:	138					
LAG-3	:	-----	:	138					
LZQ-1	:	-----	:	138					
LZQ-2	:	-----	:	138					
KMY-1	:	-----	:	138					
KMY-2	:	-----	:	138					
MYZ-1	:	-----	:	138					
MYZ-2	:	-----	:	138					
MYZ-3	:	-----	:	138					
GXL-1	:	-----	:	138					
GXL-2	:	-----	:	138					
XJX-1	:	-----	:	138					
XJX-2	:	-----	:	138					
XJX-3	:	-----	:	138					
XY-1	:	-----	:	138					
YHL-1	:	-----	:	138					
YHL-2	:	-----	:	138					
YHL-3	:	-----	:	138					
WCN-2	:	-----	:	138					
WCN-3	:	-----	:	138					
ZFJ-4	:	-----	:	138					
ZMF-1	:	-----	:	138					
ZMF-2	:	-----	:	138					
ZMF-3	:	-----	:	138					
ZMF-4	:	-----	:	138					
ZMF-5	:	-----	:	138					

Figure 1

TP53 exon8 G/C to A/T mutations in R/R DLBCL. TP53 exon8 G/C to A/T mutation was detected in R/R DLBCL. Total DNA were extracted from FFPE DLBCL tissues using QIAamp DNA FFPE Tissue Kit. TP53 exon8 was amplified by 3D-PCR and sequenced by Sanger sequencing method. All sequences were aligned and analyzed by Clustal and Genedoc software.

	780	*	800	*	820	*	840	
TP53 :	GGAAGACTCCAGTGGTAATCTACTGGGACGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTGGGAGAGACCGGC :							847
CZQ-1 :	-----							67
CZQ-2 :	-----							67
CZQ-3 :	-----						T..	67
CZQ-4 :	-----						T	67
LAG-1 :	-----				T.			67
LAG-2 :	-----			T.				67
LAG-3 :	-----						A..	67
LZQ-1 :	-----				T.			67
LZQ-2 :	-----				T.			67
KMY-1 :	-----						T	67
KMY-2 :	-----					A.	T	67
MYZ-1 :	-----							67
MYZ-2 :	-----							67
MYZ-3 :	-----						T..	67
GXL-1 :	-----						T	67
GXL-2 :	-----	T.						67
XJX-1 :	-----							67
XJX-2 :	-----				T.			67
XJX-3 :	-----							67
XY-1 :	-----						T	67
YHL-1 :	-----						T	67
YHL-2 :	-----							67
YHL-3 :	-----						T..	67
WCN-2 :	-----							67
WCN-3 :	-----						A..	67
ZFJ-4 :	-----							67
ZMF-1 :	-----							67
ZMF-2 :	-----							67
ZMF-3 :	-----							67
ZMF-4 :	-----					T.		67
ZMF-5 :	-----						A.T	67

	*	860	*	880	*	900	*	920	
TP53 :	GCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGAGCTGCCCCCAGGGAGCACTAAGCGAGCACTG :								924
CZQ-1 :	A.....T.....							-----	138
CZQ-2 :	A.....							-----	138
CZQ-3 :								-----	138
CZQ-4 :								-----	138
LAG-1 :	T.....							-----	138
LAG-2 :	A.....							-----	138
LAG-3 :	T.....A.....							-----	138
LZQ-1 :								-----	138
LZQ-2 :								-----	138
KMY-1 :	T.....							-----	138
KMY-2 :								-----	138
MYZ-1 :	A.....							-----	138
MYZ-2 :								-----	138
MYZ-3 :								-----	138
GXL-1 :	A.....							-----	138
GXL-2 :	T.....T.....							-----	138
XJX-1 :	A.....							-----	138
XJX-2 :								-----	138
XJX-3 :	...G...A...							-----	138
XY-1 :								-----	138
YHL-1 :								-----	138
YHL-2 :	A.....							-----	138
YHL-3 :	A.....							-----	138
WCN-2 :	A.....							-----	138
WCN-3 :								-----	138
ZFJ-4 :	T.....							-----	138
ZMF-1 :	A.....							-----	138
ZMF-2 :	T.....T.....A.....							-----	138
ZMF-3 :	T.....						A	-----	138
ZMF-4 :	T.....							-----	138
ZMF-5 :								-----	138

Figure 1

TP53 exon8 G/C to A/T mutations in R/R DLBCL. TP53 exon8 G/C to A/T mutation was detected in R/R DLBCL. Total DNA were extracted from FFPE DLBCL tissues using QIAamp DNA FFPE Tissue Kit. TP53 exon8 was amplified by 3D-PCR and sequenced by Sanger sequencing method. All sequences were aligned and analyzed by Clustal and Genedoc software.

Figure 2

P53 mutants in R/R DLBCL. [A] P53 mutants were detected in R/R DLBCL. P53 protein sequences were translated by Genedoc software based on DNA sequences and aligned by Clustal and Genedoc software. [B] Distribution of p53 point mutants in R/R DLBCL. Numbers means the total number of patients that the given point mutant was detected. [C] and [D] TP53 mutation rates in R/R and non-R/R DLBCL groups. Frequencies were calculated as percentage. Differences between compared groups of patients were assessed by Maximum Likelihood Chi-square test using IBM SPSS Statistics 20.

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Figure 3

Influence of TP53 exon8 mutation on clinical outcome of DLBCL. [A] CR rates in different groups base on TP53 status. CR status was evaluated based on PET-CT scan according to Lugano response criteria for non-Hodgkin's lymphoma. Differences between compared groups of patients were assessed by Maximum Likelihood Chi-square test using IBM SPSS Statistics 20. [B] PFS of different TP53 status groups. [C] Compare of PFS in IPI>2 group between TP53 hotspot mutation group and wildtype & non-hotspot mutation group. [D] Compare of PFS between IPI 0-2 group and IPI>2 with TP53 wildtype & non-hotspot mutation group. Patients were followed up for 12 months until disease progression, relapse or end of observation. PFS was defined as time to disease progression, relapse or death. PFS was estimated using the Kaplan-Meier method, and compared using the log-rank test.

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Figure 4

APOBEC3A and APOBEC3B expression in DLBCL based on Oncomine online database. [A] APOBEC3A expression in Brune Lymphoma database including DLBCL, naïve B cell, memory B cell, centrocytes and centroblast. [B] APOBEC3B expression in Brune Lymphoma database including DLBCL, naïve B cell, memory B cell, centrocytes and centroblast. [C] Comparing of APOBEC3A and APOBEC3B expression in Brune Lymphoma database. [D] APOBEC3A expression in Compagno Lymphoma database including DLBCL, naïve B cell, memory B cell and germinal center centroblasts. [E] APOBEC3B expression in Compagno Lymphoma database including DLBCL, naïve B cell, memory B cell and germinal center centroblasts. [F] Comparing of APOBEC3A and APOBEC3B expression in Brune Lymphoma database.

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Figure 5

Expression of APOBEC3B in R/R DLBCL and non-R/R DLBCL samples (IHC). [A] to [F] were R/R samples, [G] to [L] were non-R/R samples.

Figure 5

Expression of APOBEC3B in R/R DLBCL and non-R/R DLBCL samples (IHC). [A] to [F] were R/R samples, [G] to [L] were non-R/R samples.

Figure 6

APOBEC3B induces TP53 exon8 mutations in vitro. [A] 3D-PCR amplification of TP53 exon8 in APOBEC3B-inducible Pfeiffer cell (Right) and control cell (Left). Red arrows indicated DNA fragments that amplified at denature temperature of 88°C and 87°C. PCR products were separated in agarose gel electrophoresis (upper). Expression of APOBEC3B-flag was confirmed by Western Blot via anti-flag antibody (down). M: DNA ladder marker. PF: Pfeiffer. Neg: negative control. 92°C to 87°C were different denaturation temperatures in 3D-PCR. Con: Cell lysis before induction by doxycycline. d1: Cell lysis after induction by doxycycline for one day. d7: Cell lysis after induction by doxycycline for 7 days. d14: Cell lysis after induction by doxycycline for 14 days. [B] 3D-PCR amplification of MYC exon2 in APOBEC3B-inducible Pfeiffer cell. PCR products were separated in agarose gel electrophoresis (upper). Expression of APOBEC3B-flag was confirmed by Western Blot via anti-flag antibody (down). [C] Detection of APOBEC3B-flag by Western blot in APOBEC3B-inducible cell. [D] Compare of in vitro APOBEC3B-induced p53 mutants with that of R/R DLBCL samples. P53 sequences were analyzed by Clustal and Genedoc software. *: DLBCL patients. **: APOBEC3B-inducible Pfeiffer cells. ***: APOBEC3B-inducible OCI-LY10 cells. ****: APOBEC3B-inducible Hek293 cells.

Figure 7

APOBEC3B mediated proliferation and drug resistance. [A] and [B] Colony formation after induced-APOBEC3B-expression. Con: pLV-Ptight-puro vector transfected Pfeiffer cells. A3B inducible: APOBEC3B-Flag/ pLV-Ptight-puro vector transfected Pfeiffer cells. PF-con: pLV-Ptight-puro vector transfected Pfeiffer cells. PF-A3B: APOBEC3B-Flag/ pLV-Ptight-puro vector transfected Pfeiffer cells. [C] Doxorubicin resistant after induced-expression of APOBEC3B. Pfeiffer-3B-Ctrl: pLV-Ptight-puro vector transfected Pfeiffer cells. Pfeiffer-3B-OE: APOBEC3B-Flag/pLV-Ptight-puro vector transfected Pfeiffer cells. ADM: doxorubicin.

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Figure 8

p53 mutants (R273C and R282Q) induced doxorubicin resistance. [A] Effect of R273C on doxorubicin sensitivity in Ly10 cell (MTT). [B] Effect of R282Q on doxorubicin sensitivity in Ly10 cell (MTT). Ly10-Ctrl: empty vector transfected Ly10 cell. Ly10-TP53: wildtype p53 transfected Ly10 cell. Ly10-R273C: p53 R273C mutant transfected Ly10 cell. Ly10-R282Q: p53 R282Q mutant transfected Ly10 cell. ADM: doxorubicin.

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