

miR-17~92 Promotes Progression of ABC-DLBCL Lymphoma via Regulation of Canonical NF- κ B Signaling

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Abstract

Background: Activated B-cell like diffuse large B-cell lymphoma (ABC-DLBCL) is an aggressive lymphoma characterized by constitutive NF- κ B activation. Nevertheless, the role and mechanisms of miR-17~92 in contributing to the NF- κ B activation in ABC-DLBCL are still elusive.

Methods: The expression of miR-17~92 primary transcript (*MIR17HG*) and NF- κ B target genes was determined using RNA-sequencing. The expression of miR-17~92 was performed using microarray analysis. Plasmids carrying conditional over-expression and loss-of-function of miR-17~92 were respectively constructed and dual-luciferase reporter assay was used to validate the target gene of miR-17~92. Immunoprecipitation and polyubiquitination were further used to the study of potential mechanisms.

Results: Expression of *MIR17HG* was positively correlated with NF- κ B activity, miR-17~92 activated the NF- κ B signaling in ABC-DLBCL, and its over-expression promoted ABC-DLBCL cell growth, accelerated cell G1 to S phase transition and enhanced cell resistance to NF- κ B inhibitor. Importantly, miR-17~92 promoted NF- κ B activation through directly targeting multiple ubiquitin-editing regulators to lead to increase the K63-linked polyubiquitination and decrease the K48-linked polyubiquitination of RIP1 complex in ABC-DLBCL. We further found that miR-17~92 selectively activated I κ B- α and NF- κ B p65 but not NF- κ B p52/p100, and high miR-17~92 expression was also associated with poorer outcome in ABC-DLBCL patients.

Conclusions: Taken together, miR-17~92 selectively activate the canonical NF- κ B signaling via targeting ubiquitin-editing regulators to lead to constitutively NF- κ B activation and poorer outcome, which is an innovative function of miR-17~92 and previously unappreciated regulatory mechanism of NF- κ B activation in ABC-DLBCL. Targeting miR-17~92 may thus provide a novel bio-therapeutic strategy for ABC-DLBCL patients.

Background

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid malignancy, which is classified into germinal center B-cell-like (GCB), activated B-cell-like (ABC) and unclassified DLBCL based on gene expression profiling. They have markedly different clinical features, and ABC-DLBCL has distinctly worse prognosis compared to GCB-DLBCL [1]. It has been reported that constitutive NF- κ B activation plays important role in lymphomagenesis of ABC-DLBCL and somatic mutations of genes, including CD79A/B, CARD11, A20, and MYD88, which are characteristically observed in ABC-DLBCL, are directly related to the constitutive NF- κ B activation [2]. Nevertheless, close to 50% of ABC-DLBCL do not have these characteristic genetic mutations and mechanisms of NF- κ B activation in these tumors are still elusive.

NF- κ B activation is critical for B cell development and lymphoid organogenesis, and is mediated by homo- or hetero-dimers of NF- κ B family members, including NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), RelA (p65), RelB and Rel (c-Rel). Signaling pathways mediating NF- κ B activation are primarily classified

into canonical and non-canonical signaling, which involve the release of different NF- κ B dimers from inactive cytoplasmic complexes into the nucleus [3]. Canonical NF- κ B signaling relies on the phosphorylation of I κ Bs, particularly I κ B- α , leading to nuclear translocation of various NF- κ B dimers, predominantly the p50/p65, however, non-canonical NF- κ B signaling leads to nuclear translocation of the p52/RelB dimer through a special mechanism which relies on the inducible procession of p100 instead of I κ B- α . Until now, which signaling of them mediates the constitutive NF- κ B activation still remains unclear in ABC-DLBCL. In addition, the interactions between microRNAs and NF- κ B signaling have been also confirmed, suggesting that dysfunction of these interactions contributes to the development of NF- κ B “driven” inflammation and tumors [4, 5]. MicroRNA-17 ~ 92 (miR-17 ~ 92) is an oncomiR cluster consisting of six distinct microRNAs, including miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92, which fall into four microRNA families (miR-17, miR-18, miR-19 and miR-92). MiR-17 ~ 92 is processed from the transcript of *MIR17HG* (also known as *C13orf25*), which is frequently amplified in lymphoma, leukemia and some solid tumors [6, 7]. MiR-17 ~ 92 is essential for B-cell development and its absence leads to inhibit B-cell development at pro-B to pre-B transition [8, 9], suggesting an important link between miR-17 ~ 92 and its functions during B-cell lymphopoiesis. Studies have also further shown that miR-17 ~ 92 targets distinct genes, including PHLPP2, PTEN, Bim, CDKN1A/p21, CD22, FCGR2B and Chek2, which contribute to the diverse pathways in different B-cell lymphoma subtypes [10–13]. But its exact function and mechanisms in DLBCL, especially in ABC-DLBCL in which NF- κ B signaling is constitutively activated, are still unclear.

Here, we uncovered that miR-17 ~ 92 selectively activated the canonical but not non-canonical NF- κ B signaling in ABC-DLBCL through directly targeting multiple ubiquitin-editing proteins, which led to regulate the K63- and K48-linked polyubiquitination of receptor-interacting protein 1 (RIP1) complex. Activation of the canonical NF- κ B signaling further promoted tumor cell proliferation and chemoresistance as well as poorer survival. Our findings provided the rationale for targeting miR-17 ~ 92 as innovative therapeutics in ABC-DLBCL.

Materials And Methods

Patients

The diagnosis of two cohorts of 38 non-GCB and 26 ABC-DLBCL patients, who were from the Tianjin Medical University Cancer Institute and Hospital for discovery cohort and University of Nebraska Medical Center for validation cohort, respectively, was confirmed by an expert panel of hematopathologists based on WHO criteria and gene expression profiling. The study was approved by the ethics committee of Tianjin Medical University Cancer Institute and Hospital and University of Nebraska Medical Center. The informed consent was obtained from each participant.

Cell lines and primary normal B cells

The human ABC-DLBCL (U2932, Ly3, TMD8 and Ly10) and HEK293T cell lines were obtained and cultured in their respective medium supplemented with 10%~20% serum, and primary centroblasts

(CD77⁺ CD38^{hi}), as normal B cells, were isolated and purified from fresh tonsils using magnetic microbeads (Miltenyi Biotec Inc., Auburn, CA, USA) as previously described [14]. Details were provided in the Supplementary material.

RNA extraction, RNA-sequencing and microarray analysis

Total RNA was respectively extracted from fresh frozen tissues of 38 non-GCB DLBCL patients using a RNeasy Mini Kit (Qiagen, Hilden, Germany) for RNA-sequencing and 26 ABC-DLBCL patients using mirVana™ miRNA isolation kit (Life Technologies, Grand Island, NY, USA) for microarray analysis of miRNA expression. And then all the total RNA were treated with RNase-free DNase I for 30 min at 37°C to remove residual DNA. Details were described in the Supplementary material.

Plasmids construction and NF-κB activity assay

The Tet-on recombinant plasmids including conditional over-expression of miR-17 ~ 92, which was named as pTIP-miR-17 ~ 92, and conditional loss-of-function of miR-17 ~ 92, which was named as pTRIPZ-miR-17 ~ 92 sponge, were respectively constructed as our previously report [10]. The diagram and map of these plasmids were shown in Supplementary Fig. S1. NF-κB activity was measured using the NF-κB Signal reporter together with pTRIPZ-miR-17 ~ 92 sponge for transient cell transfection. Further details were provided in the Supplementary material.

Establishment of ABC-DLBCL cell lines with conditional over-expression or loss-of-function of miR-17 ~ 92

Virus was packaged into HEK293T cells and the virus pseudovirus particles were collected to infect the target cells to generate the ABC-DLBCL cells with conditional over-expression or loss-of-function of miR-17 ~ 92, which were further screened with puromycin and sorted by fluorescent expression using a FACSVantage (Bectom Dickinson, USA). Details were described in the Supplementary material.

Wild-type and mutant Luc-Target gene-3'UTR plasmids and luciferase reporter assays

Luc-Target gene-3'UTR plasmids (wild-type, WT) were constructed and the binding site's seed sequence of miR-17 ~ 92 in Luc-Target genes-3'UTR plasmids were mutated using QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, USA) to generate corresponding mutant plasmids (mutant, Mut). All plasmids were sequenced to confirm their identity. The primers were listed in Supplementary Table S1. Luciferase reporter assays were used to validate the direct target genes of miR-17 ~ 92. Details were provided in the Supplementary material.

Assays of cell growth, cycle and apoptosis, and real-time quantitative PCR

Cell counting and flow cytometry (Bectom Dickinson, CA, USA) were used to evaluate the growth, cycle and apoptosis. Three major miRNA numbers including miR-17-5p, miR-19b, miR-92a in miR-17 ~ 92 were

determined using RT-qPCR, of which the gene-specific primers were purchased from Applied Biosystems (Foster City, USA). Six NF- κ B downstream transcriptional target genes, including TNIP1, TNF α , NFKB2, CD83, BIRC3 and IRF1, were also determined using RT-qPCR, of which the primers were listed in Supplementary Table S2. Further details were described in the Supplementary material.

Immunoprecipitation, polyubiquitination of RIP1 complex and immunoblotting

RIP1 complex was immunoprecipitated from stably transduced ABC-DLBCL cell lines and the pull-down was immunoblotted to detect the K63- and K48-linked polyubiquitinations. Proteins were detected by immunoblotting. Details were described in the Supplementary material.

Survival and statistical analysis

Statistical analysis was performed using SPSS (v26.0). Survival analyses were performed using Kaplan-Meier curves and log-rank test. Two-sided $P < 0.05$ was considered as statistically significant.

Results

MiR-17 ~ 92 enhances the NF- κ B signaling in primary Non-GCB DLBCL tumors and ABC-DLBCL cells

DLBCL are subclassified into GCB- and ABC-DLBCL based on gene expression profiling [1]. During routine pathology diagnosis, DLBCL are usually subclassified into GCB- and non-GCB DLBCL according to Hans' algorithm [15]. To characterize the relationship between miR-17 ~ 92 and NF- κ B activity, we performed RNA-sequencing in 38 non-GCB DLBCL patients treated with R-CHOP, and found that miR-17 ~ 92 primary transcript (*MIR17HG*) was positively associated with the expression of a series of genes (Fig. 1, $0.011 \leq P \leq 0.037$), which had been confirmed to be NF- κ B downstream transcriptional target genes, including TNIP1, TNF- α , NFKB2, CD83, BIRC3, IRF-1, IRF-4, CCR7, NFKB1, CFLAR and TNFAIP2 [16–19]. These results suggested that miR-17 ~ 92 enhanced the NF- κ B signaling in primary non-GCB DLBCL tumors.

To further evaluate whether miR-17 ~ 92 activated the NF- κ B signaling in cells, we examined the effect of miR-17 ~ 92 on NF- κ B activity. As shown in Fig. 2B, the luciferase activity of NF- κ B Signal reporter significantly increased after TNF- α treatment for 18h, and the increase in NF- κ B luciferase activity was significantly attenuated (approximately 67.32%) with co-expression of miR-17 ~ 92 sponge, which suppressed miR-17 ~ 92 function ($P < 0.01$), suggesting miR-17 ~ 92 loss-of-function suppressed NF- κ B activity in HEK293T cells. To investigate whether miR-17 ~ 92 regulated NF- κ B activity in ABC-DLBCL cells, we examined the expression of miR-17 ~ 92 in them. Supplementary Fig. S2A showed that miR-17 ~ 92 cluster, including miR-17-5p, miR-19b and miR-92a, highly expressed in four ABC-DLBCL cell lines than primary normal B cells. U2932 and Ly3 had higher miR-17 ~ 92 expression, and they were selected to construct the conditional loss-of-function cells, whereas LY10 and TMD8 were selected to construct the conditional over-expression cells. These transduced cells were further confirmed by RT-qPCR

(Supplementary Fig. S2B-C) and enhanced RFP or GFP assay (Supplementary Fig. S3). We then determined the expression of six NF- κ B downstream transcriptional target genes in these stably transduced ABC-DLBCL cells, and found that the expression of these genes significantly decreased in U2932 and Ly3 cells upon miR-17 ~ 92 loss-of-function. Conversely, the levels of them significantly augmented in Ly10 and TMD8 cells upon miR-17 ~ 92 over-expression (Fig. 2C-D). These findings suggested that miR-17 ~ 92 indeed activated the NF- κ B signaling in multiple types of cells, including ABC-DLBCL cells. Taken together, miR-17 ~ 92 enhanced the NF- κ B signaling in primary non-GCB DLBCL tumors and ABC-DLBCL cells.

MiR-17 ~ 92 promotes tumor cell proliferation and enhances chemoresistance to NF- κ B inhibitor in ABC-DLBCL cells

Next, we constructed the stably transduced cells to investigate the cell functions of ABC-DLBCL, which were induced by miR-17 ~ 92-mediated NF- κ B activation. We found that miR-17 ~ 92 over-expression in Ly10 and TMD8 cells promoted cell growth, whereas miR-17 ~ 92 loss-of-function in U2932 and Ly3 cells inhibited cell growth (Fig. 3A). Meanwhile, miR-17 ~ 92 over-expression in LY10 and TMD8 cells promoted G1 to S phase transition; whereas miR-17 ~ 92 loss-of-function in U2932 and Ly3 cells blocked G1 to S phase transition, which resulted in G1 phase retention (Fig. 3B). BMS-345541 (abbreviated as BMS, Calbiochem, San Diego, USA) is a highly selective IKK inhibitor, which inhibits the NF- κ B dependent transcription [20]. We also found that BMS treatment inhibited cell growth via blockading NF- κ B activity in ABC-DLBCL cells (Fig. 3C, comparing control cells with or without BMS treatment). MiR-17 ~ 92 loss-of-function in U2932 and Ly3 cells enhanced the BMS-induced inhibition, whereas miR-17 ~ 92 over-expression in Ly10 and TMD8 cells partially attenuated the BMS-induced inhibition (Fig. 3C). Moreover, miR-17 ~ 92 loss-of-function sensitized the U2932 and Ly3 cells to apoptosis induced by BMS treatment; conversely, miR-17 ~ 92 over-expression rescued the Ly10 and TMD8 cells, at least partially, from BMS-induced apoptosis (Fig. 3D). Taken together, after augmenting the NF- κ B activation, miR-17 ~ 92 promoted the cell proliferation and chemoresistance in ABC-DLBCL cells.

MiR-17 ~ 92 directly targets multiple ubiquitin-editing regulators

To expose the molecular mechanism for miR-17 ~ 92 enhancing NF- κ B activity in ABC-DLBCL, we integrated literatures and searched for potential miR-17 ~ 92 target genes in NF- κ B pathway through combinatorial prediction using TargetScan 7.0 and miRanda databases (Supplementary Table S3), and found that TNFAIP3 (also known as A20), CYLD, Rnf11 and KDM2A were the top four predicted miR-17 ~ 92 target genes according to the comprehensive scores. TNFAIP3, CYLD and Rnf11 were involved in editing ubiquitin as NF- κ B negative regulators, whereas KDM2A was mainly involved in histone demethylation. Herein, the ubiquitin-editing regulators, including TNFAIP3, CYLD and Rnf11, were further validated and investigated in the process of NF- κ B activation by miR-17 ~ 92. Integration of highly conserved sites of the 3'UTR of TNFAIP3, CYLD and Rnf11 binding to the seed sequence of miR-17 ~ 92

was listed in Supplementary Fig. S4. We constructed two TNFAIP3-3'UTR luciferase reporter plasmids containing TNFAIP3-3'UTR conserved miR-18a/-19a(b) binding sites, two CYLD-3'UTR luciferase reporter plasmids containing CYLD-3'UTR conserved miR-19a(b) binding sites, and three RNF11-3'UTR luciferase reporter plasmids containing Rnf11-3'UTR conserved miR-19a(b)/-92a binding sites. The corresponding point mutation plasmids of them were also constructed (Supplementary Fig. S5). Luciferase reporter assays revealed that Luc-TNFAIP3-3' UTR plasmid 2 (P2), which harbored putative binding sites for miR-19a/b (Fig. 4A1), exhibited 38% lower luciferase activity compared to pGL3P vector, but Luc-TNFAIP3-3' UTR P2 mutant returned to similar luciferase activity to pGL3P vector (Fig. 4A2). MiR-17 ~ 92 over-expression further decreased luciferase activity by 27% in wild-type but not mutant (Fig. 4A3), whereas miR-17 ~ 92 loss-of-function increased luciferase activity by 56% in wild-type but not mutant (Fig. 4A4). These results suggested that TNFAIP3-3' UTR P2 but not P1 fragment (data not shown) was the direct target region of miR-17 ~ 92. Similarly, we verified that CYLD-3'UTR P1 but not P2 fragment, which harbored putative binding sites for miR-19a/b (Fig. 4B1-4), and RNF11-3'UTR P3 but not P1 and P2 fragments, which harbored putative binding sites for miR-92a (Fig. 4C1-4), were the direct target regions of miR-17 ~ 92. Therefore, our results demonstrated that multiple ubiquitin-editing regulators, including TNFAIP3, CYLD and Rnf11, were the direct targets of miR-17 ~ 92.

MiR-17 ~ 92 down-regulates multiple ubiquitin-editing proteins and activates the canonical NF- κ B signaling in ABC-DLBCL cells

We further examined the role for miR-17 ~ 92-mediated ubiquitin-editing protein translations after targeting these genes. Figure 5A displayed that miR-17 ~ 92 loss-of-function up-regulated the levels of ubiquitin-editing proteins, including A20, CYLD and Rnf11, in U2932 and Ly3 cells; whereas miR-17 ~ 92 over-expression down-regulated these protein levels in Ly10 and TMD8 cells. Because these ubiquitin-editing proteins are the NF- κ B negative regulators, the NF- κ B signaling will be activated after down-regulating these proteins by miR-17 ~ 92. Next, we investigated that which one of canonical and non-canonical NF- κ B pathways would be activated by miR-17 ~ 92 in ABC-DLBCL cells. Figure 5B revealed that miR-17 ~ 92 loss-of-function decreased the protein levels of phosphorylated-I κ B- α and -p65 in U2932 and Ly3 cells, whereas the level of phosphorylated-p52/p100 was not changed. Conversely, miR-17 ~ 92 over-expression upregulated phosphorylated-I κ B- α and -p65 levels in Ly10 and TMD8 cells, but not the level of phosphorylated-p52/p100. These results revealed that miR-17 ~ 92 selectively activated the canonical but not non-canonical NF- κ B signaling in ABC-DLBCL.

MiR-17 ~ 92 is reported to also amplify the B-cell receptor (BCR) signaling via inhibiting the ITIM proteins [11]. BCR signaling is one of the NF- κ B upstream pathways. We therefore selected the Ibrutinib, which is a highly selective and irreversible Bruton tyrosine kinase (BTK) inhibitor, to block the BCR signaling and further confirm the approach for miR-17 ~ 92 activating the canonical NF- κ B signaling in ABC-DLBCL. Supplementary Fig. S6 revealed that Ibrutinib blocked the BCR signaling and inhibited the expression of phosphorylated-I κ B- α and -p65, and miR-17 ~ 92 loss-of-function further amplified the inhibition effect in U2932 cells. Conversely, miR-17 ~ 92 over-expression partially reversed the inhibition effect in TMD8 cells.

These results suggested that miR-17 ~ 92 selectively activated the canonical NF- κ B signaling via targeting ubiquitin-editing proteins, including A20, CYLD and Rnf11, approach but not only BCR signaling in ABC-DLBCL.

MiR-17 ~ 92 regulates the K63-and K48-linked polyubiquitination in ABC-DLBCL cells

Rnf11 and A20 are involved in ubiquitination and interaction with RIP1 [21, 22] and RIP1 polyubiquitination is further involved in NF- κ B activity [22–24]. CYLD is an ubiquitin-specific-processing protease according to the UniProt/SwissProt Protein Knowledgebase (<http://www.uniprot.org/uniprot/Q9NQC7>). We further exposed the ubiquitin-regulated mechanism of miR-17 ~ 92 in the process of the canonical NF- κ B activation in ABC-DLBCL cells. Figure 6A showed that K63-linked polyubiquitination of RIP1 complex was up-regulated in a time-dependent manner upon miR-17 ~ 92 over-expression in TMD8 cells. Figure 6B revealed that miR-17 ~ 92 loss-of-function in U2932 cells significantly decreased the K63-linked polyubiquitination and enhanced the K48-linked polyubiquitination. Conversely, miR-17 ~ 92 over-expression in TMD8 cells significantly increased the K63-linked polyubiquitination and attenuated the K48-linked polyubiquitination. Previous report showed that ubiquitin-activating enzyme (E1) inhibitors should, in principle, block all functions of ubiquitination and are valuable tools for studying ubiquitination [25]. After blockading the polyubiquitination using PYR-41, which is the first E1 inhibitor, neither miR-17 ~ 92 loss-of-function nor miR-17 ~ 92 over-expression changed the level of phosphorylated-I κ B- α and -p65 in U2932 and TMD8 cells (Supplementary Fig. S7), suggesting that PYR-41 inhibited the effect for miR-17 ~ 92 regulating the polyubiquitination of RIP1 complex in this process of the canonical NF- κ B activation in ABC-DLBCL cells. Taken together, miR-17 ~ 92 promoted the formation of K63-linked polyubiquitination and decreased the K48-linked polyubiquitination of RIP1 complex via targeting multiple ubiquitin-editing proteins to activate the canonical NF- κ B signaling in ABC-DLBCL cells.

MiR-17 ~ 92 is correlated with poorer outcome in ABC-DLBCL patients

To validate the correlations between miR-17 ~ 92 expression and overall survival, we performed the microarray analysis of miRNA expression in 26 ABC-DLBCL patients treated with R-CHOP and found that patients with high miR-17 ~ 92 expression tended to have poorer overall survival; however, there were too few patients for a statistical significance (Fig. 6C, $P > 0.05$). Moreover, among 38 non-GCB DLBCL patients studied by RNA-sequencing, 34 completed clinical follow-up (range, 0.5–89.1 months). We found that patients with high miR-17 ~ 92 primary transcript expression had significantly poorer overall survival (Fig. 6D, $P = 0.043$).

Discussion

Studies have demonstrated that characteristic “driver” genes, including CD79A/B, CARD11, A20 and MYD88, frequently mutate in approximate 50% ABC-DLBCL patients to further result in the constitutive

NF- κ B activation [2, 26–28]. Nevertheless, for those ABC-DLBCL patients without characteristic genetic “driver” mutations, mechanisms of NF- κ B activation are still elusive. Although a report showed that miR-17 ~ 92 could induce DLBCL through regulating several pathways including BCR signaling, which further activated the NF- κ B pathway [29]. However, the BCR signaling is only one of the NF- κ B upstream pathways. It remains poorly understood whether miR-17 ~ 92 can activate the NF- κ B signaling via other upstream approaches in DLBCL, especially in ABC-DLBCL. Our results demonstrated that miR-17 ~ 92 could activate the NF- κ B signaling to lead to constitutively NF- κ B activation through directly targeting multiple ubiquitin-editing proteins in ABC-DLBCL, which was different from the BCR approach. Hence, our findings are significant, since it implies that miR-17 ~ 92 is an important and previously unappreciated regulatory mechanism during NF- κ B activation for ABC-DLBCL.

In this study, three ubiquitin-editing proteins, including TNFAIP3, CYLD and Rnf11, were preferentially predicted and validated to be the direct target genes of miR-17 ~ 92 in the process of NF- κ B activation in ABC-DLBCL. However, whether other regulators, including Tax1bp1, Itch and Traf3 which are not the ubiquitin-editing proteins, are also the direct target genes of miR-17 ~ 92 to contribute to the NF- κ B activation in ABC-DLBCL is unclear. It needs to be further validated in future. Besides, the binding of TNF α to TNF receptor can induce the trimerization and then recruits several proteins, including RIPs, TRADD, TRAF2/5, cIAP1/2, NEMO and others, to form complex (herein named RIPs complex). RIPs are the important and true adaptors in NF- κ B signaling by interacting with upstream signaling cassettes through well-characterized protein-binding domains, and recruiting IKK α / β through NEMO binding [30]. RIPs consist of seven RIP family members (RIP1 ~ 7), but RIP1 is a key regulator of NF- κ B activation [30, 31]. After formation of RIP1 complex, cIAP1/2 and Ubc5 catalyze the RIP1 polyubiquitination. Alternatively, TRAF2 and TRAF5 can also be polyubiquitinated due to their RING domains [32, 33]. In this study, RIP1 antibody was applied to pull down the entire RIP1 complex and then it was confirmed to be polyubiquitinated in this process of NF- κ B activation by miR-17 ~ 92 in ABC-DLBCL. It means that any members of RIP1 complex are possible to be polyubiquitinated. It is unclear that which member of RIP1 complex is indeed polyubiquitinated. Further studies using high throughput analysis of post-translational modifications of proteins may help to answer this key question.

In addition, our previous studies using miRNA array analysis demonstrated that miR-17 ~ 92 overexpressed in various B-cell lymphomas, including ABC-DLBCL [14]. In this study, we also found that four ABC-DLBCL cell lines showed high miR-17 ~ 92 level using RT-qPCR method. Another report displayed that amplification of *C13orf25* locus on chromosome 13 was frequently detected in GCB-DLBCL but not ABC-DLBCL [34]. Actually expect for gene amplification, miR-17 ~ 92 over-expression is also collaboratively regulated by a number of oncogenic transcription factors and histone modification, including MYC, E2F, Spi-1, Fli-1 and H3K4 trimethylation [7, 35]. Our previous study also revealed that miR-17 ~ 92 over-expression might not correlate well with the *C13orf25* gene amplification, in which miR-17 ~ 92 expression was the highest in DHL16 cells, but without *C13orf25* amplification based on Chip-chip assay [14]. Therefore, miR-17 ~ 92 over-expression in ABC-DLBCL may be secondary to the activation of c-MYC and/or other oncogenic signaling pathways, since miR-17 ~ 92 is one of the c-MYC downstream targets [36], which is often upregulated in ABC-DLBCL. Otherwise, miR-17 ~ 92 sponge was used as miR-

17 ~ 92 competitive inhibitor to investigate the miR-17 ~ 92 loss-of-function in ABC-DLBCL cells in this study. Actually, miRNA sponges have been increasingly applied in miRNA loss-of-function studies due to its high specificity and strong inhibition of target miRNAs, which suppress miRNA targets at least as strongly as chemically modified antisense oligonucleotides [37, 38].

Conclusion

In summary, this study demonstrated that miR-17 ~ 92 selectively activated the canonical but not non-canonical NF- κ B signaling via directly targeting multiple ubiquitin-editing proteins, including TNFAIP3, CYLD and Rnf11, leading to increase the K63-linked polyubiquitination and attenuate the K48-linked polyubiquitination of RIP1 complex, and activation of the canonical NF- κ B signaling further promoted cell proliferation and enhanced chemoresistance in ABC-DLBCL (Fig. 6E). Our findings imply that targeting miR-17 ~ 92 may provide a novel bio-therapeutic strategy for ABC-DLBCL patients.

Abbreviations

DLBCL: Diffuse large B-cell lymphoma; ABC-DLBCL: Activated B-cell like diffuse large B-cell lymphoma; Non-GCB DLBCL: Non-germinal center B-cell-like diffuse large B-cell lymphoma; GCB-DLBCL: Germinal center B-cell-like diffuse large B-cell lymphoma; miRNA or miRs: microRNAs; NF- κ B: nuclear factor kappa-B; RIP1: receptor-interacting protein 1; WHO: World Health Organization; RT-qPCR: reverse transcription quantitative polymerase chain reaction;

Declarations

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Authors' contributions

Kai Fu, Huilai Zhang and Huaqing Wang conceived and designed research. Xianhuo Wang, Xiaoyan Zhang and Chengfeng Bi performed experiments and interpreted results of experiments. Xianhuo Wang and Xiaoyan Zhang analyzed data and prepared figures. Xianhuo Wang and Xin Huang performed statistical and bioinformatics analysis. Wing C. Chan and Julie M. Vose provided material support. Timothy W. McKeithan and Bin Meng provided suggestions and reviewed pathology. Xianhuo Wang and Kai Fu drafted paper, edited and revised manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

The study was approved by the ethics committee of Tianjin Medical University Cancer Institute and Hospital and University of Nebraska Medical Center. The informed consent was obtained from each participant.

Competing interests

The author declares no competing interest exists.

Consent for publication

Not applicable.

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Figures

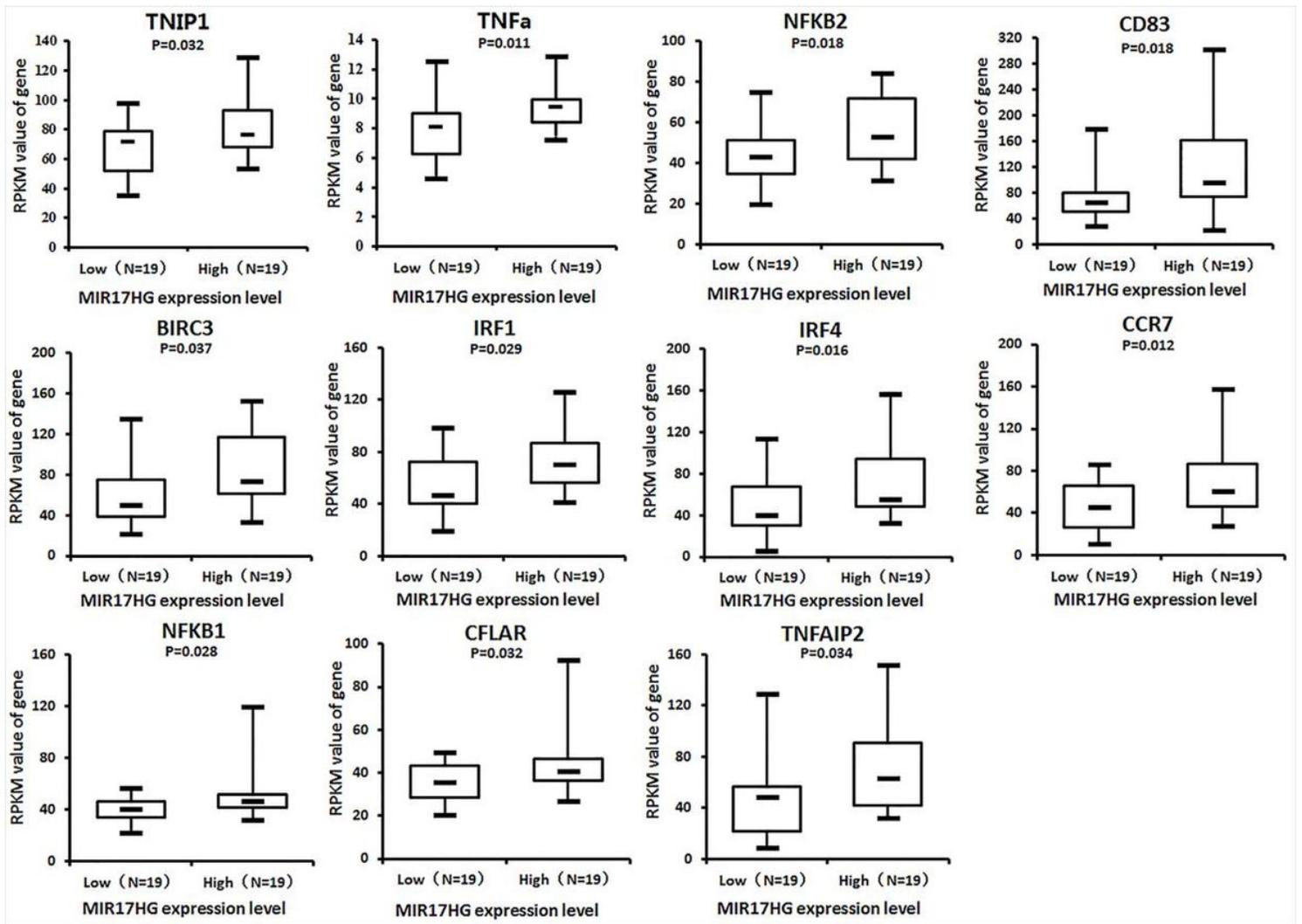


Figure 1

The correlations between MIR17HG expression and the expression of NF-κB downstream transcriptional target genes, including TNIP1, TNF-α, NFKB2, CD83, BIRC3, IRF-1, IRF-4, CCR7, NFKB1, CFLAR and TNFAIP2 based on RNA-sequencing in 38 non-GCB DLBCL patients. All samples were classified into two groups according to MIR17HG expression level: low (N=19) and high (N=19). Box plots of all gene expression were shown as median, 25th and 75th percentiles.

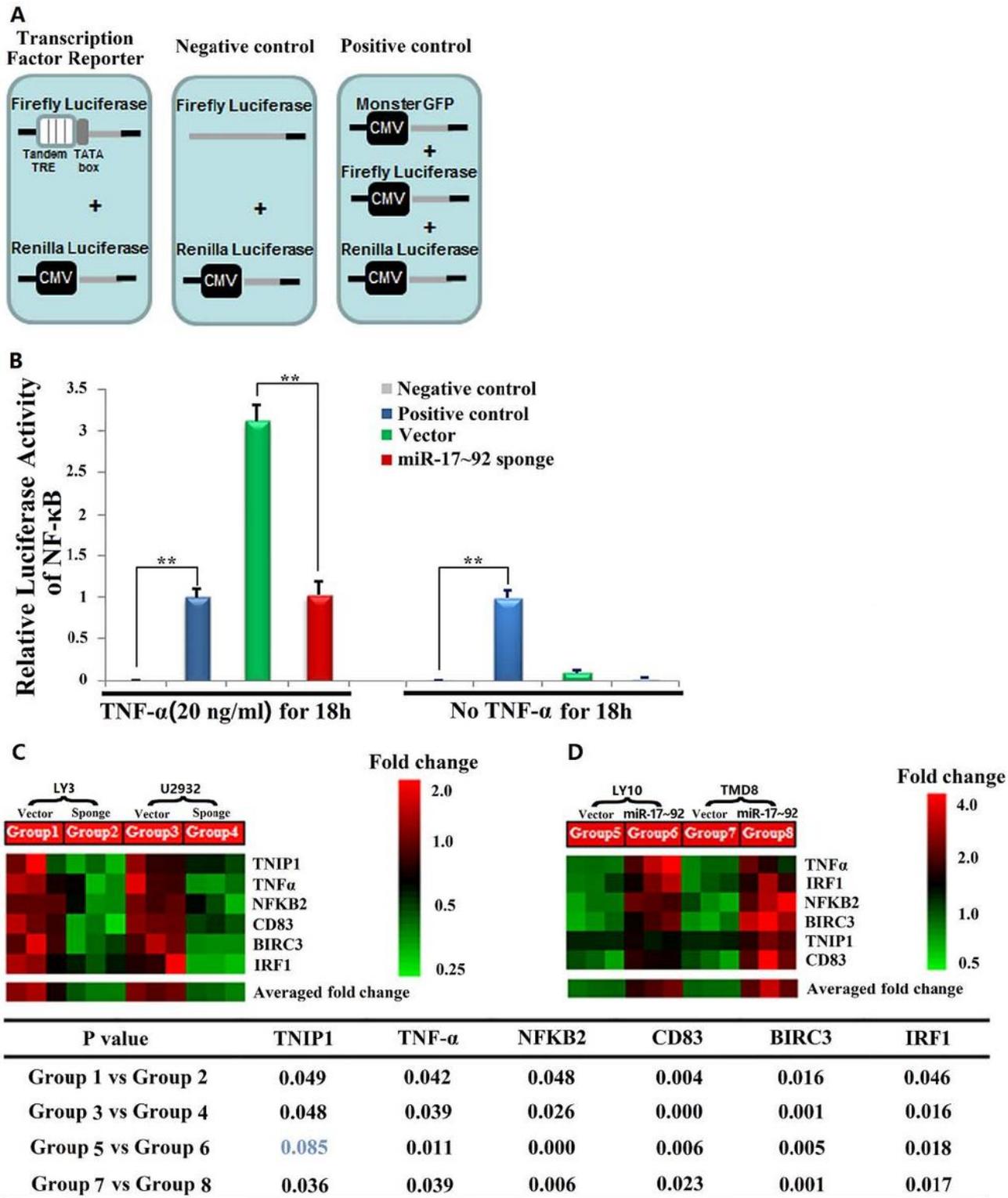


Figure 2

MiR-17~92 and NF- κ B activity. a: The schematic representation of NF- κ B Signal reporter, positive control and negative control constructs. b: NF- κ B luciferase activity in HEK293T cells which were exposed to 20 ng/ml TNF- α (left) or without TNF- α (right) for 18h after co-transfecting negative control (gray bar), positive control (blue bar), NF- κ B Signal reporter and vector (green bar), and NF- κ B Signal reporter and pTRIPZ-miR-17~92 sponge plasmids (red bar). NF- κ B luciferase activity significantly increased after

transfecting the positive control (blue bar) than negative control (gray bar) with or without TNF- α treatment ($P < 0.01$), suggesting that this system worked very well. c and d: Heat-map of the expression of six NF- κ B downstream transcriptional target genes, including TNIP1, TNF- α , NFKB2, CD83, BIRC3 and IRF-1, in the conditional loss-of-function of miR-17~92 ABC-DLBCL cells (Ly3-sponge and U2932-sponge) and the conditional over-expression of miR-17~92 ABC-DLBCL cells (Ly10-miR-17~92 and TMD8-miR-17~92). P values of these genes expression between two groups were shown in table. Experiments were performed in triplicate. ** $P < 0.01$.

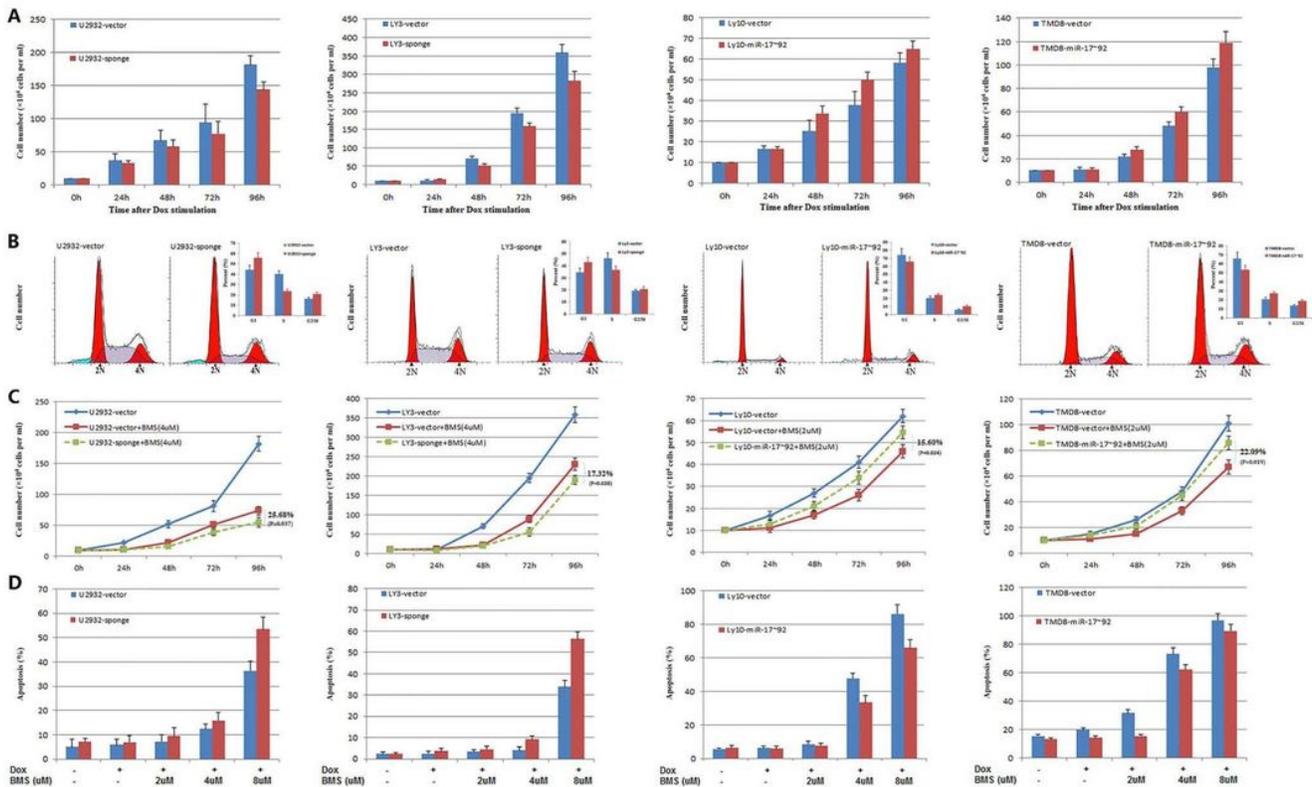


Figure 3

MiR-17~92 promotes cell proliferation and enhances chemoresistance to BMS 345541 treatment in ABC-DLBCL cells. a: Cell growth of four ABC-DLBCL cell lines stably expressing either miR-17~92 sponge or miR-17~92 (red), and control vector (blue). b: Cell cycle of four ABC-DLBCL cell lines stably expressing either miR-17~92 sponge or miR-17~92, and control vector. c: Cell growth of four ABC-DLBCL cell lines stably expressing either miR-17~92 sponge or miR-17~92 (green), and control vector (red), which were treated with BMS. Blue curves represented all stably transduced cells expressing control vector without BMS treatment. d: Percentage of apoptotic cells treated with BMS in all four ABC-DLBCL cells with over-expression or loss-of-function of miR-17~92. Experiments were performed in triplicate. Values represented mean + s.d.

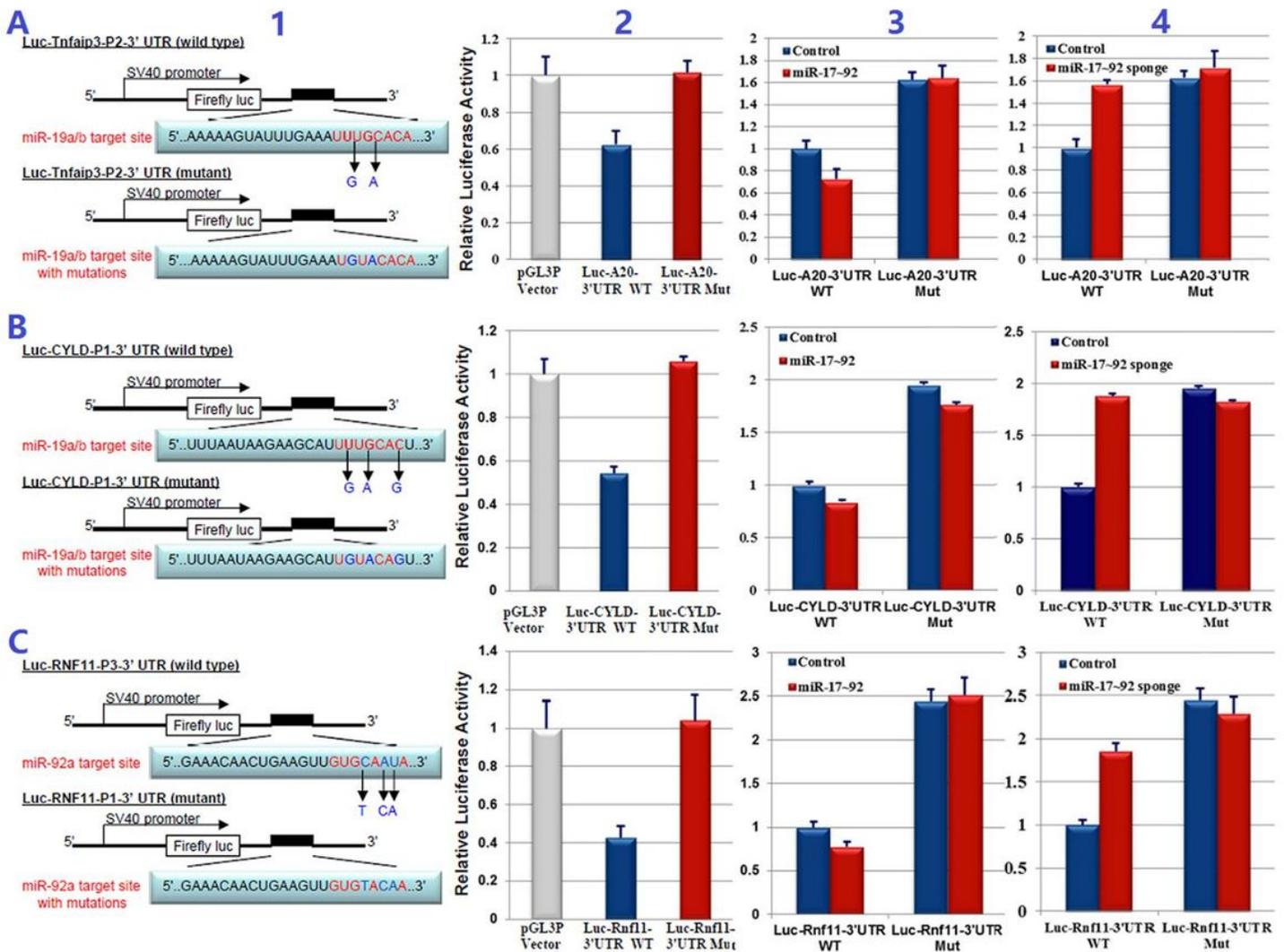


Figure 4

MiR-17~92 directly targets multiple ubiquitin-editing genes. a1: A map of Luc-TNFAIP3-3' UTR P2 fragment wild-type (WT) and mutation (Mut) which harbored putative binding sites for miR-19a/b. a2: Luciferase activity of reporter plasmids in HEK293T cells after transfecting Luc-TNFAIP3-3'UTR-WT or Mut (P2 fragment). HEK293T cells expressed some endogenous miR-17~92. a3: Luciferase activity of reporter plasmids in HEK293T cells after co-transfecting Luc-TNFAIP3-3'UTR WT or Mut (P2 fragment) along with pTIP-miR-17~92 plasmid or pTIP control. a4: Luciferase activity of reporter plasmids in HEK293T cells after co-transfecting Luc-TNFAIP3-3'UTR WT or Mut (P2 fragment) along with pTRIPZ-miR-17~92 sponge plasmid or pTRIPZ control. b1-4 and c1-4: were similar to A1-4, but the fragment of target genes were respectively CYLD-3'UTR P1 and RNF11-3'UTR P3. Experiments were performed in triplicate. Values represented mean + s.d.

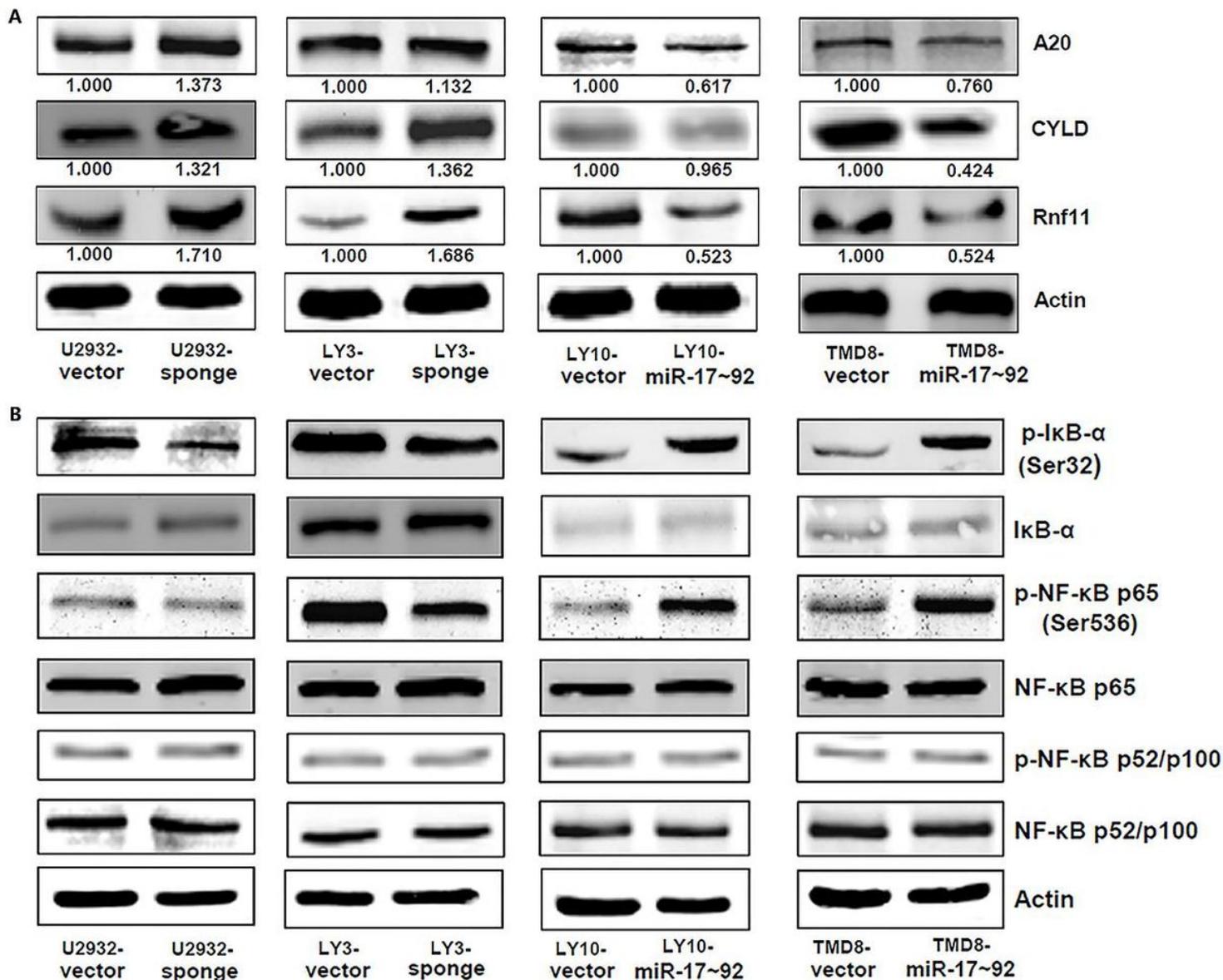


Figure 5

The regulatory effects between miR-17~92 and ubiquitin-editing proteins or NF-κB signaling. a: Expression of A20, CYLD and Rnf11 proteins in ABC-DLBCL cell lines, which were respectively transduced pTRIPZ-miR-17~92 sponge or pTIP-miR-17~92 plasmids. b: Expression of IkB-α, NF-κB p65, NF-κB p52/p100 and their phosphorylation levels in ABC-DLBCL cell lines, which were respectively transduced pTRIPZ-miR-17~92 sponge or pTIP-miR-17~92 plasmids. Total proteins were isolated from cells after inducing with 1μg/ml doxycycline for 48h.

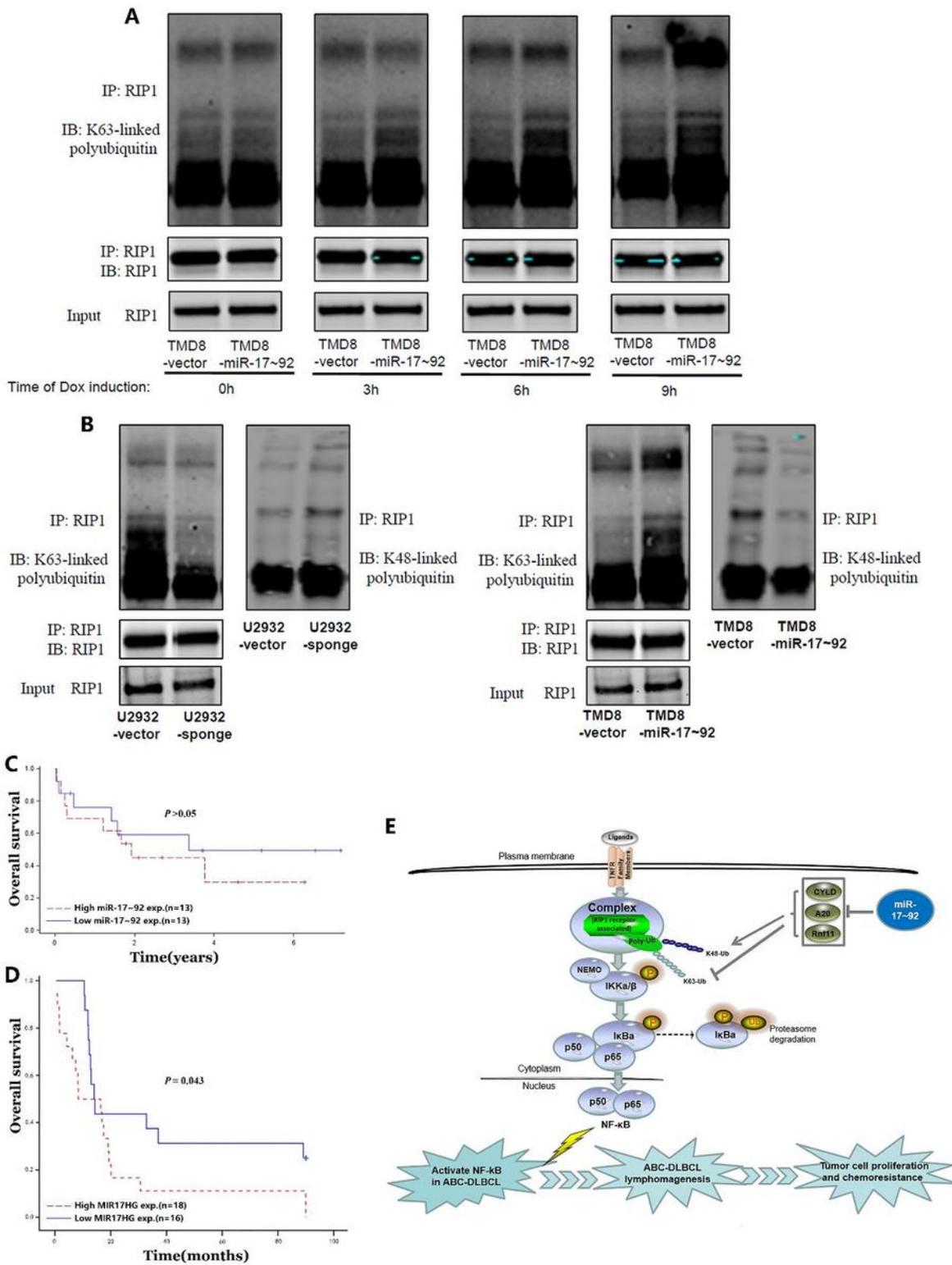


Figure 6

The regulatory effects between miR-17~92 and the K63- and K48-linked polyubiquitination of RIP1 complex, and the correlations between miR-17~92 and overall survival in ABC-DLBCL patients. a: The kinetics of K63-linked polyubiquitination level of RIP1 complex upon miR-17~92 over-expression in TMD8 cells after inducing with 1 μg/ml doxycycline for 0h, 3h, 6h and 9h. Immunoblotting with RIP1 antibody confirmed equal amount of these proteins. b: Expression of K63- and K48-linked polyubiquitination of

RIP1 complex in stably transduced U2932-miR-17~92 sponge and TMD8-miR-17~92 cells, which were induced with 1 µg/ml doxycycline for 9h. c: Kaplan-Meier curves of 26 ABC-DLBCL patients with low (N=13) and high (N=13) miR-17~92 expression. MiR-17~92 expression represented the mean values of miR-17, miR-18a, miR-19, miR-20a and miR-92 expression. d: Kaplan-Meier curves of 34 non-GCB DLBCL patients with low (N=16) and high (N=18) miR-17~92 primary transcript expression. e: A model of the role of miR-17~92 in the process of NF-κB activation in ABC-DLBCL.

Supplementary Files

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