Leukemia Research 54 (2017) 30–38

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

The relationship of REL proto-oncogene to pathobiology and chemoresistance in follicular and transformed follicular lymphoma

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ARTICLE INFO

Article history:
Received 8 November 2016
Received in revised form
15 December 2016
Accepted 2 January 2017
Available online 9 January 2017

Keywords:
REL
tFL
Amplification
Genotoxicity-induced NF-κB pathway
Chemoresistance

ABSTRACT

Follicular lymphoma (FL) is a common type of indolent lymphoma that occasionally transforms to more aggressive B-cell lymphomas. These transformed follicular lymphomas (tFL) are often associated with chemoresistance whose mechanisms are currently unknown. *REL*, a proto-oncogene located on frequently amplified 2p16.1-p15 locus, promotes tumorigenesis in many cancer types through deregulation of the NF-κB pathway; however, its role in FL pathobiology or chemoresistance has not been addressed. Here, we evaluated *REL* gene copy number by q-PCR on FFPE FL tumor samples, and observed *REL* amplification in 30.4% of FL cases that was associated with weak elevation of transcript levels. PCR-Sanger analysis did not show any somatic mutation in FL tumors. In support of a marginal oncogenic role, a REL-transduced FL cell line was positively selected under limiting serum conditions. Interestingly, reanalysis of previously reported gene expression profiles revealed significant enrichment of DNA damage-induced repair and cell cycle arrest pathways in tFL tumors with high REL expression compared to those with low REL expression consistent with the critical role of c-REL in genotoxicity-induced NF-κB signaling, which was reported to lead to drug resistance. In addition to DNA damage repair genes such as ATM and BRCA1, anti-apoptotic BCL2 was significantly elevated in REL-high FL and tFL tumors. Altogether these data suggest that other genes located in amplified 2p16.1-p15 locus may have more oncogenic role in FL etiology; however, high REL expression may be useful as a predictive biomarker of response to immunochemotherapy, and inhibition of c-REL may potentially sensitize resistant FL or tFL cells to chemotherapy.
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1. Introduction

Follicular lymphoma (FL) is the most common indolent lymphoma in the world [1]. It has a heterogeneous clinical course with ~10 year median survival after diagnosis [2]. Histological transformation (HT) to more aggressive malignancies collectively called as transformed follicular lymphomas (tFLs) such as diffuse large B-cell lymphoma (DLBCL) or Burkitt's lymphoma (BL) is observed in 10–60% of the FL patients [3]. Transformed follicular lymphomas (tFLs) are associated with shorter overall survival and immunochemotherapy resistance [4].

Neoplastic transformation of germinal center (GC) B cells are considered to be responsible for FL tumorigenesis [5]. t(14;18)(q32;q21) translocation contributes to neoplastic transformation of GC B cells in majority of the cases by bringing the IgH enhancer upstream of BCL2 [6] and thereby upregulating BCL2 expression [7], a pro-survival gene known to inhibit apoptosis [8]. Studies performed in BCL2 transgenic mice revealed that overexpression of BCL2 is not enough –by itself- suggesting other oncogenic events

Abbreviations: ECOG, Eastern Cooperative Oncology Group; FL, follicular lymphoma; FFPE, formalin-fixed paraffin-embedded; IPI, international prognostic index; LDH, lactate dehydrogenase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PMIG, pMSCV-IRES-GFP; tFL, transformed follicular lymphoma.

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http://dx.doi.org/10.1016/j.leukres.2017.01.001
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are needed for complete establishment of FL [9]. A recent study that included whole-exome sequencing on 10 FL-tFL tumor pair revealed recurrent mutations of genes in chromatin modifiers, JAK-STAT or NF-κB pathway [10] suggesting that deregulation of these pathways may have a role in initiation or progression of FL tumors.

REL amplification is frequently observed in many lymphoma types including DLBCL [11], classical Hodgkin's lymphoma [12] or primary mediastinal B-cell lymphoma [13]. On the other hand, copy number variation analysis of FL or tFL tumors showed discrepant results with respect to the presence of amplification of 2p16.1-p15 locus that includes *REL* [14–16]. A recent report based on high-throughput SNP array analysis of CNVs in FL tumor samples revealed that 22.2% of FL and 35.6% of tFL cases, have 2p16.1-p15 gain/amplification that involves *BCL11A* in addition to *REL* proto-oncogene [17]. Altogether, these studies suggest that there may be one or more oncogene candidate in this recurrently amplified locus. However, whether REL contributes to FL pathogenesis has not been addressed previously.

c-REL (protein encoded by *REL* gene) is a transcription factor, and acts as a mediator of the NF-κB signaling pathway by forming dimers with other REL family members or with each other [18]. In B-cells, c-REL was shown to promote proliferation and survival [19]. Its overexpression/activation has been shown to contribute to neoplastic transformation in several solid tumors or hematologic malignancies [20]. In addition, c-REL overexpression was shown to correlate with chemotherapy resistance in serous epithelial ovarian cancer [21]. Indeed, previous reports showed that NF-κB pathway activation may be responsible for chemotherapy resistance in many cancer types including cervical carcinoma [22] and gastric cancer [23].

Here we showed frequent amplification of *REL* in FL tumors, and evaluated whether any association is present between amplification and clinical variables. We also showed moderate positive selection of REL-transduced FL cell line under limiting serum conditions. More importantly, we observed that tFL tumors with high REL expression has activation of DNA damage-induced cell cycle arrest and repair pathways, with upregulation of ATM, BRCA1 and BCL2, which may be a useful predictive biomarker of response to immunochemotherapy or therapeutic target for overcoming chemoresistance in these tumors.

2. Materials and methods

2.1. Patient samples and cell lines

Twenty-five follicular lymphoma tumor biopsies used in this study were collected at West China Sichuan University Hospital during routine diagnosis. The cases were reviewed by at least two hematopathologists with consensus diagnosis. Four μM thick sections were obtained from tissue blocks of FL biopsies fixed with buffered formalin and embedded in paraffin (FFPE). For each patient, the diagnostic tumor sample was mounted on each of two slides for DNA or RNA isolation, and stored at 4 °C to decrease the possibility of nucleic acid degradation. Histopathological and clinical characteristics of FL patients of this study are shown in Table S1. Additionally, publicly available data [17] of 42 tFL patients whose biopsies were collected by the Lymphoma/Leukemia Molecular Profiling Project (LLMPP) consortium or the University of Nebraska Medical Center (UNMC) were used in the current study. Diagnoses of these tFL cases were performed by a panel of LLMPP hematopathologists. A patient was diagnosed as tFL if a DLBCL occurred in the patient who was concurrently or previously diagnosed with FL. The transformed FL vs. high histopathological grade FL distinction was established by diagnosing a case as high grade (i.e. stage 3A or 3B) FL if a higher number of large cells were observed without loss of follicular histology. By contrast, a transformed FL case did no longer show follicular pattern and usually represented by a DLBCL. The characteristics of these cases are shown in Table S2.

KARPAS-422 [24], HEK293T and SU-DHL-4 [25] cell lines are gifts from Dr. Anna Scuto at City of Hope Medical Center. KARPAS-422 and SU-DHL-4 cell line were cultured in RPMI medium supplemented with 10% FBS, penicillin G (100 units/mL), streptomycin (100 μg/mL), and L-glutamine. Cell culture medium used for HEK393T cells was the same except that DMEM was used instead of RPMI. All cells were kept at a humidified incubator at 37 °C in 5% CO_2.

2.2. Evaluation of REL gene copy number with q-PCR

REL copy number analysis of FFPE FL tumors was performed with quantitative real-time PCR (qPCR) using primers designed against the *REL* genomic DNA isolated with QIAamp DNA FFPE Tissue Kit (Qiagen Inc., Germany) by applying the same qPCR-based method used earlier for the detection of mono-allelic deletion of *HACE1* [26] in natural killer/T cell lymphomas. Forty ng genomic DNA was used as template for q-PCR reactions that were performed with DyNAmo HS SYBR Green qPCR Kit master mix (ThermoFisher Scientific Inc.) using Roche LightCycler480 thermal cycler. ΔΔCt method was used for quantification of copy numbers. Gene copy number of *REL* was calibrated to that of *RPS13* reference gene [27]. Then, *REL* genomic copy number of FL tumors was normalized to that of normal human NK cells activated with IL2 for 3 days, which was available from a previous study [26]. The cut-off to define *REL* gain/amplification was set as two-fold increase. The *REL* and *RPS13* genomic q-PCR primers used for copy number analysis are as follows: REL genomic q- PCR forward: 5′-CCCTTGGTAACAGAATCCCTATT- 3′, REL genomic q-PCR reverse: 5′-CTGACTTTCAACTGGGCCTTA-3′; RPS13 genomic q-PCR forward: 5′-CGACGTGAAGGAGCAGATTTA-3′; RPS13 genomic q-PCR reverse: 5′-CACGAGGACAGGCGAAATAG-3′.

2.3. Comparison of copy number and mRNA expression of REL in transformed follicular lymphomas

Copy number and gene expression values of REL were obtained for 42 tFL cases with both SNP array (Affymetrix Mapping 250 K Nsp SNP Array) and DNA microarray (GeneChip Human Genome U133 Plus version 2.0) data available in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) database with the following accession numbers: GSE81183 and GSE81184. Log2 intensities of copy number variation data of SNP_A-2301401 and SNP_A-2094019 probe intensities determined with Affymetrix Genotyping Console software were used to estimate copy number of *REL*. SNP_A-2301401 and SNP_A-2094019 correspond to rs12474254 and rs13419008 single nucleotide polymorphisms (SNPs), respectively, and they are located ~43 kb and ~81 kb upstream of the human *REL* gene based on the UCSC Genome Browser (hg38). Intensity values of these SNP probes were highly concordant (Fig. S1) and their means were used to estimate *REL* copy number in 42 tFL tumors.

REL mRNA expression of 42 tFL cases in the NCBI GEO database (accession code: GSE81184) was analyzed with NCBI GEO2R gene expression analysis tool [28] using log2-normalized median centered gene expression profile (GEP) data.

2.4. Generation and ectopic expression of c-REL in a FL cell line and evaluation of cell growth

The directional cloning procedure followed to clone REL coding sequence into MSCV-IRES-GFP (PMIG) is as follows: REL coding sequence was amplified from pcDNA-REL-FLAG (Addgene plasmid

27253) by performing high fidelity PCR using PfuUltra II Fusion HS DNA Polymerase (Agilent technologies). The PCR product and PMIG plasmid were digested with NotI and SalI restriction enzymes (NEB Inc., Germany) to generate compatible sticky ends for cloning. Digested products were then purified with QIAquick PCR Purification Kit (Qiagen) and ligated with T4 DNA ligase (NEB). Colony screen was performed with PCR and diagnostic restriction mapping. In addition, Sanger sequencing of the insert was performed to validate the positive REL-PMIG clones. Retroviral transduction of FL cell lines with PMIG or REL-PMIG construct was performed as described previously using spinocculation procedure [29]. Five days post-transduction, % GFP^+ cells were determined with a FACSCanto II analyzer or FACSAria III flow cytometer (BD Biosciences).

2.5. *Gene expression and gene set analysis of follicular and transformed follicular lymphomas with high or low REL expression*

The normalized DNA microarray data of 42 tFL cases and 69 FL cases were obtained from NCBI GEO datasets using the following accession codes: GSE81183 and GSE55267. Affymetrix HG U133 Plus 2.0 chips were used as the DNA microarray platform for these cases. GEO2R web-based tool was used to determine REL mRNA expression in tFL cases reported in a previous study [17]. All available tFL cases (NCBI GEO accession code: GSE81183) were subdivided as REL-high (n = 10) and REL-low (n = 10) groups based on the level of REL transcript expression. Similarly, previously reported microarray data (GSE55267) of FL tumor samples were subdivided into two groups based on high or low REL mRNA expression with each group having 15 cases. After that, REL-high and REL-low group for tFL cases were compared with each other using GSEA [30] to identify significantly enriched gene sets in tFL cases with high REL expression. GSEA analysis was run with default methods, and the following gene sets database was evaluated: c2.v2.symbols (curated gene sets). GEO2R bioinformatics tool was applied on normalized DNA microarray data of tFL or FL tumors with high or low REL expression to determine the mRNA expression of BRCA1, RAD51, CHEK2, RAD17, ATM, ATR and BCL2.

Experimental methods for a) mutation analysis of REL in FL tumors; b) q-RT-PCR; c) REL expression analysis in paired FL-tFL cases; d) evaluation of growth of a REL-transduced FL cell line; e) visualization of c-REL occupation on promoter of target genes by ChIP-Seq and f) statistical analysis are described in the Supplementary methods.

3. Results

3.1. REL *is frequently amplified in follicular lymphoma tumors*

We performed quantitative PCR (q-PCR) on genomic DNA from tumor biopsies to evaluate whether *REL* is specifically amplified in FL tumors or not. Consistent with a previous report [17] that showed frequent amplification of 2p15-p16.1 in FL and tFL cases, q-PCR showed frequent amplification of *REL* in at least 7 of 23 (30.4%) of FL tumors (Fig. 1A). Four of 23 (17.3%) cases were in the borderline (i.e. 1.5–2 fold increase in copy number compared to normal cells) which may be due to presence of stromal DNA. We then evaluated *REL* copy number status in two FL cell lines (i.e. KARPAS-422 and SU-DHL-4), and observed that SU-DHL-4 cell line may have *REL* amplification (Fig. S2).

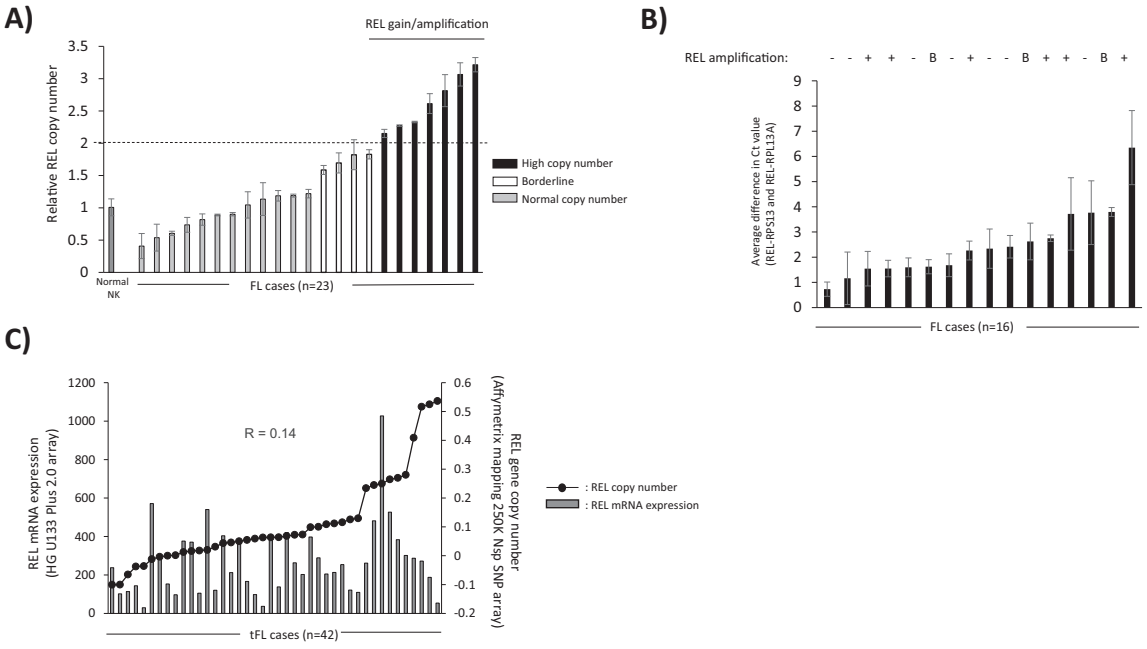

Fig. 1. The relationship between REL amplification and its mRNA expression in FL and tFL cases. (A) q-PCR was applied on gDNA isolated from FL tumors using *REL* specific primers. *RPS13* housekeeping gene, located at 11p15.1 with no known genomic abnormality in FL tumors, was used to calibrate *REL* copy number. Normal primary human NK cells activated with IL2 for 3 days, which was available from a previous study (Küçük et al. Clin. Cancer Res. 2015) was used for normalization of the copy number of *REL* in FL tumors. FL tumors with >2 fold copy number were considered to have gain or amplification. Horizontal, dashed line shows the threshold for copy number increase. (B) REL mRNA expression in FL tumors were evaluated with q-RT-PCR. Delta Ct value was calculated by taking average of Ct differences of REL with one of each of two different housekeeping genes for each FL case [i.e. average of (Ct_{REL}-Ct_{RPS13}) and (Ct_{REL}-Ct_{RPL13A})]. Means ± SD represent combination of two independent repeat of q-PCR reactions using primers for REL and each housekeeping gene. REL locus amplification status based on q-PCR is indicated on the graph with '+', '–' or 'B' signs that means REL is 'amplified', 'not amplified' or 'amplification status is borderline', respectively. (C) REL copy number and its mRNA expression for 42 tFL cases whose normalized expression and SNP-array copy number values available as GSE81184 in NCBI GEO database were calculated to evaluate correlation of amplification and expression. mRNA expression is based on the REL probe set (206036_s_at) value of the HG U133 Plus 2.0 array; and *REL* copy number estimate is based on the average of Affymetrix Mapping 250 K Nsp SNP Array log2 values of SNP_A-2301401 and SNP_A-2094019 probes that are available in the GSE67385 dataset.

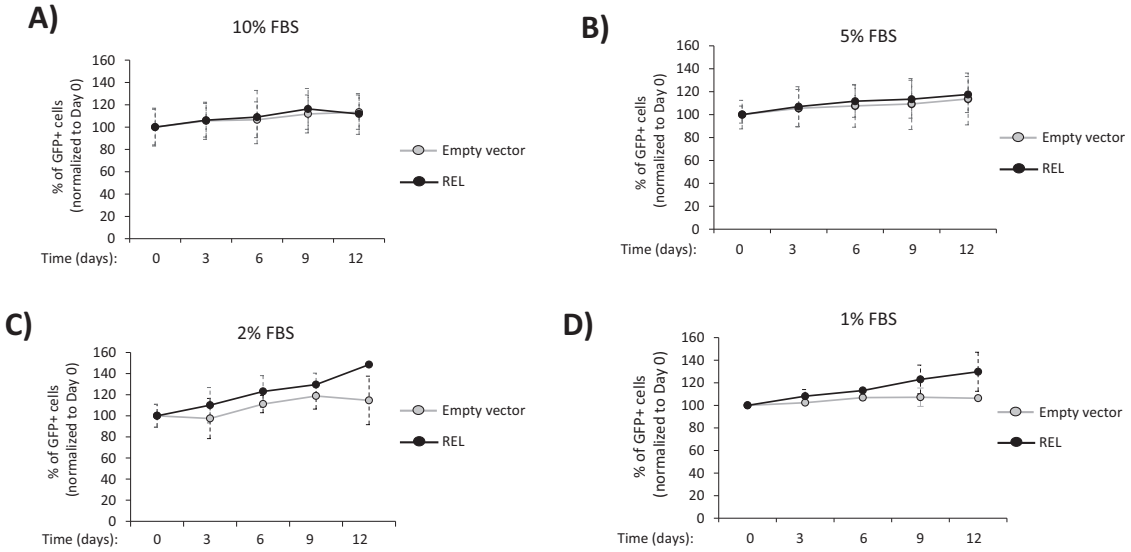

Fig. 2. Ectopic expression of REL is associated with positive selection of a FL cell line in limiting serum concentrations. The percentage of GFP⁺ cells was determined with flow cytometry in 3 day-intervals after transduction of KARPAS-422 with empty vector or REL. Serum concentration was either kept as 10% (A) or decreased to 5% (B) or 2% (C) FBS after transduction. (D) In another experiment, the percentage of GFP⁺ cells was determined in empty vector or REL-transduced KARPAS-422 cell line cultured in 1% FBS concentration post-transduction. The percentage of GFP⁺ cells determined in tracked populations was normalized to day 0 levels. Day 0 represent 8 days post-transduction for transduced cells cultured in 10%, 5% and 2% FBS, and represent 5 days post-transduction for cells cultured in 1% FBS concentrations. Serum concentrations were reduced at day 0 for each group with limiting FBS. The average% of GFP⁺ cells at day 0 for PMIG or REL transduced cells is 13% or 6.5%, respectively. Data represent means ± SD of two biological replicates.

3.2. *Relationship between* REL *amplification and clinical parameters*

Next, we evaluated whether *REL* copy number correlates with clinical variables, and observed no association between *REL* amplification and the evaluated clinical parameters, that is, presence of B symptoms, gender, age, disease stage, LDH levels, ECOG performance, largest tumor size, IPI score, and therapy response (Table S3). We were not able to evaluate the relationship between REL amplification and FL patient survival as the diagnosis of these cases were relatively recent.

3.3. *Relationship between* REL *amplification, its mRNA expression and histopathological grade*

To address whether *REL* amplification correlates with gene expression, we performed q-RT-PCR experiment. Side-by-side comparison of *REL* amplification detected by q-PCR and REL mRNA expression detected by q-RT-PCR did not reveal any notable relationship (Fig. 1B) in FL tumors with both data available. Next, we obtained the log2 intensities of REL probes (i.e. SNP_A-2301401 and SNP_A-2094019) exported from Genotyping Console Software that were available for 42 tFL cases as Affymetrix Mapping 250 K Nsp SNP Array data. We also obtained the mRNA expression data of GeneChip® Human Genome U133 Plus 2.0 Array for the same 42 tFL cases. Side-by-side comparison of the REL amplification and its mRNA expression of these cases revealed a weak (R = 0.14) positive correlation (Fig. 1C) suggesting that *REL* amplification may not lead to higher transcript expression in FL tumors. Consistent with this possibility, we did not observe increase of REL mRNA expression in FL tumors (n = 63) compared to its expression in normal B cells (n = 6) (Fig. S3).

Next we evaluated whether there is any association between REL transcript levels and histopathological grade of the FL cases which would suggest a possible etiological role for REL during histologic transformation if both pre and post-transformation levels of it are high. First, we compared REL mRNA expression determined with q-RT-PCR in low and high-grade (i.e. stage 3A/3B) FL cases, and did not observe any significant difference in expression levels (Fig. S4). We then re-analyzed REL mRNA expression in 12 paired patient biopsies obtained before and after histological transformation [31], and observed that transcript expression levels of REL were in general higher relative to those in the pool of cell lines used for comparison in both pre- or post-transformation biopsies (Fig. S5A). However, REL mRNA expression levels did not change in a specific direction when post-histological transformation levels were compared to those of pre-transformation (Fig. S5B).

3.4. *Mutation analysis of REL in FL tumor samples*

We used PCR-Sanger to evaluate whether there are somatic mutations in coding sequences of *REL* to address whether REL is activated through somatic mutations. As mutations in conserved coding sequences of proto-oncogenes are more likely to change the gene function, we screened for only conserved coding sequences. Consistent with a recent NGS-based mutation screen of 10 FL/tFL tumors [10], we did not observe any REL mutation (SNV, in-del etc.) in these FL cases.

3.5. *Ectopic expression of REL in a FL cell line is associated with moderate positive selection under limiting serum concentrations*

To address whether elevated REL expression promotes cell growth in FL cells, we ectopically expressed empty vector or c-REL in KARPAS-422, the FL cell line with no *REL* amplification (Fig. S2), through retroviral transduction and quantified the% of GFP⁺ cells at regular time intervals using flow cytometry to observe whether there is any positive selection of REL-transduced cells (Fig. S6). When cells were cultured under regular (i.e.10% FBS) serum concentrations until 17 days post-transduction (day 12), we did not observe any difference in the% of GFP⁺ cells between REL or empty vector transduced KARPAS-422 cells (Fig. 2A). To address whether c-REL promotes cell growth when apoptosis is induced by low growth factor concentrations, we then switched the cells to culture medium with 5% or 2% FBS 5 days after transduction (day 0). There was no positive selection of REL-transduced cells cultured

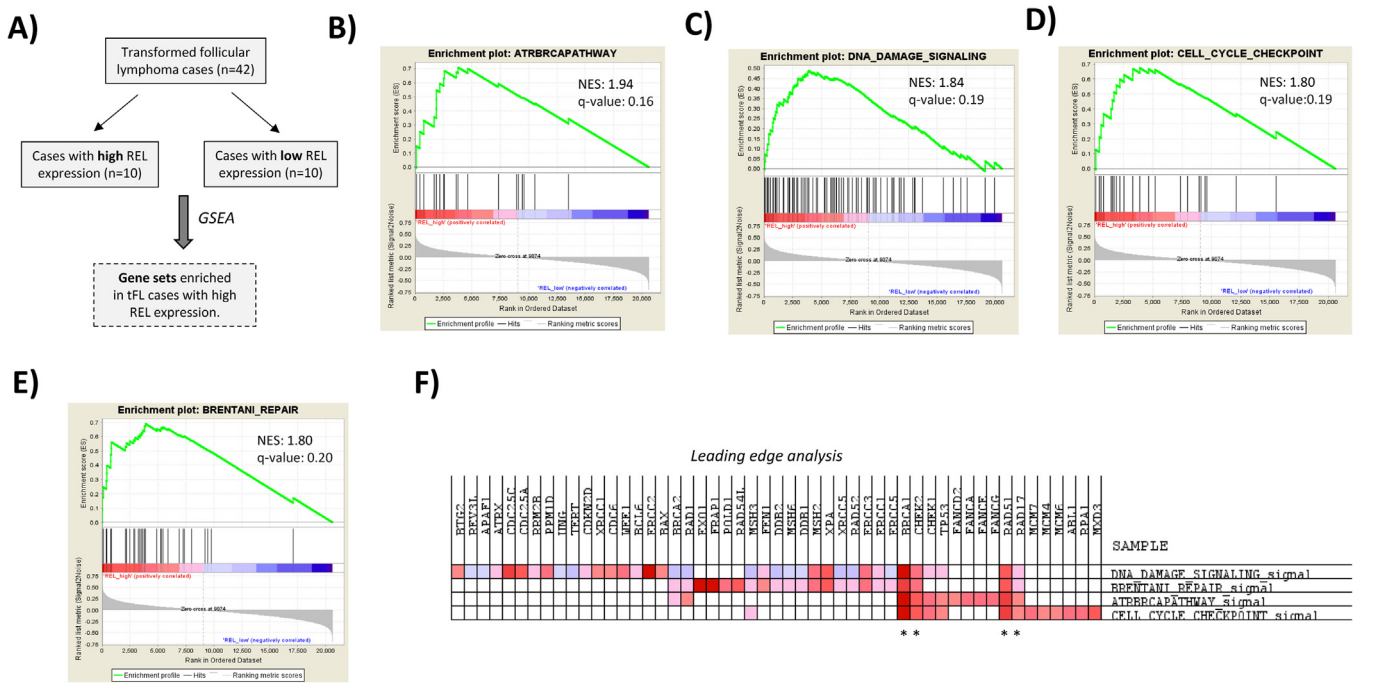

Fig. 3. Gene set analysis of transformed follicular lymphoma tumors with high REL expression shows activation of DNA damage-mediated cell cycle arrest and DNA repair pathways. (A) The diagram showing how tFL cases were separated as two distinct groups for GSEA pathway analysis based on REL expression levels. Each group has 10 tumor samples as explained in 'materials and methods'. (B–E) GSEA results showing significantly enriched gene sets in tFL cases with high REL expression (REL-high tFL) compared to those with low REL expression (REL-low tFL). The gene sets shown were enriched at significance levels of $p < 0.001$ and FDR <0.025. F) Leading edge analysis displaying heatmap of genes present in the leading edge of at least one of the four gene sets shown in B–E. Red color indicates high mRNA expression. *: Genes present in all four gene sets.

in 5% FBS concentrations (Fig. 2B) whereas moderate level of positive selection was observed in REL-transduced KARPAS-422 cells compared to empty vector transduced ones in 2% FBS concentrations (Fig. 2C). Similar results were obtained when transduced cells were cultured in 1% FBS concentrations after transduction (Fig. 2D). Ectopic expression of REL was shown with q-RT-PCR on FACS sorted empty vector or REL-transduced KARPAS-422 cells (Fig. S7).

3.6. *Transformed follicular lymphomas with high REL expression is associated with chemoresistance-related signaling pathways*

Next we performed pathway analysis using the gene expression profile of previously reported tFL tumors [17] by performing GSEA on tFL cases with high or low REL mRNA expression (Fig. 3A). Interestingly, we observed DNA damage signaling, DNA repair, and cell cycle arrest associated gene sets as the significantly enriched ones in REL-high tFL tumors compared to REL-low tFL tumors (Figs. 3 B–E and S8). After that, we performed leading edge analysis and observed that BRCA1, CHEK2, RAD51 and RAD17 genes are involved in all these four pathways suggesting possibly more critical roles for these genes in DNA damage-induced NF-kB signaling pathway (Fig. 3F).

We then analyzed the mRNA expression level of these four genes (i.e. BRCA1, CHEK2, RAD51 and RAD17) individually in tFL and FL tumor samples with high or low levels of REL expression and observed significant upregulation of RAD51, RAD17 in both FL and tFL cases whereas BRCA1 was upregulated in tFL cases, and CHEK2 was upregulated only in FL tumors (Fig. 4A–D). ChIP-Seq analysis of a lymphoblastoid cell line whose chromatin was immunoprecipitated with a c-Rel antibody revealed ChIP-Seq peaks on the promoter of these four genes raising the possibility that c-REL may directly transcriptionally regulate them (Fig. S9). We also detected overexpression of two critical genes of double stranded DNA damage repair (i.e. ATM and/or ATR) in REL-high FL or tFL cases compared to the REL-low ones (Fig. 5A, B). Also, we observed high BCL2 –a known direct transcriptional target of c-REL-expression in tFL cases with high REL mRNA levels (Fig. 5C).

4. Discussion

The weak positive correlation observed for *REL* amplification and its transcript expression in FL tumors may be related to other factors that regulate REL expression such as negative autoregulation of the REL promoter [32] or transcriptional upregulation of REL in a subset of tFL tumors by DNA damage-induced signaling. This observation suggests that the co-amplified genes of 2p16.1 locus other than *REL* such as *BCL11A* proto-oncogene [33] may be more critical for promoting FL tumorigenesis but future studies with high numbers of FL tumor samples may be needed to clarify this possibility. The lack of somatic mutations in the conserved coding sequence of FL cases is consistent with the findings of a previous report on FL and tFL tumor samples that utilized NGS-based methodologies [10], and it further decreases the possibility of REL proto-oncogene activation through genetic mechanisms in FL cases. On the other hand, we observed ectopic c-REL expression marginally promoted growth under limiting serum concentrations suggesting that high expression of REL may at least have a moderate role in enhanced cell survival and/or proliferation when centrocytes/centroblasts undergo clonal selection for higher antigen affinity that accompanies high rates of apoptosis in germinal centers [5].

R-CVP (rituximab, cyclophosphamide, vincristine and prednisolone) is the standard front-line therapy for symptomatic FL patients [34]. However, immunochemotherapy (ICT) resistance is commonly observed (15–20%) in R-CVP treated patients, and histologic transformation to high grade lymphomas (i.e. tFLs) is significantly more frequent in these cases [4]. Cyclophosphamide has genotoxic effects on DNA that triggers DNA damage response (DDR) pathways thereby halting cell cycle and inducing apoptosis

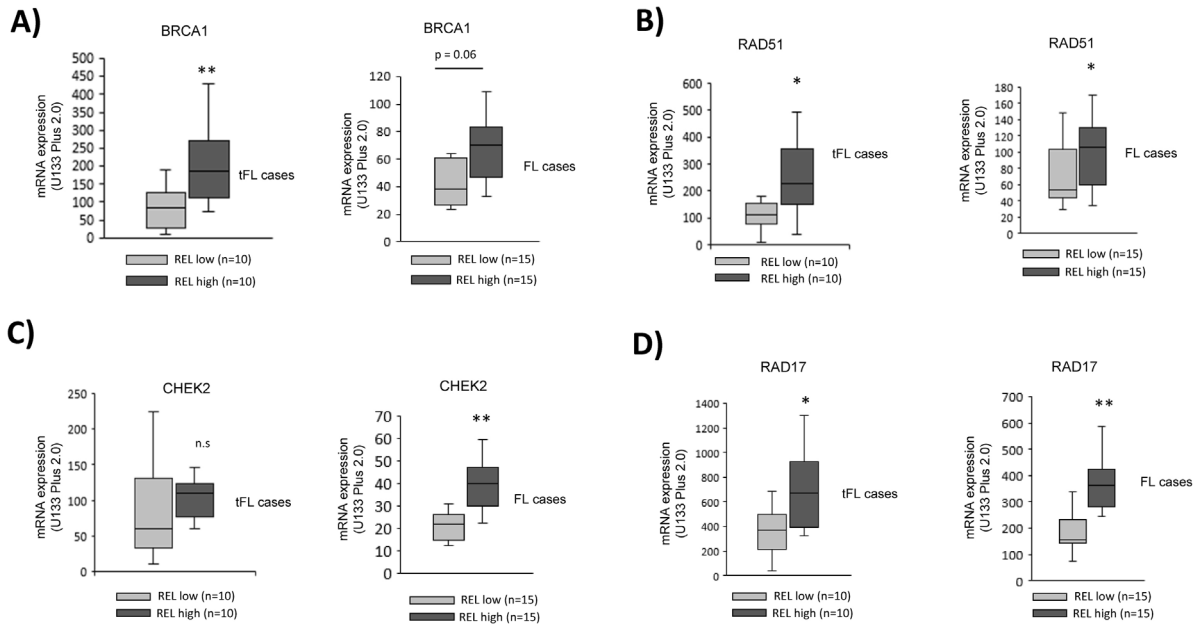

Fig. 4. Critical genes involved in DNA damage-induced repair and cell cycle arrest are upregulated in follicular and transformed follicular lymphomas with high REL expression. Transcript expression of BRCA1, CHEK2, RAD51 and RAD17 that are involved in all four pathways by GSEA in REL-high tFL tumors was evaluated by GEO2R program on normalized HG U133 Plus 2.0 microarray data in 42 tFL tumors and 63 FL tumors based on REL mRNA expression (A–D, left and right panels, respectively). Boxplots show median numbers, first and third quartiles, maximum and minimum values for mRNA expression of these genes in tFL and FL cases that were divided into two groups based on high and low REL mRNA expression. *: $p < 0.05$; **: $p < 0.01$.

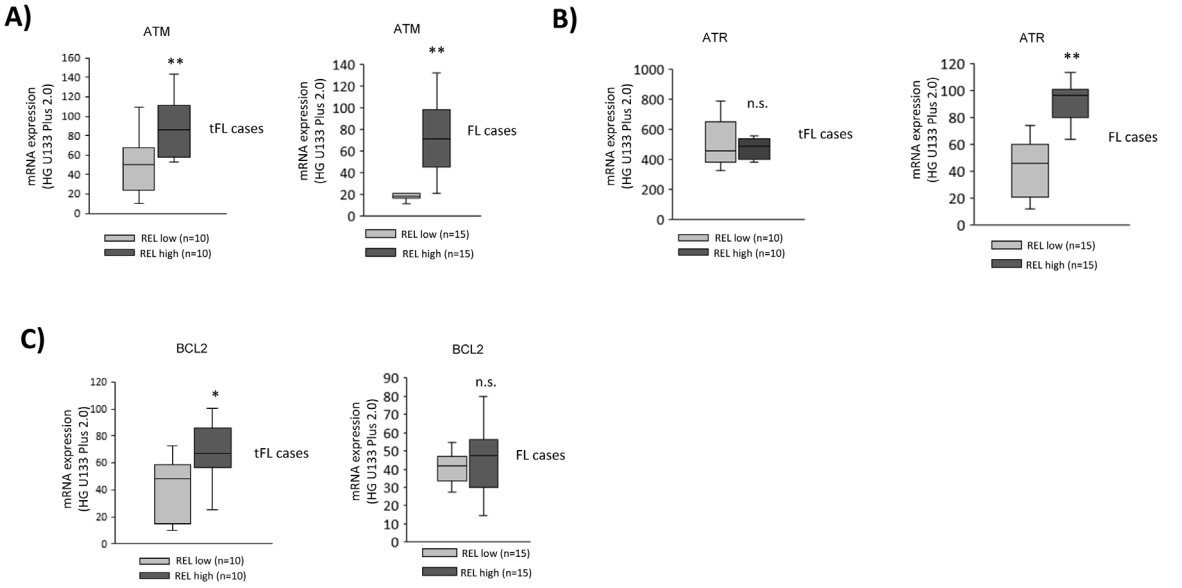

Fig. 5. ATM, ATR and BCL2 are co-expressed with REL in follicular and transformed follicular lymphoma cases. Boxplots showing the mRNA expression levels of ATM (A), ATR (B) and BCL2 (C) genes in follicular and transformed follicular lymphomas analyzed using DNA microarray data as in Fig. 4. *: $p < 0.05$; **: $p < 0.01$.

as it causes formation of DNA crosslinks and double strand breaks [35] that need to be repaired by homologous recombination [36]. R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) is an effective and commonly used ICT regimen for tFL patient treatment [37]. Importantly, R-CHOP includes doxorubicin that induces DNA double strand breaks (DSB), and causes tumor cell cytotoxicity [38]. Similarly, cisplatin, a chemotherapeutic drug used for salvage therapy of tFL patients [39], was shown to activate DNA damage-induced apoptosis [40]. Given that NF-κB pathway inhibition was reported to sensitize human breast cancer cells to doxorubicin; and that c-REL is involved in this sensitization [41], inhibition of NF-κB pathway by targeting c-REL may sensitize FL or tFL cells to R-CVP and R-CHOP, respectively. In fact, inhibition of DNA damage repair pathways associated with genotoxicity induced by chemotherapeutic agents have been used as a strategy to generate synthetic lethality in cancer therapeutics [42].

High basal REL expression in a group of tFL cases with concomitant activation of genotoxicity-induced NF-κB pathway suggests that high NF-κB activity may be associated with immunochemotheraphy resistance in tFL patients. Importantly, previous reports showed that constitutive NF-κB activity in pancreatic carcinoma cells accounts for the observed chemoresistance [43]. However, further investigation will need to be performed to address the relationship between REL expression and chemore-

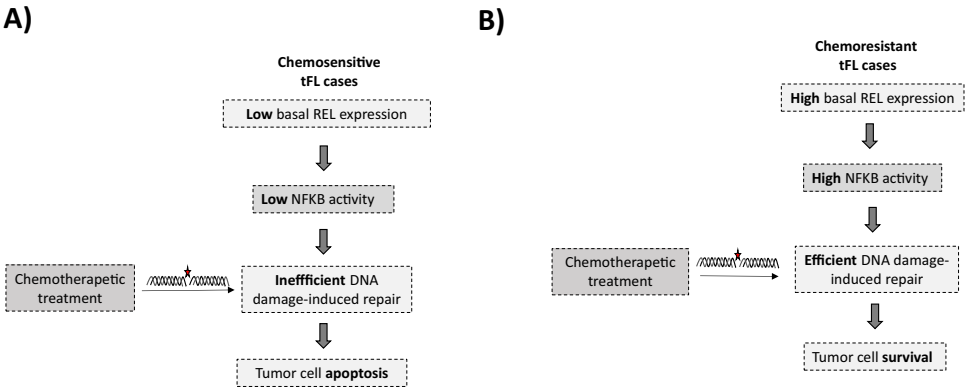

Fig. 6. A model for targeting transformed follicular lymphomas with low REL expression. Based on the proposed model, tFL tumors with low basal levels of REL expression cannot repair DSBs and undergo apoptosis. On the contrary, tFL tumors with high basal REL expression can activate DNA-damage induced repair pathway and cell cycle arrest; thereby promoting cell survival leading to chemoresistance.

sistance in the light of clinical data of tFL patients. Of note, the mutational status of P53 (i.e. WT vs. deleted or mutated) is critical when using REL as a predictive biomarker of chemosensitivity in tFL cases as P53 mutations were observed in 25–30% of tFL tumors [44]. Indeed, a recent report showed that in P53-mutated DLBCL cases, high c-REL expression was associated poor overall survival [45].

Deregulation of the NF-κB pathway was observed in many cancer types including several lymphoid neoplasms [46–49]. Of note, by forming dimers with other REL family proteins, c-REL was shown to participate in DNA damage-induced NF-κB pathway activation and subsequent cell cycle arrest that allows sufficient time for DNA repair to ensure genome stability [50]. Interestingly, *ex vivo* cultured follicular lymphoma cells stopped proliferation, upregulated NF-κB pathway, and showed resistance to doxorubicin and bendamustine when cultured as aggregates [51]. In addition to upregulation of ATM that detects double-strand DNA damage [52] or BRCA1 and RAD51 that are involved in repair of DSBs by homologous recombination [53], we also observed high BCL2 expression in REL-high tFL cases, an anti-apoptotic gene whose high expression was reported to confer chemoresistance in non-Hodgkin lymphoma cell lines [54]. Therefore, our observation that tFL tumors with high REL expression have simultaneous activation of cell cycle arrest and DNA repair pathways compared to tFL tumors with low REL expression is a critical observation, and suggests that inhibition of the NF-κB pathway in tFL tumors with high REL expression may overcome chemotherapy resistance in these patients (Fig. 6). Of note, tumor cell chemosensitization through NF-κB inhibition was shown for several cancer types using *in vitro* or *in vivo* models [55].

In conclusion, we observed activation of genotoxicity-induced NF-κB pathway that may potentially be associated with chemoresistance in tFL tumors and that may be used as a predictive biomarker of chemoresistance in these malignancies such that personalized approaches of therapy can be tailored to individual tFL patients, which aim to chemosensitize these tumors.

Competing interests

The authors declare no competing interests.

Ethics, consents and permissions

The patient samples used in this study were approved by the internal review board at the West China Hospital of Sichuan University. All patients consent to participate in the study.

Consent to publish

Not applicable.

Funding

This study has been supported by Science Academy's Young Scientist Awards Program (BAGEP) (C.K.); The Scientific and Technological Research Council of Turkey (TÜBİTAK) 2232, project 115C006 (C.K.).

Authors' contributions

X.H., E.B.: performed experiments and analyzed the data; G.H: Performed PCR experiments; J.L., B.A.: Compiled and organized patient biopsies and associated clinical data; A.S: Reviewed and edited the manuscript; provided KARPAS-422, DHL4 and 293T cell lines; B.A., K.O: Analyzed Sanger sequencing results; W.Z.: Provided all FL patient biopsies and histopathological and clinical data for FL tumor cases, edited the manuscript; C.K.: designed, conceived the project, analyzed data and wrote the manuscript.

Acknowledgements

We'd like to thank Dokuz Eylul University iBG-izmir flow cytometry and cell sorting core facility for technical assistance, and Dr. Alyssa Bouska (University of Nebraska Medical Center) for providing the overall survival data of the tFL cases.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2017.01.001.

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