DNA dependent Protein Kinase (DNA-PKcs) mediated transcriptional regulation of TOP2β drives chemoresistance in leukemia

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Introduction

Initially, 60-70% of the adult patients with Acute Myeloid Leukemia (AML) respond well to the standard chemotherapeutic regimen of cytarabine and anthracyclines. However, because of aggressive relapse in more than 80% of patients, 5-year survival of AML patients significantly decreases to approximately 1 in 4[1,2]. Although the outcome of AML therapy has improved over the years, resistance to chemotherapy remains a major challenge and is responsible for relapse. Acquired therapy resistance has been extensively studied and defined as an outcome of deregulation in multiple biological processes. We have earlier reported that the resistance mechanism acquired by an initial drug-tolerant state (or early drug-resistant state-EDRP) is different from that of a late drug-resistant state (LDRP). These highly aggressive relapse (late acquired resistant cells) evolves to acquire multiple pathways of resistance. Among them, down-regulation of topoisomerases 2beta is the major factor being anthracycline target[3].

Anthracycline's primary mechanism of action is inhibition of the topoisomerase II (TOP2) enzyme[4]. Alterations in both subtypes of topoisomerase II are reported; decreased levels of TOP2B and presence of truncated non-functional TOP2A[5,6]. The in -vitro cellular model of anthracycline developed in our lab provides us with the accessibility of resistant cells at different stages of evolution during acquired resistance[3]. We, consistent with earlier reports, observe the downregulation of TOP2B. However, how it is downregulated is still not addressed. We hypothesized that in relapse or resistant cells, the quantitative changes in TOP2B protein either through downregulation of transcription or copy number changes are driving the anthracycline resistance. Thus, the question we asked is whether anthracyclines can induce DNA damage even in the absence of its target- TOP2B and how the TOP2B is regulated in relapse.

In this study, using the in-vitro resistance cellular model and patient samples, we report that anthracyclines do not induce DNA damage in relapse cells. However, we found out that a kinase central to DNA double-strand break repair- DNA-PKcs (DNA dependent protein kinase catalytic subunit) is upregulated, and inhibition of DNA-PKcs makes relapse cells susceptible to anthracycline. Interestingly, we observed that inhibition of DNA-PKcs induces the expression of TOP2B, rendering the susceptibility to the drug. Taken together, we identify a non-canonical role of DNA-

PKcs, a protein central to the DNA damage response in mediating acquired resistance to anthracyclines in AML by acting as a transcriptional repressor of anthracycline target -TOP2B. We also demonstrate DNA-PKcs as a novel therapeutic target for refractory relapse cells.

Material and Methods

Cells lines and cell culture

HL-60, THP-1, and K562 cell lines were obtained from NCCS Pune, HL-60/MX2 cell line was obtained from ATCC (CRL 2257™). STR authenticated cells were maintained in RPMI with 10% FBS and antibiotics.

Immunofluorescence/colocalization

Cells fixed with chilled methanol were spread on coverslips and followed by permeabilization by 0.25% TritonX-100. Cells were then blocked by 1% horse serum in PBS. After overnight primary antibody incubation at 4°C and PBS washes, cells were incubated with secondary antibody either with Alexa Fluor 488 or AF633. Cells were washed with PBS again and then mounted with Vectashield mounting medium containing 0.01ug/ul DAPI. Imaging was done using Zeiss LSM 780 Confocal Microscope, and its quantification was done using ImageJ software.

Ethical Approval

Bone marrow biopsy FFPE blocks were acquired from the Pathology Department-Tata Memorial Hospital retrospectively after the approval by the institutional ethics committee (TMC-IEC III). Written informed consent was waived by the committee for retrospective FFPE blocks collections.

MTT assay and Trypan blue staining

$1X10^4$ cells were treated with doxorubicin (Pfizer Laboratories), mitoxantrone (Sun pharmaceuticals), NU7026 (Sigma), and Butyrolactone 3 (ab141255) at different concentrations. Treated cells were counted after trypan blue staining, or MTT assay was performed using the manufacturer's protocol.

Cell Cycle Analysis and Drug uptake

Doxorubicin treated cells were harvested at 5min, 12hr, and 24hr treatment points. These cells were then stained with propidium iodide, and acquisition for PI staining was done by flow cytometer (BD FACS Calibur™). Similarly, cells were analyzed for drug uptake at 480/580 nm.

Immunoblotting

Cell lysates prepared in SDS-laemmli lysis buffer were loaded and resolved on SDS-PAGE and transferred to nitrocellulose membrane. These membranes were probed with different primary antibodies. Blots were developed using ECL reagent (BioRad) and BioRad Chemidoc machine.

RT qPCR

From the cell pellet resuspended in TRI Reagent (Thermo Fisher), RNA was isolated using the choloroform-isopropanol method. Quantified and 1ug RNA was used for cDNA preparation using Superscript III First-Strand kit (Takara). In Roche LifeCycler480 qPCR machine, quantitative real-time PCR was performed. GAPDH or RPL19 was used as the internal control.

Neutral comet assay

The protocol was followed exactly as published in Salunkhe et al. earlier from our lab[3]. Briefly, 1×10^4 cells resuspended in PBS were diluted in warm LMPA at 42°C such that 25ul has approx. 100 cells. The volume of 25ul was spread on 1%agarose-coated slides and incubated with lysis buffer at 4°C. It was immediately followed by electrophoresis for 10-15min at a constant voltage of 15V. Slides were then dehydrated and stained with PI. Images were taken in a fluorescence upright microscope and quantified using Open Comet software.

siRNA knockdown of PRKDC and TOP2B

$0.2X10^6$ cells/ml were seeded per well in a 6-well plate and transfected with siRNA at 80 pmols and 50 pmols (for PRKDC and TOP2B, respectively) per well concentration using the standard 6-well protocol for Lipfectamine3000 reagent. Cells were collected 72 hr post-transfection and tested for knockdown of respective proteins by qPCR and western blotting. Knockdown cells were seeded at treated with Mitoxantrone for drug toxicity assay using trypan blue staining for 48hrs.

Immunohistochemistry

FFPE blocks of bone marrow biopsies were obtained after ethics committee approval. 4µM thickness sections were fixed on poly-l-lysine-coated glass slides and rehydrated in xylene followed by 10 min incubation in 100%, 90%, and 70% ethanol. After tap water washing, for antigen retrieval, slides in Tris-EDTA buffer (pH = 9) were kept in the pressure cooker for 15 min. Slides were then washed using TBS-Tween buffer and then blocked with 1% horse serum in PBS for 30 min at room temperature. Sections were incubated overnight with anti-DNA-PK, anti-TOP2B, and anti-GCN5 antibodies at 1:100 dilution for each. After overnight primary antibody incubation at 4°C and PBS washes, cells were incubated with secondary antibody either with Alexa Fluor 488 or AF633. Cells were washed with PBS again and then mounted with Vectashield mounting medium containing 0.01ug/ul DAPI. Imaging was done using Zeiss LSM 780 Confocal Microscope, and its quantification was done using ImageJ software.

Chromatin Immunoprecipitation and qPCR

Chromatin from the HL60 and HL-60/MX2 cells was isolated and sonicated to get the band size ranging from 300-1500bp. Chromatin immunoprecipitation will be done using Active Motif's Hi-ChIP- IT Kit. The protocol was followed as per the instructions

of the manufacturer. Briefly, 70-80% confluent cells were fixed using 1% formaldehyde and then lysed using the buffers provided in the kit and was then sonicated using Biorupter to get sheared chromatin of 300-1500bp size. Sheared chromatin was used for immunoprecipitation by anti-DNA-PKcs and anti-GCN5. Immunoprecipitated chromatin from both sets was reverse cross-linked and purified to recover DNA for qPCR quantification using primers (+100 to -1300bp to TSS) of TOP2B and PRKDC, respectively.

Cloning of 5'- serially deleted TOP2B fragments and its cloning into pGL3-Basic vector

To generate 5'-serially deleted TOP2B promoter fragments, HL60/MX2 genomic DNA was used as a template, and primers specific for sequence +100 to -1045 bp to TOP2B TSS and containing Kpn1 and XhoI sites were amplified. This was then ligated into Kpn1, and XhoI digested pGL3-Basic vector. The cloned fragments were then confirmed by insert specific PCR and sequencing. The first vector having region +100 to -1045 was named as F1 (1145bp), and this was used as the template for generating F2 (1036bp) constructs which were then used similarly for F3(547bp), then F4(351bp) constructs.

Luciferase promoter assay

Transient transfections of TOP2B 5'-serially deleted promoter constructs were carried out in 293FT cells. The cells were seed in a 96-well plate for each treatment group in technical triplicate at a density of 5000 cells per well. Along with co-transfection of 200ng promoter constructs and 100ng of pRL-TK control vector, pGL3 basic vector (Negative control), pRL-TK (Positive control), and tdRED-empty vector (transfection control) were also transfected using Lipofectamine3000 transfection reagent. 30µM NU7026 and DMSO as vehicle control were added 24hr post-transfection, for 48 hr. This is followed by a dual-luciferase assay as per the manufacture's instruction (Promega, USA).

Copy number analysis using qPCR

Primers specific for exon-intron junctions were used to amplify the seven random such junctions encompassing whole gene length. Genomic DNA was used as the template for SYBR-green based qPCR of the exon-intron junction. Relative quantification using GAPDH as an internal control was done. $\Delta\Delta$Ct values were multiplied by 2 to get copy number; values >4 were called gain in copy and values <1.5 were called loss in copy[7].

Differential Gene analysis

Analysis methodology for differentially expressed genes was followed as described in Salunkhe et.al. JCS 2021[8]. Briefly, the raw reads were aligned to the reference human genome HG19 using the TopHat alignment tool, and the differential genes were identified using the Cufflinks package. Significant differentially expressed genes

were identified based on their log fold changes (≥1.5 for upregulated genes and ≤-1.5 for downregulated genes. The p-value and FDR q-value cut-off of 0.05 was used for assessment.

Result

1. **DNA dependent protein kinase over-expression in resistant/relapse cells**

The anthracycline resistant cells THP-1 LDRP and HL-60/MX2 cells, both developed from THP-1 and HL-60 sensitive parent cells, do not incur DNA damage[3]. The downregulation of anthracycline target-TOP2B in HL-60/MX2 cells[5] and THP1-LDRP cells is thought to be one of the resistance mechanisms. To investigate whether other resistance mechanisms are also responsible, we looked for differences in DNA damage repair (DDR) proteins. There was no significant difference in expressions of most of the key proteins involved in DDR. However, interestingly we found that DNA-PKcs was upregulated in resistant cells at transcript and protein level independent of DNA damage.

Furthermore, we performed transcriptome data analysis of the whole transcriptome from the study done by Li S et al. (dbGaP accession phs001027.v2.p1)[9] and by Chiu YC et al. Eur J haemtolo.2019[10]. Both databases had whole transcriptome data of 19 paired patients (Diagnosis and Relapse) and 18 patients who responded to therapy (CR) compared to 18 those who did not respond (non-CR), respectively. DNA-PKcs was significantly overexpressed in both datasets. We also observed DNA-PKcs overexpression in bone marrow biopsies of AML patients accrued at our Centre.

2. **DNA-PKcs inhibition makes resistant cells susceptible to anthracyclines**

We investigated the effect of DNA-PKcs inhibition in resistant cells. In agreement with earlier report[11], we also found that treatment with DNA-PK kinase inhibitor (NU7026) makes resistant cells susceptible to anthracyclines and undergo DNA damage to the extent of cell death. It also decreases the tumorigenicity of HL-60/MX2 cells in mice. Although the inhibitor activity is concentration-specific, we validated non-specificity by knocking down DNA-PK by siRNA mechanism. We observe a similar effect upon knockdown. We further wanted to understand the mechanism by which cells are now susceptible as their target-TOP2B is already absent. Thus, we checked for other possible mechanisms of action of anthracyclines, such as increased production of reactive oxygen species (ROS). We hypothesized that there is an increased ROS accumulation to the level that it might be toxic to the cells in the absence of DNA-PK activity. However, we do not see any increase in ROS levels. To our surprise, we observed an increase in TOP2B expression upon either DNA-PKcs activity inhibition or its expression. Moreover, upon further knockdown of TOP2B, we saw the rescue of resistance.

3. **DNA-PKcs mediates TOP2B expression regulation**.

Considering the inverse relation of the DNA-PKcs and TOP2B expression in resistant cell lines and relapsed AML patients. We next performed a luciferase-based promoter assay to explore whether an increase in DNA-PKcs level might be repressing the TOP2B gene transcription. We cloned a region of -1145bp to +200bp relative to

TOP2B TSS upstream to the *luc* gene in pGL3.Basic vector (F1_TOP2B). It was co-transfected with renilla vector pRL-TK in 293FT cells or glioblastoma cell line U87-MG, and these cells were treated with NU7026. We observed a significant increase (more than 1.5-fold) in promoter activity upon inhibiting DNA-PKcs kinase activity. We did ChIP-qPCR for TOP2B regulatory sequences in HL-60 and HL-6/MX2 cells, taking a cue from this result. Upon immunoprecipitation by DNA-PKcs, we analyzed TOP2B sequences (+100 to -1300bp relative to TSS) using qPCR. We observed that in HL-60/MX2 cells, significant enrichment of the region between -350 to -936. Together with promoter analysis, ChIP-qPCR data shows that DNA-PK enrichment to the region upstream of TOP2B TSS has a repressive effect. To know the specific region, the DNA-PK is binding, we serially deleted the 5` sequences from F1_TOP2B and cloned them to the pGL3-basic vector. Three different constructs were made, F2_TOP2B, F3_TOP2B, and F4_TOP2B, and co-transfected with renilla vector pRL-TK in 293FT cells. We observed that deleting the -593 to -300 region upstream to TOP2B NU7026 does not affect promoter activity, indicating that this sequence is essential for DNA-PKcs to have its repressive effect on TOP2B promoter activity.

4. GCN5 mediates DNA-PKcs expression

To investigate the mechanism of DNA-PKcs overexpression in the HL-60/MX2 cells, we looked for a common regulatory mechanism of gene expression regulation. We analyzed the copy number of DNA-PKcs in both resistant cells HL-60/MX2 and THP1-D7 cells using qPCR of randomly chosen seven exons of the PRKDC gene along with FGFR1 exon as a control. We could not find any copy number alterations in either of the genes in both resistant cells. Further, we checked for any mutations in the promoter region of the *PRKDC* gene responsible for enhanced gene transcription. We analyzed the Sanger sequencing data using Mutation Surveyor and observed G>C transversion and T>C transition at the distal promoter region in HL-60 and HL-60/MX2 cells, respectively. However, both of these mutations were annotated as non-functional. We then looked for epigenetic differences in both cells by analyzing various histone modifiers and histone modification levels. Increased expression of *KAT2A* (GCN5 protein) and increased acetylation marks in resistant cells hint that open chromatin at the *PRKDC* promoter region might be the reason for DNA-PKcs increased expression. Treatment of cells by pan-HDAC inhibitor increased the expression of DNA-PKcs, which validated our hypothesis that increased acetylation modulates *PRKDC* gene expression. Also, upon knockdown of KAT2A or its inhibition using BL3 (HAT inhibitor), we see significant downregulation of PRKDC at the transcript level. We observed that H3K27Ac marks were significantly increased in the PRKDC promoter region in the UCSC browser and ChIP atlas analysis shows KAT2A enrichment in the same region. It indicated that GCN5 might be acting as a transcription factor along with histone acetylase. We, therefore, did ChIP-qPCR of GCN5 and looked for the *PRKDC* promoter region by qPCR. It also shows increased enrichment at the same region (chr8: 47960713-47960939) of the PRKDC gene.

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Statistical Analysis

Data from three independent biological replicates were considered unless stated otherwise for the calculation of significance. Using GraphPad Prism software, the two-tailed Student's t-test or one way-ANOVA in the case of multiple groups were applied for the analysis. Significant results considered if $p < 0.05$.

Discussion

We identify a non-canonical role of DNA-PKcs, a kinase central to the DNA damage response in mediating acquired resistance to anthracyclines in AML. It acts as a transcriptional repressor of anthracycline target gene-TOP2B. We also demonstrate DNA-PKcs as a novel therapeutic target for refractory relapse cells.

High mortality in AML is due to acquired chemoresistance and relapse, emphasizing the need for better therapeutic strategies. Using the clinically relevant resistance cellular models model systems and patient samples, we demonstrate that relapse or late resistant cells do not incur DNA double-strand break by modulating DNA-PKcs expression via KAT2A mediated transcriptional activation. Pharmacological inhibition or genetic perturbation of DNA-PKcs causes re-expression of TOP2B, thereby making cells sensitive to anthracyclines. It was surprising because earlier reports suggest that anthracycline resistance is primarily due to the presence of the non-functional TOP2A subtype. Reports suggest that the presence of truncated TOP2A protein in resistant cells because of the retention of intron 19 or intron 33.[6] However, consistent with Harker et al. original report characterizing HL-60/MX2 cells, we also saw downregulation of TOP2B expression at mRNA and protein levels [5]. Also, the acquired resistance is reversible in the absence of the drug, which indicates no genetic changes responsible for the resistance. Also, extensive studies suggest DNA-PKcs overexpression to cause poor therapy response and adverse prognosis in various cancers, including AML[12–18]. Consistent with earlier reports, we also observed the overexpression of DNA-PKcs and downregulation of TOP2B. The surprising finding was the re-expression of TOP2B in the absence of DNA-PKcs or its kinase activity. We showed mechanistically how DNA-PKcs are regulating TOP2B and how DNA-PKcs itself is getting overexpressed in the resistant cells.

The absence of DNA binding domain in the DNA-PKcs protein intrigues us how it is binding to TOP2B promoter region. We show that it is interacting with other transcription factors, and along with that, it is binding to the region, thereby inhibiting gene transcription. Nevertheless, in conclusion, we provide a mechanistic explanation of the DNA-PKcs overexpression and resistance to anthracycline in the absence of TOP2B. We believe that these findings are clinically relevant, and it merits further investigations regarding DNA-PKcs interactors and the establishment of findings in perspective of *in-vivo* significance.

Impact of the research in the advancement of knowledge or benefit to mankind

Therapy resistance is an outstanding question in the field of leukemia. Of 70-80% of patients that go into remission upon chemotherapy, almost all come back with aggressive refractory relapse, thus significantly decreasing the 5-year survival of the patients to 28.3% (SEER Cancer Statistics Review 2019). The acquired resistance is responsible for AML resistance and relapse making it mandatory to target these cells to prevent relapse.

This research extends the fundamental knowledge of how resistant relapse cells survive DNA damage-based chemotherapy in AML. This is the first report to provide molecular insights into the DNA damage repair independent role of DNA-PKcs in refractory relapse cells. Furthermore, understanding the DNA-PKcs mediated resistance mechanisms in acquired resistant cells will identify novel pathways that can be targeted to induce the death of resistant relapse cells.

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