

LETTER TO THE EDITOR

NF- κ B Pathway Activation Mutations Linked to Failure of CpG-Stimulated Karyotyping in DLBCL

Xiaojun Chen^{1,2,3} | Chunxiao Yang^{1,2} | Xingping Lang^{1,2} | Huafei Chen^{1,2} | Shouli Wang³ | Bingzong Li⁴  | Sheng Xiao⁵ 

¹Advanced Molecular Pathology Institute of Soochow University and SANO, Suzhou, China | ²Suzhou Sano Precision Medicine Ltd, Suzhou, China | ³Department of Pathology and Pathophysiology, Soochow University School of Biology and Basic Medical Sciences, Suzhou, China | ⁴Department of Hematology, The Second Affiliated Hospital of Soochow University, Suzhou, China | ⁵Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, USA

Correspondence: Bingzong Li (lbzwz0907@hotmail.com) | Sheng Xiao (sxiao2025@126.com)

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Dear Editors,

Chromosomal rearrangements are common in lymphomas and frequently result in the formation of fusion genes or the deregulated, constitutive expression of oncogenes, thereby activating oncogenic signaling pathways [1]. Patients with diffuse large B cell lymphoma (DLBCL), featuring *MYC*, *BCL2*, and/or *BCL6* rearrangements, have inferior outcomes and are referred to as double-hit or triple-hit lymphomas [2]. Most rearrangements involve immunoglobulin (*IG*) loci primarily *IGH*, but also *IGL* and *IGK* [3]. These fusion events have important implications for disease classification and prognostic stratification.

New technologies have emerged to detect chromosome rearrangements using molecular-based techniques such as optical genome mapping (OGM), genomic proximity mapping (GPM), and DNA next-generation sequencing (DNA NGS). However, these DNA-based assays are not single-cell level and may fail to detect chromosome abnormalities when tumor cell percentage is low. Additionally, the *IG* loci are typically highly repetitive, further complicating their detection via molecular methods such as FISH or NGS. Given these limitations, karyotyping remains an essential technique for detecting both chromosome structural rearrangements and chromosome numerical changes [4].

However, karyotype analysis faces its own challenges when tumor cells fail to divide in culture while normal hematopoietic cells continue to proliferate, resulting in normal karyotypes that do not reflect the underlying malignancy. Current

lymphoma karyotyping protocols rely on CpG oligonucleotide stimulation to promote B-cell proliferation [5]. However, our findings indicate that approximately 40% of DLBCL cases failed karyotyping. Targeted sequencing revealed that karyotype failure was associated with mutations in *MYD88*, *CD79B*, and/or *PIM1*, leading to constitutive activation of NF- κ B signaling. Because CpG stimulation acts upstream of these molecules, tumor cells with constitutive NF- κ B activation do not respond to CpG stimulation. Furthermore, these CpG-non-responsive DLBCL cells fail to undergo active division during the prolonged in vitro culture required for CpG stimulation, leading to karyotyping failure. In such cases, successful karyotyping can be achieved using short-term culture without CpG stimulation.

We analyzed 263 patients of mature B-cell tumors diagnosed according to WHO criteria, and using both 24-h unstimulated and 72-h CpG-stimulated cultures for karyotyping. Overall, 143 patients (54.37%) showed abnormal karyotypes. Among them, abnormalities were detected in 102 patients using CpG-stimulated cultures only (71.33%), 7 patients using unstimulated cultures (4.90%) only, and 34 patients with abnormalities in both conditions (23.78%) (Table S1). In 217 non-DLBCL lymphoma patients, including Burkitt lymphoma (BL), chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), follicular lymphoma (FL), lymphoplasmacytic lymphoma/Waldenström macroglobulinemia (LPL/WM), marginal zone lymphoma (MZL), and unclassified lymphomas, all karyotype abnormalities ($n=106$) were found in CpG-stimulated or both culture types, with none detected using unstimulated cultures alone.

TABLE 1 | Chromosome abnormalities detected solely by unstimulated cultures but not in CpG-stimulated cultures (CpG oligonucleotides CpG-ODN, pokeweed mitogen (PWM), and phorbol 12-myristate 13-acetate (PMA)) in DLBCL.

Case	Unstimulated	CpG + PWM + PMA
1	47,XY,add(1)(p36),+3,t(3;14)(q27;q32),add(9)(p23),del(21)(q22)[1] ^a /46,XY[9]	46,XY[20]
2 ^b	47,Y,der(X)t(X;11)(p22;q13),+add(1)(p13),del(6)(q21q23),+der(6;13)(p10;q10),+i(7)(p10),-8,der(11)add(11)(p13)add(11)(q21),del(16)(q21),der(17)t(8;17)(q11.2;p13),der(19)t(11;19)(q12;q13)hsr(11)(q13)[cp2]/46,XY[18]	46,XY[20]
3 ^b	47,Y,der(X)t(X;11)(p22;q13),+add(1)(p13),del(6)(q21q23),+der(6;13)(p10;q10),+i(7)(p10),-8,der(11)add(11)(p13)add(11)(q21),del(16)(q21),der(17)t(8;17)(q11.2;p13),der(19)t(11;19)(q12;q13)hsr(11)(q13)[cp3]/46,XY[7]	46,XY[20]
4	51,XY,+3,del(3)(p13p21),del(3)(q21q23),del(4)(q27q31),+18,+18,add(22)(p11.2),+2mar[cp4]/52,idem,+11[cp4]/46,XY[2]	46,XY[20]
5	47,XY,add(1)(q32),+del(1)(p12),-8,t(9;22)(p24;q11.2),add(13)(q32),add(15)(p13),+21[5]/46,XY[15]	46,XY[20]
6	84-86 < 4n>,XXYY,+Y,add(1)(p36),i(1)(q10),-2,der(3)t(3;3)(p21;q12),-4,-6,i(6)(p10),-8,-9,-12,-14,-19,-21,-22,-22,+2-4mar[cp5]/46,XY[5]	No metaphase
7	47-48,X,-Y[9],t(2;13;15)(q35;q32;q22),+16,add(18)(p11.3),+mar[cp15]/46,XY[5]	46,XY[20]

^aFISH confirmed the presence of *IGH::BCL6* gene rearrangement and increased BCL6 copy number (+3).

^bCases #2 and #3 were 2 samples from the same patient, collected 5 months apart.

In patients with abnormalities detected in both culture conditions ($n = 17$), CpG-stimulated cultures consistently showed a higher proportion of abnormal metaphases compared with unstimulated cultures ($p < 0.001$). These results align with previous findings and confirm that these tumors should be cultured under CpG-stimulation conditions [6].

Among 46 DLBCL samples, 37 had abnormal karyotypes. Within this group, 13 were abnormal in CpG-stimulated cultures (35.14%), 7 in unstimulated cultures (18.92%), and 17 in both (45.95%). Among the 17 cases with abnormalities detected in both conditions, unlike non-DLBCL, the proportion of abnormal metaphases showed no significant difference in DLBCL ($p = 0.843$), suggesting biological heterogeneity within DLBCL. Further analysis of these 17 cases, based on the relative proportion of abnormal metaphases under the two culture conditions, revealed two distinct patterns: approximately half (9/17, 52.94%) showed a significantly higher proportion of abnormal metaphase cells in CpG-stimulated cultures (CpG-responsive, $p = 0.007$), while the remaining cases (8/17, 47.06%) showed the opposite trend, with a higher proportion in unstimulated cultures (CpG-unresponsive, $p < 0.001$). Overall, 15 of 37 samples (40.54%) showed no additional benefit from CpG stimulation (CpG-unresponsive group), including 7 cases detected only in unstimulated cultures and 8 cases with higher proportions of abnormal metaphases in unstimulated cultures. The remaining cases were therefore classified as CpG-responsive. Representative metaphase spreads are shown in Figure 1A,B.

To investigate why a subset of DLBCL samples failed to respond to CpG stimulation, we hypothesized the role of underlying mutations affecting NF- κ B signaling. CpG oligonucleotides normally enhance B-cell proliferation by binding to TLR9 and triggering NF- κ B signaling [7]. We performed targeted DNA NGS testing on 36 of 37 DLBCL samples that had abnormal karyotypes and found mutations in 29 of them. Notably, alterations in *MYD88*, *PIM1*, and *CD79B*-key regulators of

NF- κ B-were significantly more common in cases unresponsive to CpG stimulation. Specifically, *MYD88*, *PIM1*, and *CD79B* mutations were present in 10, 7, and 5 DLBCL patients who were unresponsive to CpG, compared to only 3, 0, and 0 patients that were responsive to CpG ($p = 0.009$, 0.002 , and 0.017). Co-occurring mutations in *MYD88* and *PIM1/CD79B* were identified in 7 of the 14 CpG-unresponsive DLBCL patients, while none of the 15 CpG-responsive patients showed this pattern ($p = 0.002$) (Figure 1C). Interestingly, *MYD88* mutations were also present in 8 of 9 LPL/WM samples. However, all abnormal karyotypes in these patients were obtained from CpG-stimulated cultures rather than unstimulated cultures. Unlike in DLBCL, *PIM1* and *CD79B* mutations were absent in LPL/WM cases (Figure 1C). This suggests that the *MYD88* mutations alone may induce partial NF- κ B activation, allowing further response to CpG stimulation.

Mechanistically, mutations in *MYD88*, *CD79B*, and *PIM1* activate NF- κ B signaling through distinct pathways (Figure 1D). *MYD88* L265P mutation, located within the Toll/Interleukin-1 receptor (TIR) domain, leads to the constitutive activation of MYD88 that is independent of ligand binding to TLRs or interleukin-1 receptors (IL-1Rs). This activation recruits Interleukin-1 receptor-associated kinase (IRAK) and TNF receptor-associated factor 6 (TRAF6). TRAF6 undergoes ubiquitination, activating TGF- β -activated kinase 1 (TAK1) and the I κ B kinase (IKK) complex (IKK $\alpha/\beta/\gamma$). Phosphorylation of I κ B α by the IKK complex promotes its degradation, thereby releasing NF- κ B dimers to enter the nucleus and drive transcription of genes involved in inflammation, cell survival, and proliferation [8].

CD79B, a complex component of B-cell receptor (BCR), is essential for initiating signaling upon antigen binding. Mutations in *CD79B* cause ligand-independent BCR activation, triggering a cascade through Syk, Bruton's Tyrosine Kinase (BTK), and phospholipase C γ 2 (PLC γ 2). This cascade leads to inositol trisphosphate (IP3) and diacylglycerol (DAG)

production, calcium mobilization, and activation of protein kinase C (PKC). This in turn activates the NEMO complex and the NF- κ B pathway [9].

PIM1 contributes to NF- κ B activation by promoting I κ B α phosphorylation. Mutant PIM1 enhances I κ B α degradation, thereby releasing NF- κ B dimers, allowing them to enter the nucleus and activate gene transcription [10].

Compared to LPL/WM, which typically harbors only the MYD88 L265P mutation, DLBCL often carries multiple mutations affecting NF- κ B signaling, including those in MYD88, CD79B, and PIM1. Acting through distinct mechanisms, these mutations collectively drive more robust and sustained NF- κ B signaling [11]. Consequently, LPL/WM cells may still respond to CpG stimulation, while DLBCL cells with constitutive NF- κ B activation often fail to respond. In these cases, extended culture (e.g., 3 days) with CpG stimulation may result in reduced cell division. However, a shorter overnight culture can effectively capture the typically abundant endogenous dividing cells, facilitating successful karyotyping.

We found that patients in the CpG-unresponsive group exhibited increased genomic instability, characterized by highly complex karyotypes (≥ 5 chromosomal aberrations). The average number of chromosomal abnormalities was significantly higher in the unresponsive group compared to the responsive group (14 vs. 8, $p=0.007$). Such extreme karyotypic complexity has been associated with aggressive disease behavior and inferior clinical outcomes in DLBCL [12]. These findings suggest that a lack of response to CpG stimulation may be associated with high-risk genetic features and could potentially contribute to risk stratification.

In conclusion, while CpG stimulation is a standard protocol for karyotyping mature B-cell lymphomas, our findings suggest that approximately 40% of DLBCL cases, often harboring multiple NF- κ B pathway-related gene mutations, may benefit from short-term, unstimulated cultures. Furthermore, a lack of response to CpG stimulation may also be associated with increased genomic instability and potentially poorer prognosis. Given the variability in mutation profiles at the start of culture, we recommend performing both unstimulated and CpG-stimulated cultures simultaneously to capture chromosomal abnormalities in all DLBCL cases.

Author Contributions

S.X. and B.L. designed and directed the study; X.C. performed laboratory work and wrote the manuscript. C.Y., X.L., H.C., S.W., and B.L. performed laboratory work. All authors critically reviewed and approved the final version of the manuscript.

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The authors have nothing to report.

Ethics Statement

This study was approved by the Medical Ethics Committee of the Second Affiliated Hospital of Soochow University (Approval No. JD-LK-2017-024-01).

Consent

Written informed consent was obtained from all participants included in the study. All methods were performed in accordance with the relevant guidelines and regulations.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

Xiaojun Chen
Chunxiao Yang
Xingping Lang
Huafei Chen
Shouli Wang
Bingzong Li
Sheng Xiao

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Detection rates of abnormal karyotypes in mature B-Cell neoplasms under different culture conditions.