Role of Rac1-Pak pathway in aggressive b-cell lymphoma

Tian Tian
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Role of Rac1-PAK Pathway in Aggressive B-cell Lymphomas

By

Tian Tian

A DISSERTATION

Presented to the Faculty of

The University of Nebraska Graduate College

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

Pathology & Microbiology Graduate program

Under the Supervision of Professor Kai Fu

University of Nebraska Medical Center,

Omaha, Nebraska

Dec, 2018

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Role of Rac1-PAK pathway in Aggressive B-cell Lymphomas

Tian Tian

University of Nebraska Medical Center, 2018

Advisor: Kai Fu, M.D. Ph.D.

Aggressive B-cell lymphomas are diverse group of neoplasms that arise at different stages of B-cell development and by various mechanisms of neoplastic transformation. Aggressive B-cell lymphomas include many types, subtypes and variants of diffuse large B-cell lymphoma (DLBCL), Burkitt lymphoma (BL), mantle cell lymphoma (MCL) and B lymphoblastic lymphoma. The treatment of patients with aggressive B-cell lymphomas remains a clinical challenge. Conventional chemotherapeutic regimens, mainly based on the CHOP (cyclophosphamide, hydroxydaunorubicin, oncovin and prednisone) combination, obtain a relatively high number of complete or partial clinical responses (20-80%), but the tumor relapses in most patients, who will die of the disease. Addition of rituximab, a chimeric monoclonal antibody directed against CD20, to this combination increases the overall survival, and the response rate and duration. However, different clinical evolution suggests that not all patients need the same therapy.

Rac1, a small guanosine triphosphatase (GTPase), has been found to be dysregulated in many human malignancies, including solid tumors and chronic myeloid leukemia. However, its status in MCL remains unknown. Here, we found Rac1 is overexpressed at both mRNA and protein levels in MCL cell lines and cases, and over-expression is correlated with poor prognosis. Functional study showed that inhibiting Rac1 by shRNA or specific inhibitor NSC 23766 significantly inhibited cell viability and enhanced chemotherapy drug Adriamycin-induced cytotoxic effect in MCL cells. Mechanistic studies
showed diminished activity of several oncogenic pathways (AKT/mTOR, NF-κB and ERK) upon Rac1 inhibition. However, as a GTPase, Rac1 is hard to target. Group I P21-associated kinases (PAKs) are well known downstream of Rac1. Here, we demonstrated overexpressed and highly activated Pak2 signaling in MCL, and in DLBCL and BL as well. Inhibition of Pak2, not Pak1, by siRNA or inhibitor FRAX 597 significantly inhibited lymphoma cells viability, through diminishing activity of AKT/mTOR and NF-κB pathways, which furtherly confirmed the pro-survival role of Rac1-Pak2 axis in lymphoma situation. Synergistic effect between FRAX 597 and Adriamycin or ibrutinib was shown in MCL. Moreover, FRAX 597 could overcome the ibrutinib resistant in MCL and ABC-DLBCL. Collectively, studies suggest targeting Rac1-Pak2 as a promising strategy in clinical treatment of aggressive B-cell lymphomas.
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<td>ABPP</td>
<td>Activity-based proteomics</td>
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<td>AID</td>
<td>Autoinhibitory domain</td>
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<td>AKT</td>
<td>Protein kinase B</td>
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<td>APC</td>
<td>Antigen presenting cells</td>
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<td>ARHGEF1</td>
<td>RHO guanine nucleotide exchange factor 1</td>
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<td>ATM</td>
<td>Ataxia-telangiectasia mutated</td>
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<td>BAD</td>
<td>BCL2 antagonist of cell death</td>
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<td>BAM32</td>
<td>B lymphocyte adaptor molecule of 32kDa</td>
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<td>BCL2</td>
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<td>CDK</td>
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<td>CHOP</td>
<td>Cyclophosphamide, hydroxydaunorubicin, oncovin and prednisone</td>
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<td>Combination index</td>
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<td>CLPs</td>
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<td>DLBCL</td>
<td>Diffuse large B-cell lymphoma</td>
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<td>Dox</td>
<td>Doxycycline</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
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<td>EFS</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<td>EZH2</td>
<td>Enhancer of zeste homolog 2</td>
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<td>FDC</td>
<td>Follicular dendritic cells</td>
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<td>FLIP</td>
<td>FADD-like apoptosis regulator</td>
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<td>GAPs</td>
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<td>Germinal centers</td>
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<td>Germinal center B-cell-like</td>
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<td>GEFs</td>
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<td>Glutathione-S-transferase</td>
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<td>GTPases</td>
<td>Guanosine triphosphatase</td>
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<td>HGBL</td>
<td>High-grade B-cell lymphoma</td>
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<td>HSCs</td>
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<td>ID3</td>
<td>Inhibitor of DNA Binding 3</td>
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<td>IgD</td>
<td>Immunoglobulin D</td>
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<tr>
<td>IgH</td>
<td>Immunoglobulin heavy</td>
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<td>IGHV</td>
<td>IgH Variable-region</td>
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<td>IgL</td>
<td>Immunoglobulin light</td>
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<td>IL-7R</td>
<td>Interleukin-7 receptor</td>
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<td>IRAK1</td>
<td>Interleukin 1 receptor-associated kinase</td>
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<tr>
<td>IRF4</td>
<td>Interferon-regulatory factor 4</td>
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<td>ISMCN</td>
<td>In situ mantle cell neoplasia</td>
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<td>ITAMs</td>
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<td>JAK-STAT3</td>
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<td>JNK</td>
<td>c-JUN NH2-terminal kinase</td>
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<td>LinB220KIT^*SCA^*FLT3^<em>CD34^</em></td>
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<td>LLMPP</td>
<td>Lymphoma/leukemia Molecular profiling project</td>
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<td>LMPPs</td>
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<td>MALT</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MCL</td>
<td>Mantle cell lymphoma</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<td>MYD88</td>
<td>Myeloid differentiation primary response protein 88</td>
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<td>MZ</td>
<td>Marginal-zone</td>
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<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
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<td>NHL</td>
<td>Non-Hodgkin's lymphomas</td>
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<td>OS</td>
<td>Overall survival</td>
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<td>PAK</td>
<td>p21-activated kinases</td>
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<td>Paired box 5</td>
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<td>PFS</td>
<td>Progression-free survival</td>
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<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<td>Description</td>
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<td>PKC</td>
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<td>Phosphatidylinositol-3,4,5-triphosphate</td>
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<td>Protein tyrosine kinase</td>
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<td>Ribosomal protein S6</td>
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<td>S1PR2</td>
<td>Sphingosine-1-phosphate receptor 2</td>
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<td>SHM</td>
<td>Somatic hypermutation</td>
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<td>sIgM</td>
<td>Surface immunoglobulin M</td>
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<td>SOX11</td>
<td>Sex determining region Y-box 11</td>
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<td>Description</td>
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<tr>
<td>SYK</td>
<td>Spleen tyrosine kinase</td>
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<tr>
<td>TCF3</td>
<td>Transcription factor 3</td>
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<tr>
<td>TMAs</td>
<td>Tissue micro-arrays</td>
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<tr>
<td>TME</td>
<td>Extrinsic tumor microenvironment</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>XBP1</td>
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Acknowledgements

First and foremost, I would like to express my sincere gratitude to my advisor, Dr. Kai Fu, for his continued support and guidance throughout graduate school. He provided me with a wonderful research environment where I can work on a topic that was of great interest to me. He taught me to think critically about each experiment. His hard working inspires me to be a better researcher.

I would also like to thank my committee members, Dr. Javeed Iqbal, Dr. John Davis, Dr. Timothy Greiner and Dr. Ying Yan for their valuable time, precious suggestions and kind advices regarding the topic of my research. I am especially grateful to Dr, Yan for her mentorship, support and trust in my abilities. She is not only a great supervisor, but also a wonderful mentor in life. She shared so many ideas about science, culture differences and choices with me. She taught me far beyond the lab.

As a Fu lab member, I have been lucky to work with so many intelligent researches. I would like to thank all current and previous members of the Fu lab for being great colleagues and being helpful in various ways. In particular, I am grateful for the help I received from Dr. Ting Lu, Dr. Cuijuan Zhang, Dr. Yuhua Huang and Dr. Lifang Huang. I would like to reserve special thanks for Lab instructor, Dr. Chengfeng Bi, who gave so much instructions and help on my projects. Without his help, I could not make it. In addition, I would like to thank my fellow graduate students, Dr. Xuan Zhang and Dr. Cheng Wang for their companion and help during these years.

I would like to thank my wonderful friends, Alice Wu, Cheng Wang and Yangyuna Yang, who provided me with countless experience outside of laboratory that made my life during graduate study valuable and entertaining. They are so supportive during the most difficult periods of time in graduate school. They will forever be my closest friends.
I would like to thank Tuire Cechin from PAMM graduate program and Lenal Bottoms and Hannah Tong from APRDP program for their excellent administrative supports. I would also like to thank Dr. Rakesh Singh, director of our PAMM program, for his tremendous support.

I would also like to acknowledge the funding I received from the Chinese Scholarship Council for providing financial support for the first four years of my graduate study.

Last but not the least, I would like to thank my parent, Shihua Tian and Qiong Yang, for being always supportive for my decisions. Thank you very much for all the unconditional love, tremendous support, and understanding.
Chapter 1 Introduction
B cells development, maturation and differentiation

B cells are generated from hematopoietic stem cells (HSCs) and develop in the bone marrow before they migrate into the blood to reach peripheral lymphoid organs. Of note, end-stage B cells (plasma cells), which develop following the activation of mature B cells by antigen in peripheral lymphoid organs, are crucial for mediating humoral immune responses, return and colonize the bone marrow.

The development of B cells happens in an ordered series of stages by the support of specific bone marrow environment. In developmental pathway from HSC to early B-cell precursors, there are several precursor-cell subsets. HSC, highly purified as lineage (Lin)B220KITSCA1fms-related tyrosine kinase 3(FLT3)CD34 cells, can give rise to all blood-cell lineages. Cells that are LinB220KITSCAFLT3CD34 have been shown to lack erythroid-cell and megakaryocyte potential but retain myeloid-cell and lymphoid-cell potential, these are termed lymphoid-primed multipotent progenitors (LMPPs). LMPPs might represent a first step towards commitment to the lymphoid-cell lineage. Arising from LMPPs is a cell population known as common lymphoid progenitors (CLPs), which are LinKITlowSCA1low interleukin-7 receptor (IL-7R) or LinAA4.1SCA1lowIL-7R cells and can generate B- and T-lineage cells but not myeloid-lineage cells. The existence of these multipotent intermediates raises the possibility that their differentiation into B cells is induced by the components of the specific niches.

B-cell precursors, consisting of cells that are negative for cell-surface immunoglobulin but positive for the B-cell-lineage marker B220, were divided into four subsets according to their differential expression of a range of cell-surface markers during development in the bone marrow. These four subsets were termed fractions A, B, C and D. Because fraction A cells have been shown to progress to fraction B or C phenotypes in in vitro stromal-cell culture, fraction A cells have been suggested to be the earliest identifiable
B-cell precursors and subsequently been termed pre-pro-B cells. B cells undergo immunoglobulin-gene rearrangement during their differentiation; B-cell precursors first assemble a heavy-chain DJ rearrangement, which is then followed by heavy-chain variable (V)DJ rearrangements\(^9,10\). Because most pre-pro-B cells lack heavy-chain DJ rearrangements, development of the early stages of B-cell precursors is likely to be independent of immunoglobulin-gene rearrangement. Fractions B and C consist mainly of large mitotically active cells, which have heavy-chain DJ or VDJ rearrangements and are termed pro-B cells\(^11\). Fraction D consists mainly of small resting cells, termed pre-B cells. Immature B cells, which express cell-surface IgM but not IgD, are generated from the cells in fraction D and then exit the bone marrow and reach the peripheral lymphoid organ, where they mature into peripheral mature B cells.

B cells that have successfully undergone V(D)J recombination that express functional B cell receptors (BCR) migrate as naïve B cells from bone marrow to the secondary lymphoid organs, including the spleen, lymph nodes, Peyer’s patches and tonsils\(^12,13\). Here, germinal centers (GC) are developed. Naïve B cells first migrate to the T-cell zone of lymphoid tissue, in where B cells become fully activated by their interaction with CD4\(^+\) T cells and antigen presenting cells (APC). The most important and well-studied costimulatory receptor-ligand interaction is that between the tumor necrosis factor (TNF)-receptor family member CD40, which is expressed by all B cells, and its ligand CD154 expressed by CD4\(^+\) T cells\(^14\). Activated B cells can then either develop directly into antibody-secreting cells in specialized extrafollicular sites, such as the medullary cords of lymph nodes, or mature into GC-precursor B cells and move to the primary follicle, which is made of recirculating IgM\(^+\)IgD\(^+\) B cells within a network of follicular dendritic cells (FDCs)\(^15\). Here, B cells start to proliferate rapidly and push the IgM\(^+\)IgD\(^+\) B cells aside to form the mantle zone around the GC, which yielded the secondary follicle. The charac-
teristic structure of the GC, a dark zone and a light zone, becomes apparent after a few days of vigorous proliferation. Histologically, dark zone consists of almost exclusively of densely proliferating B cells known as centroblasts, whereas in the light zone, the B cells, known as centrocytes, are intermingled with FDCs, T cells and macrophages. Functionally, the dark zone is the site of B cell division and somatic hypermutation (SHM), whereas the light zone is where B cells undergo activation and selection based on the affinity of their BCR. Cycling of centroblasts and centrocytes between dark and light zones seems to be mediated by signals from the BCR and other GC resident cells\textsuperscript{16-18}. These signals involve signal transduction molecules and nuclear transcriptional effectors that are often structurally and functionally altered during lymphomagenesis\textsuperscript{17}. Antigen-selected centrocytes eventually differentiated into memory B cells or plasma cells.

Inducing GC B cells to differentiate into plasma cells or memory cells requires various signals. The first event is the functional inactivation of the transcriptional repressor paired box 5 (PAX5), which is essential for maintain the identity of mature B cell, including naïve, GC and memory B cells. A second event that is required for post-GC differentiation is the downregulation of B-cell lymphoma 6 (BCL6), which is thought to terminate the GC transcriptional programme and allow for GC B cells to differentiate into memory B cells or plasma cells. Moreover, the expression of B lymphocyte-induced maturation protein 1 (BLIMP1), interferon-regulatory factor 4 (IRF4) and X-box-binding protein 1 (XBP1) are all jointly required for this differentiation process\textsuperscript{19}. All those pathways must be orderly regulated to maintain the normal immune response.

**BCR structure and signaling**

For effective humoral immunity, mature B cells must respond to foreign antigens and generate antigen-specific effector cells. This latter stages of B cell maturation requires the BCR complex. The BCR is required also during early B-cell ontogeny for
maintenance. In other words, the BCR has two main roles. The first is to transmit signals that regulate B-cell fate decisions. The second is to mediate antigen processing, leading to the presentation of antigen to T cells, which allows full activation of B cells in the effector phase. These two roles are interrelated, but are under distinct genetic control.

The BCR complex is made up of immunoglobulin heavy (IgH) and light (IgL) chains associated with two signaling components, Igα and Igβ. After BCR ligation by antigen, both the protein tyrosine kinase (PTK), spleen tyrosine kinase (SYK), and Src-family PTKs such as LYN, are activated initially\textsuperscript{20}. Then LYN phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of Igα and Igβ, which, in turn, recruit and facilitate the activation of SYK and the TEC-family PTK, bruton tyrosine kinase (BTK). Phosphatidylinositol 3-kinase (PI3K) and phospholipase Cy2 (PLCy2) are important downstream effectors of BCR signaling. B cell adaptors, such as B-cell linker (BLNK) and BAM32 (B lymphocyte adaptor molecule of 32kDa), fine-tune BCR signals by efficiently connecting the above kinases with the effectors. PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PtdInsP2) to produce phosphatidylinositol-3,4,5-triphosphate (PtdInsP30), which, in turn, recruits some BCR signaling components and also activates downstream kinase such as protein kinase B (AKT). Activation of PLCy2 induce the release of intracellular calcium and activation of protein kinase C (PKC), both of which are crucial for the activation of mitogen-activated protein kinase (MAPK)-family kinases, such as extracellular signal-regulated kinase (ERK), c-JUN NH2-terminal kinase (JNK) and p38 MAPK, and transcription factors, including nuclear factor-κB (NF-κB) and nuclear factor of activated T cells (NFAT)\textsuperscript{21,22}.

The BCR induced signaling pathways are crucial for the survival and proliferation of B cells. These include the PI3K, RAS-RAF-ERK and NF-κB pathway. Firstly, PI3K-AKT pathway is a primary candidate for mediating B-cell survival in the base of treat-
ment of B cells with inhibitors of PI3K leading to an increase in BCR-induced cell death. AKT functions to protect against BCR-induced cell death and to promote B-cell survival in the resting state. The specific mechanism is by inhibiting activation of the pro-apoptotic B-cell lymphoma 2 (BCL2)-family member BAD (BCL2 antagonist of cell death) and inhibiting glycogen synthase kinase 3 (GSK3) kinase in B cells. Secondly, the activation of ERK is regulated in a PLCγ2-dependent manner, also in a PLCγ2-independent manner, most probably through the RAS-RAF pathway. Although it is clear that ERK is involved in the survival of other cell types, its role in immature B cells is unclear and needed to be clarified. Thirdly, NF-κB can protect many cells from pro-apoptotic signals. Activation of PLCγ2 is absolutely required for BCR-induced NF-κB activation in B cells. Activation NF-κB, on the one hand, could induce the expression of anti-apoptotic BCL2-family members, such as BCL2, BCL-XL (B-cell lymphoma-extra large) and A1, on the other hand, could increase the cyclin D2 expression. Normally, BCR expression at the cell surface might generate a basal “tonic” signal, which include the activation of NF-κB, which is sufficient for cell survival. This is the case for immature B cells. However, in mature B cells, basal NF-κB signaling acts as a maintenance signal for cell survival. Moreover, BCR ligation probably induces additional survival signals, such as AKT, ERK and NF-κB to override death signals.

BCR complex plays an important role in directing generating mature B cells subsets. Productive variable-region and joining-region rearrangements of the immunoglobulin light chain (V,L rearrangements) are completed in pre-B cells, and expression of the BCR drives pre-B cells to the immature B-cell stage. This process could generate potentially harmful, self-reactive B cells. An important role of BCR at this stage is to induce efficient elimination of these potentially harmful cells. Immature B cells that have passes successfully through developmental checkpoints in the bone marrow emigrate subse-
quent to the spleen as transitional B cells, which then become mature B cells. Transitional B cells can be divided further into two subsets, type 1 (T1) and T2\textsuperscript{23}. T1 cells are recent emigrants from the bone marrow, which means that their phenotype is related closely to that of immature B cells in the bone marrow. T1 cells express no or low levels of immunoglobulin D (IgD), CD21 and CD23, but high levels of surface immunoglobulin M (sIgM). Only a small percentage of T1 cells can successfully enter B-cell follicles become T2 cells. T2 cells have a mixed phenotype between that of T1 B cells and mature follicular B cells. They still express high levels of sIgM, but also express high levels of surface (s)IgD, CD21, and CD23. BCR signaling pathways are different in T1 and T2 cells. After BCR crosslinking, T2 cells express much higher level of cyclin D2 or A1 and active AKT compared with T1 cells. Studied have shown that BCR signaling is required for the T1 to T2 transition. BCR signals facilitate T2-cell generation and maintain T2-cell survival perhaps by enhancing the activation of NF-κB. Final step, transition from the T2 to mature B cells, also requires BCR signals\textsuperscript{23}. Mature B cells are classified into at least three subsets, follicular B cells, marginal-zone (MZ) B cells and peritoneal B cells\textsuperscript{24}. Studied have indicates that BCR signaling induced by self-ligands is required for the generation and maintenance of all three mature subsets. Moreover, some ongoing BCR signals from foreign ligands or antigens are required for B cells to survive.

**Aggressive B-cell lymphomas and its classification**

Aggressive B-cell lymphomas are diverse group of neoplasms that arise at different stages of B-cell development and by various mechanisms of neoplastic transformation. The aggressive B-cell lymphomas include many types, subtypes and variants of diffuse large B-cell lymphoma (DLBCL), Burkitt lymphoma (BL), mantle cell lymphoma (MCL) and its blastoid variant, and B lymphoblastic lymphoma\textsuperscript{25}.

*Mantle cell lymphoma*
MCL classically has been recognized as an aggressive but incurable small B-cell lymphoma. MCL is developed in a linear fashion from naïve B cells. MCL has two subtypes, which are recognized with different clinic-pathological manifestations and molecular pathogenic pathways. Classical MCL is composed of IgH Variable-region (IGHV)-unmutated or minimally mutated B cells that express SOX11 (sex determining region Y-box 11) and typically involves lymph nodes and other extranodal sites. Acquisition of additional molecular abnormalities can lead to even more aggressive blastoid or pleomorphic MCL. Other MCL develop from IGHV-mutated SOX11 B cells which leads to leukemic nonnodal MCL with the peripheral blood, bone marrow and spleen involvement. These cases are frequently clinically indolent, however, will become aggressive after secondary abnormalities. In situ MCL is now to be called in situ mantle cell neoplasia (ISMCN), emphasizing the low rate of progression. It is characterized by the presence of cyclin D1+ cells.

**Diffuse large B-cell lymphoma, NOS**

Based on gene expression profiling (GEP), DLBCL can be divided into two subgroups, germinal center B-cell-like (GCB) and activated B-cell-like (ABC) DLBCL. This is cell-of-origin classification. A significant advance in recent years has been the better understanding of MYC alterations in large B cell lymphomas. MYC is rearranged in 5% to 15% of DLBCL NOS, is frequently associated with BCL2 or, to a lesser extent, BCL6 translocation, in the so-called “double-hit” or “triple-hit” lymphomas. This type of lymphoma is included in the updated WHO classification in the new category of high-grade B-cell lymphoma (HGBL), with rearrangements of MYC and BCL2 and/or BCL6. MYC protein expression is detected in a much higher proportion of DLBCL (30%-50%) and is associated with concomitant expression of BCL2 in 20% to 35% of cases. Most of these tumors do not carry MYC/BCL2 chromosomal alterations and have been named "double-
expressor lymphoma.” Cutoff to define these cases for MYC expression is 40%, for BCL2 expression is 50%. According to 2016 WHO, double expression of MYC and BCL2 proteins without gene aberrations should be considered a prognostic indicator in DLBCL, NOS but not a separate category.

*Burkitt lymphoma*

BL cells are derived from the GC B cells. These tumors carry specific chromosome translocations, that is, t(8;14)(q24;q32) or its variants. Mutations in the transcription factor 3 (TCF3) or its negative regulator ID3 (inhibitor of DNA Binding 3) occur in about 70% of sporadic and immunodeficiency-related BL and 40% of endemic cases. The subset of lymphomas that resemble BL morphologically, to a large extent phenotypically and by GEP, but lack MYC rearrangements, has been included in a new entity in the 2016 WHO classification called Burkitt-like lymphoma with 11q aberration.

*Cellular origin and genetic alterations in aggressive B-cell lymphomas*

*Mantle cell lymphoma*

MCL represents 5%-10% of all non-Hodgkin’s lymphomas (NHL), and predominantly occurs in males with advanced age. The neoplastic cells in MCL are mature B-lymphocyte that expressed genes normally detected in naïve B cells, such as IgD and the T-cell-associated CD5 antigen, and tend to colonize the mantle zone of the lymphoid follicles. These propose the term MCL and suggest that the cell origin of MCL is naïve B cells.

MCL is genetically characterized by the t(11;14)(q13;q32) translocation that juxtaposes the proto-oncogene CCND1, which encodes cyclin D1, at chromosome 11q13, to the Ig heavy chain gene at chromosome 14q32. As a consequence of the translocation, cyclin D1, which is not expressed in normal B lymphocytes, becomes constitutively
overexpressed. This genetic alternation is thought to be the primary event in the pathogenesis of the tumor, probably facilitating the dysregulation of the cell cycle at the G1-S phase transition. Cyclin D1 participates in the control of the G1 phase by binding to cyclin-dependent kinase (CDK) 4 and CDK6. Cyclin D1-CDK4 and cyclin D1-CDK6 complexes could phosphorylate retinoblastoma 1 (RB1) and lead to the inactivation of its suppressor effector on cell cycle progression. The overexpression of MCL cells also titre p27 into CDK4-cyclin D1 complexes rendering p27 incapable of inducing G1 cell cycle arrest. There is a small subset of tumors negative for cyclin D1 and t(11;14) but with a morphology, phenotype, global-expression profile and secondary genetic events undistinguishable from conventional MCL, suggesting that they correspond to the same disease. However, these cases have high expression of cyclin D2 or cyclin D3. These cases are uncommon but highlight the relevance of the oncogenic dysregulation of the G1 phase of the cell cycle in the pathogenesis of MCL. Despite the important role of the t(11;14) translocation and cyclin D1 overexpression in the development of MCL, several observations suggest that these mechanisms may not be sufficient for the full transformation of the cells and the aggressive behavior of the tumor. Cytogenetic studies aimed at identifying secondary genetic alternations have revealed that MCL is one of the malignant lymphoid neoplasms with the highest level of genomic instability. The recurrent aberrations of certain chromosomal regions have been identified, such as losses of 11q and 13q, gains of 3q and 12q and losses of 9p, 9q and 17p. Alterations of the 8q24 locus, including the t(8;14)(q24;q32) translocation that activates MYC, are uncommon but have been identified in MCL with a very rapid clinical evolution. Besides these chromosomal imbalances, MCLs present frequent tetraploid clones that are more common in pleomorphic and blastoid variants than in cases with typical morphology.
The potential targets genes in the chromosomal regions altered in MCL are mainly belonging to two pathogenic pathways: the cell cycle and the DNA damage response. In cell cycle dysregulation, highly proliferative MCL carry two main oncogenic pathways alteration, INK4a-CDK4-RB1 and ARF-MDM2-p53, which are responsible for the control of the cell cycle and senescence. Tumor obtains a selective advantage inactivating those pathways. For DNA damage response pathways alteration, deletion of the ataxiatelangiectasia mutated (ATM) gene is the most frequent event. The inactivation of ATM facilitates the development of genomic instability and increases tumorigenesis in these B cells. In addition, MCL may also have activated cell survival mechanisms. Specifically, amplification of anti-apoptotic BCL2 and homozygous deletions of BIM, a gene of the pro-apoptotic BH3-only family, have been observed in several MCL cell lines. Constitutive activation of NF-κB has been detected in MCL cell lines and primary tumor with overexpression of downstream targets such as FADD-like apoptosis regulator (FLIP). The AKT survival pathway is also activated in MCL, which might be associated with loss of phosphatase and tensin homologue (PTEN) expression. Currently, new drugs targeting these mechanisms are increasingly available\textsuperscript{26,32}.

**Burkitt lymphoma**

BL includes sporadic, endemic and HIV associated forms. It is an aggressive malignancy derived from GC dark zone B cells, as indicated by the presence of mutated IgV sequences and its transcriptional signature. All endemic and 1/3 of all sporadic and AIDS-associated BL cases are infected by the Epstein-Barr virus (EBV). Genetic alternations in BL lead to the aberrant expression of MYC and to the activation of the PI3K signaling pathway, both of which are not part of the physiological programme of the dark zone B cells.
Translocations of the MYC oncogene into the immunoglobulin loci are present in 100% of cases and therefore are the hallmark of BL. The translocation leads to the ectopic and constitutive expression of MYC as a result of the escape from the BCL6-mediated transcriptional repression that normally prevents MYC expression in dark zone B cells. MYC is a nuclear phosphoprotein that functions as a sequence-specific DNA-binding transcriptional regulator to control proliferation, cell growth, differentiation and apoptosis, all of which are implicated in carcinogenesis. MYC promotes genomic instability by inducing replication stress, which is particularly dangerous when activated in highly proliferative dark zone B cells. Approximately 70% of BL have either mutation of TCF3, which encodes the transcription factor E2A, or inactivating mutations in ID3, which prevent its regulation of E2A. The resulting dysregulated activity of E2A seems to promote antigen-independent “tonic” BCR signaling. The PI3K pathway happens to be a key component of tonic BCR signaling. Whereas other pathways, including NF-κB or MAPK, which are essential effectors of the antigen-dependent BCR pathway, are shown to be dispensable. Besides, 1/3 of individuals with BL have inactivating mutations of several tumor suppressors including tumor protein 53 (TP53), PTEN and cyclin-dependent kinase inhibitor 2A (CDKN2A)\textsuperscript{17,18}.

Diffuse large B cell lymphoma

DLBCL is the most common form of B-NHL and account for ~40% of all new diagnoses of B-NHL in adulthood. It includes cases that arise de novo as well as cases that derive from the clinical evolution of various less aggressive B-NHLs, such as follicular lymphoma and chronic lymphocytic leukemia. Based on gene expression profiles, DLBCLs correspond to B cells arrested by transformation events that occur at various stages of the GC transit. In particular, GCB-like subtype DLBCL resemble light zone B cells, whereas ABC-like DLBCLs seem to derive from GC cells arrested during the early
stages of post-GC plasma cell differentiation (plasmablasts). These molecular phenotypes have prognostic value; ABC-DLBCL has a more aggressive clinical course and more unfavorable outcome than GCB-DLBCL. Consistent with their distinct molecular phenotype and clinical course, each DLBCL subtype shows predominant or exclusive genetic aberrations, although they still share some pathogenetic alternations, including those involving chromatin modifiers, BCL6 dysregulation and immune recognition.

The pathogenesis of the GCB subtype of DLBCL is poorly understood. A few biologically relevant lesions have been identified, but each of them is present only in a fraction of cases. Chromosomal translocations involving MYC and BCL2, which are analogous to those that characterize BL and follicular lymphoma, are detected in ~10% and ~40% of GCB-DLBCL, respectively. The co-occurrence of lesions affecting the MYC and BCL2 genes is associated with a particularly poor prognosis. In addition, two programmes seem to be affected with some specificity in GCB-DLBCL: EZH2 (enhancer of zeste homolog 2)-driven epigenetic changes and B cell migration. EZH2 gene is mutated in ~21% of GCB-DLBCL cases and its mutations result in a gain-of-function phenotype. Mice studies engineered to express the mutation develop GC hyperplasia, which indicates that mutations in EZH2 contribute to lymphomagenesis. Moreover, mutations of several chemokines and receptors in GC have been shown in 30% GCB-DLBCL, including sphingosine-1-phosphate receptor 2 (S1PR2) and P2Y purinoceptor 8 (P2RY8), GNA13 (which encodes Gα13) and ARHGEF1 (which encodes RHO guanine nucleotide exchange factor 1). All those mutations contribute to the loss of confinement of B cells to the GC. In addition, they provide an explanation for the ability of GCB-DLBCL cells leave their tissue of origin and travel to distant sites, such as bone marrow.

The pathogenesis of ABC-DLBCL is characterized by two main events: the constitutive activation of NF-κB and the blockade of terminal differentiation to plasma cells.
Both events can be achieved by distinct genetic lesions. Constitutive NF-κB signaling is sustained by a variety of genetic alterations. In ~20% of ABC-DLBCL cases, mutations in CD79A and/or CD79B (which encodes components of the BCR complex) contribute to chronic BCR signaling by preventing endocytosis of the receptor and/or by blunting the activity of LYN, which is a negative regulator of the pathway. Activating mutations targeting CARD11 (recruiting domain-containing protein 11) in ~10% ABC-DLBCL lead to hype-responsiveness of the signal transduction complex CARD11-BCL10-MALT1 to activate NF-κB independently of upstream signals, including BCR. ~35% ABC-DLBCL cases carry MYD88 (myeloid differentiation primary response protein 88) mutation. On the one hand, MYD88 mutation leaded to gain the ability of spontaneously assembling a complex containing interleukin 1 receptor-associated kinase (IRAK1) and IRAK4, which leads to activation of NF-κB. On the other hand, MYD88 mutations induce Janus kinase (JAK)-signal transducer and activator of transcription 3 (STAT3) signaling. Besides these mutations, the inactivation of TNFAIP3 (TNF alpha induced protein 3) gene, which encodes a key negative modulator of NF-κB pathway, has been found in 30% of ABC-DLBCL cases, thus preventing termination of NF-κB responses. All those alterations described above could not exclude the possibility that constitutive activation of NF-κB may occur as a consequence of chronic stimulation of the BCR by antigens or autoantigens. The inability of ABC-DLBCL cells to terminally differentiate seems to depend on two mechanisms that are mostly mutually exclusive and that converge on the negative regulation of the plasma cell master regulator BLIMP1. Bi-allelic inactivation of PRDM1 (PR domain zinc finger protein 1) is observed in ~30% of ABC-DLBCL cases. Alternatively, BCL-6 dysregulation by chromosomal translocations, which are more frequently in ABC-DLBCL than GCB-DLBCL, also leads to constitutive repression of PRDM1 by BCL6. Studies have shown that mice with GC B cells that carry inactivated PRDM1 develop
ABC-DLBCL that shows constitutive NF-κB activation, which indicates that both of these pathways are required for this type of lymphomagenesis\textsuperscript{17,18}.

**Current therapies and challenges for aggressive B-cell lymphomas management**

The treatment of patients with aggressive B-cell lymphomas remains a clinical challenge. Conventional chemotherapeutic regimens, mainly based on the CHOP (cyclophosphamide, hydroxydaunorubicin, oncovin and prednisone) combination, obtain a relatively high number of complete or partial clinical responses (20-80%), but the tumor relapses in most patients, who will die of the disease. Addition of rituximab, a chimeric monoclonal antibody directed against CD20, to this combination increases the overall survival, and the response rate and duration. Other intensive chemotherapy regimens such as hyper-CVAD and combination with rituximab or stem cell transplantation have also been used for initial treatment.

In the past few years, new strategies have been developed that target crucial biological cellular pathways and that may change the management and outcome of patients.

*Proteasome inhibitors*

Proteasome targets such as cyclin D1, TP53 and NF-κB are altered in multiple types of B cell lymphomas, justifying the emergence of the proteasome as an attractive target for aggressive B-cell lymphomas treatment. Clinical evidence demonstrated that bortezomib (first-in-class proteasome inhibitor) treatment induces a high percentage of responses and superior overall survival in MCL and ABC-DLBCL compared with GCB-DLBCL.

*BCR signaling inhibitor*

BTK has been shown to be an essential component of chronic active BCR signaling, which is a key survival pathway in the ABC-DLBCL. Potent BTK pharmacologic in-
hibitor (ibrutinib) kills ABC cells, which had been identified in many studies. In addition, a moderate to strong BTK expression has been found in MCL. Ibrutinib inhibited the viability of MCL cell line in concentration- and time-dependent manners, also induced a concentration-dependent apoptosis. Studies also demonstrated that ibrutinib treatment resulted in significant improvement in progression-free survival (PFS) and good tolerability in patients with relapsed or refractory MCL. Currently, the challenge for ibrutinib is that both primary and secondary resistance has been reported in various lymphomas. Resistance may arise due to mutations that impair the affinity of ibrutinib for BTK, or due to alterations in pathways downstream of BTK and may confer BCR signaling independence in resistant clones.

**Apoptosis inhibitor**

Apoptosis execution depends on the activation of caspases by either the intrinsic (mitochondrial) pathway or the extrinsic pathway that requires activation of the TNF family of cell death receptors (FAS, DR4 and DR5) by their related ligands (FAS ligand) and TNF-related apoptosis inducing ligand (TRAIL). BCL2 family proteins are the most prominent regulators of the intrinsic apoptotic pathway. Preclinical data from lymphoma cell lines and primary tumor samples indicate high efficacy of BCL2 inhibitor against lymphoma. In parallel, the extrinsic pathway has emerged as a potential target in therapeutics, particularly because chemo-refractory cells often have defects in their intrinsic pathway. Agents such as TRAIL or anti-DR4 and 5 humanized antibodies that selectively kill tumor cells have recently been shown to be effective in lymphoid tumors.

**Inhibition of survival pathways**

PI3K-AKT-mTOR (mammalian target of rapamycin) acts as a central controller of multiple growth signaling pathways. mTOR inhibitors have arisen as promising agents
for cancer therapy, such as Temsirolimus, which has shown anti-tumor activity in lymphoma\textsuperscript{35}.

Aggressive B-cell lymphomas follow a very rapid progression. At present, the therapeutic approaches are relatively similar in all patients, and the different strategies do not consider this potential variation. However, different clinical evolution suggests that not all patients need the same therapy. Developing precise and reliable criteria to predict such disparate clinical behavior and adjusting the best therapeutic strategy for each individual patient are needed. Promisingly the increasing number of drugs that target specific molecular mechanisms is opening new perspective for lymphoma patients, but the evaluation of these approaches will require a correct stratification of the patients according to the specific biological risk of their disease. Moreover, deciphering the pathogenesis of B-cell lymphoma is needed to identify novel treatable targets for better therapeutic intervention\textsuperscript{32,36}.

**Rho-family GTPases (guanosine triphosphatase) and their roles in cancer**

Rho-family GTPases have been recognized as crucial signal-transducing proteins for almost 20 years. It comprised a family of 23 proteins that are related in primary sequence. Phylogenetic analysis shows that these proteins cluster into several subfamilies based on sequence similarity. The prototypical members of the family, Rac1 (Ras-related C3 botulinum toxin substrate 1), CDC42 (Cell division control protein 42) and RHOA (Ras homolog gene family, member A), can bind both GTP and GDP. When bound to GTP they are active and can transduce signals by binding to effector proteins. By contrast, the GDP-bound forms do not bind effector proteins and are generally assumed to be inactive. However the Rnd (Rho-related GTP-binding protein family), RhoBTB (Rho-related BTB domain-containing protein family), RHOD, RHOU and RHOV GTPases are atypical and do not conform to this stereotypical mechanism of regulation.
as they are mainly GTP bound. They might instead be regulated by expression, phos-
phorylation or stability\textsuperscript{37,38}.

The switch between the GDP- and GTP- bound forms of typical Rho GTPase is
regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating pro-
teins (GAPs). When in the GTP-bound active form, Rho family GTPase transduced sig-
nals by binding to effector proteins. These in turn are involved in many cellular process-
es including regulation of the actin cytoskeleton, microtubule dynamics, cell division, mi-
gration and adhesion. There are 79 identified GEFs for Rho family GTPases in the
mammalian genome. 65 Rho family GAPs are known in mice.

A branch of Rho GTPases are involved in tumorigenesis. There is increasing ev-
idence that Rho proteins are dysregulated during tumor progression and that this corre-
lated with poor prognosis. On the one side, Rho proteins can promote cell-cycle pro-
gression, which is controlled by CDKs. Rho proteins affect CDK activity by regulating the
levels of cyclin D1, as well as P21\textsuperscript{WAF1} and P27\textsuperscript{KIP1}, which bind to and modulate CDK ac-
tivity\textsuperscript{39}. On the other side, evidence showed that RHO proteins might protect cells
against apoptosis. Additionally, modulation of Rho protein activity can promote the me-
tastasis of tumor cells by disrupting epithelial-sheet organization, increasing cell motility
and promoting the degradation of the extracellular matrix. Given the involvement of Rho
proteins in cancer, they might make good therapeutic targets.

Overview of the chapters

The overall goal is to study the role of Rac1-PAK (p21-activated kinases) axis in
aggressive B-cell lymphomas, particularly MCL, and identify potential candidates in ef-
fective therapy or combination therapy.
In Chapter 2, Rac1 has been identified as commonly activated in MCL. In addition, we have demonstrated Rac1 play a tumorigenesis role in MCL by activating multiple oncogenic pathways, such as AKT/mTOR and NF-κB pathway, and inhibition of Rac1 enhanced the cytotoxic effect induced by Adriamycin. The evidence supported a concept of targeting Rac1 might be a potential strategy for treating MCL. However, one problem is that Rac1 is GTPase, which is hard to target. Considering the function of Rac1 is through activation of PAKs, known as Rac1-PAK axis, and PAK is a kinase, which makes targeting PAK a better choice. In Chapter 3, we have identified that inhibiting Rac1-Pak2 axis, not Pak1, significantly inhibit the tumor cells growth in MCL, also in other aggressive B-cell lymphoma cells. Additionally, synergistic effect was observed between PAK inhibitor and Adriamycin or Ibrutinib in MCL. Moreover, PAKs inhibitor could overcome ibrutinib resistant in treating ibrutinib-resistant MCL and ABC-DLBCL cell lines. In Chapter 4, the major conclusion was summarized and future directions were discussed. Chapter 5 and Chapter 6 are materials and methods section and bibliography section, respectively.
Chapter 2 Rac1 is a novel therapeutic target in mantle cell lymphoma

Parts of this chapter are derived from:

Summary

Rac1 is a small guanosine triphosphatase (GTPase), which plays a critical role in cell survival, migration and transformation. Rac1 has been found to be dysregulated in many human malignancies, including solid tumors, chronic myeloid leukemia, and peripheral T cell lymphomas. However, its status in MCL remains unknown. Here, we investigated the expression of Rac1 in MCL and found it is overexpressed at both mRNA and protein levels. Furthermore, the level of Rac1 protein in MCL primary tumors is correlated with poor prognosis of MCL patients. To determine the significance of Rac1 dysregulation in MCL, we knocked down Rac1 in MCL cell lines using shRNA. The result shows that decreasing Rac1 level in MCL cells significantly inhibited the proliferation and enhanced chemotherapy drug Adriamycin (doxorubicin)-induced cytotoxic effect. Consistently, inhibition of Rac1 using a specific inhibitor NSC 23766 in MCL cells showed a similar effect as Rac1 knockdown by shRNA. Mechanistic studies demonstrated that inhibition of Rac1, either by shRNA or by chemical inhibitor, substantially diminished the activity of signaling pathways downstream of Rac1, including pathways of AKT/mTOR, NF-κB and ERK. Collectively, the studies of the report suggest targeting Rac1 as a promising strategy in clinical treatment of MCL.
INTRODUCTION

MCL is an aggressive hematological malignancy, comprising 6-8% of human B-cell lymphomas. Cytogenetically, MCL is characterized by the chromosomal translocation t(11;14)(q13;q32), which results in aberrant expression of cyclin D1. Rituximab (R)-CHOP, which combines rituximab, cyclophosphamide, hydroxydaunorubicin, oncovin and prednisone, is the most common chemotherapeutic treatment approach used to treat MCL. Despite the improvement of the overall survival with intensive frontline chemotherapy, it remains an incurable disease and most patients are destined to relapse after initial therapy. The median survival of patients with MCL ranges between 3 and 5 years. Therefore, new strategies are urgently needed for the treatment of MCL. Increasingly, seeking for the drivers of MCL pathogenesis will elucidate potential targets for therapeutic intervention of this disease.

Rac1, a member of the Rho family, is small GTPase. Studies have shown that Rac1 plays a critical role in cell proliferation, migration and survival. Rac1 bears two conformational states, inactive GDP-bound form and active GTP-bound form. The transition of Rac1 between these two forms is regulated by its GEFs and GAPs. GEFs promote Rac1 activation by driving the switch of GDP to GTP-bound activated form. Oppositely, GAPs inactivate Rac1 through GTP hydrolysis, by which Rac1 switches to GDP-bound inactivated form. In the active state, Rac1 interacts with multiple effectors and activates numerous downstream signaling pathways, such as PI3K/AKT, AMPK and PAK/ERK pathways.

Overexpression of Rac1 has been reported in several types of solid tumors, including breast cancer and pancreatic cancer. In these tumors, activation of AKT, NF-κB and c-RAF/MEK/ERK pathways play a major role in Rac1 promoted oncogenesis, survival and metastasis. In hematological malignancies, studies have shown that
Rac1 GTPase activated by BCR-ABL represented a novel target in Chronic Myeloid Leukemia (CML). Treatment with the specific Rac1 inhibitor NSC 23766 remarkably suppressed the growth of primary bone marrow cells from patients with blast phase CML as well as leukemia cells ectopically expressing BCR-ABL T315I\textsuperscript{56}.

Interestingly, AKT signaling is one of the most commonly dysregulated oncogenic pathways in MCL. Constitutively activation of PI3K/AKT/mTOR pathway has been shown to contribute to survival in a subset of MCL\textsuperscript{57-59}. These signaling results in the activation of a number of well-known target genes and pathways, including the NF-κB pathway, a second major oncogenic signaling pathway postulated to be activated independently in MCL\textsuperscript{59}. Studies also showed that ERK1/2 pathway is critical to the proliferation and survival of MCL through inhibiting BCL family BCL-x\textsuperscript{l}\textsuperscript{60}. Cross talk between these pathways has been reported previously in a number of studies\textsuperscript{59}.

Despite the widely study of Rac1 in solid tumor and myeloid malignancies, its role in MCL remains unclear. In the current study, we have assessed the expression of Rac1 in MCL cell lines and tissues, and its correlation with clinical outcome. We also investigated the effects of Rac1 on its downstream oncogenic signaling pathways, as well as the impact of Rac1 on cell proliferation, survival and anticancer drug response in MCL cells. Adriamycin, the core agent of R-CHOP regimen, was used as standard treatment in the drug combination part.

RESULTS

Rac1 is overexpressed in MCL patient samples and cell lines

GEP was done on total 41 MCL cases from LLMPP (Lymphoma/leukemia Molecular profiling project) database, and expression of all members of Rho GTPases family was shown in figure 2.1A. Next, we compared the expression levels of those GTPases
in MCL and in naïve B cells. We found that three genes, which are RHOU, RHOBTB3 and Rac1, displayed substantial higher expression in MCL cases than in naïve B cells (p<0.05) (Figure 2.1A). Heat-map was also shown here to emphasize the relative expression difference (Figure 2.1B). Of these three genes, Rac1 has an overall higher expression mRNA level in MCL cases than the other two. Combined with the relative higher expression of Rac1 in MCL than naïve B cells (Figure 2.1C), which make Rac1 be a potential research candidate in the following study. Moreover, this result was further confirmed by analyzing 64 MCL cases from online database (Figure 2.2). We also examined the levels of Rac1 mRNA and protein in a panel of 6 MCL cell lines (Granta-519, Jeko-1, JVM2, Maver-1, Mino and Z138). The result showed that, while Rac1 mRNA is expressed at higher levels in 4 of the MCL cell lines (Jeko-1, Maver-1, Mino and Z138) than Naïve B cells (Figure 2.3A), the protein level is markedly increased in all of the tested MCL cell lines compared to the normal control Naïve B cells (Figure 2.3B). Moreover, the Rac1-GTP level also increased in MCL cell lines compared to the normal control, which means that Rac1 is activated in all MCL cell lines (Figure 2.3B). It is worth noting that the mRNA expression of Rac1 is not well correlated with its protein level, implying that post-transcriptional or translational regulation plays a part in Rac1 expression in MCL cells.

To confirm the up-regulation of Rac1, we performed immunohistochemistry (IHC) analysis of Rac1 in 32 cases of MCL. In normal lymphoid follicles, mantle zones were negative for Rac1 (Representative Figure 2.4A-B), whereas 18 cases of MCL (56%) showed positive reactivity to Rac1, with six respective cases fell into weak, medium and strong staining (cut-off value 30%) (Table 2.1 and Figure 2.4C-D). Furthermore, we analyzed the prognosis of these MCL cases, and found that Rac1 positive staining was associated with shorter overall survival (OS, p=0.039) in MCL patients (Figure 2.5). These data suggest that dysregulation of Rac1 signaling is involved in MCL progression.
Knockdown of Rac1 inhibits the proliferation and increase the cytotoxic effect of Adriamycin

To confirm the significance of Rac1 dysregulation in MCL, the 41 cases were then divided into three groups based on Rac1 mRNA level and correlated with cell proliferation. As shown in supplemental Figure 2.6, higher Rac1 level was significantly correlated with higher levels of the proliferation signature, as established in our previous study (p<0.001). Next, we transduced MCL cell lines Z138 and Mino, which express high levels of both Rac1 and Rac1-GTP (Figure 2.3B), with Rac1-shRNA by inducible retroviral vector. Upon doxycycline (Dox) induction, Rac1 protein level was decreased by 55% and 50% in Z138 and Mino cells, respectively, compared to the vector controls (Figure 2.7A-B). Notably, knockdown of Rac1 decreased cell proliferation by 43% (p<0.001) and 24% (p=0.002) in Z138 and Mino cells, respectively, after three days of continuous culture (Figure 2.8A-B). However, Rac1 knockdown did not induce any cell apoptosis in those two MCL cell lines (Figure 2.9A-B).

We also examined the effect of Rac1 on chemotherapy drug Adriamycin-induced cytotoxic effect in MCL cells. As shown in Figure 2.9A-B, even if Rac1 knockdown itself has little effect on the apoptosis in Z138 and Mino cells, when combined with Adriamycin, Rac1 knockdown cells exhibited an enhanced Adriamycin-induced apoptosis, with a nearly 2-fold increase in apoptosis observed in the Rac1 knockdown cells treated with 50nM Adriamycin compared to the control cells received the same treatment.

We next explored the molecular mechanism underlying the functional role of Rac1 dysregulation. For the study, we analyzed several Rac1-regulated oncogenic pathways in the cells transduced with Rac1-shRNA versus control-shRNA-transduced cells. As results shown in Figure 2.10, Rac1 knockdown substantially diminished the phosphorylation of AKT at both T308 and S473 residues. Consistently, AKT downstream
target mTORC1 was also exhibited a decreased activity, as shown by diminution in phosphorylation of RPS6 (Ribosomal protein S6). Because AKT activation is commonly observed in MCL and considered as a core driven signaling, these data suggest that Rac1 overexpression plays an important part in the development of MCL. In addition, phosphorylation of RelA/p65 and ERK1/2, indicative of activation of these proteins, were also decreased in the MCL cells upon Rac1 knockdown (Figure 2.10). Collectively, these results suggest an important role for Rac1 overexpression in the hyper-activation of multiple oncogenic pathways in MCL.

**Rac1 inhibition by NSC 23766 reduces viability of MCL cells and Rac1 overexpression rescue the inhibition effect of NSC 23766**

To validate the effect of Rac1 inhibition, we used NSC 23766, a commonly used specific Rac1 inhibitor\textsuperscript{55,61,62}. Rac1 activity assay demonstrated that the EC\textsubscript{50} (Inhibition of Rac1 activity in cells by 50%) for NSC 23766 is approximate 25 μM (Figure 2.11). We then treated Z138 and Mino cells with the drug. As shown in Figure 2.12A, the cell proliferation of both cell lines were substantially inhibited by NSC 23766 in dose dependent manner within its linear dose range for Rac1 inhibition. Nevertheless, normal naïve B cells were barely affected by NSC 23766 treatment, even at the 100 μM concentration (Figure 2.12B). We also checked MCL cells death induced by NSC 23766. After treated with NSC 23766, we observed significantly increased cell apoptosis by 25 μM and 50 μM (Figure 2.13). These results suggested that Rac1 targeting provides a specific toxic effect toward only lymphoma cells, which is consistent with our previous results showing that Rac1 was upregulated in MCL and involved in the activation of pro-survival oncogenic pathways.

Next, we overexpressed Rac1 in Z138, as well as JVM2 cells that express lower level of both Rac1 and Rac1-GTP (Figure 2.14). The result showed that Rac1 overex-
pression substantially rescued the cells from the inhibitory effect of NSC 23766 (Figure 2.15 A-B). These results further supported the pro-tumor role of Rac1 and targeting Rac1 provides a specific toxic effect toward MCL lymphoma cells.

We next examined the effect of NSC 23766 on the major oncogenic signaling pathways downstream of Rac1. As shown in Figure 2.16, similar to the effect of Rac1 knockdown by shRNA, inhibition of Rac1 by NSC 23766 in Z138 and Mino cells markedly reduced the pathway activities of AKT/mTORC1 [p-AKT (T308), p-AKT (S473), p-PRS6 (S235/S236) and p-4E-BP1 (T37/T46)], NF-κB [P65 (S536)] and ERK [ER1/2 (T202/T204)] following treatment with NSC 23766. Together, these data validate the effect of Rac1 knockdown by shRNA and suggest a role for Rac1 in promoting tumor progression in MCL.

**Inhibition of Rac1 by NSC 23766 enhances cytotoxic effect of Adriamycin in MCL cells**

In view of chemotherapy by Adriamycin being the standard approach to treat MCL, we test the effect of Rac1 inhibition on the cytotoxic effect of Adriamycin in MCL cells. Z138 and Mino cells were treated with Adriamycin and NSC 23766, individually or in combination. Cell viability assay showed that the inhibition of Rac1 by NSC 23766 significantly increased the cytotoxic effect of Adriamycin especially after 72h of treatment (5.65 ± 0.05 vs 3.12 ± 0.02, p=0.007 for Mino; 10.8 ± 0.38 vs 4.2 ± 0.07, p<0.001 for Z138), although synergy of two drugs was not observed (Figure 2.17A). We also assessed the effect of Rac1 inhibition on apoptosis induction following Adriamycin treatment. For Z138 cells, combination of 10 µM NSC and 25 nM Adriamycin induced apoptosis approximately twice than that by single Adriamycin treatment (10.05 ± 0.05 vs 23.7 ± 1.3, p=0.009; Figure 2.17B). For Mino cells, combination of 25 µM NSC 23766 and 75 nM Adriamycin induced 20% more cell apoptosis than that by individual Adriamycin.
treatment (34.6 ± 3.4 vs 58.1 ± 0.5, p=0.02; Figure 2.17B). These data suggests that 
Rac1 inhibition could increase the cytotoxic effect of Adriamycin, further supporting its 
application in clinical practice.

**Rac1 is overexpressed in other B-cell lymphoma cell lines and inhibiting Rac1 could diminish cell viability of those lymphoma cell lines**

As we known that various dysfunctional survival pathways, like PI3K/AKT/mTOR 
and NF-κB, has been demonstrated in DLBCL and BL. Rac1 positively regulated AKT, 
ERK and NF-κB pathways, which were confirmed in our previous result. To determine if 
inhibition of Rac1 induced some tumor suppression effect in other lymphomas, we 
checked Rac1 expression in other B-cell lymphoma cell lines. Two ABL-DLBCL (Ly3 and 
TMD8), two GCB-DLBCL (DHL16 and Ly19) and two BL (Daudi and GA10) cell lines 
were chosen. Western blot showed increased Rac1 protein level in all six lymphoma 
cells (Figure 2.18).

Next, to identify the role of overexpressed Rac1 signaling in other lymphomas, 
Rac1 siRNA was used to knock down Rac1 in DLBCL and BL cell lines. Three cell lines 
we chose here were TMD8, Ly19 and Daudi cells that all have high expressed Rac1 pro-
tein level (Figure 2.18). Knockdown efficiency of siRNA reached over 50% in all cell lines 
(Figure 2.19A). Notably, Rac1 knockdown significantly decreased cell viability in all three 
cell lines compared to their respective controls (6.86 ± 0.10 vs 4.44 ± 0.27 for Daudi at 
72h, p<0.001; 7.70 ± 0.17 vs 3.97 ± 0.08 for TMD8 at 72h, p<0.001; 8.27 ± 0.02 vs 5.86 
± 0.08 for Ly19 at 72h, p<0.001, Figure 2.19B). Rac1 inhibitor, NSC 23766, was further 
used here to treat those three cells. Our data showed that at 1μM and 10 μM NSC 
23766 significantly inhibited the cell viability of lymphoma cells (Figure 2.20).

Molecular mechanism was further explored. Similarly, Rac1 knockdown substan-
tially diminished the phosphorylation of AKT at both T308 and S473 residues. Consist-
ently, AKT downstream target mTORC1 was also exhibited a decreased activity, as shown by diminution in phosphorylation of RPS6. In addition, phosphorylation of RelA/p65, indicative of activation of NF-κB pathway, was also decreased in the lymphoma cells upon Rac1 knockdown (Figure 2.21). These results gave us an initial concept that Rac1 signaling could be a potential target in the DLBCL and BL system.

**DISCUSSION**

The study of MCL, including multi-disciplinary research from molecular biology to the clinic, has improved the understanding of the MCL pathogenesis and given a new sight to design novel clinical therapeutic strategies. However, it is still a challenge to define a standard therapy because of the heterogeneous biology and clinical presentation of MCL patients. Thus, there is a critical need for a clear understanding of the pathogenic mechanisms of MCL in order to identify attractive and promising targets.

Rac1 has been defined as an oncogene for more than a decade with most of the studies focusing on solid tumor. In this study, we demonstrate that Rac1 protein is aberrantly overexpressed in primary MCL tumors and established MCL cell lines (see Figure 1). Importantly, high Rac1 protein level is correlated with poor overall survival of MCL patients (see Figure 1h), making the examination of Rac1 level valuable in prognosis prediction. Immunohistochemical staining is a practical way to evaluate the expression level of Rac1, however, due to the limited number of cases being included in current study, the significance of the Rac1 positive proportion and intensity have not been well characterized. Further study involving larger cohorts of cases are encouraged to delineate Rac1 expression with disease progression in more detail.

Gene rearrangement involving *Cyclin D1* is the hallmark of MCL. However, it has demonstrated that simple Cyclin D1 overexpression is insufficient to induce the onset of MCL tumor, raising the importance of other mechanisms in MCL. Consistently, several
core oncogenic pathways including AKT and NF-κB signaling have been found to be dysregulated without correlated genomic aberration in MCL, which implies an interactive activation of pathway network in cancerous state. In this study, we demonstrate that Rac1 is directly associated with the activation of several oncogenic pathways in MCL, suggesting that it locates at the central zone of pathway network, although the mechanism underlying Rac1 overexpression in MCL remains unclear. Previous studies have demonstrated that endogenous Rac1 is activated by the BCR signaling and is required for the activation of BCR subsequent signal transduction. Considering the constitutive activation of the BCR signaling in MCL and its wide connections with other oncogenic pathways, it is likely that Rac1 plays an important role in mediating both of the tonic and chronic activating signals for MCL cells. Future studies are expected to elucidate the Rac1 regulation network and its significance in signaling integration.

We found that the inhibition of Rac1 brought about only cytostatic effect. This is in agreement with the defect of the pro-apoptotic machinery in MCL, such as BCL-2 and MCL1 overexpression, that simple inhibition of common oncogenic pathways is insufficient to induce apoptosis. Notably, inhibition of Rac1 either by shRNA or NSC 23766 significantly enhanced the cytotoxic effect of Adriamycin (see Figure 2 & 4), potentiating the combination of Rac1 inhibition with standard chemotherapy in MCL treatment. In the current study, we tested the Rac1 specific inhibitor NSC 23766 in MCL cells, which showed remarkable inhibition on cell viability upon single drug treatment and increased apoptosis when combined with Adriamycin. Although the high effective dose (micromolar range) of NSC 23766 may impede its application in clinical practice, the data obtained with it provides evidence supporting a proof-of-concept to this hypothesis, which will be tested upon new Rac1 inhibitors with improved EC50 become available.
Moreover, we did some initial study of Rac1 in other aggressive B-cell lymphomas, like DLBCL and BL. Overexpressed Rac1 was also been found in other lymphoma cell lines. In addition, inhibition of Rac1, either by siRNA or specific inhibitor, brought cytostatic role in those lymphoma cells. The possible explanation is that the downstream pathways of Rac1, AKT and NF-κB pathways are well-known oncogenic pathways in lymphomas. Further studies are needed to present the solid evidence on the potential of inhibiting Rac1 in lymphoma treatment.
Figure 2.1 Expression of Rho GTPases in human primary MCL tumors.

(A) mRNA Expression of all Rho GTPases members in human primary MCL tissues from the LLMPP database. (B) Relative mRNA expression level of each Rho GTPase in MCL cases compared with in naïve B cell, shown in fold changes. (C) Analysis of Rac1 mRNA level in human primary MCL tissues from the LLMPP database. Expression levels are the values of probes in affymetrix HG-U133-plus 2.0 arrays. Data are shown as mean ± SEM. P value was calculated using the two-tailed student’s t test. * indicates P < 0.05.
Figure 2.2 Expression of Rho GTPases in 64 online MCL cases.

Relative mRNA expression level of each Rho GTPase in MCL cases compared with in naïve B cell, shown in fold changes. Expression levels are the values of probes in affymetrix HG-U133-plus 2.0 arrays.
Relative expression, N=64
Figure 2.3 Rac1 is overexpressed in MCL cell lines.

(A) Rac1 mRNA level in MCL cell lines and naïve B cells. (B) Upper panel: Rac1-GTP, Rac1-total and β-actin protein expression in MCL cell lines was analyzed by western blot; lower panel: quantification of Rac1 protein level by Odyssey CLx system (LI-COR). The calculation is based on the ratio between Rac1 signal and that of β-Actin. The experiments were repeated three times, and an average ration to that of the naïve B cells is shown.
Figure 2.4 Representative images of the IHC staining of Rac1 in tissues.

Representative images of Rac1 IHC staining in tonsil tissues (A) and negative staining (B). (C and D) Representative image of Rac1 IHC staining in MCL case tissues. W= weak Rac1 staining, S= strong Rac1 staining.
Figure 2.5 Association of Rac1 protein level with overall survival (OS) in MCL patients.
Overall Survival

Time (months)

Negative (n=14)
Positive (n=18)

P=0.039
Figure 2.6 Positive correlation was existed between Rac1 mRNA expression and proliferation rate in primary MCL tumors.  

41 MCL cases with GEP data were divided into three groups by Rac-1 mRNA expression. Heat map illustrated the proliferation signature of Rac1-lower and higher mRNA expression cases.
Figure 2.7 Knocking down of Rac1 in two MCL cell lines.

Rac1 was knocked down by Dox inducible shRNA in Z138 cells (A) and Mino cells (B). The protein level was determined by western blot after 24h of 1 μg/ml Dox treatment. The relative Rac1 levels in the Dox treated samples were determined by normalization of the Rac1 levels with their respective β-Actin levels.
A  Z138

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Actin

B  Mino

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Actin
Figure 2.8 Rac1 promotes cell proliferation and survival in MCL.

Cell viability of Rac1 knockdown Z138 (A), Mino (B) cells and vector control cells were determined for 3 days by Presto Blue cell proliferation assay. Define the cells number of each group on day 0 as 1 and the y-axis stands for the increased folds of cell numbers on each day compared to the day 0. The experiments were repeated three times in four replicates with similar results obtained. Data shown are the average of three experiments and are presented as mean ± SEM. P value stands for the difference between Rac1 shRNA Dox (+) and control Dox (+). **, P≤0.01; ***, P≤0.001.
A

Z138

Cell Viability (folds of cell seeded) vs Days

Control (Dox -)  Control (Dox +)  Rac1 shRNA (Dox -)  Rac1 shRNA (Dox +)

B

Mino

Cell Viability (folds of cell seeded) vs Days

Control (Dox -)  Control (Dox +)  Rac1 shRNA (Dox -)  Rac1 shRNA (Dox +)
Figure 2.9 Rac1 knocking down increases the toxicity of Adriamycin in MCL.

Z138 (A) and Mino cells (B) transduced with Rac1-shRNA or control-shRNA were treated with 1 μg/ml of Dox for 24h, and then treated with increasing concentrations of Adriamycin for 48h. Apoptosis was determined by Annexin V and 7-AAD staining followed by flow cytometry measurement. The experiments were repeated three times with similar results obtained. Data shown are the average percentage of three experiments and are presented as mean ± SEM. P value stands for the difference between Rac1-shRNA Dox (+) and control Dox (+). *, P≤0.05; **, P≤0.01; ***, P≤0.001.
Figure 2.10 Pathway changes in Rac1 knocking down MCL cells.
Rac1-shRNA expressing and control cells were examined for phosphorylation of AKT, RPS6, NF-κB (p65), ERK1/2 and β-Actin by immunoblotting after 24h Dox induction. Ctr, control.
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Figure 2.11 EC50 of Rac1 inhibitor, NSC 23766, in MCL cell line.

Mino cells were treated with NSC 23766 at the indicated concentrations for 2h. Cell lysates were subjected to Rac1 activity assay as described in *Materials and Methods*. Relative Rac1 activity in each sample is calculated by normalizing the Rac1-GTP level with total Rac1 level.
Figure 2.12 Inhibition of Rac1 by NSC 23766 induces cytostatic effect in MCL cells.

(A) Z138 and Mino cells were treated with NSC 23766 at indicated concentrations for 3 days and assessed for cell proliferation by Presto Blue assay. Define cells number of each group on day 0 as 1 and Y-axis stands the increased folds of cells number on day 3 compared to the day 0. (B) Naïve B cells were treated with 100 µM NSC23766 for the indicated number of days and cell growth was determined by Presto Blue assay. Define cells number of each group on day 0 as 1 and Y-axis stands the increased folds of cells number on each day compared to the day 0. The experiments were repeated three times in four replicates with similar results obtained, and an average Presto Blue fluorescence unit is shown. PFUs: Presto Blue fluorescent units.
**A**

NSC 23766

- 0
- 1
- 10
- 25
- 50
- 100

Cell proliferation (folds of cell seeded)

Z138 Mino

**B**

Naive B cells

- None
- NSC

100 μM NSC 23766

Days 0 1 2 3

Cell proliferation (folds of cell seeded)
Figure 2.13 NSC 23766 induced cell apoptosis in MCL cell lines.

Z138 and Mino cells were treated with increasing concentrations of NSC 23766 for 48h. Apoptosis was determined by Annexin V and 7-AAD staining followed by flow cytometry measurement. The experiments were repeated three times with similar results obtained. Data shown are the average percentage of three experiments and are presented as mean ± SEM.
Figure 2.14 Overexpression of Rac1 in MCL.

Exogenous Rac1 was transfected by pcDNA3-EGFP-Rac1-wt in Z138 and JVM2 cells. The protein level was determined by Western blot. The relative Rac1 levels in the Rac1 vector transfected cells were determined by normalization of the Rac1 levels with their respective β-Actin levels.
Figure 2.15 Overexpression of Rac1 could partially rescue the cytotoxic effect of NSC 23766.

Cell viabilities of Rac1 overexpressed Z138 cells (A) and JVM2 cells (B) and vector control cells were determined for 3 days after treated with NSC 23766 at indicated concentration by Presto Blue cell proliferation assay. Define the cells number of each group on day 0 as 1 and the y-axis stands for the increased folds of cell numbers on the 3rd day compared to the day 0. The experiments were repeated three times in four replicates with similar results obtained. P value stands for the difference between Rac1 transfected group and control group upon NSC 23766 treatment. **, P≤0.01; ***, P≤0.001.
Cell proliferation (folds of cell seeded)

A

NSC23766 (μM)

B

NSC23766 (μM)
Figure 2.16 Oncogenic pathway changes upon NSC 23766 treatment in MCL.

Z138 and Mino cells were treated with 50 µM NSC 23766 for the indicated time and followed by Western blot analyses for the indicated protein phosphorylation and total levels.
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Figure 2.17 Inhibition of Rac1 by NSC 23766 enhances the cytotoxic effect of Adriamycin.

Z138 (A) and Mino (B) cells were treated with NSC 23766 and/or Adriamycin for 48h and apoptosis determined by Annexin V and PI staining, as described in Materials and Methods. The experiments were repeated three times with similar results obtained. Data shown are the average of three experiments and are presented as mean ± SEM. *, P≤0.05; **, P≤0.01.
A

Apoptosis (%)

0 5 10 15 20 25 30

Control NSC Adriamycin NSC +Adriamycin

Z138

B

Apoptosis (%)

0 10 20 30 40 50 60 70

Control NSC Adriamycin NSC +Adriamycin

Mino
Figure 2.18 Rac1 is overexpressed in other lymphoma cell lines.

Upper panel: Rac1-total and β-Actin protein expressions in ABC-DLBCL, GCB-DLBCL, BL cell lines and normal B cells were analyzed by western blot; lower panel: quantification of Rac1 protein level by Odyssey CLx system (LI-COR). The calculation is based on the ratio between Rac1 signal and that of β-Actin. The experiments were repeated three times, and an average ration to that of the normal B cells is shown.
Figure 2.19 Rac1 knockdown induced cytostatic effect in DLBCL and BL cell lines.

(A) Rac1 was knocked down by siRNA in Daudi, TMD8 and Ly19 cell lines. The protein level was determined by western blot after 48h of siRNA transfection. The relative Rac1 levels in the siRNA samples were determined by normalization of the Rac1 levels with their respective β-Actin levels. (B) Cell viability of Rac1 knockdown cells and control cells were determined for 3 days by Presto Blue cell proliferation assay. Define the cells number of each group on day 0 as 1 and the y-axis stands for the increased folds of cell numbers on each day compared to the day 0. The experiments were repeated three times in four replicates with similar results obtained. Data shown are the average of three experiments and are presented as mean ± SEM. P value stands for the difference between Rac1 siRNA and control. ***, P≤0.001.
Figure 2.20 Inhibition of Rac1 by specific inhibitor, NSC 23766, induces cytostatic effect in DLBC and BL cell lines.

Ly19, TMD8 and Daudi cells were treated with NSC 23766 at indicated concentrations for 3 days and assessed for cell viability by Presto Blue assay. Define cells number of each group on day 0 as 1 and Y-axis stands the increased folds of cells number on day 3 compared to the day 0. The experiments were repeated three times in four replicates with similar results obtained, and an average Presto Blue fluorescence unit is shown.
Cell lines
Ly19  TMD8  Daudi
Cell proliferation
(Folds of cell seeded)
NSC 23766(uM)

Cell lines
Ly19  TMD8  Daudi
Cell proliferation
(Folds of cell seeded)
NSC 23766(uM)
Figure 2.21 Pathway changes in Rac1 knockdown DLBCL and BL cell lines.

Rac1-siRNA expressing and control cells were examined for AKT/mTOR, NF-κB (p65) pathways and β-Actin by immunoblotting 48h after transfection. Ctr, control.
**Table 2.1.** Rac1 protein expression in MCL tissues

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</tr>
<tr>
<td></td>
<td>55%-75%</td>
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<td></td>
<td>30%-55%</td>
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<td>Negative (&lt;30%)</td>
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Chapter 3 Rac1-Pak2 axis is a potential therapeutic target in aggressive B-cell lymphomas
Summary

Previously, we demonstrated that Rac1 is a potential therapeutic target in MCL. However, it is hard to target Rac1, as a GTPase. PAKs are a family of serine/threonine protein kinases comprised of six isoforms (PAK1-6). Only group I PAKs (Pak1, 2 and 3) are activated by Rac1. When activated by mutation, overexpression, or by upstream elements such as Rac1, most PAK isoforms have oncogenic signaling effects in cells, including the acquisition of growth signal autonomy, evasion of apoptosis, and promotion of invasion and metastasis. However, no study about PAKs has been done on hematological malignancies. Here, we investigated the expression of PAKs in MCL. We found overexpressed Pak1 and Pak2 mRNA and protein level and highly activated Pak2 signaling in MCL. Furthermore, we extended our studies to other aggressive B-cell lymphomas, which showed the same results. To determine the significance of PAKs dysregulation in aggressive B-cell lymphomas, we knocked down Pak1 and Pak2 separately in lymphoma cell lines using siRNA. The result shows that decreasing Pak2 level, not Pak1, in cells significantly inhibited the cell proliferation. Consistently, inhibition of group I PAKs using a specific inhibitor FRAX 597 in aggressive B-cell lymphomas cell lines showed a similar cytostatic effect as Pak2 knockdown by siRNA. Moreover, synergistic effect was observed between FRAX 597 and Adriamycin or ibrutinib in MCL cell lines. FRAX 597 could overcome the ibrutinib resistant in MCL and ABC-DLBCL resistant cell lines. Mechanistic studies demonstrated that inhibition of Pak2, either by siRNA or chemical inhibitor, substantially diminished the activity of signaling pathways downstream of Rac1-Pak axis, including AKT/mTOR and NF-κB pathways. Collectively, the studies of the report suggest targeting Rac1-Pak2 could be a promising strategy in clinical treatment of aggressive B-cell lymphomas.
Introduction

The small GTPases RAS, Rac, Rho and CDC42 orchestrate many of the hallmarks of cancer. These proteins act as molecular switches existing in two conformational states, GDP and GTP bound. The exchange of GDP for GTP is accelerated by the association of GEPs. RAS mutation disrupt the subsequent hydrolysis of GTP and cause Ras to remain its activated GTP-bound state, are found in about 20% of tumors. Upon activation, small GTPases interact with downstream effectors to elicit their responses. The p21-activated kinases (PAKs) are among the best characterized effectors of Rac and CDC42.

PAKs are a family of serine/threonine protein kinases comprised of six isoforms (PAK1-6). By sequence and structure, the six mammalian PAKs can be categorized into two subgroups: group I (PAK1-3) and group II (PAK4-6). All PAKs possess a conserved C-terminal serine/threonine kinase domain with a single phosphorylation site and an N-terminal regulatory domain. The regulatory domain of group I PAKs is structurally distinct from that of group II PAKs, consistent with the different mechanisms regulating activity of these proteins.

Group I PAKs are thought to be regulated via a trans auto-inhibition mechanism. The N-terminal p21-GTPases binding domain (GBD) overlaps with an autoinhibitory domain (AID). PAKs folds into an inactive homodimer, wherein the AID domain binds to the kinase domain of its partner. Binding of active Rho GTPases such as CDC42 and Rac1 to the GBD, and coincident binding of phosphoinositide to an adjacent segment rich in basic amino acids, leads to dissociation of the AID from the kinase domain reorganization of the dimer and subsequent autophosphorylation. When the phosphorylated kinase domain binds to a substrate, it adopts a monomeric conformation. Subse-
quent autophosphorylation at multiple sites stabilizes this catalytically active state. However, the mechanism of activation of group II PAKs is less clear. Unlike group I PAKs, the kinase domain of the group II PAKs is constitutively phosphorylated. Hence, transition to the active form likely depends on conformational changes. Currently, it was believed that the AID in the N-terminus that inactivates the kinase domain in cis, until binding of GRP-CDC42.

Mounting evidence that cancer is a disease of dysregulated signaling pathways has led to development of signaling-based targeted therapies for various human tumor types. PAKs are positioned at the intersection of a number of signaling pathways required for oncogenesis. When activated by mutation, overexpression, or by upstream elements such as Rac, most PAK isoforms have oncogenic signaling effects in cells, including the acquisition of growth signal autonomy, evasion of apoptosis, and promotion of invasion and metastasis. There is little evidence for cancer cells having activating mutation in PAK genes although a mutation was found in the kinase domain of PAK4 in a colorectal tumor sample. However, PAK family members are amplified, overexpressed or hyper-activated in a number of human tumors. Moreover, several distinct molecular mechanisms caused aberrant PAK signaling in cancer, including gene amplification and alteration of upstream regulators, such as Rac1. Studies have shown that in most cell types, PAK isoforms promote cell cycle progression when overexpressed and hinder such progression when removed or inhibited. The specific mechanism are understood in some detail, as PAKs activate components of the ERK, AKT and Wnt signaling pathways, all of which are closely tied to cell cycle progression. In addition, several members of the PAK family have been shown to inhibit apoptosis. Some of these effects are mediated by phosphorylation of BAD, which renders this protein unable to bind BCL2 and participate in apoptotic signaling. It has been also shown that PAK activates the NF-
κB pathway, which is relevant to several aspects of tumorigenesis, including cell survival, angiogenesis and inflammation\textsuperscript{77}.

All those data with regard to cancers is on the role of Pak1 in various solid tumor, such as breast cancer and pancreatic cancer. No study about PAKs has been done on hematological malignancies. Our previous studies showed Rac1 play a pathogenesis role in MCL and could be a potential therapeutic target in treating MCL. In view of group I PAKs are activated by Rac1, whereas group II PAKs are activated only by CDC42, group I PAKs will be our focus in the following study. Moreover, considering that Pak3 is preferentially expressed in neuronal cells and involved in synapse formation\textsuperscript{79,80}, Pak1 and Pak2 will be our emphases of this Chapter. Furthermore, we extended our study to the other aggressive B-cell lymphomas in this Chapter.

RESULTS

PAKs is overexpressed and activated in aggressive B-cell lymphomas patient samples and cell lines

By analyzing the GEP data of 41 MCL cases, we found that Pak1 and Pak2 mRNA are overexpressed in MCL samples, compared to Naïve B cells (p<0.005) (Figure 3.1A). Figure 3.1B showed Pak2 is the one who has the highest mRNA expression in MCL for Group I PAKs. Next we examined protein levels of Pak1, Pak2 and its activation in a panel of 6 MCL cell lines (Granta-519, Jeko-1, JVM2, Maver-1, Mino and Z138) and two GCB-DLBCL cell lines (DHL16 and LY19), two ABC-DLBCL cell lines (LY3 and TMD8) and two Burkitt lymphoma cell lines (Daudi and GA10). The result showed that, while Pak1 is expressed at higher levels in 3 of the MCL cell lines (Granta 519, JVM2 and Z138) than in Naïve B cells (Figure 3.2), Pak2 is over-expressed in all MCL cell lines compared to the normal control Naïve B cells (Figure 3.2). Moreover, we observed a re-
markably higher expression of p-Pak2 level in two active phosphorylation sites (Ser141 and Thr423), but not p-Pak1 level, in all MCL cell lines than in normal naïve B cells. In DLBCL cell lines and Burkitt lymphoma cell lines, we observed the similar phenomenon, that remarkably expressed Pak1, Pak2 protein and highly activated Pak2 signaling (indicated by increased phosphorylated level) was existed compared with normal B cells (Figure 3.3). All those data indicated that constitutively expressed and activated Pak2 signaling was existed in aggressive B-cell lymphomas.

To confirm the up-regulation of PAKs, we performed IHC analysis of PAKs in one MCL TMA and two DLBCL TMAs. We observed negative staining of Pak1 in both MCL and DLBCL tissues (Figure 3.4A). However, over-expressed Pak2 protein was found in both MCL and DLBCL cases shown in Figure 3.4B. We also observed strong p-PAK staining in our lymphoma tissues (Figure 3.4C). Considering that p-Pak2 level is so much higher than p-Pak1 in our previous western blot experiment, we can assume that strong p-PAK staining was mainly due to the high level of p-Pak2 level in lymphoma tissue. All these IHC results pointed out that Pak2 signaling is constitutively expressed and activated in lymphoma tissues, and highly activated Pak2 signaling may play a tumorigenesis role in lymphoma.

**Knockdown of Pak2, not Pak1, inhibits the proliferation and induces cytostatic effect in aggressive B-cell lymphomas through inhibiting various downstream oncogenic targets**

To determine the specific role of Pak1 and Pak2 in MCL and who is the major one contributing transmitting Rac1 signaling in MCL, we transfected Z138 cell line, which expressed high level of Pak1 and Pak2, with Pak1 and Pak2 siRNA respectively. We observed the knockdown efficiency reached over 50% in Z138 cells for each siRNA (Figure 3.5 A-B). Notably, knockdown of Pak2 significantly decreased cell proliferation in
Z138 cells compared to the control group (9.49 ± 0.27 vs 5.93 ± 0.23 for siRNA1, 5.19 ± 0.29 for siRNA2 at 72h, p<0.001, Figure 3.6A). However, Pak1 knockdown did not induce any cytostatic effect in Z138 cells (7.24 ± 0.19 vs 6.90 ± 0.32 for siRNA1, 6.84 ± 0.05 for siRNA2 at 72h, p>0.05, Figure 3.6B). These data gave us a hint that Pak2 might be the one who contributed to transmit activated Rac1 signaling in MCL system. To explore the dysregulated pathway changes in Pak2 knockdown cells and confirm the role of PAKs in transmitting Rac1 activated signaling, we checked the well-studied Rac1 downstream pathways mentioned previously. As results shown in Figure 3.7A, Pak2 knockdown substantially diminished the activation of AKT/mTORC1 pathway, as shown by diminution in phosphorylation of AKT at both T308 and S473 residues, and p-RPS6 in Z138 cells. In addition, p-P65 was also decreased in Pak2 knockdown Z138 cells, which indicated the decreased activation of NF-κB signaling. However, there is no obvious pathway changes in Pak1 knockdown Z138 cells (Figure 2.7B). These results indicated that the function role of Rac1 is mainly through Pak2, not Pak1, and Rac1-Pak2 play a tumorigenesis role by affecting various oncogenic pathways in MCL.

We also did the same knockdown study on other lymphoma cell lines, ABC-DLBCL cell line TMD8, GCB-DLBCL cell line Ly19 and BL cell line Daudi. Transfection induced the knockdown efficiency reaching over 50% in three cell lines for each siRNA (Figure 3.8 A-B), excepting Pak2 siRNA2 in Ly19 cells, which was not working very efficiently (Figure 3.8A). For the cell viability study, similarly we observed that Pak2 knockdown (9.32 ± 0.11 vs 5.39 ± 0.23 for siRNA1, 5.81 ± 0.07 for siRNA2 at 72h in Daudi, p<0.001; 8.67 ± 0.20 vs 4.34 ± 0.22 for siRNA1, 4.19 ± 0.15 for siRNA2 at 72h in TMD8, p<0.001; 8.52 ± 0.08 vs 6.45 ± 0.07 for siRNA1, P=0.009, 8.32 ± 0.08 for siRNA at 72h in Ly19, p>0.05, Figure 3.9A), not Pak1 knockdown (Figure 3.9B), remarkably inhibited the lymphoma cells growth. This result confirmed the pro-survival role of Pak2 in lym-
phoma system. Western blot also showed that Pak2 knockdown decreased the activation of Akt-mTORC1 and NF-κB signaling pathways (Figure 3.10). Above results indicated that in lymphoma system, Pak2 is the major one who transmitting activated Rac1 signaling and contribute to the lymphoma survival through activating various pro-survival oncogenic pathways.

**PAKs inhibition by chemical inhibitor leads to inhibition of MCL cell proliferation and acts synergistically with Adriamycin treatment**

Knockdown of Pak2 results in the inhibition of lymphoma cell growth and multiple downstream targets. There are several PAKs-inhibitors available for studies. FRAX 597 is a potent group I PAKs inhibitor. Therefore, we determined whether we could achieve the growth inhibition and blockage of the downstream targets of Pak2 in aggressive B cell lymphoma with FRAX 597.

MCL cell lines, Z138 and Mino, were treated with FRAX 597 at different concentrations and cultured for 3 days, cell viability was detected on day 3. We observed significantly decreased cell viability in both cell lines when treated with Pak inhibitor at 1μM (Figure 3.11A-B). Previously, we demonstrated that Rac1 could sensitize the cytotoxicity of chemo-drug Adriamycin. Here, we further treated Z138 and Mino with Adriamycin combined with FRAX 597. Not surprisingly, we observed synergistic effect between Adriamycin and FRAX 597 shown by combination index (CI) level lower than 1 (Figure 3.11 A-B). We also detected the apoptosis in combination of FRAX 597 500 nM with Adriamycin 12.5 nM. Western blot showed significantly increased cleaved-caspase 3 level in combination group compared with single drug alone (Figure 3.11C).

To further confirm the pathway changes upon Pak2 inhibition, Mino cells and Z138 cells were harvested after 24h treatment by 1 μM FRAX 597 and western blots
were performed. FRAX 597 treatment inhibited the phosphorylation of AKT and mTORC1 downstream targets, RPS6 and 4E-BP1. We also observed a downregulation of the phosphorylation of p65 (NF-κB pathway), p42/44 (ERK pathway) (Figure 3.12). All those pathways are supposed to play an important role in promoting cell proliferation and inhibiting apoptosis. In addition, a decreased cyclin D1 level was found after FRAX 597 treatment (Figure 3.12). These data are consistently with previous knockdown results and further confirmed the pathogenic role of Rac1-Pak2 pathway in MCL.

**FRAX597 leads to inhibition of DLBCL and BL cell viability**

Furthermore, we did the same study on other B cell lymphomas, such as DLBCL and BL. Not surprisingly, we observed the remarkably proliferation-inhibition effect of FRAX597 in LY3 and TMD8 cell lines, DHL16 and Ly19 cell lines, Daudi and GA10 cell lines (Figure 3.13). Specifically, 1μM FRAX 579 almost stop the lymphoma cells proliferation (Figure 3.13).

**Synergistic effect was found between FRAX 597 and ibrutinib in Ibrutinib sensitive MCL cell lines and FRAX 597 could overcome ibrutinib resistant in resistant MCL cell lines**

In the treatment of refractory MCL, promising results have been achieved with ibrutinib. However, the biggest problem for ibrutinib in the clinic scenario is drug resistant. Moreover, ibrutinib resistant usually means potentially untreatable. Studies showed that sustained PI3K-AKT activity and activation of the alternative NF-κB signaling pathway can lead to primary or secondary resistance to ibrutinib. As PAK inhibition resulted in the suppression of multiple oncogenic pathways including AKT/mTOR and NF-κB signaling pathways, we questioned whether resistance to ibrutinib could be overcome by FRAX 597 treatment, and if there is synergistic effect between ibrutinib and
FRAX 597 treatment in MCL. We chose four MCL cell lines whose resistance to ibrutinib is known and shown in table 3.1: Mino and Maver-1 (which are responsive to ibrutinib), Z138 (with a TRAF2 mutation contributing alternative NF-κB activation\(^\text{86}\)) and JVM2 (which are resistant to ibrutinib). Cells were treated with escalating doses of ibrutinib and FRAX 597 for 72h and Presto Blue assays were performed. We observed a synergistic effect existed in Mino cells with CI values less than 0.3 (Figure 14A) and in Maver-1 cells (Figure 14B) with CI values less than 0.4 in all combination groups. Additionally, resistance to ibrutinib could be overcome by combined with FRAX 507 shown by CI values less than 0.7 in JVM2 cell line (0.23 for the last combination group) (Figure 3.15A); CI values less than 0.9 for the first two combination groups and less than 0.2 for the last combination group in Z138 cell line (Figure 3.15B).

**FRAX 597 could overcome ibrutinib resistant in resistant ABC-DLBCL cell lines**

Ibrutinib has also been widely used in ABC-DLBCL\(^\text{87}\). However, MYD88\(^\text{TIR mutations}\) (not combined with CD79 A/B mutations) contribute to the primary resistance to ibrutinib in ABC-DLBCL\(^\text{88}\). To testify if resistance to ibrutinib could be overcome by combined with FRAX 597 in ABC-DLBCL, two ibrutinib resistant cell lines, Ly1\(^\text{87}\) and Ly3\(^\text{33}\), were chosen. We observed a synergistic effect at some combination conditions in those two cell lines (Figure 3.16). Specifically, for Ly3 cell line we observed synergistic effect on all combination groups with CI value less than 0.5 (Figure 3.16A). For Ly1 cells, synergistic effect happened on 0.5+0.1, 1+1 and 2+2 combination groups with CI value less than 0.71 (Figure 3.16B). These results indicated that resistance to ibrutinib could be overcome by combined with PAKs inhibitor in ABC-DLBCL.

**DISCUSSION**
DLBCL and MCL are aggressive subtypes of NHL with diverse clinical and molecular characteristic. Significant proportions of DLBCL and almost all patients with MCL ultimately develop resistant disease. Introduction of rituximab (R) to the standard CHOP regimen in DLBCL and MCL has significantly improved outcomes. However, patients with high-risk disease continue to have unfavorable outcomes, underscoring the biologic heterogeneity of this disease. There is a critical need for a clear understanding of the pathogenic mechanisms of DLBCL and MCL in order to identify attractive and promising targets.

Our previous study has identified aberrantly over-expressed Rac1 in MCL cases and cell lines, and the overexpression correlated with higher proliferation rate and poor prognosis of MCL patients. In addition, Rac1 inhibition by shRNA or inhibitor significantly decreased MCL cell proliferation and increased the cytotoxicity of chemo-drug Adriamycin. We also extended Rac1 study to DLBCL and BL. Similar results indicated that Rac1 also contribute to the dysregulated oncogenic pathways in DLBCL and BL, and play a tumorigenesis role. However, Rac1 is a member of Rho GTPase, which is hard to target. There is no potent Rac1 specific inhibitor that can be used in further in vivo study. Studies have showed that PAKs are among the best-characterized effector of Rac1. Therefore, we are trying to identify if activated Rac1 signal is through PAKs and targeting PAKs might be a better choice.

PAK serine/threonine kinases have recently been found to be key regulators of cancer-cell signaling networks. They exert regulatory control over essential biological processes. For group I PAKs, Pak1 is the most established isoform in human cancers, like breast cancer, colon cancer, and ovarian cancer. The roles of Pak2 are beginning to be discovered. In our study, we demonstrated that Pak2 protein were aberrantly overexpressed in primary MCL and DLBCL tumors and established MCL and DLBCL
cell lines. Moreover, highly activated Pak2 were found in both MCL, DLBCL cell lines and clinical cases shown by overexpressed phosphorylation level of Pak2. All these indicated that hyper-activated Rac1-pak2 signaling are existed in aggressive B-cell lymphomas.

Gene rearrangement is a hallmark of lymphoma, such as involving Cyclin D1 is the hallmark of MCL, involving MYC is the hallmark of BL. However, it has been demonstrated that Cyclin D1 overexpression alone is insufficient to induce the onset of MCL, raising the importance of other mechanisms in MCL lymphomagenesis. Aberrant activities of PI3K/AKT/mTOR and NF-κB pathways have been reported in various types of B-cell neoplasms. Constitutively activation of NF-κB has frequently been observed in follicular lymphoma, DLBCL, mucosa-associated lymphoid tissue (MALT) lymphoma, multiple myeloma and MCL, as well as MCL cell lines, in which inhibition of this constitutive activation induces growth arrest and apoptosis. Activation of PI3K/AKT/mTOR pathway is one of the most common defects in human malignancies, including BL, MCL and Hodgkin's lymphoma. The repeated discovery of the involvement of NF-κB and PI3K/AKT/mTOR in distinct forms of B-cell neoplasia underscores the importance of these signaling pathways in B-cell transformation. Additionally, evidences showed that NF-κB and PI3K pathways are interconnected in various lymphoma models, such as iMyc B cell lymphoma. Signaling through each of these molecules is required for tumor maintenance and Myc expression, and combined inhibition results in additive suppression of tumor growth. In this study, we demonstrated that Pak2 is directly associated with the activation of several pro-survival oncogenic pathways mentioned above in MCL, as well as in DLBCL and BL, suggesting that it locates at the central node of pathway network. Moreover, these pathways have also been demonstrated as the downstream pathways of Rac1, which means that Pak2 functioned as transmitting activated Rac1
signal in aggressive B-cell lymphomas. This indicated that inhibiting Rac1-Pak2 axis could be a way to inhibit lymphoma by shutting down constitutively activated various oncogenic pathways all at once.

Not surprisingly, we found that the inhibition of Pak2, not Pak1, brought about a cytostatic effect, which consistent with the above pathway changes. Notably, inhibition of Pak2 either by siRNA or by specific inhibitor FRAX 597 significantly inhibited cell growth of MCL cell lines, also DLBCL and BL cell lines. As we known, ibrutinib, available BTK-inhibitor, could achieve significant efficacy in the treatment of refractory or relapsed MCL. However, somatic mutations in NF-κB regulatory genes can confer resistance to ibrutinib treatment in MCLs\textsuperscript{100}. Interestingly, Pak2 inhibition resulted in the inhibition of the NF-κB signaling pathways not only in the MCL cell lines Mino, but also in Z138, which has a known TRAF2 mutation (contribute to the Ibrutinib resistant). Combined treatment of ibrutinib and FRAX 597 turned out to be synergistic and ibrutinib resistance in Z138 and JVM2 cells could be overcome by FRAX 597 through inhibition of the NF-κB signaling pathway. For ABC-DLBCL, constitutive activation of BCR leads to activation of the NF-κB pathway via a cascade of kinases, such as SYK, BTK, protein kinase C-β (PKCβ)\textsuperscript{101}. Inhibition of BTK with ibrutinib has demonstrated an overall response rate of 41% in ABC-DLBCL. However, patients with CD79B\textsuperscript{WT} and MYD88\textsuperscript{mut} or CARD11\textsuperscript{mut} did not respond to ibrutinib, indicating the need for therapies targeting BCR-independent activation of NF-κB induced by these mutations. Our results showed FRAX 597 could overcome ibrutinib resistance in two ibrutinib-resistant ABC-DLBCL cell lines, Ly3 and Ly1. This conclusion indicated that PAK inhibition could be a potential therapeutic approaches that can combined with ibrutinib in MCL and ABC-DLBCL patients. Moreover, FRAX 597 combined with another chemo-drug Adriamycin also induced synergistic effect in
MCL. All these results indicated the potential of PAK inhibitor as combination treatment agent in the future study.

In summary, Pak2 inhibition by siRNA and inhibitor demonstrated promising anti-tumor growth activities in MCL, DLBCL and BL cells. The potential mechanism is Pak2 inhibition leads to significant inhibition of two important survival pathways: the PI3K/AKT and NF-κB pathways, which are the two most important survival pathways in aggressive B-cell lymphomas. PAK inhibitor combined with ibrutinib or Adriamycin induced synergistic effect in MCL. Furthermore, PAK inhibitor could overcome ibrutinib resistant in resistant MCL and ABC-DLBCL cell lines. Therefore, a molecularly based clinical exploration of FRAX 597 in combination with standard of care chemotherapy, ibrutinib, or other targeted therapies is a promising combination therapeutic approach for the treatment of aggressive B-cell lymphomas.
Figure 3.1 Expression of PAKs in human primary MCL tumors.

(A) Analysis of Pak1 and Pak2 mRNA level in human primary MCL cases from the LLMPP database. (B) mRNA expression of all group I PAKs members in MCL cases. Expression levels are the values of probes in affymetrix HG-U133-plus 2.0 arrays. Data are shown as mean ± SEM. P value was calculated using the two-tailed student’s t test. ** indicates P < 0.001.
A

Relative mRNA expression

**Naive B cells**

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**P<0.005**

B

mRNA expression (From GEP)

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**P<0.005**
Figure 3.2 PAKs are overexpressed and constitutively activated in MCL cell lines.

Pak1, Pak2, p-PAKs and β-Actin protein expression in MCL cell lines was analyzed by western blot.
Figure 3.3 Over-expressed and activated Pak2 signaling in aggressive B-cell lymphomas cell lines.

Pak1, Pak2, p-PAKs and β-Actin protein expression in MCL cell lines was analyzed by western blot.
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- Pak1 (Ser144)
- Pak2 (Ser141)
- Pak1 (Thr423)
- Pak2 (Thr402)
Figure 3.4 Representative images of the IHC staining of PAKs and p-PAK in tissues.

Representative images of Pak1 staining (A), Pak2 staining (B) and p-PAK staining in MCL case tissues.
Figure 3.5 Pak1 and Pak2 knocking down in Z138 cell line.

(A) Pak1 was knocked down by two different Pak1 siRNAs (siRNA 1 and 2) transfection in Z138 cells. (B) Pak2 was knocked down by two different Pak2 siRNAs (siRNA 1 and 2) transfection in Z138 cells. The protein level was determined by western blot 48h after transfection. The relative Pak1 and Pak2 levels in the knockdown group were determined by normalization of the Pak1 and Pak2 levels with their respective β-Actin levels.
Figure 3.6 Pak2 promotes cell proliferation and survival in MCL.

(A) Cell proliferation of Pak2 siRNAs group (siRNA1 and siRNA2) and control group in Z138 cell line was determined for 3 days by Presto Blue cell proliferation assay. (B) Cell proliferation of Pak1 siRNAs group (siRNA1 and siRNA2) and control group in Z138 cell line were determined for 3 days by Presto Blue cell proliferation assay. Define the cells number of each group on day 0 as 1 and the y-axis stands for the increased folds of cell numbers on day 3 compared to the day 0. The experiments were repeated three times in four replicates with similar results obtained. Data shown are the average of three experiments and are presented as mean ± SEM.  P value stands for the difference between Pak2 knockdown group and control group. ***P≤0.001.
Cell proliferation (Folds of cell seeded)

A

Control
Pak1 siRNA 1
Pak1 siRNA 2

B

Control
Pak2 siRNA 1
Pak2 siRNA 2

***
Figure 3.7 Pathway changes of Pak2 knockdown and Pak1 knockdown in MCL cell line Z138.

Pak2-siRNA expressing and control cells (A), Pak1-siRNA expressing and control cells (B) were examined for phosphorylation of PAKs, AKT, RPS6, NF-κB (p65) and β-Actin by immunoblotting 48h after transfection. Ctr, control.
A

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Figure 3.8 PAKs knocking down in DLBCL cell lines (TMD8 and Ly19 cell lines) and BL (Daudi cell line).

(A) Pak2 was knocked down by two different siRNAs in Daudi, TMD8 and Ly19 cell lines. 
(B) Pak1 was knocked down by two different siRNAs in TMD8 and Ly19 cell lines. The protein level was determined by western blot 48h after siRNA transfection. The relative Pak2 or Pak1 level in the knockdown group were determined by normalization of the Pak2 or Pak1 level with their respective β-actin levels.
### A

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Figure 3.9 Pak2 knockdown, not Pak1 knockdown, induced inhibition of cell viability of DLBCL cell lines.

(A) Cell proliferation of Pak2 siRNAs group (siRNA1 and siRNA2) and control group in TMD8, LY19 and Daudi cell lines were determined for 3 days by Presto Blue cell proliferation assay. (B) Cell proliferation of Pak1 siRNAs group (siRNA1 and siRNA2) and control group in TMD8 and Ly19 cell lines were determined on day 3 by Presto Blue cell proliferation assay. Define the cells number of each group on day 0 as 1 and the y-axis stands for the increased folds of cell numbers on day 3 compared to the day 0. The experiments were repeated three times in four replicates with similar results obtained. Data shown are the average of three experiments and are presented as mean ± SEM. P value stands for the difference between Pak2 knockdown group and control group. ***P≤0.001. **P≤0.01.
Figure 3.10 Pathway changes in Pak2 knocking down aggressive B-cell lymphoma cell lines.

Pak2-siRNA expressing and control cells were examined for phosphorylation of PAKs, AKT, RPS6, NF-κB (p65) and β-Actin by immunoblotting 48h after transfection. Ctr, control.
Figure 3.11 Inhibition of Rac1 by FRAX 597 induces cytostatic effect in MCL cells and synergistic effect was found between FRAX 597 and Adriamycin in MCL cell lines.

Z138 (A) and Mino (B) cells were treated with FRAX 597 single drug, and combined with Adriamycin, at indicated concentrations for 3 days and assessed for cell viability by Presto Blue assay. Define cells number of each group on day 0 as 1 and Y-axis stands the increased folds of cells number on day 3 compared to the day 0. The experiments were repeated three times in four replicates with similar results obtained, and an average Presto Blue fluorescence unit is shown. PFUs: Presto Blue fluorescent units. Combination index (CI) between these two drugs was calculated by using Compusyc software. (C) Cells were treated with FRAX 597 at 500nM group, Adriamycin at 12.5 nM group and combination group. Western blot showed the cleave-caspase 3 level in those groups.
Figure 3.12 Oncogenic pathway changes upon FRAX 597 inhibition in MCL.

Mino and Z138 cells were treated with 1 μM FRAX 597 for 24h and followed by Western blot analyses for the indicated protein phosphorylation and total levels.
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Figure 3.13 Inhibition of PAKs by specific inhibitor, FRAX 597, induces cytotoxic effect in DLBC and BL cell lines.

Ly19, TMD8 and Daudi cells were treated with FRAX 597 at indicated concentrations for 3 days and assessed for cell viability by Presto Blue assay. Define cells number of each group on day 0 as 1 and Y-axis stands the increased folds of cells number on day 3 compared to the day 0. The experiments were repeated three times in four replicates with similar results obtained, and an average Presto Blue fluorescence unit is shown.
FRAX 597 (uM)

Cell proliferation
(Folds of cell seeded)

FRAX 597 (uM)

Cell lines

Ly19  TMD8  Daudi

0  0.125  0.25  0.5  1  2
Figure 3.14 Synergistic effect was existed between FRAX 597 and ibrutinib in ibrutinib sensitive cell lines, Mino and Maver-1.

Mino (A) and Maver-1 (B) cells were treated with FRAX 597 or ibrutinib single drug and combination at indicated concentrations for 3 days and assessed for cell viability by Presto Blue assay. Define cells number of each group on day 0 as 1 and Y-axis stands the increased folds of cells number on day 3 compared to the day 0. The experiments were repeated three times in four replicates with similar results obtained. Combination index (CI) between these two drugs was calculated by using Compusyc software.
Figure 3.15 PAK inhibitor, FRAX 597, could overcome ibrutinib resistant in resistant MCL cell lines, JVM2 and Z138.

JVM2 (A) and Z138 (B) cells were treated with FRAX 597 or ibrutinib single drug and combination at indicated concentrations for 3 days and assessed for cell viability by Presto Blue assay. Define cells number of each group on day 0 as 1 and Y-axis stands the increased folds of cells number on day 3 compared to the day 0. The experiments were repeated three times in four replicates with similar results obtained, and an average Presto Blue fluorescence unit is shown. Combination index (CI) between these two drugs was calculated by using Compusyc software.
A  

**JVM2**

Cell proliferation

Ibrutinib (µM)  
FRAX 597 (µM)

Ibr  
FRAX 597  
Ibr +FRAX 597

CI  0  0.61  0.57  0.23

B  

**Z138**

Cell proliferation

Ibrutinib (µM)  
FRAX 597 (µM)

Ibr  
FRAX 597  
Ibr +FRAX 597

CI  0  0.74  0.8  0.19
Figure 3.16 Synergistic effect was found between FRAX 597 and ibrutinib in ibrutinib-resistant ABC-DLBCL cell lines.

Ly3 (A) and Ly1 cells (B) were treated with FRAX 597 or ibrutinib single drug and combination at indicated concentrations for 3 days and assessed for cell viability by Presto Blue assay. Define cells number of each group on day 0 as 1 and Y-axis stands the increased folds of cells number on day 3 compared to the day 0. The experiments were repeated three times in four replicates with similar results obtained, and an average Presto Blue fluorescence unit is shown. CI between these two drugs was calculated by using Compusyc software.
A

Ly3

B

Ly1

Cell proliferation

Ibrutinib (µM) 0 0.001 0.01 0.1 1
FRAX 597 (µM) 0 0.125 0.25 0.5 1
CI 0 0.23 0.43 0.37 0.5

Cell proliferation

FRAX 597 (µM) 0 0.125 0.25 0.5 1 2
Ibrutinib (µM) 0 0.001 0.01 0.1 1 2
CI 0 1 1.25 0.51 0.39 0.71
### Table 3.1 IC50 of ibrutinib and resistant status in MCL cell lines

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<tr>
<td>Z138</td>
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Chapter 4 Major conclusions and future directions
Major conclusions

This dissertation describes the exciting discovery of highly activated Rac1-Pak2 axis and their tumorigenesis role in aggressive B cell lymphomas, including MCL, DLBCL and BL. The function of Rho GTPase family member in lymphoma is not clear. In Chapter 2, we did GEP in 41 MCL cases and analyzed the whole Rho GTPase members, turned out Rac1 is the only GTPase that has a relatively higher mRNA expression in MCL cases than in normal control naïve B cells. Further study confirmed the expression in the protein level by showing that higher total-Rac1 level and Rac1-GTP expression in MCL cases and cell lines than in normal control. In addition, Rac1 higher expression correlated with the higher proliferation rate of MCL cases and the poor overall survival in MCL patients. The following functional study demonstrated that inhibiting Rac1 brought out cytostatic effect in MCL cell lines, and sensitized the cytotoxicity effect of chemodrug. We also extended our Rac1 study to the DLBCL and BL. We demonstrated that inhibiting Rac1 could also induce cytostatic effect by inhibiting various oncogenic pathways in DLBCL and BL.

In Chapter 3, we described the discovery of Pak2, not pak1, is the one that passed the activated Rac1 signal to the downstream various oncogenic pathways in aggressive B cell lymphomas. Inhibiting Pak2 decreased the lymphoma cells viability by diminishing the activation of AKT/mTOR and NF-κB pathways, which are well known as two important survival pathways in aggressive B-cell lymphomas. Combination treatment study demonstrated synergistic effect was existed between PAK inhibitor and ibrutinib or Adriamycin in MCL cell lines and ibrutinib resistance could be overcome by combined with PAKs inhibition in resistant MCL and ABC-DLBCL cell lines. In summary, we present a proof-of-concept that targeting Rac1-Pak2 axis might be a potential approach used in combination treatment to treat relapsed and refractory MCL and DLBCL, two
subtypes of NHL with AKT/mTOR and NF-κB pathways dysregulations, where PAKs inhibitor is therapeutically relevant.
Future directions

Generate secondary resistant to ibrutinib in MCL and ABC-DLBCL cell lines and identify the resistant overcome by PAKs inhibitor

Ibrutinib is a novel BTK inhibitor that has shown an unprecedented overall response rate and progression-free survival in relapsed/refractory MCL patients and in ABC-DLBCL. Clinically, ibrutinib rapidly induced lymphocytosis and lymph node shrinkage. Unfortunately, despite the dramatic responses to ibrutinib, resistance inevitably develops. Approximately 43% of MCL patients have shown partial or complete lack of response to ibrutinib and experience disease progression within 12 months of treatment. Our study has demonstrated that primary resistant to ibrutinib (JVM2, Z138, LY3 and Ly1 cells) could be overcome by PAK inhibitor. In the future direction, we will determine if PAK inhibition could overcome acquired ibrutinib resistant.

Two approaches will be used. Traditionally, acquired drug resistance would be studied by chronically exposing cancer cell lines in vitro to increasing does of drug. Therefore, firstly, we will generate acquired ibrutinib resistant Mino, Maver-1 and TMD8 cell lines. ABPP (activity-based proteomics) will be used to compare changes in relative kinase activities between resistant cells and parental cells at a system level. Studies have mentioned that BTK-independent kinases or BTK bypass pathways drive ibrutinib resistant, such as PI3K-AKT-mTOR-related kinases. We will confirm this in our future study and try to find more potential targeted kinases. PAKs inhibitor will be used to identify if PAKs inhibition could overcome the acquired ibrutinib resistance.

Secondly, drug resistance is heavily influenced by the extrinsic tumor microenvironment (TME). TME-mediated resistance is a form of de novo drug resistance that protects tumor cells from the effects of diverse therapies. Lymph node stromal cells (HK
cells) and bone marrow stromal cells (HS5) will be used to co-culture with Mino, Maver-1 and TMD8 cell lines. ABPP will be used to examine the kinase response profiles in those cells modulated by stroma. Combining the protein kinase reprogramming data of acquired drug resistance cells, a major kinase network can be formed. Moreover, this result can be instrumental in the identification of potential drug targets. Also, PAKs inhibitor will be used here to determine if PAKs inhibition could improve resistance mediated by lymphoma-stromal cell interactions.

**Identification of Pak2-interacting protein profiles in MCL**

To identify Pak2-interacting proteins, a combination of co-immunoprecipitation assay (Co-IP) with mass spectrometry (MS) analysis will be conducted in the future.

Pak2 knock down Mino and Z138 stably expressing an N-terminal HA-tagged full-length human Pak2 cDNA (HA-Pak2) will be generate using a retroviral system. The following Pak2 immunoprecipitation (IP) analysis will be performed using anti-HA affinity matrix. To map out the molecular interaction of Pak2, anti-HA Co-IP products will be examined by MS analysis. This approach will prove that the direct interaction between Pak2 and specific proteins and give clues about the other potential binding interfaces.

**Study of combination effects of PAKs inhibitor and BTK inhibitor in animal models**

Our *in vitro* study had demonstrated that synergistic effect was existed between FRAX 597 and ibrutinib or Adriamycin in lymphoma cell lines. FRAX 597 could also overcome the ibrutinib resistant in MCL and ABC-DLBCL resistant cell lines. All these *in vitro* data indicated the potential use of FRAX 597 as drug combination agent. Therefore, *in vivo* study is needed to be done to further confirm the in vitro study.
Cell line-derived xenograft and patient-derived xenograft mice models will be used in the future study. Mice will be randomly divided into 4 groups and treated with vehicle, ibrutinib 25 mg/Kg oral gavage daily alone, FRAX 597 25 mg/Kg intraperitoneal injection daily alone and ibrutinib + FRAX 597 (ibrutinib 25 mg/kg oral gavage daily with FRAX 597 25 mg/Kg intraperitoneal injection daily). Tumor volumes will be examined.
Chapter 5 Materials and methods
Cell lines and culture medium

MCL cell lines (Jeko-1, JVM2, Mino, Mauer-1 and Z138 cells), ABC-DLBCL cell lines (TMD8 and Ly3 cells), GCB-DLBCL cell lines (LY19 and DHL16 cells) and BL cell lines (GA10 and Daudi cells) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Invitrogen). Granta-519 cells and GP2-293T cells were cultured in DMEM culture medium with 10% FBS. Naïve B cells were isolated from peripheral blood mononuclear cells using a naïve B cell isolation kit (Miltenyl Biotec, CA). Normal B cells were isolated from normal tonsil using a CD19 isolation kit (Miltenyl Biotec, CA).

Chemical reagents

Rac1 inhibitor NSC 23766 was purchased from Tocris Biosciences (Ellisville, MO). PAK inhibitor FRAX 597 was purchased from Tocris Biosciences (Ellisville, MO). BTK inhibitor ibrutinib was purchased from Selleckchem (Houston, TX).

Immunohistochemistry

Tissue from 32 MCL cases and 2 Tonsils were obtained from the University of Nebraska Medical Center (UNMC), embedded in paraffin and arranged into tissue microarrays (TMAs). TMAs were deparaffinized in xylene followed by rehydration through graded ethanol. Endogenous peroxidase was blocked using peroxidase-quenching solution (SuperPicture3rd Gen IHC Detection Kit, Life Technology) for 10 min. The slides were then boiled in citrate buffer (pH 6.0, Dako) for 1 hour, allowed to cool and rinsed with PBS. The sections were incubated overnight at 4 °C with primary antibody (Rac1 1:100, #23A8 from EMD MILLIPORE) in Signal Stain antibody diluent (Cell Signaling Technology). Slides were then washed with PBS and incubated with HRP polymer conjugate (SuperPicture3rd Gen IHC Detection Kit) for 1 h at room temperature, followed by PBS wash and then stained with DAB reagent. Photographs were taken using Roche Ventana Scan HT. All clinical samples and features of the patients were retrieved from
the clinical database of Department of Pathology and Microbiology at UNMC. This study was approved by the institutional review board of UNMC.

**Gene expression profiling (GEP)**

Previously, the LLMPP conducted a comprehensive GEP of MCL specimens. As a result, a set of proliferation genes (proliferation signature) were identified representing a biological integrator of oncogenic events associated with cell proliferation, which allowed the definition of prognostic subgroups that differ in median survival by more than 5 years\textsuperscript{102,103}. In our study, GEP of 41 molecularly defined MCL cases from the LLMPP were analyzed for Rac1 expression. Moreover, patients were divided into three groups according to their Rac1 mRNA expression levels (high, median and low Rac1 groups, see Table 1), then gene expression-based proliferation signatures between Rac1 high and low groups was analyzed.

**Antibodies and immunoblotting**

Cell lysates were separated and immunoblotted by using Bolt\textsuperscript{®} Bis-Tris system (Life technology, Carlsbad, CA). Imaging and quantitative analyses were performed by Odyssey CLX system (LI-COR Biosciences, Lincoln, NE). The antibodies for immunoblots are as follows: AKT (#4685), p-AKT (#4060), p-AKT (#13038), p-RPS6 (#4858), RPS6 (#2217), 4E-BP1 (#9644), p-4E-BP1 (#2855), p-ERK1/2 (#4370), ERK1/2 (#4695), p-P65 (#3033), P65 (#8242), cleaved-caspase (#9664) and PAK1/2/3 antibody sampler kit (#4750) were all from Cell Signaling Technology (Danvers, MA). β-Actin (#SC-47778) was from Santa Cruz Biotechnology (Dallas, TX). Rac1 (#23A8) was from EMD MILLIPORE (Billerica, Massachusetts). Anti-Pak1 (phospho S144) + Pak2 (phospho S141) + Pak3 (phospho S154) antibody (ab4040795) was from Abcam (Cambriage, MA).
Quantitative reverse transcription PCR (qRT-PCR)

RNA was isolated using The Quick-RNA™ MiniPrep (Plus) kit (ZYMO Research). The SuperScript® III First-Strand Synthesis System (Invitrogen) was used for cDNA synthesis. Real-time PCR was performed using DyNAmo HS SYBR Green qPCR kit (Thermo Scientific Inc.) with CFX connect (Bio-Rad, Hercules, CA) real-time thermocycler. The ΔCT method was used to calculate the relative mRNA expression level by normalizing with the expression of housekeeping gene RPL13A. QRT-PCR primers used in this study are as follows: Rac1 forward: CTGATGCAGGCCATCAAGT; Rac1 reverse: TCTCCAGGAAATGCATTGGT. RPL13A forward: AGATGGCGGAGGTGCAG; RPL13A reverse: GGCCCAGCAGTACCTGTGA.

Rac1 activity assay

A glutathione-S-transferase (GST)-PAK1 (PAK-CRIB domain) fusion protein, containing the Rac1 binding region from human PAK1B, was used to determine Rac1 activity as described. Mino cells were pretreated with serum starvation (1% FBS containing medium) for 24h, followed by treated with different concentrations of NSC 23766 for 2 hours. This was then followed by adding epidermal growth factor (EGF) (Sigma-Aldrich) at 100 ng/mL for 15 min, re-suspension in lysis buffer (50 mM Tris-HCL (pH 7.4), 100 mM NaCL, 2 mM MgCl₂ and 1 mM benzamidine; 1% NP-40; 10% glycerol; 1 µg/mL leupeptin, pepstatin, and aprotinin) and centrifugation at 1500 rpm for 5 min at 4°C. For negative control, supernatant protein was pretreated with 10mM EDTA (Boston BioProducts, Inc., Ashland, MA) and 1 mM GDP (20-177, EMD Millipore) for 15 min at 30°C with rotation. Equal amounts of supernatant protein (including negative control tube) were incubated with the GST-PAK1 fusion protein bound to glutathione-coupled Sepharose beads at 4°C for 1h with rotation to capture GTP-bound Rac1. Beads were
washed 3 times with lysis buffer and eluted in Laemmli buffer (60 mM Tris (pH 6.80), 2% sodium dodecyl sulfate, 10% glycerin, 0.1% bromophenol blue). SDS-PAGE was performed on samples using a 4-20% gel and bound Rac1 analyzed by immunoblotting.

**Rac1 shRNA knockdown in MCL cell lines**

For the inducible shRNA expression, miR-E based shRNA was cloned into retroviral vector p-Retro-x (Clontech, CA). Virus was packaged by co-transfecting GP2-293T cells with constructed vectors and pCL-ampho. After 48h of transfection, virus-containing medium was collected and precipitated with PEG at 4°C overnight. Targeted cells were then transduced with concentrated lentivirus with TransDux (System Biosciences, CA) and incubated for 48h followed by selection with puromycin. shRNA expression was initiated by Dox at 1 µg/mL and the knockdown efficiency was determined by Western blot after 24h of induction. In all, two shRNAs were tested and the one with the highest knockdown efficiency was chosen for subsequent functional studies. The Rac1-shRNA sequences are shRNA1: gggcatattaattcatttta, and shRNA2: cgacagtgcactgtgaccaa.

**Rac1 overexpression in MCL cell lines**

For transduction, pcDNA3-EGFP-Rac1-wt was modified into a retroviral expression plasmid pMIP-w containing WRE element and puromycin resistant gene as selection marker, which as modified from pMIG-w, a gift from Luk Parijs (Addgene plasmid #12282). Expression plasmid was mixed with pCL-Ampho retrovirus packaging vector (Novus) in the presence of TransIT-LT1 transfection reagent, and then were transfected into GP2-293T cells. Virus containing culture medium was collected from GP2-293T at 48h and 72h post transfection. Virus was concentrated using PEG-it Virus Precipitation
Solution (System Biosciences) and resuspended with RPMI medium and Transdux (System Biosciences) to infect MCL cell lines (JVM2 and Z138 cells).

**Rac1, Pak1 and Pak2 silencing**

To inhibit expression of Rac1, Pak1 or Pak2 gene, a pool of specific siRNAs was purchased from IDT and transfected using NEON Transfection System (Invitrogen, Carlsbad, CA). In detail, $4 \times 10^5$ were transfected with a 10 µl-tip at 40nM concentration of siRNA pool, or with mock transfection buffer alone. After electroporation, cells were seeded into cell culture six-well plates and incubated for 48 h before additional treatments.

**Analysis of viability and apoptosis**

Cell proliferation and viability were determined by Presto Blue cell proliferation/viability assay kit (Invitrogen, CA) following the manufacturer’s instructions. Fluorescence intensity was measured using Plate reader Infinite® 200PRO (Tecan, Switzerland) at 560 nm for excitation and 590 nm for emissions. Apoptosis was determined by Annexin V-FITC and propidium iodide (PI), or Annexin V-PE and 7-AAD staining (BD Pharmingen) according to the manufacturer’s instructions followed by quantification using a FACS Calibur flow cytometer (BD Biosciences).

**Statistical analyses**

For correlation and survival analyses, patients were divided into two groups according to Rac1 IHC staining (cutoff: 30%). Pearson's w2-test was used to analyze correlations between Rac1 status and OS or event free survival (EFS). Survival was estimated using the Kaplan–Meier method, and the comparison between study groups was
performed with the log-rank test. The end-point EFS was calculated as time from diagnosis to the first documented recurrence.

For in vitro experiments, group comparisons were evaluated using the two-tailed, unpaired $t$-test (equal variance). All statistical analyses were performed using SPSS 16.0 and are shown as means ± SD (standard deviation). P-values <0.05 was considered significant.
Chapter 6 Bibliography


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76. Rane CK, Minden A. P21 activated kinases: structure, regulation, and functions. Small GTPases;5.


