Rac1 is a novel therapeutic target in mantle cell lymphoma

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Mantle cell lymphoma (MCL) is an aggressive B-cell lymphoma, comprising 6–8% of human B-cell non-Hodgkin lymphomas1. R-CHOP, which combines rituximab with cyclophosphamide, hydroxydaunorubicin (Doxorubicin/Adriamycin), oncovin (Vincristine) and prednisone, is the most common regimen employed for treating MCL. Nevertheless, most patients are destined to relapse after initial therapy2, highlighting the urgent need for new therapeutic strategies. Here, we explore a member of Rac family of small guanosine triphosphatases (GTPase), Rac1, as novel target for MCL treatment.

Rac family proteins cycle between active GTP-bound and inactive GDP-bound states. Studies have shown that Rac1 plays a critical role in a variety of cellular responses, including cell proliferation and survival, gene transcription, adhesion, motility and forming of the actin cytoskeleton3. Overexpression of Rac1 has been reported in several types of solid tumors, including breast cancer and pancreatic cancer4,5. In hematological malignancies, several types of solid tumors, including breast cancer and acute leukemia in a murine model in vivo6. However, the role of Rac1 in lymphoma thus far has not been clearly defined.

Rac1-GTP interacts with multiple effectors and activates numerous downstream signaling pathways such as PI3K/Akt/mTOR pathway not only contribute to aggressiveness of MCL, but also crosstalk with other oncogenic pathways such as NF-κB signaling pathway8,9. In addition, ERK1/2 pathway is also critical to the proliferation as well as survival of MCL tumor cells through inhibition of BCL-2 family member BCL-XL10. These findings suggest that Rac1 is likely to play an important role in the pathogenesis of MCL.

By analyzing the gene expression profiling (GEP) data of 41 MCL cases, we found that Rac1 mRNA is overexpressed in MCL tumor samples (Fig. 1a). We also examined the levels of Rac1 mRNA and protein in a panel of MCL cell lines. The results showed that Rac1 mRNA is overexpressed in four of six MCL cell lines (Jeko-1, Mino and Z138) compared to naive B cells (Fig. 1b), while the Rac1-GTP protein level is markedly increased in all tested MCL cell lines compared to naive B cells (Fig. 1c). 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 upregulation of Rac1, we performed immunohistochemical (IHC) analysis in 32 MCL cases. In normal lymphoid tissue, mantle zones of follicles were negative for Rac1 (Fig. 1d), whereas 18 cases of MCL (18/32; 56%) showed positive expression for Rac1, with six cases each falling into weak, medium and strong staining groups, respectively (cutoff value 30%) (Supplemental Table 1 and Fig. 1f, g). Furthermore, we correlated Rac1 expression with clinical outcome and found that Rac1 positivity was strongly associated with shorter overall survival (OS, p = 0.039) (Fig. 1h).

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 Fig. 1, higher Rac1
level was significantly correlated with higher levels of the proliferation signature, as established in our previous study \((p < 0.001)^{11}\). Next, we transduced MCL cell lines Z138 and Mino, which express high levels of both Rac1 and Rac1-GTP, with Rac1-shRNA by inducible retroviral vector. Upon doxycycline (Dox) induction, Rac1 protein
Fig. 2 (See legend on next page.)
level was decreased by 55% and 50% in Z138 and Mino cells, respectively, compared to the vector controls (Fig. 2a). Notably, knockdown of Rac1 decreased cell viability by 43% \((p < 0.001)\) and 24% \((p = 0.002)\) in Z138 and Mino cells, respectively, after 3 days of continuous culture (Fig. 2b). To confirm the result, we deployed a commonly used selective inhibitor NSC23766 \(^{12}\) to inhibit Rac1. After 2 h incubation, half maximal effective concentration \((EC_{50})\) of NSC23766 toward Rac1 inhibition was observed at 25 μM (Supplemental Fig. 2a). Cell viability of Z138 and Mino were substantially inhibited by NSC23766 in a dose-dependent manner within its linear dose range for Rac1 inhibition (Fig. 2c). Notably, naïve B cells were barely affected by NSC23766 treatment, even at the 100 μM concentration (Supplemental Fig. 2b). Moreover, we overexpressed Rac1 in Z138, as well as JVM2 cells that express lower level of both Rac1 and Rac1-GTP (Fig. 2d). The result showed that Rac1 overexpression substantially rescued the cells from the inhibitory effect of NSC23766 (Fig. 2e). These results suggest that targeting Rac1 provides a specific toxic effect toward MCL lymphoma cells.

In view of Adriamycin being a core reagent of R-CHOP regimen, we tested the combination effect of Rac1 depletion with Adriamycin in MCL cells. As shown in Fig. 2f, knockdown of Rac1 in MCL cells had little effect on cell survival. However, when combined with Adriamycin, Rac1 knockdown cells exhibited a nearly 2-fold increase in apoptosis compared to the control cells. Similarly, the addition of NSC23766 to Adriamycin significantly increased the inhibitory effect after 72 h treatment \((3.65 ± 0.05)\) vs. \(3.12 ± 0.02\) folds increase in apoptosis for Mino, \(p = 0.007\); \(10.8 ± 0.38\) vs. \(4.2 ± 0.07\) folds increase for Z138, \(p < 0.001\), though synergy effect was not clearly demonstrated (Supplemental Figure 3a). We also specifically investigated the cytotoxic effect and found that the combination treatment induced apoptosis ~2.36-fold and 1.68-fold higher in Z138 and Mino cells, respectively, than that of Adriamycin alone treatment (Supplemental Figure 3b). These data suggest that Rac1 inhibition enhances the cytotoxic effect of Adriamycin, further supporting its significance in clinical practice.

To explore the molecular mechanism underlying the functional roles of Rac1 dysregulation, we investigated several oncogenic pathways of MCL upon Rac1 depletion. As shown in Supplemental Figure 4a, Rac1 knockdown substantially diminished the phosphorylation of Akt at both T308 and S473 residues. Consistently, Akt downstream target mTORC1 also exhibited a decreased activity, as shown by diminution in phosphorylation of RPS6 \((S235/S236)\). In addition, phosphorylation of RelA/p65 \((S536)\) and ERK1/2 \((T202/T204)\), indicative of activation of these proteins, were also decreased upon Rac1 knockdown. Similar effect was observed when MCL cells treated with NSC23766 (Supplemental Figure 4b). These results suggest that Rac1 overexpression plays important role in the hyper-activation of multiple oncogenic pathways in MCL.

Gene rearrangement involving Cyclin D1 is the hallmark of MCL. However, it has been demonstrated that Cyclin D1 overexpression alone is insufficient to induce the onset of MCL \(^{13}\), raising the importance of additional mechanisms in MCL lymphomagenesis. Consistently, several core oncogenic pathways including Akt and NF-κB signaling have been found to be dysregulated without correlated genomic aberrations in MCL, which implies an interactive activation of pathway networks in the cancerous state. Here we demonstrated that Rac1 is directly associated with the activation of several prosurvival oncogenic pathways in MCL, suggesting that it locates at the central node of pathway network. However, the mechanism underlying Rac1 overexpression in MCL remains unclear. Previous studies have demonstrated that
endogenous Rac1 is activated by B-cell antigen receptor (BCR) signaling and is required for the subsequent activation of BCR downstream signal transduction\textsuperscript{14}. Considering the constitutive activation of the BCR signaling in MCL\textsuperscript{15} and its wide connections with other oncogenic pathways, it is likely that Rac1 is an important integrator of activating signals in 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 only brought cytoplastic effect. This is in agreement with the notion that defects of the pro-apoptotic machinery, such as BCL-2 and MCL1 overexpression, render MCL tumor cells resistance to chemotherapy-induce apoptosis. Notably, inhibition of Rac1 significantly enhanced the cytotoxic effect induced by Adriamycin, potentiating the combination of Rac1 inhibition with R-CHOP in clinical practice. Although in vivo studies are needed to demonstrate the effectiveness, our findings provide evidence supporting a proof-of-concept to target Rac1 as a novel therapeutic strategy for the treatment of MCL.

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Conflict of interest
The authors declare that they have no conflict of interest.

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