Fermitin family member 2 promotes melanoma progression by enhancing the binding of p-α-Pix to Rac1 to activate the MAPK pathway

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We identified fermitin family member 2 (FERMT2, also known as kindlin-2) as a potential target in A375 cell line by siRNA library screening. Drugs that target mutant BRAF kinase lack durable efficacy in the treatment of melanoma because of acquired resistance, thus the identification of novel therapeutic targets is needed. Immunohistochemistry was used to identify kindlin-2 expression in melanoma samples. The interaction between kindlin-2 and Rac1 or p-Rac/Cdc42 guanine nucleotide exchange factor 6 (α-Pix) was investigated. Finally, the tumor suppressive role of kindlin-2 was validated in vitro and in vivo. Analysis of clinical samples and Oncomine data showed that higher levels of kindlin-2 predicted a more advanced T stage and M stage and facilitated metastasis and recurrence. Kindlin-2 knockdown significantly inhibited melanoma growth and migration, whereas kindlin-2 overexpression had the inverse effects. Further study showed that kindlin-2 could specifically bind to p-α-Pix(S13) and Rac1 to induce a switch from the inactive Rac1-GDP conformation to the active Rac1-GTP conformation and then stimulate the downstream MAPK pathway. Moreover, we revealed that a Rac1 inhibitor suppressed melanoma growth and metastasis and the combination of the Rac1 inhibitor and vemurafenib resulted in a better therapeutic outcome than monotherapy in melanoma with high kindlin-2 expression and BRAF mutation. Our results demonstrated that kindlin-2 promoted melanoma progression, which was attributed to specific binding to p-α-Pix(S13) and Rac1 to stimulate the downstream MAPK pathway. Thus, kindlin-2 could be a potential therapeutic target for treating melanoma.

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INTRODUCTION

Melanoma is one of the most malignant cutaneous cancers. Its occurrence has increased in the past several decades, especially in the Western world. Melanoma with early metastasis can develop rapidly and eventually cause death [1–4]. For advanced or recurrent melanoma, surgery alone does not have a good therapeutic effect; thus, other therapeutic options, including radiotherapy, immunotherapy, immune checkpoint inhibitor therapy, and molecular targeted therapy, are needed [5–9]. PD-1 immune checkpoint blockade therapy has already been used in melanoma; however, advanced metastatic melanoma exhibits a high rate of innate resistance (60–70%) to this treatment [10]. A high percentage of melanomas harbor BRAF mutations and RAS mutations, which affect the downstream MAPK pathway to influence melanoma progression [11, 12]. These results provide reasonable evidence to support combination therapy with the BRAF inhibitor dabrafenib and the MEK inhibitor trametinib, and this combination has been proven to improve survival in patients with BRAF V600E/K mutations [13, 14]. Because of acquired resistance to BRAFV600E inhibitors, the long-lasting therapeutic effect of BRAFV600E inhibitors is limited; moreover, acquired resistance to MAPK-targeted therapy results in failure to respond to anti-PD-1 therapy. Thus, finding a combination of molecular targeted therapies that more successfully inhibit the MAPK signaling pathway is essential [10, 15, 16]. Our previous siRNA library screening identified numerous new proteins implicated in melanoma progression [17]. Among these proteins, fermitin family member 2 (FERMT2, also known as kindlin-2) was selected for functional evaluation in melanoma growth and metastasis. Kindlin-2, is a member of the kindlin family, which contains three members that have a highly conserved FERM domain and usually function to link the cell membrane to the cytoskeleton and as molecular linkers [18]. Recently, some studies have discovered that kindlin-2 mutation or dysregulation can promote the development of certain cancers, including breast cancer, hepatocellular carcinoma (HCC), esophageal cancer, prostate cancer, gastric cancer and glioma progression [19–25]. In our study, we have explored the potential molecular mechanisms by which kindlin-2 regulates the growth and metastasis of melanoma and, more importantly, evaluated its clinical significance to identify an
improved option for melanoma treatment. Our results demonstrated that kindlin-2 knockdown inhibited cell proliferation and metastasis by hindering the binding of Rac1 and p-α-Pix; thus, Rac1 could not be activated to promote MAPK pathway signaling. Rac1 is a member of the typical Rho guanosine triphosphate phosphohydrolase (GTPase) family and can cycle between an active, guanosine triphosphate (GTP)-bound conformation and an inactive, guanosine diphosphate (GDP)-bound conformation. This process is regulated by guanine nucleotide exchange factors (GEFs), which lead to Rac1 activation, and by GTPase-activating proteins (GAPs), which inactivate Rac1 [26]. α-Pix is a GEF that can activate p21 Rac1 and Cdc42 but not RhoA [27]. We discovered that high kindlin-2 expression can promote the binding of p-α-Pix to Rac1 and then activate the MAPK pathway. Recent studies showed that kindlin-2 promoted tumor growth and progression [28, 29]. In our study, we confirmed the role of kindlin-2 in melanoma progression, and also discovered that Rac1 inhibition could hinder the growth and metastasis of melanoma. Moreover, the combination of a Rac1 inhibitor and vemurafenib (a drug that targets mutant BRAF kinase but lacks long-lasting efficacy because of acquired resistance [30]) suppressed melanoma growth and metastasis to a higher degree than either agent as monotherapy. Thus, we identified kindlin-2 as a potential therapeutic target for melanoma.

RESULTS

Association between kindlin-2 expression and clinicopathological features of melanoma patients

We screened an siRNA library targeting 46,000 human genes in A375 melanoma cells. Kindlin-2 knockdown by siRNA significantly suppressed cell viability by 59.22% (P = 0.0001188), indicating that kindlin-2 might be a potential target in melanoma. Then, the relationship between the clinicopathological characteristics of melanoma patients and kindlin-2 protein expression was analyzed. Among the 82 patients with melanoma, 54 showed strong kindlin-2 expression, 28 showed weak kindlin-2 expression, and 28 showed negative kindlin-2 expression. The proportion of scores and representative examples of kindlin-2 expression in melanoma tissues are shown in Figure S1. The data in Table 1 indicate that a high kindlin-2 expression level might be related to T3-T4-stage (P < 0.05) and M1-stage (P < 0.05) disease. However, no evidence of an obvious association between kindlin-2 protein expression and sex (P = 0.109), age (P = 0.1646), tumor location (P = 0.4444), N stage (P = 0.6925) or TNM stage (P = 0.0602) was observed.

Transcription levels of kindlin-2 in patients with melanoma

The Oncomine database suggested that patients with metastasis had higher kindlin-2 mRNA levels than those without metastasis (Fig. S2A). High kindlin-2 mRNA expression was found to be correlated with M1-stage disease (Fig. S2B). In addition, kindlin-2 mRNA levels were significantly increased in patients with melanoma recurrence (Fig. S2C).

Kindlin-2 promoted the proliferation of melanoma cells

First, we determined the expression levels of kindlin-2 in human melanoma cell lines (A375, A875, MelWo, WM35, SK-Mel-2, and SK-Mel-28) by Western blotting (Fig. 1A). Kindlin-2 expression was detected in the cytoplasm in different cell lines (Fig. 1B, C). Then, we suppressed kindlin-2 expression in the MeWo and WM35 cell lines and overexpressed kindlin-2 in the A375 and A875 cell lines. Kindlin-2 knockdown via specific shRNAs suppressed its expression, whereas kindlin-2 overexpression significantly increased its expression (Fig. 1D). Kindlin-2 knockdown obviously inhibited the proliferation of melanoma cells and resulted in a marked decrease in the colony formation rates of MeWo and WM35 cells. In contrast, kindlin-2 overexpression dramatically increased the proliferation of melanoma cells and promoted colony formation (Fig. 1E, F). Flow cytometric analysis demonstrated that the reduction in kindlin-2 protein expression led to an increased rate of apoptosis in the melanoma cell lines MeWo and WM35 (Fig. 1G). Collectively, these results indicate that kindlin-2 plays a key role in mediating melanoma cell proliferation and growth.

Kindlin-2 influenced the cell cycle in melanoma cells

Compared with the control groups, groups with kindlin-2 suppression exhibited an increased proportion of G1 phase cells but a decreased proportion of S phase and G2 phase cells. In contrast, kindlin-2 overexpression led to a decrease in the proportion of G1 phase cells and an increase in the proportion of S phase and G2 phase cells. Thus, kindlin-2 knockdown induced G1 phase cell cycle arrest, while kindlin-2 overexpression promoted cell division (Fig. 2A).

Kindlin-2 knockdown inhibited the migration but not the invasion potential of melanoma cells

After interference, the MeWo and WM35 cell lines exhibited an apparent decrease in migration but not invasion compared with the control cells (P < 0.05), while the A375 and A875 cell lines showed increased migration but not invasion after kindlin-2 overexpression (Fig. 2B-D). The results of anokis assays showed that the anokis rates were significantly increased in shkindlin-2 cells (MeWo and WM35) in suspension, kindlin-2 overexpression markedly reduced the anokis rates in the A375 and A875 cell lines (Fig. 2E). Analysis of the epithelial markers E-cadherin and ZO-1 and the mesenchymal markers N-cadherin and vimentin revealed that kindlin-2 overexpression reduced E-cadherin and ZO-1 expression and increased N-cadherin and vimentin expression. However, kindlin-2 knockdown resulted in the inverse effects (Fig. 2F). Then, we knockdowned the expression of E-cadherin and ZO-1 by siRNA, respectively, the expression of kindlin-2 had no

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**Table. 1.** Correlation analyses of kindlin-2 protein expression in relation to clinicopathologic variables.

| Clinical factor | Kindlin-2 expression high | Low | p     |
|-----------------|---------------------------|-----|-------|
| Gender          |                           |     |       |
| Male            | 35                         | 13  | 0.1090|
| Female          | 19                         | 15  |       |
| Age             |                           |     |       |
| <53             | 26                         | 18  | 0.1646|
| ≥53             | 28                         | 10  |       |
| Location        |                           |     |       |
| Body            | 26                         | 11  | 0.4444|
| The Four limbs  | 28                         | 17  |       |
| T               |                           |     |       |
| T1/T2           | 6                          | 10  | 0.0077*|
| T3/T4           | 48                         | 18  |       |
| N               |                           |     |       |
| N0/N1           | 51                         | 27  | 0.6925|
| N2/N3           | 3                          | 1   |       |
| M               |                           |     |       |
| M0              | 45                         | 28  | 0.0245*|
| M1              | 9                          | 0   |       |
| TNM             |                           |     |       |
| I/II            | 38                         | 25  | 0.0602|
| III/IV          | 16                         | 3   |       |

*p < 0.05, statistically significant.
change (Fig. S3). That is, kindlin-2 can mediate cell-matrix adhesion itself, also it can influence cell-cell adhesion by affecting the expression of E-cadherin and ZO-1.

Kindlin-2 bound independently to Rac1 and α-Pix
To identify the proteins that interact with kindlin-2 in melanoma cells, we performed immunoprecipitation combined with mass spectrometry in A375 cells with an anti-kindlin-2 antibody. Rac1, a member of the Rac subfamily of Rho family small GTPases with strong evidence of its dysregulation in cancer, was found to be a candidate kindlin-2-interacting protein. Moreover, α-Pix, a GEF (activator) of the Rho family small GTP-binding protein family members Rac1 and Cdc42, was also found to be a candidate (Figs. S4, S5). The interaction between kindlin-2 and Rac1 and the
Kindlin-2 influenced the cell cycle and the migration but not the invasion of melanoma cells. A The percentage of melanoma cells in each phase was quantified. B The migration ability of melanoma cells with kindlin-2 overexpression or knockdown was assessed by measuring the average gap width (μm) in a wound-healing assay. C The migration ability of melanoma cells with kindlin-2 overexpression or knockdown was measured by a Transwell assay. D The invasive ability of melanoma cells with kindlin-2 overexpression or knockdown was measured by a Transwell assay. E Anoikis in melanoma cells with kindlin-2 overexpression or knockdown was measured by FACS analysis, and the relative percent of apoptotic cells was calculated. F Levels of epithelial-mesenchymal transition (EMT) markers in melanoma cells with kindlin-2 overexpression or knockdown were measured by Western blots. The data are presented as the means ± SDs of three independent tests. *P < 0.05, **P < 0.01, ***P < 0.001, significant differences between the treatment groups and control groups.
Fig. 3  Kindlin-2 bound independently to Rac-1 and α-Pix. A The interactions between kindlin-2 and Rac1 and between kindlin-2 and α-Pix were confirmed by co-IP in melanoma cells. B The interactions between kindlin-2 and Rac1 and between kindlin-2 and α-Pix were confirmed by colocalization as observed by confocal microscopy in melanoma cells. C kindlin-2 binding to Rac-1 and α-Pix in vitro, respectively, using the GST pull-down assay. D Binding affinities of Rac-1 and α-Pix for the kindlin-2 determined via Isothermal Titration Calorimetry (ITC). E The interaction between Rac1 and α-Pix was confirmed by co-IP in melanoma cells with kindlin-2 overexpression or knockdown.
interaction between kindlin-2 and α-Pix were further confirmed by co-IP and immunofluorescence colocalization analyses in different melanoma cell lines. The results showed that kindlin-2 and Rac1 were mainly localized in the cytosol and α-Pix was localized in the cytoplasm. Rac-1 and kindlin-2, α-Pix and kindlin-2 were co-localized in the cytoplasm (Figs. 3A, B, S6). In addition, GST pull-down assay revealed that kindlin-2 can directly bind to Rac-1 and α-Pix, respectively (Fig. 3C). Also, we utilized isothermal titration calorimetry to quantitatively measured the affinity the affinities of kindlin-2 for Rac-1 and α-Pix. Kindlin-2 was found to bind to Rac-1 with $K_d = 14.9 \pm 4.2 \mu M$. The $K_d$ value of kindlin-2 and α-Pix is $21.6 \pm 3.9 \mu M$ (Fig. 3D). To verify whether kindlin-2 influenced the binding of Rac-1
and α-Pix, we overexpressed the kindlin-2 protein and found that the binding of Rac1 and α-Pix strengthened, while suppressing kindlin-2 expression weakened the binding of Rac1 and α-Pix (Fig. 3E).

**Kindlin-2 activates the MAPK pathway by promoting the binding of Rac1 to p-α-Pix**

We analyzed the activation of Rac1 and found that Rac1 activation was significantly increased after kindlin-2 overexpression in the A375 and A875 cell lines but substantially decreased after kindlin-2 suppression in the MeWo and WM35 cell lines. We also conducted integrin activity assays, and found that overexpression of kindlin-2 in A375 cells could promote the integrin activity, but the GDP-Rac1 level cannot be affected by the inhibition of integrin (Fig. S7). As the downstream effect of Rac1-GTP is MAPK pathway activation, Western blotting was carried out to detect alterations in the MAPK pathway. Kindlin-2 knockdown in MeWo dramatically suppressed the phosphorylation of the JNK, p38, and ERK proteins, but had nearly no effect on the levels of total JNK, p38, and ERK, while in WM35 melanoma cells (harbored the BRAF mutations) with kindlin-2 knockdown, the phosphorylation of the JNK and p38 proteins were decreased, but had nearly no effect on the levels of total JNK and p38. In contrast, kindlin-2 overexpression in A375 and A875 cells significantly increased the phosphorylation levels of the JNK, p38, and ERK proteins but, similarly, did not change the levels of total JNK, p38, and ERK. These findings indicate that the MAPK signaling pathway is likely involved in the kindlin-2-mediated promotion of melanoma growth and metastasis. However, the expression of α-Pix was not significantly altered (Fig. 3A, B). Thus, we knocked down α-Pix by siRNA in A375 cells (Fig. 4C). After interference, Rac1 activation was markedly decreased; moreover, the phosphorylation levels of the JNK and p38 proteins were decreased, but the levels of total JNK and p38 were barely affected (Fig. 4D). To determine whether kindlin-2 can bind only p-α-Pix, alkaline phosphatase was used to dephosphorylate α-Pix. After phosphatase treatment, the binding of kindlin-2 to α-Pix was abrogated in melanoma cell lines (Fig. 4E). To investigate the binding mode of kindlin-2 to Rac1 as well as to p-α-Pix, docking simulation studies were carried out. First, phosphorylation sites on α-Pix and the binding domain of p-α-Pix on kindlin-2 were predicted (Table S1, Table S2). To identify the phosphorylation sites on α-Pix, the predicted phosphorylation sites (S13, S19, T9, and T23) were individually mutated in A375 cells. Unlike wild-type (WT) α-Pix, α-Pix S13A failed to activate Rac1 and interact with kindlin-2 (Fig. 4G, G). To discover the binding domains on kindlin-2 for p-α-Pix and Rac1, kindlin-2 truncation mutants were constructed. The kindlin-2 fragment containing residues 328-499 pulled down p-α-Pix (Fig. 4H, I), and the fragment containing residues 1-105 pulled down Rac1 (Fig. 4J, K). Moreover, when the residues 1-177 of Rac1 was deleted, cell growth and cell migration were suppressed, while cell apoptosis was promoted (Fig. 4B, D, E).

**Suppression of RAC1 reversed the overexpression of kindlin-2**

1A-116, an inhibitor of RAC1, was used to treat A375 cells. The appropriate dose and treatment time of 1A-116 were 5 μg/ml and 12 h, respectively (Fig. 5A). After kindlin-2 overexpression, the expression of Rac1-GTP and the phosphorylation of the JNK and p38 proteins were suppressed, but the levels of total RAC1, JNK and p38 were barely affected (Fig. 5B). After the residues 1-177 of Rac1 was deleted, cell growth and cell migration were suppressed, while cell apoptosis was promoted (Fig. 5C, D, E). After overexpression of kindlin-2, the phosphorylation levels of the JNK and p38 proteins were decreased, but the levels of total JNK and p38 were barely affected (Fig. 5D). The proportion of G1 phase cells was increased after Rac1 inhibition, while that of S phase and G2 phase cells was decreased (Fig. 5E). After treatment with 1A-116, melanoma cells exhibited an apparent weakening of migration but not on the level of invasion (Fig. 5F, G, S11). The results of the anokits assays showed that the anokik rate was significantly increased after the application of 1A-116 (Fig. 5H).

**Kindlin-2 knockdown and RAC1 inhibition suppressed melanoma growth in a mouse xenograft model**

Kindlin-2 knockdown inhibited tumor growth, while kindlin-2 overexpression promoted tumor growth; however, this promotion was reversed by the RAC1 inhibitor. Daily treatment of mice with compound 1A-116 at a dose of at least 3 mg/kg body weight/day markedly reduced tumor formation. In addition, none of these treatments significantly affected the body weight of the mice, and no other signs of acute or delayed toxicity were observed in the mice during treatment (Fig. 5I-Q). In addition, Western blot analysis of cell lysates from the xenografted tumor tissues showed that silencing kindlin-2 in mice inoculated with kindlin-2 knockdown tumor cells led to decreased phosphorylation levels of the JNK and p38 proteins, while kindlin-2 overexpression led to increased phosphorylation levels of the JNK and p38 proteins; however, this increase was inhibited by 1A-116 (Fig. 5J).

**Kindlin-2 knockdown and RAC1-GTP inhibition suppressed melanoma metastasis in a mouse model of metastatic melanoma**

We found that compared to control animals, mice that received kindlin-2-knockdown tumor cells (MeWo) exhibited a weakened...
metastatic ability and fewer lung metastatic nodules (Figs. 6A–E, S13A). However, the metastatic ability was strengthened, and the number of lung metastasis nodules was substantially increased in mice injected with kindlin-2-overexpressing A375 cells; moreover, daily treatment of the mice with compound 1A-116 at a dose of at least 2 mg/kg body weight/day significantly reduced the formation of total metastatic lung colonies (Figs. 6F–J, S13B).

**Combination of the Rac1 inhibitor and vemurafenib strengthened the therapeutic effect of each monotherapy in melanoma**

The A375 cell line in our study harbors a BRAF mutation, and we combined vemurafenib (20 mg/kg) and the Rac1 inhibitor to treat melanoma [31]. Compared with vemurafenib or Rac1 inhibitor treatment alone, the combination of the Rac1 inhibitor and vemurafenib significantly reduced melanoma growth (Fig. 7A–D).
In addition, none of these treatments, whether monotherapy or combination therapy, significantly affected the body weight of the mice (Fig. 7F). Regarding the metastatic capacity, the inhibitory effect in the combination treatment group was better than that in the vemurafenib treatment group (Figs. 7F-J, S14).

DISCUSSION
Recently, progress has been made in understanding the structure and biological functions of the kindlin-2 protein. Certain cancers, such as breast cancer, HCC, esophageal cancer, prostate cancer and gastric cancer, as well as glioma, have been found to be related to kindlin-2 dysfunction. The responsible mechanisms include the following: enhancing cell proliferation and migration by stabilizing DNMT1 or driving cancer progression by activating the TGF-β/CSF-1 signaling axis in breast cancer, promoting invasion and metastasis by activating Wnt/β-catenin signaling in HCC, activating the β-catenin/YB-1/EGFR pathway to promote progression in glioma, facilitating invasiveness via the NF-kB pathway to upregulate MMP-9 and MMP-2 expression in prostate cancer, and accelerating invasion in gastric cancer by phosphorylating integrin β1 and β3 [19–25]. In our study, we found that kindlin-2 can affect the proliferation and migration of melanoma cells, which was consistent with some research. However, our results did not discover that kindlin-2 affected melanoma cell invasion, considering that this was related to slight differences in the grouping and statistical methods of the experiments. Many physiological processes, including mesenchymal stem cell differentiation, podocyte morphology acquisition, cell spreading, vascular barrier function, cardiac function and chondrogenesis, are affected by kindlin-2 mutation or dysregulation [32–37]. In malignant melanoma, kindlin-2 was identified as a candidate target from an siRNA library screen, and we found that in melanoma, kindlin-2 knockdown inhibited cell proliferation, promoted apoptosis, and suppressed growth and metastasis, while kindlin-2 overexpression resulted in the inverse effects. Further analysis showed that kindlin-2 overexpression promoted the phosphorylation of proteins (p-p38, p-JNK, and p-ERK) in the MAPK pathway; the responsible molecular mechanism is the strengthening of Rac1 and p-α-Pix (S13) binding mediated by high kindlin-2 expression. Then, the inactive, GDP-bound Rac1 conformation undergoes a switch to the active, GTP-Rac1 conformation and stimulates the downstream MAPK pathway, which leads to the growth and metastasis of melanoma. Importantly, we discovered that kindlin-2 can bind to only p-α-Pix (S13) and not to unphosphorylated α-Pix or α-Pix phosphorylated at other sites—most likely, α-Pix can bind to kindlin-2 only when the conformation of α-Pix transitions after phosphorylation at S13. Moreover, we revealed that the fragment of kindlin-2 containing residues 328-499 can bind to p-α-Pix and that the fragment of kindlin-2 containing residues 1-105 can bind to Rac1. In addition, our clinical data and Oncomine database strengthened the evidence that higher kindlin-2 expression predicts more advanced T stage and M stage and facilitates metastasis and recurrence. However, it made more sense to compare the expression of kindlin-2 in the N stages or M stages within the stage III and IV sub-cohorts. Therefore, more detailed patient information was needed for analysis.

To the best of our knowledge, this study is the first to document that kindlin-2 plays a role in the growth and metastasis of melanoma by strengthening the binding of Rac1 and p-α-Pix (S13) and subsequently stimulating the downstream MAPK pathway. Furthermore, we attempted to determine the clinical therapeutic significance of our findings. First, the Rac1 inhibitor 1A-116 was used to treat kindlin-2-overexpressing A375 cells, and this treatment greatly reversed the effects of kindlin-2 overexpression on melanoma cell growth and metastasis. In animal studies, further evidence was obtained to demonstrate that kindlin-2 knockdown can dramatically suppress the growth and metastasis of melanoma. However, kindlin-2 overexpression definitely promoted the growth and metastasis of melanoma, but this promotive effect was hindered by the Rac1 inhibitor.

The RAS-RAF-MEK-ERK pathway is a kinase cascade that controls multiple vital cellular processes, such as cell cycle progression, survival and migration [38, 39]. Drugs targeting this pathway have achieved effective outcomes in the treatment of certain cancers with BRAF mutations, including colorectal cancer, hairy cell leukemia, melanoma, thyroid cancer, non-small cell lung cancer, etc. [40–44]. Vemurafenib was discovered as a highly specific BRAFV600 kinase inhibitor and produced a notable response in advanced melanoma patients with the BRAFV600 mutation [45–47]. However, tumor resistance occurs rapidly in some patients; unfortunately, searching for the appropriate combination therapy for melanoma remains challenging, and widespread metastasis is acknowledged as the main cause of death in melanoma patients [48, 49]. In our research, kindlin-2 was found to enhance the binding of Rac1 and α-Pix and subsequently stimulate the MAPK pathway, and the Rac1 inhibitor was proven to significantly affect the growth and, more importantly, the metastasis of melanoma both in vivo and in vitro. Thus, we tried to determine whether the combination of
the Rac1 inhibitor and vemurafenib could improve outcomes. Encouragingly, the combination therapy group exhibited a markedly greater therapeutic effect than the vemurafenib and Rac1 inhibitor monotherapy groups. Dysregulation of Rac1 activity has been found in certain cancers, including melanoma, breast cancer, colorectal cancer, gastric cancer, etc., and Rac1 has been discovered to influence cell proliferation, adhesion, migration, and invasion in the progression of certain cancers [50–57]. As previously reported, activation of Rac1 can promote MAPK pathway signaling; thus, we speculated that the combination of the Rac1 inhibitor and vemurafenib, which provided a double hit to the MAPK pathway, might result in improved therapeutic outcomes, and the results of our animal studies validated this hypothesis. Thus, melanomas with high kindlin-2 expression and BRAF mutations can be treated more effectively with the combination of a Rac1 inhibitor and vemurafenib than with either monotherapy.

In summary, our study demonstrated a substantial potential oncogenic role of kindlin-2 in melanoma. Kindlin-2 can bind specifically to p-α-Pix (S13) and Rac1 and subsequently enhance the binding of p-α-Pix (S13) to Rac1 to induce the switch of Rac1 from the inactive, Rac1-GDP conformation to the active, Rac1-GTP conformation. In turn, the downstream MAPK pathway is stimulated to promote the growth and metastasis of melanoma. Furthermore, we revealed that Rac1 inhibition can reverse melanoma growth and metastasis caused by high kindlin-2 expression, and combination therapy with a Rac1 inhibitor and vemurafenib can result in a better therapeutic outcome than monotherapy in melanoma patients with high kindlin-2 expression and BRAF mutations (Fig. 8). Collectively, these findings indicate that kindlin-2 is a potential diagnostic biomarker and therapeutic target for melanoma.

**MATERIALS AND METHODS**

**Cell culture**

Six melanoma cell lines (A375, A875, MeWo, WM35, SK-Mel-2, and SK-Mel-28) were cultured for the following experiments. The BRAF mutational status of melanoma cell lines were displayed in Table S3.

**Rac1 activation assay with magnetic bead pulldown**

The following protocol was followed: cells were cultured and rinsed twice with ice-cold PBS. Then, ice-cold leupeptin (0.5 ml per 150-mm tissue
culture plate) was added, and cells were detached by scraping and lysed. Next, the cell lysates were transferred to microfuge tubes on ice, and 0.5 ml of each cell extract was aliquoted to a microfuge tube. A total of 10 µl (10 µg) of Rac1/Cdc42 Assay Reagent (PAK-1 PBD-conjugated magnetic beads) was added to each tube and incubated for 45 min at 4 °C. The beads were pelleted by centrifugation (10 s, 14,000×g, 4 °C), and the supernatant was removed and discarded. The beads were washed 3 times with leupeptin and resuspended in 40 µl of 2× Laemmli buffer. Next, 2 µl of 1 M dithiothreitol was added and boiled for 5 min, and the beads were pelleted by centrifugation. The supernatant and beads were mixed, and 20 µl of the mixture was loaded on a polyacrylamide gel for SDS-PAGE. After that, the proteins were transferred to a membrane. After the above steps, the membranes were blocked with 5% skim milk (w/v) at room temperature for 1 h and incubated overnight at 4 °C with an anti-Rac1 antibody (diluted to 1 µg/ml). Secondary antibodies were then added and incubated for 1 h at room temperature. Protein-antibody complexes were then detected by chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo, USA).

**Xenograft animal experiment and tissue processing**

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, revised 1996) and the Institutional Ethical Guidelines for Animal Experiments developed by Sun Yat-sen University. Melanoma cells (5 × 10⁶ in 100 µl of PBS) were injected subcutaneously into the left flank of female athymic nude mice aged 3–4 weeks, and tumors developed at the injection sites after 1 week. When the formed tumors reached 100 mm³, the animals were divided randomly into different groups with 6 mice per group. Certain groups were treated with different concentrations (1, 2, 3, 4, 5, and 6 mg/kg) of 1A-116 or with vemurafenib (20 mg/kg) daily via intratumoral injection [58]. The experiment was terminated 18 days after tumor cell inoculation. Tumor sizes were measured using Vernier calipers every three days, and tumor volumes were calculated as follows: \( V = \frac{\text{width}^2 \times \text{length}}{2} \). At the termination of the experiment, the mice were sacrificed, and the tumors were excised from each mouse and weighed. A portion of the tumors was fixed with 10% formalin and used to prepare tumor tissue lysates for western blot analysis. No blinding was done.

**Metastasis model**

A tail vein injection model was used for lung colonization assays. All female athymic nude mice aged 3–4 weeks were divided randomly into different groups with six mice per group. All mice were injected via the tail vein with 1×10⁵ luciferase and GFP-positive melanoma cells in 0.1 ml of serum-free medium. On the day of cell inoculation, bioluminescence imaging using the IVIS® Lumina II system (Caliper Life Sciences, Hopkinton, MA) was performed to verify a fluorescence signal in all mice after intraperitoneal injection of 4.0 mg of luciferin (Gold Biotech) in 50 µl of saline. The metastases were monitored using the IVIS® Lumina II system (Caliper Life Sciences, Hopkinton, MA) daily via intratumoral injection [58]. The experiment was terminated 18 days after tumor cell inoculation. Tumor sizes were measured using Vernier calipers every three days, and tumor volumes were calculated as follows: \( V = \frac{\text{width}^2 \times \text{length}}{2} \). At the termination of the experiment, the mice were sacrificed, and the tumors were excised from each mouse and weighed. A portion of the tumors was fixed with 10% formalin and used to prepare tumor tissue lysates for western blot analysis. No blinding was done.
Kindlin-2 can bind specifically to p-α-Pix (S13) and Rac1, enhance the binding of p-α-Pix (S13) to Rac1 to induce the switch of Rac1 from the inactive, Rac1-GDP conformation to the active, Rac1-GTP conformation. Then, the downstream MAPK pathway is stimulated to promote the growth and metastasis of melanoma. The combination of Rac1 inhibitor and vemurafenib can reverse melanoma growth and metastasis caused by high GDP conformation to the active, Rac1-GTP conformation. Then, the downstream MAPK pathway is stimulated to promote the growth and metastasis of melanoma. The combination of Rac1 inhibitor and vemurafenib can reverse melanoma growth and metastasis caused by high

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