Abstract. Neuroblastoma is the most common extracranial solid tumor in childhood, whose molecular mechanism on clinically heterogeneous behavior is still unclear. Receptor for activated C-kinase 1 (RACK1) has been shown to be involved in the regulation of growth and migration in many types of cells. Two types of human neuroblastoma cell lines, SK-N-SH and SK-N-BE(2), were used to explore the role of RACK1 in neuroblastoma cell migration and proliferation. Cell migration and proliferation were detected by a transwell system and colorimetric bromodeoxyuridine (BrdU) ELISA, respectively. The expressions of RACK1, N-Myc, phospho-Src(Tyr416), phospho-Src(Tyr527), phospho-Akt, phospho-ERK1/2 and phospho-p38 were analyzed by Western blotting. The expression of RACK1 was then repressed by target RNA interference (RNAi) in both types of neuroblastoma cells, and the expression of signaling molecules, migration and proliferation of cells was analyzed. Compared with SK-N-SH, the migration and proliferation of SK-N-BE(2) cells was remarkably higher which was accompanied by higher expression of RACK1 and phospho-Src(Tyr416). RACK1 RNAi repressed cell migration and proliferation, and decreased the expression of phospho-Src(Tyr416) in both cell lines. In summary, RACK1 was expressed in the neuroblastoma cells and positively regulated cell migration and proliferation probably via modulating the activation of Src on Tyr416 residue.

Introduction

Neuroblastoma, a neoplasm of peripheral neural crest origin, is the most common extracranial solid tumor in childhood and the most frequently diagnosed neoplasm during infancy (1,2). Despite current therapeutic advances and basic mechanism investigations, neuroblastoma remains a complex medical challenge with the unpredictable clinical course and dismal overall outcome for advanced-stage disease (3-5).

Extraordinary migration and proliferation plays an important role in invasive and malignant of neuroblastoma cells (6,7). It is proposed that finding key point proteins or signaling molecules that participated in the invasive potential, such as migration and proliferation, of neuroblastoma cells, might be a potential therapeutic targets and might be a way to overcome current clinical dilemma of neuroblastoma treatments (8).

Receptors for activated C-kinases (RACK1) is the founding member of the family of intracellular receptors of activated protein kinase C (PKC) (9,10). The human RACK1 gene (Gene Bank accession no. GNB2L1) is highly conserved, includes eight exons and seven introns and is located on chromosome 5 (5q35.3). The gene product is a 36-kDa cytosolic protein that contains seven tryptophan-aspartate repeats, a domain involved in protein-protein interactions. RACK1 is widely expressed in the nervous system (11). Several studies suggested that RACK1 are involved in numerous key cellular functions, such as cellular apoptosis (9) and migration (12). It had been reported that the expressions of RACK1 mRNA and protein were upregulated in damaging and repairing process (13) and during angiogenesis in carcinomas (14). In light of the role in cell growth, apoptosis and proliferation, there are accumulating interest focused on the oncogenic role of RACK1 signaling (15-19). Hence, we hypothesized that RACK1 might play an important role in the development and progression of the neuroblastoma. However, few works were carried out to evaluate the expression and function of the RACK1 in the growth and migration of neuroblastoma.

In the present research, the expression of RACK1, and the migration and proliferation in two kinds of human neuroblastoma cell lines, SK-N-SH and SK-N-BE(2), were detected. The possible role of RACK1 in migration and proliferation of neuroblastoma cells were further analyzed after RACK1 gene silence. Our results could provide a useful tool for the evaluation of the invasive potential of neuroblastoma and may provide a new therapeutic target.

Materials and methods

Cell culture. The SK-N-SH and SK-N-BE(2) cell lines were purchased from cell bank of Chinese Academy of
Science. SK-N-SH is a MYCN gene (gene name of N-Myc) non-amplified cell line while SK-N-BE(2) is a MYCN gene amplified cell line (20). SK-N-SH was cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin and SK-N-Be(2) was cultured in advanced DMEM/Nutrient Mixture F-12 (DMEM/F12, Invitrogen) containing 10% FBS (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C in a humidified incubator of 95% air and 5% CO₂.

RNA interference. The mRNA sequences of human RACK1 [NM_006098, Homo sapiens guanine nucleotide binding protein (G protein), β polypeptide 2-like 1 (GNB2L1)] and N-Myc [NM_006098, Homo sapiens v-myelocytomatosis viral related oncogene, neuroblastoma derived (avian) (MYCN)] were acquired from NCBI GenBank. Small interfering RNAs (siRNA) target against human RACK1 and N-Myc mRNA were designed and synthesized by GenePharma Biological Co. (Shanghai, China). The double strands of siRNA for RACK1 were: 5'-CCACCUUUGUUAGCCGAGUTT-3' and 5'-ACA UCGCUAAACAAGUGGTTT-3'; siRNA for N-Myc were: 5'-GGGACGAGAGACUCUUTC-3' and 5'-UAGAA GUCUACUUCCGUCCGTT-3'. For RNA interference (RNAi) studies, SK-N-SH and SK-N-BE(2) were seeded at a density of 2.0x10⁵ cells per well in 6-well plate and grown in 10% FBS/ DMEM and 10% FBS/DMEM/F12, respectively. After seeding for 24 h, the cells were transfected with siRNA and Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instruction. Briefly, 100 nmol siRNA and 5 µl Lipofectamine™ 2000 were diluted in opti-MEM (Invitrogen), serum- and antibiotic-free, to a final volume of 800 µl. After mixing for 20 min at room temperature, the siRNA/Lipofectamine 2000 mixture was added dropwise onto the cells and incubated for 6 h at 37°C in a humidified CO₂ incubator. Following incubation, the mixture was replaced with complete medium [10% FBS/DMEM for SK-N-SH and 10% FBS/DMEM/F12 for SK-N-Be(2)] for 24 h. Non-silencing siRNA that does not recognize any known homology to human genes was used as a mock control.

Real-time PCR and Western blotting. The mRNA expressions of RACK1 in different human neuroblastoma cell lines were detected by real-time PCR (Bio-Rad). Specific primers for N-Myc were synthesized by Sangon Biological Co. (Shanghai, China) (sense: 5’-GCTGGGTCACGGAGATGCT-3’; antisense: 5’-CCCACUUUGUUAGCGAUGUTT-3’) and 5’-ACA UCGCUAAACAAGUGGTTT-3’. For RNA interference (RNAi) studies, SK-N-SH and SK-N-BE(2) were seeded at a density of 2.0x10⁵ cells per well in 6-well plate and grown in 10% FBS/ DMEM and 10% FBS/DMEM/F12, respectively. After seeding for 24 h, the cells were transfected with siRNA and Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instruction. Briefly, 100 nmol siRNA and 5 µl Lipofectamine™ 2000 were diluted in opti-MEM (Invitrogen), serum- and antibiotic-free, to a final volume of 800 µl. After mixing for 20 min at room temperature, the siRNA/Lipofectamine 2000 mixture was added dropwise onto the cells and incubated for 6 h at 37°C in a humidified CO₂ incubator. Following incubation, the mixture was replaced with complete medium [10% FBS/DMEM for SK-N-SH and 10% FBS/DMEM/F12 for SK-N-Be(2)] for 24 h. Non-silencing siRNA that does not recognize any known homology to human genes was used as a mock control.

Cell migration assay. Cell migration cell assay was performed using the Transwell (Costar) system, which allows cells to migrate through a polycarbonate membrane with a pore size of 8 µm in 6-well plates. Briefly, cells were plated at a concentration of 1.0x10⁵ cells/ml on the upper chamber and the lower chamber was filled with 800 µl 20% FBS/DMEM for SK-N-SH and 20% FBS/DMEM/F12 for SK-N-BE(2) to serve as the chemoattractant. After 6 h of incubation at 37°C in a humidified CO₂ incubator, cells were fixed in 4% paraformaldehyde for 20 min, permeabilized for 5 min with 0.4% Triton X-100 in PBS and dyed with Ehrlich's haematoxylin solution for 30 min. The number of migrated cells was counted under a microscope (magnification x200, Olympus). Six randomly chosen fields were evaluated per transwell membrane.

Cell proliferation assay. Cell proliferation was analyzed using a colorimetric bromodeoxyuridine (BrdU) kit (Roche Diagnostics). SK-N-SH and SK-N-BE(2) were seeded at a density of 1.0x10⁵ cells per well in 96-well plates and grown in 10% FBS/DMEM and 10% FBS/DMEM/F12, respectively. 6 h prior to the end of RNAi, BrdU labeling reagent was added into the culture medium (1:1000) and labeled according to the manufacturer's instructions. Briefly, the cells were fixed with FixDenat solution for 30 min at room temperature and then incubated with anti-BrdU peroxidase working solution (freshly diluted 1:100) for 90 min. Following 3 rinses with washing buffer, 100 µl/well substrate solution was added onto the cells and incubated for 20 min at room temperature. Thereafter, 25 µl H₂SO₄, 1 mol/l, were added to each well and the 96-well plates were shaken at 300 r.p.m. The absorbance at 450 nm was measured in an ELISA plate reader (Bio-Rad 680).

Statistical analysis. Each experiment was performed at least in triplicates and all values are expressed as mean ± SD. The one-way ANOVA was used to compare the results between the two groups. A value of P<0.05 was considered significant.

Results

High migration and proliferation properties of SK-N-BE(2) with high expression of RACK1. The mRNA and protein expres-
sions of RACK1 in SK-N-BE(2), the tumorigenic, aggressive and MYCN gene amplified neuroblastoma cell line, were both significantly higher than that in SK-N-SH, the less aggressive and MYCN gene non-amplified (P<0.01, Fig. 1). The migration property of SK-N-BE(2), analyzed by Transwell system, was notably higher than that of SK-N-SH (P<0.05, Fig. 2A). Cell proliferation was evaluated by measuring BrdU incorporation in newly synthesized DNA. As shown in Fig. 2B, in comparison with SK-N-SH, the proliferation of SK-N-BE(2) was also notably higher (P<0.05). These data suggested that RACK1 might play a role in neuroblastoma growth and migration.

Expressions of N-Myc, phospho-Src, phospho-Akt, phospho-ERK1/2 and phospho-p38 in SK-N-SH and SK-N-BE(2). To evaluate the possible molecules involved in differential migration and proliferation of SK-N-BE(2) and SK-N-SH, the expression of N-Myc and the activations of Src, Akt, ERK1/2 and p38 were assessed by their specific antibody and phospho-antibodies individually. The phospho-antibodies recognize the phosphorylated Tyr416 and Tyr527 residue on Src, Thr308 residue on Akt, Thr180 and Tyr182 residues on p38, Thr202 and Tyr204 residues on ERK1/2. These phosphorylated residues are the critical sites required for the activation of these factors, respectively.

As shown in Fig. 3, the expression of N-Myc was significantly higher in MYCN amplified SK-N-BE(2) than that in MYCN non-amplified SK-N-SH (P<0.01). The phosphorylation of Src on Tyr416 residue was also increased in SK-N-BE(2) (P<0.01, Fig. 3), while on Tyr527 residue the phosphorylation was similar between the two cell lines (P>0.05, Fig. 3). The phosphorylation on Akt was remarkably decreased in SK-N-BE(2) in comparison with SK-N-SH (P<0.01, Fig. 3). The expressions of phospho-ERK1/2 and phospho-p38 in SK-N-BE(2) had no significant difference from that in SK-N-SH (P>0.05, Fig. 3).

The protein expressions of RACK1, N-Myc, phospho-Src(Tyr416) were changed similar to, while phospho-Akt was opposite to, the migration and proliferation of cells. Therefore, it provided evidence that these factors might participate in the modulation of migration and proliferation of neuroblastoma cells, but phospho-ERK1/2 and phospho-p38 might not.

Repressed expression of RACK1 on SK-N-BE(2) downregulates the migration and proliferation of cell and decreases the phosphorylation of Src(Tyr416). In comparison with the mock control, the decreased expression of RACK1 (P<0.01, Fig. 4) by target RNAi significantly repressed migration and proliferation of SK-N-BE(2) (P<0.05, Fig. 5). Specific RACK1 RNAi also significantly suppressed the phosphorylation of Src on Tye416 residue (P<0.05, Fig. 4), but had no specific effects on the expression of N-Myc and phosphorylation of Src on Tye527 residue, Akt, ERK1/2 and p38 (P>0.05, Fig. 4).

Repressed expression of RACK1 on SK-N-SH decreases the proliferation of cells and the phosphorylation of Src(Tyr416). In SK-N-SH, RACK1 ‘knock-down’ by RNAi (P<0.01, Fig. 6) notably repressed the phosphorylation of Src on Tye416 residue (P<0.05, Fig. 6), while the expression of phosphorylation of Src on Tye527 residue, Akt, ERK1/2 and p38 were also similar between the RACK1 RNAi and the mock control (P>0.05, Fig. 6). Target RACK1 RNAi also decreased the cell proliferation (P<0.05, Fig. 7B) and
migration, but the decreased effect on cell migration was not significant (P>0.05, Fig. 7A).

Repressed expression of N-Myc downregulates the migration and proliferation of SK-N-BE(2), decreases expressions of RACK1, phosphor-Src(Tyr416), but increases expression of phospho-Akt. The role of N-Myc in the regulation of neuroblastoma cell proliferation and migration has been confirmed by several studies (21,22). We then using RNAi techniques to suppress the expression of N-Myc in SK-N-BE(2) in order to detect the relation between N-Myc expression and RACK1 expression.

When the expression of N-Myc was remarkably suppressed by RNAi in comparison with the mock control (P<0.01, Fig. 4), the migration and proliferation of SK-N-BE(2) were significantly decreased (P<0.05, Fig. 5). The expressions of RACK1 and phospho-Src(Tyr416) were also decreased by N-Myc RNAi (P<0.05, Fig. 4), while expression of phospho-Akt was increased (P<0.05, Fig. 4). The expressions of phospho-Src(Tyr527), phospho-ERK1/2 and phospho-p38 were not changed by N-Myc RNAi in SK-N-BE(2) (P>0.05, Fig. 4).

Figure 3. Expressions of N-Myc, and phosphorylations of Src(Tyr416), Src(Tyr527), Akt, ERK1/2 and p38 in SK-N-SH and SK-N-BE(2). The expression of N-Myc was significantly higher in SK-N-BE(2) than that in SK-N-SH, accompanied by remarkably increased expression of phospho-Src(Tyr416) and decreased expression of phospho-Akt. Top panel, the blots from a typical study; the bottom histogram shows the relative intensities of N-Myc which were densitometrically quantified by relating to GAPDH, and the relative intensities of phospho-Src(Tyr416), phospho-Src(Tyr527), phospho-Akt, phospho-ERK1/2 and phospho-p38, were densitometrically quantified by relating to their individual total proteins, respectively. Values shown are the mean ± SD for each condition from 4 independent experiments. *P<0.05 vs. SK-N-SH, **P<0.01 vs. SK-N-SH.

Figure 4. The effects of N-Myc and RACK1 target RNAi on expressions of N-Myc and RACK1, and phosphorylation of Src(Tyr416), Src(Tyr527), Akt, ERK1/2 and p38 in SK-N-BE(2). The expression of RACK1 was decreased by target N-Myc RNAi, but the expression of N-Myc was not changed by target RACK1 RNAi. The phosphorylation of Src Tyr416 residues were decreased by both N-Myc and RACK1 RNAi, while the phosphorylation of Akt was only induced by N-Myc RNAi. The phosphorylation of Src Tyr527 residues, ERK1/2 and p38 were not changed by N-Myc nor RACK1 RNAi. Values shown are the mean ± SD for each condition from 4 independent experiments. *P<0.05 vs. the mock control, **P<0.01 vs. the mock control.

Figure 5. The effect of N-Myc and RACK1 target RNAi on migration and proliferation of SK-N-BE(2). (A) Compared with the mock control, both N-Myc and RACK1 target RNAi repressed the migration ability of SK-N-BE(2), which was determined by Transwell assays. The number of cells illustrates the migration ability. Bar, 50 µm. Histogram shows fold changes in cell migration relative to the mock control. Values shown are the mean ± SD for each condition from 3 independent experiments and 6 images were random acquired in each independent experiment. *P<0.05 vs. the mock control. (B) Both N-Myc and RACK1 target RNAi decreased the proliferation ability of SK-N-BE(2), which was determined by BrdU ELISA. Histogram shows fold changes in cell proliferation relative to the mock control. Values shown are the mean ± SD for each condition from 4 independent experiments. *P<0.05 vs. the mock control.
The results revealed that the high expression of N-Myc in SK-N-BE(2) was a crucial inducer for cell migration and proliferation. The expression of RACK1 and phosphorylation of Src(Tyr416), Akt, ERK1/2 and p38 in SK-N-SH. Similar to the variation of signaling molecules in SK-N-BE(2), the expression of RACK1 was decreased by target RNAi. The phosphorylation of Src Tyr416 residues were decreased by RACK1 RNAi. The phosphorylation of Src Tyr527 residues, Akt, ERK1/2 and p38 were not changed by RACK1 RNAi. Values shown are the mean ± SD for each condition from 4 independent experiments. *P<0.05 vs. the mock control, **P<0.01 vs. the mock control.

The expression of RACK1 was affected by N-Myc expression, but RACK1 had no effect on the expression of N-Myc. The present data suggest that RACK1 ‘knock-down’ and N-Myc ‘knock-down’ had similar effects on SK-N-BE(2) migration, proliferation and Src Tyr416 phosphorylation, but different on Akt phosphorylation. Hence, RACK1 might be a downstream signaling molecule of N-Myc, and contributed to the migration and proliferation of SK-N-BE(2) induced by N-Myc. The phosphorylation of Src on Tyr416 residue might be involved in this process, but Akt might not.

Discussion

Hyper-proliferation and hyper-migration behaviors induced by various oncogenic proteins are the most prominent and well-characterized features of neuroblastoma cells, and are fundamental to tumor growth and metastasis (6,7). Finding new regulating mechanism might provide a useful tool for the evaluation of the invasive potential of neuroblastoma and might provide a new therapeutic target.

In this study, our results revealed that RACK1 was expressed in neuroblastoma and was involved in regulation of tumor cell migration and proliferation. Differential expression of RACK1 in the human neuroblastoma cell lines, SK-N-SH and SK-N-BE(2), were accompanied with differential abilities of migration and proliferation. The expression of RACK1, cell migration and proliferation were significantly higher in SK-N-BE(2), an aggressive and MYCN gene (gene name of N-Myc) amplified neuroblastoma cell line, than that in SK-N-SH, a less aggressive and MYCN non-amplified neuroblastoma cell line. The RACK1 gene silencing by RNAi decreased the proliferation ability of SK-N-SH, which was determined by BrdU ELISA. Histogram shows fold changes in cell proliferation relative to the mock control. Values shown are the mean ± SD for each condition from 4 independent experiments. *P<0.05 vs. the mock control.

Figure 6. The effects of RACK1 target RNAi on expression of RACK1, and phosphorylation of Src(Tyr416), Src(Tyr527), Akt, ERK1/2 and p38 in SK-N-SH. Similar to the variation of signaling molecules in SK-N-BE(2), the expression of RACK1 was decreased by target RNAi. The phosphorylation of Src Tyr416 residues were decreased by RACK1 RNAi. The phosphorylation of Src Tyr527 residues, Akt, ERK1/2 and p38 were not changed by RACK1 RNAi. Values shown are the mean ± SD for each condition from 4 independent experiments. *P<0.05 vs. the mock control, **P<0.01 vs. the mock control.

Figure 7. The effect of RACK1 target RNAi on migration and proliferation of SK-N-SH. (A) Compared with the mock control, RACK1 RNAi had no significant effect on cell migration, which was determined by Transwell assays. The number of cells illustrates the migration ability. Bar, 50 µm. Histogram shows fold changes in cell migration relative to the mock control. Values shown are the mean ± SD for each condition from 3 independent experiments and 6 images were random acquired in each independent experiment. *P<0.05 vs. the mock control. (B) Compared with the mock control, RACK1 target RNAi decreased the proliferation ability of SK-N-SH, which was determined by BrdU ELISA. Histogram shows fold changes in cell proliferation relative to the mock control. Values shown are the mean ± SD for each condition from 4 independent experiments. *P<0.05 vs. the mock control.
Our results revealed that the phosphorylation of Tyr416 residue on Src, but not Tyr527 residue, was regulated by RACK1 RNAi in both SK-N-SH and SK-N-BE(2). Since, Tyr416 for kinase active and Tyr527 for kinase inactive (25), repressed expression of RACK1 resulted in a decreased activation of Src kinase via decreased phosphorylation on Tyr416 residue of Src.

Src is a non-receptor protein tyrosine kinase, whose activity and expression has been strongly implicated in the development, maintenance, progression, and metastatic spread of several human cancers (26). Overexpression or aberrant activation of Src has been detected in neuroblastoma (27). Downregulation of Src activity was associated with apoptosis of neuroblastoma cells (28). There are reports that RACK1 negatively regulated cell growth and migration via Src-dependent pathways (9,12).

MYCN gene has been proven to be a critical gene for stimulation of neuroblastoma growth and migration (21,22). Our study by RNAi showed that, in SK-N-BE(2), whose N-Myc expression was in a high level, the N-Myc RNAi and RACK1 RNAi had similar effects on cell migration and proliferation. The repressed expression of N-Myc reduced the expression of RACK1, while the repressed expression of RACK1 had no specific effect on expression of N-Myc. These results suggested that N-Myc might be an upstream regulator of RACK1 in MYCN gene amplified SK-N-BE(2) cells, which subsequently induced migration and proliferation.

Increasing evidence has been reported on the activation of Akt playing an important role in promoting survival of neuroblastoma (23,24,29). Our results revealed that the activation of Akt was increased by downregulation of N-Myc in MYCN gene amplified neuroblastoma cells, which might be involved in the upregulation of cell proliferation. These anti-apoptosis and pro-proliferation effects of Akt activation might contribute to tumor growth. In our present research, the activations of Akt and ERK1/2 were not changed by the repressed expression of RACK1, which were not consistent with the effect of RACK1 in other tumor cells, such as human colon cells (9) and breast carcinoma (17). The exact molecular mechanism among N-Myc, RACK1 and Akt activation, and their roles in apoptosis, proliferation and migration of neuroblastoma cells still needs further research in the future.

In summary, our data suggested that RACK1 is expressed in the neuroblastoma cells, and positively regulated cell migration and proliferation via modulating the activation of Src on Tyr416 residue. In MYCN gene amplified neuroblastoma cells, RACK1 might be a downstream signaling molecule of N-Myc and involved in tumor cell migration and proliferation regulated by N-Myc. Our findings may provide a tool for the evaluation of the invasive potential of neuroblastoma and may provide a new therapeutic target.

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