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Identification of integrin α1 as an interacting protein of protein tyrosine phosphatase PRL-3 ☆

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Abstract

PRL-3 is a newly identified protein tyrosine phosphatase associated with tumor metastasis. It is over-expressed in various cancers, such as colorectal cancer, gastric cancer, and ovarian cancer, and is correlated with the progression and survival of cancers. Although PRL-3 plays a causative role in promoting cancer cell invasion and metastasis, the molecular mechanism is unknown. To investigate PRL-3's roles in tumorigenesis and signal transduction pathway, we screened the human placenta brain cDNA library with the bait of PRL-3 in yeast two-hybrid system. Then we identified integrin α1 as a PRL-3-interacting protein for the first time, and verified this physical association with pull-down and co-immunoprecipitation assays. Furthermore, we found that PRL-3 could down-regulate the tyrosine-phosphorylation level of integrin β1 and increased the phosphorylation level of Erk1/2. Our present discovery will provide new clues for elucidating the molecular mechanism of PRL-3 in promoting cancer invasion and metastasis.

Keywords: Protein tyrosine phosphatase PRL-3; Integrin; Interaction; Erk1/2; Phosphorylation

PRLs (PRL-1, -2, and -3) are a subgroup of non-classical protein tyrosine phosphatases (PTPs), characteristic of the CAAX sequences at the C-terminal for prenylation. It is located at the cytoplasmic membrane when prenylated and in the nucleus when non-preylated. All PRLs have significant sequence homology to dual-specific phosphatase PTEN and Cdc14 in regions other than the conserved PTP signature motif [1,2]. Protein tyrosine phosphorylation is a major post-transcriptional modification, which plays a fundamental role in regulating diverse physiological and pathological processes, such as cell growth, differentiation, carcinogenesis, and so on [3,4]. The dynamic and reversible protein tyrosine phosphorylation changes are precisely regulated by protein tyrosine kinases and phosphatases, which exert like a switch to control the balances of physiological activities in cell. When the balance is destroyed, signal transduction network becomes disrupted and contributes abnormal cell properties, for example, tumorigenesis [5]. Many PTKs are thought as oncogenes, because their abnormal activations usually promote tumorigenesis, such as Ras and Src. Meanwhile, PTPs used to be thought of as the "off" switch of PTKs, because of their roles in reversing the actions of protein tyrosine kinases. However, more and more studies show that some PTPs might have positive roles in carcinogenesis. For example, PTPα activates Src kinase by dephosphorylating its C-terminal inhibitory phosphorylated tyrosine, thereby promotes cell transformation [6–8]. PRL-3 is such a newly identified PTP associated with tumor metastasis. Our previous study and other groups' reports showed that *PRL-3* was up-regulated in colorectal cancer, gastric cancer, and ovarian cancer with metastasis, and was correlated with caner progression and pathological stage [9–13]. Over-expression of *PRL-3* could not only transform Chinese hamster ovary cell (CHO) and mouse melanoma cell B16, but

☆ PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; MAPK, mitogen-associated protein kinase; Erk1/2, extracellular-signal-related kinase; GST, glutathione *S*-transferase.

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also enhanced their motility and invasive ability. Moreover, *PRL-3* facilitated the formation of lung and liver metastases in mice. These studies demonstrate that *PRL-3* plays a causative role in tumor metastasis, and is a potential marker for clinical evaluation for tumor aggressiveness [14–16]. However, the molecular mechanism of *PRL-3* promoting the migration and invasion is unclear, and no PRL-3-interacting protein has been reported so far. In this study, we have identified integrin α1 as a PRL-3-interacting protein in yeast two-hybrid system, and confirmed this association with pull-down and co-immunoprecipitation assays in vivo. Furthermore, we found that *PRL-3* down-regulated the tyrosine-phosphorylation level of integrin β1, and enhanced the phosphorylation level of Erk1/2.

Materials and methods

Cell culture and antibodies. Flp-In-T-REx-293 cells (Invitrogen, CA) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 15 μg/ml Blasticidin and 100 μg/ml Zeocin (Invitrogen). African green monkey kidney cell line COS-7 (ATCC, VA) was cultured in DMEM and Ham's F12K medium (Invitrogen), respectively. All cell lines were maintained in humidified chamber with 5% CO_2 at 37 °C.

Anti-integrin β1 (MAB 1951) and anti-phosphotyrosine (MAB3080) antibodies were purchased from Chemicon International. Antibodies against Myc and GST tags were obtained from TianGen (Beijing, China). Antibodies against Erk1/2 (sc-94), phosphorylated Erk1/2 (sc-7383), and β-Actin (sc-1616) were products of Santa Cruz Biotechnology. Monoclonal antibody 3B6 against PRL-3 was prepared as previously described [17].

Generation of stably expressing PRL-3 cell line Flp-In-T-REx-293. For generation of stably expressing PRL-3 cell line Flp-In-T-REx-293, the full-length cDNA of *PRL-3* was cloned as described before [17] and inserted into plasmid pcDNA5/FRT/TO at *Bam*HI and *Xho*I sites. Plasmid pcDNA5/FRT/TO-*PRL-3* and pOG40 were co-transfected into Flp-In-T-REx-293 cells at the rate of 9:1 with Lipfectamine 2000 (Invitrogen). Forty-eight hours later, cells were split and selected in DMEM with 15 μg/ml Blasticidin, 100 μg/ml Zeocin, and 100 μg/ml hygromycin B. The expressions of PRL-3 of clones were assayed with antibody 3B6.

Yeast two-hybrid screen. The yeast two-hybrid screen was performed as dictated in the MATCHMAKER Two-Hybrid user manual (Clontech Laboratories, CA). The full-length cDNA of *PRL-3* was inserted in yeast expression vector pGBKT7 at *Bam*HI and *Sal*I sites. Yeast strain AH109 was co-transformed with plasmid pGBKT7-*PRL-3* and pACT2 pre-inserted with placenta brain cDNA library (Clontech, Catalogue Nos. HY402AH) in sequential transformation procedure. The co-transformants were plated on SD minimal medium lacking tryptophan, leucine, histidine, and adenine. After incubated at 30 °C for 5 days, clones growing on selection medium were analyzed for the β-galactosidase activity by colony-lift filter assay. The plasmids of blue positive clones were rescued and verified further with yeast mating. In brief, yeast strain AH109 transfected with BD-PRL-3 or control was mated with strain Y187 transfected with AD candidate. Then the β-galactosidase activity of the co-transformants growing on selection medium was analyzed again. The sequences of positive clones were searched for homology with the BLAST algorithm through NCBI web site.

Pull-down and co-immunoprecipitation. Integrin α1 cDNA fragment was digested form yeast vector pACT2 and inserted into eukaryotic vector pCMV-Myc (Clontech) at *Eco*RI and *Xho*I sites. The full-length cDNA of *PRL-3* was inserted into the C-terminal of glutathione *S*-transferase (GST) tag in eukaryotic expression plasmid pEBG at *Bam*HI and *Not*I sites. COS-7 cells were co-transfected with pEBG-*PRL-3* and pCMV-Myc-integrin α1 plasmids. For pull-down, the supernatant of cell lysates was incubated with glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1 h at 4 °C with gently rotation. The beads were washed three times with PBS and re-suspended in Laemmli loading buffer. Then the supernatant of boiled beads was subjected to 12% sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) and immunoblotted with anti-Myc antibody. For immunoprecipitation, Sepharose 4B beads conjugated to protein G (Amersham Pharmacia Biotech) were incubated with anti-Myc antibody for 1 h at 4 °C and were washed three times with PBS. Then the beads were incubated with the supernatant of cell lysates overnight at 4 °C, washed with PBS, and analyzed by immunoblotting with anti-GST antibody.

RNA interfering. Human *PRL-3*-specific and negative control siRNAs were designed and synthesized in Genechem (Shanghai, China). The sequence of *PRL-3*-specific siRNA was as follows: 5′-TCACCTAC CTGGAGAAATA-3′, which was designed to target the two splices of human *PRL-3* (GenBank Accession Nos. NM032611 and NM007079). For RNA interference, 100 nM *PRL-3*-specific or control siRNA was transfected into 293-PRL-3 cells with Lipfectamine 2000 for 6 h. After cultured for 48 h, cells were harvested.

RT-PCR. After interfered with *PRL-3*-specific or control siRNA for 48 h, the total RNA of 293-PRL-3 cells was extracted with Trizol reagent (Invitrogen) according to the manufacturer's protocol and were reverse-transcribed to cDNA. Then semi-nested PCR was used to amplify *PRL-3* as previously described [17].

Phosphorylation assay and Western blot. For over-expressing PRL-3, Flp-In-TMT-RExTM-293-PRL-3 or control cells were treated with 1 μg/ml tetracycline for 12 h. Then cells were lysed in RIPA lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsufonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin) for 20 min at 4 °C. The supernatant of lysates was collected after centrifugation at 12,000*g* for 20 min at 4 °C, and then incubated with corresponding antibody for immunoprecipitation or subjected to Western blot. Adsorbed beads or cell lysates were immediately boiled for 10 min and subjected to 12% SDS–PAGE and electro-blotted to a nitrocellulose member. Non-specific binding was blocked with 5% non-fat milk in PBS overnight at 4 °C and was rinsed twice with 0.1% Tween 20/PBS. Then the membrane was incubated with antibody against phosphorylated protein at room temperature for 1 h, followed by HRP-labeled anti-IgG for 1 h. Reaction product was visualized with enhanced chemoluminescence according to the kit instructions (Amersham Pharmacia Biotech). The result was documented on an X-ray film. For knocking down the expression of *PRL-3*, 293-PRL-3 cells were induced with 1 μg/ml tetracycline for 12 h, and then transfected with *PRL-3*-specific or control siRNAs for 48 h. Cells were harvested and treated as above.

Results

Isolation of integrin α1 as a PRL-3 interaction protein in yeast two-hybrid system

To search for PRL-3-interacting proteins, full-length PRL-3 was used as a bait to screen the human placenta brain library in yeast two-hybrid system. At first, AH109 transformed with pGBKT7-PRL-3 were prepared to competent cells. The expression of PRL-3 in AH109-PRL-3 was confirmed with 3B6 antibody (Fig. 1A) and BD-PRL-3 alone did not activate reporter genes (data not shown). Then human placenta brain library plasmids were transformed into AH109-PRL-3 and the co-transformants were selected on SD minimal medium lacking tryptophan, leucine, histidine, and adenine. In all, there were 64 clones analyzed for the β-galactosidase activity, of which we obtained five distinct positive clones. BLAST analyses identified one single open reading frame corresponding to the 3′-end 1364 base pairs of integrin α1 cDNA (GenBank

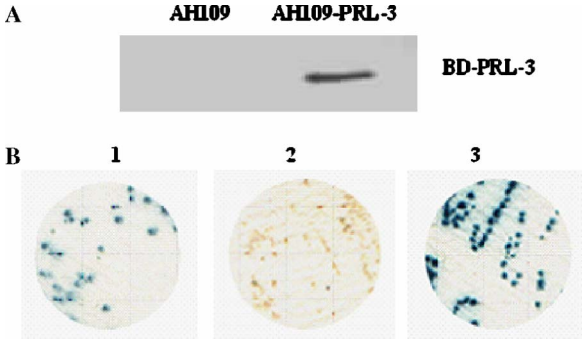

Fig. 1. Screening of the PRL-3-interacting protein in yeast two-hybrid system. (A) The expression of PRL-3 in yeast strain AH109. AH109 cells were transformed according to the small-scale LiAc yeast transformation procedure. The transformants were lysed by ultrasonic and subjected to Western blot with the antibody 3B6 against PRL-3. AH109 un-transformed was used as negative control. (B) Two-hybrid screen was performed as described in the manual of MATCHMARKER Two-Hybrid System. The positive clone obtained from primary screen was verified further with yeast mating. Yeast strain Y187 transformed with pACT2-integrin α1 was mated with strain AH109 transformed with pGBKT7-*PRL-3* (lane 1). The β-galactosidase activity of the co-transformants growing on selection medium was analyzed. As negative control, Y187 transformed with pACT2-integrin α1 was mated with AH109 transformed with pGBKT7 (lane 2). As positive control, Y187 transformed with BD-murine p53 fusion plasmid pVA3-1 was mated with AH109 transformed with AD-SV40 large T-antigen fusion plasmid pTD1-1 (lane 3).

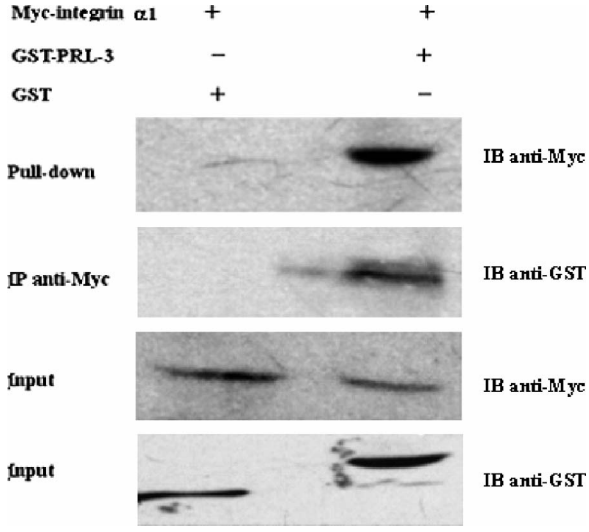

Fig. 2. Interaction of PRL-3 with integrin α1 in mammalian cells. COS-7 cells transiently co-transfected with pCMV-Myc-integrin α1 with pEBG-*PRL-3* or with pEBG were lysed in RIPA buffer. For pull-down assay, glutathione–Sepharose beads were incubated with cell lysates and immunblotted by anti-Myc antibody (the first panel). For co-immunoprecipitation assay, anti-Myc antibody was incubated with cell lysates and immunoblotted by anti-GST antibody (the second panel).

Accession No. NM181501). This sequence matches to the 445 amino acid residues in the C-terminal of integrin α1, including integral cytoplasmic region and partial extracellular region. Positive clones were subjected to yeast mating further. The mating results showed that the interaction of AD-integrin α1 with BD-PRL-3 initiated the expression of β-galactosidase, which hydrolyzed substrate X-Gal and released blue products (lane 1 of Fig. 1B). As the negative control, Y187 transformed with pACT2-integrin α1 was mated with AH109 transformed with pGBKT7. The co-transformants growing on SD/-Leu-Trp, but not on SD/-Leu-Trp-His-Ade, and the β-galactosidase activity of the former were negative (lane 2 of Fig. 1B).

Physical association of PRL-3 with integrin α1 in vivo

To further confirm the interaction of PRL-3 with integrin α1, the integrin α1 fragment from yeast vector pACT2 was inserted into eukaryotic plasmid pCMV-Myc. PRL-3 was fused with GST in eukaryotic expression plasmid pEBG, which has a more active promoter than CMV promoter. Then COS-7 cells co-expressing Myc-integrin α1 (52 kDa) with GST-PRL-3 (48 kDa) were analyzed by pull-down and co-immunoprecipitation assays. Mock vector pEBG was used as negative control, which expresses GST tag of about 26 kDa. As shown in Fig. 2, glutathione–Sepharose beads could pull-down GST-PRL-3 with Myc-integrin α1, while GST alone could not interact with Myc-integrin α1 (the first panel). Conversely, anti-Myc antibody could co-immunoprecipitate Myc-integrin α1 with GST-PRL-3, but not with GST (the second panel). Input expression of Myc-integrin α1, GST-PRL-3, and GST was showed in the third and the fourth panels. These results demonstrated that PRL-3 could interact with integrin α1 in vivo.

PRL-3 down-regulated the tyrosine-phosphorylation level of integrin β1

Because PRL-3 could interact with integrin α1, we wondered whether this physical interaction could affect the tyrosine phosphorylation of integrin. It has been known that integrin α1β1 is a receptor for collagen I, collagen IV, and laminin [18]. Integrins are heterodimeric molecules composed of α and β subunits, both of which have a long extracellular domain binding to ECM, a transmembrane domain, and a short cytoplasmic domain of about 20–70 amino acid residues [19]. Integrin α1 has no tyrosine in the cytoplasmic region, but integrin β1 has (Fig. 3). So

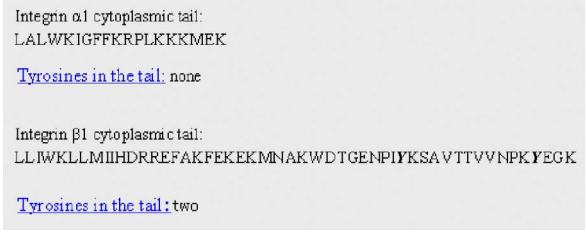

Fig. 3. Structure analyses of the cytoplasmic tails of integrin α1 and β1. The full-length amino acid sequences of integrin α1 and β1 were submitted to and analyzed on the web site of PSORT II Prediction and SMART. Tyrosine was marked with bold italic Y.

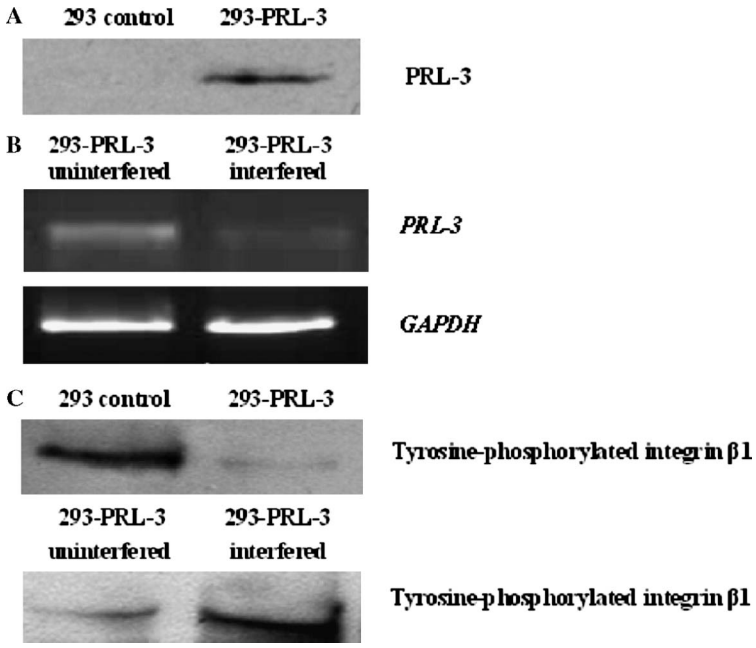

Fig. 4. PRL-3 decreases the tyrosine-phosphorylation level of integrin β1. (A) Flp-In-T-REx-293-PRL-3 cells were induced with 1 μg/ml tetracycline and the expression of PRL-3 was detected with anti-PRL-3 antibody 3B6. (B) *PRL-3*-specific siRNA or negative control siRNA was transfected into 293-PRL-3 cells, respectively. Then RT-PCR assay was performed to determine the *PRL-3* mRNA level. *GAPDH* was used as control. (C) Anti-integrin β1 antibody was used to immunoprecipitate the endogenous integrin β1 of 293 cells when *PRL-3* was over-expressed or knocked down, and antibody against phosphorylated tyrosine was used to analyze the tyrosine-phosphorylation levels.

we were interested in the influence of PRL-3 on the tyrosine phosphorylation of integrin β1. Endogenous PRL-3 is hardly to be detected in most cell lines (data not shown). So we first constructed Flp-In-T-REx-293-PRL3 cell line, which over-expressed PRL-3 when induced by tetracycline, while 293 transfected with mock vector pcDNA5/FRT/TO was used as control (Fig. 4A). Anti-integrin β1 antibody was used to immunoprecipitate the endogenous integrin β1 of equal amount lysates of 293-control and 293-PRL-3 cells. Then the tyrosine-phosphorylation level of the precipitated integrin β1 was detected with anti-phosphotyrosine antibody. As shown in the upper panel of Fig. 4C, when PRL-3 was over-expressed, the amount of tyrosine-phosphorylated integrin β1 of 293-PRL-3 cells was decreased. Contrarily, when the *PRL-3* expression in 293-PRL-3 was knocked down significantly with siRNA (Fig. 4B), the tyrosine-phosphorylated integrin β1 was increased dramatically (the bottom panel of Fig. 4C).

PRL-3 enhanced the phosphorylation level of Erk1/2

The activation of MAPK (Erk1/2) pathway is a common route transcriptionally regulating genes critical for cell growth and differentiation. Integrin-mediated cell adhesion could activate MAPK including Erk1/2, c-Jun kinase, and p38 MAPK [20]. Thus, we postulated that the signal transduction pathway of MARK could be influenced by PRL-3 possibly. To verify this hypothesis, we examined the expression levels of Erk1/2 and their phosphorylation status in 293 when *PRL-3* was over-expressed or interfered.

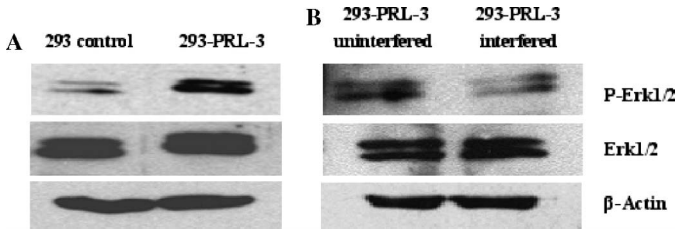

Fig. 5. PRL-3 increases the phosphorylation of Erk1/2. The phosphorylation level of Erk1/2 was assessed with anti-Erk1/2 or anti-p-Erk1/2 antibodies. 293 cells were treated as described in Materials and methods. (A) 293 control and 293-PRL-3 cells were lysed, and immunoblotted by anti-Erk1/2 or anti-p-Erk1/2. The loading amounts were equated with anti-β-Actin antibody. (B) 293-PRL-3 cells were transfected with *PRL-3*-specific siRNA or control siRNA, and treated as above.

When PRL-3 was over-expressed, the phosphorylation level of Erk1/2 in 293-PRL-3 cells was up-regulated (the upper panel of Fig. 5A). By contraries, the amount of phosphorylated Erk1/2 was lowered slightly when *PRL-3* was knocked down in 293-PRL-3 cells (Fig. 5B).

Discussion

There are strong evidences supporting that *PRL-3* plays a causative role in promoting cancer cell motility and invasion [14,16]. At present, the studies on *PRL-3* are mainly focused on its correlation with cancer on cellular and organic levels, but the mechanisms are unclear. Therefore, identifying PRL-3-interacting protein is important for understanding PRL-3's molecular roles in carcinogenesis

and signaling pathway. Using yeast two-hybrid system, we have demonstrated that integrin α1 interacts with PRL-3. Integrins are a large family of cell surface receptors involved in adhesion, migration, and other crucial physical processes. Furthermore, they influence on tumorigenicity ranging from local tumor growth to metastasis [21]. While the cytoplasmic tail of α subunit regulates integrin functions by directly initiating signaling events or modulating the signaling and ligand binding of β subunit, the cytoplasmic tail of β subunit plays a central role in integrin function [19,22,23]. Because the cytoplasmic tail of α1 subunit has no tyrosine residue, we analyzed the influence of PRL-3 on the tyrosine-phosphorylation level of integrin β1. We found that PRL-3 could down-regulate the tyrosine phosphorylation of integrin β1. It was reported that the binding ability of integrin β1 to talin, a key effector of integrin-mediated signal transduction pathway, was inhibited when the tyrosine in the NPXY motif of β1 cytoplasmic tail was phosphorylated [24]. So we also investigated the effect of PRL-3 on signaling pathway. MAPK cascade is an important signal transduction pathway triggered by integrin β1 [25,26]. In the present study, we demonstrated that PRL-3 increased the phosphorylation level of Erk1/2. However, more evidences are needed to elucidate whether the influence of PRL-3 on the phosphorylation of integrin β1 or Erk1/2 is dependent on integrin α1 or β1, and whether these effects are direct or indirect.

In summary, this is the first report demonstrating that PRL-3 can interact with integrin α1, down-regulate the tyrosine-phosphorylation level of integrin β1, and enhance the phosphorylation of Erk1/2. This study provides new clues for understanding the molecular mechanism of PRL-3 in promoting migration and metastasis.

Acknowledgments

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