Phosphorylated T567 ezrin is associated with merlin expression in KIT-mutant gastrointestinal stromal tumors

WEN-HUI WENG[1,2*], CHUN-NAN YEH[3*], YUNG-FENG CHENG[1], GOVINDA LENKA[2] and YI-WEI LIAW[1]

[1]Department of Chemical Engineering and Biotechnology, Graduate Institute of Biotechnology; [2]College of Engineering, Energy and Optoelectronic Materials Program, National Taipei University of Technology, Taipei; [3]Department of Surgery, Chang Gung Memorial Hospital, Chang Gung University, Taoyuan, Taiwan, R.O.C.

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Abstract. Membrane-cytoskeleton linker organizer ezrin is a member of the ERM (ezrin-radixin-moesin) protein family. It has been suggested as an important element in the oncogenic process, particularly in conferring a metastatic ability on tumor cells. We hypothesized that the KIT oncogenic form is one of the proteins that modulates expression of the ezrin protein via phosphorylated ezrin at different residues; furthermore, it may interact with the protein merlin, and promoting tumor development via the PI3K or MAPK pathway. In the present study, we observed that differential expression of ezrin was a common feature in gastrointestinal stromal tumors (GISTs). We further demonstrated that cases exhibiting expression of phosphorylated Thr567 in the ezrin protein were associated with immunoactivities of KIT and merlin expression (p=0.039 and 0.013, respectively). In conclusion, GISTs harbor activation of KIT protein may induce phosphorylation of the downstream protein ezrin at certain residues, thereby triggering subsequent signal transduction cascades and driving downstream pathways of tumor progression. However, a larger series of tumor samples should be analyzed in future studies, as well as the identification of phosphorylated sites to determine the role of ezrin in tumor progression thus shedding light on clinical outcomes.

Introduction

Investigations spanning almost a decade reveal that the membrane-cytoskeleton linker protein ezrin plays an important role in promoting tumor metastasis (1,2). It is therefore generally considered to be one of the predictive prognostic biomarkers in various cancer types, including osteosarcoma, breast cancer, colorectal carcinoma, soft tissue sarcoma and serous ovarian carcinoma (3-10). Ezrin protein is a member of the ERM (ezrin-radixin-moesin) group of proteins that are produced from the *VIL2* (Ctytovillin) gene, which is a cyclic AMP-dependent protein kinase anchoring protein. Activation of ezrin is known to be caused by phosphorylation at certain residues, in turn interacting directly with the actin by the C-terminus, and connecting with several transmembrane proteins or membrane-associated partners via the amino-terminal FERM (four-point-one, ezrin, radixin, moesin) domain (11). There are reports indicating that the functions of ezrin particularly operate in the regulation of epithelial cell morphogenesis, cell-cell and cell-matrix adhesion proceed through various pathways (12). Furthermore, the unexpected phosphorylation at certain residues of ezrin is vital for tumor progression. Monni *et al* observed that phosphorylated ezrin at the Tyr353 and Tyr146 residues may induce tumor cell apoptosis and promote cell proliferation in murine erythroleukemia, respectively (13). In addition, the binding of phosphatidylinositol 4,5-bisphosphate (PIP2) with ezrin N-terminal ERM association domain is necessary for the subsequent phosphorylation at Thr567, which is involved in the subsequent activation process to unmask both membrane and actin binding sites (14), which then extend to interact with CD43 and CD44 (15-18). Moreover, some of the studies further indicated that a mutant form of ezrin by mimic a phosphorylated residue ezrin T567D may maintain the protein in an open conformation that will further trigger the activity of the Rac1 pathway (but not RhoA or Cdc 42) (19,20).

Conversely, merlin acts as an inhibitor of small G-protein activation, a role more like a 'gatekeeper', in many types of tumors. The protein merlin is encoded by the tumor-suppressor gene *Neurofibromin 2 (NF2)*, from which multiple isoforms are generated after transcripts undergo alternative splicing. However, only isoform I functions as a tumor-suppressor protein, and the activitity is according to the phosphorylated status (21,22). The location of the merlin protein in cells is rather similar to ezrin. They are commonly present at the membrane-cytoskeleton interface underneath the plasma membrane, cell-cell junctions, as well as actin-rich sites (23); thus the structure of merlin shares N-terminal

Correspondence to: Dr Wen-Hui Weng, Department of Chemical Engineering and Biotechnology, Graduate Institute of Biotechnology, National Taipei University of Technology, 1, Sec. 3, Chung-Hsiao E. Rd., Taipei 10608, Taiwan, R.O.C.
E-mail: wwhlab@gmail.com

*Contributed equally

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sequence homology with the ERM protein family, except that it lacks the actin binding region. The behavior of merlin is extremely opposite to ezrin proteins, while phosphorylated-open forms exhibit loss of the function of the protein. Therefore, to decide whether the merlin or ezrin protein binds to the transmembrane proteins, such as CD44, depends on their status of phosphorylation. According to a report from Ponta *et al*, merlin negatively regulates RAC1; it is considered to be a protein which competes with the function of ezrin (11).

Based on Monni *et al*, ezrin protein may be a downstream target of the KIT gene, a receptor tyrosine kinase (RTK) (13). Other studies have also provided strong evidence regarding the possible mechanism of the mutant KIT, and have all indicated that it may affect the downstream signaling transduction; to regulate the activation of ezrin may be one of the possibilities, which eventually results in cell proliferation, anti-apoptosis or induction of cell migration. GISTs is one of the diseases related to KIT expression, which is characterized by the presence of KIT (CD117) protein activation in 75-90% of cases (24-33). Therefore, in the present study, we hypothesized that oncogenic KIT mutations may regulate ezrin and/or merlin expression in human GIST cells. In addition, the status of phosphorylated ezrin at different residues may offer a valuable piece of information in KIT-related tumor progression.

Materials and methods

Clinical samples and cell lines. A total of 13 fresh frozen tumor samples, characterized as GISTs, were kindly provided by Dr Chun-Nan Yeh of the Surgical Department of Chang Gung Memorial Hospital, Taiwan. All clinical samples had the approval of the research ethics committee and informed patient consent. The human c-*KIT*-positive GIST882 and c-*KIT*-negative GIST62 cell lines were also included as internal controls for detection of KIT expression. It is known that the GIST882 cell line with a homozygous exon 13 missense mutation, encoding a K642E results in KIT expression (34); the GIST62 cell line used to harbor an in frame exon 11 mutation after passage subsequently loses KIT expression (35). Both cell lines were grown in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) containing 20% FBS. The A431 human epithelial carcinoma and human embryonic kidney 293 (HEK293) cell lines were used as positive controls for detection of ezrin or merlin protein expression, respectively. It is known that the A431 cell line contains phosphorylated p81-ezrin on serine and threonine residues. However, after treatment with 100 ng/ml EGF p81-ezrin may become phosphorylated on tyrosine or phosphorylation of threonine residues increases (36,37).

Protein extraction. The lysate preparation of fresh tumor samples and cell line samples was carried out for the Western blot analysis. Fresh tissue samples were intially maintained at a low temperature to homogenize the tissues before being washed 3 times with ice-cold PBS, and then lysed in Pro-Prep™ protein extraction solution (iNtRON Biotechnology Inc., Seongnam, Korea) containing phosphatase inhibitor cocktail (Thermo Scientific, USA) according to the manufacturer's instructions (iNtRON Biotechnology Inc.). In brief, to induce cell lysis, the cells were incubated for 10-30 min on ice, followed by centrifugation at 13,000 rpm, at 4˚C for 5 min then stored at -20˚C. The concentration of protein was determined using the Bradford method and detected by operating the Thermo Scientific NanoDrop 2000 protein assay.

Western blot assays. All of the protein samples were resolved by 8-10% SDS-polyacrylamide gel electrophoresis and then transferred to PVDF membranes (Millipore Corporation, Billerica, MA, USA). Western blotting was performed with anti-ezrin (mouse monoclonal antibody, 1:300 dilution) (Lab Vision Corp, Fremont, CA, USA.), anti-phospho-ezrin (Tyr353) (rabbit polyclonal antibody, 1:1000 dilution) (Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phospho-ezrin (Tyr146) (rabbit polyclonal antibody, 1:400 dilution) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) anti-phospho-ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) (41A3) (rabbit monoclonal antibody, 1:1000 dilution) (Cell Signaling Technology Inc.), anti-Kit (C-19) (rabbit polyclonal antibody, 1:300 dilution) (Santa Cruz Biotechnology Inc.), and anti-NF2 (A-19) (rabbit polyclonal antibody, 1:300 dilution) (Santa Cruz Biotechnology Inc.) antibodies. ß-actin antibody was used as the loading control (Novus Biologicals, Littleton, CO, USA) for each set of experiments.

Statistical analysis. To investigate the correlation between the protein expression of KIT and the status of phosphorylated ezrin at the Thr567, Tyr146 and Tyr353 residues, as well as merlin expression, the samples were divided into KIT-positive (10 cases) or KIT-negative (3 cases) groups to correlate with the other proteins as shown in Table I. The Chi-square test was performed, and P-values <0.05 were considered to denote statistical significance.

Results

Western blot analysis. In the present study, Western blot analysis was performed to detect protein expression of KIT, total ezrin, phosphorylated status at ezrin Thr567, Tyr146, Tyr353 residues, and merlin. The results showed that 10 out of 13 (77%) GIST samples exhibited KIT expression. In addition, ezrin protein was expressed at different levels in all of the samples (100%) (Table I). Particularly, case no. 5 exhibited high expression when compared to the others (Fig. 1). Notably, when comparing the different residues Thr567, Tyr146 and Tyr353 and expression of phosphorylated ezrin, the high percentage of cases showed phosphorylation of ezrin at the Thr567 residue (85%). Interestingly, merlin expression was commonly observed in the tumors that simultaneously expressed both KIT and ezrin proteins (cases 3-5) (Fig. 1).

Chi-square test analysis. To investigate the correlation between the expression of KIT and the status of phosphorylated ezrin at the different residues, as well as merlin in the GIST samples, the Chi-square test showed KIT expression in the GIST cases was highly associated with phosphorylated ezrin at Thr567 or merlin expression (both P=0.039). Notably, all of the cases that contained phosphorylated ezrin at Tyr146 also exhibited expression of merlin (Table I).

Table I. Correlation between the expression of KIT and different phosphorylation sites of ezrin and merlin in GISTs.

Protein	GISTs (n)						Rate of expression (%)
	KIT		P-value	Merlin		P-value	
	+	-		+	-		
Ezrin							
Expressed	10	3	-	11	2	-	100
p-ezrinThr567							
Expressed	10	1	**0.039**	11	0	**0.013**	85
Not expressed	0	2		0	2		15
p-ezrinTyr146							
Expressed	5	0	0.231	5	0	0.487	39
Not expressed	5	3		6	2		61
p-ezrinTyr353							
Expressed	4	1	0.835	5	0	0.487	39
Not expressed	6	2		6	2		61
Merlin							
Expressed	10	1	**0.039**				85
Not expressed	0	2					15

n, the number of cases; +, positive expression of protein; -, negative expression of protein; P-values <0.05 are indicated in bold.

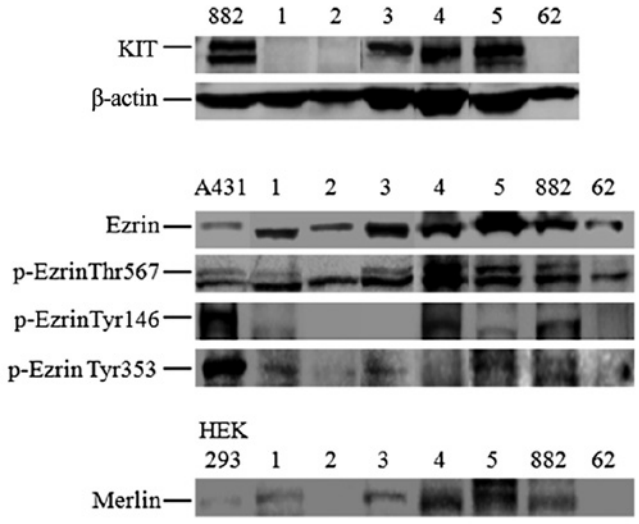

Figure 1. Western blot analysis showed the expression of KIT, p-ezrinThr567, p-ezrinTyr146, p-ezrinTyr354 and merlin in the GIST samples. GIST882 and GIST62 cell lines were used as KIT-positive and -negative internal controls, respectively. A431 and HEK293 cell lines were used as the positive controls to detect expression of total ezrin or phosphorylated ezrin and merlin, respectively. ß-actin served as the loading control.

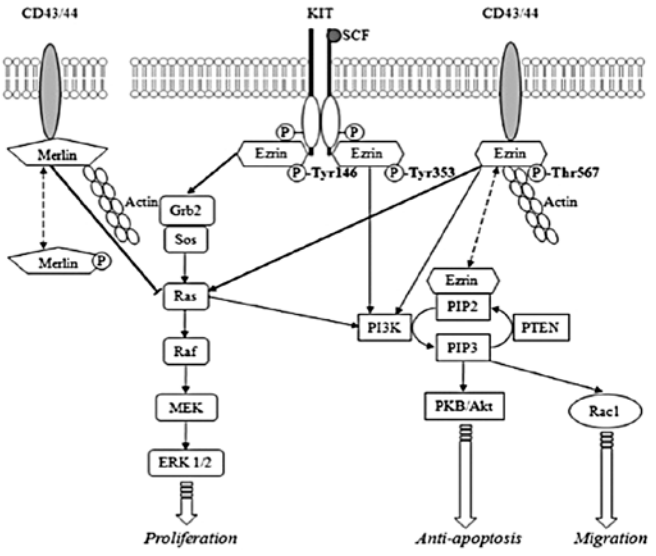

Figure 2. Schematic illustration of the proposed mechanism of ezrin protein phosphorylation upon the activation of (mutant) KIT. KIT ligand/receptor activity induces phosphorylation of ezrin at Tyr146 and Tyr353, which exerts RAS/MAPK and PI3K signaling transduction pathways. Hence, p-ezrinThr567 indirectly affects RAC1 activation through the PI3K pathway. Whereas merlin was presented as a negative regulated form, p-ezrinTyr146 and p-ezrinThr567 were triggered and mediated the RAS/MAPK pathway as indicated by bold arrows.

Discussion

In the present study, different expression levels of ezrin total protein were observed in all samples (Table I). A high correlation between the expression of KIT and phosphorylated ezrin at Thr567 or merlin was noted (P=0.039 and 0.013, respectively). A recent study provided evidence that ezrin overexpression in GISTs may be an independent adverse prognostic indicator (29). Hence, in several types of cancers such as sarcoma, mesenchymal tissue tumors, hepatocellular carcinoma and prostate cancer, ezrin was found to play an important role in tumor progression and was highly

associated with tumor metastasis (9,38,39). Monni *et al* found that phosphorylated ezrin at certain residues, for example, p-ezrinTyr146 and p-ezrinTyr353, acted as a downstream effector of oncogenic tyrosine kinases in a study of murine erythroleukemia (13). Moreover, KIT ligand/stem cell factor (SCF) may induce phosphorylation on threonine residue of ERM proteins, besides, the increasing of Rac1 activation through the activation of PI3K was also demonstrated in human melanocytes (40). Accordingly, we proposed that ezrin is an important target to further drive tumor progression, particularly in tumors associated with oncogenic KIT mutants, such as GISTs.

Thus, in the present study, a total of 13 clinical GIST cases were investigated, and 10 cases confirmed to be KIT-positive were included. Expression of p-ezrinThr567 and merlin were simultaneously present in the KIT-positive GIST samples (P=0.039). We presumed that KIT-related tumors may simultaneously express both ezrin and merlin, although they have an opposite function as cell proteins. The underlying variant pathways may rely on KIT activation; in turn ezrin activity is regulated by the turnover of phosphorylation on the Thr567 residue in ezrin protein. This mechanism may play a key role in triggering downstream pathway activities. This finding was supported by Di Cristofano *et al* and Cui *et al* who evaluated p-ezrinTyr353 and p-ezrinThr567 expression in osteosarcoma and pancreatic ductal adenocarcinoma, respectively, and pinpointed that p-ezrinThr567 expression could be observed mostly in tumors and related to tumor progression. However, there is no strong evidence supporting the promotion of tumor metastatis (41,42).

Moreover, we further observed that the cases exhibiting expression of p-ezrinTyr146, which were all associated with the expression of p-ezrinThr567. To our knowledge, the potential mechanism of tumoral development regarding p-ezrinTyr146, may be via the MEK pathway cascade, in turn promoting tumor cell proliferation (Fig. 2). Similar evidence has been provided in leukemic cells in both *in vitro* and *in vivo* studies (13). Thus, we presumed that while the tumors presented phosphorylation at both the Tyr146 and Thr567 ezrin protein residues, tumor development might be through the MEK signaling pathway promoting tumor cell proliferation. On the other hand, for the tumors exhibiting phophorylation at Thr567 of the ezrin protein only, the later signaling transduction might guide tumor cells to the consequences of anti-apoptosis or tumor migration via the PI3K pathway (Fig. 2). Accordingly, we concluded that tumor progression of KIT-related tumors, such as GISTs, strongly relies on the activities of phosphorylated ezrin residues, and the consequent activities of the ezrin protein may provide a novel clinical application.

Regarding the correlation between ezrin and merlin, due to the highly similar structure of the proteins, merlin shares its domain organization of NH2-terminal sequence homology with ERM proteins, but does not contain a canonical actin-binding motif at its C-terminus. Thus, the phosphorylated merlin protein structure presumably opens and forms heterodimers with ezrin or other ERM proteins, and localizes at the cell cortex. Therefore, all of these may bind to identical or similar proteins of the plasma membrane (43). Once merlin protein is unable to bind either upstream of the SOS or downstream of the Ras and Rac, this may lead to cell proliferation or transformation (23,44). Our present study demonstrated that the merlin-positive cases were specifically associated with p-ezrinThr567 in GISTs (P=0.013), but were not correlated with p-ezrinTyr353 and p-ezrinTyr146 expression. Thus, we speculated that the p-ezrinThr567 residue may play a crucial role in the interaction with merlin, and then directly modulates RAS protein activity further triggering the downstream MEK signaling transduction pathway (Fig. 2). Since one of the functions of merlin is to promote PDGFR degradation, it appears to suppress the activation of the MAPK and PI3K signaling pathways (46). The loss of function of the merlin protein in tumors may result by either phosphorylation or formed deficiency of the truncated protein that further leads to tumor progression (45). Although all of our samples presented ezrin total protein expression, we then further determined that the phosphorylated ezrin residue site was correlated with merlin expression. The corresponding phosphorylated status of merlin, or the gene structure of merlin may need to be confirmed in future studies as well.

In summary, further investigation with different phosphorylation sites to determine the role of ezrin in tumor progression is needed, and larger series of tumor samples are necessary in future studies. The results may shed light on clinical outcome.

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