Wild-type KIT overexpression in colon cancer:

Protein Kinase C-δ-mediated recycling of KIT

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Wild-type KIT overexpression in colon cancer: Protein Kinase C-δ-mediated recycling of KIT

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마지막으로 부족한 딸이지만 항상 최고라 말해주시는 아버지, 학위기간 동안 저보다 더 힘들었을 어머니, 때론 친구같이 때론 선배같이 많은 도움 준 오빠, 언니께 끝없는 감사와 사랑을 드립니다.

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ABSTRACT

Wild-type KIT overexpression in colon cancer: Protein Kinase C-δ-mediated recycling of KIT

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Directed by Professor Hoguen Kim

Abnormal signaling through receptor tyrosine kinase (RTK) moieties is important in tumorigenesis and drug targeting of colorectal cancers (CRCs). Wild type KIT (WT-KIT), a RTK that is activated upon binding with stem cell factor (SCF), is highly expressed in some colon cancers; however, little is known about the functional role of SCF-dependent KIT activation in colon cancer pathogenesis. We aimed to elucidate the conditions and roles of WT-KIT activation in colon cancer tumorigenesis.

CRCs with KIT expression were characterized by immunoblotting and immunohistochemistry. The biological alterations after KIT-SCF binding were analyzed with or without protein kinase C (PKC) activation. We found that WT-KIT was expressed in a subset of colon cancer cell lines and was activated by SCF, leading to activation of downstream AKT and ERK signaling pathways. We also demonstrated that KIT expression gradually decreased after prolonged SCF stimulation due to lysosomal degradation. Degradation of WT-KIT after SCF binding was significantly rescued when PKC was activated. We also demonstrated the involvement of activated PKC-δ in the recycling of WT-KIT. We further showed that a subset of CRCs exhibit expressions of both WT-KIT and activated PKC-δ and that expression of KIT is correlated with poor patient survival (P=.004).

Continuous downstream signal activation after KIT-SCF binding is accomplished through PKC-δ-

mediated recycling of KIT. This sustained KIT activation may contribute to tumor progression in a subset of colon cancers with KIT expression, and might provide the rationale for a therapeutic approach targeting KIT.

Keywords: protein kinase C-δ, KIT expression, KIT recycling, colon cancer_

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Ⅰ. INTRODUCTION

The genetic aberrations of colon cancers have been well characterized. Recent colon cancer genome analysis studies demonstrated that most colorectal cancers show similar patterns of genomic alteration, and mutations of *APC*, *TP53*, *SMAD4*, *PIK3CA* and *KRAS* were frequently identified[1]. These mutations contribute to the development of biologically aggressive colorectal carcinomas and are directly linked to dysregulation of signaling pathways involving Wnt/β-catenin, PI3K, and MAPK in colon cancers[2].

Among the signaling pathways that are active in colorectal cancers (CRCs), activation of signaling through the receptor tyrosine kinase (RTK) moieties of growth factor receptors plays a crucial role in colon cancer tumorigenesis and drug targeting. Activation of epidermal growth factor receptor (EGFR) is particularly relevant in CRCs. EGFR overexpression occurs in 60 to 80% of CRCs and Cetuximab, a monoclonal antibody to EGFR, has been shown to be clinically effective in CRCs [3]. KIT

is a RTK that is expressed in some epithelial cell lineages during embryogenesis. KIT and its ligand, stem cell factor (SCF), are essential for the maturation of some primitive cells during embryonic development and aberrant expression of KIT and SCF has been reported in human malignant tumor cells derived from epithelial cell lineages that express KIT during embryogenesis such as breast, lung, and prostate [4-6]. In addition, previous studies demonstrated that some human CRCs express high levels of KIT and SCF relative to normal mucosa cells [7, 8].

Activation of KIT in tumors is achieved in two different ways. Activation through the acquisition of activating mutations is common and has been reported in gastrointestinal stromal tumor (GIST), acute myelogenous leukemia (AML), and mastocytosis [4]. The molecular mechanisms of this type of activation are well established and imatinib, which targets activated KIT molecules, is effective in the control of tumors with *KIT* mutations [9, 10]. The other pathway involves ligand-dependent activation of wild type KIT (WT-KIT). To date, SCF is the only identified ligand that binds to KIT. Binding of SCF to WT-KIT leads to receptor dimerization and induces activation of downstream signals [11, 12]. Although the molecular characteristics of KIT activation after SCF binding are well established, little is known about the functional roles of SCF-induced WT-KIT activation in cancers.

In this study, we analyzed the biological alteration of KIT after SCF binding in WT-KIT expressing colorectal cancer cells as well as its impact on downstream signaling. Based on our findings, we propose that sustained activation of downstream signals after KIT and SCF binding can be accomplished by PKC-δ-mediated recycling of WT-KIT.

Ⅱ. MATERIALS AND METHODS

1. Cell lines and culture

Five human colon cancer cell lines (DLD-1, HCT116, SNUC4, Colo320DM, and Ls174T) were selected from 11 colon cancer cell lines after initial screening of KIT expression by reverse transcription-polymerase chain reaction (RT-PCR). DLD-1, Colo320DM, and Ls174T were selected as KIT-expressing colon cancer cell lines and HCT116 and SNUC4 were selected as control cell lines lacking KIT expression. HeLa was also used as a control cell line. Cells were purchased from the Korean Cell Line Bank (Cancer Research Institute, Seoul, Korea). All cells were grown in RPMI and Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA) and 1% penicillin/streptomycin at 37℃ in 5% CO2.

2. Construction of expression vectors

An expression vector for *KIT* cDNA containing a FLAG tag was constructed using pCMV vector and *KIT* coding regions amplified by PCR using cDNA from DLD-1 cells. The generation of PKC constructs was described previously [13].

3. Transfections

All transfection experiments were carried out using OPTI-MEM and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). On the second day after transfection, cells were harvested and proteins were extracted for Western blot analysis.

4. Western blotting

Whole cell lysates were prepared using passive lysis buffer (Promega, Madison, WI, USA) with a protease inhibitor cocktail (Roche, Meylan, France). The membranes were incubated with primary antibodies against GAPDH (Trevigen, Gaithersburg, MD, USA); FLAG (Sigma-Aldrich, Poole, Dorset, UK); KIT (Dako, Cambridge, UK); ERK, phospho-ERK, HA (Santa Cruz Biotechnology, SantaCruz, CA, USA); phospho-KIT (Invitrogen); and AKT, phospho-AKT, PKC-δ, phospho-PKC-δ (Cell Signaling, Danvers, MA, USA) for 2 hours at room temperature. Western blot images were analyzed with a LAS-4000 Mini camera (Fujifilm, Tokyo, Japan).

5. Immunoprecipitation

For immunoprecipitation, lysates were precleared and immunoprecipitated with anti-FLAG M2-agarose affinity gel (Sigma-Aldrich) at 4℃ or incubated with various antibodies and captured with Protein A/G beads. The immune complexes bound to the affinity gel were washed three times and boiled with 100 mM Tris-HCl/1% SDS to elute the complexes.

6. Immunofluorescence

Cells grown on slides were rinsed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 minutes, and permeabilized in 0.3% Triton X-100 in PBS. After blocking of non-specific sites with 2% bovine serum albumin for 1 hour, the slides were incubated with primary antibody for 1 hour. Cells were washed and incubated for 50 minutes with the appropriate fluorescent-labeled secondary antibody (Invitrogen). All images were obtained using a LSM700 confocal microscope (Carl Zeiss, Jena, Germany).

7. Quantitative RT-PCR

The primers for semiquantitative RT-PCR and qRT-PCR were designed using Primer 3 database (http://frodo.wi.mit.edu/primer3/). RNA was isolated from cells using Trizol (Invitrogen) and reverse

transcription was performed using M-MLV reverse transcriptase (Invitrogen). RT-PCR was performed using AmpliTaq Gold® 360 DNA Polymerase (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed using the ABI PRISM 7500 Sequence Detector (Applied Biosystems) and SYBR Premix Ex Taq II (TaKaRa, Japan). The amount of target mRNA was normalized to that of *GAPDH* mRNA. The sequences of the primers used are listed in Table 1.

Table 1. Primers of the genes used for RT-PCR analysis and siRNA sequences

Primers for **RT-PCR**		
KIT	forward	5'-GCCTTTTCCGTGATCCATTCA-3'
	reverse	5'-GGCGACGAGATTAGGCTGTTA-3'
GAPDH	forward	5'-ATGGGGAAGGTGAAGGTCG-3'
	reverse	5'-GGGGTCATTGATGGCAACTATA-3'
Primers for **qRT-PCR**		
KIT	forward	5'-GTTCTGCTCCTACTGCTTCGC-3'
	reverse	5'-TAACAGCCTAATCTCGTCGCC-3'
GAPDH	forward	5'-CCAGTGCAAAGAGCCCAAAC-3'
	reverse	5'-GCACGGACACTCACAATGTTC-3'
siRNA sequences		
KIT		5'-CUGUAGAUUCUGUGGAACA-3'
PKC-δ		5'-CUCAUGGUACUUCCUCUGU-3'
Non-specific RNA		5'-CCUACGCCACCAAUUUCGU-3'

8. Patients and tissue samples

Colorectal carcinoma tissue samples from 250 patients with primary colorectal carcinomas of stages I to IV were used in this study. The specimens were obtained from the archives of the Department of

Pathology, Yonsei University, Seoul, Korea and from the Liver Cancer Specimen Bank of the National Research Resource Bank Program of the Korea Science and Engineering Foundation of the Ministry of Science and Technology. Colon cancer tissues were subjected to immunohistochemical analysis for KIT using a tissue microarray. Histologic analysis was performed by two pathologists (HyK. and Ho K.) without any knowledge of the molecular data. Patient data were collected retrospectively from hospital records and the Korea National Statistics Office. All patients had undergone colorectal resection between 2004 and 2006 and fresh snap-frozen samples were obtained immediately at the time of surgery. The median follow-up time after surgery was 59.2 months. Among these 250 colon cancers, a total of 32 sporadic colorectal carcinomas (22 of which showed KIT expression by immunohistochemical analysis) and 32 matched non-tumorous colonic mucosal tissues were subjected to Western blotting analysis. All of the carcinoma samples comprised greater than 70% tumor cells, and none of the patients had received neoadjuvant chemotherapy. Authorization for the use of these tissues for research purposes was obtained from the Institutional Review Board of Yonsei University of College of Medicine.

Ⅲ. RESULTS

1. Identification of WT-KIT expression in colon cancer cell lines

We examined KIT expression in a control cell line (HeLa) and five colon cancer cell lines (DLD-1, HCT116, SNUC4, Colo320DM, and Ls174T). KIT expression was identified in three of the six cell lines (DLD-1, Colo320DM, and Ls174T) by RT-PCR, qRT-PCR (Fig. 1A). KIT protein expression in these three cell lines correlated with the mRNA levels (Fig. 1B). No mutations were found in exons 9, 11, 13, and 17, which are sites of previously identified *KIT* mutations (data not shown). Loss of KIT expression following treatment with *KIT*-specific siRNA was confirmed (Fig. 1C). These findings indicate that WT-KIT is strongly expressed in a subset of these colon cancer cell lines.

2. SCF induces KIT activation and subsequently activates AKT and ERK signaling pathways

We next examined whether WT-KIT is activated after SCF treatment of DLD-1, Colo320DM, and Ls174T cell lines. In all three cell lines, treatment with SCF induced KIT activation in a dose-dependent manner. SCF also induced activation of the well-known downstream molecules AKT and ERK that are related to cell survival and proliferation, respectively, as assessed by Western blotting measuring the phosphorylated forms of these proteins (Fig. 1D). Activation of AKT and ERK after SCF treatment was abolished by KIT siRNA, demonstrateng that activation of AKT and ERK was exclusively dependent on activated KIT (Fig. 1E). In contrast to the cell lines with WT-KIT expression, SCF treatment did not affect downstream signaling pathways in GIST882 (*KIT*-K642E-GIST) and HMC-1 (*KIT*-D816V-mast cell) cells that express mutant KIT (Fig. 1F). These findings confirm that SCF activates WT-KIT and downstream signaling pathways in colon cancer cell lines but has no effect on mutant KIT expressed in other cell lines. We next evaluated the time course of SCF stimulation by examining the status of KIT activation 5 and 15 minutes after SCF treatment and found that levels of phospho-KIT, phospho-AKT, and phospho-ERK gradually increased over this period. However, the expression of KIT was markedly decreased 15 minutes after SCF treatment (Fig. 1G).

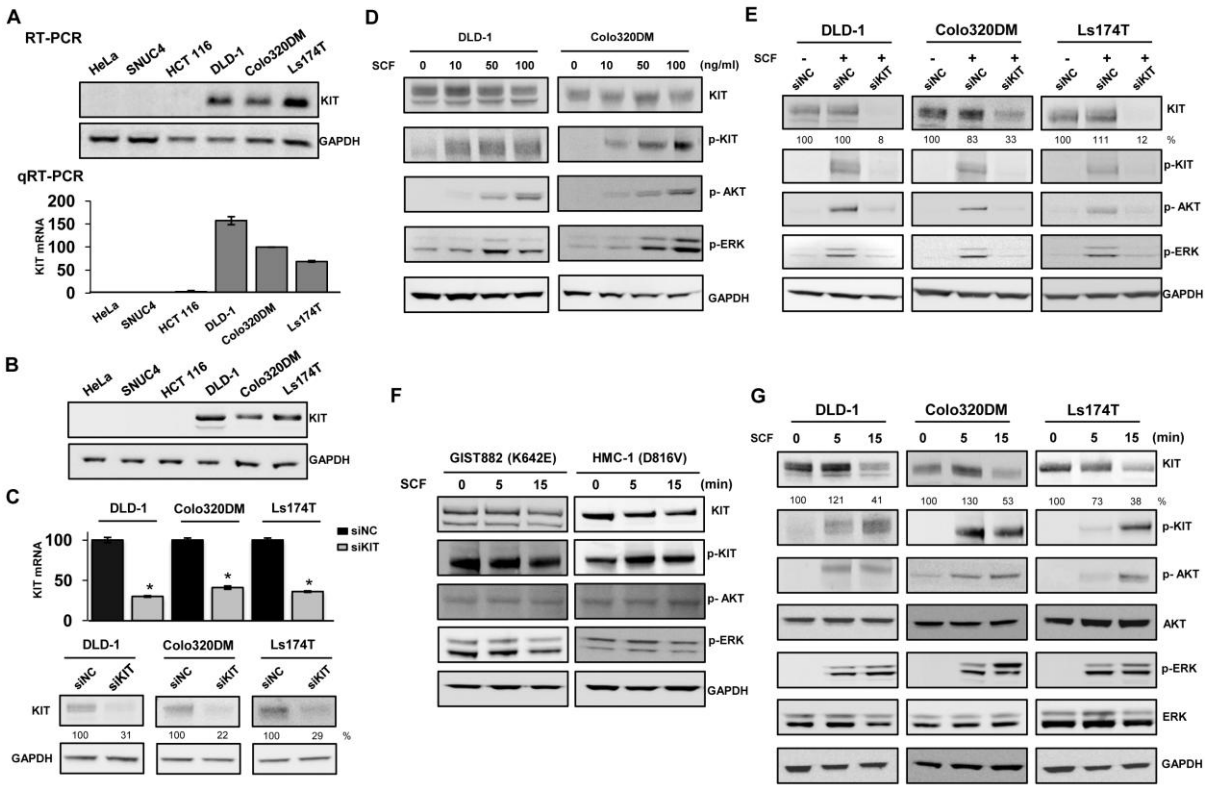

Figure 1. KIT expression and SCF treatment induced activation of downstream signaling pathways in colon cancer cells. (A) RT-PCR and real-time PCR analysis of KIT gene expression in HeLa, SNUC4, HCT116, DLD-1, Colo320DM, and Ls174T cells. KIT mRNA was expressed in DLD-1, Colo320DM, and Ls174T cells. Data are shown as mean ± standard deviation (SD; n=3). (B) Western blot analysis of KIT expression. KIT protein expression correlated with mRNA expression. (C) Inhibition of KIT expression by KIT-specific siRNA. KIT mRNA and protein were measured by real-time PCR and Western blot analysis 72 hours after transfection. GAPDH was used as a loading control. (D) Expression of KIT, phospho-KIT, phospho-AKT, and phospho-ERK increased with increasing SCF concentration in two KIT-expressing cell lines. (E) Analysis of phospho-KIT, phospho-AKT, and phospho-ERK expression after SCF treatment. None of the induced phospho-proteins were detected after KIT knockdown by treatment with siRNA. (F) No changes in the expression of KIT, phospho-KIT, phospho-AKT, and phospho-ERK were found after SCF treatment in two tumor cell lines with KIT mutation. (G) Expression of phospho-KIT, phospho-AKT, and phospho-ERK after SCF treatment

in three KIT-expressing cell lines. Increased levels of phospho-KIT, phospho-AKT, and phospho-ERK were observed 5 and 15 minutes after SCF treatment. In contrast, the level of KIT protein was decreased 15 minutes after SCF treatment.

3. Activated KIT proteins are degraded by the lysosomal degradation pathway

Having demonstrated down-regulation of WT-KIT protein 15 minutes after SCF treatment, we measured the mRNA and protein levels of KIT during a 2-hour period after SCF treatment. *KIT* mRNA expression levels in DLD-1, Colo320DM, and Ls174T cell lines were constant during this time (Fig. 2A) whereas the levels of KIT protein were decreased at 1 hour after SCF treatment. The expression of phospho-KIT was markedly increased 10 minutes after SCF treatment, but was barely detectable at 1 and 2 hours (Fig. 2B). In contrast to the gradual down-regulation of WT-KIT proteins after SCF treatment, there was no change in the expression of mutant KIT or phospho-KIT proteins in GIST882 and HMC-1 cells (Fig. 3C). It is well known that RTKs are internalized into vesicles and transferred via early and late endosomes for degradation in the lysosome upon the appropriate stimulus. Normally, this endocytotic process is constantly ongoing in the cell. To confirm whether lysosomal degradation is responsible for the gradual decrease in WT-KIT after SCF binding, we designed a rescue assay using inhibitors known to block two major protein degradation pathways: Bafilomycin A1 was used to block lysosomal degradation and MG132 was used to block the proteasomal pathway. Cells were treated with SCF alone or with the respective inhibitors for 1 hour and then expression of KIT protein was analyzed. The results showed that KIT proteins were barely rescued after inhibition of proteasomal degradation (Fig. 2D) whereas there was significant rescue of KIT protein (up to 100% compared to the control) after Bafilomycin A1 treatment, indicating that the down-regulation of activated WT-KIT is mainly dependent on the lysosomal degradation pathway (Fig. 2E). We next validated the lysosomal degradation of KIT by analyzing the intracellular location of KIT after SCF binding. In the resting state, KIT proteins were mainly distributed on the plasma membrane of DLD-1 cells. After SCF stimulation, rapid internalization of KIT was observed. To

identify the location of KIT after SCF stimulation, we compared the localization of KIT and LAMP-1 (lysosomal associated membrane protein 1). Without SCF stimulation KIT was rarely co-localized with LAMP-1; however, the majority of KIT proteins clearly showed co-localization with LAMP-1 in cells treated with SCF (Fig. 2F). Taken together, these findings indicate that WT-KIT is activated by SCF binding, internalized, and finally targeted to the lysosomes for degradation.

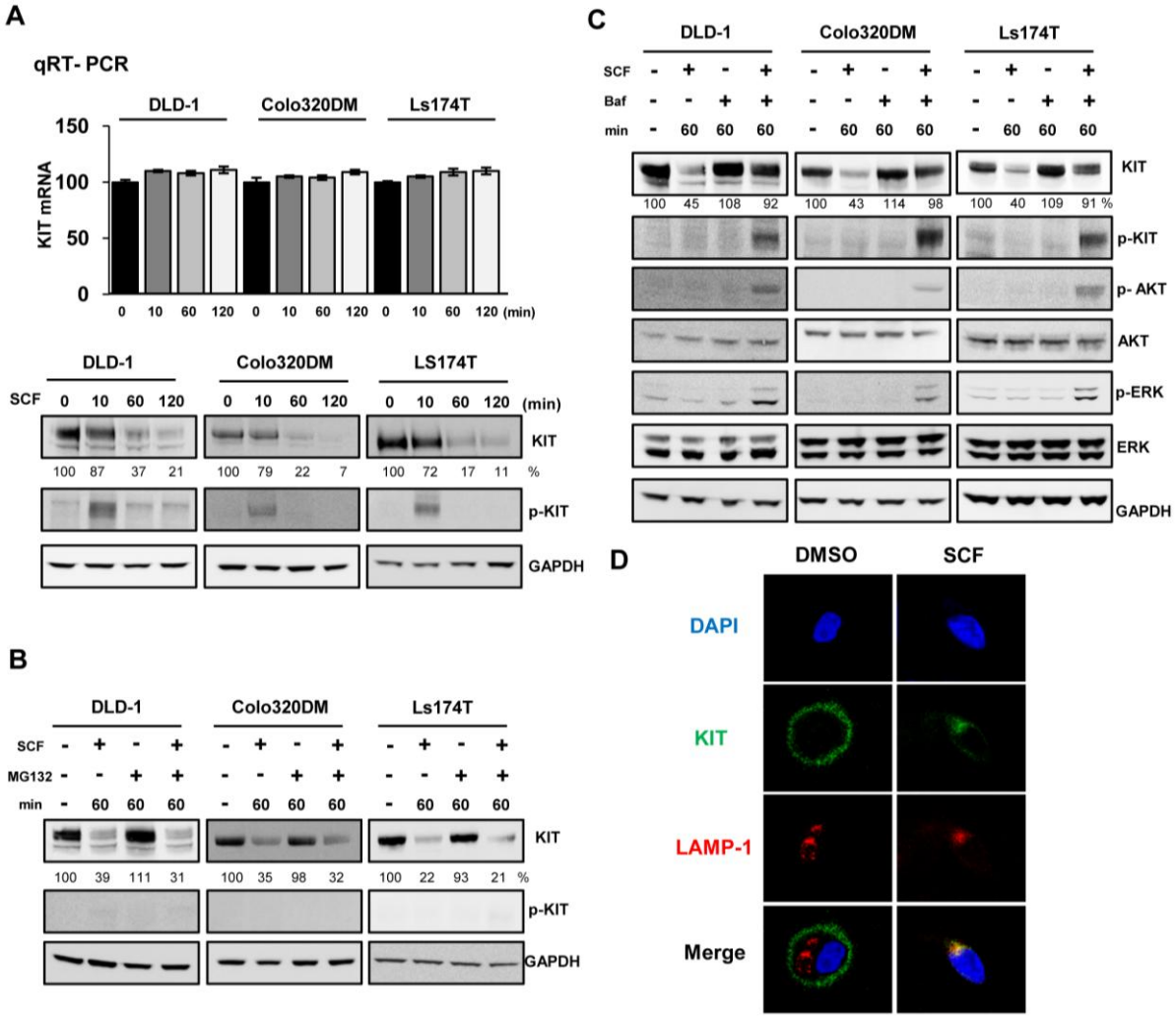

Figure 2. Activated KIT proteins are degraded through the lysosomal pathway. A, mRNA and protein expression levels of KIT in DLD-1, Colo320DM, and Ls174T cells after SCF treatment were analyzed at the indicated times by real-time PCR and Western blotting. Data are shown as mean ± SD (n=3). The mRNA levels of KIT were constant in all three cell lines during the experiment, however KIT protein was barely detectable 60 and 120 minutes after SCF treatment. B, Treatment with MG132

for 60 minutes had no effect on SCF-induced KIT degradation in all three cell lines. C, Treatment with Bafilomycin A1 (Baf) rescued the KIT protein level and stabled levels of p-KIT, p-AKT, and p-ERK in all three cell lines. D, Immunostaining of KIT and LAMP1 in DLD-1 cells treated with or without SCF. DLD-1 cells were stimulated by SCF for 1 hour prior to immunostaining for KIT (green), LAMP-1 (red), and staining with DAPI (blue). Co-localization of KIT and LAMP-1 is evident as a yellow signal in cells treated with SCF.

4. PKC activation rescues KIT from lysosomal degradation

Given the rapid degradation of WT-KIT in colon cancer cell lines after SCF treatment, we speculated on the roles of SCF-KIT activation in CRC tumorigenesis. If SCF-KIT activation plays an important role in CRC tumorigenesis, constant activation of SCF-KIT would be required. Because WT-KIT proteins are degraded in the lysosome after binding of SCF, we hypothesized that activated WT-KIT proteins are recycled in tumor cells and thus constantly contribute to CRC tumorigenesis. We further suspected that protein kinase C (PKC) activation might be involved in the recycling of WT-KIT protein because it has been reported that other RTK receptors such as EGFR and platelet-derived growth factor (PDGF) β-receptor are recycled by PKC activation [14, 15]. To test the involvement of PKC in the regulation of KIT, we treated the three KIT-expressing cell lines with phorbol 12-myristate 13-acetate (PMA), a PKC activation factor, after SCF activation. We found that the level of KIT proteins was dramatically rescued after PMA treatment, and that activated forms of KIT, AKT, and ERK were stabilized even 2 hours after SCF treatment in all three cell lines (Fig. 3A). When DLD-1 and Colo320DM cells were concomitantly treated with PMA and Gö6983, a PKC inhibitor, KIT and the downstream proteins were no longer stabilized (Fig. 3B).

To confirm that activation of PKC rescues KIT from degradation, we evaluated the intracellular location of KIT with or without PKC activation. Before SCF stimulation most of the KIT proteins were localized at the plasma membrane. After SCF stimulation, cytoplasmic translocation of KIT was evident and only a scant amount of KIT was present in the plasma membrane. When the cells were

concomitantly treated with SCF and PMA, more KIT protein was detected on the plasma membrane than in cells treated with SCF only, suggesting that PMA-mediated PKC activation leads to recycling of KIT proteins in the late endosome. In addition, we observed very little co-localization of KIT proteins with LAMP-1 in the cells treated concomitantly with SCF and PMA (Fig. 3C).

To validate the endosomal recycling of KIT after PMA stimulation, we analyzed the co-localization of KIT and Rab11, a well-known recycling endosome marker. When Ls174T cells were treated with SCF alone, very little co-localization of Rab11 and KIT was identified; however, in cells treated concomitantly with SCF and PMA a large proportion of KIT proteins co-localized with Rab11 (Fig. 3D). These findings indicate that PKC activation plays a role in sustained KIT activation by inhibiting KIT degradation and instead recycling the KIT proteins.

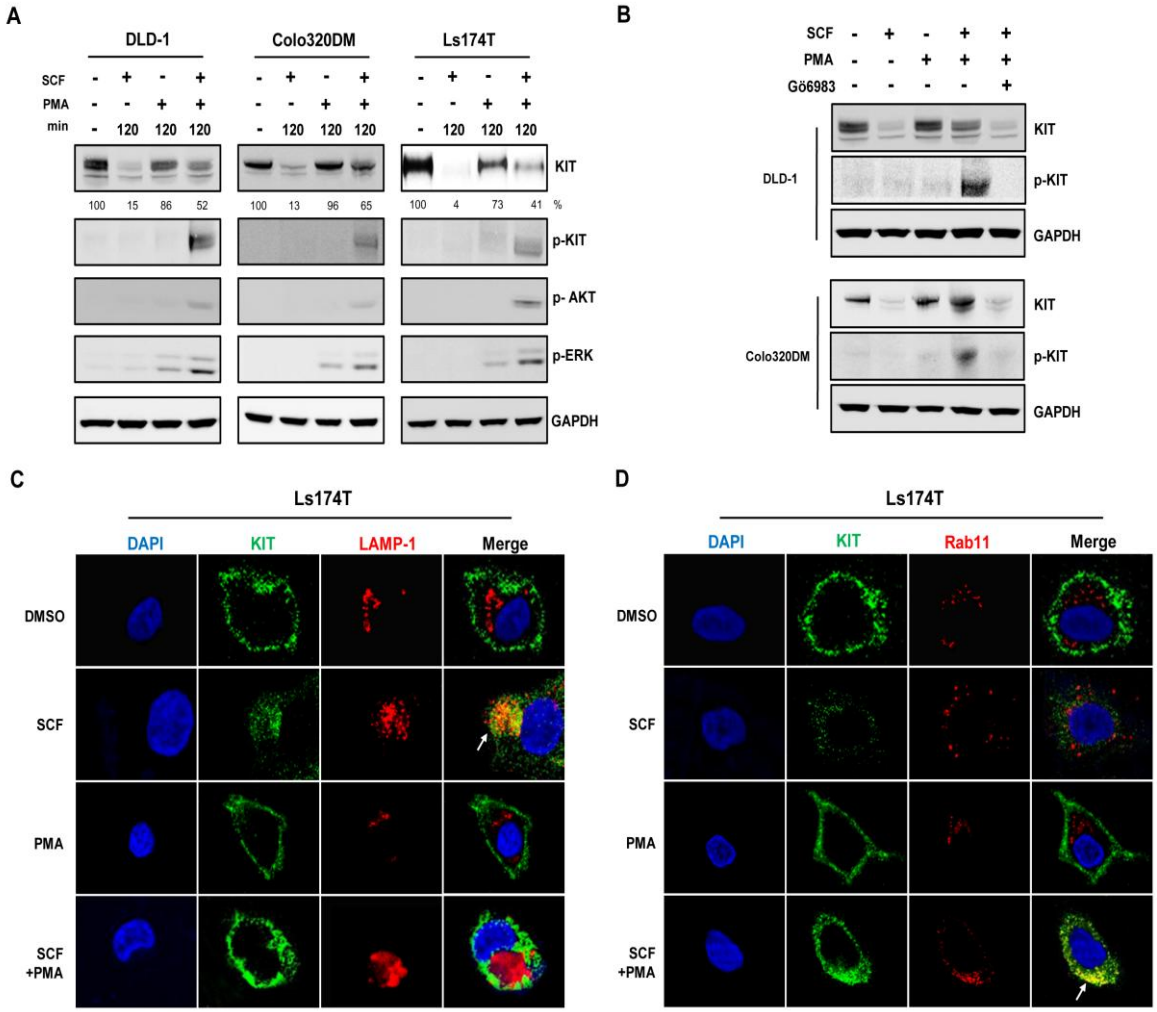

Figure 3. PKC activation rescues KIT from lysosomal degradation. (A), to activate PKC, cells were pretreated with 300nM PMA for 30 minutes and then incubated with or without SCF for 2 hours. All three cell lines showed expression of KIT, phospho-KIT, phospho-AKT, and phospho-ERK after treatment with SCF and PMA. (B), rescue of KIT expression was not detected in the cells treated with Gö6983, a PKC inhibitor in DLD-1 and Colo320DM cells. C and D, Ls174T cells were pretreated with 300nM PMA and incubated with SCF for 1 hour. Cells treated with only SCF were used as a control. Cells were stained for KIT (green), DAPI (blue), and LAMP-1 (red) (C) or Rab11 (red) (D). In cells treated concomitantly with SCF and PMA, membrane expression of KIT was constantly detected (C) and co-localization of LAMP1 and KIT was rarely detected. Co-localization of KIT and Rab11 was evident (D).

5. PKC-δ directly binds and rescues WT-KIT

Having identified a role of PKC in constant SCF-KIT activation in colon cancer cells, we investigated which isoform of PKC contributes to KIT recycling by co-immunoprecipitation assays using synthetic KIT and PKC proteins. *In vitro* binding assays performed after treatment with SCF and PMA showed that WT-KIT directly and exclusively binds to PKC- δ (Fig. 4A). To further examine the interaction between KIT and PKC-δ, we performed binding assays in the presence or absence of SCF and/or PMA. Binding of KIT to PKC-δ was only observed after treatment with both SCF and PMA, indicating that activated PKC-δ is involved in KIT recycling (Fig. 4B). Interestingly, the binding of KIT and PKC-δ is exclusively SCF-dependent; no binding between KIT and PKC-δ was found in cells without SCF treatment. To confirm that activation of PKC-δ can rescue KIT, cells were transfected with control siRNA or PKC-δ-specific siRNA before treatment with SCF and PMA. The rescue of KIT was significantly reduced (8%) in the cells transfected with siPKC-δ compared with control cells (Fig. 4C), indicating that KIT recycling depends on activated PKC-δ.

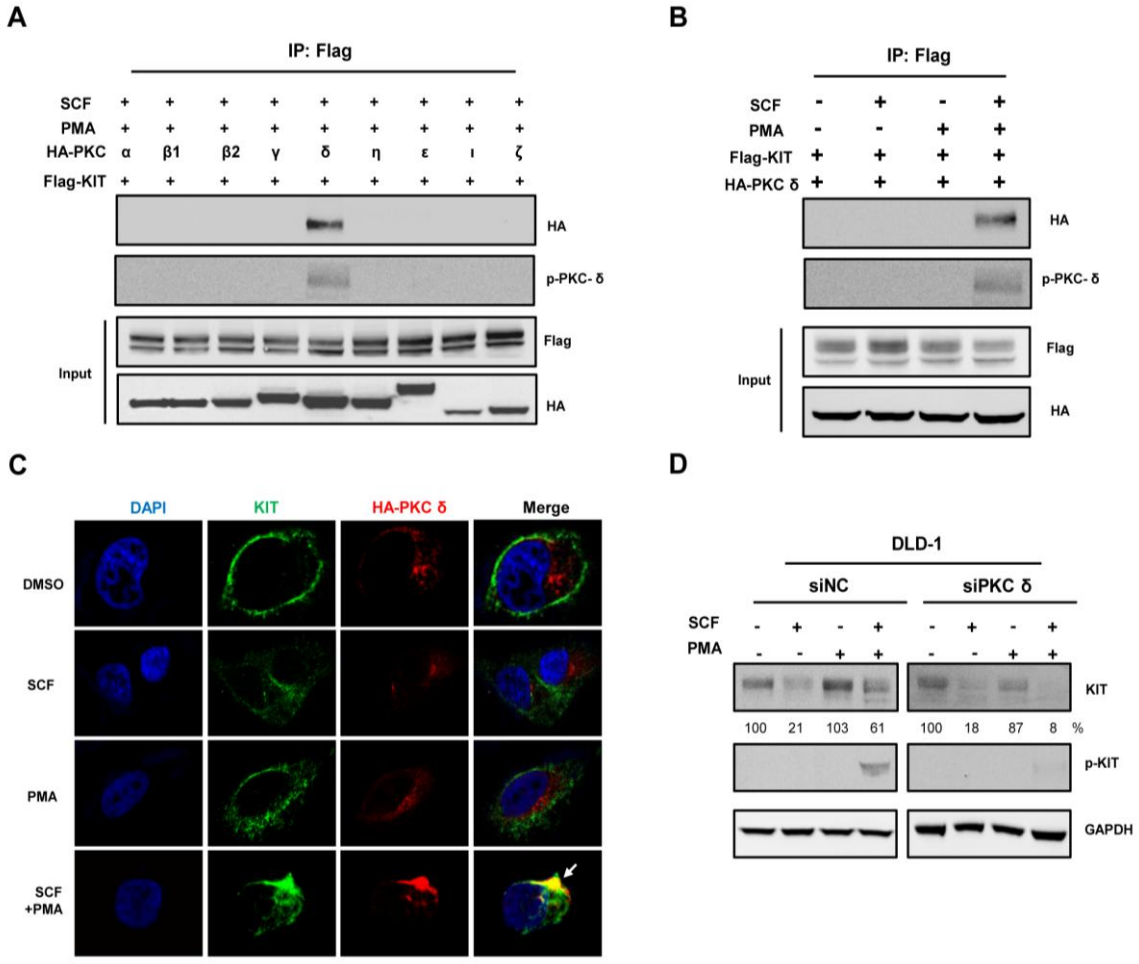

Figure 4. PKC-δ directly binds WT-KIT and rescues KIT from degradation. A, Synthetic WT-KIT protein directly bound to synthetic PKC-δ protein in vitro. HA-PKC isoenzyme constructs were co-transfected with Flag-KIT into HeLa cells. Cells were pretreated with 300nM PMA and incubated with SCF for 2 hours. B, Binding between KIT and PKC-δ was evident only in cells treated with SCF and PMA. C, HA-PKC-δ was co-transfected with Flag-KIT into HeLa cells. Cells were pretreated with 300nM PMA and incubated with or without SCF for 1 hour. Cells were stained for KIT (green), HA-PKC-δ (red) and DAPI (blue). In cells treated with SCF and PMA, co-localization of PKC- δ and KIT was evident (yellow). D, Inhibition of PKC-δ resulted in the loss of rescued KIT in DLD-1 cells treated with SCF and PMA. DLD-1 cells transfected with control siRNA or siPKC-δ were pretreated with 300nM PMA and incubated with or without SCF for 2 hours.

6. KIT and PKC-δ are activated in KIT-expressing colon cancer tissues

Although the expression of KIT in colon cancer cell lines has previously been reported, KIT expression in colon cancer tissues was not well characterized. We evaluated KIT expression in 250 CRC tissues by immunohistochemistry and detected expression in 47 cases. In Western blot analysis of 22 of the 47 KIT-immunopositive cases, 18 cases (81.8%) showed strong KIT expression compared with the normal matched mucosa (Fig. 5A). In contrast, Western blotting did not show increased KIT expression in 10 KIT-immunonegative colon cancers (data not shown). We also demonstrated that most of the KIT protein expressed in colon cancer tissues is activated by detecting phospho-KIT expression in 16 of the 22 KIT-immunopositive colon tissues (72.7%). The expression of KIT and activated KIT in tumor tissues was significantly higher than in normal tissue (Fig. 5B and C). Additionally, the expression of phospho-PKC-δ was increased in the CRC tissues with KIT expression and there was a linear correlation between KIT expression and phospho-PKC-δ expression (r=0.5119, *P*<.001; Fig. 5D).

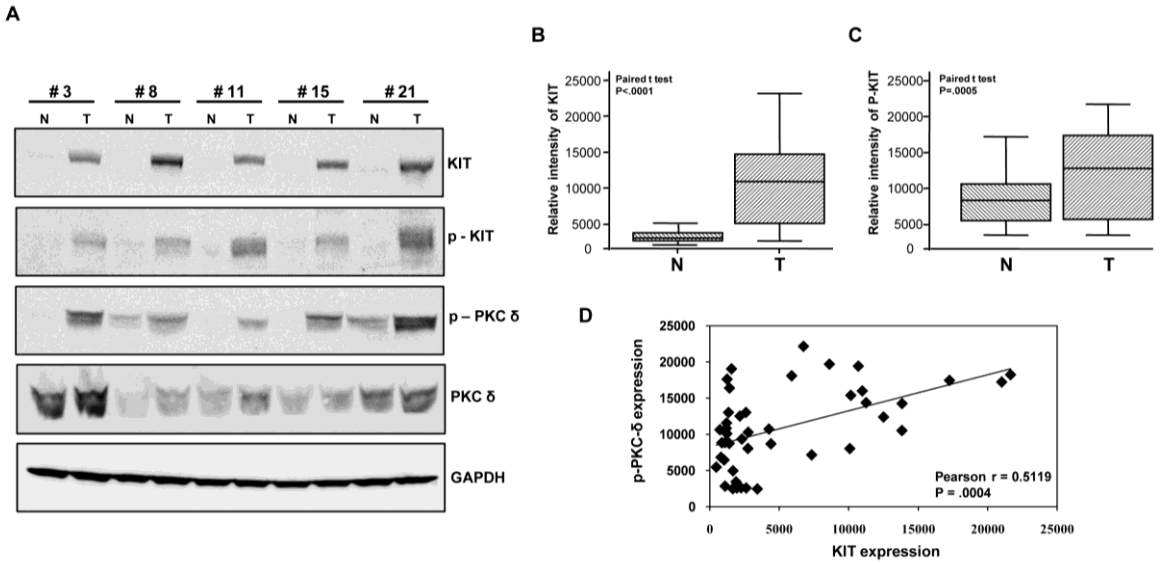

Figure 5. KIT and PKC-δ are concomitantly activated in colon cancer tissues. (A) Western blot analysis of KIT, phospho-KIT, PKC-δ, phospho-PCK-δ and GAPDH expression in human colon cancer tissues (T) and matched non-tumorous colonic mucosal tissues (N). (B-C) expression of KIT

(B) and phospho-KIT (C) in 22 KIT-immunopositive colon cancer tissues (T) compared with non-tumorous colonic mucosal tissues (N). Statistical analysis was performed with a paired t-test (P<.0001 for KIT and P =.0005 for phospho-KIT). (D) Quantification of Western blotting analysis of KIT and phospho-PCK-δ showed that expression of KIT correlated with expression of phospho-PCK-δ (r=0.5119, P=.0004).

7 Clinicopathologic characteristics of colon cancers with KIT expression

Lastly, we analyzed the clincopathologic characteristics of CRCs with KIT expression among 250 CRCs using tissue microarray. Expression of KIT was not identified in normal colonic mucosa (Fig. 6A) and most of the CRCs tested (Fig. 6B). The majority of tumors with KIT expression showed expression in the tumor cell cytoplasm (Fig. 6C and D). The clinicopathologic characteristics of the 250 patients with CRCs according to KIT expression are described in Table 2. No significant correlation between KIT expression and clinicopathologic variables was found except for increased serum CEA level in CRCs with KIT expression (Table 2). We also evaluated KIT as a prognostic factor and found that the expression of KIT correlated with poor survival. The overall cumulative survival rates for patients with KIT expression (n=47) and without KIT expression (n=203) were 68.1 and 85.7%, respectively (*P*=.004; Fig. 6E). When we analyzed the correlation between KIT expression and patient survival for the subgroup of patients with stage II and III disease, the overall cumulative survival rates for stage II and III patients with KIT expression (n=34) and without KIT expression (n=161) were 79.4% and 92.5%, respectively (*P*=.017, Fig. 6F).

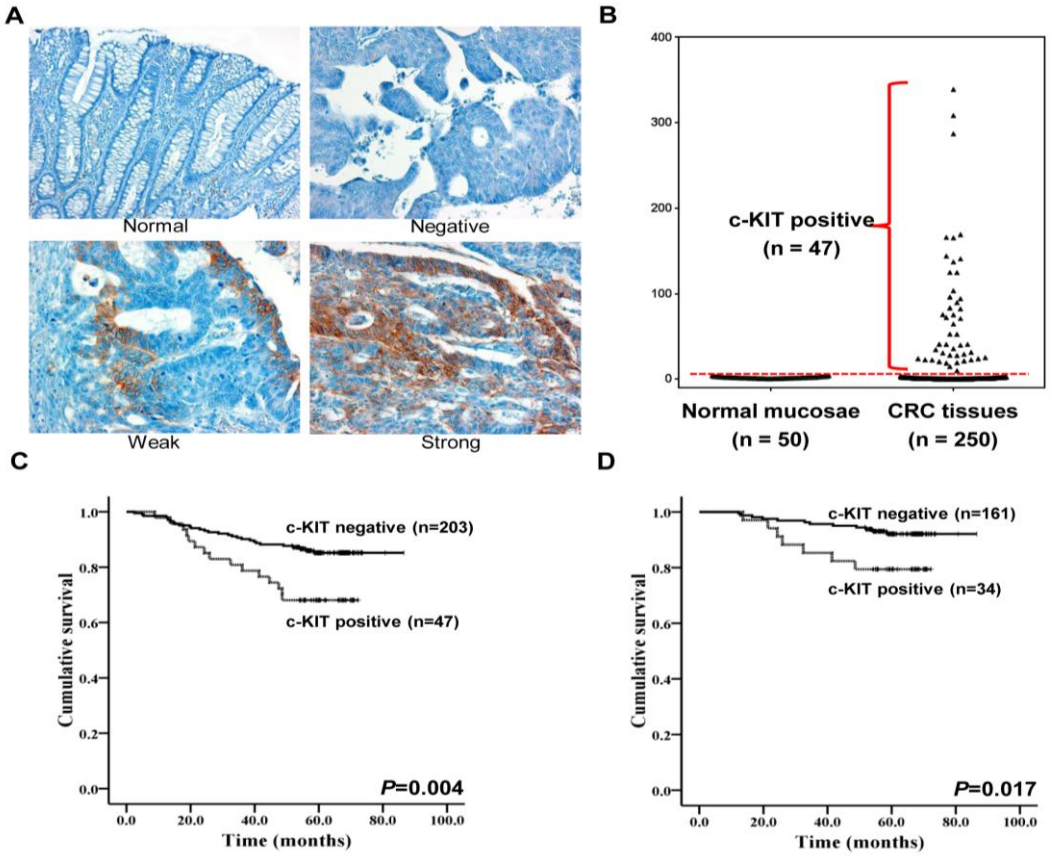

Figure 6. Clinicopathologic characteristics of colon cancers with KIT expression. A, Immunohistochemical analysis of KIT protein expression in colon cancer tissues. Normal colonic mucosa (Normal) and most of the colon cancer tissues tested did not express KIT protein (Negative). Expression of KIT protein was evident in some colon cancers (Weak). In such cases, KIT protein was detected in the membrane and cytoplasm of tumor cells (Strong). B, The amount of KIT expression was analyzed by using the ImageJ program and categorized as KIT positive and negative. C, Overall survival according to KIT expression in colorectal cancers (n=250). The survival rate of colon cancer patients according to KIT expression was analyzed by the Kaplan-Meier method. Colon cancer patients with KIT expression showed poorer prognosis than patients whose tumors did not express KIT. D, overall survival curve according to KIT expression for patients with stages II and III colorectal cancers (n=195).

Table 2. Clinicopathological characteristics of 250 colorectal cancers according to KIT expression

Category	Variables	Case no. (n=250)	KIT expression				P-value
			Positive (%) (n=47)		Negative (%) (n=203)		
Age (years)			60.8 ± 10.7		60.1 ± 11.8		.393
Gender							
	Male	160	32	(68.1)	128	(63.1)	.517
	Female	90	15	(31.9)	75	(36.9)	
Location							
	Colon	162	29	(61.7)	133	(65.5)	.064
	Rectum	88	18	(38.3)	70	(34.5)	
Size							
	≤ 5 cm	142	28	(59.6)	114	(56.2)	.670
	> 5 cm	108	19	(40.4)	89	(43.8)	
Differentiation grade							
	Well	26	3	(6.3)	23	(11.3)	.705
	Moderate	192	41	(87.4)	151	(74.4)	
	Poor	32	3	(6.3)	29	(14.3)	
Tumor Stage							
	I	29	4	(8.5)	25	(12.3)	.247
	II	106	21	(44.7)	85	(41.9)	
	III	89	13	(27.7)	76	(37.4)	
	IV	26	9	(19.1)	17	(8.4)	
MSI status*							
	MSS/MSI-low	190	40	(88.9)	150	(81.1)	.215
	MSI-high	40	5	(11.1)	35	(18.9)	
CEA level							
	≤ 5 ng/ml	178	25	(53.2)	153	(75.4)	.002
	> 5 ng/ml	72	22	(46.8)	50	(24.6)	
p53 expression							
	Negative	122	26	(55.3)	96	(47.3)	.321
	Positive	128	21	(44.7)	107	(52.7)	

MSS, microsatellite stability; MSI, microsatellite instability; CEA, carcinoembryonic antigen
* 230 out of 250 cases were included in the MSI study

Ⅳ. Discussion

KIT, a member of the RTK family, is a highly oncogenic tyrosine kinase that is involved in the activation of major signal transduction pathways. The best-known downstream signal transduction pathways of KIT are PI3 kinase-AKT, Src family kinase, Ras-ERK, and JAK-STAT [11, 16, 17]. As we have described previously, KIT can be activated in two ways: WT-KIT is activated by binding of SCF whereas mutant KIT is constantly activated in the absence of SCF [11, 17, 18].

In contrast to tumorigenesis induced by activation of mutant KIT, the molecular characteristics of activation of WT-KIT and its role in tumorigenesis are poorly understood. Although expression of WT-KIT has been reported in many tumors, including CRCs [7, 18-20], its role in tumorigenesis and the therapeutic efficacy of inhibition of activated KIT have not been characterized. If activated WT-KIT plays a role in tumorigenesis, it is reasonable to assume that the downstream signaling pathway of activated WT-KIT would be the same or similar to that of mutant KIT and the activation would be constant, as for mutant KIT. SCF is the only known ligand for WT-KIT [17]. We and others have previously confirmed that the signaling pathways downstream of the activation of WT-KIT by SCF binding are the same as those activated [11, 18]. In this study, we provide the first evidence that WT-KIT can be constantly activated through recycling of KIT. We initially found that the effects of KIT activation were temporary because KIT was degraded in lysosomes. When we continuously stimulated KIT-expressing cell lines with SCF, the exp ression of KIT decreased according to the time of SCF treatment and activation of the downstream signaling pathways was also decreased. Although these findings suggest a physiological role of WT-KIT activation, they raise questions regarding the role of SCF-KIT activation in tumorigenesis. KIT is internalized by clathrin-mediated endosytosis after SCF treatment, resulting in its degradation in the lysosome [21]. Although we observed degradation of activated KIT in colon cancer cell lines, we hypothesized that KIT could be recycled in the tumor cells and thus be continuously active after SCF treatment. Some other RTKs are known to be rapidly recycled back to the membrane in cancer cells instead of being degraded. For example, activation of PKC-α is a critical step in sorting the PDGF β-receptor towards Rab4a-dependent recycling [15]. Recent

studies also suggest that recycling of PDGFR drives the invasion of glioblastoma cells, and recycling of EGFR coupled with α5β1 integrin induces the migration of ovarian cancer cells [22, 23]. However, little is known about recycling of KIT and the impact of KIT-SCF activation on colorectal cancer progression. We therefore investigated the possibility that KIT is recycled in KIT-expressing colon cancer cell lines. In the case of EGFR recycling, PKC is known to be involved in the inhibition of EGFR degradation [14]. We initially activated PKC by treating colon cancer cells with PMA and found that KIT was not degraded after prolonged SCF stimulation but instead constantly activated the downstream signaling pathways. These findings indicate that KIT-SCF might contribute to colon cancer tumorigenesis through PKC activation and subsequent KIT recycling. We further demonstrated that PKC-δ is responsible for KIT recycling by showing direct binding of KIT and PKC-δ in vitro and concomitant overexpression of KIT and PKC-δ in a subset of colon cancer tissues. PKCs play important roles in the regulation of proliferation, differentiation, tumor promotion, apoptosis, and angiogenesis [24, 25]. Various factors such as growth factors, tumor promoters, chemotherapeutic agents, and ras protein induce activation of PKCs [26-28]. Activation of PKC-δ by substance P-induced proinflammatory signaling in human colonocytes [29] and by a hypoxic microenvironment has been reported [30]. Therefore, it is likely that endogenous PKC-δ is activated and functions in KIT recycling in the microenvironment of CRCs. Our findings indicate that CRCs expressing WT-KIT can constantly generate activated SCF-KIT signaling as a result of KIT recycling.

In addition to demonstrating expression of endogenous KIT and PKC-δ in CRCs tissues, we found that colon cancer patients with KIT-expressing tumors showed a worse prognosis than patients without KIT expression. These findings suggest that the activated downstream signals of KIT-SCF induce rapid colon cancer progression, and also provide rationales for anti-KIT therapy. Because the downstream signaling pathways of KIT-SCF binding are same or similar to those of activated mutant KIT, activation after KIT-SCF binding can be inhibited at post-transcriptional and/or post-translational levels. We previously showed that specific inhibition of *KIT* by transfection with microRNA decreased induction of p-AKT an d p-ERK by SCF stimulation in cells expressing WT-

KIT [31]. These findings suggest that inhibition of *KIT* mRNA might be a novel therapeutic tool in tumor cells expressing KIT. Moreover, post-translational inhibition of KIT by Imatinib treatment could be applied to colon cancers with WT-KIT expression. In addition to these approaches of direct inhibition of activated KIT, it is possible that activated WT-KIT could be regulated by interrupting the recycling pathway. Future studies into modulating PKC-δ activity in tumors expressing WT-KIT might provide novel therapeutic tools for cancer treatment.

In conclusion, we propose KIT-SCF activation and PKC-δ-induced KIT recycling as a novel mechanism of tumor progression in colon cancers with WT-KIT expression.

Ⅴ. Conclusion

In this study shows that WT-KIT is expressed in a subset of colon cancer cell lines and is activated by SCF, leading to activation of downstream AKT and ERK signaling pathways. We also demonstrated that WT-KIT expression gradually decreased after SCF stimulation due to lysosomal degradation. We provide the first evidence that PKC-δ directly binds to activated WT-KIT and inhibits WT-KIT degradation. We further demonstrate that WT-KIT and PKC- δ are concomitantly activated in colon cancer tissues expressing WT-KIT, and that expression of KIT correlates with poor patient survival. Our findings indicate that continuous downstream signal activation after SCF-KIT binding is accomplished through PKC-δ-mediated recycling of WT-KIT and that sustained KIT activation contributes to tumor progression in colon cancers with KIT expression.

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ABSTRACT (IN KOREAN)

대장암에서 정상형 KIT 단백질 과발현:

Protein Kinase C-δ를 통한 KIT recycling

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박 미 선

종양 유전자중 하나인 KIT 단백질은 RTK (Receptor Tyrosine Kinase)의 하나로서, 세포막에 존재하며 세포증식에 대한 신호를 조절하는 물질로 암 발생 기전과 이를 억제한 치료법 개발에 성공한 대표적인 물질로 알려져 있다. KIT 유전자 활성은 돌연변이에 의한 KIT 단백질 활성과 정상형 KIT 단백질이 stem cell factor(SCF) 등과 결합하여 활성화되는 두 가지 경우가 알려져 있으며, 모두 암 발생과 진행에 관여한다고 추정된다. 인체에 발생한 종양 중에서는 acinic cell tumor 등을 포함한 많은 종양에서 정상 KIT 단백질 과발현이 발견되었고, 일부 대장암에서도 KIT 단백질 과발현이 발견되었다. 그러나 어떤 이유로 KIT 단백질이 과발현 되는지 와 어떻게 암 발생과 진행에 기여하는지는 아직 보고된 바 없다. 따라서 본 연구에서는 대장암에서 과발현된 KIT 단백질이 SCF에 의해 활성화 되면 대장암 발생과정에 어떤 부분에서 기여하는지 밝히고자 하였다. 본 연구에서는 먼저 정상 KIT 단백질이 발현되는 대장암 세포주 3개(DLD-1, Colo320DM 그리고 Ls174T)를 qRT-PCR과 Western blotting analysis 를 통해 밝혔으며 이 세포주에 SCF처리시 PI3 kinase-AKT와 Ras-ERK 신호가 활성화 되는 것을 확인하였다. 그러나 PI3 kinase-

AKT와 Ras-ERK 신호 활성화는 SCF를 긴시간 처리 시 곧 사라지며, KIT 단백질 역시 분해되어 사라지는 것을 확인하였다. 이러한 KIT 단백질 분해는 lysosome inhibitor인 Bafilomycin A1을 처리하자 회복하는 것을 확인함으로써 lysosome degradation pathway를 따르는 것을 규명하였다. 최근 일부 RTK가 이러한 lysosome 분해를 거치지 않고 재활용 됨으로써 암 발생과 진행에 기여할 것이라 보고되고 있었다. 본 연구자는 KIT 단백질 역시 Protein Kinase C-δ(PKC-δ) 활성화 시 분해되지 않고 세포막으로 다시 재활용 되는 것을 처음으로 밝혔으며, 이렇게 재활용된 KIT 단백질은 SCF와 지속적 결합을 통해 PI3 kinase-AKT와 Ras-ERK 신호의 지속적인 활성화를 유도한다. 이러한 현상이 대장암 환자 조직에서도 발생하는지 확인하기 위해 총 250개 대장암 조직에서 Immunohistochemistry 를 통해 47개 KIT-immunopositive cases 를 선택했다. 이렇게 선택된 조직 중 Western blotting 을 통해 확인한 결과 18 case에서 정상 조직보다 대장암 조직에서 KIT 단백질 발현이 더 높은 것을 확인할 수 있었으며 이러한 환자의 생존률이 KIT 단백질 발현이 낮은 환자보다 좋지 않음을 규명하였다. 또한 KIT 단백질이 발현하는 조직에서 phospho-PKC-δ 역시 비례하게 발현하는 것을 통해 대장암 세포주 뿐만 아니라 실제 대장암 조직에서도 KIT 단백질이 phospho-PKC-δ를 통한 재활용을 통해 대장암 발생과 진행에 기여 할 것이라 사료된다.

핵심되는 말 : protein kinase C-δ, KIT 유전자 발현, KIT 재활용, 대장암

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