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Original Article

Peptides containing the MXXCW motif inhibit oncogenic RET kinase activity with a novel mechanism of action

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Abstract: REarranged during Transition (RET) is a tyrosine kinase associated with the development of several malignancies. Identification of RET kinase inhibitors promises valuable therapeutic tools for the intervention of RET-driven tumors. Most currently available tyrosine kinase inhibitors target the ATP binding site, but there are several drawbacks of these ATP-competitive drugs. Therefore, there is a need to develop new kinase inhibitors with alternative mechanisms of action. We have previously reported that a conserved cysteine in the MXXCW motif of RET is crucial to the disulfide-bonded dimerization-linked activation of RET kinases. Reagents which bind to this cysteine may inhibit the activity of RET kinases through disulfide-bond mediated dimerization. Here, we examine the potential of MXXCW motif-containing peptides as candidate kinase inhibitors. We demonstrate that MXXCW motif-containing peptides bind to RET in a redox-sensitive manner and block enzymatic activity, causing inhibition of the RET-dependent activity of extracellular signal-regulated kinases and effectively reducing the malignant potential of RET-papillary thyroid carcinoma-1 (PTC)-expressing cells. These motif-containing peptides were also found to be effective against the drug resistant mutant of RET. The inhibition of RET kinase activity by these peptides resulted in suppression of RET-PTC-1-mediated cancer growth. The great potency of these cysteine targeted peptides could indicate promising approaches for novel molecular-targeted therapies for RET-associated cancers.

Keywords: RET, cysteine, peptide, tyrosine kinase inhibitor

Introduction

The c-*RET* proto-oncogene encodes a receptor tyrosine kinase-REarranged during Transition (RET)-which can be physiologically activated by the binding of a corresponding glial cell line-derived neurotrophic factor (GDNF) to the GDNF-family receptor α [1]. Ligand-receptor binding results in RET protein dimerization and activation of the kinase, followed by transphosphorylation of tyrosine residues in the intracellular domain. It has also been shown that RET can be activated by genomic rearrangement or point mutations [2]. Activating germline point mutations of c-*RET* underpin a number of hereditary neoplastic disorders such as multiple endocrine neoplasia (MEN) type 2, a group of cancer syndromes characterized by medullary thyroid carcinoma and pheochromocytoma [2]. Genomic rearrangements of *RET* have been reported to result in the expression of constitutively active RET fusion kinases [3] and *promote the development of cancer.* Rearrangements of this gene are frequently observed in papillary thyroid carcinoma (PTC) [4, 5], and recently, *RET* fusion genes have been identified in a small fraction of lung adenocarcinoma patients [6-8]. Deregulation of RET tyrosine kinase has been implicated in sporadic medullary thyroid cancer [9], breast cancer [10], pancreatic cancer [11], colorectal cancer [12], and leukemia [13]. In addition, it has been reported that exposure to ionizing radiation increases the risk of PTC with *RET* translocations [14].

Tyrosine kinases are important therapeutic targets for cancer treatment and various inhibitors have been developed. Currently, the most of the

kinase inhibitors are ATP-competitive inhibitors that target the ATP binding site [15]. The limitations of these inhibitors, such as poor selectivity and drug resistance, mean that development of new inhibitors is of high importance. However, there are relatively few examples of drugs with alternative mechanisms of action.

Protein tyrosine kinases are known to be activated by phosphorylation of major autophosphorylation sites. We previously demonstrated that a conserved cysteine residue (C376 of mutant RET-PTC-1 fusion protein, equivalent to C987 of c-RET) in the alpha-helix H region of the catalytic domain is crucial for the disulfide bond-mediated dimerization-linked activation of RET kinases [16-18] as well as the tyrosine phosphorylation-dependent activation mechanism [19]. Substitution of C376 of RET-PTC-1 with alanine results in the prevention of disulfide-bonded homodimer formation, with concomitant almost complete inactivation of RET proteins [16-18]. As well as this cysteine, the surrounding amino acids are highly conserved in an MXXCW motif. These facts suggest that this cysteine and the MXXCW motif are essential for maintaining tyrosine kinase function and structure. Reagents that bind to cysteine in the MXXCW motif may disrupt the disulfide-bonded dimerization and, therefore, activity of kinases.

In this study, we investigated the inhibitory effects of MXXCW motif-containing peptides on RET kinase, and evaluated the antitumor activity of the peptides on RET-PTC-1-expressing cells to determine their potential as drug candidates for RET-mediated cancers.

Materials and methods

Plasmid construction and transfection

The cysteine residue (C987 in c-RET) in the MXXCW motif is highly conserved among tyrosine kinases, and 96% of human tyrosine kinases carry an equivalent cysteine to C987. However, there is another conserved cysteine (C976) in c-RET, and 80% of human tyrosine kinases also contain the MXXCW motif within a CXXXXXXXMXXCW motif [20]. To induce expression of the MLQCW (MXXCW motif of RET protein) peptide in RET-bearing cells, we generated three 6× His-tagged fusion peptide expression vectors. The first peptide was MLQCW, which consisted of the region from M984 to W988 of c-RET (**Figure 2A**, sequence A). We also designed peptides that were longer peptides than these five amino acids, derived from the RET cytoplasmic fragment that included MLQCW. The second peptide that we designed (denoted 976C-988W) was composed of 13 amino acids from C976 to W988 of c-RET (**Figure 2A**, sequence B). The third (denoted 976C-1042D) consisted of the region from C976 to 1042D, located immediately upstream of next cysteine residue (C1043 in c-RET) (**Figure 2A**, sequence C). We fused these three peptides with a sequence derived from the pcDNA3.1/His vector (Thermo Fisher Scientific, Waltham, MA). Two complementary oligonucleotide strands were designed with appropriate overlapping restriction sites. The oligonucleotides were mixed in equimolar concentrations. These duplexes were annealed and ligated into a pcDNA3.1/His C vector. The cDNA fragment encoding amino acids from M984 to W988 of c-RET (His_6-MLQCW) was cloned in the EcoRI sites of pcDNA3.1/His C vector. The cDNA fragment encoding amino acids from C976 to W988 of c-RET (His_6-976C-988W) was cloned in the EcoRI-XhoI site of the same vector. The 976C-1042D fragment of c-RET was amplified using PCR and inserted into EcoRI-NotI site of the pcDNA3.1/His C vector (His_6-976C-1042D).

We inserted either *RET-PTC-1* cDNA [5] or cDNA of *RET-PTC-1* in which Valine 804 (numbering refers to wild-type RET, equivalent to V193 of RET-PTC-1) was mutated to methionine (denoted RET-PTC-1 V804M) into an APtag-1 vector containing the Moloney murine leukemia virus long terminal repeat, as described previously [18]. The *RET-PTC-1* V804M mutant was prepared using a KOD Plus Mutagenesis Kit (TOYOBO, Osaka, Japan) according to the manufacturer's instructions.

Oligonucleotides were purchased from Hokkaido System Science and are detailed in **Table 1**. All constructs were confirmed by sequencing. Each plasmid was transfected into cells using Lipofectamine 3000 reagent, following manufacturer's instructions (Thermo Fisher Scientific).

Cell culture

The NIH3T3 fibroblasts and cells transfected with *RET-PTC-1* have been described previously

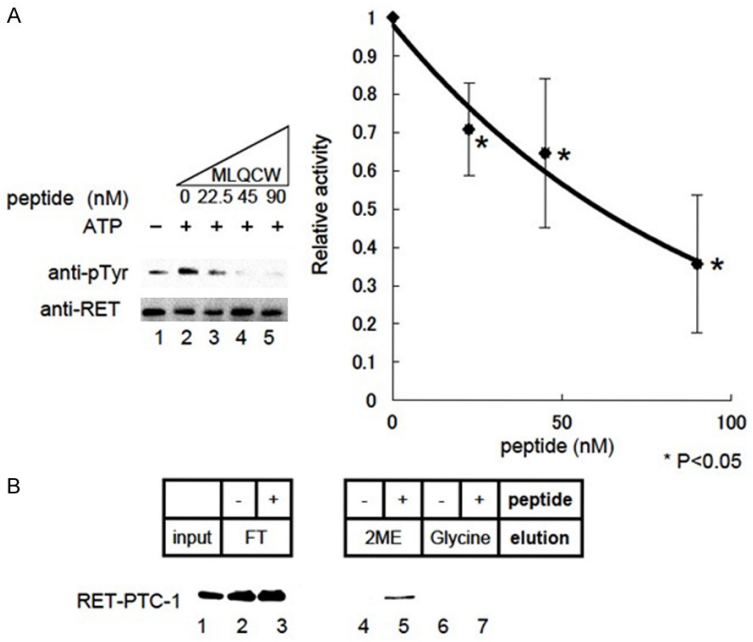

Figure 1. Synthetic peptides can bind to RET protein via disulfide bonds to inhibit kinase activity. Lysates from RET-PTC-1-over-expressing cells were subjected to an in vitro kinase assay after immunoprecipitation with anti-RET antibodies and addition of various concentrations of MLQCW peptide. The beads with bound RET proteins were then subjected to western blotting with anti phosphotyrosine (A, top) and anti-RET (A, bottom). Levels of phosphorylation were quantified by densitometry. Mean ratios of relative activity were plotted. Error bars indicate standard error of the mean (SEM, n=4). *Steel test, P<0.05 compared with the no-peptide control. Beads with bound MLQCW peptide were incubated with lysates from RET-PTC-1-over-expressing cells. They were then washed and eluted with reducing buffer (2-mercaptoethanol) and then elution buffer (Glycine). Proteins in the eluates were analyzed by western blotting with anti-RET antibodies (B). Lanes 1-3 represent 2% of protein inputs and the flow-through fraction (FT).

[18]. The TT (CR1803) and MCF7 (RCB1904) cells were provided by the American Type Culture Collection and RIKEN BRC, respectively. Transfectans and NIH3T3 cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 8% bovine calf serum (BCS; GE Healthcare, Buckinghamshire, England), 100 units/ml penicillin (FUJIFILM Wako Pure Chemical Corporation), and 100 μg/ml streptomycin (FUJIFILM Wako Pure Chemical Corporation). Both TT and MCF7 cells were cultured in RPMI-1640 medium (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% Fetal bovine serum (FBS; GE Healthcare), 100 units/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Western blotting, immunoprecipitation, and kinase assay

Western blotting and immunoprecipitation were performed as described previously [17]. For the kinase activity assay, immunoprecipitated RET proteins were washed three times with 1 ml lysis buffer (10 mM Tris-HCl, pH 8.0, 1% Triton-X-100, 150 mM NaCl, 1 mM EDTA, 0.5 mM Na_3VO_4) and three times with 1 ml kinase buffer (30 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$), and were suspended in 30 μl kinase buffer with 1 mM ATP (Sigma-Aldrich, St. Louis, MO). The mixture was incubated for 30 minutes in a 30°C water bath, after which the reaction was terminated by adding sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% Glycerin, 5% 2-mercaptoethanol [2ME]). The immunoprecipitates were then boiled for 5 minutes and loaded onto SDS polyacrylamide gels. The molecular sizes of proteins were estimated by comparison with protein standards (Bio-Rad Laboratories, Hercules, CA).

The anti-RET antibody was obtained from Immuno-Biological Laboratories (Catalog No. 18-128) and Cell Signaling Technology (#14698). Anti-phospho-RET (Y905) (#3221), anti-phosphotyrosine (#9441), anti-phospho-extracellular signal-regulated kinase (ERK) (#4370), and anti-ERK (#9102) antibody were obtained from Cell Signaling Technology (Danvers, MA). MLQCW peptides were synthesized at Greiner Bio-One (Kremsmünster Austria). We purchased GDNF from R&D Systems, Inc. (Minneapolis, MN). Densitometric analysis was performed using Scion Image software (Scion Corporation, Frederick, MD).

Peptide immobilization and pulldown experiments

Synthetic peptides (10 mg) were immobilized onto NHS-Activated Agarose dry resin (15 mg)

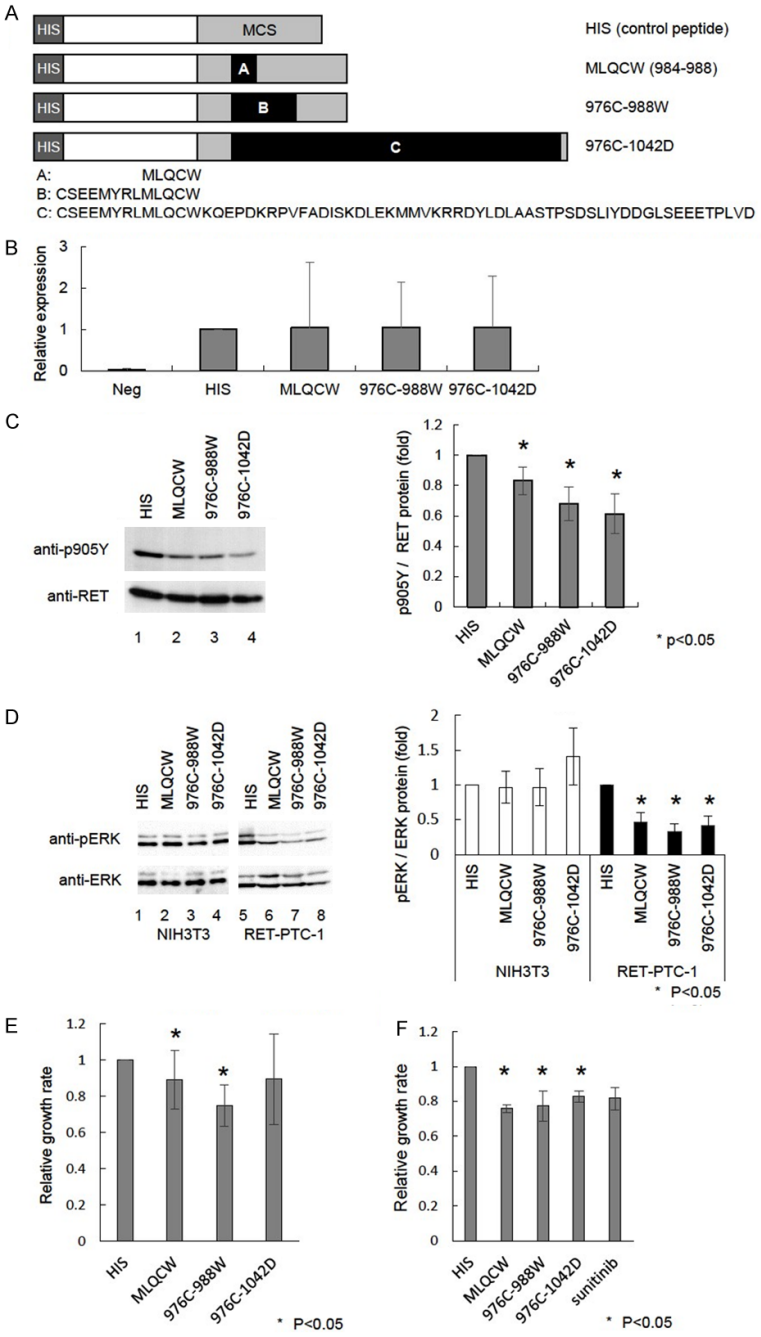

Figure 2. Inhibition of RET-PTC protein activity and downstream signaling by expression of peptides in cells. Schematic illustration of MLQCW motif-containing peptide expression vector constructs (A). Cells that over-expressed RET-PTC-1 were transiently transfected with each peptide expression vector or with the control pcDNA 3.1His vector. Cells were cultured for 2 days and transcript expression levels of His-tagged peptides were analyzed by real-time PCR. Data (mean ± standard deviation, n=6) are shown as relative mRNA amounts in relation to the house-keeping gene *hypoxanthine phosphoribosyltransferase (Hprt),* and normalized to the mean of the control pcDNA 3.1His vector-transfected cells (B). Lysates from original (D lane1-4) or RET-PTC-1-expressing (C and D lane 5-8) NIH3T3 cells that had been transfected with each peptide expression vector or control pcDNA 3.1His vector were subjected to western blotting with anti-phospho-RET (p905Y) (C, top), anti-RET (C, bottom), anti-phospho-ERK (D, top), or anti-ERK (D, bottom) antibodies. Bar graphs illustrate the mean ratios of relative phosphorylation quantified using densitometry (C and D). Error bars indicate standard error of the mean (SEM, C: n=5, D: n=4). Anchorage-dependent growth of transiently peptide-transfected cells was measured using a Cell Counting Kit 8 (E). Anchorage-independent growth rates of transiently peptide-transfected cells or cells treated with 1 μM sunitinib and grown on ultra-low attachment plates were measured using a Cell Counting Kit 8 (F). Data (mean ± standard error of the mean, E: n=6, F: n=4) are normalized to the mean of the control pcDNA 3.1His vector-transfected cells. *Steel test, P<0.05 compared with pcDNA 3.1His vector-transfected cells. Abbreviations: MCS, multiple cloning site.

according to the manufacturer's instructions (Thermo Fisher Scientific). The RET-PTC-1 over-expressing NIH3T3 cells were lysed by incubation in lysis buffer. Lysates were centrifuged at 21,000×g for 30 minutes at 4°C, and the supernatant collected. Protein content was quantified using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). Cell lysate with a total of 4.5 mg protein was incubated with the beads. After over-night incubation, the beads were washed five times with 1 ml PBS. Bound proteins were eluted with *reducing buffer* (5% 2ME) and then with elution buffer (0.1 M Glycine-HCl pH 2.1). Eluted fractions were loaded onto SDS-polyacrylamide gels, and western blot analysis was performed with anti-RET antibodies.

Quantitative real-time polymerase chain reaction

Total RNA was extracted using the High Pure RNA Isolation

Table 1. List of oligonucleotides used in this study

	Sequence (5' to 3')	Purpose
MLQCW top strand	aattcatgcttcaatgctggg	Gene expression
MLQCW bottom strand	aattcccagcattgaagcatg	Gene expression
976C-988W top strand	aattctgcagcgaggagatgtaccgcctgatgctgcaatgctggc	Gene expression
976C-988W bottom strand	tcgagccagcattgcagcatcaggcggtacatctcctcgctgcag	Gene expression
976C-1042D forward	ccggaattctgcagcgaggagatgtaccgc	Gene expression
976C-1042D reverse	ttttcctttgcggccgcttagtccaccagcggtgtc	Gene expression
V804M forward	gagtacgccaaatacggctccctgc	Site directed mutagenesis
V804M reverse	catgattaataggagcgggccatcctggc	Site directed mutagenesis
Hprt forward	ctttgctgacctgctggatt	Determination of expression level of Hprt in real-time PCR
Hprt reverse	tatgtcccccgttgactgat	Determination of expression level of Hprt in real-time PCR
His-tagged peptide forward	gcatgactggtggacagcaa	Determination of expression level of His-tagged peptide in real-time PCR
His-tagged peptide reverse	tcctggtaccttatcgtcatcgt	Determination of expression level of His-tagged peptide in real-time PCR

Kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. Synthesis of cDNA was carried out by reverse transcription of total RNA, using the Prime Script RT Master Mix (TAKARA BIO, Shiga, Japan). Real-time quantitative PCR with SYBR green was performed using the FastStart Universal SYBR Green Master (Roche Diagnostics) in the 7500 Real-Time PCR system (Thermo Scientific). The primers used are listed in **Table 1**.

Proliferation assay, growth curve, and soft agar assay

To measure anchorage-dependent proliferation, cells were cultured at concentrations of 3×10^3 cells per well on a 96-well plate for 2 days. To measure anchorage-independent proliferation, cells were cultured at concentrations of 2×10^4 cells per well on a 96-well ultralow-attachment plate (Corning, Corning, NY) for 5 days. All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. Growth analyses were performed by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) assay using a Cell Counting Kit 8 (DOJINDO LABORATORIES, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, CCK-8 solution (10 µl) was added to each well and the absorbance of WST-8 formazan was measured at 450 nm using a microplate reader. Sunitinib was purchased from Cell Signaling Technology.

To generate growth curves, cells were seeded in 24-well plates at 2×10^4 cells per well. Cells were counted daily after trypsinization for a total of four days using a standard hemocytometer. Soft agar assay was performed as described previously [18]. Briefly, cells were seeded at a concentration of 2.5×10^4 cells per 35-mm dish. Bottom layers were made up of 0.72% agar in DMEM supplemented with 8% BCS. Cells were resuspended in a top layer of 0.36% agar in 8% BCS-DMEM. All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. The number of colonies with diameters larger than 0.1 mm was assessed after 14 days.

Tumorigenicity in nude mice

A total of 5×10^5 cells were suspended in 200 µl of DMEM and inoculated subcutaneously into the right dorsal portion of 6-week-old male BALB/cSlc-nu/nu mice (Japan SLC, Shizuoka, Japan). The length (longest dimension), width (shorter dimension), and height (longest diameter of tumor perpendicular) of visible tumors were measured and tumor volume was calculated using the following formula: tumor volume (mm^3) = length (mm) × width (mm) × height (mm) ×0.5. Mice were maintained in a humidity-(50±10%), light/dark cycle-(12/12 hours) and temperature-(22±2°C) controlled pathogen-free animal facility. All experimental studies involving the mice were performed in accordance with The Animal Care and Use Committee (approval No. 201910006) of Chubu University approved this study.

Results

Inhibition of kinase activity in solution

The autophosphorylation of RET proteins decreased significantly following addition of the MLQCW peptide in a concentration-dependent

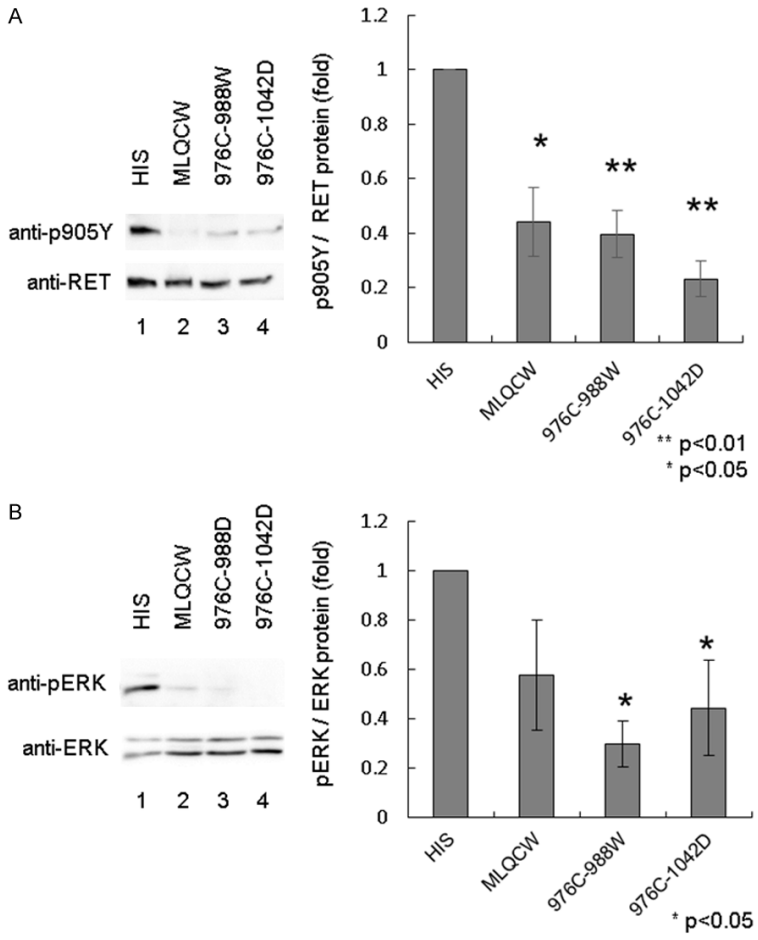

Figure 3. Inhibition of RET-PTC V804M protein activity and downstream signaling by motif-containing peptides in cells. We transfected RET-PTC-1-V804M-overexpressing NIH3T3 cells with either an MLQCW motif-containing peptide expression vector or control pcDNA 3.1His vector. Cell lysates were subjected to western blotting with anti-phospho-RET (p905Y) (A, top), anti-RET (A, bottom), anti-phospho-ERK (B, top), or anti-ERK (B, bottom) antibodies. Bar graphs illustrate the mean ratios of relative phosphorylation quantified using densitometry. Error bars represent the standard error of the mean (A: n=7, B: n=6). Significant differences (*P<0.05, **P<0.01) to pcDNA 3.1His vector-transfected cells were identified using the Steel test.

manner (**Figure 1A**). The IC50 of the MLQCW peptide for RET-PTC-1 was about 61.2 nM.

The binding of MLQCW peptide to RET-PTC-1 protein was confirmed (**Figure 1B**), as RET protein bound to the MLQCW peptide immobilized on beads and was eluted by a disulfide bond-reducing agent (2ME).

Inhibition of kinase activity in RET-expressing cells

The mRNA expression levels from all three peptide expression vectors and the control pcDNA3.1/His vector were comparable following transfection into RET-PTC-1-expressing NIH3T3 cells (**Figure 2B**). The activity of RET in cells was evaluated by monitoring the phosphorylation status of Y905 in the RET kinase domain. Tyrosine 905 (numbering refers to wild-type RET, equivalent to Y294 of RET-PTC-1) is thought to be the primary autophosphorylation site of RET, which plays an indispensable role in activation of RET kinase [21, 22]. Phosphorylation of Y905-a hallmark of RET activation-was significantly decreased in cells expressing any of the three inhibitory peptides (**Figure 2C**).

Oncogenic *RET* proteins such as RET-PTC-1 activate several signal-transduction cascades including the mitogen-activated protein kinase kinase (MEK)-*ERK* pathways [23]. Examination of downstream signaling of RET-PTC-1 revealed that phosphorylation of ERK in RET-PTC-1 over-expressing cells was repressed by the inhibitory peptides (**Figure 2D**). However, suppression was not observed in original NIH3T3 cells, which do not express RET kinase protein (**Figure 2D**).

Transfection of the three inhibitory peptide expression plasmids resulted in suppression of anchorage-dependent and independent growth in RET-PTC-1-expressing NIH3T3 cells (**Figure 2E** and **2F**). The inhibitory effects of the peptides were comparable with 1 μM sunitinib (**Figure 2F**), which has been reported to inhibit the transforming effects of RET-PTC [24].

Effect on enzyme activity of the gatekeeper mutant

Next, we investigated the effects of the motif-containing peptides on RET gatekeeper mutant (RET-PTC-1 V804M) activity. As **Figure 3A** illustrates, MLQCW-motif-containing inhibitory peptides reduced the activity of RET-PTC-1 V804M. Phosphorylation of ERK in RET-PTC-1-V804M-

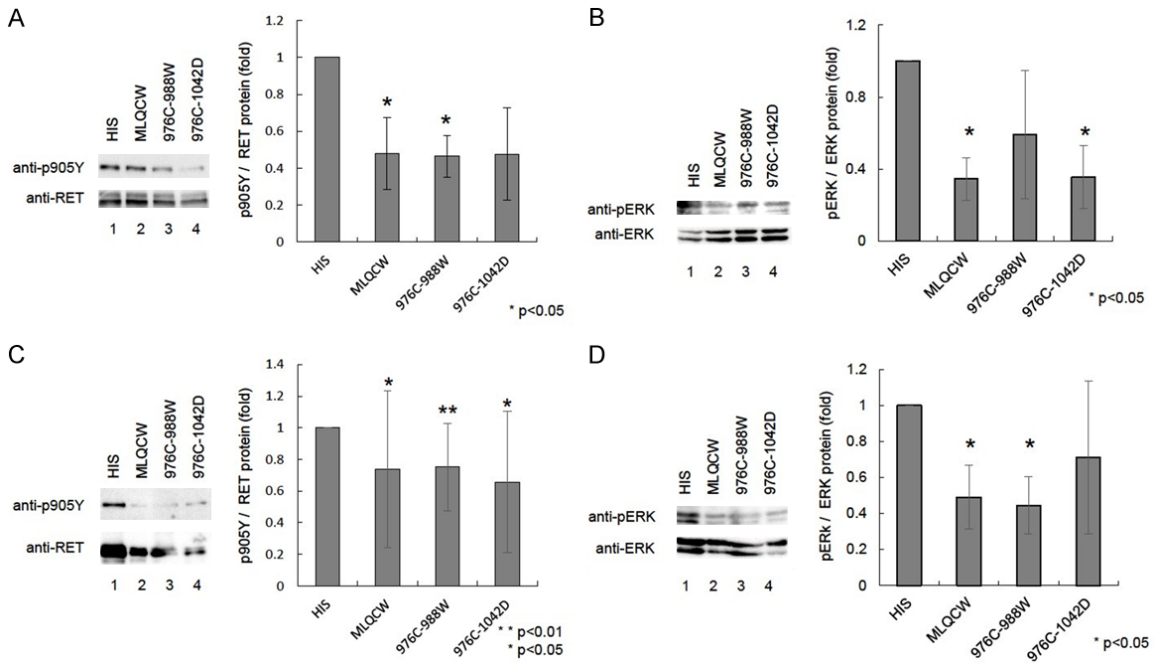

Figure 4. Inhibition of kinase activity by expression of peptides in human cancer cell lines. Cells were transfected with each MLQCW motif-containing peptide expression vector or control pcDNA 3.1His vector, and TT cells were cultured for 2 days after transfection (A and B). Peptide-transfected MCF7 cells were stimulated with 10 ng/ml GDNF for 20 minutes (C and D). Lysates from cells were subjected to western blotting with anti-phospho-RET (p905Y) (A and C, top), anti-RET (A and C, bottom), anti-phospho-ERK (B and D, top), or anti-ERK (B and D, bottom) antibodies. Bar graphs illustrate the mean ratios of relative phosphorylation quantified using densitometry. Error bars indicate standard error of the mean (SEM, A: n=4, B: n=4, C: n=6, D: n=6). Significant differences (*P<0.05, **P<0.01) from the pcDNA 3.1His vector-transfected cells were identified using the Steel test.

mutant-expressing cells was also suppressed by the expression of inhibitory peptides (**Figure 3B**).

Inhibition of kinase activity in human cancer cell lines

Transfection of any of the three inhibitory peptide expression vectors into human thyroid carcinoma TT cells suppressed RET kinase activity, indicated by the reduction in RET Y905 phosphorylation, as well as phosphorylation of ERK (**Figure 4A** and **4B**). The MLQCW motif-containing peptides also inhibited ligand-induced RET and downstream phosphorylation of ERK in MCF7 human breast cancer cells (**Figure 4C** and **4D**).

Suppression of RET protein dimerization and malignancy

We further investigated the inhibitory properties of the peptides by generating stable transfectants that expressed RET-PTC-1 and one of the inhibitory peptides. His-tagged peptides showed almost identical expression in all the clones that were screened by real-time PCR (**Figure 5A**). Expression of the three inhibitory peptides reduced the phosphorylation of RET at Y905, whereas expression of the His-tagged control peptide did not (**Figure 5B**).

A small amount of RET-PTC-1 was found to be dimerized in non-transfected control cells under non-reducing conditions (lane 1 of **Figure 5C**). The dimerization of RET protein was reduced in inhibitory peptide-expressing cells (**Figure 5C**). The proportion of the dimer was slightly decreased in the control peptide-expressing cells compared with parental cells, but this difference was not statistically significant.

The growth of cells was significantly inhibited by expression of any of the three inhibitory peptides (**Figure 6A**). Stable expression of the control peptide tended to reduce the malignancy of cells and decrease RET protein dimerization.

The expression of RET-PTC oncogenes induces cell transformation in NIH3T3 cells [18, 25]. This transforming potential is linked to the con-

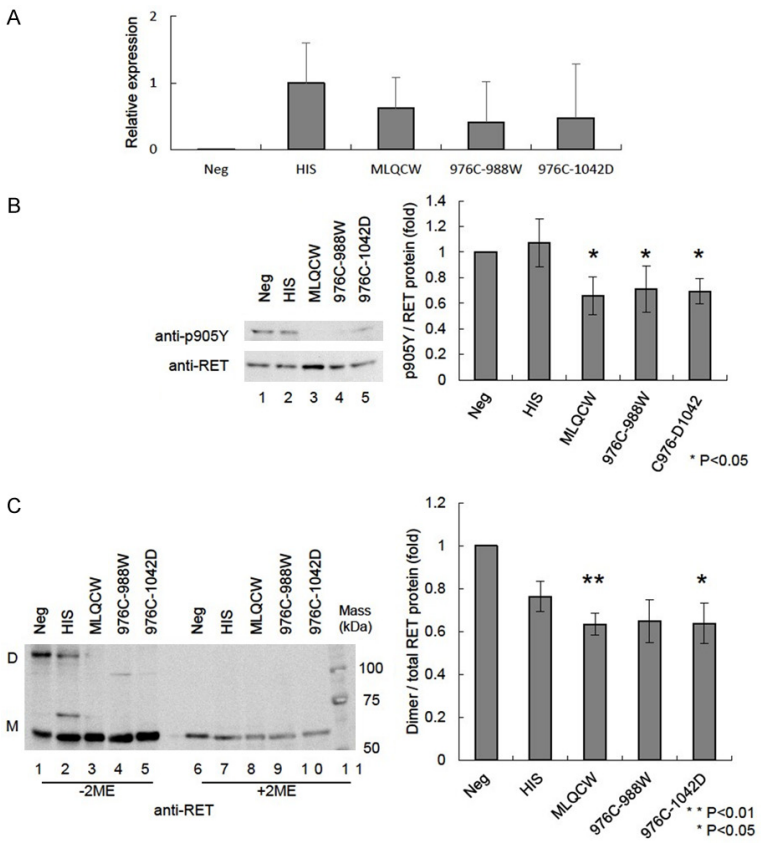

Figure 5. Decrease of kinase activity and protein dimerization by expression of peptides in stably transfected cells. Cells overexpressing RET-PTC-1 were transfected with an MLQCW motif-containing peptide expression vector or control pcDNA 3.1His vector. Expressions of His-tagged peptides were analyzed using real-time PCR on cDNAs from stably transfected cells. Results shown are representative of more than eight individual cells. Data (mean ± standard deviation) are shown as relative amount of mRNA in relation to the house-keeping gene *Hprt* and normalized to the mean of the control pcDNA 3.1His vector-transfected cells (A). Lysates from RET-PTC-1-expressing cells transfected with peptides or the no peptide control (Neg) were subjected to western blotting with anti-phospho-RET (p905Y) (B, top) and anti-RET (B, bottom and C) antibodies. SDS-PAGE was done under ordinary reducing (B and C lane 6-10) or non-reducing (without 2-mercaptoethanol; C lane 1-5) conditions. The letter M indicates the 55 kDa monomeric RET (estimated by comparison with protein standards in lane 11); D, 110 kDa dimer RET. Levels of phosphorylation (B) and dimer relative to total RET protein amounts (C) were quantified by densitometry. Data (mean ± standard error of the mean) are normalized to the mean of no peptide-transfected control cells (B: n=12, C: n=8). Significant differences (*P<0.05, **P<0.01) from the control (Neg) were identified using the Steel test.

stitutive kinase activation. It is well known that original NIH3T3 cells fail to grow when suspended in agar gel. However, when these cells are transformed, they are able to grow in the gel. In our study, NIH3T3 cells transfected with RET-PTC-1 formed colonies in soft agar gel. As shown in **Figure 6B**, the transforming activities of RET-PTC-1 decreased upon expression of the inhibitory peptides, although expression of the His-tagged control peptide had no significant effect.

Reduction of RET protein-induced tumorigenicity in nude mice

Alleles of *RET-PTC* induce tumor formation in nude mice when introduced into NIH3T3 cells [25]. As **Figure 7A** illustrates, tumors that expressed MLQCW motif-containing peptides tended to grow slower than the tumors that did not express peptides, although the differences in tumorigenicity were not statistically significant at the study endpoint due to variability between individuals (**Figure 7B**). At the same time, expression of these peptides delayed RET-PTC-1-induced tumor onset (**Figure 7D**).

Discussion

In this study, we demonstrate that peptides containing the MXXCW motif inhibit the catalytic and transforming activities of RET tyrosine kinase, and reduce the development of RET-induced tumors. The MLQCW peptide interacted with RET-PTC protein via disulfide bonds, which resulted in inhibition of kinase activity. The inhibitory activity of the MLQCW peptide was comparable to the known RET kinase inhibitors sorafenib, sunitinib, cabozantinib, and vandetanib (reported IC50 values: 50 nM [26], 224 nM [24], 4 nM [27], and 100 nM [25], respectively).

The potent inhibition of RET by the peptides in this study was confirmed in NIH3T3 cells that expressed oncogenic RET-PTC. Our results suggest that the three MLQCW motif-containing inhibitory peptides suppress RET-PTC-1 activity by disrupting RET protein dimerization. It has

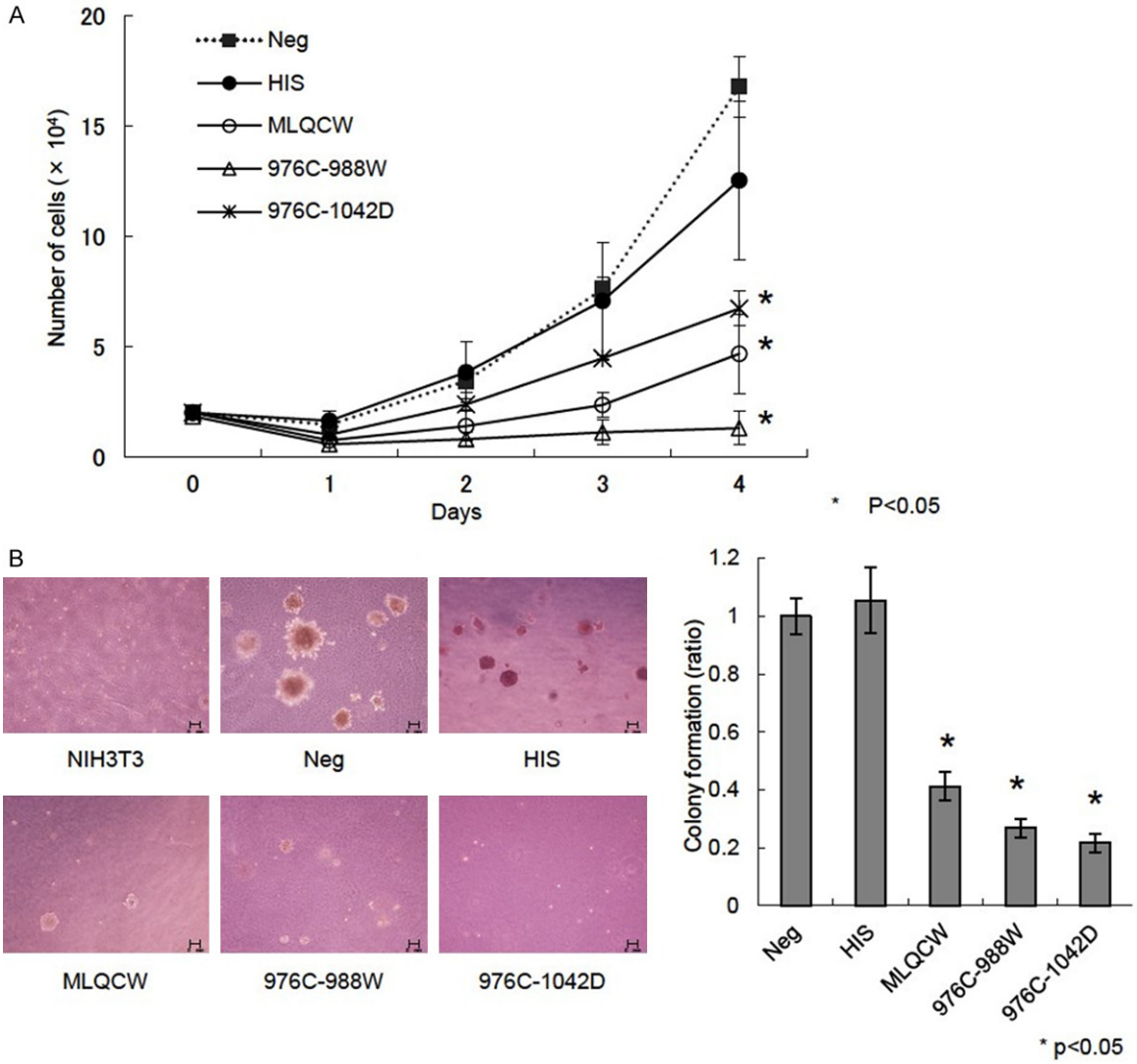

Figure 6. Decrease of anchorage-independent and dependent proliferation by expression of peptides in RET-PTC-1 over-expressing cells. Cells that over-expressed RET-PTC-1 and had been stably transfected with MLQCW motif-containing peptide vectors or the no-peptide control (Neg) were cultured for 4 days and the number of cells counted each day. The means ± standard error of the mean are reported (A, n=4). The same cells were grown in soft agar and photographed after 14 days of culture (B). The number of colonies with diameters larger than 0.1 mm was determined in 26 fields of view from four different dishes (B, right). Data (mean ± standard error of the mean) are normalized to the mean of the no-peptide-transfected control cells. Bar =0.1 mm. Significant differences (*P<0.05) from the control (Neg) were identified using the Steel test.

been reported that administration of histidine increases the antioxidant capacity [28], and so it is possible that, in the case of His-tagged control vector expression, the histidine-induced antioxidant capacity of cells may have resulted in the reduced the formation of disulfide-bonded RET dimers that we observed. The three peptides also inhibited RET activity in TT cells, which were derived from a medullary thyroid carcinoma harboring a constitutively activate MEN2A-type mutation, and in MCF7 cells, in which RET activation has previously been shown to stimulate cell proliferation [10]. These data demonstrate that the motif-containing peptides inhibit not only RET-PTC fusion kinase, but also RET kinases with other activating mutations and ligand-induced RET activation.

Dimerization of RET is predicted to be mediated by fusion partners, and, in the case of RET-PTC protein, is thought to be essential for activation of the kinase [29]. The RET-MEN2A protein contains a point mutation in the cysteine-rich region of extracellular domain. This cysteine

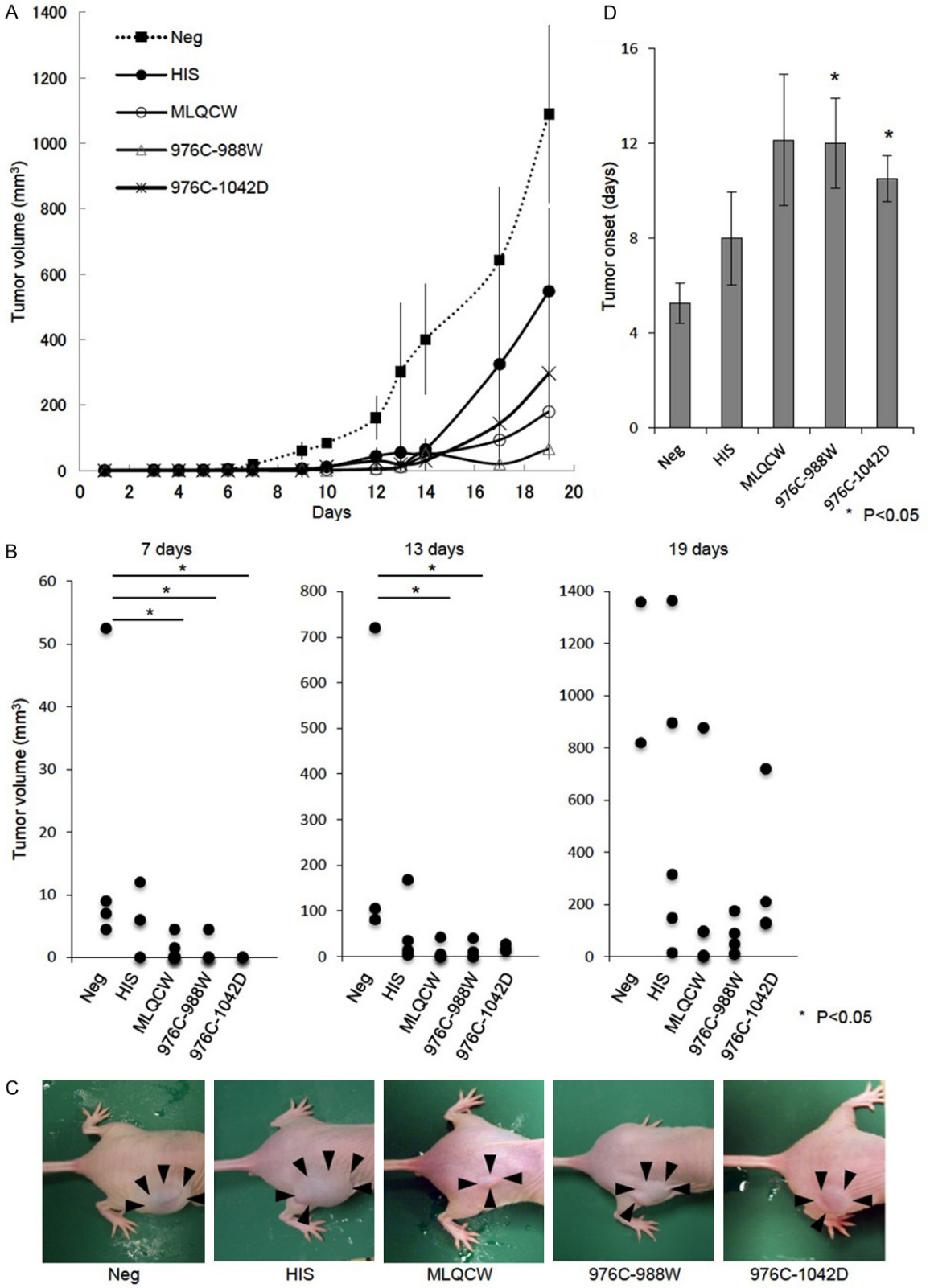

Figure 7. Inhibition of tumor growth in mice. A total of 5×10^5 of RET-PTC-1 over-expressing cells that had been stably transfected with a peptide vector or the no-peptide control (Neg) were injected subcutaneously into nude mice. Tumor volumes were measured, and the means ± standard error of the mean are reported (A). The tumor volume of each animal was at 7, 13, and 19 days after the tumor injection was plotted (B). Representative photograph taken at the study endpoint. Arrowheads indicate a tumor (C). Tumor onset was monitored by palpation and the mean ±

standard error of the mean plotted (D). Significant differences (*P<0.05) from the control (Neg) were identified using the Steel test. (Neg: n=4, HIS: n=7, MLQCW: n=7, 976C-988W: n=6, 976C-1042D: n=4).

mutation induces dimerization and activation of RET through the formation of disulfide bonds between unpaired cysteine residues of two RET proteins [30]. The binding of ligands to the extracellular domain also triggers dimerization. Therefore, regardless of whether the dimerization site is located in the extracellular or intracellular domain, intracellular expression of cysteine-targeted peptides could inhibit RET kinase activation.

In the present study, MLQCW motif-containing peptides inhibited RET-dependent cell proliferation and tumorigenicity in mice. This suggests that such peptides may have a clinical role in the treatment of patients with RET-associated cancers. Clinical use of tyrosine kinase-based therapies in cancer treatment is widespread. Various compounds have been reported to inhibit oncogenic RET including PP1 [31], PP2 [32], vandetanib [25], imatinib [33], CEP-751 [34], RPI-1 [35], sunitinib [24], semaxanib [36], sorafenib [37], NVP-AST487 [38], cabozantinib [27], and lenvatinib [39]. However, of the known kinase inhibitors, only vandetanib, sorafenib, and lenvatinib are indicated for the treatment of thyroid cancers. Although several RET-selective inhibitors such as BLU667 and LOXO-292 have recently been identified [40, 41], many RET kinase inhibitors were developed for other tyrosine kinases. Since the ATP binding site is highly conserved among many kinases, optimization of selectivity is one of the most important tasks in kinase inhibitor development [42], as additional activities can result in off-target pharmacology. To improve efficacy and toxicity profiles, it is highly desirable to develop RET-selective inhibitors for the treatment of RET-induced tumors.

Analyses of a gene data base (Swiss-Prot Data base) have shown that the cysteine of the MXXCW motif is conserved in all except one of the 82 human protein tyrosine kinases [43]. Therefore, the cysteine-dependent activation mechanism is potentially important for many other tyrosine kinases besides RET. However, MXXCW motif-containing peptides are expected to bind to kinase more specifically than ATP-competitive inhibitors, because there are no other conserved amino acids around this motif in the C-terminus of the kinase domain [44]. Indeed, we demonstrate here that the three inhibitory peptides had no effect on ERK phosphorylation in NIH3T3 cells. This suggests that these peptides cannot inhibit ERK or its upstream kinases in cells that do not express RET. Moreover, the growth of RET-PTC-expressing NIH3T3 cells was effectively suppressed by the peptides; an effect that was not observed in original NIH3T3 cells (data not shown). These data indicate that the peptides *specifically inhibit* RET kinase, with little effect on other kinases involved in NIH3T3 cell growth. The three peptides examined in this study were similarly effective in inhibiting RET kinase activity, although further investigation into whether there is a distinct specificity for other tyrosine kinases among these peptides may be required.

Prolonged exposure to tyrosine kinase inhibitors in patients with RET-associated tumors can lead to acquired resistance. In particular, point mutations at the so-called “gatekeeper position” are commonly found in protein kinases that are targeted in cancer therapy [45]. These mutations result in reduced binding of the inhibitor to the hinge region of the ATP pocket due to steric hindrance [46]. The V804M and V804L gatekeeper mutations in the RET kinase domain have also been reported to confer drug resistance [47]. The identification and development of inhibitors that are less *susceptible* to such mutations are, therefore, the focus of many research projects. Since the MXXCW motif is located distally to the ATP binding site, binding of these peptides to the kinase is unlikely to be affected by the major drug-resistance mutations that are currently known. Indeed, our results indicate that MLQCW motif-contained peptides reduce the kinase activity of a RET gatekeeper mutant. Combination therapies involving ATP-competitive inhibitors and cysteine-targeted drugs need to be planned in advance of clinical application.

Cysteine residues possess an aliphatic thiol (SH) which has unique reactivity [48] and can be targeted with thiol-reactive molecules. It is recognized that irreversible kinase inhibitors have several potential advantages over conventional reversible kinase inhibitors including prolonged pharmacodynamics, improved selectivity, and the potential to overcome resistance

mutations [49]. This design paradigm has been successfully applied to drug candidates for epidermal growth factor receptor (EGFR) and a few related protein kinases [49]. Many irreversible kinase inhibitors bind to a specific rare cysteine residue at the ATP binding site, which is conserved among 10 kinases. The MXXCW motif-containing peptides were predicted to bind to the cysteine in helix H, which is located in the C-terminus of the kinase domain. Regardless of the particular site, cysteine-binding materials would have the common advantage of irreversible inhibition.

In conclusion, MXXCW motif-containing peptides could represent promising candidates for novel tyrosine kinase inhibitors that have a distinct mode of action and could expand the range of options for molecular-targeted cancer therapy. New cysteine-targeted RET inhibitors could have a large clinical impact on RET-driven cancer treatment.

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Disclosure of conflict of interest

None.

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