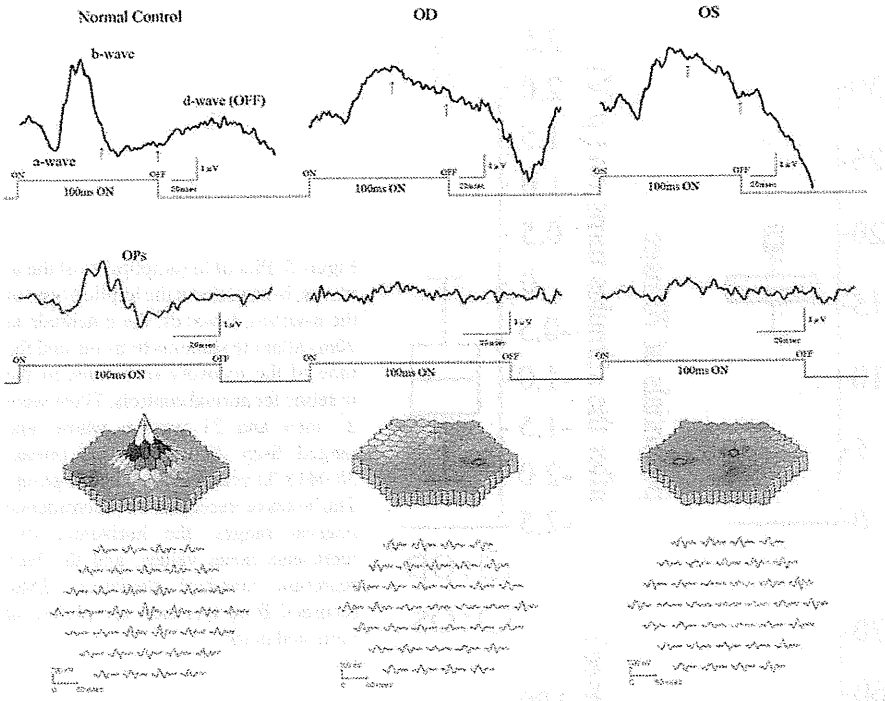

Figure 4. Results of focal macular electroretinograms (ERGs) and multifocal ERGs. Focal macular ERGs and oscillatory potentials recorded from a normal subject and this case are shown (top). The amplitude of the a-wave of this case was severely reduced, and the plateau region was significantly elevated (arrows). The topographic map and the local responses of multifocal ERGs recorded from the normal subject and this case are shown (bottom). The amplitudes in the foveal area were severely reduced in this case.

arrow). To analyze this characteristic, we quantified the potentials at 70 ms after the stimulus was turned on, and the recovery time of the descending slope of b-wave to the baseline from the peak of the b-wave. We calculated the 95% confidence intervals (CI) for the amplitudes of the a-waves and b-waves, the implicit times of the a-waves and b-waves, the potentials at 70 ms after the stimulus turns on, and the time of the recovery of the b-waves to the baseline obtained from the normal controls (Figure 5). Among these six parameters, the amplitudes of the a-waves, the implicit times of the b-waves, the potentials at 70 ms after the stimulus was turned on, and the time of the recovery of the descending slope of the b-wave to the baseline obtained from both eyes of this case were outside the range of the standard deviation and the 95% CI of the normal controls (Figure 5). Especially, the amplitudes of the a-waves, the potentials at 70 ms after the stimulus was turned on, and the time of the recovery of the descending slope of the b-wave to the baseline obtained from this case were severely affected. The amplitudes of the mfERGs in the foveal area were severely reduced in this case (Figure 4).

Molecular genetic findings: Mutation analysis of the *RP1L1* gene in this case showed three missense mutations. There was a c.2578 C>T in exon 4 with a substitution of tryptophan (TGG) for arginine (CGG) at amino acid position 860, a c. 3596 C>G in exon 4 with a substitution of cysteine (TGT) for serine (TCT) at amino acid position 1199, and a c. 4484 C>G in exon 4 with a substitution of arginine (CGC) for proline (CCC) at amino acid position 1495. The amino acid substitution at position 860 and 1495 has already been reported in the SNP database and is found in a high percentage of the normal population. A mutation at amino acid position 1199 has not been reported in the SNP database or in earlier reports (Figure 6A). The serine at position 1199 is well conserved among the RP1L1 family in other species (Figure 6B). This mutation was predicted to be probably damaging with a score of 0.999 by PolyPhen-2. The SIFT tool analysis revealed a score of 0 and predicted that the replaced amino acid is potentially damaging and would not be tolerated. PMut predicted that this mutation is pathological. Align GVGD predicted this mutation as class C65, which means it most likely interferes with the protein function. Out of five computational assessments, only MutationTaster predicted this mutation as a polymorphism. We confirmed that the mutation in this case was segregated with the disease in one affected member and one unaffected member of the family (Figure 6C). The unaffected member of the family in Case 1 underwent clinical examination, including BCVAs, slit-lamp biomicroscopy, fundus ophthalmoscopy, OCT, and focal ERGs. All examination findings were normal. This mutation was not present in 300 control alleles. This mutation p.S1199C has been registered in GenBank with accession number AB684329.

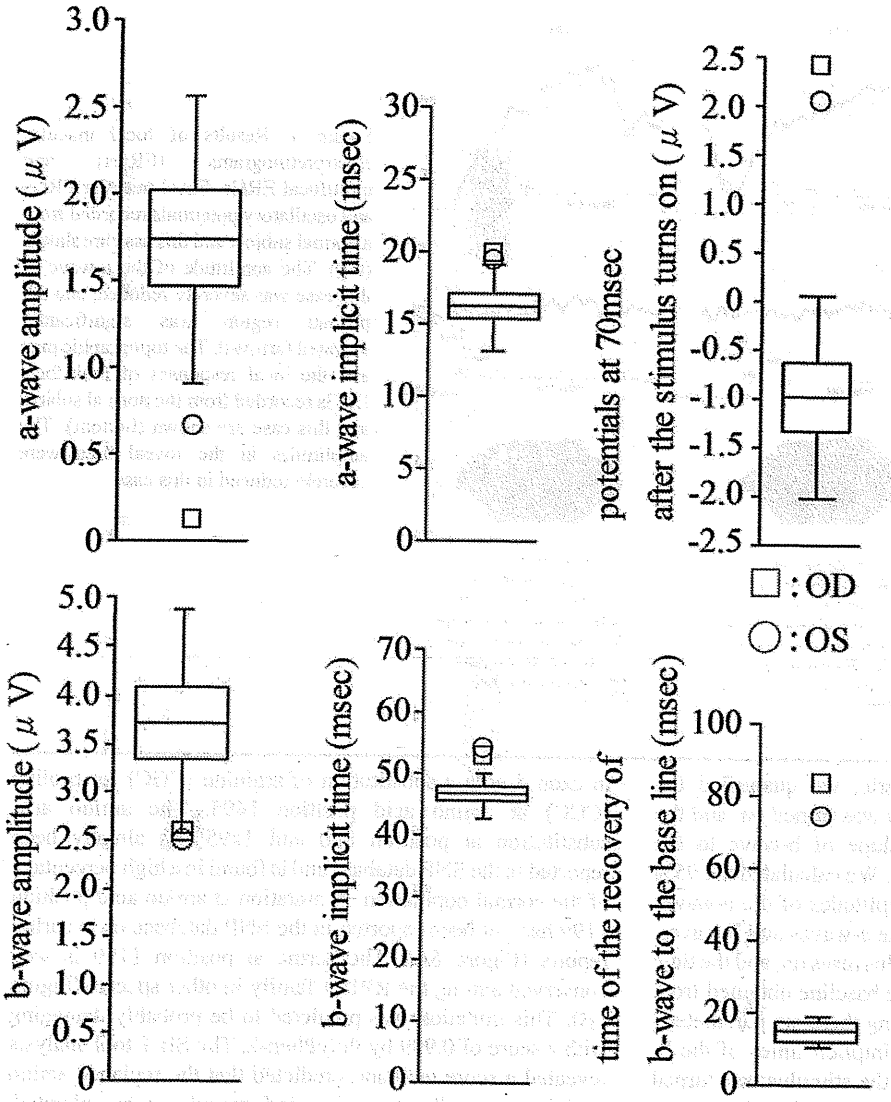

Figure 5. Plot of the amplitudes of the a-waves, b-waves, and the implicit time of the a-waves, b-waves, the potentials at 70ms after the stimulus turns on, and the time of the recovery of b-wave to the baseline for normal controls. There were 25 men and 21 women whose age ranged from 23 to 60 years (mean, 38.04±8.33 years) in this control group. The boxes represent the 95% confidence interval ranges, the horizontal line represents mean values, and the bars represent standard deviation. Data recorded from this case are plotted at indicated mark.

Bowne et al. [11] reported that *RP1L1* mRNA is variable due to the presence of a 48 bp polymorphic coding repeat. They reported that as many as six 48 bp repeats have been observed in normal controls. In this case, one allele contains a 48 bp repeat, and the other allele contains three 48 bp repeats (Figure 6D). There are variations of only two amino acids in the length polymorphism region from this case compared to the reference sequence (NP_849188). One variation with the substitution of E to G in the 14th amino acid of the length polymorphism region was in a previous report [12] (AAN86962, AAN86963, and AAN86964). The other variation with the substitution of G to V in the ninth amino acid of the length polymorphism region was found in more than 10 normal control alleles from a Japanese population. These variations of the length polymorphisms of *RP1L1* with one and three repeats have been registered in GenBank with accession numbers AB684331 and AB684332, respectively.

DISCUSSION

The mutation found in the *RP1L1* gene in this case was a missense mutation with cysteine substituted for serine at amino acid position 1199. This residue is well conserved among the RP1L1 family in other species, suggesting the importance of this amino acid residue for RP1L1 function. Four out of five computational analysis tools predicted this mutation is damaging to the protein function. We did not find this mutation in the sister of the patient with normal vision, although she was the only other family member we were able to test. To decide whether this mutation was pathogenic, we need to examine more family members and a larger number

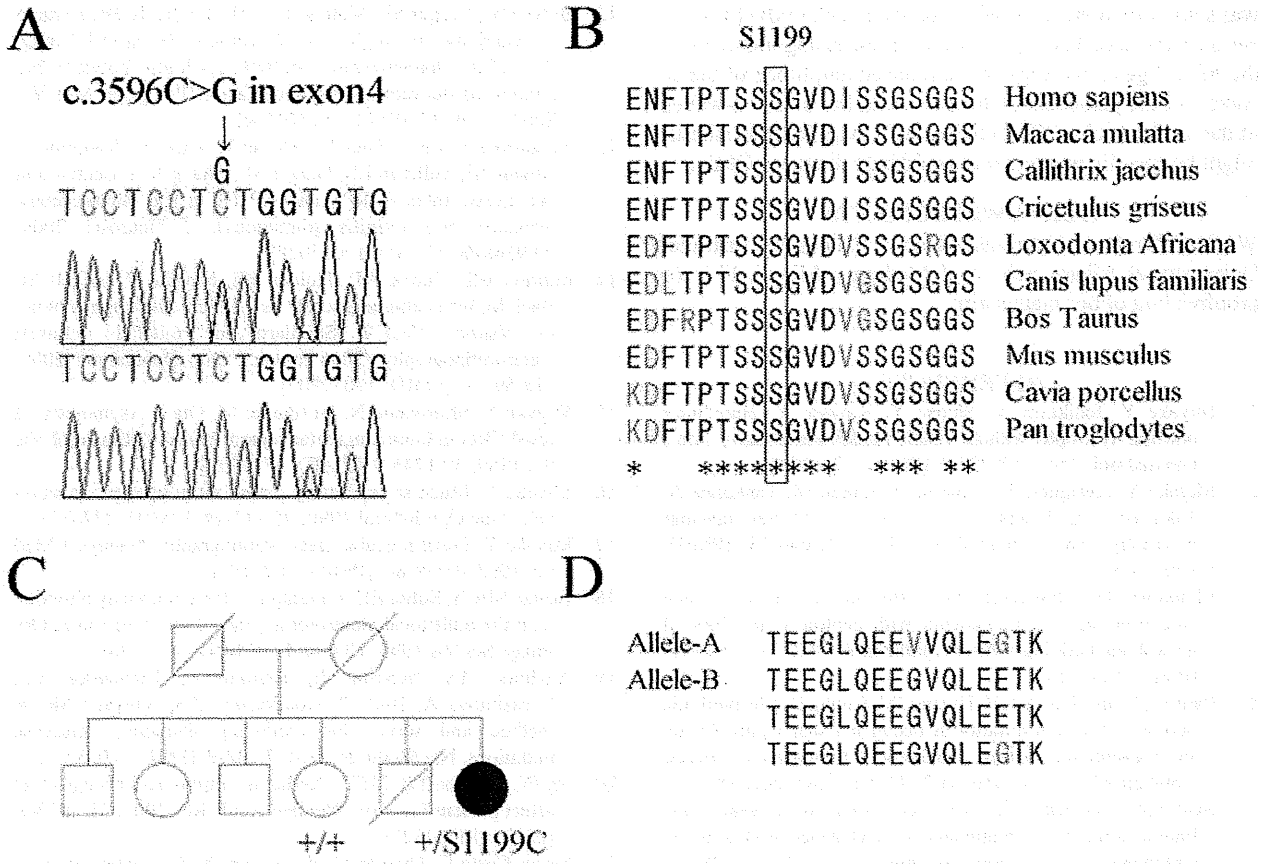

Figure 6. DNA analysis for c.3596C>G mutation and deduced amino acids of length polymorphism region of the RP1-like protein 1 (*RP1L1*) gene and the pedigree of the family with *RP1L1* gene mutation. **A**: Sequence chromatograms for this case (top) and the normal control (bottom) are shown. This case had a c.3596 C>G mutation in exon 4. **B**: Alignment of S1199 in the RP1L1 family proteins. Amino acid-sequence alignments of RP1L1 from 10 species reported in the NCBI database are shown. Amino acid residues of S1199 in humans and conserved residues from other species are boxed. The asterisks indicate completely conserved residues. S1199 is well conserved in all species reported. **C**: We confirmed that the mutation in Case 1 was segregated with the disease in one affected member and one unaffected member of the family. **D**: Deduced amino acids (AA) of repeated regions of the RP1L1 length polymorphism. In this case, one allele contains a 16 AA, and the other allele contains three 16 AA repeats. Variations of amino acids from reference sequence of RP1L1 are shown in red. Those variations are within normal limits.

of normal controls. However, the phenotype of this case was typical of OMD, and thus the mutation in this case was most likely pathogenic.

The photoreceptor IS/OS junction and the COST line can be detected in the SD-OCT images of normal eyes [25-28]. Recently, several degrees of disruption of the IS/OS junction and/or COST line in the SD-OCT images of patients with OMD have been reported [4-8]. In our case, the IS/OS junction and the COST line appeared blurred in the SD-OCT images similar to previous reports.

Researchers have emphasized that the key to differentiating OMD from other diseases, such as optic neuritis or psychological disorders, is the recording of focal macular ERGs from the central retina [1-3]. Focal macular ERGs have a unique waveform when elicited by long-duration stimuli [29]. As shown in this patient, the waveform of focal macular ERGs recorded from patients with OMD with long-duration stimuli had a depolarizing pattern, simulating the ERG waveforms observed after the hyperpolarizing bipolar cell activity is blocked [30-33]. Researchers have demonstrated that by blocking hyperpolarizing bipolar cells with cis-2,3-piperidine dicarboxylic acid or kynurenic acid in monkeys, the a- and d-waves of photopic ERGs become smaller and the plateau between the b- and d-waves remains elevated above the baseline potential [34]. Full-field cone ERG in some human retinal dystrophies show a similar depolarizing pattern [29,35]. Kondo et al. [29] reported similar focal macular ERGs elicited with 100 ms stimuli from a patient with glittering crystalline deposits in the posterior fundus. The waveform of the focal macular ERGs of this case

was similar to those reported for patients with OMD [31-33]. Because this case had a putative disease-causing mutation of the *RP1L1* gene, we suggest the reduced amplitude of the a-wave and the persistent plateau between the b- and d-waves of the focal macular ERGs elicited with long-duration stimuli might be specific markers that could help diagnose OMD.

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Novel Complementary Peptides to Target Molecules

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Abstract. *We generated an evolutionary computer program that generates complementary peptide (C-pep) sequences, with the potential to interact with a target peptide, by comparing several physico-chemical parameters of each pair of the complementary peptides being analyzed. We generated C-peps to target several molecules. About 30% of synthesized C-peps interfered with the function of their targets. C5a stimulates generation of TNFα and other inflammatory cytokines. Inhibition of C5a should be effective against sepsis, which impairs the status of cancer-bearing patients. One of the inhibitory C-peps of C5a, termed AcPepA, was effective in Cynomolgus monkeys intravenously infused with a lethal dose of bacterial LPS (4 mg/kg) destined to die. The monkeys were rescued by intravenous administration of 2 mg/kg/h of AcPepA. The excellent therapeutic effect of AcPepA is likely to be due to restriction of high mobility group box 1 (HMGB1) surge induced by the effect of C5a on C5L2, which is the second C5a receptor, since the released HMGB1 has the capacity to stimulate TLR4 as an endogeneous ligand resulting in further activation of inflammatory cells to release inflammatory cytokines forming a positive feedback circuit of inflammation.*

After proposal of the possible role of antisense peptides for molecular interaction among proteins by Blalock *et al.* (1) in 1984, the theory was reviewed later (2, 3). Many examples of sense–peptide and antisense–peptide relationships have been found between receptors and their protein ligands (4-8). We speculated that such interactions between sense and antisense peptides should play a role in formation of the tertiary structure of proteins. We developed a novel computer program named ANTIS to find antisense peptide sequences between proteins to be compared (9). By analyzing intramolecular antisense peptides within a single protein molecule, we found that there are an appreciable number of sense and antisense peptide pairs within a protein molecule and we designated these as antisense homology boxes (AHB) (9). Using ANTIS, we analyzed sense and antisense peptide relationships in the endothelin receptor (ETR) molecule and between endothelin and ETR. One of the AHB peptides of ETR, named ETR-P1/fl, had the capacity to interfere with the function of ETR (10). We expected that it would be possible to generate candidates of complementary peptide reactive to a target amino acid sequence based upon the sense-antisense amino acid relationship. We generated an evolutionary computer program that runs on any PC, and generates complementary peptide (C-pep) sequences, with the potential to interact with a target peptide, by comparing several physico-chemical parameters of each pair of the complementary peptides being analyzed (11). With the program named MIMETIC, we generated complementary peptides (C-peps) to HIV-reverse transcriptase (11, 12), procarboxypeptidase R, thrombomodulin (13), and C5a anaphylatoxin (14) as listed in Table I. About 30% of the synthesized peptides interfered with the function of their target molecules. Out of 19 complementary peptides (C-peps) targeted to C5a anaphylatoxin, 7 exhibited an inhibitory effect.

C5a is a 74 amino acid peptide released from the fifth component of complement (C5) by C5 convertase generated during complement activation (15). C5a anaphylatoxin is considered to be an effective target for treatment of hyperinflammation since C5a stimulates generation of tumor necrosis factor alpha (TNFα) and other inflammatory cytokines (16-18). Although C5a generated *in vivo* is regulated by carboxypeptidase N and more efficiently by carboxypeptidase R (CPR) (19, 20), excessive generation of

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Key Words: Antisense pepitide, complementary peptide, anaphylatoxin, inflammation, sepsis, cytokine storm.

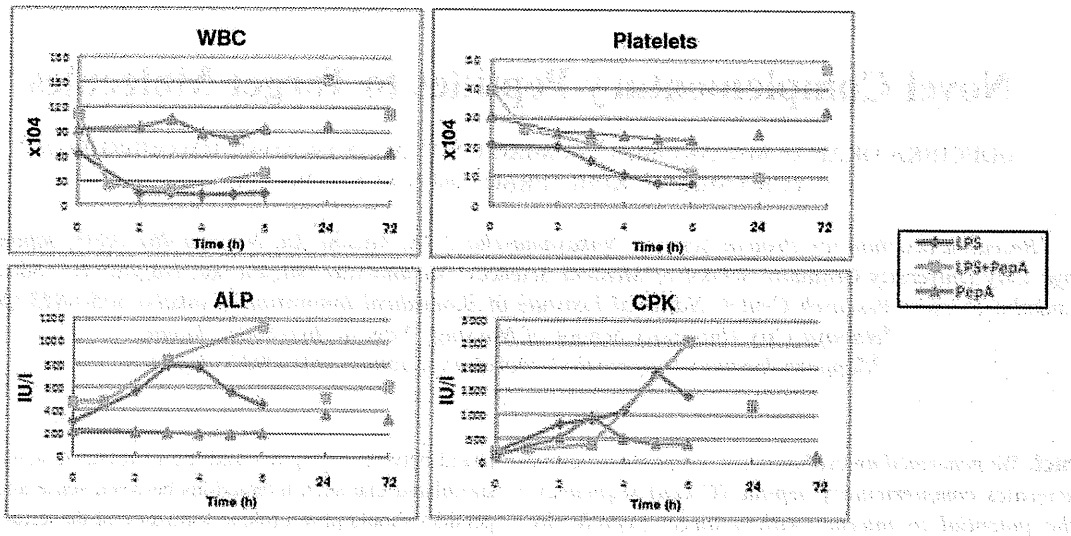

Figure 1. *Clinical features in peripheral blood of endotoxin-shocked monkeys. Extensive leukopenia (WBC), and thrombopenia (platelets), increased alkaline phosphatase (ALP), as well as increased creatinine phosphokinase (CPK), were observed in blood from AcPepA-treated monkeys and in untreated monkeys following LPS injection. Esssentially no significant changes were observed in a monkey treated with AcPepA alone without LPS injection.*

C5a appears to exceed the capacity of CPR, since administration of lipopolysaccharide (LPS) at a lethal dose to rats exhausted CPR capacity before death (21). However, attempts to restrict the effect of C5a with C5a receptor (C5aR) antagonists would not be successful because C5aR is not only expressed on inflammatory leukocytes, but also on many other cell types (17). Furthermore, C5aR numbers increase in an acute inflammatory state (22).

On the other hand, antibodies to C5a have been demonstrated to be effective in treating experimental primate models of sepsis (16, 23), indicating that C5a inhibitors should be useful for treatment of patients suffering from hyperinflammation such as in sepsis and multiple organ failure (24). If an inhibitor of C5a has a therapeutic effect on sepsis, which impairs the status of cancer-bearing patients, the inhibitor could be beneficial for the cancer patients in order to improve their performance status.

AHB in C5aR, and between C5aR and C5a were analyzed by ANTIS program, and we found that amino acids 37 to 53 of C5a (RAARISLGPRCIKAFTE) comprise an antisense peptide to AHB peptides (9) of the C5aR, and this has been designated PL37 (25). This region of C5a is presumed to be a potential site for C5aR stimulation (26). Using the computer program MIMETIC (11), we generated 19 C-peps to PL37. One of the 7 inhibitory C-peps to PL37 which interfered with C5a function was termed PepA (ASGAPAPGPAGPLRPMF) (14). To improve stability, we modified PepA by acetylation of its *N*-terminal alanine generating acetylated PepA (AcPepA) which was more stable in animal experiments (27). In preliminary experiments with human lung tissues, AcPepA successfully suppressed the allergic response *in vitro* (28). Therefore, we performed experiments in *Cynomolgus* monkeys *in lieu* of using humans.

Table I. *Inhibitory capacity of complementary peptides (C-pep) to target molecules. Complementary peptides designed by MIMETIC program were synthesized and their inhibitory capacity on the function of target molecules determined. About 30% of these peptides interfered with the activity of target molecules.*

Target molecule	Activity of target	Number of C-pep	
		Tested	Effective (%)
HIV-RT*	Enzyme activity	10	3 (30%)
ProCPR **(TAFI)	Enzyme activity	10	3 (30%)
Thrombomodulin	Cofactor activity***	3	2 (67%)
C5a anaphylatoxin	Bioactivity	19	7 (37%)

*RT: Reverse transcriptase (12); **ProCPR: procarboxypeptidase R; ***cofactor activity for thrombin (13).

Materials and Methods

Peptides. PepA (ASGAPAPGPAGPLRPMF) whose *N*-terminal alanine is acetylated (AcPepA) was synthesized and purified (over 95% purity) by Biologica Co. Ltd. (Nagoya, Japan). The peptide was dissolved in saline at a concentration of 2 mg/ml and passed through a 0.22 μm Millipore filter prior to administration intravenously with an automated injection pump.

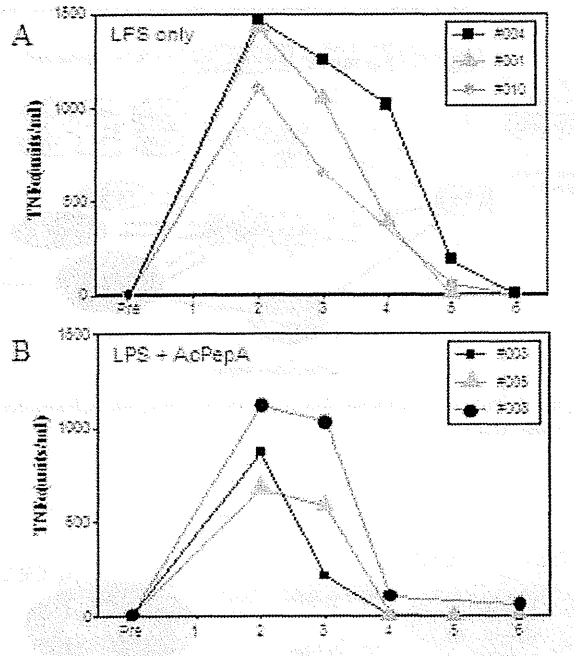

Figure 2. *TNFα levels in plasma of monkeys injected with 4 mg/kg LPS (A) and monkeys treated with AcPepA following the LPS injection (B). AcPepA t (2 mg/kg/h) was intravenously infused from 30 min to 6 h following LPS injection. AcPepA treatment suppressed the TNFα level to approximately 60% those of the untreated monkeys. TNFα in plasma disappeared within 4 h after LPS injection in AcPepA-treated monkeys, whereas this took 5 h in untreated monkeys.*

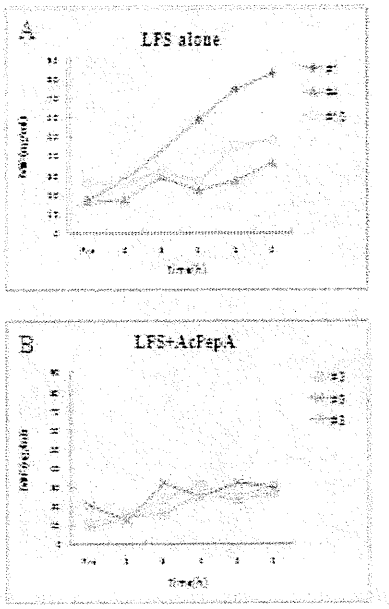

Figure 3. *Macrophage migration inhibitory factor (MIF) levels in plasma. Monkeys injected with LPS alone (#1, #4, #10; A), and monkeys treated with AcPepA following the LPS injection (#3, #5, #8; B) were tested for their MIF levels. MIF of AcPepA-treated monkeys remained at low levels (less than 30 ng/ml), whereas that of untreated monkeys increased to over 30 ng/ml following LPS injection.*

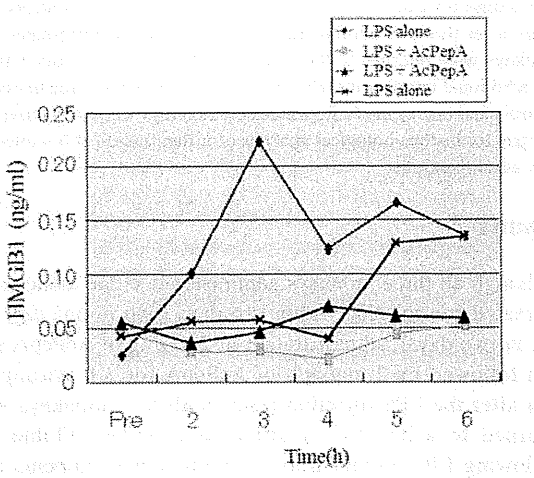

Figure 4. *Increase of HMGB1 in plasma of monkeys injected with LPS. Monkeys injected with LPS alone (#1, #10) showed increased HMGB1 levels in plasma, while AcPepA treatment following LPS injection (#5, #8) did not cause an increase (34).*

Monkeys. Cynomolgus monkeys were supplied from a breeding colony maintained at the Corporation for Production and Research of Laboratory Primates (CPRLP), Tsukuba, Japan. The Institutional Animal Ethical Committee of the Choju Medical Institute, Fukushimura Hospital, and the Institutional Animal Care Use Committees of the Tsukuba Primate Research Center, National Institute of Biomedical Innovation approved the study protocol. Animals weighed 4 to 5.5 kg, had hematocrits exceeding 36% and were free of infection, including tuberculosis. Animals were held for one month prior to LPS-lethal shock studies at CPRLP.

Titration of cytokine levels in plasma. TNFα in monkey plasma was determined using an ELISA kit purchased from Quantikine Immunoassay (Minneapolis, MN, USA). Macrophage inhibitory factor (MIF) was determined by use of an ELISA kit (29) prepared by Sapporo Immuno Diagnostic Laboratory (Sapporo, Japan). For high mobility group box 1 (HMGB1) determination, an ELISA kit from Shino-Test Co. (Sagamihara-shi, Kanagawa, Japan) was used.

Treatment of Cynomolgus monkeys. Fourteen monkeys were used for the experiment, and 13 were administered a lethal dose of LPS (4 mg/kg) sufficient to kill a monkey within 2 days, and 1 monkey was not administered LPS as an untreated control. Following sedation using ketamine hydrochloride (14 mg/kg, subcutaneously), monkeys were anesthetized with sodium pentobarbital administered through the capalic vein *via* a percutaneous catheter to maintain light surgical anesthesia. Oral intubation allowed animals to breathe spontaneously. Under anesthesia with sodium pentobarbital, 13 monkeys were intravenously administered 4 mg/kg LPS within 30 min. Thirty minutes after the LPS injection, 8 of the 14 animals were

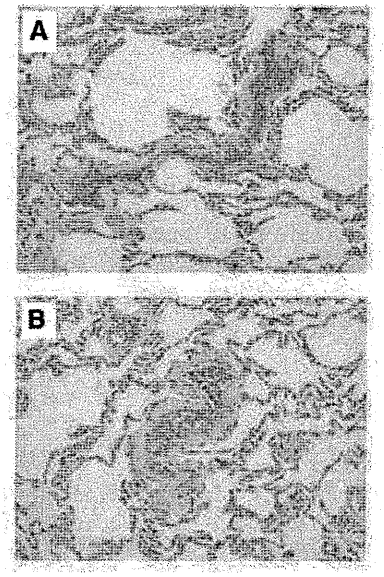

Figure 5. *Inflammation in lungs of monkeys 6 h after injection of a lethal dose of LPS observed in AcPepA-treated monkey (B) was to the same extent as that in untreated monkeys after LPS injection (A). (Magnification: ×200).*

administered 2 mg/kg of AcPepA in 2 min followed by 2 mg/kg/h of AcPepA for 3 h. The other 6 LPS-injected monkeys were injected with saline instead of AcPepA treatment as untreated controls. Six hours after the LPS administration, anesthesia was terminated and monkeys were returned to their cages to observe their status without any additional interference. However, half (3 animals) of the untreated controls and one of the AcPepA-treated monkeys were euthanized for autopsy for histopathological analysis of inflammation at 6 h after the LPS administration.

Results

Although all three monkeys administered saline alone as an untreated control died within two days (two in one day and one in two days), administration of 2 mg/kg of AcPepA in 2 min followed by 2 mg/kg/h of AcPepA for 3 h starting 30 min after the LPS injection rescued all of 7 monkeys who returned to a healthy condition in two days (Table II). Following LPS administration, significant leukopenia and thrombopenia were observed in peripheral blood obtained 6 h after the LPS injection both from monkeys treated with AcPepA and from control monkeys treated with saline instead of AcPepA (Figure 1). The increased TNFα level in plasma obtained during experiments in AcPepA-treated monkeys was lowered by only about 30% compared with that of untreated monkeys (Figure 2). The increase in the level of MIF (Figure 3) and HMGB1 (Figure 4) after LPS injection tended to be suppressed in the AcPepA-treated monkeys. Some of the monkeys were sacrificed under

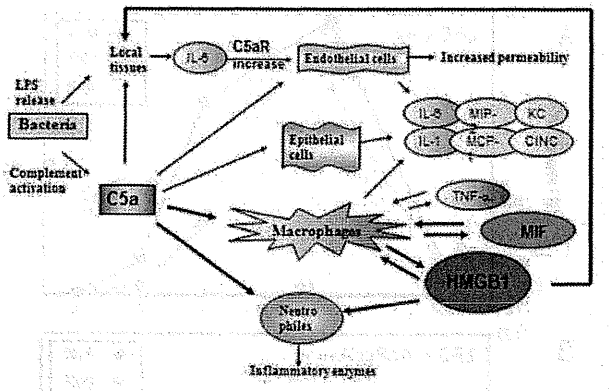

Figure 6. *Role of C5a anaphylatoxin in induction of an inflammatory cytokine 'storm'.*

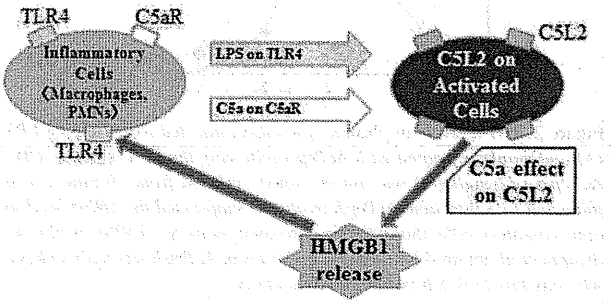

Figure 7. *Possible role for C5a in a positive feedback inflammatory circuit. Following bacterial infection, LPS stimulates TLR4, and C5a generated during complement activation stimulates C5aR, resulting in expression of C5L2 on leukocyte membranes. Stimulation of C5L2 by C5a on activated leukocytes induces release of HMGB1 which then reacts with TLR-4 on other leukocytes, as did LPS, resulting in further recruitment of activated leukocytes that express C5L2. These reactions create an inflammatory amplification circuit (34).*

anesthesia 6 h after LPS administration in order to perform autopsies. Pathological analysis of organ tissues showed serious inflammatory changes, including leukocyte infiltration, to the same extent in the lungs of both treated and untreated monkeys (Figure 5).

Discussion

The monkeys treated with AcPepA might have escaped induction of a feedback inflammatory circuit which was progressing gradually in LPS-treated monkeys at a late stage of the endotoxin shock syndrome as a vicious circle (Figure 6). This may be because HMGB1 has the capacity to stimulate toll-like receptor 4 (TLR4) and TLR2 as an endogenous stimulator (30, 31).

Table II. *Therapeutic effect of AcPepA on monkeys inoculated with a lethal dose of lipopolysaccharide (LPS) (4 mg/kg).*

	LPS alone	LPS and AcPepA	AcPepA alone
Decreased blood pressure	4/6	3/7	0/1
Increase in body temperature	5/6	3/7	0/1
Leukopenia	6/6	7/7	0/1
Increased CPK	6/6	7/7	0/1
Death	3/3	0/7	0/1
Euthanized at 6 h*	3	1	

CPK: Creatinine phosphokinase; *for histopathological analysis.

Consequently, inhibition of HMGB1 release presumably rescued animals suffering from septic vicious circle (32, 33). Therefore, suppression of HMGB1 in monkeys treated with AcPepA (Figure 4) could explain the extreme therapeutic effect of AcPepA on endotoxin shock in these animals (34). In other words, continuous generation of C5a by LPS or bacteria in monkeys receiving as well as possibly in patients with sepsis likely induce a cytokine 'storm' amplified by the release of HMGB1, resulting in a lethal effect on the host as a vicious circle of inflammation. The suppression of HMGB1 induction by inactivation of C5a could directly correlate with the survival observed following AcPepA treatment of monkeys injected with a lethal dose of LPS. Furthermore, AcPepA was shown to suppress pathophysiological events and prolonged survival time of piglets with sepsis induced by cecal ligation and perforation (CLP) (35, 36). Survival times were longer in the AcPepA-treated group than in the group treated with CLP alone (19.3 h±2.7 h *vs*. 9.9 h±0.7 h, $p<0.005$). In this case, AcPepA also delayed the HMGB-1 surge (36).

Therefore, suppression of C5 anaphylatoxin by AcPepA interferes with the induction of a cytokine 'storm'. Since C5a has the capacity to cause release of HMGB1 following stimulation of the second C5a receptor, termed C5L2, generated on activated monocytes (37-39), inhibition of C5a successfully interferes with the above release (Figure 7) (34).

Therefore, AcPepA would be beneficial for treatment of patients with sepsis and could be administered in large amounts at an acute stage, with little likelihood of an overdose, since the half-life of AcPepA in rats is 2.5 min. Administration of AcPepA to cancer patients at their terminal stage of their disease could improve their performance status.

Acknowledgements

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Targeting the hedgehog signaling pathway with interacting peptides to Patched-1

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Abstract
Background The hedgehog (Hh) signaling pathway is aberrantly activated in many cancers. Overproduction of sonic hedgehog (Shh), a ligand in the Hh pathway, increases Hh signaling activity by inhibiting Patched-1 (Ptch1), a suppressive receptor in the Hh pathway. The purpose of this study was to establish a novel strategy for treating pancreatic cancer and other Hh-dependent cancers through control of the tumor-suppressive function of Ptch1.
Methods We synthesized seven interacting peptides to the amino-acid sequence of the Ptch1 docking site for Shh. Human pancreatic cancer cell lines (AsPC-1, SUIT2) were cultured in the presence or absence of the peptides. Cell proliferation was assessed by cell counting and by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The activity of the Hh pathway was estimated by real-time polymerase chain reaction of the target gene product Gli1. To confirm their anti-tumor activity in vivo, the effect of the peptides in a mouse model of pancreatic cancer was determined. Finally, the Hh signaling activity of the xenograft was examined.
Results Three of the interacting peptides to Ptch1 suppressed the proliferation of the two pancreatic cancer cell lines and decreased the expression of Gli1, both in vitro and in vivo.
Conclusions This study suggests that interacting peptides to Ptch1 may be a new tool for controlling the Hh-dependent growth of pancreatic cancer.

Keywords Pancreatic cancer · Patched 1 protein · Peptides · Signal transduction

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Introduction

Pancreatic cancer is one of the most lethal of all malignancies. The therapeutic options for patients with unresectable, metastatic, or recurrent disease are extremely limited, and few of these patients survive for 5 years [1]. Recently, gemcitabine and S1 have become available for these patients; however, although the prognosis of pancreatic cancer patients has improved slightly, it still remains poor [2, 3]. Thus, the development of novel therapeutics for pancreatic cancer is urgently required.

Recently, several molecular targeting drugs have been developed and some are now clinically available. However, these drugs generally target growth factor receptors or components of the signaling pathway connecting growth factors and Ras, a small GTP-binding protein. KRAS encodes Ras, which mediates the signaling pathway between extracellular growth-factor stimulation and cell-cycle activation [4]. Unfortunately, more than 90% of pancreatic cancers carry mutations in the KRAS oncogene. This is the highest proportion of KRAS alterations found in any human tumor type [5]. Mutated KRAS gene products constitutively activate Ras protein, resulting in the

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constitutive activation of downstream pathways [5]. From the viewpoint of Ras mutation, the current development of molecular targeting drugs has still not been of major benefit to pancreatic cancer patients.

The hedgehog (Hh) signaling pathway has a crucial role in embryonic development, tissue regeneration, and carcinogenesis [6]. Recently, the Hh signaling pathway has been reported to be aberrantly activated in pancreatic cancer, in a ligand-dependent manner [6–12]. Without ligand stimulation, the transmembrane protein Patched-1 (Ptch1) suppresses another transmembrane protein Smoothened (Smo), which is the activator of the Hh signaling pathway. The Hh signaling pathway has three kind of ligands, Sonic Hh (Shh), Desert Hh (Dhh), and Indian Hh (Ihh). In pancreatic cancer cells, aberrantly overproduced Shh binds to Ptch1 and activates Smo, resulting in the translocation of Gli (glioma-associated oncogene) from the cytoplasm to the nucleus. Gli1, one of the three transactivators of the Hh signaling pathway, allows transcription of target genes of the Hh signaling pathway, including Gli1 itself. Thus, Gli1 is frequently used as a marker of activation of the Hh signaling pathway [13].

Suppression of the activated Hh signaling pathway is effective in controlling the development of many cancers in experimental studies [6–8, 12, 14, 15]. However, to date no drug has been developed to control the Hh signaling pathway and suppress cancer growth at the clinical level. We previously reported that anti-Ptch1 polyclonal antibodies raised against the docking site of Shh suppressed both Hh signaling activity and cancer cell growth [13]. However, polyclonal antibodies are far from being used clinically, and the generation of hybrid antibodies is costly.

We have developed an evolutionary computer program named MIMETIC that generates interacting peptide sequences, with the potential to interact with a target peptide. Thus, we have generated interacting peptides to HIV-reverse transcriptase [16, 17], procarboxypeptidase R, thrombomodulin [18], and C5a anaphylatoxin [19]. About 30% of the synthesized peptides interfered with the function of their target molecules [20]. In this study, we set out to generate synthetic interacting peptides to the short amino acid sequence of Ptch1, for which we had already raised antibodies [13], and we investigated the anti-cancer effect of these peptides in vitro and in vivo.

Materials and methods

Design and synthesis of interacting peptides to Ptch1

The target amino acid sequence of Ptch1 was selected according to our previous study [13]. Interacting peptides to the target sequences of Ptch1 were designed by the Institute for Protein Science (Nagoya, Japan), according to the method reported previously [16, 21]. Briefly, we used the MIMETIC program to design peptides that would interact with a target amino acid. This software employs a genetic algorithm that generates a series of increasingly optimized peptides to a target by random alteration of amino acids for 5,000 generations. Every peptide sequence generated in this manner is assigned a score based on several physico-chemical parameters, including hydropathic complementarity optimization, average structural similarity optimization, minimization of bulky side chain interference, and backbone alignment. Following the final generation, the program re-arranges the peptides into a list according to a scoring method for "goodness of fit" to the target. Eventually, seven peptides with high scores were selected, and the selected peptides were synthesized by Sigma-Aldrich Japan (Tokyo, Japan).

Cells and cell culture conditions

The human pancreatic cancer cell lines, AsPC1 and SUIT2, were maintained at 37°C under a humidified atmosphere of 5% CO_2 and 95% air in RPMI1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin; Meijiseika, Tokyo, Japan) [4, 6].

Proliferation assay

The pancreatic cancer cell lines, AsPC1 and SUIT2, were seeded in 24-well plates at a concentration of 1×10^4 cells per well for cell counting with a Coulter counter (Beckman Coulter, Fullerton, CA, USA), and in 96-well plates at a concentration of 3×10^3 cells per well for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, in complete culture medium. After overnight incubation, the medium was changed to fresh medium containing various concentrations of peptides for the MTT assay or 10 μg/mL of peptides for cell counting. Cells were incubated for 72 h (MTT assay), or for 48 and 72 h (cell counting). Then, cells were harvested by trypsin and viable cells were subjected to the MTT assay or cell counting using a Coulter counter [9].

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA, USA) and quantified by spectrophotometry (Ultrospec 2100 Pro; Amersham Pharmacia Biotech, Cambridge, UK). RNA (700 ng) was treated with DNase, and reverse transcribed to cDNA using the Quantitect Reverse Transcription Kit (Qiagen) according to the

manufacturer's protocol. Reactions were run with SYBR Premix Ex Taq (Takara Bio, Otsu, Japan) on a DNA Engine Opticon 2 System (MJ Research, Waltham, MA, USA). pGli1-GFP (green fluorescent protein) was serially diluted in 10-fold increments and amplified with the primer pairs to generate a standard curve for Gli1. Each sample was run in triplicate. All primer sets amplified fragments that were 200 base pairs long. The sequences of the primers used were as follows: beta-actin, forward, 50-TTG CCG ACA GGA TGC AGA AGG A-30, reverse, 50-AGG TGG ACA GCG AGG CCA GGA T-30; and Gli1, forward, 50-GGT TCA AGA GCC TGG GCT GTG T-30, reverse, 50-GGC AGC ATT CTC AGT GAT GCT G-30. The quantity of gene in a given sample was normalized to the level of β-actin in that sample [12].

Table 1 Sequences of interacting peptides to Patched-1 (Ptch1)

Peptide	Sequence	Length
A	GAPGRPAGGGRRRRTG GLRRAAAPDRDY	28 mer
B	DNTRYSPPPPYSSHS	15 mer
C	DTLSCQSPESTSSTRD	16 mer
D	PTPSPEPPPSVVR	13 mer
F	EGHSGPSNRARWGPRGARSH NPRNPASTAMGSSVPG	36 mer
G	FAPVLDGAVSTLLGV	15 mer
H	ESRHHPPSNPRQQPH	15 mer

Immunostaining of cultured cells

AsPC1 cells (2 × 104/well) were seeded onto a cover glass (Asahi Techno Glass, Chiba, Japan) in 24-well plates and incubated overnight. Then the old medium was changed to fresh medium containing 10 μg/mL of the indicated peptides. After incubation for 24 h, the slides were air-dried and immersed in 8% formaldehyde for 30 min. Primary antibodies were incubated overnight at 4°C. The primary antibody used was Shh (N-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at the concentration of 1:250. Secondary antibodies (rabbit anti-goat immunoglobulin; Nichirei, Tokyo, Japan) were applied for 1 h at room temperature. After mounting in Vectorshield Mounting Medium (Vector Laboratories, Burlingame, California, USA), samples were visualized under a laser-scanning confocal fluorescence microscope (LSM-GB200 System; Olympus Optical, Tokyo, Japan).

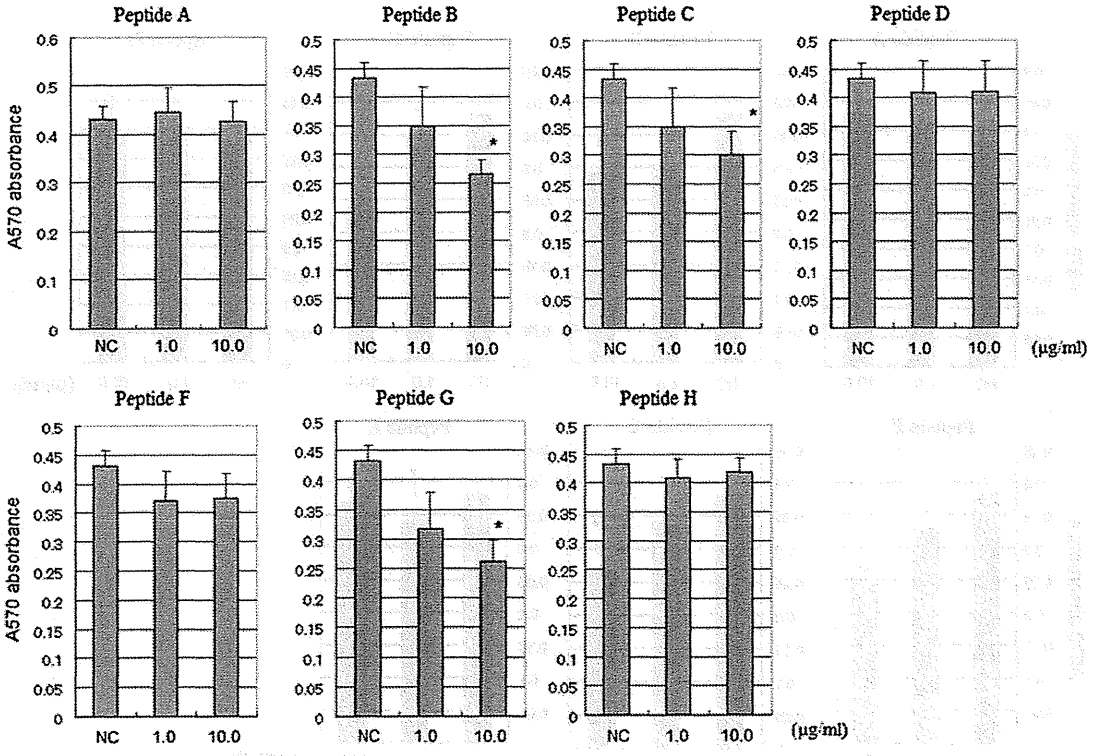

Fig. 1 Biological screening of interacting peptides using pancreatic cancer cell line AsPC1. AsPC1 cells were incubated with two concentrations of peptides, 1.0 or 10.0 μg/mL, and subjected to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Peptides B, C, and G showed a suppressive effect on cell growth in a dose-dependent manner. Results are expressed as means ± standard deviation (SD). *NC* normal control (buffer alone). $^*P < 0.05$

Animal model and peritoneal transplantation of AsPC1

The procedures followed for the animal experiments were in accordance with the guidelines for the care and use of laboratory animals of our institution or the guidelines of the National Animal Welfare Committee. Four- to six-week-old female NOD/severe combined immunodeficiency (SCID) mice were purchased from Japan SLC (Hamamatsu, Japan). The mice were housed in laminar flow cabinets under specific pathogen-free conditions in facilities approved by Kyushu University. AsPC1 or SUIT2 cells were suspended in a serum-free-RPMI/Matrigel (BD Biosciences Japan, Tokyo, Japan) mixture (1:1 volume, total 1×10^5 cells/0.4 mL) and then injected into the gluteal region of the mice with a 27-gauge needle. After tumor formation, 200 μg of peptide was injected into the tumor site with a 27-gauge needle once a day for 5 days. Tumor formation, the size of the tumors, and body weight of the mice were determined every 2 days for 46 days [22].

Statistical analysis

Student's *t*-test was used for statistical analysis. All calculations were carried out using StatView 5.0J software (Abacus Concepts, Berkeley, CA, USA). *P* values of <0.05 were considered significant [12, 23].

Results

Sequences of interacting peptides to Ptch1

Using the MIMETIC program, seven peptide sequences were designed as interacting peptides to Ptch1 (peptides A–H; Table 1).

Interacting peptides to Ptch1 suppressed pancreatic cancer cell proliferation in vitro

Peptides B, C, and G showed suppressive effects on cell growth in a dose-dependent manner (Fig. 1). We repeated the biological screening using SUIT2. Peptides B, C, F, and G suppressed SUIT2 cell growth in a dose-dependent manner (Fig. 2). This result was similar to that for AsPC1, with the exception of peptide F.

To further confirm the biological effect of the peptides as detected by the MTT assay, pancreatic cancer cells were incubated with the peptides, and subjected to Coulter counting. Consistent with the result of the MTT assay, peptides B, C, and G showed a suppressive effect on the proliferation of AsPC1 and SUIT2, and peptide F had an effect only on SUIT2 (Fig. 3a, b).

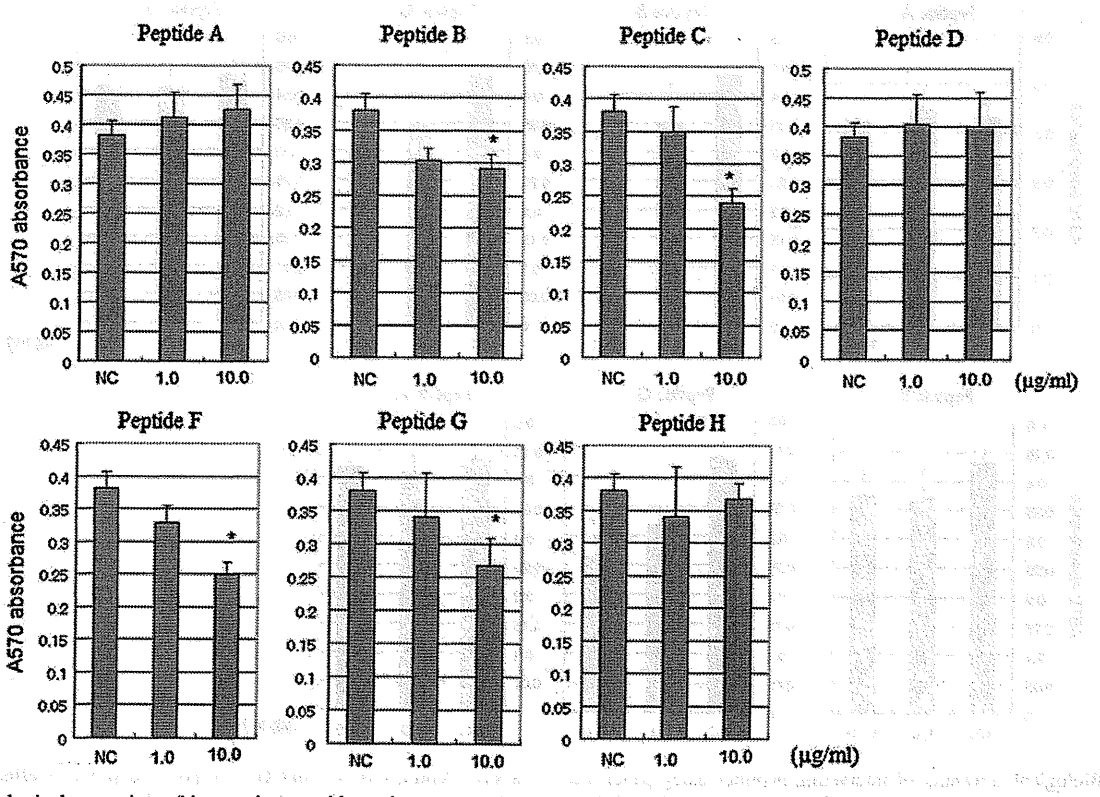

Fig. 2 Biological screening of interacting peptides using pancreatic cancer cell line SUIT2. SUIT2 cells were incubated with two concentrations of peptides, and subjected to the MTT assay. Peptides B, C, F, and G suppressed SUIT2 cell growth in a dose-dependent manner. Results are expressed as means ± SD, $^*P < 0.05$

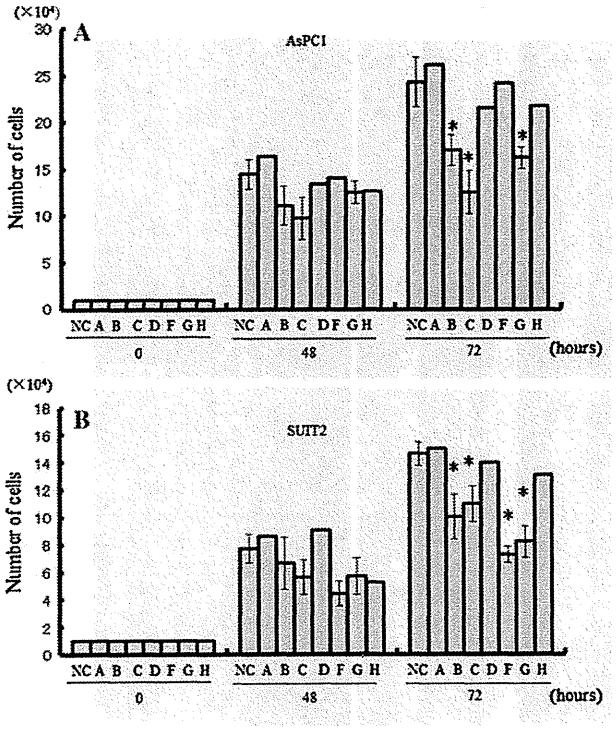

Fig. 3 Interacting peptides suppressed the proliferation of pancreatic cancer cells. Pancreatic cancer cells, AsPC1 (**a**) and SUIT2 (**b**), were incubated with the interacting peptides, and then subjected to Coulter counting. Consistent with the result of the MTT assay, peptides B, C, and G showed a suppressive effect on the proliferation of AsPC1 and SUIT2, and peptide F had an effect on SUIT2 only. Results are expressed as means ± SD. $^*P < 0.05$

Interacting peptides to Ptch1 suppressed Hh signaling pathway

To confirm that the biological effect of the peptides on cancer cell growth was caused by attenuation of the activity of the Hh signaling pathway, we examined the effect of the synthesized peptides on this pathway. Because Gli1 is not only the transcription factor of the Hh signaling pathway but also a target gene of the Hh pathway, we used the Gli1 mRNA expression level as a marker of Hh pathway activity. As peptides B, C, and G had been confirmed to suppress the growth of AsPC1, we co-incubated AsPC1 with each of these peptides. Peptide A was also co-incubated with AsPC1 independently as a negative control. After a 72-h incubation,, mRNA was prepared from the cultured cells and subjected to quantitative analysis by real-time RT-PCR. As expected, the mRNA level of Gli1 was significantly suppressed by all three peptides B, C, and G, in contrast with the mRNA level of cells co-incubated with peptide A or without peptides (Fig. 4a).

As four peptides, B, C, F, and G, showed a suppressive effect on the proliferation of SUIT2, we examined the effect of these four peptides on SUIT2, and also used peptide A as a negative control. Real-time RT-PCR showed that the mRNA level of cells cultured with all four peptides, B, C, F, and G, was significantly suppressed compared with that in cells cultured with peptide A or without peptides (Fig. 4b). Our data strongly suggested that the suppressive effect of the interacting peptides to Ptch1 on tumor cell growth was induced by attenuating the activity of the Hh signaling pathway.

Interacting peptides to Ptch1 inhibited the interaction between Ptch1 and Shh

The interacting peptides to Ptch1 were too small to be stained by antibodies, so we confirmed the specificity of peptides to Ptch1 protein by the effect that peptides competitively inhibited the interaction between Ptch1 and Shh. AsPC1 cells were incubated with peptide B, C, or G. Then the staining levels of Shh around the AsPC1 cells were examined (Fig. 5). Staining levels of AsPC1 cells with peptides B, C, and G were lower than that of cells with peptide A, the control peptide. This finding supported the idea that peptides B, C, and G specifically interacted with Ptch1 at the designated docking site with Shh.

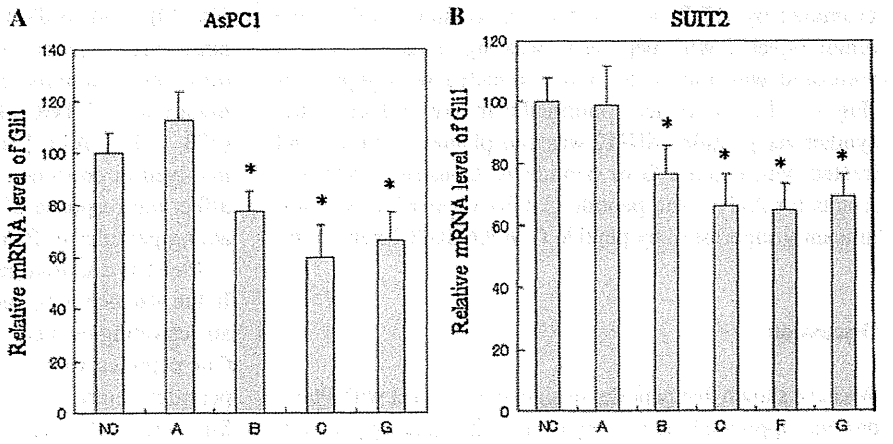

Fig. 4 Interacting peptides suppressed the activity of the hedgehog (Hh) signaling pathway. AsPC1 (**a**) and SUIT2 (**b**) cells were incubated for 72 h with the interacting peptides. The mRNA level of Gli1 was significantly suppressed by peptides B, C, and G in AsPC1 and SUIT2, and by peptide F in SUIT2. Results are expressed as means ± SD. $^*P < 0.05$

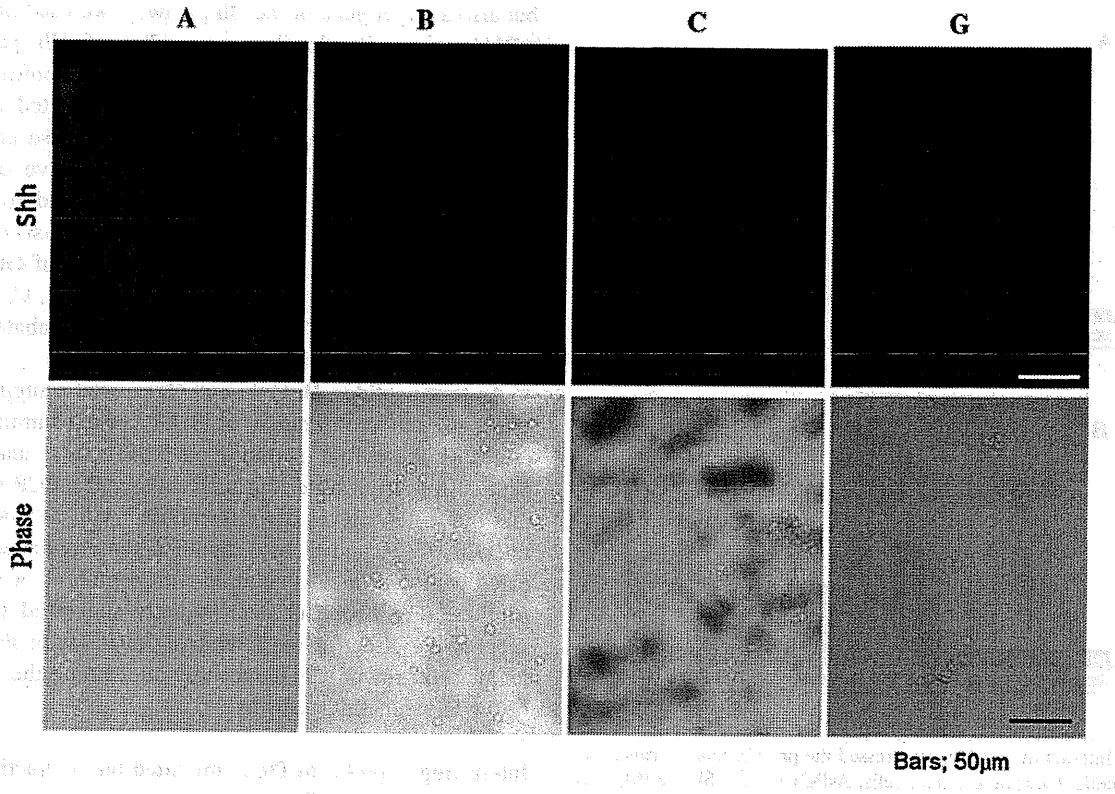

Fig. 5 Interacting peptides to Patched-1 (Ptch1) inhibited the interaction between Ptch1 and sonic hedgehog (*Shh*). AsPC1 cells were incubated with the peptides, A, B, C, and G as indicated. Then the cells were stained with Shh (*upper panels*). *Lower panels* show phase-contrast images (*Phase*) of peptides A, B, C, and G (*from left to right*). *Bars* 50 μm

Interacting peptides to Ptch1 suppressed pancreatic tumor growth in vivo

We confirmed the anti-cancer effect of peptides in vivo using AsPC1 cell xenografts. When the transplanted tumors had grown sufficiently to be palpable, peptide A or C was injected into the mice. The tumor size was measured every 2 days and the tumor volume was deduced from the measured data. The tumor volume was suppressed by peptide C in comparison to peptide A (Fig. 6a, b). The tumors were finally harvested and the Gli1 mRNA expression level was examined by RT-PCR. The Gli1 expression level of the tumor injected with peptide C was significantly reduced compared with that in the tumor injected with peptide A (Fig. 6c, d). To further confirm the in vivo effect of the synthesized peptides, SUIT2 was transplanted into mice and treated with peptide G or peptide A. Consistent with the results for AsPC1 and peptide C, Gli1 expression was significantly suppressed by peptide G in the SUIT2 xenograft.

Discussion

We have shown that peptides designed to interact with Ptch1 protein suppressed the activity of the Hh signaling pathway in pancreatic cancer cells, and suppressed the proliferation of pancreatic cancer cells. The anti-cancer effect of the peptides was further confirmed in vivo in a xenograft of the pancreatic cancer cell lines, AsPC1 and SUIT2. Finally, the mRNA expression of Gli1 in the xenograft was found to be reduced by the complementary peptides.

Three interacting peptides, B, C, and G, showed the same effect on both AsPC1 and SUIT2. However, peptide F showed a suppressive effect only on SUIT2, and the effect was reproducible. Recently, signal cross-talk between the Hh signaling pathway and Ras or p53 has been reported [24–30]. Both AsPC1 and SUIT2 have the same mutated amino acid sequence in Ras G12D [31]. However, they have different mutations in p53, as AsPC1 has a frame-shift mutation (135 TGC-GC), and SUIT2 has a point mutation (273 CGT-CAT) [31]. The different status of signals involved in cross-talk with the Hh signaling pathway may affect the response of pancreatic cancer cells to the interacting peptides to Ptch1.

Ptch1 is a unique receptor of the Hh signaling pathway. In the absence of ligand stimulation, Ptch1 suppresses Smo, an activating element of the Hh signaling pathway [32–36]. Our peptides were designed to interact with Ptch1. If the peptides interact with Ptch1 to suppress Ptch1 function, Smo and the Hh signaling pathway may be suppressed. We

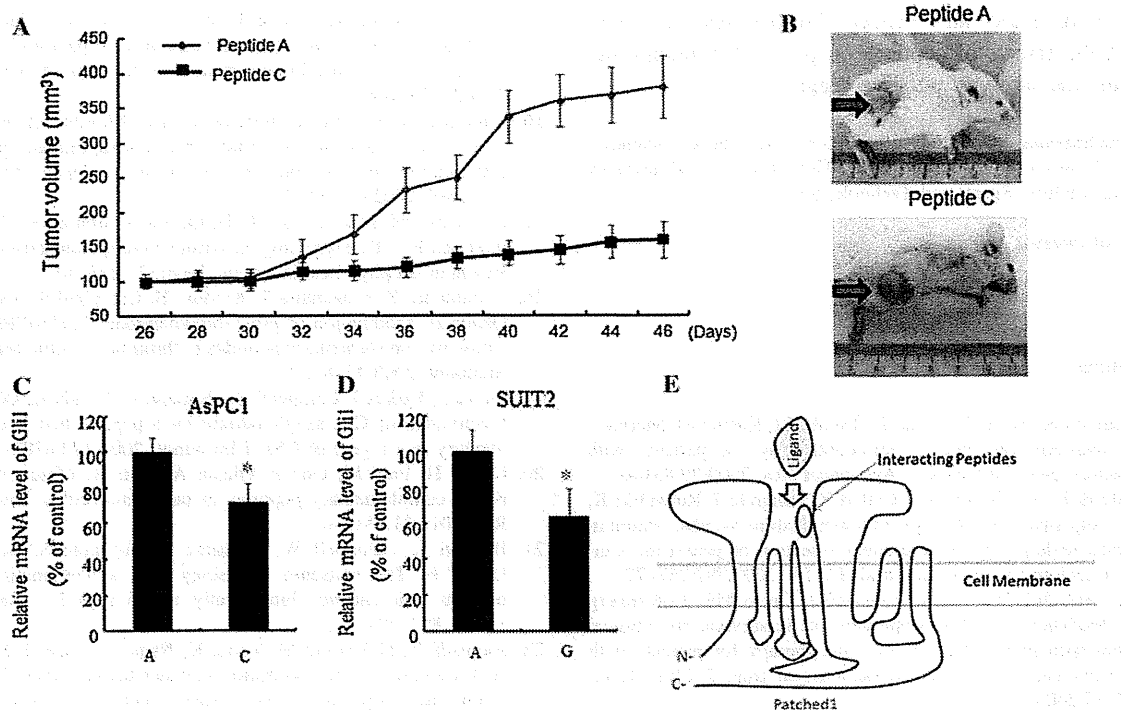

Fig. 6 Interacting peptides suppressed pancreatic tumor growth in vivo. AsPC1 tumor volume was suppressed by peptide C in comparison to peptide A (**a**). *Arrows* indicate implanted tumors (**b**). Gli1 expression in the tumor injected with peptide C was significantly decreased compared with that in the tumor injected with peptide A (**c**). In SUIT2 tumors, peptide G similarly reduced Gli1 expression (**d**). Results are expressed as means ± SD. $^*P < 0.05$. **e** Schema of the interacting peptide and Ptch1. The site of the interacting peptide corresponds to the target amino acid sequence of Ptch1. The schema indicates the putative mechanism, i. e., that the peptide interferes with the interaction between Ptch1 and the ligand of the Hh signaling pathway. *N* amino terminus, *C* carboxy terminus

aimed to suppress the activity of the Hh signaling pathway to control the proliferation of the pancreatic cancer cells. Thus, the target of the peptides was the short amino acid sequence of Ptch1 (KADYPNIQH) which was located in the putative docking site of the Hh ligand and Ptch1 (Fig. 6e) [37]. Furthermore, this Ptch1 sequence was previously selected for generating antibodies. As the site of the sequence was determined to be hydrophilic, the protein structure suggests it may not be folded inwards [37], and may enable peptides to access Ptch1 easily.

Controlling the Hh signaling pathway may contribute not only to controlling the proliferation of cancer cells, but also to controlling other significant events essential for carcinogenesis. For example, inhibition of the Hh signaling pathway has been shown to reduce the expression of the transcription factor snail, allowing the upregulation of E-cadherin, resulting in the inhibition of epithelial-to-mesenchymal transition and the reduction of in vitro invasive capacity [32]. Nagai et al. reported that blockade of the Hh pathway by cyclopamine inhibited pancreatic cancer cell invasion in association with the decreased expression of matrix metalloproteinase-9 [15]. These findings indicate that suppression of the Hh signaling pathway may be a putative therapeutic method to suppress both tumor growth and tumor invasion.

The Hh signaling pathway was first reported to be reactivated in an autocrine signaling manner, although it originally functioned as a paracrine signal. Recently, several studies have added new knowledge of the paracrine networks of the Hh signaling pathway in cancer tissue. Olsen et al. reported that Hh-interacting protein was highly expressed in endothelial cells but was downregulated during angiogenesis and in several human tumors [38]. Guimaraes et al. [39] reported that Shh increased the mRNA levels of vascular endothelial growth factor, stromal cell-derived factor-1, and angiopoietin-1. Nakamura et al. [40] reported that pancreatic cancer cell-derived Shh induced angiotensin-1 and insulin-like growth factor-1 production in bone marrow-derived pro-angiogenic cells, resulting in their enhanced migration and capillary morphogenetic activity.

It is possible that the Hh signaling pathway is reactivated in pancreatic cancer and controls several kinds of cells in cancer tissue for cancer development, just as it is originally activated and controls several kinds of cells in fetal tissue [41]. The studies cited here highlight the significance of controlling the Hh signaling pathway in the

treatment of pancreatic cancer. Further research may improve the effects of peptides on pancreatic tumors and determine the safety of putative drugs.

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Conflict of interest None.

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