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<th><strong>Title</strong></th>
<th>Tumor Suppressor Protein And Nucleotide Encoding Same</th>
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TUMOR SUPPRESSOR PROTEIN AND NUCLEOTIDE ENCODING SAME

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Field of Classification Search 514/2; 424/184.1

References Cited
U.S. PATENT DOCUMENTS

OTHER PUBLICATIONS
Kaiser (Science, 2006, 313, 1370).*
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ABSTRACT
The invention provides a method for suppressing tumor cell growth in a patient, comprising: administering to the patient an effective amount of an expression vector including a poly-nucleotide encoding a tumor suppressor protein having SEQ ID NO: 1 under conditions wherein the expression vector incorporates itself into the tumor cell genome and inhibits cell proliferation or induces cell death.

The invention further provides a method for inhibiting tumor cell proliferation in a tumor cell population comprising: administering to the tumor cell population an amount of a composition comprising a tumor suppressor protein having SEQ ID NO: 1 effective to inhibit tumor cell proliferation therein.

4 Claims, 11 Drawing Sheets
Fig. 1A

Fig. 1B
Fig. 1C

Fig. 1D
Fig. 1E

Fig. 1F
Fig. 2D

MCF-7

Fig. 2E

Hep-G2
Fig. 4
TUMOR SUPPRESSOR PROTEIN AND NUCLEOTIDE ENCODING SAME

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Patent Application No. 60/814,315, filed Jun. 16, 2006, the entire contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to a tumor suppressor protein useful as an anti-cancer agent, to nucleotides that encode for such tumor suppressor protein, and the expression vectors including the foregoing nucleotide.

BACKGROUND OF THE INVENTION

In 2002, it was estimated that there were 10.9 million new cases of cancer and 6.7 million cancer-related deaths worldwide. Globally, prostate cancer has become the third most common cancer in men, with half a million new cases each year, amounting to about 10% of all male cancers. It is the most common cancer after skin cancer, and is the second leading cause of cancer-related death in men in the United States. Breast cancer is the most frequent cancer of women, accounting for 23% of all cancers. Liver cancer is the fifth most common cancer in the world, and most of the liver cancer cases occur in developing countries, especially the Far East and Southeast Asia. The prevalence of liver cancer in developing countries and the emergence of prostate cancer and breast cancer as public health problems in developed countries is due to a number of factors and will put tremendous pressure on the healthcare systems to provide new and effective treatments.

Current understanding of cancer cell biology has allowed scientists to develop a rational approach to combat cancer by using a combination of anti-cancer drugs, which would inactivate and/or activate, respectively, multiple targets in cell growth-promoting and growth-inhibitory signaling pathways. Clearly, there is an unmet clinical need to develop novel therapeutic agents which can act effectively alone and/or in combination with conventional radiation or chemotherapy to halt or reverse the progression of advanced cancer. Such demand has fueled the search for novel endo-/paracrine growth-promoting and growth-inhibitory signaling pathways important in cancer pathogenesis, which may yield new therapeutic agents or targets for anti-cancer drug discovery and development.

PDZ domain-containing protein 2 (PDP2) (also named KIAA0300, PIN-1, PAPIN, activated in prostate cancer (AIPC), and PDZ domain-containing protein 3 (PDZK3)), is a six-PDZ (for PSD95, Discs-large, and ZO-1) domain protein which is expressed in multiple tissues. Though proteins containing PDZ domains have been shown to bind specific C-terminal protein sequences of transmembrane receptors or ion channels, and are believed to be involved in mediating intracellular protein-protein interactions, protein scaffolding and intracellular signaling, the functions of PDZD2 in humans are as yet little understood.

SUMMARY OF THE INVENTION

The present invention relates to sPDPZD2, a cleavage product of PDZD2, and a method for inhibiting or retarding cancer growth comprising administering an effective cancer growth inhibiting or retarding amount of this compound to a patient.

Accordingly, the present invention provides a tumor suppressor protein, a polynucleotide encoding the protein, an expression vector containing the polynucleotide and a cell transformed with the expression vector. The present invention also provides a method for suppressing proliferation of cancer cells. The present invention further provides a pharmaceutical composition for preventing or treating cancer comprising the polynucleotide in a pharmaceutically acceptable carrier. In accordance with one aspect of the present invention, the invention provides a tumor suppressor protein isolated from humans which has the amino acid sequence of SEQ ID NO: 1.

The present invention also provides growth-inhibitory functions for human sPDPZD2 protein on prostate, breast and liver cancers. The present invention further provides for a method of inhibiting the growth of a prostate cancer cell line in a mammal comprising administering to the mammal of recombinant human sPDPZD2 to inhibit the cancer growth.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows the expression and secretion of sPDZD2/sPDZD2 in prostate cancer cells. The presence of PDZD2 and sPDZD2 in cell lysates and concentrated conditioned media of (Lane 1) LNCaP, (Lane 2) DU145, (Lane 3) PC-3, and (Lane 4) 22Rv1 cells were detected by immunoblotting, using rabbit anti-PDZD2 antiserum (1:10,000 dilution).

FIG. 1B shows the expression and secretion of PDZD2/sPDZD2 in breast and liver cancer cells. The presence of PDZD2 and sPDZD2 in cell lysates and concentrated conditioned media of (Lane 1) Hep-G2 and (Lane 2) MCF-7 cells were detected by immunoblotting, using rabbit anti-PDZD2 antiserum (1:10,000 dilution).

FIG. 1C shows the effects of caspase-3 inhibitor Z-DEVD-FMK on PDZD2/sPDZD2 expression and secretion. DU145 cells were treated with (Lane 1) 10 μM Z-FA-FMK (an inactive analog of Z-DEVD-FMK), or (Lane 2) 10 μM Z-DEVD-FMK for 48 hours. Cell lysates were harvested and the conditioned media were concentrated before detection of PDZD2 and sPDZD2 using rabbit anti-PDZD2 antiserum (1:10,000 dilution).

FIG. 1D shows the effects of caspase-3 inhibitor Z-DEVD-FMK on PDZD2/sPDZD2 expression and secretion. PC-3 cells were treated with (Lane 1) 10 μM Z-FA-FMK (an inactive analog of Z-DEVD-FMK), or (Lane 2) 10 μM Z-DEVD-FMK for 48 hours. Cell lysates were harvested and the conditioned media were concentrated before detection of PDZD2 and sPDZD2 using rabbit anti-PDZD2 antiserum (1:10,000 dilution).

FIG. 1E shows the effects of caspase-3 inhibitor Z-DEVD-FMK on PDZD2/sPDZD2 expression and secretion. 22Rv1 cells were treated with (Lane 1) 10 μM Z-FA-FMK, (an inactive analog of Z-DEVD-FMK), or (Lane 2) 10 μM Z-DEVD-FMK for 48 hours. Cell lysates were harvested and the conditioned media were concentrated before detection of PDZD2 and sPDZD2 using rabbit anti-PDZD2 antiserum (1:10,000 dilution).

FIG. 1F shows the effects of caspase-3 inhibitor Z-DEVD-FMK on PDZD2/sPDZD2 expression and secretion. MCF-7 and Hep-G2 cells were treated with (Lanes 1 and 3) 10 μM Z-FA-FMK, (an inactive analog of Z-DEVD-FMK), or (Lanes 2 and 4) 10 μM Z-DEVD-FMK for 48 hours. Cell lysates were harvested and the conditioned media were concentrated before detection of PDZD2 and sPDZD2 using rabbit anti-PDZD2 antiserum (1:10,000 dilution).
FIG. 1C shows the effects of caspase-3 inhibitor Z-DEVD-FMK on cell growth. DU145 cells were treated with 10 μM Z-DEVD-FMK, 10 μM Z-FA-FMK, or vehicle for 24 and 48 hours. Cell proliferation was monitored by MTS assays. Data are shown as mean ± S.E. The data were analyzed with one way ANOVA followed by Tukey’s test. Two group comparisons were analyzed by unpaired Student’s t test. The level of significance for all statistical analyses was set at P<0.05. ***P<0.01 and ****P<0.001 compared to control.

FIG. 11 shows the effects of caspase-3 inhibitor Z-DEVD-FMK on cell growth. PC-3 cells were treated with 10 μM Z-DEVD-FMK, 10 μM Z-FA-FMK, or vehicle for 24 and 48 hours. Cell proliferation was monitored by MTS assays. Data are shown as mean ± S.E. The data were analyzed with one way ANOVA followed by Tukey’s test. Two group comparisons were analyzed by unpaired Student’s t test. The level of significance for all statistical analyses was set at P<0.05. ***P<0.001 compared to control.

FIG. 12 shows the effects of caspase-3 inhibitor Z-DEVD-FMK on cell growth. 22Rv1 cells were treated with 10 μM Z-DEVD-FMK, 10 μM Z-FA-FMK, or vehicle for 24 and 48 hours. Cell proliferation was monitored by MTS assays. Data are shown as mean ± S.E. The data were analyzed with one way ANOVA followed by Tukey’s test. Two group comparisons were analyzed by unpaired Student’s t test. The level of significance for all statistical analyses was set at P<0.05. ***P<0.001 and ****P<0.001 compared to control.

FIG. 1J shows the effects of caspase-3 inhibitor Z-DEVD-FMK on cell growth. MCF-7 cells were treated with 10 μM Z-DEVD-FMK, 10 μM Z-FA-FMK, or vehicle for 24 and 48 hours. Cell proliferation was monitored by MTS assays. Data are shown as mean ± S.E. The data were analyzed with one way ANOVA followed by Tukey’s test. Two group comparisons were analyzed by unpaired Student’s t test. The level of significance for all statistical analyses was set at P<0.05. ***P<0.001 compared to control.

FIG. 1K shows the effects of caspase-3 inhibitor Z-DEVD-FMK on cell growth. Hep-G2 cells were treated with 10 μM Z-DEVD-FMK, 10 μM Z-FA-FMK, or vehicle for 24 and 48 hours. Cell proliferation was monitored by MTS assays. Data are shown as mean ± S.E. The data were analyzed with one way ANOVA followed by Tukey’s test. Two group comparisons were analyzed by unpaired Student’s t test. The level of significance for all statistical analyses was set at P<0.05. ***P<0.001 and ****P<0.001 compared to control.

FIG. 1L shows the effects of caspase-3 inhibitor Z-DEVD-FMK with or without exogenous sPZD2 on cell growth. DU145, PC-3, and 22Rv1 cells were incubated with 10 μM Z-DEVD-FMK, 10 μM Z-FA-FMK, 10 μM Z-DEVD-FMK plus 10-8 M sPZD2, or vehicle for 48 hours. Cell proliferation was monitored by MTS assays. Data are shown as mean ± S.E. Two group comparisons were analyzed by unpaired Student’s t test. The level of significance for all statistical analyses was set at P<0.05. ***P<0.001 compared to control.

FIG. 1M shows the effects of caspase-3 inhibitor Z-DEVD-FMK with or without exogenous sPZD2 on cell growth. MCF-7 and Hep-G2 cells were incubated with 10 μM Z-DEVD-FMK, 10 μM Z-FA-FMK, 10 μM Z-DEVD-FMK plus 10-8 M sPZD2, or vehicle for 48 hours. Cell proliferation was monitored by MTS assays. Data are shown as mean ± S.E. Two group comparisons were analyzed by unpaired Student’s t test. The level of significance for all statistical analyses was set at P<0.05. ***P<0.001 compared to control.

FIG. 2A shows the effects of sPZD2 on prostate cancer cell proliferation. DU145 cells were treated with 10-9 M, 10-8 M, and 10-7 M recombinant sPZD2 for 24 and 48 hours. The effects of sPZD2 on cell proliferation were monitored by MTS assays. Data are shown as mean ± S.E. The data were analyzed with one way ANOVA followed by Tukey’s test. Two group comparisons were analyzed by unpaired Student’s t test. The level of significance for all statistical analyses was set at P<0.05. ***P<0.001 compared to control.

FIG. 2B shows the effects of sPZD2 on prostate cancer cell proliferation. PC-3 cells were treated with 10-9 M, 10-8 M, and 10-7 M recombinant sPZD2 for 24 and 48 hours. The effects of sPZD2 on cell proliferation were monitored by MTS assays. Data are shown as mean ± S.E. The data were analyzed with one way ANOVA followed by Tukey’s test. Two group comparisons were analyzed by unpaired Student’s t test. The level of significance for all statistical analyses was set at P<0.05. ***P<0.001 and ****P<0.001 compared to control.

FIG. 2C shows the effects of sPZD2 on prostate cancer cell proliferation. 22Rv1 cells were treated with 10-9 M, 10-8 M, and 10-7 M recombinant sPZD2 for 24 and 48 hours. The effects of sPZD2 on cell proliferation were monitored by MTS assays. Data are shown as mean ± S.E. The data were analyzed with one way ANOVA followed by Tukey’s test. Two group comparisons were analyzed by unpaired Student’s t test. The level of significance for all statistical analyses was set at P<0.05. ***P<0.001 compared to control.

FIG. 2D shows the effects of sPZD2 on breast cancer cell proliferation. MCF-7 cells were treated with 10-9 M, 10-8 M, and 10-7 M recombinant sPZD2 for 24 and 48 hours. The effects of sPZD2 on cell proliferation were monitored by MTS assays. Data are shown as mean ± S.E. The data were analyzed with one way ANOVA followed by Tukey’s test. Two group comparisons were analyzed by unpaired Student’s t test. The level of significance for all statistical analyses was set at P<0.05. ***P<0.001 compared to control.

FIG. 2E shows the effects of sPZD2 on liver cancer cell proliferation. Hep-G2 cells were treated with 10-9 M, 10-8 M, and 10-7 M recombinant sPZD2 for 24 and 48 hours. The effects of sPZD2 on cell proliferation were monitored by MTS assays. Data are shown as mean ± S.E. The data were analyzed with one way ANOVA followed by Tukey’s test. Two group comparisons were analyzed by unpaired Student’s t test. The level of significance for all statistical analyses was set at P<0.05. ***P<0.001 and ****P<0.001 compared to control.

FIG. 3A shows the effects of sPZD2 on LNCaP cell growth. LNCaP cells were treated with 10-9 M, 10-8 M, and 10-7 M recombinant sPZD2 for 24 and 48 hours. The effects of sPZD2 on cell viability were monitored by trypan blue dye exclusion assays. Data are shown as mean ± S.E. Two group comparisons were analyzed by unpaired Student’s t test. The level of significance for all statistical analyses was set at P<0.05. ***P<0.001 compared to control.

FIG. 3B shows the effects of sPZD2 on LNCaP cell growth. Cell Death Detection ELISA assay Kit (Roche) was used to determine the apoptotic effects of sPZD2 on LNCaP cells. After treatment of the cells with 10-9 M and 10-7 M recombinant sPZD2 for 24 hours, the cells were lysed and the presence of nuclei acid in the cytoplasm was detected by measuring absorbance values at 405 nm. Data are shown as mean ± S.E. Two group comparisons were analyzed by unpaired Student’s t test. The level of significance for all statistical analyses was set at P<0.05. ***P<0.001 compared to control.

FIG. 4 shows the volume changes of DU145 prostate cancer xenograft tumors in nude mice given daily saline, low dose (0.084 mg recombinant sPZD2 in 0.1 ml saline/dose/
The present invention identifies the growth-inhibitory and retarding functions for human sPDZD2 protein on cancers, particularly prostate, breast and liver cancers. The present invention also provides for a method of inhibiting the growth of a prostate cancer cell line in a mammal comprising administering to the mammal of recombinant human sPDZD2 to inhibit the cancer growth.

EXAMPLE 1

Amino Acid Sequences of Human PDZD2 and sPDZD2 Protein

The primary and secondary accession numbers for the protein sequence of human PDZD2 protein as deposited in the Swiss-Prot database are, respectively, O15018 and Q9BDX4.

The tumor suppressor protein of the present invention, i.e., human sPDZD2 protein has the amino acid sequence of SEQ ID NO: 1 (one letter amino acid code) which is listed below:

SEQ ID NO: 1

LDRLCQEDYSAGQPSAVLFPETELEITFRSPPCPVQVCSPEEGCRACFG
GSQFYTSAKTPSSASDGTGAQGDLFFRSVHLDQLLVSAGDOQRMLS

Various substitution, addition and/or deletion of the amino acid residues of the protein may be done without adversely affecting the protein's function. Further, a portion of the protein may be used when a specific purpose is to be fulfilled. The term “the tumor suppressor protein of the present invention” used herein includes these modified amino acids and fragments thereof. Therefore, the present invention includes, in its scope, a polypeptide having substantially the same amino acid sequence as the sPDZD2 protein having the amino acid sequence of SEQ ID NO: 1 and a fragment thereof. As used herein, “substantially the same polypeptide” refers to a polypeptide whose amino acid sequence shows preferably 80% or more, more preferably 90% or more, or most preferably 95% or more homology to the amino acid sequence of SEQ ID NO: 1.

The sPDZD2 protein of the present invention may be encoded by a polynucleotide comprising a nucleotide sequence deduced from the amino acid sequence of the sPDZD2 protein according to the genetic code (hereinafter called “sPDZD2 gene”). It is known that several different codons encoding the same amino acid may exist due to the codon degeneracy, and, therefore, the sPDZD2 gene of the present invention may include various nucleotide sequences deduced from the amino acid sequence of the sPDZD2 protein. A preferred sPDZD2 gene has the nucleotide sequence of SEQ ID NO: 2.
The spPDZD2 gene, or the protein, of the present invention can be obtained from human tissue or synthesized using a conventional DNA or peptide synthesis method. Further, the gene thus prepared may be inserted into a conventional vector to obtain an expression vector, which may, in turn, be introduced into a suitable host, e.g., a microorganism such as an E. coli or yeast, or an animal cell such as a mouse or human cell.

The transformed host may then be used in producing the inventive DNA or protein on a large scale.

The present invention provides a method for suppressing growth of a cancer cell comprising introducing an expression vector containing the inventive spPDZD2 gene into a cancer cell to induce antiproliferation and/or apoptosis thereof. Any type of cancer cell may be used in the inventive method. Preferred are cervical, prostate, pancreatic, kidney, sarcoma, leukemia, lymphoma, liver, uterine, colorectal, lung, brain, ovary, and breast cancer cells, and more preferred is a prostate cancer cell.

The present invention also includes within its scope a pharmaceutical composition for treating or preventing cancer which comprises the inventive tumor suppressor gene as an active ingredient and pharmaceutically acceptable carriers, excipients or other additives, if necessary. The pharmaceutical composition of the present invention is preferably formulated for administration by injection with a diluent (such as normal saline, distilled water, or other aqueous vehicle with or without preservatives or other inactive ingredients) for example.

The pharmaceutical composition of the present invention is administered into a cancerous tissue of a subject in a conventional manner to slow the growth of the tissue. The amount of the tumor suppressor gene actually administered should be determined in light of various relevant factors including the condition to be treated, the chosen route of administration, the age and weight of the individual patient, and the severity of the patient's symptoms.

The invention further provides a tumor suppressor protein, a pharmaceutical composition comprising the tumor suppressor protein, together with a pharmaceutically acceptable vehicle and a method for suppressing or inhibiting tumor cell growth comprising administering an effective amount of the tumor suppressor protein in a pharmaceutically effective vehicle.

The following Examples are intended to further illustrate the present invention without limiting its scope.

**EXAMPLE 2**

**Endogenous PDZD2 Expression and spPDZD2 Secretion in Prostate, Breast and Liver Cancer Cells**

Using a specific anti-PDZD2 antiserum that recognize both the full-length and secreted forms of PDZD2, we analyzed the lysates and conditioned media from cultured DU145, PC-3, 22Rv1, LNCaP, MCT-7 and Hep-G2 cells by immunoblotting. PBS-washed native prostate, breast and liver cancer cells were incubated in their respective culture media without any added FBS for 24 hours, before the cells and conditioned media were collected for immunoblotting with the rabbit anti-PDZD2 antibody. Full-length PDZD2 (301 kDa) and spPDZD2 (37 kDa) were detected, respectively, in the cell lysates and conditioned media of all cancer cell lines. The lack of detectable expression of spPDZD2 in the cell lysates suggests that spPDZD2 is predominantly secreted rather than stored intracellularly after its cleavage from PDZD2 (FIGS. 1A and 1B).

**EXAMPLE 3**

**Construction, Expression and Purification of Recombinant Human spPDZD2 Protein**

Human spPDZD2 cDNA was amplified by PCR with a sense primer: 5'-GTT-GTT-CCG-CTT-GAC-AAG-CTC-TGG-AAC-GGC-GAT-3' (SEQ ID NO.3) and an anti-sense primer: 5'-GTT-GTT-CTC-GAG-CTA-TCA-TAG-AGA-ATG-CCT-3' (SEQ ID NO.4). The 5' ends of the sense and anti-sense amplimers were tagged with the restriction enzyme sequences of Ndel and Xhol respectively to facilitate subsequent subcloning of amplified human spPDZD2 cDNA into the expression vector pTYBI2. PCR was performed using the Advantage® 2 PCR Kit (Clontech), in a 50 µl reaction mixture contained 5 µl Advantage® 2 PCR buffer [40 mM Tris-HCl (pH 8.7), 15 mM KCl, 3.5 mM Mg(OAc)₂, 3.75 µg/ml BSA, 0.005% [w/v] 20, 0.005% NP-40], 5 µl dNTPs (10 mM each), 0.5 µl of each primer (100 pmole/µl), 1 ng KIAA0300 cDNA, and 1 µl Advantage® 2 polymerase mix. The 25 cycles of PCR amplification were preceded by a heat-denaturing step at 94° C. for 105 seconds. Each PCR cycle consisted of denaturation at 94° C. for 10 seconds, annealing at 68° C. for 30 seconds and extension at 68° C. for 60 seconds. The specific PCR product was then digested with Ndel and Xhol before gel electrophoresis and purification. The digested and purified spPDZD2 cDNA was ligated with Ndel and Xhol digested pTYBI2 vector DNA before transformation into competent DH-15a bacterial cells. The plasmid DNA pTYBI2-spPDZD2 was finally sequenced in both directions.

The IMPACT™ (Innate Mediated Purification with an Affinity Chitin-binding Tag)-CN system from New England Biolabs was used for the synthesis of recombinant human spPDZD2 protein. This system utilizes the inducible self-cleavage activity of intein to separate the target protein from the affinity tag. The pTYBI2-spPDZD2 construct was transformed into the bacterial host strain ER2566 and the recombinant spPDZD2 protein was synthesized according to the manufacturer’s instructions. Briefly, the production of the intein-spPDZD2 fusion protein was carried out by induction using 0.75 mM IPTG at 28° C. for 6 hours with constant shaking. Cells were then harvested and resuspended in column buffer [20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 1 mM EDTA, with protease inhibitors]. After sonication for 10 minutes with approximately 10 seconds per pulse to release cellular proteins, cell debris was removed by centrifugation at 4000 rpm at 4° C. for 10 minutes, followed by centrifugation at 16000 rpm at 4° C. for 15 minutes. Supernatant fluid containing the intein-spPDZD2 recombinant protein was filtered through 0.45 µm filter and then slowly loaded onto an
EXAMPLE 4
Effects of Caspase-3 Inhibitor Z-DEVD-FMK on Endogenous PDZD2 Expression and sPDZD2 Secretion, and Cancer Cell Growth

Full-length PDZD2 protein, which exhibits close sequence homology to pro-IL-16, is processed and cleaved by a caspase-dependent mechanism to generate sPDZD2. Given that cleavage of pro-IL-16 to form the corresponding secretory IL-16 cytokine is mediated by caspase-3, we tested whether or not proteolytic cleavage of full-length PDZD2 to its secreted protein sPDZD2 is caspase-3-dependent in human prostate, breast and liver cancer cells. Human prostate cancer cell lines LNCaP and LNCaP-PC3 (CRL-1740), DU145 (HTB-81), PC-3 (CRL-1435), and 22Rv1 (CRL-2505), breast adenocarcinoma cell line MCF-7 (HTB-22), and hepatocellular carcinoma cell line Hep-G2 (HEP-805) were obtained from American Type Culture Collection. DU145, PC-3, 22Rv1, MCF-7, and Hep-G2 cells were incubated under serum-free conditions for 48 hours with either 10 μM Z-DEVD-FMK (BD Biosciences), a specific inhibitor of caspase-3, or 10 μM Z-FA-FMK (BD Biosciences), a negative control peptide, before the cells and conditioned media were collected for immunoblotting with the rabbit anti-PDZD2 antibody. Cell lysates in sample buffer (0.2% SDS, 10% glycerol), 0.06 M Tris-HCl (pH 6.8), 100 mM DTT, and 0.01% bromophenol blue) were heated at 95°C for 5 min. Proteins in the conditioned media were concentrated by 50-fold using YM-10 Centrifuges (Millipore). Samples (10 μg) were resolved by SDS-PAGE and electroblotted to PVDF membranes (Millipore). The blots were blocked with 5% non-fat milk powder in TBS-T for 1 hour at room temperature and then incubated with rabbit anti-PDZD2 antibody (1:10,000 dilution) overnight at 4°C. After washing with TBS-T, the blots were incubated with secondary antibodies against rabbit immunoglobulin G (ZYMED Laboratories). Blots were stripped in 25 mM glycine buffer (pH 2.0) for 30 min for re-probing with α-tubulin (1:500 dilution, Santa Cruz Biotechnology). The signals were visualized by enhanced chemiluminescence Western blotting system (Amersham Biosciences) and densitometric scans of films of the developed blot normalized against α-tubulin were determined.

To determine whether or not the expression of PDZD2 and sPDZD2 levels in inhibitor-treated cells are associated with any changes in the cell growth rate, we also monitored DU145, PC-3, 22Rv1, MCF-7 and Hep-G2 cell proliferation by MTS-based assays. Cell proliferation was measured by a tetrazolium-based Cell Titer 96® Aqueous assay kit (Promega). Absorbance at 490 nm was recorded 3 hours after MTS addition. There were significant (P<0.01) increases in DU145 (20.1% to 22.7%), PC-3 (22.9% to 24.8%), 22Rv1 (23.4% to 26%), MCF-7 (21.5% to 37.2%), and Hep-G2 (19.3% to 30.8%) cell proliferation after the cells were treated with 10 μM of the caspase-3 inhibitor Z-DEVD-FMK for 24 and 48 hours (Figs. 1C-1K).

To examine if the observed increases in cell proliferation were due to inhibition of sPDZD2 secretion, we tested the ability of exogenously applied recombinant sPDZD2 to counteract the growth-promoting effects of the caspase-3 inhibitor. In separate sets of experiments, DU145, PC-3, 22Rv1, MCF-7 and Hep-G2 cells were incubated with 10 μM specific caspase-3 peptide inhibitor, Z-DEVD-FMK (BD Biosciences), 10 μM negative control peptide Z-FA-FMK (BD Biosciences), or vehicle for 48 hours. In addition, DU145, PC-3, 22Rv1, MCF-7 and Hep-G2 cells were each co-incubated with 10 μM Z-DEVD-FMK and 10⁻⁸ M sPDZD2 for 48 hours. As shown in Figs. 1L and 1M, the increases in DU145, PC-3, 22Rv1, MCF-7 and Hep-G2 cell proliferation induced by caspase-3 inhibitor treatment were abolished by co-incubating with 10⁻⁸ M sPDZD2. These results suggest that the antiproliferative effects were predominantly mediated by sPDZD2 instead of PDZD2.

EXAMPLE 5
Effects of Recombinant sPDZD2 on Cancer Cell Growth In Vitro and In Vivo

For in vitro studies, DU145, PC-3, 22Rv1, MCF-7 and Hep-G2 cells (2x10⁶/ml) were seeded in 96-well plates and were incubated with or without 10⁻⁸ M, 10⁻⁷ M, and 10⁻⁶ M purified recombinant sPDZD2, or vehicle (20 mM Tris-HCl, pH 8, 1 mM EDTA) for 24 and 48 hours before the cells were processed for cell proliferation studies. Cell proliferation was measured by a tetrazolium-based Cell Titer 96® Aqueous assay kit (Promega). Absorbance at 490 nm was recorded in 5 hours after MTS addition. Cell viabilities of DU145, PC-3, 22Rv1, MCF-7 and Hep-G2 cells, incubated with or without 10⁻⁹ M, 10⁻⁸ M, and 10⁻⁷ M purified recombinant sPDZD2 for 24 and 48 hours, were measured by trypsin blue dye exclusion assays. The number of viable cells was counted using hemocytometers. Any apoptotic effect on cancer cells induced by sPDZD2 was measured by the Cell Death Detection ELISA²ując assay kit (Roche), which detects the pres-
ence of mono- and oligonucleosomes in the cytoplasm of the cells after lysis. Briefly, the cancer cells (1x10^5/ml) were seeded in 24-well plates and treated with 10^{-8} M and 10^{-7} M sPDZD2 for 24 hours. After treatment, cells were harvested and any apoptosis was detected according to manufacturer’s instructions. Recombinant sPDZD2 induced a significant (P<0.001) concentration-dependent inhibition of cell proliferation in DU145, PC-3, 22RV1, MCF-7 and Hep-G2 cells (FIGS. 2A-2E).

Treatment of DU145 cells with 10^{-8} M and 10^{-7} M sPDZD2 for 24 hours resulted in, respectively, 25.8% and 29.9% decreases in cell proliferation (P<0.001). Treatment of PC-3 cells with 10^{-8} M and 10^{-7} M sPDZD2 for 24 hours resulted in, respectively, 13.5% and 22.6% decreases in cell proliferation (P<0.01). Treatment of 22RV1 cells with 10^{-8} M and 10^{-7} M sPDZD2 for 24 hours resulted in, respectively, 12.7% and 15.4% decreases in cell proliferation (P<0.001). Treatment of MCF-7 cells with 10^{-8} M and 10^{-7} M sPDZD2 for 24 hours resulted in, respectively, 14.5% and 23.2% decreases in cell proliferation (P<0.01). Similar treatment of Hep-G2 cells with 10^{-8} M and 10^{-7} M sPDZD2 for 24 hours resulted in, respectively, 21% and 23.1% decreases in cell proliferation (P<0.01). On the other hand, DU145 cell proliferation exhibited 38.6% and 43.5% decreases (P<0.001) after the cells were treated with 10^{-8} M and 10^{-7} M sPDZD2, respectively. For 48 hours, PC-3 cell proliferation exhibited 23.8% and 41.6% decreases (P<0.001) after the cells were treated with 10^{-8} M and 10^{-7} M sPDZD2, respectively. For 48 hours, 22RV1 cell proliferation exhibited 21.7% and 27.7% decreases (P<0.001) after the cells were treated with 10^{-8} M and 10^{-7} M sPDZD2, respectively. For 48 hours. Similarity, incubation of MCF-7 cells with 10^{-8} M and 10^{-7} M sPDZD2 for 48 hours resulted in 13.7% and 21.4% decreases (P<0.001) in cell proliferation respectively. For Hep-G2 cells, 17% and 22.2% decreases (P<0.001) in cell proliferation were, respectively, observed after the cells were treated with 10^{-8} M and 10^{-7} M sPDZD2 for 48 hours. (FIGS. 2A, 2B, 2C, 2D, and 2E). No changes in DU145, PC-3, 22RV1, MCF-7 and Hep-G2 cell viability were detected (see Table 1 which shows the effects of recombinant sPDZD2 on the viability of human prostate, breast and liver cancer cell lines).

### TABLE 1

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<th>10^{-8} M sPDZD2</th>
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<td><strong>Control</strong></td>
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<tr>
<td>24 hrs.</td>
<td>95.02 ± 0.47%</td>
<td>94.66 ± 0.69%</td>
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<td>48 hrs.</td>
<td>95.69 ± 0.72%</td>
<td>95.09 ± 1.02%</td>
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<td><strong>(A) DU145</strong></td>
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<td>24 hrs.</td>
<td>94.21 ± 0.52%</td>
<td>95.03 ± 1.00%</td>
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<td>48 hrs.</td>
<td>97.50 ± 0.44%</td>
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<td><strong>(B) PC-3</strong></td>
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<td>24 hrs.</td>
<td>96.81 ± 0.39%</td>
<td>96.01 ± 0.38%</td>
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<td>48 hrs.</td>
<td>94.66 ± 0.44%</td>
<td>93.02 ± 0.47%</td>
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<td><strong>(C) 22RV1</strong></td>
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<td>24 hrs.</td>
<td>93.78 ± 0.31%</td>
<td>93.38 ± 0.54%</td>
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<tr>
<td>48 hrs.</td>
<td>94.82 ± 0.28%</td>
<td>93.64 ± 0.60%</td>
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*P<0.001, DU145, (B) PC-3, (C) 22RV1, (D) MCF-7, and (E) Hep-G2 cells were incubated with 10^{-8} M or 10^{-7} M recombinant sPDZD2 for 24 and 48 hours. After treatment, the cells were harvested by trypsinization (0.25%, w/v) and collected by centrifugation at 180 x g for 5 minutes. The viability of the cells was measured by trypan blue exclusion assay. Data shown are mean ± S.E.

In contrast, incubation of LNCaP cells with 10^{-7} M sPDZD2 for 24 hours resulted in 26.2% decrease in cell viability (P<0.001), whereas LNCaP cell viability exhibited 14.7% and 18.6% decreases (P<0.001) after treatment with 10^{-8} M and 10^{-7} M sPDZD2, respectively, for 48 hours (FIG. 3A).

The observed decreases in LNCaP cell viability in response to sPDZD2 were found to be due to apoptosis induction (FIG. 3B), as measured by Cell Death Detection ELISA PLUS assay kit (Roche). Significant (P<0.001) increases in absorbance at 405 nm, which reflects an increase in mono- and oligonucleosomes in the cell cytoplasm, were observed in LNCaP cells treated with 10^{-7} M and 10^{-7} M sPDZD2 for 24 hours.

For in vivo studies, DU145 cancer cells (5x10^6) in 0.2 ml FBS-containing EMEM medium were injected subcutaneously into the right flank of each male BALB/c athymic nude mice (weight, 20±2 g). After cancer cell injection, the mice were randomly divided into 3 groups, namely the control, low-dose treatment, and high-dose treatment groups. Ten days after tumor cell implantation, each mouse of the low-dose treatment group and high-dose treatment group was injected, respectively, once daily by the intraperitoneal route with 0.084 mg (in 0.1 ml saline) and 8.4 mg (in 0.1 ml saline) recombinant sPDZD2 for 58 days. The width and length of the tumor in millimeters were measured weekly by a caliper, when the implanted tumor became measurable. The tumor volume was calculated according to the following formula: Volume = \( (\text{d} \times \text{D}^2) / 2 \), where d is the shorter width and D was the longer length. Changes in tumor volumes in nude mice given daily recombinant sPDZD2 injections are shown in FIG. 4. Compared with saline-treated group, significant decreases (*)P<0.05) in tumor volumes were detected in animals treated with high dose recombinant sPDZD2 at 39 days, 46 days, 53 days, 60 days and 67 days after tumor cell implantation. No significant differences were found between tumor volumes of animals treated with low-dose recombinant sPDZD2 and saline-treated group. The tumor growth suppressive function of recombinant human sPDZD2 protein demonstrated in vitro as described above was validated in vivo.
Leu  Asp  Lys  Leu  Cys  Ser  Glu  Asp  Tyr  Ser  Ala  Gly  Pro  Ser  Ala  Val  
1    5    10    15  
Leu  Phe  Lys  Thr  Glu  Leu  Glu  Ile  Thr  Pro  Arg  Arg  Ser  Pro  Gly  Pro  
20   25   30  
Pro  Ala  Gly  Gly  Val  Ser  Cys  Pro  Glu  Lys  Gly  Asn  Arg  Ala  Cys  
35   40   45  
Pro  Gly  Gly  Ser  Gly  Pro  Lys  Thr  Ser  Ala  Ala  Glu  Thr  Pro  Ser  Ser  
50   55   60  
Ala  Ser  Asp  Thr  Gly  Glu  Ala  Ala  Gin  Asp  Leu  Pro  Phe  Arg  Arg  Ser  
65   70   75   80  
Trp  Ser  Val  Asn  Leu  Asp  Gin  Leu  Val  Ser  Ala  Gly  Asp  Gin  Gin  
85   90   95  
Arg  Leu  Gin  Ser  Val  Leu  Ser  Ser  Val  Gly  Ser  Thr  Ile  Leu  
100  105  110  
Thr  Leu  Ile  Gin  Glu  Ala  Lys  Ala  Gin  Ser  Glu  Asn  Glu  Glu  Asp  Val  
115  120  125  
Cys  Phe  Ile  Val  Leu  Asn  Arg  Lys  Glu  Gly  Ser  Gly  Leu  Gly  Phe  Ser  
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Val  Ala  Gly  Thr  Asp  Val  Glu  Pro  Lys  Ser  Ile  Thr  Val  His  Arg  
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Gln  Glu  Pro  Pro  Thr  Ala  Asn  Gly  Lys  Gly  Leu  Ser  Arg  Lys  Thr  
225  230  235  240  
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275  280  285  
Arg  Val  Tyr  Lys  Gly  Ala  Ala  Gin  Ala  Gly  Ile  Ile  Glu  Ala  
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Gly  Asp  Glu  Ile  Leu  Ala  Ile  Asn  Gly  Lys  Pro  Leu  Val  Gly  Leu  Met  
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### US 7,807,626 B2

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The invention claimed is:

1. A method for inhibiting prostate cancer cell proliferation in a prostate cancer cells comprising:
   administering to the prostate cancer cells an amount of a composition comprising a prostate cancer cell suppressor protein consisting of SEQ ID NO: 1 effective to induce prostate cancer cell apoptosis.
2. A method for causing prostate cancer cell apoptosis in a prostate cancer cells comprising:
   * * * * *