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(54) Title: CONTACTIN-1 (CNTN1) FOR USE IN METHODS OF DIAGNOSIS AND TREATMENT OF PROSTATE CANCER

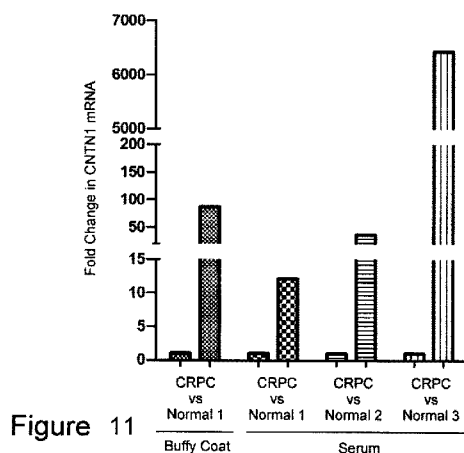


Figure 11

(57) Abstract: A method of determining prostate cancer, or progression, recurrence or metastasis of prostate cancer in a male mam-
mal is provided. The method comprises the steps of determining in a biological sample obtained from the mammal the expression
level of CNTN1 in the sample, comparing the CNTN1 expression level to a control level, and determining that the mammal has pro-
state cancer when the expression level of CNTN1 in the sample is greater than the control level.

CONTACTIN-1 (CNTN1) FOR USE IN METHODS OF DIAGNOSIS AND TREATMENT OF PROSTATE CANCER

Field of the Invention

[0001] The present invention generally relates to diagnostic and therapeutic methods for prostate cancers, and more particularly, to the use of the cell surface neural cell adhesion factor, contactin 1 (CNTN1), in such methods.

Background of the Invention

[0002] Prostate cancer (PC) is the most common malignancy and the second leading cause of cancer-associated death for men in the developed world. The number of cases diagnosed has increased significantly since the introduction of prostate specific antigen (PSA) testing in the late 1980s. The PSA test allows for early detection and results in the decline of metastatic PC cases at diagnosis from 20% before 1990 to 3.4% in the 1990s, and thus contributes to the decrease of PC-associated mortality. This knowledge is supported by a large screen trial that involved 182,000 patients. However, the common use of PSA also resulted in the over-diagnosis of many patients with clinically insignificant disease, contributing to overtreatment. Although 30% of patients with Gleason score (GS) 6 tumors will progress to a clinically significant stage, only 6.8% of 11,892 men across 36 clinical sites in the USA elected for active surveillance. Collectively, the current system saves lives at the expense of compromising the quality of life for many others. Furthermore, the current strategy waits for rising PSA levels (biochemical recurrence/BCR) following radical prostatectomy (RP) and radiation therapy (RT) before initiating aggressive treatments for patients with advanced PC, although evidence suggests greater benefits during early intervention. Despite these problems, PSA remains the only clinically-used biomarker. Collectively, the lack of prognostic biomarkers compromises patients' quality of life, increases financial burden for families and society, and reduces therapy efficiency.

[0003] Prostate cancer progresses from high-grade prostatic intra-epithelial neoplasia (HGPIN), locally invasive carcinoma to metastatic cancer. Metastatic PC is commonly treated by androgen ablation, pioneered by Charles Huggins in 1941. While the therapy is initially effective in 80% of patients, castration resistant PC (CRPC) almost inevitably arises; the median survival

time for these patients is approximately one year. Metastasis thus starts the fatal process of PC. Although the mechanisms responsible for developing metastasis are poorly understood, accumulating evidence reveals a critical role of cancer stem cells (CSCs) in cancer progression and metastasis. Tumors consist of heterogeneous cell populations, in which a minor group of cells (CSCs) are the unit of evolution selection. Prostate stem cells (PSC) have been identified in both humans and mice. Akin to orchiectomy leading to robust prostate involution, androgen-removal results in marked PC regression. Both PSC and Prostate Cancer Stem Cells (PCSCs) are androgen-independent. While PSCs regenerate the prostate, PCSCs produce recurrent castration resistant prostate cancer (CRPC). The signatures of PCSCs are associated with PC bone metastasis and poor prognosis.

[0004] Despite this knowledge, how PCSCs promote PC progression and metastasis remains unclear. However, it is becoming apparent that the integration of a complex network of signals is essential. As a result of signal integrations, cellular alterations occur that promote PC progression and metastasis; the typical alterations for epithelium-originated tumors include the reduction of cell-cell adhesion in part due to the downregulation of E-cadherin, a typical event of epithelial-mesenchymal transition (EMT). In addition to the reduction of E-cadherin, elevation of cell adhesion molecules (CAMs), especially Ig-like neural cell adhesion molecules, plays an important role in cancer progression and metastasis.

[0005] Given the foregoing, it would be desirable to identify specific cellular alterations indicative of prostate cancer progression and/or metastasis.

Summary of the Invention

[0006] It has now been determined that expression of the protein, contactin 1 (CNTN1), is indicative of prostate cancer, promotes prostate cancer tumor initiation, progression and recurrence, and promotes metastasis in mammals.

[0007] Thus, in one aspect of the invention, a method of determining prostate cancer, or progression, recurrence or metastasis of prostate cancer, in a male mammal is provided. The method comprises the steps of determining in a biological sample obtained from the mammal the expression level of CNTN1 in the sample, comparing the CNTN1 expression level to a control

level, and determining that the mammal has prostate cancer when the expression level of CNTN1 in the sample is greater than the control level.

[0008] In another aspect of the invention, a method of treating prostate cancer in a mammal is provided comprising administering to the mammal a CNTN1 inhibitor.

[0009] In another aspect, a composition comprising a CNTN1 inhibitor and a pharmaceutically acceptable carrier is provided.

[0010] In a further aspect of the invention, a method of determining tamoxifen resistance in breast cancer is provided, comprising determining the level of CNTN1 in breast cancer cells, comparing the CNTN1 level to a control level, and determining that the mammal has tamoxifen resistant breast cancer when the level of CNTN1 in the sample is greater than the control level.

[0011] These and other features of the invention can become more apparent from the following descriptions in which reference is made to the appended drawings.

Brief Description of Drawings

[0012] Figure 1 shows CNTN1 expression promotes prostate cancer cell invasion. A) microarray analysis on gene expression between DU145 and DU145 PCSCs was performed three times. The average levels of CNTN1 mRNA in both cell lines were graphed. *:p<0.05 by a 2-tailed student t-test. B) RNA was isolated from the indicated DU145 cell lines and examined by real-time PCR for CNTN1. β -actin was used as an internal control. Experiments were repeated three times. CNTN1 mRNA in DU145 PCSCs is shown as a fold change to DU145 cells (mean $\pm$ s.d.). *:p<0.05 by a 2-tailed student t-test. C) cell lysates were collected from the indicated cell lines and examined for protein expression by western blot for CNTN1 and actin. Experiments were repeated at least three times; representative image from a single repeat is shown. M: monolayer cells. D) whole DU145 spheres were prepared onto slides by cytopsin and stained by immunofluorescence for CNTN1 expression. Similar images were obtained from multiple spheres; a typical image is included. E) RNA was isolated from PC3 cells cultured in the presence of 10% FBS (PC3) and from PC3 cells cultured in serum free PCSC conditions (PC3 SF), followed by the examination of CNTN1 using real-time PCR. β -actin was used as an

internal control. CNTN1 mRNA in PC3 SF is shown as a fold change to PC3 cells (mean $\pm$ s.d.). Experiments were repeated two times. F) western blot analysis of CNTN1 in DU145 EV, CNTN1, parental (Par) cells from which spheres were derived, and sphere cells. G) DU145 EV and DU145 CNTN1 cells were examined for their ability to pass through a control and matrigel membrane. Experiments were repeated three times. Typical images from one experiment are shown (left panel). Cell invasion was quantified (right panel). *:p<0.05 by a 2-tailed student t-test.

[0013] Figure 2 shows Ectopic CNTN1 does not affect DU145 cell proliferation. 1000 cells of DU145 EV and CNTN1 cells were seeded in triplicate in 96 well plates, followed by determining cell proliferation daily using WST-1 for 5 days. Experiments were repeated 3 times.

[0014] Figure 3 shows the characterization of the impact of CNTN1 on LNCaP C4-2 cell biology. A) CNTN1 was stably overexpressed in LNCaP C4-2 cells by EV and CNTN1 retrovirus. The expression of ectopic CNTN1 was confirmed by western blot. B) LNCaP C4-2 EV and CNTN1 cells were seeded into 6 well plates at 10^3 cells/well, cultured for two weeks, and stained with crystal violet. C) LNCaP C4-2 EV and CNTN1 cells (5×10^4 cells) were analyzed for their ability to pass through a matrigel membrane (8 μ m). D) LNCaP C4-2 EV and CNTN1 cells were serum starved over night, stimulated with 10% FBS for the indicated timepoints, and examined by western blot for serine 473-phosphorylated AKT (pAKT) and AKT. Experiments were repeated twice; typical images from a single repeat are shown.

[0015] Figure 4 shows CNTN1 affects AKT activation and E-cadherin expression. A) DU145 EV and CNTN1 cells were serum starved over night followed by the addition of media with 10% FBS for the indicated timepoints. AKT activation was examined by western blot for serine 473-phosphorylated AKT (pAKT). Experiments were repeated twice; typical images from a single repeat are shown. B) DU145 sphere cells were infected with shCTRL (control shRNA) and shCNTN1, followed by western blot examination for the indicated proteins (inset). Experiments were repeated three times; the levels of pAKT were standardized to total AKT and quantified (means $\pm$ s.d.). *:p<0.05 by a 2-tailed student t-test. Quantification of E-cadherin expression in DU145 EV and CNTN1 cells (D) and in DU145 spheres shCTRL and shCNTN1

cells (E) are shown. Quantifications were derived from three independent repeats. *: $p < 0.05$ by a 2-tailed student t-test.

[0016] Figure 5 shows CNTN1 promotes xenograft tumor formation. The indicated sphere cells (A) and monolayer cells (B) were subcutaneously implanted into NOD/SCID mice at 10^4 sphere cells or 10^6 monolayer cells per mouse. Four or five mice were used per line. Xenograft tumors were monitored weekly; tumor volumes were graphed (means $\pm$ s.d.). *: $p < 0.05$ by a 2-way ANOVA.

[0017] Figure 6 shows CNTN1 increases lung metastases in mice. A) tumor volumes were measured using ImageJ and graphed (means $\pm$ s.d.). *: $p < 0.05$ by 2-tailed student t-test. B) dot plot distribution of tumor volumes for DU145 EV and DU145 CNTN1 for volumes of 0-15 mm³ and 16+ mm³. Mean is represented by the horizontal line. *: $p < 0.05$ by a 2-tailed student t-test.

[0018] Figure 7 shows CNTN1 associates with prostate cancer progression and biochemical recurrence. A) H-Score was calculated for each sample and grouped into Gleason stages. Staining intensity was graphed (mean $\pm$ s.d.). *: $p < 0.05$, **: $p < 0.01$ by two-tailed student t-test. D) Kaplan-Meier analysis of biochemical recurrence-free survival in CNTN1-positive (n=385) versus CNTN1-negative (n=91) patients ($p < 0.05$ by a log-rank test).

[0019] Figure 8 shows CNTN1 affects gene expressions that function in tumorigenesis. A) a heatmap of microarray data for DU145 cells over expressing CNTN1 and B) CNTN1 knockdown in DU145 PCSCs. C) Top diseases and biological functions for differentially expressed genes for DU145 PCSCs shCTRL and shCNTN1 and DU145 EV and CNTN1. D, analysis of PTEN, BCHE and TMPRSS2-ERG transcripts using NanoString technology for 7 paired non-tumor and prostate cancer tissues.

[0020] Figure 9 shows the number of differentially expressed genes for A) DU145 PCSCs shCTRL compared to shCNTN1 and B) DU145 EV compared to CNTN1 associated with disease and function.

[0021] Figure 10 shows CNTN1 represses BCHE expression. RNA was isolated from DU145 monolayer, sphere cells (A), DU145 EV and CNTN1 cells, and LNCaP C4-2 EV and

CNTN1 cells (B). A, Microarray analysis on gene expression between DU145 monolayer and DU145 PCSCs revealed a 63 fold down-regulation of BCHE in DU145 PCSCs. *:p<0.05 by a two tailed student t-test. B, real-time PCR analysis for BCHE and β -actin on overexpression cell lines. *:p<0.05 by a two tailed student t-test.

[0022] Figure 11 shows a robust elevation of blood CNTN1 in a patient with castration resistant prostate cancer. Blood samples were collected from three volunteers and a patient with castration resistant prostate cancer. Total RNA was purified from blood cells (Buffy coat) and serum. Real time PCR reactions were performed to amplify CNTN1 and actin. The respective CNTN1 mRNA was normalized to the corresponding actin mRNA. Patient's CNTN1 was expressed as fold changes in reference to normal individuals.

[0023] Figure 12 shows the expression of CNTN1 in a panel of breast cancer cell lines. Western blot analysis was performed to detect CNTN1 and actin.

[0024] Figure 13 shows that ectopic CNTN1 confers MCF7 cells resistant to tamoxifen-induced cytotoxicity by graphically illustrating quantification of surviving MCF7 EV and CNTN1 cells in response to tamoxifen treatment from 3 independent repeats. *p<0.05 by 2-tailed student t-test.

[0025] Figure 14 shows elevation of CNTN1 in tamoxifen resistant MCF7 cells. Tamoxifen resistant MCF7 cells (Tam-R) were produced by culturing in the presence of 0.1 μ M tamoxifen for 12 months. The status of tamoxifen resistance was confirmed. Real time PCR were performed on both MCF7 and MCF7 Tam-R cells to analyze CNTN1 expression. The level of CNTN1 in MCF7 Tam-R cells was expressed as fold changes to it in MCF7 cells.

[0026] Figure 15 illustrates the amino acid sequence of A) human CNTN1 isoform 1, B) human CNTN1 isoform 3, C) mouse CNTN1, and the mRNA sequences for D) human CNTN1 isoform 1, and E) mouse CNTN1.

Detailed Description of the Invention

[0027] A method of determining prostate cancer in a male mammal is provided. The method comprises the steps of determining in a biological sample obtained from the mammal the

expression level of CNTN1, comparing the CNTN1 expression level to a control level, and determining that the mammal has prostate cancer when the level of CNTN1 in the sample is greater than the control level.

[0028] The term “Contactin-1”, or CNTN1, refers to a neural cell adhesion protein having the amino acid sequence shown in Fig. 20A, e.g. isoform 1, including functionally equivalent variants such as other isoforms, e.g. isoform 2 in which residues 11-21 of isoform 1 are missing; and orthologs such as CNTN1 from other mammals, e.g. mouse CNTN1 ortholog as shown in Fig. 20, and other functionally equivalent variants, including fragments. The term “functionally equivalent” refers to CNTN-1 variant proteins that retain the same or similar function and/or activity as human CNTN-1 (isoform 1) and which, as a result, would similarly be expressed in connection with prostate cancer. Functionally equivalent variants may include a native CNTN1 including one or more amino acid deletions, insertions or substitutions (such as, but not limited to, conservative amino acid substitutions in which one or more amino acids is replaced with a similarly charged amino acid, e.g. substitution of a basic amino acid such as histidine, lysine and arginine; a negatively charged amino acid such as glutamic and aspartic acid; a hydrophobic amino acid such as alanine, valine, leucine, isoleucine, tryptophan, methionine, and isoleucine; and substitution of an uncharged amino acid such as threonine, glutamine, asparagine and serine). Examples of CNTN-1 variants include CNTN-1 in which the proline at position 794 is changed to histidine, or in which the valine at position 798 is changed to leucine, or a variant in which the glutamic acid at position 824 is changed to glycine. Proteolytic fragments of CNTN-1 may result from its proteolytic breakdown, and thus, may be detectable in blood as representative of CNTN-1 expression.

[0029] The term “prostate cancer” refers to any cancer of the prostate gland, including but not limited to, adenocarcinoma and neuroendocrine prostate cancer, which may become castration resistant following androgen deprivation therapy.

[0030] To conduct the method, a biological sample is obtained from a male mammal. The term “biological sample” is meant to encompass any human sample that may contain an increased expression level of CNTN1, protein or nucleic acid (genomic or mRNA), including circulating biological fluids such as, but not limited to, blood, plasma/serum, urine and

cerebrospinal fluid, lymphatic fluid, bone marrow, lymph nodes and tissue biopsies, e.g. prostate tumour or prostate tissue, may also be used. The sample is obtained in a manner well-established in the art. The term “mammal” is used herein to refer to both male human and non-human mammals, such as domestic animals such as dogs, cats, horses and the like, as well as other animals.

[0031] Once a suitable biological sample is obtained, it is analyzed to determine the expression level (or concentration) of CNTN1, including either the presence of CNTN1 protein or CNTN1-encoding nucleic acid (e.g. mRNA), in the sample. Prior to analysis, the sample may be subject to processing such as extraction, filtration, centrifugation or other sample preparation techniques to provide a sample that is suitable for further analysis. For example, biological fluids may be filtered or centrifuged (e.g. ultracentrifugation) to remove solids from the sample to facilitate analysis. Tissue samples may be subject to extractions in order to provide an analyzable sample. As one of skill in the art will appreciate, CNTN1 expression level may be determined using one of several techniques established in the art that would be suitable for detecting a protein or nucleic acid encoding the protein in a biological sample, including mass spectrometry, chromatographic techniques such as high performance liquid and gas chromatography, immunoassay or enzyme-based assays with colorimetric, fluorescence or radiometric detection, electrophoretic techniques, and nucleic acid hybridization techniques which may include PCR.

[0032] The expression level CNTN1 in a sample may be measured by immunoassay using an antibody specific to CNTN1. The antibody binds to the biomarker and bound antibody is quantified by measuring a detectable marker which may be linked to the antibody or other component of the assay, or which may be generated during the assay. Detectable markers may include radioactive, fluorescent, phosphorescent and luminescent (e.g. chemiluminescent or bioluminescent) compounds, dyes, particles such as colloidal gold and enzyme labels. The term “antibody” is used herein to refer to monoclonal or polyclonal antibodies, or antigen-binding fragments thereof, e.g. an antibody fragment that retains specific binding affinity for the target biomarker. Antibodies to the target biomarkers may be raised using techniques conventional in the art. For example, antibodies may be made by injecting a host animal, e.g. a mouse or rabbit, with the antigen (target biomarker), and then isolating antibody from a biological sample taken

from the host animal. Alternatively, CNTN1 antibodies may be commercially obtained, e.g. from Sigma Aldrich, Sino Biological Inc., Atlas Antibodies, etc.

[0033] As will be appreciated by one of skill in the art, different immunoassays may be used to determine CNTN1 expression level in a sample, including indirect immunoassay in which CNTN1 is non-specifically immobilized on a surface; sandwich immunoassay in which the CNTN1 is specifically immobilized on a surface by linkage to a capture antibody bound to the surface; and a competitive binding immunoassay in which a sample is first combined with a known quantity of CNTN1 antibody, and then the sample is exposed to immobilized CNTN1 which competes with the sample to bind any unbound antibody. Enzyme Linked ImmunoSorbent Assay (ELISA) may also be used to determine the expression level of CNTN1 in a sample. In this case, CNTN1 is generally immobilized on a solid support, complexed with an antibody which is itself linked to an enzyme indicator, such as horseradish peroxidase (HRP), alkaline phosphatase (AP), β -galactosidase, acetylcholinesterase and catalase. Detection may then be accomplished by incubating this enzyme-complex with a substrate for the enzyme that yields a detectable product.

[0034] In one embodiment, sphere-forming cells are first isolated from the biological sample and determination of CNTN1 expression level in the sphere-forming cells is conducted as described. Sphere-forming cells may be prostate cancer stem cells which are able to grow in suspension (i.e. floating) in these 3-D sphere-shaped structures and are associated with prostate cancer. Sphere-forming cells may be isolated using established methods. For example, biopsied cells may be dissociated, and then cultured in a serum-free media supplemented with EGF and optionally other nutrients (e.g. BSA, vitamins) for a sufficient period of time, e.g. 10-12 days. This isolation step functions to concentrate potential target sphere-forming or cancer stem cells to facilitate the analysis.

[0035] Once the expression level of CNTN1, protein or CNTN1-encoding nucleic acid, in the sample is determined, the expression level is compared to a CNTN1 control level to determine the difference or statistical significance between the determined CNTN1 or CNTN1 nucleic acid level and the control level. The term "control level", as it is used herein, is the level of CNTN1 protein or nucleic acid detected in a sample from a healthy unaffected subject who

does not have prostate cancer. Preferably the control value is a mean control value obtained from a healthy population of matched subjects (*e.g.* age-, and/or ethnically-matched to a population). CNTN1 expression levels may also be compared to a control level of one or more housekeeping or reference genes. The term “housekeeping genes” as used herein is meant to refer to genes that encode protein products that are not connected to, involved in or required for processes specific to prostate cancer cells, and thus, exhibit a fixed expression level in cancerous and non-cancerous cells. Examples of suitable housekeeping genes include, but are not limited to, genes encoding ACTB (Beta-actin), GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), RPLP0 (60S acidic ribosomal protein P0), GUSB (beta-glucuronidase), beta-tubulin, ATP-binding cassette, sub-family F member 1, and TFRC (transferring receptor 1). In a comparison of the expression level of CNTN1 to one or more housekeeping genes, a determination of an increase in transcript abundance or expression has been determined to be indicative of prostate cancer.

[0036] A determination that a male mammal has prostate cancer, or has a greater than 50% chance of prostate cancer developing, recurring or metastasizing, is made when the difference in the level of CNTN1 in a biological sample is statistically greater than the CNTN1 control level, or the control level of one or more housekeeping genes. The determination of statistical significance is well-established in the art. Statistical significance is attained when the *p*-value is less than the significance level. The *p*-value is the probability of observing an effect given that the null hypothesis is true whereas the significance or alpha (α) level is the probability of rejecting the null hypothesis given that it is true. Generally, a statistically significant difference, *i.e.* an increase in CNTN1 expression level, is an increase in the CNTN1 expression level from the control level of at least about 10%, or greater, *e.g.* at least about 20%, 30%, 40%, 50% or greater, for example, an increase in CNTN1 expression of 80% or greater as compared to the control.

[0037] In addition, the present method may also be used to predict disease progression and/or monitor treatment response to therapy. In this regard, for a patient determined to have prostate cancer, the present method may subsequently be used to determine CNTN1 expression level as described. The determination of CNTN1 expression level is determined on at least two occasions. In this case, the difference in a first CNTN1 expression level from a control level

(which may be a baseline level previously determined in the patient) is determined (a first difference) and compared to a subsequent difference (second difference) which is the difference between a subsequent determined CNTN1 expression level and the control level. If the difference in CNTN1 expression level increases over time (difference 1 is less than difference 2), this indicates that the disease is progressing (or treatment is not effective), while no change in the difference of CNTN1 expression levels over time indicates that the disease is not progressing (or treatment may be effective), and a decrease in the difference of CNTN1 expression levels over time indicates disease remission (or treatment is effective).

[0038] The present method advantageously provides a means to determine risk of, progression of and/or metastasis of, prostate cancer in a patient. CNTN1 exists at the surface of tumor cells in prostate cancer. Its surface presence and its association with prostate cancer stem cells, the source of the disease progression and metastasis, make CNTN1 an ideal candidate to be used to diagnose patients who are at risk of, or may have prostate cancer, and thus, are at risk of developing metastatic prostate cancer.

[0039] In another aspect of the invention, a method of treating prostate cancer in a mammal is provided comprising administering to the mammal a CNTN1 inhibitor.

[0040] In one embodiment, CNTN1 activity may be inhibited at the protein level, for example, using inhibitors designed to block CNTN1 either directly or indirectly. CNTN1 inhibitors may include biological compounds, and synthetic small molecules or peptide mimetics, for example, based on such biological compounds. Biological CNTN1 inhibitors also include immunological inhibitors such as monoclonal antibodies prepared using the well-established hybridoma technology as described above. CNTN1 antibodies can also be conjugated with a toxic anti-tumour agent such that the antibody delivers the anti-tumour agent to CNTN1-positive prostate cancer cells.

[0041] Expression of CNTN1 may also be inhibited at the nucleic acid level using nucleic acid-based inhibitors such as anti-sense inhibitors, and RNA interference inhibitors, e.g. siRNA, shRNA and the like. CNTN1-encoding nucleic acid molecules may be used to prepare antisense oligonucleotides effective to bind to CNTN1 nucleic and inhibit the expression thereof. The term "antisense oligonucleotide" as used herein means a nucleotide sequence that is

complementary to at least a portion of a target CNTN1 nucleic acid sequence. The term "oligonucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars, and intersugar (backbone) linkages. The term also includes modified or substituted oligomers comprising non-naturally occurring monomers or portions thereof, which function similarly. Such modified or substituted oligonucleotides may be preferred over naturally occurring forms because of properties such as enhanced cellular uptake, or increased stability in the presence of nucleases. The term also includes chimeric oligonucleotides which contain two or more chemically distinct regions. For example, chimeric oligonucleotides may contain at least one region of modified nucleotides that confer beneficial properties (e.g. increased nuclease resistance, increased uptake into cells) as well as the antisense binding region. In addition, two or more antisense oligonucleotides may be linked to form a chimeric oligonucleotide.

[0042] The antisense oligonucleotides of the present invention may be ribonucleic or deoxyribonucleic acids and may contain naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The oligonucleotides may also contain modified bases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-tri-fluoromethyl uracil and 5-trifluoro cytosine. Other antisense oligonucleotides of the invention may contain modified phosphorous, oxygen heteroatoms in the phosphate backbone, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. For example, the antisense oligonucleotides may contain phosphorothioates, phosphotriesters, methyl phosphonates and phosphorodithioates. In addition, the antisense oligonucleotides may contain a combination of linkages, for example, phosphorothioate bonds may link only the four to six 3'-terminal bases, may link all the nucleotides or may link only 1 pair of bases.

[0043] The antisense oligonucleotides of the invention may also comprise nucleotide analogs that may be better suited as therapeutic agent. An example of an oligonucleotide

analogue is a peptide nucleic acid (PNA) in which the deoxyribose (or ribose) phosphate backbone in the DNA (or RNA), is replaced with a polyamide backbone which is similar to that found in peptides. PNA analogues have been shown to be resistant to degradation by enzymes and to have extended lives in vivo and in vitro. PNAs also form stronger bonds with a complementary DNA sequence due to the lack of charge repulsion between the PNA strand and the DNA strand. Other oligonucleotide analogues may contain nucleotides having polymer backbones, cyclic backbones, or acyclic backbones. For example, the nucleotides may have morpholino backbone structures (U.S. Pat. No. 5,034,506). Oligonucleotide analogues may also contain groups such as reporter groups, protective groups and groups for improving the pharmacokinetic properties of the oligonucleotide. Antisense oligonucleotides may also incorporate sugar mimetics as will be appreciated by one of skill in the art.

[0044] Antisense nucleic acid molecules may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art based on a given CNTN1 nucleic acid sequence such as that provided herein. The antisense nucleic acid molecules of the invention, or fragments thereof, may be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed with mRNA or the native gene, e.g. phosphorothioate derivatives and acridine substituted nucleotides. The antisense sequences may also be produced biologically. In this case, an antisense encoding nucleic acid is incorporated within an expression vector that is then introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense sequences are produced under the control of a high efficiency regulatory region, the activity of which may be determined by the cell type into which the vector is introduced.

[0045] In another embodiment, RNA silencing technology can be applied to inhibit expression of CNTN1. Application of nucleic acid fragments such as siRNA and shRNA fragments that correspond with regions in a CNTN1 transcript and which selectively target a CNTN1 transcript may be used to block CNTN1 expression. Such blocking occurs when the siRNA or shRNA fragments bind to the CNTN1 transcript thereby preventing translation thereof to yield functional CNTN1. siRNA, small interfering RNA molecules, or shRNA, small hairpin RNA molecules, corresponding to CNTN1 mRNA are made using well-established methods of

nucleic acid syntheses as outlined above with respect to antisense oligonucleotides. The effectiveness of selected siRNA and shRNA to block CNTN1 expression can be confirmed using a CNTN1-expressing cell line. Briefly, selected siRNA/shRNA may be incubated with a CNTN1-expressing cell line under appropriate growth conditions. Following a sufficient reaction time, i.e. for the siRNA or shRNA to bind with CNTN1 mRNA to result in decreased CNTN1 expression, the reaction mixture is tested to determine if such a decrease has occurred. Suitable siRNA/shRNA will prevent processing of the CNTN1 transcript to yield functional CNTN1 protein. This can be detected by assaying for CNTN1 activity in a cell-based assay, for example, to identify expression of a reporter gene that is regulated by CNTN1 binding, as described in more detail herein.

[0046] It will be appreciated by one of skill in the art that siRNA/shRNA fragments useful in the present method may be derived from specific regions of CNTN1-encoding nucleic acid which may provide more effective inhibition of gene expression, for example, the 3' end of the transcript, including the 3' untranslated portion. In addition, as one of skill in the art will appreciate, useful siRNA fragments may not correspond exactly with a CNTN1 target gene, but may incorporate sequence modifications, for example, addition, deletion or substitution of one or more of the nucleotide bases therein, provided that the modified siRNA retains its ability to bind to the target CNTN1 gene. Selected siRNA fragments may additionally be modified in order to yield fragments that are more desirable for use. For example, siRNA fragments may be modified to attain increased stability in a manner similar to that described for antisense oligonucleotides.

[0047] Once prepared, oligonucleotides determined to be useful to inhibit CNTN1 gene expression, such as antisense oligonucleotides, shRNA and siRNA, or other CNTN1 inhibitors as described, may be used in a therapeutic method to treat a mammal having prostate cancer. In one embodiment, a selected oligonucleotide may be introduced into tissues or cells of the mammal using techniques in the art including vectors (retroviral vectors, adenoviral vectors and DNA virus vectors) or by using physical techniques such as microinjection. In other embodiments, a CNTN1 inhibitor may be administered by any route suitable to come in contact with and inhibit target CNTN1. Examples of suitable administrable routes include, but are not limited to, oral, subcutaneous, intravenous, intraperitoneal, intranasal, enteral, topical, sublingual, intramuscular, intra-arterial, intramedullary, intrathecal, inhalation, ocular,

transdermal, vaginal or rectal means. Depending on the route of administration, the inhibitor may be coated or encased in a protective material to prevent undesirable degradation thereof by enzymes, acids or by other conditions that may affect the therapeutic activity thereof.

[0048] The selected CNTN1 inhibitor may be used alone or combined with at least one pharmaceutically acceptable adjuvant, in the treatment of metabolic syndrome in accordance with an embodiment of the invention. The expression "pharmaceutically acceptable" means acceptable for use in the pharmaceutical and veterinary arts, i.e. not being unacceptably toxic or otherwise unsuitable. Examples of pharmaceutically acceptable adjuvants are those used conventionally with peptide- or nucleic acid- based drugs, such as diluents, excipients and the like. Reference may be made to "Remington's: The Science and Practice of Pharmacy", 21st Ed., Lippincott Williams & Wilkins, 2005, for guidance on drug formulations generally. The selection of adjuvant depends on the intended mode of administration of the composition. In one embodiment of the invention, the compounds are formulated for administration by infusion, or by injection either subcutaneously or intravenously, and are accordingly utilized as aqueous solutions in sterile and pyrogen-free form and optionally buffered or made isotonic. Thus, the compounds may be administered in distilled water or, more desirably, in saline, phosphate-buffered saline or 5% dextrose solution. Compositions for oral administration via tablet, capsule or suspension are prepared using adjuvants including sugars, such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and derivatives thereof, including sodium carboxymethylcellulose, ethylcellulose and cellulose acetates; powdered tragacanth; malt; gelatin; talc; stearic acids; magnesium stearate; calcium sulfate; vegetable oils, such as peanut oils, cotton seed oil, sesame oil, olive oil and corn oil; polyols such as propylene glycol, glycerine, sorbitol, mannitol and polyethylene glycol; agar; alginic acids; water; isotonic saline and phosphate buffer solutions. Wetting agents, lubricants such as sodium lauryl sulfate, stabilizers, tableting agents, anti-oxidants, preservatives, colouring agents and flavouring agents may also be present. Creams, lotions and ointments may be prepared for topical application using an appropriate base such as a triglyceride base. Such creams, lotions and ointments may also contain a surface active agent. Aerosol formulations may also be prepared in which suitable propellant adjuvants are used. Other adjuvants may also be added to the composition regardless of how it is to be administered, for example, anti-microbial agents may be added to the composition to prevent microbial growth over prolonged storage periods.

[0049] As one of skill in the art will appreciate, the amount of inhibitor administered to a mammal to treat prostate cancer will vary with a variety of factors such as the individual and condition being treated, as well as the inhibitor utilized. Generally, a dosage of inhibitor, including oligonucleotide or other inhibitors (such as antibody inhibitors or other inhibitors as above) sufficient to result in silencing of CNTN1 expression to result in inhibition of target prostate cancer cells is used, for example, an amount of oligonucleotide to result in at least about 50% CNTN1 gene silencing, or a greater amount of gene silencing, e.g. about 60%, 70%, 80%, 90% or more.

[0050] As one of skill in the art will appreciate, a CNTN1 inhibitor may be administered to a mammal in conjunction with a second therapy or therapeutic agent to facilitate treatment of the prostate cancer. The second therapy may be administered simultaneously with the inhibitor, either in combination or separately. Alternatively, the second therapy may be administered prior or subsequent to the administration of the CNTN1 inhibitor. In one embodiment, the second therapy is a chemotherapeutic agent that is also useful to treat prostate cancer, or to treat side effects associate therewith. The second therapy may also be androgen deprivation therapy.

[0051] In another aspect of the invention, a method of determining tamoxifen resistance in breast cancer is provided, comprising determining the level of CNTN1 in breast cancer cells, comparing the CNTN1 level to a control level, and determining that the mammal has tamoxifen resistant breast cancer when the level of CNTN1 in the sample is greater than the control level. CNTN1 has been determined to facilitate the development of tamoxifen resistance in breast cancer. CNTN1 was detected in breast cancer cells, including an aggressive HER2+ breast cancer cell line (HCC1954). Ectopic expression of CNTN1 increases tamoxifen resistance in tamoxifen sensitive cells, while tamoxifen resistant cells express higher levels of CNTN1 than a control level expressed in tamoxifen sensitive cells (e.g. by at least about 10% or greater, e.g. by 20%, 30%, 40%, 50% or greater).

[0052] The above disclosure generally describes aspects and embodiments of the present invention. It is believed that one of ordinary skill in the art could, using the preceding description, make and use the invention, e.g. practice the methods of the present invention. A more complete understanding can be obtained by reference to the following specific examples.

These examples are described solely to illustrate preferred embodiments of the present invention and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Other generic configurations would be apparent to one skilled in the art. Documents such as literature references, patents or patent applications, referred to herein are hereby incorporated by reference.

[0053] Although specific terms have been used in these examples, such terms are intended in a descriptive sense and not for purposes of limitation. Methods referred to but not explicitly described in the disclosure and these examples are reported in the scientific literature and are well known to those skilled in the art.

Example 1

Methods:

[0054] Cell lines and plasmids - All cell lines used was purchased from American Type Culture Collection (ATCC) and cultured in MEM (DU145), F12 (PC3), RPMI-1640 (LNCaP, HCC1954, BT549, BT474, T47D, ZR-75-1) and DMEM (MCF7, MDAMB453) supplemented with 10% FBS (Sigma Aldrich) and 1% Penicillin-Streptomycin (Life Technologies). The androgen-independent LNCaP derivative C4-2 was kindly provided by Dr. Martin Gleave at The University of British Columbia, Vancouver, BC, Canada (as described in Thomas et al. *Mol Cancer Ther* 10, 347-59 (2011)). C4-2 was cultured in RPMI-1640 media supplemented with 5% FBS and 1% Penicillin-Streptomycin. CNTN1 shRNA was purchased from Santa Cruz Biotechnology (Santa Cruz) and CNTN1 isoform 3 cDNA was purchased from Open Biosystems.

Generation of DU145 spheres

[0055] DU145 prostate cancer stem-like cells (spheres) were isolated and propagated as previously published (Rybak et al. *Biochim Biophys Acta* 1813, 683-694 (2011)). Briefly, DU145 monolayer cells were individualized with TrypLE Express solution (Life Technologies) and subsequently resuspended at a density of 5,000 cells/mL in serum-free (SF) media (DMEM/F12 at a 3:1 mixture) (Life Technologies) supplemented with 0.4% bovine serum

albumin (BSA) (Bioshop Canada Inc) and 0.2× B27 minus Vitamin A (Life Technologies) in T75 flasks. Typical spheres were formed in 10 to 12 days.

Knockdown of CNTN1 in spheres

[0056] Hairpin shRNAs (control/Ctrl and CNTN1) were expressed by a retroviral-based shRNA vector (Santa Cruz Biotechnology). Knockdown of CNTN1 was carried out according to published conditions (Yan et al. *PLoS One* 8, e65463 (2013)). Briefly, a gag-pol-expressing vector, a rev-expressing vector and an envelope-expressing vector (VSV-G) (Stratagene) were transiently co-transfected with a designed retroviral plasmid into 293T cells. The virus-containing medium was harvested 48 hours later, filtered through a 0.45 µM filter, and centrifuged at 20,000 g for 120 minutes to concentrate the retrovirus. Polybrene (10 µg/ml, Sigma Aldrich) was added before infection and cells were selected for stable integration with puromycin (1µg/mL, Sigma Aldrich).

Retroviral overexpression of CNTN1

[0057] Human CNTN1 isoform 3 cDNA was further modified to generate the full length isoform 1 of CNTN1 as previously described (Yan et al. *PLoS One* 8, e65463 (2013)). Briefly, PCR primers were synthesized flanking the C-terminus fragment present in isoform 1, but missing in isoform 3. The C-terminus fragment was then ligated with the commercially purchased isoform 3. The full length isoform 1 cDNA for CNTN1 was subsequently cloned into a retroviral vector, pBabe. Overexpression of CNTN1 was carried out using a gag-pol expressing vector and an envelope expressing vector (VSV-G) (Stratagene, Mississauga, ON). All steps were carried out in the same manner described above for the knockdown of CNTN1. The pBabe vector without CNTN1 was used as an empty vector control.

Gene expression analysis

[0058] Total RNA was isolated from DU145 and DU145 prostate cancer stem-like cells with TRIZOL (Life Technologies). Gene expression was examined using the Affymetrix Human Gene 1.0 ST microarrays, which was purchased through the University Health Network Microarray Center (UHNMAC, www.microarrays.ca, Toronto, ON). Procedures were carried out at UHNMAC according to the protocol detailed by Affymetrix. Functional analysis of

differentially expressed genes was carried out using Ingenuity Pathway Analysis to determine top diseases and biological functions.

Western blot analysis

[0059] Cell lysates were prepared in a buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM NaF, 1 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 2 μ g/ml leupeptin and 10 μ g/ml aprotinin (Sigma Aldrich). A total of 50 μ g of cell lysates were separated on SDS-PAGE gel and transferred onto Amersham hybond ECL nitrocellulose membranes (Amersham). Membranes were blocked with 5% skimmed milk and then incubated with the indicated antibodies at 4°C overnight. Appropriate HRP-conjugated secondary antibodies were incubated for one hour at room temperature. Signals were detected using an ECL Western Blotting Kit (Amersham). The primary and secondary antibodies and the concentrations used were: anti-CNTN1 (1:200, R&D systems, AF904), Anti-AKT Ser473 phosphorylation (1:1000, Cell Signaling, 9271), anti-AKT (1:1000, Santa Cruz Biotechnology, 1618), anti-E-cadherin (1:2500, BD Biosciences, 610181), anti-actin (1:1000, Santa Cruz Biotechnology, 1615), anti-goat (1:3000, Santa Cruz Biotechnology, sc2473), anti-mouse (1:3000, GE Healthcare, NA931V) and anti-rabbit (1:3000, GE Healthcare, NA934V).

Collection of primary and metastatic PC

[0060] Prostate tissues were collected from patients who underwent prostate biopsies or radical prostatectomy at St. Joseph's Hospital in Hamilton, Ontario, Canada under the approval from the local Research Ethics Board (REB# 11-3472) and with consent from patients.

Immunohistochemistry (IHC)

[0061] Slides were deparaffinized in xylene, cleared in ethanol series and subjected to endogenous peroxidase quenching and heat-treated for 30 minutes in sodium citrate buffer (pH = 6.0) in a food steamer. Sections were blocked for 1 hour at room temperature in 10% normal goat serum. Primary antibody specific for CNTN1 (1:10, Sigma Aldrich, HPA041060) and PAP (1:300, Abcam, ab109004) was incubated with the sections overnight at 4°C. Negative controls were incubated with a non-specific rabbit IgG. Biotinylated secondary IgG and Vector ABC

reagent (Vector Laboratories) were subsequently added according to the manufacturer's instructions. Washes were performed with PBS. Chromogen reaction was carried out with diaminobenzidine (Vector Laboratories), and counterstained with hematoxylin (Sigma Aldrich). Images were taken with a light microscope (Olympus, BX41).

Nanostring gene expression

[0062] RNA was isolated from 10µm sections of FFPE prostatectomies using the High Pure FFPE RNA isolation kit following the manufacturer's protocol. Total RNA was sent to the Farncombe Metagenomics Facility at McMaster University for analysis using the nCounter Elements tagset. RNA probes were synthesized for the target genes in collaboration with technicians at Nanostring Technologies. Raw gene counts obtained from the nCounter system were imported into the nSolver software. Raw counts for each gene tag and samples were normalized against positive controls and three endogenous reference genes; ATP-Binding Cassette, sub-family F member 1, Glyceraldehyde-3-Phosphate Dehydrogenase and β -tubulin. Normalization was carried out by dividing the raw counts of each target gene by the geometric mean of the positive controls followed by the geometric mean of the endogenous reference genes. The fold change was determined by the ratio of the normalized gene counts in the tumor and normal tissues.

Tissue Microarray (TMA) staining and analysis

[0063] TMA 2 and TMA 5 were obtained from the Cooperative Prostate Cancer Tissue Resource. TMA2 was organized according to Gleason score and consisted of 1,128 prostate tissue cores that were derived from 250 PC tumors, 32 non-tumor cases, and 58 high grade prostatic intra-epithelial neoplasia (HGPIN) cases. TMA5 is a large patient cohort that was organized according to the biochemical recurrence of PC. Biochemical recurrence in Cooperative Prostate Cancer Tissue Resource was defined as an increase in serum PSA levels > 0.6 ng/ml (single value) or consecutive rise in serum PSA levels between 0.4 and 0.6 ng/ml after radical prostatectomy. TMA5 contained 1,616 PC tissue cores that were derived from 404 patients based on their PC biochemical recurrence. IHC was carried out as described above with the following modifications. Primary antibody specific for CNTN1 (1:3000, Sigma Aldrich, HPA041060) was incubated with the sections overnight at 4°C. This was followed by the use of a TSA™ Plus

Biotin kit (Perkin Elmer) according to the manufacturer's protocol. TMA slides were scanned using a ScanScope and analyzed using ImageScope software (Aperio). All spots (stained cores) were also manually examined to exclude those spots that were either scratched or contained no prostate glands (only stromal tissue). All spots were also manually scored. The scores obtained using the ImageScope software were representative of the scores obtained manually. Scores obtained using Imagescope software were converted to HScore using the formula $[(HScore = \% \text{ positive} \times (\text{intensity} + 1))]$. H-Scores were subtracted from the H-Scores obtained from stroma regions which were used for background subtraction. Scores were assigned to a scale of 0-3 (0-negative or background staining, 1-weak staining, 2-mod. staining, 3-strong staining). Positive staining was classified as moderate to strong staining with an H-score > 20.

Immunofluorescence staining

[0064] DU145 spheres were prepared onto slides by cytopsin and allowed to air dry for one hour prior to staining. Cells were washed with 1x PBS and fixed with 4% paraformaldehyde. Primary antibody goat anti-CNTN1 (1:50, R&D systems, AF904) was incubated overnight at 4°C, washed with 1xPBS and incubated with subsequent secondary antibodies for one hour. Cells were mounted with DAPI mounting medium (Vector Labs). Images were taken with a fluorescence microscope (Carl Zeiss, Axiovert 200).

Real time PCR analysis

[0065] Total RNA was isolated using TRIZOL (Life Technologies). Reverse transcription was carried out using superscript III (Life Technologies) according to the manufacturer's instruction. PCR primers used are as follows: Cntn1 (forward) 5' CAATAGTGCAGGGTGTGGAC 3' (SEQ ID NO: 1) and (reverse) 5' TGGCTAGGAGGTGCTTTCTT 3' (SEQ ID NO: 2). BChe (forward) 5' ACAGGCCAGCTTGTGCTATT 3' (SEQ ID NO: 3) and (reverse) 5' CAAAAGCCGAGGAAATTT TG 3' (SEQ ID NO: 4). Actin (forward) 5' ACCGAGCGCGGCTACAG 3' (SEQ ID NO: 5) and (reverse) 5' CTTAATGTCACGCACGATTTC 3' (SEQ ID NO: 6). Real-time PCR was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) in the presence of SYBR-green according to the manufacturer's instructions (Applied Biosystems). All samples were run in triplicate.

Cell proliferation assay

[0066] A total of 1000 cells of DU145 EV and DU145 CNTN1 cells was seeded into a 96 cell plate and incubated at 37°C for 5 days. Proliferation was measured using the WST-1 cell proliferation assay kit (Millipore) according to the manufacturer's instructions. Absorbance readings were measured with a plate reader at 420nm.

Colony Formation

[0067] Cells were seeded at the indicated number of cells per well in triplicates and allowed to grow for 1 week. Media was changed every 2 days. Cells were stained with crystal violet (0.5%).

Invasion Assay

[0068] Modified boyden chambers were commercially purchased consisting of inserts with an 8µm pore membrane coated with Matrigel (BD Biosciences) placed in a 24-well plate. Invasion assays were performed according to the manufacturer's procedure. Briefly, matrigel inserts were given 2 hour to rehydrate at 37°C prior to use in the presence of 0.5 ml of medium. Complete medium (0.5 ml) supplemented with 10% fetal bovine serum (FBS) was placed in the lower chamber. A total of 5×10^4 cells were seeded into the top chamber of the insert in 0.5 ml of serum-free medium for 22 hours. Cells that passed through the membranes were fixed and stained with crystal violet (0.5%, Sigma Aldrich). Percentage of invasive cells was calculated by dividing the number of cells passing through the 8 µm pore size matrigel membrane by the number of cells migrating through the control membrane and multiplying by 100.

Formation of mice xenografts and lung metastasis

[0069] A total of 10^6 DU145 EV (n=4) and DU145 CNTN1 (n=5) and 10^4 DU145 shCTRL (n=4) and shCNTN (n=6) spheres were individualized and resuspended in media/matrigel mixture (1:1 volume), followed by implantation of 0.1mL of this mixture subcutaneously (s.c.) into flanks of 8-week-old male NOD/SCID mice (The Jackson Laboratory). Mice were inspected for tumor appearance, by observation and palpation, and tumor growth was measured weekly using a caliper. Tumor volumes were determined using the standard formula: $L \times W^2 \times 0.52$, where

L and W are the longest and shortest diameters, respectively. For the generation of lung metastasis, 10^6 individualized DU145 EV (n=4) and CNTN1 (n=5) cells was resuspended into 0.3mL of PBS and injected through the lateral tail vein of NOD/SCID mice. Mice were scarified at 16 weeks post injection and lungs harvested and examined for tumor nodules. Tumour volumes were photographed and measured using ImageJ. All animal work was carried out according to experimental protocols approved by the McMaster University Animal Research Ethics Board.

Statistical analysis

[0070] Statistical analysis was performed using student t-test unless otherwise specified. Two-way ANOVA was used to determine significance for tumour growth. For comparison between H-score intensity and PC Gleason scores, a one-way ANOVA was performed. For comparison between CNTN1 staining for non-cancer versus cancer and between PC Gleason a chi-square test for trend was performed. A log-rank test was performed to assess statistical significance between survival curves. All tests were two tailed. A p-value <0.05 was considered statistically significant for all tests.

Results:

CNTN1 promotes prostate cancer cell invasion but not proliferation

[0071] It is becoming increasingly clear that cancer stem cells (CSCs) are a major contributor to cancer progression and metastasis. Sphere cells were isolated from DU145 cells. These sphere cells are likely PCSCs because of their elevated (100 fold) ability for tumor initiation in NOD/SCID mice, as well as spheres can be re-isolated from xenograft tumors. To examine whether DU145 sphere cells (PCSCs) were associated with unique PC-promoting factors, gene expression between DU145 non-stem (monolayer) and PCSCs was profiled. Among a set of genes whose expression was altered was a top candidate, CNTN1. Its expression was increased approximately 10 fold in sphere cells compared to monolayer cells (Fig 1A). The upregulation was confirmed by both real-time PCR (Fig 1B) and western blot (Fig 1C). Consistent with CNTN1 as a cell surface adhesion protein in neural cells, the surface presence of CNTN1 in DU145 sphere cells was apparent under both immunofluorescence (Fig 1D) and immunohistochemistry staining against a non-specific IgG control. To consolidate CNTN1

upregulation in PCSCs, it was shown that both LNCaP and PC3 cells did not express CNTN1 when cultured in the presence of 10% fetal bovine serum (FBS), a situation similar to DU145 monolayer cells (Fig 1C). However, a robust increase in CNTN1 mRNA was detected in PC3 cells cultured under serum free (PC3-SF) PCSC-culturing conditions (Fig 1E). PC3-SF cells do not proliferate slower than PC3 cells cultured in a FBS-containing medium (data not shown), adopted a different morphology, and formed significantly more colonies, suggesting that PC3-SF cells acquired PCSC characteristics. Under SF-PCSC culture conditions, LNCaP did not survive (data not shown). Taken together, the above observations reveal the upregulation of CNTN1 in PCSCs.

[0072] The apparent cell surface localization of CNTN1 in DU145 spheres suggests CNTN1 as being functional. To address this possibility, ectopic CNTN1 was expressed in DU145 monolayer cells to a level comparable to the endogenous levels in DU145 sphere cells (Fig 1F). In comparison to DU145 empty vector (EV) cells, ectopic CNTN1 did not alter cell proliferation (Fig 2), but significantly enhanced DU145 cell invasion (Fig 1G). To further support these observations, a CNTN1-expressing stable line was also constructed in LNCaP C4-2 cells (Fig 3A). In comparison to the EV cells, ectopic CNTN1 did not dramatically affect the cell's ability to form colonies (Fig 3B), but promoted LNCaP C4-2 cell's invasion ability (Fig 3C). Collectively, thus, CNTN1 promotes prostate cancer cell invasion.

CNTN1 enhances AKT activation and reduces E-cadherin expression in PC cells

[0073] One of the major pathways affecting cell invasion is that of PI3K/AKT. To examine the impact of CNTN1 on AKT activation, an enhancement of AKT activation was observed in DU145 CNTN1 and LNCaP C4-2 CNTN1 cells in comparison to the respective EV cells in response to serum stimulation (Fig 4A, Fig 3E), despite that ectopic CNTN1 did not affect the basal levels of AKT activation in both lines (Fig 3D). Conversely, knockdown of CNTN1 in DU145 PCSCs reduced AKT activation (Fig 4B). To further determine the relevance of CNTN1-affected AKT activation during prostate tumorigenesis, the status of AKT activation in xenograft tumors produced by DU145 cells was examined and it was found that CNTN1 expression was modulated. In comparison to DU145 EV cell-derived xenograft tumors, a clear elevation of AKT activation was detected in DU145 CNTN1 monolayer cell-produced xenograft tumors.

Furthermore, reduction of AKT activation in xenograft tumors produced by CNTN1-knocked down DU145 sphere cells was observed.

[0074] Another key event leading to increases in the invasive ability of epithelial cell-originated tumors is the loss or reduction of E-cadherin. The possibility that CNTN1 may reduce E-cadherin levels is in line with the above observation in which CNTN1 activated AKT and that AKT plays a role in E-cadherin downregulation. In supporting this scenario, overexpression of CNTN1 reduced E-cadherin levels in DU145 monolayer cells in comparison to DU145 EV cells (Fig 4C); conversely, knockdown of CNTN1 in DU145 sphere cells led to E-cadherin upregulation (Fig 4D). Furthermore, DU145 CNTN1 cell-produced xenograft tumors exhibited lower levels of E-cadherin than DU145 EV cell-originated xenograft tumors; DU145 sphere cells in which CNTN1 was knocked down produced xenograft tumors with elevated levels of E-cadherin. Taken together, these observations demonstrate that CNTN1 reduces E-cadherin expression concurrently with the upregulation of AKT activation.

[0075] CSCs are defined by their elevated ability for cancer initiation in immunocompromised mice. The specific expression of CNTN1 in DU145-derived PCSCs and its role in promoting PC cell invasion and AKT activation as observed above collectively indicate a role of CNTN1 in DU145 PCSCs-associated tumor initiation. To examine this possibility, CNTN1 was knocked down in DU145 sphere cells (Fig 4B). CNTN1 downregulation did not have an apparent effect on the cell's ability to form spheres (data not shown). However, in comparison to DU145 Ctrl shRNA sphere cells, knockdown of CNTN1 significantly reduced the cell's ability on tumor initiation (Fig 5A). CNTN1 expression was significantly lower in xenograft tumors produced by CNTN1 knockdown sphere cells. Additionally, overexpression of CNTN1 in DU145 monolayer cells enhanced xenograft tumor formation (Fig 5B). CNTN1 levels remained higher in DU145 CNTN1 cell-produced xenograft tumors than DU145 EV cell-derived tumors. CNTN1 was clearly detected at the cell surface in DU145 CNTN1 monolayer and DU145 Ctrl sphere cell produced xenograft tumors. Interestingly, despite DU145 EV monolayer cells expressing no detectable levels of CNTN1 *in vitro*, clusters of CNTN1-positive cells with membrane localization were clearly detected. On the other hand, CNTN1-negative clusters of cells were also present in DU145 CNTN1 monolayer and DU145 Ctrl shRNA sphere cell-produced

xenograft tumors. Collectively, the above results demonstrate a critical role of CNTN1 in tumor initiation for prostate cancer.

CNTN1 enhances prostate cancer metastasis

[0076] The observation that CNTN1 promotes DU145 cell invasion (Fig 1G) suggests a role of CNTN1 in PC metastasis. To examine this possibility, DU145 EV and CNTN1 cells were administrated into NOD/SCID mice via the tail vein. While DU145 EV cells formed lung metastases, it was clear that DU145 CNTN1 produced larger lung nodules, especially in the 16mm³ or larger (16+mm³) tumor group (Fig 6A). As expected, CNTN1-positive cell clusters were detected in both DU145 EV and CNTN1-produced lung metastases, but the latter contained more CNTN1-positive cells with higher levels. Overall, a greater number of lung nodules were generated in both the $\leq 15\text{mm}^3$ and 16+mm³ categories for DU145 CNTN1 cells (Fig 6B).

CNTN1 associates with prostate cancer progression

[0077] To further support a role of CNTN1 in promoting prostate cancer tumorigenesis, its expression in primary prostate cancer tissues was examined. An anti-CNTN1 (Sigma) antibody specifically recognized tumor-associated CNTN1 in IHC staining, evidenced by no detectable staining with control IgG (data not shown) and the positive signals could be competed out by a CNTN1 peptide. In a limited number of patients consisting of three Gleason Score (GS) 6-7, six GS 8-10, 3 pairs of local and lymph node metastasis, and 9 bone metastases were examined; CNTN1 was stained either negative or at very low levels in normal prostate glands and positively in advanced (GS8-10) prostate carcinomas. The cell surface presence of CNTN1 was clearly observed in both primary and the matched lymph node metastasis and in bone metastases. The bone metastases were confirmed as being originating from prostate cancer, as evidenced by the positive staining for prostatic acid phosphatase. CNTN1 expression was examined using two large tissue microarrays (TMAs), TMA2 and TMA5, obtained from the Cooperative Prostate Cancer Tissue Resource. TMA2 was organized according to PC progression; TMA5 was arranged based on outcome. TMA2 had 1,128 prostate tissue cores covering 250 patients; TMA5 contained 1,616 prostate tissue cores derived from 404 patients (<http://www.cpcetr.net/tma.html>). All tissue cores were visually inspected. Typical staining for non-tumor tissue, high grade PIN (HGPIN), GS6 and GS9 carcinomas shows the trends of elevating CNTN1 expression following

PC progression. Quantification of CNTN1 staining was derived from 637 patients from those whose GS score was obtained, and revealed that levels of CNTN1 expression increased following prostate cancer progression from GS5/6 carcinomas to GS7, and to GS8/9 carcinomas (Fig 7A). The number of CNTN1-positive cases was also increased following the progression from non-tumor prostate tissues to HGPINs and to carcinomas (Table 1).

Among carcinomas of different severities, the percentage of CNTN1 positive tumors increased from GS5 to GS9 carcinomas (Table 1). Furthermore, Kaplan-Meier survival analysis revealed that patients with CNTN1-positive prostate cancer were associated with decreased biochemical recurrence free survival (Fig. 7B). Collectively, this evidences that CNTN1 expression associates with prostate cancer progression and the reduction of biochemical recurrence-free survival.

Table 1 **CNTN1 is associated with prostate cancer progression**

	Positive (%)	Negative (%)	<i>p</i> -value*
Non-Tumour	26	74	<0.05
HGPIN	33	67	
Cancer	42	58	
Gleason 5	50	50	<0.01
Gleason 6	81	19	
Gleason 7	95	5	
Gleason 8	89	11	
Gleason 9	90	10	

* chi-square test for trend. Total number of patients: 637.

CNTN1 affects pathways that are important for tumorigenesis

[0078] Prostate cancer progression and metastasis is a complex process involving numerous factors. CNTN1 has been found to enhance tumor initiation and metastasis, and to be associated with PC progression and biochemical recurrence indicating that CNTN1 may alter the expression of tumorigenesis-relevant genes. In line with this possibility, analysis of gene expression using the Affymetrix platform revealed the alterations of the expression of numerous genes by ≥ 1.5 fold following ectopic expression of CNTN1 in DU145 monolayer cells and knockdown of CNTN1 in DU145 spheres (Fig 8A, B; Table 1). The expected changes of CNTN1 in the overexpression and knockdown cells were confirmed (Fig 8A, B; Table 1). Knockdown of CNTN1 in spheres predominantly affected the disease of cancer, while overexpression of

CNTN1 in DU145 monolayer cells alerted genes involved in cancer, immunological disorders, and inflammation (Fig 8C, top panels); the latter two processes are well known to impact tumorigenesis. The main physiological processes affected by these candidate genes are organism and organ development (Fig 8C, middle panels). Consistent with CNTN1 as a neural cell adhesion protein, these genes contribute to neurological disorders (Fig 8C, top right panel) and nervous system development (Fig 8C, middle left panel). Knockdown of CNTN1 in DU145 sphere cells affected the expression of genes functioning in cell-cell communications and maintenance, is consistent with CNTN1 being a cell surface protein and with PCSCs' role in maintaining a tumor mass (Fig 8C, bottom left panel). The overexpression of CNTN1 in DU145 monolayer cells altered the expression of genes functioning in cell movement (Fig 8C, bottom right panel) and supports its role in promoting DU145 cell's invasion (Fig 1G). In summary, knockdown of CNTN1 in DU145 spheres (PCSCs) affected genes largely functioning in tumorigenesis, while overexpression of CNTN1 in DU145 monolayer cells altered genes involved in multiple processes, including tumor cell invasion (Fig 9). Collectively, these observations demonstrate a general role of CNTN1 in promoting PC progression.

[0079] Decreasing butyrylcholinesterase (BChE) activity in blood correlates with PC progression and bone metastasis. BChE is at the top of the list of altered/downregulated genes upon CNTN1 overexpression (Fig 8A, Table 2). BChE was also robustly reduced in DU145 sphere cells compared to DU145 monolayer cells (Fig 10A). The reduction of BChE in DU145 and LNCaP C4-2 CNTN1 cells in comparison to the respective EV cells was then determined (Fig 10B). More importantly, in the analysis of BChE expression in 7 paired non-tumor prostate and prostate carcinoma tissues using the NanoString Technology, Bche mRNA was reduced in 5 out of 7 patients; the levels of downregulation exceeded that of PTEN (Fig 8D). The validity of the assay was further made certain by the demonstration of the immense increases of TMPRSS2-ERG in prostate carcinomas (Fig 8D), the recurrent fusion event detected in more than 50% of prostate cancer.

Table 2A Affymetrix gene expression changes for CNTN1 overexpression and knockdown

Gene Expression Changes for DU145 EV compared to DU145 CNTN1					
Gene Symbol	RefSeq	FC ^a	Gene Name	Gene Description	References
CNTN1	NM_001843NM_175038	3.4581987	contactin 1	Cell adhesion molecule involved in neural development (1-3)	
BCHE	NM_000055	-2.7004411	butyrylcholinesterase	Catalyzes the hydrolysis of butyrylcholine. Abnormally (4-7)	
COTL1 pseudogene	NM_021149.3	2.2320216	coactosin-like 1 (Dictyostelium) (COTL1) pseudogene	Pseudogene of COTL1. COTL1 binds to actin filaments (8, 9)	
IFITM3	NM_021034	-1.8500125	interferon induced transmembrane protein 3 (1-3U)	Anti-viral activity amongst members of the Bunyavirus (10-14)	
DND1	NM_194249	1.8486362	dead end homolog 1 (zebrafish)	RNA binding protein that regulates microRNA. Impo (15-17)	
PYY	NM_004160	-1.822233	peptide YY	Enterohormone secreted by the GI tract in (18-23)	
FKBP1A	NM_000801NM_054014	-1.8034257	FK506 binding protein 1A, 12kDa	Immunophilin protein that binds to FK506 and rapam (24, 25)	
IL6	NM_000600	1.782458	interleukin 6 (interferon, beta 2)	Cytokine involved in inflammation. Associated with (26-29)	
FMN1	NM_001277313	-1.7491621	formin	Important in limb formation. Regulate actin polymer (30-33)	
RPL26L1	NM_016693	-1.7240521	ribosomal protein L26-like 1	Function unknown	
UQCRCF51	NM_006603	-1.7004228	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide	Subunit of cytochrome bc1 complex in the mitochondr (34-37)	
BRD9	NM_023924NM_0010098	1.6760395	bromodomain containing 9	BRD recognizes acetylation motifs. BRD9 function u (38)	
OR10Z1	NM_001004478	1.6704825	olfactory receptor, family 10, subfamily Z, member 1	Receptors responsible for smell. Interacts with odoran (39)	
SEPT9	NM_001113491.1	-1.6519994	septin 9	Important in cytokinesis and cell cycle control. Assoc (40-43)	
C15orf48	NM_032413NM_197955	-1.6516455	chromosome 15 open reading frame 48	Expressed in the esophagus, stomach, small intestine (44, 45)	
PAGE2B	NM_001015038	-1.6485145	P antigen family, member 2B P antigen family, member 2 (pre)	Primary expressed in testes (46)	
EIF5A	NM_001143760.1	-1.643807	eukaryotic translation initiation factor 5A	Upregulated expression is associated with tumorigen (47-49)	
LCE2C	NM_178429	-1.6245959	late cornified envelope 2C	Precursors of the cornified envelope of the stratum (50)	
TUBB4Q	NM_020040	-1.5992213	tubulin, beta polypeptide 4, member Q	Subtype of tubulin (51)	
MAL2	NM_052886	-1.5878217	mal, T-cell differentiation protein 2	Component of lipid raft. Reported to be associated wi (52-55)	
ARID3B	NM_006465	-1.5865202	AT rich interactive domain 3B (BRIGHT-like)	Upregulated in cancer. DNA binding protein that reg (56, 57)	
CYHR1	NM_001129838.1	-1.5823977	cysteine/histidine-rich 1 hypothetical protein LOC100132129	Identified to bind to galectin-3 by a yeast two-hybrid (58, 59)	
CDRT1/IRIM16	NM_006382	1.5586788	CMT1A duplicated region transcript 1 tripartite motif contain	Member of the F-box protein. F-box proteins acts as (60)	
DEX1	NM_014015	-1.5537672	dexamethasone-induced transcript	Gluocorticoid induced protein coding gene. Frequent (61, 62)	
KIR2DS2/KIR3DL1NM_02312NM_014511		1.5519682	killer cell immunoglobulin-like receptor, two domains, short cy	Associated with rheumatoid arthritis. Membrane bon (63, 64)	
MIPEP	NM_178540	-1.5480449	mitochondrial intermediate peptidase collagen triple helix re	Involved in processing and maturation of oxidative pl (65, 66)	
SDHD	NM_003602	1.5452253	succinate dehydrogenase complex, subunit D, integral membra	Complex in respiratory chain for oxidation of succina (67-69)	
CST6	NM_001323	-1.533219	cystatin EM	Associated with breast cancer. Member of the cystein (70-73)	
OSTbeta	NM_178859	1.5300515	organic solute transporter beta	Subunit of the organic solute transporter for bile acid (74-76)	
CCL3	NM_003983	-1.522853	chemokine (C-C motif) ligand 3	Plays a role in the inflammatory response. Binds recep (77-79)	
PLK2	NM_006622	1.5172838	polo-like kinase 2 (Drosophila)	Serine/threonine protein kinase important in normal c (80-82)	
PIGF	NM_173074NM_002643	1.5123001	phosphatidylinositol glycan anchor biosynthesis, class F	Protein involved in synthesis of glycosylphosphatidyl (83, 84)	
PLEKHA3	NM_019091	-1.5078716	pleckstrin homology domain containing, family A (phosphoino	Regulates transport from golgi to cell surface membra (85-87)	
METTL1	NM_023033	1.5031359	methyltransferase like 1	Associated with tRNA m ⁶ G-methyltransferase activity (88, 89)	

^a Fold change relative to control

Table 2B

Gene Expression Changes for Spheres shCNTN1 compared to Spheres shCNTN1					
Gene Symbol	RefSeq	FC ^a	Gene Name	Gene Description	References
HAPLN1	NM_001884	5.1425776	hyaluronan and proteoglycan link protein 1	Component of cartilage extracellular matrix. Stabilize (90-92)	
CNTN1	NM_001843NM_175038	-4.5103955	contactin 1	Cell adhesion molecule involved in neural development (1-3)	
CLDN16	NM_006580	-4.0595984	claudin 16	Component of tight junctions in epithelial or endothel (93-95)	
SNORA23	NM_002962	2.1323938	small nucleolar RNA, H/ACA box 23	Non protein coding RNA required for rRNA maturation (96)	
KIR3DL1/KIR3DC1	NM_014431	1.9214258	killer cell lectin-like receptor subfamily C, member 4 killer ce	Important in regulating NK cell functions against tum (97, 98)	
EYA4	NM_004100NM_172105	-1.591951	eyes absent homolog 4 (Drosophila)	Potential transcriptional activator. Important for eye d (99-101)	
CDR1	NM_004065	1.8635496	cerebellar degeneration-related protein 1, 34kDa	Autoantibodies against CDR1 found in paraneoplastic (102-104)	
MYO15B	NM_003587	1.7702606	myosin XVb pseudogene	Similar to myosin XVa. Originated from genomic du (105)	
RAB43/ISY1	NM_198490	-1.7690837	RAB43, member RAS oncogene family ISY1 splicing factor 1	Regulator of vesicle movement and membrane traffi (106, 107)	
FAM130A2	NM_024969	-1.7602352	family with sequence similarity 130, member A2	Possible role in apoptosis. Binds to sequence AGAG (108, 109)	
MYO10	NM_012334	-1.6957346	myosin X	Unconventional myosin ubiquitously expressed at lo (110-112)	
CCDC51	NM_024681	-1.6933301	coiled-coil domain containing 51	Function unknown. No data available	
AKR1C2	NM_001354NM_205845	-1.6885077	aldo-keto reductase family 1, member C2 (dihydrodiol dehydro	Converts aldehydes and ketones to respective alcohol (113-115)	
TACSTD2	NM_002353	1.6491949	tumor-associated calcium signal transducer 2	Highly expressed in human carcinomas. Transmembr (116-119)	
CGA	NM_000735	1.6415079	glycoprotein hormones, alpha polypeptide	Alpha subunits for glycoprotein hormones, chorionic (120, 121)	
POP4	NM_006627	-1.6358767	processing of precursor 4, ribonuclease P/MRP subunit (S. cere	Subunit of the small nucleolar ribonucleoprotein com (122-124)	
CYP24A1	NM_000782	1.6325915	cytochrome P450, family 24, subfamily A, polypeptide 1	Catalyzes reactions including drug metabolism and (125-127)	
HIF1AN	NM_017902	-1.6303127	hypoxia-inducible factor 1, alpha subunit inhibitor	Negative regulator of hypoxia inducible factors. Hyd (128, 129)	
GUCY1B3	NM_000957	-1.6150277	guanylate cyclase 1, soluble, beta 3	Encodes subunit for soluble guanylate cyclase. Cataly (130)	
MET	NM_006245	1.5930927	met proto-oncogene (hepatocyte growth factor receptor)	Aberrant activation of MET in cancer. Proto-oncogen (131-133)	
PSMG3	NM_032302	-1.5859141	proteasome (prosome, macropain) assembly chaperone 3	Chaperone protein required for assembly of 20S prote (134)	
MPZL2	NM_144765NM_005797	-1.5702941	myelin protein zero-like 2	Member of the immunoglobulin superfamily. Express (135)	
RDH10	NM_172037	1.5671868	retinol dehydrogenase 10 (all-trans)	Converts all trans retinol to all trans retinal (136, 137)	
SOX2	NM_003106	-1.550381	SRY (sex determining region Y)-box 2	Transcription factor involved in the regulation of emb (138, 139)	
RTKN2	NM_145307	-1.548975	rotectin 2	Target peptide for Rho-GTPase. May play a potential (140)	
LAMA3	NM_198129NM_000227	1.5477984	laminin, alpha 3	Component of basement membrane. Implicated in can (141-143)	
CCDC88C	NM_001080414	-1.5476444	coiled-coil domain containing 88C	Negative regulator of Wnt signalling pathway and int (144)	
FLJ32810	NM_152432.2	-1.5416094	Homo sapiens Rho GTPase activation protein 42	Rho-specific GAP in smooth muscle cells. Important (145)	
ECHDC1	NM_001002030NM_0184	1.5412538	enoyl Coenzyme A hydratase domain containing 1	Expressed abundantly in brown adipose tissue, liver a (146)	
ZNF622	NM_033414	-1.537156	zinc finger protein 622	Identified as a substrate for MPK38 (147)	
DCLK1	NM_004734	-1.5336214	doublecortin-like kinase 1	Regulates microtubule polymerization. Putative cancer (148-151)	
ARL2/SNX15	NM_001667	1.5294169	ADP-ribosylation factor-like 2 sorting nexin 15	Regulates mitochondrial morphology, motility and A' (152, 153)	
TNS4	NM_032865	1.5292526	tenascin 4	Functions as an oncogene. Promotes migration (154-157)	
DISP2	NR_015403NR_037218	-1.5255487	dispatched homolog 2 (Drosophila) hypothetical LOC728211	Required for normal hedgehog signaling (158)	
SORL1	NM_0238105	1.5234814	soritin-like receptor, (LDR class) A repeats-containing	Association with Alzheimer's disease. Potentially imp (159-161)	
SDF2L1	NM_022044	-1.5223639	stromal cell-derived factor 2-like 1	ER localized protein. Function unclear. Increased in r (162, 163)	
SLC6A8/SLC6A1	NM_005629NR_003083	1.5091225	solute carrier family 6 (neurotransmitter transporter, creatine), 1	Transport creatine in and out of cells. Mutations lead (164, 165)	

Expression of CNTN1 in the blood of prostate cancer patients

[0080] Circulating prostate tumor cells (CTCs) and disseminated bone marrow prostate tumor cells (DTCs) display great prognostic significance. However, both CTCs and DTCs are heterogeneous cell populations; the current detection of CTCs relies on the presence of epithelial specific cell surface proteins, especially EpCAM. As the subpopulation of CTCs and DTCs with metastatic potential may undergo epithelial-mesenchymal transition (EMT), those positive for epithelial cell surface antigens may not be the source of metastasis. This possibility is supported by the detection of CTCs in 24 – 74% and DTCs in approximately 50% of PC patients, demonstrating the need for detecting aggressive cells/PCSCs in CTCs and DTCs to improve their prognostic potentials. CNTN1 is thus attractive for these applications. CNTN1 is thus attractive for these applications. A robust presence of CNTN1 in the blood of a patient with castration-resistant prostate cancer was clearly detected in comparison to 3 normal controls (Fig 11).

CNTN1 contributes to the development of tamoxifen resistance in breast cancer

[0081] Breast cancer is the major malignancy affecting women worldwide. The disease shares similarities with the male-specific prostate cancer. While prostate cancer depends on androgen, a predominant proportion of breast cancer requires estrogen signaling. The biosynthesis of both hormones are intimately related. Approximately 70% of breast cancers are estrogen receptor (ER) positive; these cancers are commonly treated by the disruption of ER signaling using tamoxifen. However, resistance commonly occurs, and is a major clinical problem in breast cancer therapy. Unfortunately, the mechanisms leading to tamoxifen resistance remains unclear.

[0082] The demonstrated role of CNTN1 in promoting prostate cancer prompted an examination of its potential role in enhancing resistance to tamoxifen. In the initial examination of a panel of breast cancer cell lines, CNTN1 was clearly detected in some lines, including a HER2+ line, HCC1954 (Fig 12). Ectopic expression of CNTN1 in CNTN1-negative MCF7 cells substantially elevated tumour cell survival to the cytotoxicity imposed by tamoxifen treatment (Fig 13). Tamoxifen-resistant MCF7 cells were produced by prolonged culturing (12 months) of the cells in the presence of low levels of tamoxifen. In comparison to tamoxifen-sensitive MCF7 cells, the resistant cells displayed 1.5 fold higher CNTN1 expression (Fig 14). Collectively, this indicates the role of CNTN1 in enhancing the resistance of breast cancer to tamoxifen. These

results together with the observations obtained in prostate cancer indicates that CNTN1 plays a role in the tumorigenesis of prostate and breast cancer.

[0083] To conclude, the specific expression of CNTN1 in DU145 cell-derived prostate cancer stem-like cells (PCSCs) and in PC3 cells cultured under PCSC conditions was found. *In vitro*, CNTN1 enhanced cell invasion and AKT activation, and reduced E-cadherin expression. *In vivo*, CNTN1 promoted xenograft tumor formation and lung metastasis concurrently with the upregulation of AKT activation and the downregulation of E-cadherin. In primary prostate cancer (PC), CNTN1 was detected in advanced PC, lymph node and bone metastases, and is associated with PC progression and the reduction of biochemical recurrence free survival.

CLAIMS

1. A method of determining prostate cancer, or progression, recurrence or metastasis of prostate cancer in a male mammal comprising the steps of determining in a biological sample obtained from the mammal the expression level of CNTN1 in the sample, comparing the CNTN1 expression level to a control level, and determining that the mammal has prostate cancer when the expression level of CNTN1 in the sample is greater than the control level.
2. The method of claim 1, wherein sphere-forming cells are isolated from the biological sample and determination of CNTN1 expression level in the sphere-forming cells is conducted.
3. The method of claim 2, wherein sphere-forming cells are isolated dissociating cells in the sample and culturing the dissociated cells in a serum-free media supplemented with EGF.
4. The method of claim 1, wherein the CNTN1 is selected from isoform 1, or a functionally equivalent variant thereof.
5. The method of claim 1, wherein the biological sample is selected from blood, plasma, serum, urine, cerebrospinal fluid, lymphatic fluid, bone marrow, lymph nodes, prostate tumour or prostate tissue.
6. The method of claim 1, wherein the CNTN1 expression level is determined using one or more methods selected from mass spectrometry, chromatography, immunoassay, electrophoretic techniques, and nucleic acid hybridization techniques.
7. The method of claim 1, wherein the control level is the CNTN1 expression level from a healthy unaffected individual, or a mean control value obtained from a healthy population of matched subjects.
8. The method of claim 1, wherein the control level is the level of one or more housekeeping or reference genes.
9. The method of claim 1, wherein the expression level of CNTN1 in the sample is at least 10-80% greater than the control level.

10. A method of treating prostate cancer in a male mammal comprising administering to the mammal a CNTN1 inhibitor.
11. The method of claim 10, wherein the CNTN1 inhibitor is a CNTN1 antibody.
12. The method of claim 10, wherein the CNTN1 inhibitor is an oligonucleotide inhibitor.
13. The method of claim 12, wherein the oligonucleotide inhibitor is selected from an antisense oligonucleotide, siRNA and shRNA.
14. A composition comprising a CNTN1 inhibitor and a pharmaceutically acceptable carrier.
15. A method of determining tamoxifen resistance in breast cancer, comprising determining the expression level of CNTN1 in breast cancer cells, comparing the CNTN1 expression level to a control level, and determining that the mammal has tamoxifen resistant breast cancer when the expression level of CNTN1 in the sample is greater than the control level.
16. The method of claim 15, wherein the CNTN1 is selected from isoform 1, or a functionally equivalent variant thereof.
17. The method of claim 15, wherein the control level is the CNTN1 expression level from a healthy unaffected individual, or a mean control value obtained from a healthy population of matched subjects.
18. The method of claim 15, wherein the control level is the level of one or more housekeeping or reference genes.
19. The method of claim 15, wherein the expression level of CNTN1 in the sample is at least 10-80% greater than the control level.

Figure 1

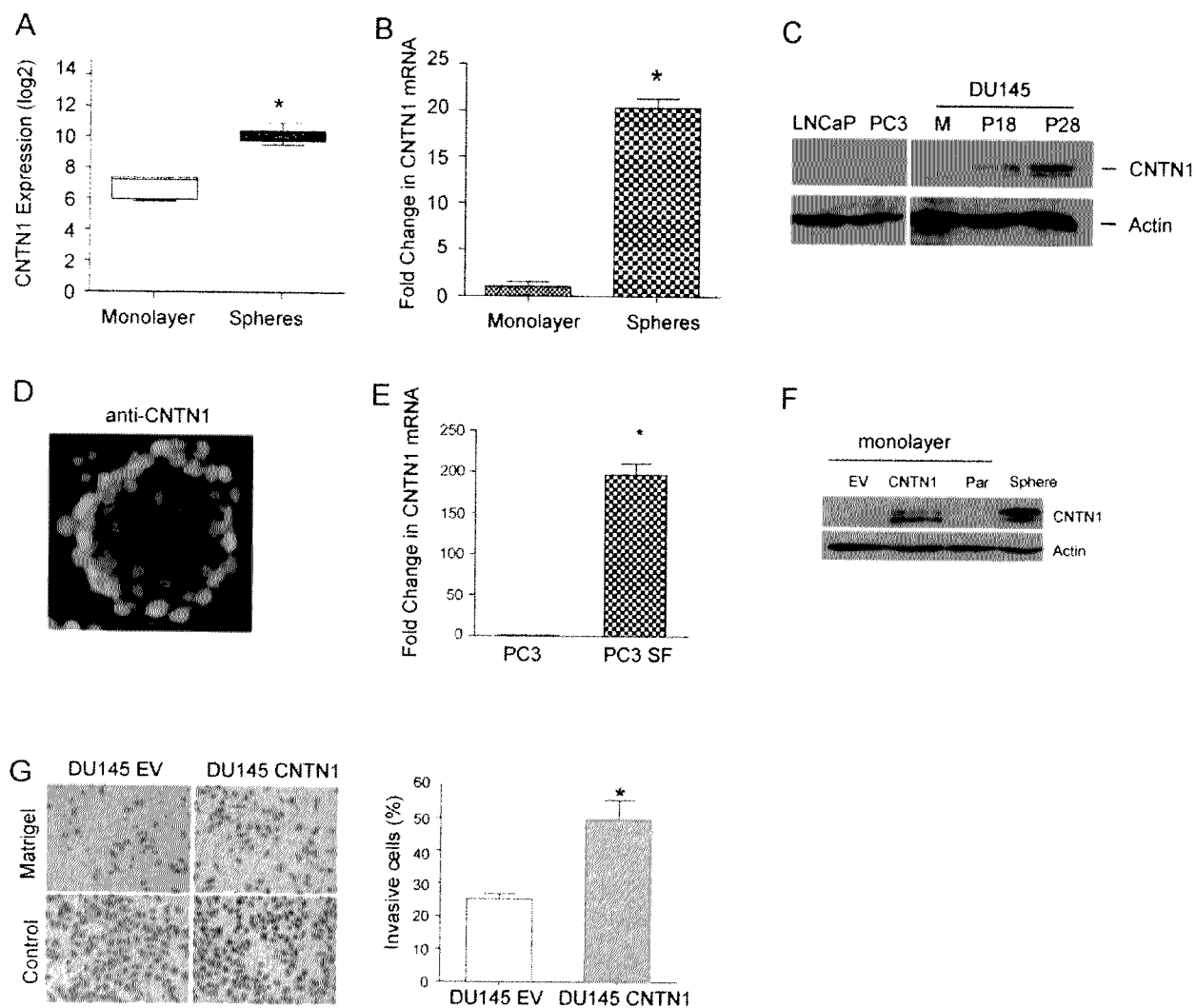


Figure 4

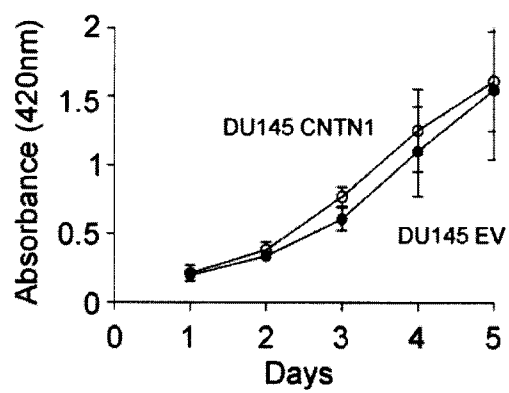


Figure 3

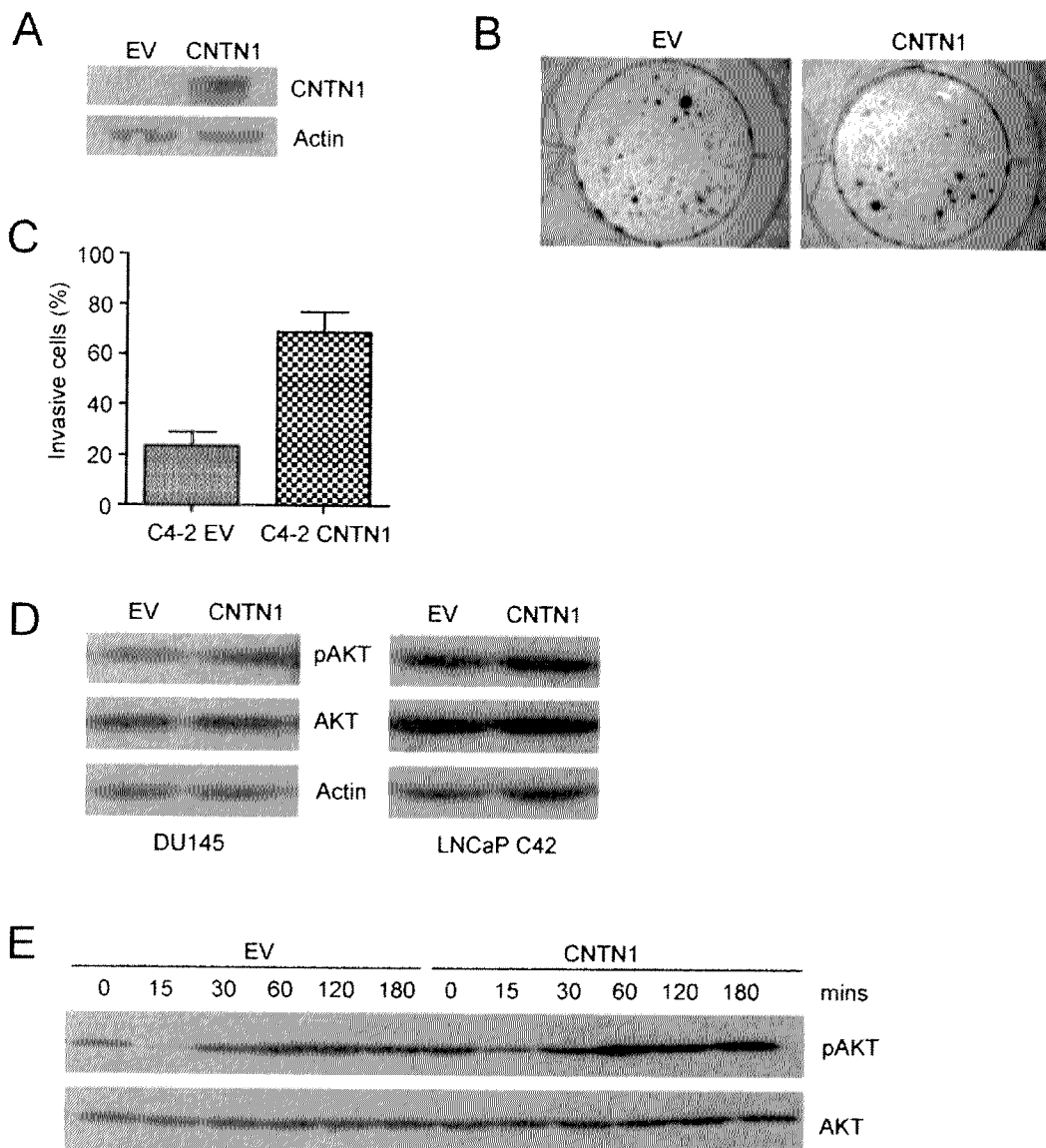


Figure 4

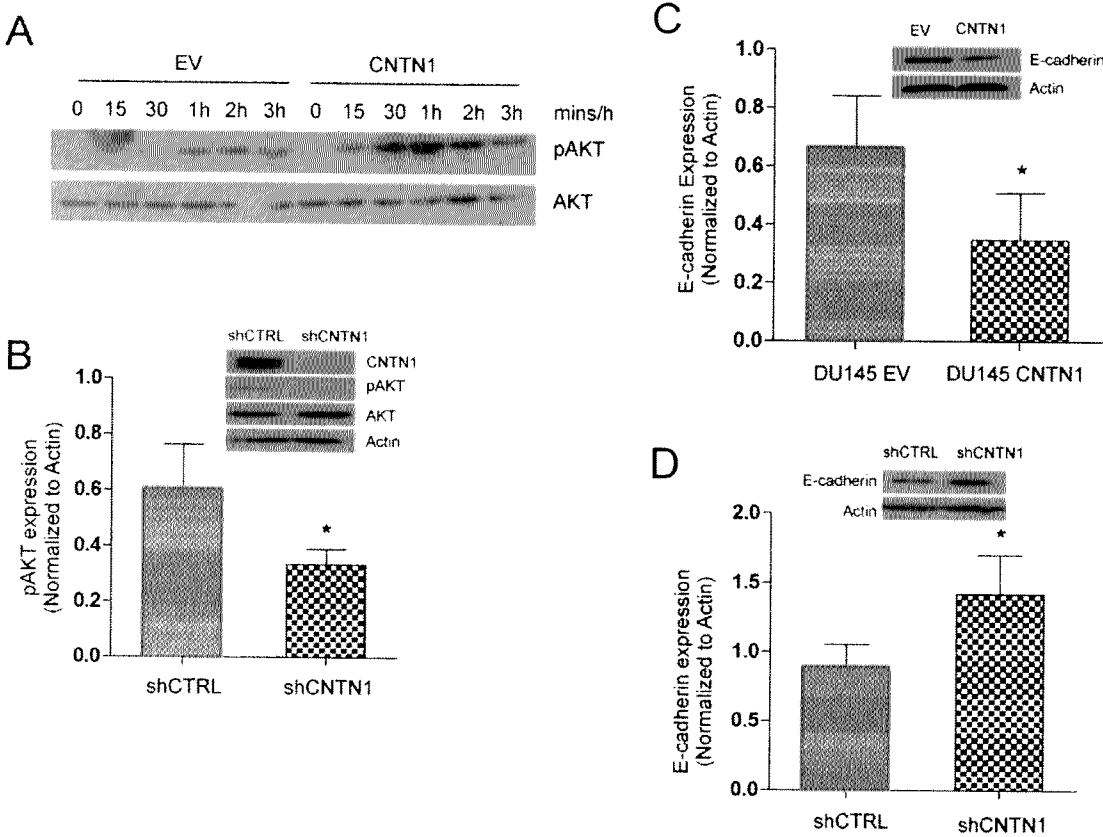


Figure 5

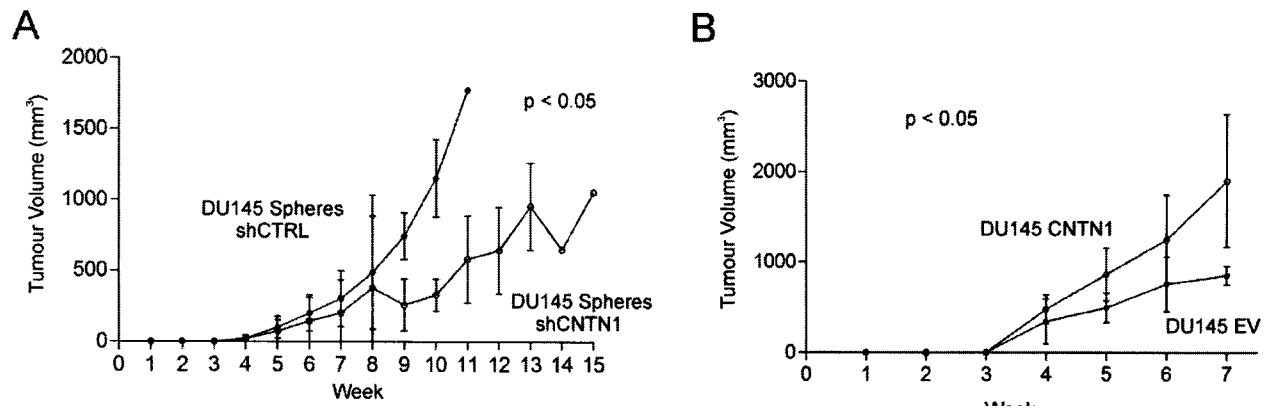
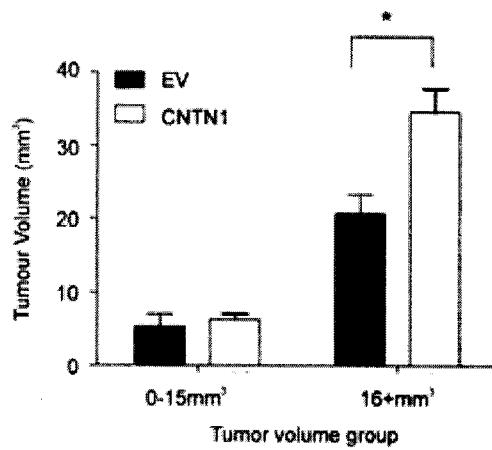


Figure 6

A



B

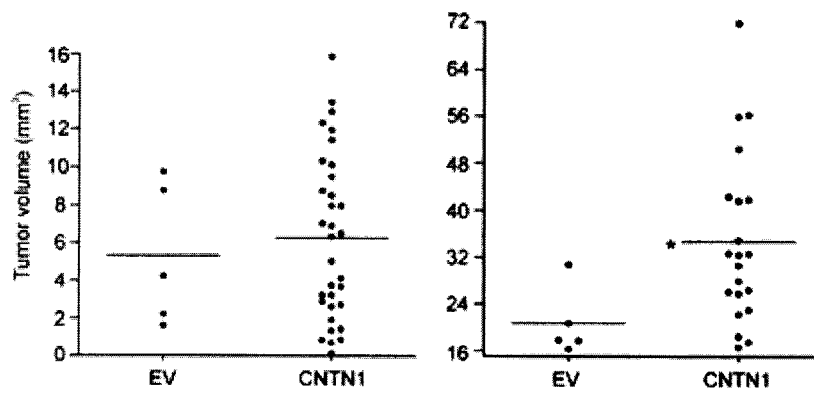


Figure 7

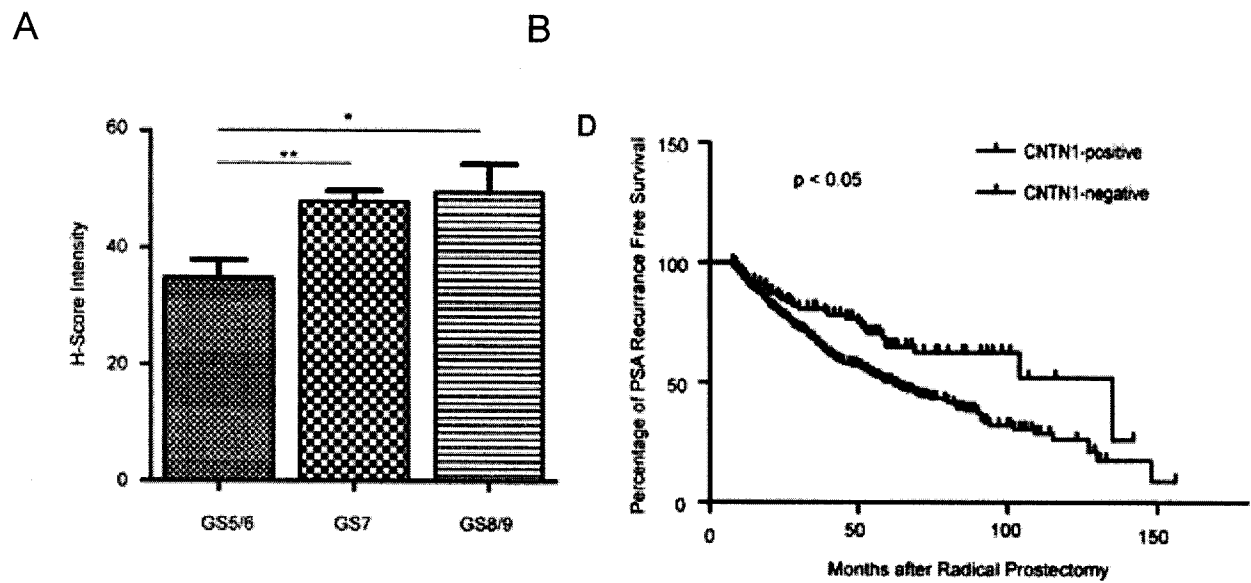


Figure 8

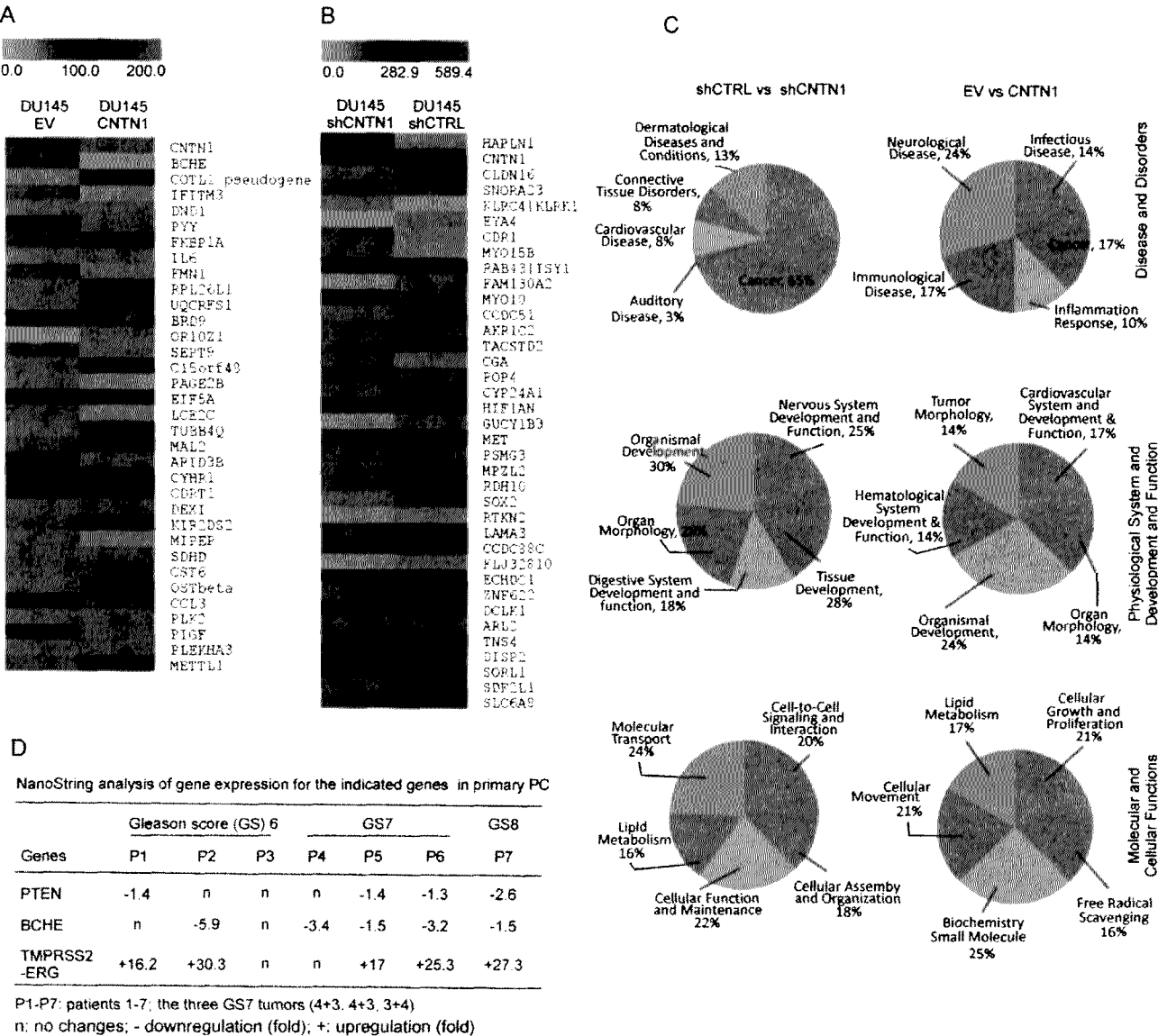
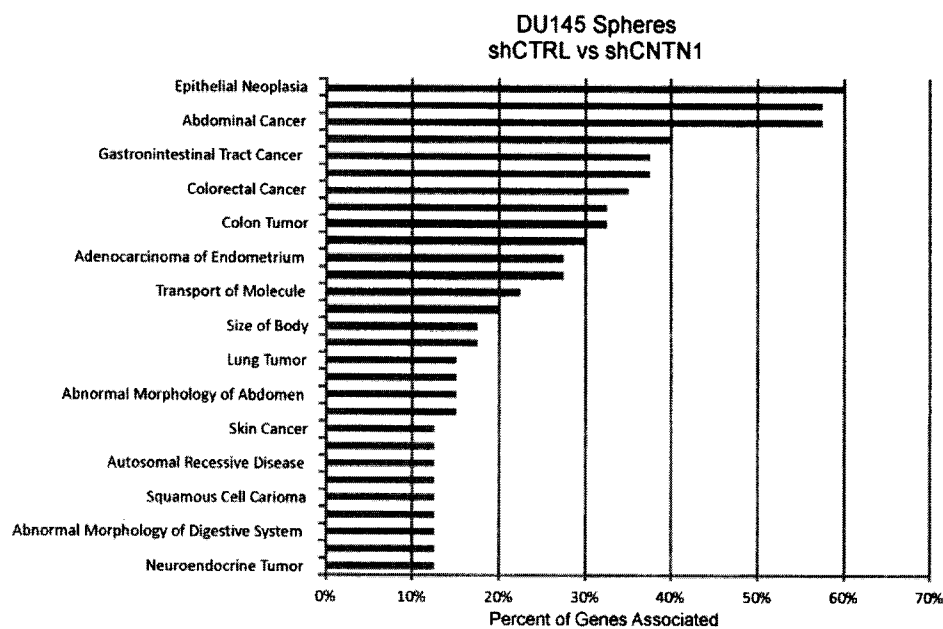


Figure 14

A



B

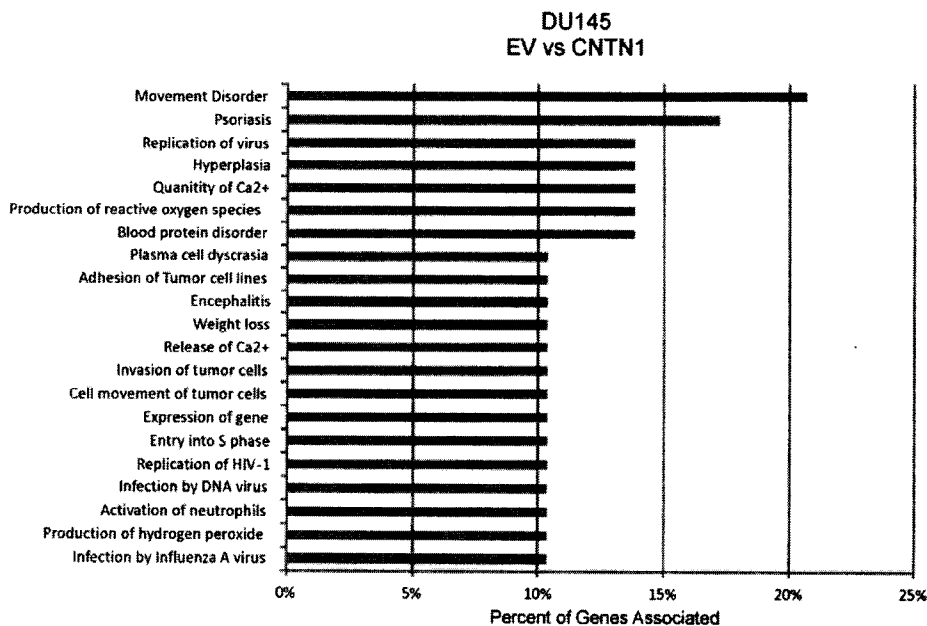
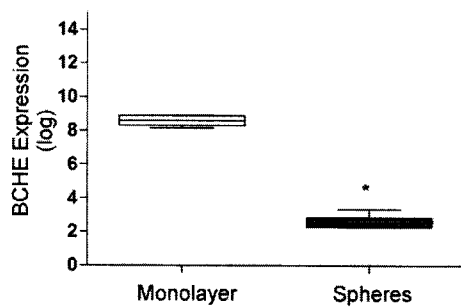


Figure 10

A



B

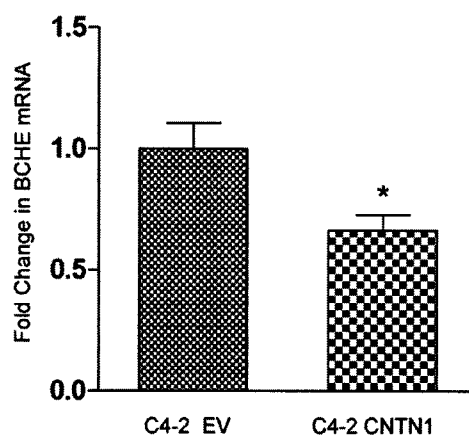
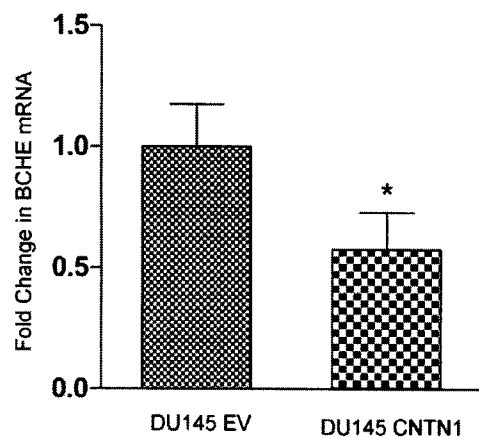


Figure 11

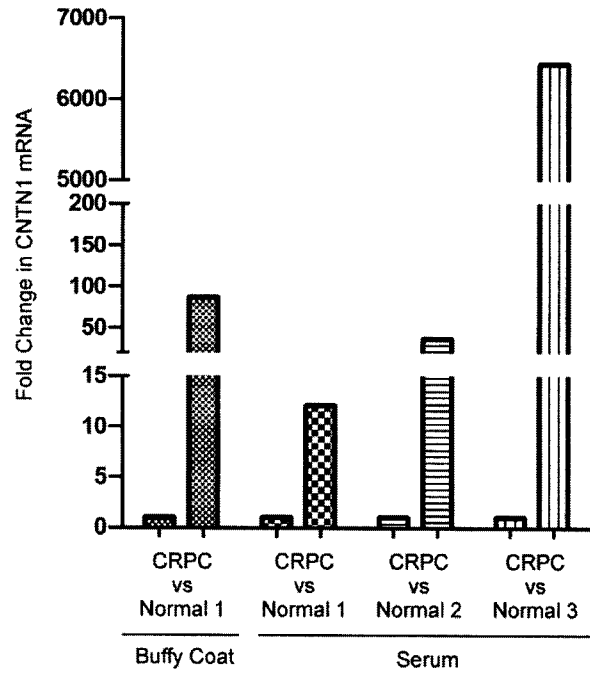


Figure 12

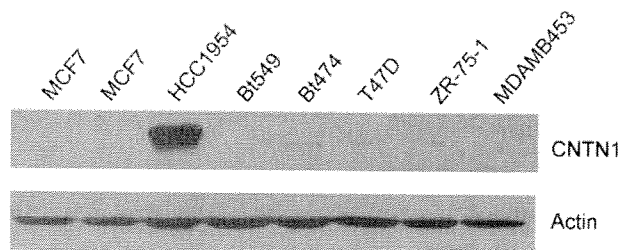


Figure 13

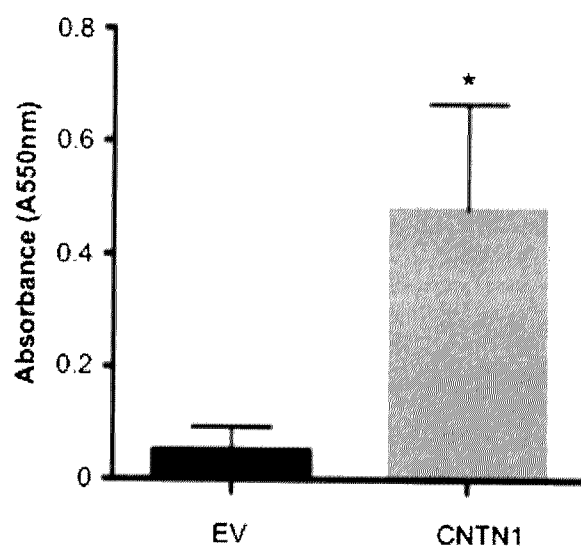


Figure 14

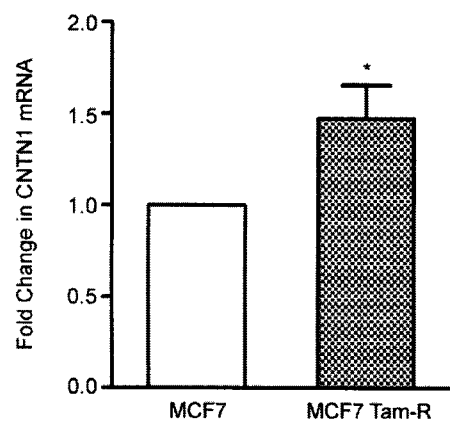


Figure 15

A) Isoform 1

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B) Isoform 3

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 itmdkrrfvs qtngnlyian veasdkgnys cfvsspsitk svfskfipli piperttkpy
 padivvqfkd vyalmgqnv tlecfalgnvp pdirwrkvle pmpstaeist sgavlkifni
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 tirwlkngya yhkgelrlyd vtfenagmyq ciaentygai yanaelkila laptfemnpm
 kkkilaakgg rviieckpka apkpkfswsk gtewlvnssr iliwedgsle innitrndgg
 iytcfannr gkanstgtlv itdptriila pinaditvge natmqcaasf dpaldltfww
 sfngyvidfn kenihiyqrnf mldsdngelli rnaqlkhagr ytctaqtivd nssasadlvv
 rgknrkgegek nmvdsflpvc aslpptw (SEQ ID NO: 8)

C) Mouse

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 vslncraras pfpvykwrnm ngdvdltndr ysmvggnlvi nnpdkqkdag vyyclasnny
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 itmdkrrfvs qtngnlyian vessdrgnys cfvsspsitk svfskfipli piperttkpy
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 qledeglyec eaenirgkdk hqariyvqaf pewvehindt evdigsdlyw pciatgkpi
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 vvrpgpppgg glriedirat svaltwrsrgs dnhspiskyti qtktilsdd wkdaktdppi
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D) Human mRNA isoform 1

```

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121 ccgcaccgag gcgagcagga gcagggaaaca ggtgtttaaa attatccaac tgccatagag
181 ctaaattctt ttttggaata ttgaaccgaa cttctactga atacaagatg aaaatgtggt
241 tgctggctcag tcatcttgtg ataatatcta ttactacctg tttagcagag ttacatggt
301 atagaagata tggcatgga gtttctgagg aagacaaagg atttggaacca atttttgaag
361 agcagccaat caataccatt tatccagagg aatcactgga agggaaaagt tcaactcaact
421 gtagggcacg agccagccct ttcccggttt acaaattggg aatgaataat ggggacgttg
481 atctcacaag tgatcgatac agtatggtag gagggaaacct tggtatcaac aaccctgaca
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661 aggtcagagt aaaagaaggg aaaggaatgg tgcttctctg tgacccccca taccattttc
721 cagatgatct tagctatcgc tggcttctaa atgaatttcc tgtatttatc acaatggata
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901 aattcatccc actcattcca atacctgaac gaacaacaaa accatatcct gctgatattg
961 tagttcagtt caaggatgta tatgcattga tgggccaaaa tgtgacctta gaatgtttt
1021 cacttggaat tcctgttccg gatatccgat ggcggaagg tctagaacca atgccaagca
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1141 aaggcatcta tgaatgtgag gctgagaaca tagaggaaa ggataaacat caagcaagaa
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1381 atgccggaat gtatcagtcg atagctgaaa acacatatgg agccatttat gcaaagtctg
1441 agttgaagat cttggcggtg gctccaactt ttgaaatgaa tcctatgaag aaaaagatcc
1501 tggctgctaa aggtggaagg gtgataattg aatgcaaac taaagctgca ccgaaaccaa
1561 agttttcatg gagtaaaggg acagagtggc ttgtcaatag cagcagaata ctcatttggg
1621 aagatggtag cttggaaatc aacaacatta caaggaatga tggaggatc tatacatgct
1681 ttgcagaaaa taacagaggg aaagctaata gcactggaac ccttgttatc acagatccta
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2521 gccgatatgt ccataaagat gaaacctgga gcccttccac tgcatttcaa gttaaagtca
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2761 gggctgcccc tgacaaagaa gaagctgcaa acagagtcca agtcaccagc caagagtact
2821 cggccaggct cgagaacctt ctgccagaca cccagtattt tatagaagtc ggggcctgca
2881 atagtgcagg gtgtggacct ccaagtgaca tgattgaggc tttaccaag aaagcacctc
2941 ctagccagcc tccaaggatc atcagttcag taaggctctg ttcacgctat ataatacct
3001 gggatcatgt cgttgacta tcaaatgaat ctacagtgac gggatataag gtactctaca
3061 gacctgatgg ccagcatgat ggcaagctgt attcaactca caaacactcc atagaagtcc

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Figure 15 Continued

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3121 caatccccag agatggagaa tacgttgtgg aggttcgcgc gcacagtgat ggaggagatg
3181 gagtgggtgtc tcaagtcaaa atttcagggtg caccacacct atccccaaagt cttctcggct
3241 taactgtgcc tgcctttggc atccttgtct acttggaatt ctgaatgtgt tgtgacagct
3301 gctgttccca tcccagctca gaagacaccc ttcaaccctg ggatgaccac aattccttcc
3361 aatttctgcg gctccatcct aagccaaata aattatactt taacaaacta ttcaactgat
3421 ttacaacaca catgatgact gaggcattcg ggaacccctt catccaaaag aataaacttt
3481 taaatggata taaatgattt ttaactcgtt ccaatatgcc ttataaacca cttaacctga
3541 ttctgtgaca gttgcatgat ttaacccaat gggacaagtt acagtgttca attcaatact
3601 ataggctgta gagtgaaagt caaatcacca tatacagggtg ctttaaattt aataacaagt
3661 tgtgaaatat aatagagatt gaaatgttgg ttgtatgtgg taaatgtaag agtaatacag
3721 tctctgttac tttcctcact gttttgggta ctgcatatta ttgaatggcc cctatcattc
3781 atgacatctt gagttttctt gaaaagacaa tagagtgtaa caaatatttt gtcagaaatc
3841 ccattatcaa atcatgagtt gaaagatttt gactattgaa aaccaaattc tagaacttac
3901 tatcagtatt cttattttca aaggaaataa ttttctaaat atttgatttt cagaatcagt
3961 tttttaatag taaagttaac ataccatata gatttttttt tacttttata ttctactctg
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4081 gtatcataat attgctatat ttgttcatat gtggagtgac aaattttgaa aagtagagtg
4141 ctcccttttt tattgagatg tgacagctct tacatgggta ggaataagtg acagttaagt
4201 gaatatcaca attactagta tggttggttt tctgcttcat tctaagtat tacgtttctt
4261 tattgcagat gtcagatcaa aaagtcacct gtaggttgaa aaagctaccg tattccattt
4321 tgtaaaaaata acaataataa taataataat aattagtttt aagctcattt cccacttcaa
4381 tgcaatactg aaaactggct aaaaatacca aatcaatata ctgctaattg tactttgaag
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4561 ctccatgggtg tgcattgaaa tgtgtttgag tgtggatgta aaagaaatcg agtaataaag
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4741 tcgtaaaaaa tatttacagg aagcaaaata gatctattac tactttaccg actttacccc
4801 ctttctttta tttgtataat tttgtacta tatatcgatg tgtaaatggt tagagtcttc
4861 attatgaaaa tatcaataaa tatttcatta gtttacattt aactctggta taaaatgaaa
4921 cttttaaaaa taagtgaat ggatgatttc ccagtggaa gtagtcaaca gtcttaagat
4981 cattgccaga tttcataaaa tatttaagta tttgaaaaag aaacaaaatg tcttcatact
5041 ttagggaaac gaataccctg tataccttct gtacaaatgt ttgtgttttc attgttacac
5101 tttgggggtt tacttttgca atgtgacca tgttgggcat ttttatataa tcaacaacta
5161 aatcttttgc caaatgcatg cttgcctttt attttcta atatgataat aacgagcaaa
5221 actgggttaga ttttgcatga aatggttctg aaaggtaaga ggaaaacaga ctttgagggt
5281 tgttttagtt tgaatttctg acagagataa agtagtttaa aatctctcgt aactgataa
5341 ctcaagcttt tcattttctc atacagttgt acagatttaa ctgggaccat cagttttaaa
5401 ctgttgtcaa gctaactaat aatcatctgc ttttaagacg aagattctga attaaaactt
5461 atataggtat agatacatct gttgtttctt tgtatttcag gaaagggtgat agtagtttta
5521 tttgatactg ataaatattg aattgatttt ttagttattt tttatcattt tttcaatgga
5581 gtagtatagg actgtgcttt gtccctttta tgaatgaaaa aattagtata aagtaataaa
5641 tgtcttatgt taccacaaga aaaa (SEQ ID NO: 10)

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E) Mouse mRNA

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1 agcattgact ccagccagcc aggttcggga atgtaaatcc acagctcaca gcccagaatg
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121 gcagaagggt ggctgaagga tgggagcggc tatgcctgcc tctgaggta cagcatgagg
181 tctttttaac ttgccttctg ctgcagaaga gaagcccat gtgtcataac tctccctgag
241 tctattcaaa ggggcaattt gaagcagata ttatagcaag atcggcagat gattcaaccc
301 acacgggtct tcatagtact caactgccat aaagctttca ttacttttgg aaactgaccc

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Figure 15 Continued

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361 atctattcaa caaaagatga aaatgccatt gctgggcagc catctcctgc taatctcctt
421 aacatcttgt ctaggagact ttacctggca cagaagatat ggatcatggag tgtctgagga
481 ggacaaagga tttggggcca tttttgaaga gcagccaatc aataccattt acccagaaga
541 gtcactggaa ggaaaagtgt cactcaactg ccgggcacgc gccagcccat ttccagttta
601 caaatggaga atgaataacg gggacgtgga cctgacaaac gatcgggtaca gtatggtcgg
661 aggaaacctt gttatcaaca acccggaaca acagaaagac gctgggggtgt actactgcct
721 ggcatccaac aactacggaa tggtcaggag cacggaggca actctgagct tcgggtatct
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3421 tgtctactcg gaattctgaa tgtaacgacc ttggctgttt tcatccctgc tcagaggatg
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Figure 15 Continued

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3541 caaattagac tctaacaagc tgttcaactg atttcccaac acatcacgac tgaggcattc
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3661 tccaatatgc cttataaaacc atttaacctg attctgtgaa agttgcatga cttaaatcaa
3721 tggacaagtt acagtgttca attcaatgcc ctaggctgta gagtgaaggt caccatacac
3781 aggtgcttta catgtttgac aagtttgtgaa atataataga gattgaaatg ttgattgtat
3841 gtagtatgtg ttcgagtaat gcagtctctt gtattagcct atatgtttgg gttcctgcct
3901 atttttgaaa gaccctcatc attcatgata ttttgagttt tccataaaaat taaaacaaaa
3961 aaaatagaaa aaaaatcatt ttgacataaa caccattacc aaatcacttt actgaatgag
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4141 ctaatgctga cgttcccatg ccacgtgagg attttttttt ttttttttac ttttgtattc
4201 taatctgaaa ttgttttgtg cttttcttct catcaatgtc aaaccacaga agctgcagcg
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4321 tagagcgctt tcattttatac tgcaatgcga tcgtctttat ccagctagaa acaagtgaca
4381 gtaaagtatc agaatctctg aatgttctca ttgttctggg tttgtgcttc atgcctaagt
4441 ataaactctc tttaacaaga ggtgtagagt caagaattca tatgcagggt tggaaaaaagc
4501 tacagaattc catttcaaga agtttaaaagc tcgtttccca tttcaatgca atactgaaaa
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4861 gctaattaga gacagagttt atcttttttag aaaagatacc ctatagggtcc ataaagaata
4921 tttaacaggaa acaaaaaatag atctattaat atgttacccc tatctttaat ttgtataatt
4981 tttgtactat atgtgtaaag gtttaaaagtc ctcatatga aaatatcaat aaatatttca
5041 ttaattttaca cttaacactt tgttatacaa atgaaacttt ttaaatcggg aaaacagatg
5101 ctctcccagt agaagcatgt caacaaacca tcgccagggt tcataaaaata tttaatgatt
5161 tgcaaaaagaa gaaaatgact tcatacttta gggagatgaa cactctgtaa gctttctgta
5221 caaacgttgt gttttccttg ttatattcgg gggttttcct ctgcatgtg gacccatgtt
5281 gggcattttt gtatcatcaa ttaacaacta catcttttgc caaatgcatg cctgcctttt
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5401 gaaagataag aaaacagact ttgaagggtg tttagtttaa atatgtggcg gatagactca
5461 actctctcat gcacaaatga cttctgcttt tcatttcctc atataattgt acagatgtga
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5581 caagataatt aattaaactt tatatagctc tagacacatc tgttgtttct ttgtatttca
5641 ggaaaggtaa tagtagtttc atttgatact gataaataac gaattgattt ttttagttat
5701 tttttatcat tttcttcaat ggagtagtat aggactgtcg tttgtccttt ttatgaatga
5761 atacaaatta taaagaaata aatgtcttac tgttgcccaa aaagtaactg tcccttc
(SEQ ID NO: 11)

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2015/050663

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **A61K 39/395** (2006.01), **A61B 5/00** (2006.01), **A61K 31/7088** (2006.01), **A61P 35/00** (2006.01),
C12Q 1/68 (2006.01), **G01N 33/574** (2006.01), **C12N 15/113** (2010.01), **C07K 16/28** (2006.01) .

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: **A61K 39/395** (2006.01), **A61B 5/00** (2006.01), **A61K 31/7088** (2006.01), **A61P 35/00** (2006.01),
C12Q 1/68 (2006.01), **G01N 33/574** (2006.01), **C12N 15/113** (2010.01), **C07K 16/28** (2006.01) .

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Databases : Scopus, Pubmed, Questel-Orbit.

Keywords: Contactin-1, CNTN1, prostate, breast, cancer, diagnosis, treatment, inhibitor, tamoxifen resistance.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YAN J et al. "Contactin-1 reduces E-cadherin expression via activating AKT in lung cancer." PLoS ONE. 28 May 2013, 8(5):e65463. doi: 10.1371/journal.pone.0065463. *the whole document*	14
X	SU JL et al. "Knockdown of conatctin-1 expression suppresses invasion and metastasis of lung adenocarcinoma." CANCER RES. 1 Mar 2006, 66(5):2553-61. *the whole document*	14
A	VINARSKAJA A et al. "DNA Methylation and the HOXC6 Paradox in Prostate Cancer." CANSERS (BASEL). 27 Sep 2011, 3(4):3714-25. *abstract*	1-19

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* "A" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier application or patent but published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" "X" "Y" "&"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art document member of the same patent family
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Date of the actual completion of the international search
16 September 2015 (16-09-2015)

Date of mailing of the international search report
14 October 2015 (14-10-2015)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 001-819-953-2476

Authorized officer

Mostapha Bayaa (819) 994-6940

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2015/050663**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim Nos.: 10-13
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 10-13 are directed to a method for treatment of the human or animal body by surgery or therapy, which the International Searching Authority is not required to search under PCT Rule 39.1(iv). However, this Authority has carried out a search based on the alleged effect or purpose/use of the product defined in claims 10-13.

2. ☒ Claim Nos.: 10-14
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 10-14 encompass a product that is not defined in clear and concise terms as required under PCT Article 6. The application provides support within the meaning of PCT Article 6 for specifically CNTN1 hairpin shRNAs. In the present case, the claim so lacks clarity and/or support, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been established for the parts of the application which appear to be clear and/or supported, namely CNTN1 hairpin shRNAs.

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.:

- Remark on Protest**
- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.