Abstract: The present invention provides methods for detecting and diagnosing cancer, such methods involving the determination of the expression level of the EZH2 gene. The present invention also provides methods for determining or assessing the prognosis of cancer based on the expression level of the EZH2 gene. Furthermore, the present invention provides methods of screening for therapeutic agents useful in either or both of the treatment and prevention of cancer and methods for either or both of treating and preventing cancer. Moreover, the present invention provides double-stranded molecules targeting the EZH2 gene, all of which are suggested to be useful in either or both of the treatment and prevention of cancer.

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Description

Title of Invention: EZH2 AS TARGET GENE FOR CANCER THERAPY AND DIAGNOSIS

Technical Field

[0001] The present invention relates to methods of detecting and diagnosing cancer as well as methods of treating and preventing cancer, particularly cancers associated with the overexpression of EZH2. The present invention also relates to methods of screening for a candidate substance for either or both of treating and preventing an EZH2-associated cancer. Moreover, the present invention relates to double-stranded molecules that reduce EZH2 gene expression and uses thereof.

[0002] PRIORITY

The present application claims the benefit of U.S. Provisional Applications No. 61/478,206, filed on April 22, 2011, the contents of which are hereby incorporated herein by reference in their entirety for all purposes.

Background Art

[0003] The emergence of effective cancer chemotherapy is one of the major medical advances in recent years. (NPL 1) Adjuvant chemotherapy for lung, breast or colon cancer can augment the survival benefit afforded by surgical management NPL 2-4) Even in patients with advanced solid tumors or recurrences following surgery, chemotherapy can increase survival and quality of life. (NPL 5) In those patients, however, the therapeutic index is narrow: responses are usually partial, and often disappointingly brief. (NPL 6) In addition, many antitumor agents have unexpected detrimental side effects, accentuating the limitation of cytotoxic chemotherapy. Therefore, it is desirable to discover novel therapeutic targets to extend the capability of cancer chemotherapy and improve patient care.

[0004] The N-terminal tails of histones are subjected to post-translational modifications, including methylation, acetylation and phosphorylation, which generate an extensive repertoire of chromatin structures. (NPL 7,8) We previously reported that SMYD3, a histone lysine methyltransferase, stimulates proliferation of cells and plays an important role in human carcinogenesis through its methyltransferase activity. (NPL 9-13) With the exception of Dotl/DOTIL, all histone lysine methyltransferases (HKMTs) contain a SET domain of about 130 amino acids. (NPL 14) The SET domain was originally identified as a domain shared by three Drosophila proteins involved in epigenetic processes: the suppressor of position-effect variegation [Su(var)3-9]; an enhancer of the eye colour mutant zeste which belongs to the PcG proteins [E(Z)]; and the homeobox gene regulator trithorax [TRX]. (NPL 15) Mammalian homologues of
Drosophila Su(var)3-9, Suv39hl and Suv39h2, were the first ones characterized as HKMTs, and they methylate histone H3 at lysine 9 (H3K9). (NPL 16) So far, nearly 40 HKMTs or potential HKMTs containing the SET domain have been identified, and some of them are shown to methylate lysine residues at codons 4, 9, 27 or 36 of histone H3 or lysine 20 of histone H4. Mammalian HKMTs can be classified into several families according to sequence similarities within their SET domain and adjacent sequences, as well as other structural features such as the presence of other defined protein domains. (NPL 14)

Citation List

Non Patent Literature


**Summary of Invention**

[0006] In order to investigate the roles of HKMTs in human carcinogenesis, the present inventors examined expression profiles of human HKMTs in clinical tissues, and found that in various types of cancers, expression levels of the enhancer of zeste homolog 2 (EZH2) were significantly up-regulated, compared with corresponding normal tissues. In particular, the present inventors have found EZH2 to be highly overexpressed in lung and other cancers, and demonstrate that EZH2 is integral to proliferation in cancer cells. qRT-PCR analysis revealed higher expression of EZH2 in clinical bladder cancer tissues than in corresponding non-neoplastic tissues (P < 0.0001), and the present inventors confirmed that a wide range of cancers also overexpress EZH2, using cDNA microarray analysis. Immunohistochemical analysis showed positive staining for EZH2 in 14 of 29 bladder cancer, 135 of 292 NSCLC and 214 of 245 colorectal cancer cases, whereas no significant staining was observed in various normal tissues. The present inventors further found elevated expression of EZH2 to be associated with poor prognosis for patients with NSCLC (P = 0.0239). In lung and bladder cancer cells overexpressing EZH2, suppression of EZH2 using specific siRNAs inhibited incorporation of BrdU and resulted in significant suppression of cell growth even though no significant effect was observed in the normal cell strain CCD-I8C0, which has undetectable EZH2.

[0007] These results demonstrate that EZH2 is a good diagnostic and prognostic marker for cancer and a good molecular target for cancer therapy.

The EZH2 gene, as well as its transcription and translation products, finds diagnostic utility as a marker for cancer and as an oncogene target, the expression and/or activity of which may be altered to treat or alleviate a symptom of cancer. Similarly, by detecting changes in the expression of the EZH2 gene and/or the activity of the EZH2
protein that arise from exposure to a test substance, various agents for treating or preventing cancer can be identified.

Accordingly, it is an object of the present invention to provide a method for diagnosing or determining a predisposition to cancer in a subject by determining the expression level of the EZH2 gene in a subject-derived biological sample, such as tissue sample. An increase in the level of expression of the gene as compared to a normal control level indicates that the subject suffers from or is at risk of developing cancer.

Moreover, the present invention relates to the discovery that the expression level of the EZH2 gene correlates to prognosis of cancer. Particularly, a higher expression level of the EZH2 gene correlates to poor prognosis of lung cancer. In contrast, a higher expression level of the EXH2 gene correlates to good prognosis of colorectal cancer. Therefore, the present invention provides a method for assessing or determining the prognosis of a patient with cancer, particularly lung cancer and colorectal cancer, which method includes the steps of detecting the expression level of EZH2 gene, comparing it to a pre-determined reference expression level and determining the prognosis of the patient from the difference between them.

It is another object of the present invention to provide a kit that includes at least one reagent for detecting a transcription or translation product of the EZH2 gene.

It is yet another object of the present invention to provide a reagent for diagnosis or detection of cancer, which comprises a nucleic acid that binds to a transcriptional product of the EZH2 gene, or an antibody that binds to a translational product of the EZH2 gene.

It is yet another object of the present invention to provide use of a nucleic acid that binds to a transcriptional product of the EZH2 gene, or an antibody that binds to a translational product of the EZH2 gene for use in the manufacture of a reagent for diagnosis or detection of cancer.

It is yet another object of the present invention to provide methods for identifying substances that binds the EZH2 protein, by contacting the EZH2 protein with a test substance and detecting the binding between the EZH2 protein and the test substance. Test substances that bind the EZH2 protein may be used to reduce symptoms of cancer, or either or both of treating and preventing cancer.

It is yet another object of the present invention to provide methods for identifying substances that inhibit the biological activity of the EZH2 protein, by contacting the EZH2 protein with a test substance and detecting the biological activity of the EZH2 protein. The biological activity of the EZH2 protein to be detected is preferably cell proliferative activity (cell proliferation enhancing activity) or methyltransferase activity (e.g., histone methyltransferase activity). A decrease in the biological activity of the EZH2 protein as compared to a control level in the absence of the test substance
indicates that the test substance may be used to reduce symptoms of cancer.

[0012] It is yet another object of the present invention to provide methods for identifying substances that inhibit the expression of the EZH2 gene or a reporter gene that is controlled by the transcription initiation region of the EZH2 gene, by contacting a test cell expressing the EZH2 gene with a test substance and determining the expression level of the EZH2 gene or the reporter gene. The test cell may be an epithelial cell, such as a cancerous epithelial cell. A decrease in the expression level of the gene as compared to a control level in the absence of the test substance indicates that the test substance may be used to reduce symptoms of cancer.

[0013] In the present invention, inhibitory effects of siRNAs on the expression of the EZH2 gene were confirmed. In particular, the inhibition of cell proliferation of cancer cells by the siRNAs is demonstrated in the Examples section. The data herein support the utility of the EZH2 gene as a preferred therapeutic target for cancer.

Thus, it is another object of the present invention to provide a double-stranded molecule that inhibits the expression of the EZH2 gene as well as an siRNAs against the EZH2 gene, and a vector encoding the double-stranded molecule. The double-stranded molecule of the present invention inhibits expression of the EZH2 gene when introduced into a cell expressing an EZH2 gene, wherein the double-stranded molecule comprises a sense strand and an antisense strand, wherein the sense strand comprises a nucleotide sequence corresponding to a target sequence selected from the group consisting of SEQ ID NOs: 13 and 16, and the antisense strand comprises a nucleotide sequence complementary to the target sequence of the sense strand so that the sense and antisense strands hybridize to each other to form the double-stranded molecule.

[0014] It is yet another object of the present invention to provide methods for treating and/or preventing cancer, including the step of administering a double-stranded molecule against EZH2 gene that inhibits the expression of the EZH2 gene or a vector encoding the double-stranded molecule to a subject.

[0015] It is yet another object of the present invention to provide a pharmaceutical composition for use in treating and/or preventing cancer, or inhibiting cancer cell growth, including the double-stranded molecule against EZH2 gene that inhibits the expression of the EZH2 gene or a vector encoding the double-stranded molecule.

It is yet another object of the present invention is to provide a use of a double-stranded molecule against EZH2 gene that inhibits the expression of the EZH2 gene or a vector encoding the double-stranded molecule for use in the manufacture of a medicament for treatment and/or prevention of cancer.

[0016] More specifically, the present invention provides the following [1] to [31]:

[1] A method for detecting or diagnosing cancer or a predisposition for developing the cancer in a subject, comprising a step of determining an expression level of an
EZH2 gene in a subject-derived biological sample, wherein an increase in said expression level as compared to a normal control level of said gene indicates that said subject suffers from or is at a risk of developing cancer, wherein said expression level is determined by any method selected from a group consisting of:
(a) detecting an mRNA of an EZH2 gene;
(b) detecting a protein encoded by an EZH2 gene; and
(c) detecting a biological activity of a protein encoded by an EZH2 gene;
[2] The method of [1], wherein said expression level is at least 10% greater than the normal control level;
[3] The method of [1] or [2], wherein the subject-derived biological sample is a biopsy specimen, saliva, sputum, blood, serum, plasma, pleural effusion or urine sample;
[4] The method of any one of [1] to [3], wherein the biological activity is cell proliferative activity or histone methyltransferase activity;
[5] A method for assessing prognosis of a subject with cancer, wherein the method comprises steps of:
(a) detecting an expression level of EZH2 gene in a subject-derived biological sample;
(b) comparing the detected expression level to a control level; and
(c) determining prognosis of the subject based on the comparison of (b);
[6] The method of [5], wherein the control level is a good prognosis control level and an increase of the expression level compared to the control level indicates poor prognosis when the cancer is lung cancer;
[7] The method of [5], wherein the control level is a poor prognosis control level and an increase of the expression level compared to the control level indicates good prognosis when the cancer is colorectal cancer;
[8] The method of [6] or [7], wherein the increase is at least 10% greater than said control level;
[9] A method of screening for a candidate substance for either or both of treating and preventing cancer, wherein said method comprises steps of:
(a) contacting a test substance with an EZH2 polypeptide or functional equivalent thereof;
(b) detecting binding between the EZH2 polypeptide or functional equivalent and the test substance; and
(c) selecting a test substance that binds to the EZH2 polypeptide or functional equivalent as a candidate substance for either or both of treating and preventing cancer;
[10] A method of screening for a candidate substance for either or both of treating and preventing cancer, wherein said method comprises steps of:
(a) contacting a test substance with an EZH2 polypeptide or functional equivalent thereof;
(b) detecting a biological activity of the EZH2 polypeptide or functional equivalent;
(c) comparing the biological activity of the EZH2 polypeptide or functional equivalent with the biological activity detected in the absence of the test substance; and
(d) selecting a test substance that suppresses the biological activity of the EZH2 polypeptide or functional equivalent as a candidate substance for either or both of treating and preventing cancer;

[11] The method of [10], wherein the biological activity is cell proliferative activity or methyltransferase activity;

[12] A method of screening for a candidate substance for either or both of treating and preventing cancer, which comprises steps of:
(a) contacting a test substance with a cell expressing an EZH2 gene;
(b) detecting expression level of the EZH2 gene;
(c) comparing the expression level with the expression level detected in the absence of the test substance; and
(d) selecting a test substance that reduces the expression level of the EZH2 gene as a candidate substance for either or both of treating and preventing cancer;

[13] A method of screening for a candidate substance for either or both of treating and preventing cancer, wherein said method comprises steps of:
(a) contacting a test substance with a cell introduced with a vector that comprises a transcriptional regulatory region of an EZH2 gene and a reporter gene expressed under control of the transcriptional regulatory region;
(b) measuring an expression level or activity of said reporter gene;
(c) comparing the expression level or activity with the expression level or activity detected in the absence of the test substance; and
(d) selecting a test substance that reduces the expression level or activity as a candidate substance for treating and/or preventing cancer;

[14] A double-stranded molecule that, when introduced into a cell expressing an EZH2 gene, inhibits expression of the gene, wherein the double-stranded molecule comprises a sense strand and an antisense strand, wherein the sense strand comprises a nucleotide sequence corresponding to a target sequence selected from the group consisting of SEQ ID NOs: 13 and 16, and the antisense strand comprises a nucleotide sequence complementary to the target sequence of the sense strand so that the sense and antisense strands hybridize to each other to form the double-stranded molecule;

[15] The double-stranded molecule of [14], wherein the sense strand hybridizes with antisense strand at the target sequence to form the double-stranded molecule having between 19 and 25 nucleotide pair in length;

[16] The double-stranded molecule of [14] or [15], wherein said double-stranded molecule is a single polynucleotide construct comprising the sense strand and the
antisense strand linked via a single-strand;

[17] The double-stranded molecule of [16], which has a general formula
5’-[A]–[B]–[A’]-3’ or 5’-[A’]-[B]–[A]-3’, wherein [A] is a sense strand comprising a nu-
cleotide sequence corresponding to a target sequence selected from the group
consisting of SEQ ID NO: 13 and 16, [B] is a single-strand and consists of 3 to 23 nu-
cleotides, and [A’] is an antisense strand comprising a nucleotide sequence com-
plementary to the target sequence of [A];

[18] A vector encoding the double-stranded molecule of any one of [14] to [17];

[19] Vectors comprising each of a combination of polynucleotide comprising a sense
strand nucleic acid and an antisense strand nucleic acid, wherein said sense strand
nucleic acid comprises a nucleotide sequence corresponding to SEQ ID NO: 13 or 16,
and said antisense strand nucleic acid consists of a sequence complementary to the
sense strand, wherein the transcripts of said sense strand and said antisense strand
hybridize to each other to form a double-stranded molecule, and wherein said vectors,
when introduced into a cell expressing EZH2 gene, inhibit the cell proliferation;

[20] A method of either of both of treating and preventing cancer in a subject,
comprising administering to said subject a pharmaceutically effective amount of a
double-stranded molecule against an EZH2 gene or a vector encoding said double-
stranded molecule, wherein the double-stranded molecule, when introduced into a cell
expressing EZH2 gene, inhibits the expression of the EZH2 gene;

[21] The method of [20], wherein the double-stranded molecule is that of any one of
[14] to [17];

[22] The method of [21], wherein the vector is that of [18] or [19];

is selected from the group consisting of AML, bladder cancer, breast cancer, cholan-
giocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal
cancer and renal cell carcinoma;

[24] A composition for either or both of treating and preventing cancer, which
comprises a pharmaceutically effective amount of a double-stranded molecule against
an EZH2 gene or a vector encoding said double-stranded molecule, wherein the
double-stranded molecule, when introduced into a cell expressing EZH2 gene, inhibits
the expression of the EZH2 gene, and a pharmaceutically acceptable carrier;

[25] The composition of [24], wherein the double-stranded molecule is that of any one
of [14] to [17];

[26] The composition of [25], wherein the vector is that of [18] or [19];

[27] The composition of any one of [24] to [26], wherein the cancer is selected from
the group consisting of AML, bladder cancer, breast cancer, cholangiocellular
carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and
renal cell carcinoma;

[28] A kit for diagnosing or detecting cancer, or assessing or determining prognosis of a subject with cancer comprising a reagent for detecting a transcription or translation product of an EZH2 gene;

[29] The kit of [28], wherein the reagent comprises a nucleic acid that binds to a transcription product of the EZH2 gene or an antibody that binds to a translation product of the EZH2 gene;

[30] A reagent for diagnosing or detecting cancer, or assessing or determining prognosis of a subject with cancer, comprising a nucleic acid that binds to a transcription product of an EZH2 gene or an antibody that binds to a translation product of the EZH2 gene; and

[31] The kit of [28] or [29], or the reagent of [30], wherein the cancer is selected from the group consisting of AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma.

[0017] One advantage of the methods described herein is that the disease is identified prior to detection of overt clinical symptoms of cancer. Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

It will be understood by those skilled in the art that one or more aspects of this invention can meet certain objectives, while one or more other aspects can meet certain other objectives. Each objective may not apply equally, in all its respects, to every aspect of this invention. As such, the preceding objects can be viewed in the alternative with respect to any one aspect of this invention.

[0018] It will also be understood that both the foregoing summary of the present invention and the following detailed description are of exemplified embodiments, and not restrictive of the present invention or other alternate embodiments of the present invention. Other objects and features of the invention will become more fully apparent when the following detailed description is read in conjunction with the accompanying figures and examples. In particular, while the invention is described herein with reference to a number of specific embodiments, it will be appreciated that the description is illustrative of the invention and is not constructed as limiting of the invention. Various modifications and applications may occur to those who are skilled in the art, without departing from the spirit and the scope of the invention, as described by the appended claims. Likewise, other objects, features, benefits and advantages of the present invention will be apparent from this summary and certain embodiments described below, and will be readily apparent to those skilled in the art. Such objects, features, benefits and advantages will be apparent from the above in conjunction with
the accompanying examples, data, figures and all reasonable inferences to be drawn therefrom, alone or with consideration of the references incorporated herein.

**Brief Description of Drawings**

[0019] Various aspects and applications of the present invention will become apparent to the skilled artisan upon consideration of the brief description of the figures and the detailed description of the present invention and its preferred embodiments that follows:

[fig.1A-C]Fig. 1 demonstrates elevated EZH2 expression in human bladder cancer. Part A depicts EZH2 gene expression in normal and tumor bladder tissues in British cases. Expression levels of EZH2 were analyzed by quantitative real-time PCR, and the result is shown by box-whisker plot (median 50% boxed). Relative mRNA expression shows the value normalized by GAPDH and SDH expressions. Mann-Whitney's U-test was used for statistical analysis. Part B depicts comparison of EZH2 expression between normal and tumor bladder tissues in Japanese patients. Signal intensity for each sample was analyzed by cDNA microarray, and the result is shown by box-whisker plot (median 50% boxed). Mann-Whitney's U-test was used for statistical analysis. Part C depicts qRT-PCR analysis performed in normal heart, normal liver, normal lung, normal kidney and randomly selected normal and tumor bladder tissues (3 cases and 5 cases, respectively).

[0020] [fig.1D-F]Part D and E depict immunohistochemical staining of EZH2 in bladder tissues. Clinical information for each section is represented above histological pictures. All tissue samples were purchased from BioChain. Original magnification, x100, x200. Part F depicts immunohistochemical analysis of EZH2 in various normal tissues. No significant staining was observed.

[0021] [fig.2A-C]Fig. 2 demonstrates that EZH2 was overexpressed in lung cancer and appears to be a prognostic marker of non small cell lung cancer. Part A depicts comparison of EZH2 expression between normal and tumor (small cell lung cancer (SCLC)) lung tissues in Japanese patients. Signal intensity for each sample was analyzed by cDNA microarray, and the result is shown by box-whisker plot (median 50% boxed). Mann-Whitney's U-test was used for the statistical analysis. Part B depicts immunohistochemical staining of EZH2 in lung tissues. Clinical information for each section is represented above histological pictures. All tissue samples were purchased from BioChain. Original magnification, x100, x400. C depicts representative cases for positive and negative EZH2 staining in lung cancer and normal lung tissues on the tissue microarray. Original magnification, x100 and x200.

[0022] [fig.2D] (D) Kaplan-Meier estimates of overall survival time of patients with NSCLC (P = 0.0239, log-rank test).
[0023] Fig. 3 demonstrates that elevated EZH2 expression was observed in the majority of colorectal cancer cases. Part A depicts expression of EZH2 in 4 normal cell lines, 3 colorectal cancer cell lines, 5 lung cancer cell lines, 3 liver cancer cell lines and 12 bladder cancer cell lines. Expression levels were analyzed by quantitative real-time PCR, and relative mRNA expression shows the value normalized by GAPDH and SDH expressions. Part B depicts immunohistochemical staining of EZH2 in colorectal tissues. Tissue samples were purchased from BioChain. Original magnification, x100, x400.

[0024] Fig. 3C-D] Part C depicts representative cases for positive and negative EZH2 staining in colorectal cancer and normal colorectal tissues. 172 Japanese cases operated at Nagasaki University Hospital were used for detailed immunopathological analysis. Part D depicts Kaplan-Meier estimates of overall survival time of patients with NSCLC (P = 0.014, log-rank test).

[0025] Fig. 4A-B] Fig. 4 demonstrates involvement of EZH2 in the cell cycle regulation of cancer cells. Part A depicts knockdown effects of siRNAs targeting EZH2 on cancer cells. Left panel depicts the results of quantitative real-time PCR showing suppression of endogenous expression of EZH2 by two EZH2-specific siRNAs (siEZH2#1 and #2) in SBC5 cells. Right panels depict western blot analysis of EZH2 in SBC5 and SW780 cells after treatment with two EZH2 siRNAs (siEZH2#1 and siEZH2#2) and two control siRNAs (siEGFP and siNC). ACTB was used as an internal control. Part B depicts effect of EZH2 siRNA knockdown on the viability of bladder cancer cell lines (SW780 and RT4) and lung cancer cell lines (A549, LC319) and a colorectal cancer cell line (HCT1 6). Relative cell number shows the value normalized to siEGFP-treated cells. Results are the mean +/- SD in three independent experiments. P-values were calculated using Student's t-test (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

[0026] Fig. 4C-D] Part C and D depict effect of EZH2 knockdown on cell cycle kinetics in A549 and SW780 cells. Cells were collected 72 h after the treatment with siRNAs, and cell cycle distribution was analyzed by flow cytometry after coupled staining with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU and 7-amino-actinomycin D (7-AAD). Left panels depict numerical analysis of the FACS results (left). Right panels depict representative histograms (right) for each experiment. Results are the mean +/- SD in three independent experiments. P-values were calculated using Student's t-test (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

[0027] Fig. 5 demonstrates that EZH2 methylates lysine 120 of histone H2B. Part A depicts in vitro methylation of histone H2B by EZH2. Recombinant His-histone H2B and 3H-SAM were incubated in the presence or absence of recombinant EZH2, and the reaction products were analyzed by SDS-PAGE followed by fluorography (upper panel). The membrane was stained with Ponceau S (lower panel). BSA was used as a
negative control. Part B depicts the MS/MS spectrum corresponding to the mono-methylated histone H2B peptide. The 14 Da increase of the Lys 120 residue was observed, demonstrating the mono-methylated Lys 120.

[0028] [fig.5C] Part C depicts the MS/MS spectrum of methylated histone H2B peptides (HAVSEGTKAVTKYTSSK).

[0029] [fig.5D-E] Part D depicts validation of an anti-mono-methylated K120 H2B antibody. Recombinant histone H2B (Millipore 14-410 or in-house) or PCNA-K248A (in-house) proteins and 3H-SAM were incubated in the presence or absence of recombinant EZH2, and the reaction products were analyzed by SDS-PAGE followed by Western blot. The membrane was immunoblotted with an anti-mono-methylated histone H2BK120 antibody (upper panel) and stained with MemCode™ Reversible Stain (lower panel). SAHH: S-adenosyl-L-homocysteine hydrolase. Part E depicts immunocytochemical analysis of HeLa cells after transfection with a pCAGGS-n3FC-EZH2 vector. Cells were stained with an anti-mono-methylated H2BK120 antibody (Alexa Fluor™ 488), an anti-FLAG antibody (EZH2, Alexa Fluor™ 594) and 4',6'-diamidine-2'-phenylindole dihydrochloride (DAPI).

[0030] [fig.6] Fig. 6 demonstrates that histone H2B K120 methylation levels are increased in cancer cells. Cell lysates of two non-tumor cell lines (CCD-18C0 and HFL1) and nine tumor cell lines (SBC5, RERF-LC-AI, HCT116, SW480, Alexander, HepG2, RT4, MCF7 and HeLa) were fractionated by SDS-PAGE and Western blot was conducted using anti-mono-methylated histone H2BK120, anti-histone H2B, anti-EZH2 and anti-ACTB. ACTB was used as an internal control.

[0031] [fig.7A] Fig. 7 demonstrates that methylation competitively antagonizes ubiquitination of Lys 120 on histone H2B. Part A depicts the effect of siEZH2 on ubiquitination of H2BK120. SBC5 cells were transfected with siNC (negative control) and siEZH2 for 48 h. Samples were immunoblotted with anti-histone H2B (upper left), anti-mono-methylated histone H2BK120 (upper right) and anti-EZH2 antibodies. The band intensity corresponding to histone H2B and mono-methylated histone H2BK120 were quantified by GS-800 (BIO-RAD), and relative H2BK120 ubiquitination showed the intensity of H2B ubiquitination normalized by histone H2B.

[0032] [fig.7B] Part B depicts immunocytochemical analysis of HeLa cells overexpressing EZH2.

[0033] [fig.8] Fig. 8 depicts mRNA expression levels of EZH2 in clinical bladder tissues analyzed by quantitative real-time PCR. Part A depicts the correlation between EZH2 expression and pathological tumor stages. Statistical analysis was done using Kruskal-Wallis test: NS, Not significant. Part B depicts the correlation between EZH2 expression and pathological tumor grades. Statistical analysis was done using Kruskal-Wallis test: NS, Not significant.
Fig. 9 depicts validation of EZH2 protein expression in various cell lines. Lysates from normal cell lines (MRC5, SAEC, **CCD-I8C0** and IMR90 cells) and cancer cell lines (A549, SBC5, LC319, HeLa, Huh7, SW780, HCT116-[, p53 null] and HCT116 ++ [p53 wild]) were immunblottedted with anti-EZH2 and -ACTB (an internal control) antibodies.

**Description of Embodiments**

Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the present invention, the preferred methods, devices, and materials are now described. However, before the present materials and methods are described, it is to be understood that the present invention is not limited to the particular sizes, shapes, dimensions, materials, methodologies, protocols, etc. described herein, as these may vary in accordance with routine experimentation and optimization. It is also to be understood that the terminology used in the description is for the purpose of describing the particular versions or embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

The disclosure of each publication, patent or patent application mentioned in this specification is specifically incorporated by reference herein in its entirety. However, nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In case of conflict, the present specification, including definitions, will control.

**Definitions**

The words "a", "an", and "the" as used herein mean "at least one" unless otherwise specifically indicated.

The terms "isolated" and "purified" used in relation with a substance (e.g., polypeptide, antibody, polynucleotide, etc.) indicates that the substance is substantially free from at least one substance that may else be included in the natural source. Thus, an isolated or purified antibody refers to antibodies that are substantially free of cellular material such as carbohydrate, lipid, or other contaminating proteins from the cell or tissue source from which the protein (antibody) is derived, or substantially free of chemical precursors or other chemicals when chemically synthesized. The term "sub-
stantially free of cellular material" includes preparations of a polypeptide in which the polypeptide is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus, a polypeptide that is substantially free of cellular material includes preparations of polypeptide having less than about 30%, 20%, 10%, or 5% (by dry weight) of heterologous protein (also referred to herein as a "contaminating protein"). When the polypeptide is recombinantly produced, it is also preferably substantially free of culture medium, which includes preparations of polypeptide with culture medium less than about 20%, 10%, or 5% of the volume of the protein preparation. When the polypeptide is produced by chemical synthesis, it is preferably substantially free of chemical precursors or other chemicals, which includes preparations of polypeptide with chemical precursors or other chemicals involved in the synthesis of the protein less than about 30%, 20%, 10%, 5% (by dry weight) of the volume of the protein preparation. That a particular protein preparation contains an isolated or purified polypeptide can be shown, for example, by the appearance of a single band following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the protein preparation and Coomassie Brilliant Blue staining or the like of the gel. In a preferred embodiment, antibodies and polypeptides of the present invention are isolated or purified.

The terms "polypeptide", "peptide", and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is a modified residue, or a non-naturally occurring residue, such as an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that similarly functions to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those modified after translation in cells (e.g., hydroxyproline, gamma-carboxyglutamate, and O-phosphoserine). The phrase "amino acid analog" refers to compounds that have the same basic chemical structure (an alpha carbon bound to a hydrogen, a carboxy group, an amino group, and an R group) as a naturally occurring amino acid but have a modified R group or modified backbones (e.g., homoserine, norleucine, methionine, sulfoxide, methionine methyl sulfonium). The phrase "amino acid mimetic" refers to chemical compounds that have different structures but similar functions to general amino acids.

Amino acids may be referred to herein by their commonly known three letter symbols or the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission.

The terms "polynucleotide", "oligonucleotide", "nucleic acid", and "nucleic acid
molecule" are used interchangeably unless otherwise specifically indicated and are similarly to the amino acids referred to by their commonly accepted single-letter codes. Similar to the amino acids, they encompass both naturally-occurring and non-naturally occurring nucleic acid polymers. The polynucleotide, oligonucleotide, nucleic acid, or nucleic acid molecule may be composed of DNA, RNA or a combination thereof.

Unless otherwise defined, the term "cancer" refers to a cancer over-expressing the EZH2 gene, for example, acute myeloblastic leukemia (AML), bladder cancer, breast cancer, cholangiocellular carcinoma, chronic myelocytic leukemia (CML), esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma. In preferred embodiments, the cancer may be lung cancer, bladder cancer or colorectal cancer.

As used herein, the term "double-stranded molecule" refers to a nucleic acid molecule that inhibits expression of a target gene, including, for example, short interfering RNA (siRNA; e.g., double-stranded ribonucleic acid (dsRNA) or small hairpin RNA (shRNA)) and short interfering DNA/RNA (siD/R-NA; e.g., double-stranded chimera of DNA and RNA (dsD/R-NA) or small hairpin chimera of DNA and RNA (shD/R-NA)). Herein, "double-stranded molecule" is also referred to as "double-stranded nucleic acid", "double-stranded nucleic acid molecule", "double-stranded polynucleotide", "double-stranded polynucleotide molecule", "double-stranded oligonucleotide" and "double-stranded oligonucleotide molecule".

As used herein, the term "target sequence" refers to a nucleotide sequence within mRNA or cDNA sequence of a target gene, which will result in suppression of translation of the whole mRNA of the target gene if a double-stranded molecule targeting the sequence is introduced into a cell expressing the target gene. A nucleotide sequence within mRNA or cDNA sequence of a gene can be determined to be a target sequence when a double-stranded molecule including a sequence corresponding to the target sequence inhibits expression of the gene in a cell expressing the gene. When a target sequence is shown by cDNA sequence, a sense strand sequence of a double-stranded cDNA, i.e., a sequence that mRNA sequence is converted into DNA sequence, is used for defining a target sequence. A double-stranded molecule is composed of a sense strand that has a sequence corresponding to a target sequence and an antisense strand that has a complementary sequence to the target sequence, and the antisense strand hybridizes with the sense strand at the complementary sequence to form a double-stranded molecule. Herein, the phrase "corresponding to" means converting a target sequence according to the kind of nucleic acid that constitutes a sense strand of a double-stranded molecule. For example, when a target sequence is shown in DNA sequence and a sense strand of a double-stranded molecule has an RNA region, base "t"s within the RNA region is replaced with base "u"s. On the other hand,
when a target sequence is shown in an RNA sequence and a sense strand of a double-stranded molecule has a DNA region, base "u"s within the DNA region is replaced with "t"s.

For example, when a target sequence is shown in the DNA sequence of SEQ ID NO: 13 or 16 and the sense strand of the double-stranded molecule has the 3' side half region composed of DNA and the 5' side half region composed of RNA, "a sequence corresponding to a target sequence" is "5'- CUAACAUAGTTAACAATA -3" (for SEQ ID NO: 13) or "5'- GACAGAAAGGGAAAGTGT -3" (for SEQ ID NO: 16).

Also, a complementary sequence to a target sequence for an antisense strand of a double-stranded molecule can be defined according to the kind of nucleic acid that constitutes the antisense strand.

For example, when a target sequence is shown in the DNA sequence of SEQ ID NO: 13 or 16 and the antisense strand of the double-stranded molecule has the 5' side half region composed of DNA and 3' side half region composed of RNA, "a complementary sequence to a target sequence" is 5'- TAGTTGT AAAACAUGGUAG-3 " (for SEQ ID NO: 13) or "5'- ACACCTTTCCCCUCUCUGUC-3" (for SEQ ID NO: 16).

On the other hand, when a target sequence is shown in the DNA sequence of SEQ ID NO: 13 or 16 and a double-stranded molecule is composed of RNA, the sequence corresponding to a target sequence is the RNA sequence of SEQ ID NO: 11 or 14, respectively, and the complementary sequence to a target sequence is the RNA sequence of SEQ ID NO: 12 or 15, respectively.

A double-stranded molecule may have one or two 3' overhangs having 2 to 5 nucleotides in length (e.g., uu) and/or a loop sequence that links a sense strand and an antisense strand to form hairpin structure, in addition to a sequence corresponding to a target sequence and complementary sequence thereto.

As used herein, the term "siRNA" refers to a double-stranded RNA molecule which prevents translation of a target mRNA. Standard techniques of introducing siRNA into the cell are used, including those in which DNA is a template from which RNA is transcribed. The siRNA includes a sense nucleic acid sequence (also referred to as "sense strand"), an antisense nucleic acid sequence (also referred to as "antisense strand") or both. The siRNA may be constructed such that a single transcript has both the sense and complementary antisense nucleic acid sequences of the target gene, e.g., a hairpin. The siRNA may either be a dsRNA or shRNA.

As used herein, the term "dsRNA" refers to a construct of two RNA molecules including complementary sequences to one another and that have annealed together via the complementary sequences to form a double-stranded RNA molecule. The nucleotide sequence of two strands may include not only the "sense" or "antisense" RNAs selected from a protein coding sequence of target gene sequence, but also RNA
molecule having a nucleotide sequence selected from non-coding region of the target
gene.

[0049] The term "shRNA", as used herein, refers to an siRNA having a stem-loop structure,
including the first and second regions complementary to one another, i.e., sense and
antisense strands. The degree of complementarity and orientation of the regions is
sufficient such that base pairing occurs between the regions, the first and second
regions are joined by a loop region, the loop results from a lack of base pairing
between nucleotides (or nucleotide analogs) within the loop region. The loop region of
an shRNA is a single-stranded region intervening between the sense and antisense
strands and may also be referred to as "intervening single-strand".

[0050] As used herein, the term "siD/R-NA" refers to a double-stranded polynucleotide
molecule which is composed of both RNA and DNA, and includes hybrids and
chimeras of RNA and DNA and prevents translation of a target mRNA. Herein, a
hybrid indicates a molecule wherein a polynucleotide composed of DNA and a polynu-
cleotide composed of RNA hybridize to each other to form the double-stranded
molecule; whereas a chimera indicates that one or both of the strands composing the
double stranded molecule may contain RNA and DNA. Standard techniques of in-
trroducing siD/R-NA into the cell are used. The siD/R-NA includes a sense nucleic acid
sequence (also referred to as "sense strand"), an antisense nucleic acid sequence (also
referred to as "antisense strand") or both. The siD/R-NA may be constructed such that
a single transcript has both the sense and complementary antisense nucleic acid
sequences from the target gene, e.g., a hairpin. The siD/R-NA may either be a dsD/
R-NA or shD/R-NA.

[0051] As used herein, the term "dsD/R-NA" refers to a construct of two molecules
including complementary sequences to one another and that have annealed together via
the complementary sequences to form a double-stranded polynucleotide molecule. The
nucleotide sequence of two strands may include not only the "sense" or "antisense"
polynucleotides sequence selected from a protein coding sequence of target gene
sequence, but also polynucleotide having a nucleotide sequence selected from non-
coding region of the target gene. One or both of the two molecules constructing the
dsD/R-NA are composed of both RNA and DNA (chimeric molecule), or alternatively,
one of the molecules is composed of RNA and the other is composed of DNA (hybrid
double-strand).

[0052] The term "shD/R-NA", as used herein, refers to an siD/R-NA having a stem-loop
structure, including the first and second regions complementary to one another, i.e.,
sense and antisense strands. The degree of complementarity and orientation of the
regions is sufficient such that base pairing occurs between the regions, the first and
second regions are joined by a loop region, the loop results from a lack of base pairing
between nucleotides (or nucleotide analogs) within the loop region. The loop region of an shD/R-NA is a single-stranded region intervening between the sense and antisense strands and may also be referred to as "intervening single-strand".

As used herein, an "isolated nucleic acid" is a nucleic acid removed from its original environment (e.g., the natural environment if naturally occurring) and thus, synthetically altered from its natural state. In the context of the present invention, examples of isolated nucleic acid include DNA, RNA, and derivatives thereof.

**1. Polynucleotide and polypeptide**

The present invention is based in part on the discovery of elevated expression of the EZH2 gene in cells from subjects of various cancers, for example, AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma.

EZH2 is a polycomb group (PcG) protein homologous to Drosophila enhancer of zeste, a histone methyltransferase associated with transcriptional repression. EZH2 has a SET domain that is typical of histone methyltransferases, and catalyses the addition of methyl groups to histone H3 at lysine 27 (H3K27).

The typical nucleotide sequences of the EZH2 gene are shown in SEQ ID NOs 1, 3, 5, 7 and 9, but not limited to those. These sequence data are also available via GenBank accession numbers: NM_00 1203247.1, NM_00 1203248.1, NM_001203249.1, NM_004456.4 and NM_152998.2, respectively.

Herein, the EZH2 gene encompasses the human EZH2 gene as well as those of other animals including, but not limited to, non-human primate, mouse, rat, dog, cat, horse, and cow, and further includes allelic mutants and genes found in other animals as corresponding to the EZH2 gene.

The typical amino acid sequences encoded the human EZH2 gene are shown in SEQ ID NO: 2, 4, 6, 8 and 10, but not limited to those.

In the context of the present invention, the polypeptide encoded by the EZH2 gene is referred to as "EZH2", and sometimes as "EZH2 polypeptide" or "EZH2 protein". One of skill will recognize that EZH2 sequences need not be limited to the above-mentioned sequences and that variants (e.g., functional equivalents and allelic variants) can be used in the present invention as described below.

According to an aspect of the present invention, functional equivalents are also included in the EZH2 protein. Herein, a "functional equivalent" of a protein is a polypeptide that has a biological activity equivalent to the protein. Namely, any polypeptides that retain the biological ability of the EZH2 protein may be used as such functional equivalents of each protein in the present invention.

The biological activities of the EZH2 protein include, for example, cancer cell proliferation activity (cancer cell proliferation enhancing activity) and methyltransferase
activity (e.g., histone methyltransferase activity). Such functional equivalents include those one or more amino acids are substituted, deleted, added, and/or inserted to the natural occurring amino acid sequence of the EZH2 protein. Alternatively, the polypeptide may be one that includes an amino acid sequence having at least about 80% homology (also referred to as sequence identity) to the sequence of the EZH2 protein (e.g., SEQ ID NO: 2, 4, 6, 8 or 10), more preferably at least about 90% to 95% homology, even more preferably 96%, 97%, 98% or 99% homology.

[0058] A polypeptide of the present invention may have variations in amino acid sequence, molecular weight, isoelectric point, the presence or absence of sugar chains, or form, depending on the cell or host used to produce it or the purification method utilized. Nevertheless, so long as it has a functional equivalent to that of the human protein of the present invention, it is within the scope of the present invention.

[0059] In other embodiments, the polypeptide can be encoded by a polynucleotide that hybridizes under stringent conditions to the natural occurring nucleotide sequence of the gene. In some embodiments, the polypeptide is encoded by a polynucleotide that shares at least about 90%, 93%, 95%, 97%, 99% sequence identity to a reference sequence of EZH2, e.g., SEQ ID NO: 1, 3, 5, 7 or 9, as determined using a known sequence comparison algorithm.

[0060] The phrase "stringent (hybridization) conditions" refers to conditions under which a nucleic acid molecule will hybridize to its target sequence, typically in a complex mixture of nucleic acids, but not detectably to other sequences. Stringent conditions are sequence-dependent and will vary in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10 degrees C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at Tm, 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times of background, preferably 10 times of background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42 degrees C, or, 5x SSC, 1% SDS, incubating at 65 degrees C, with wash in 0.2x SSC, and 0.1% SDS at 50 degrees C.

[0061] In the context of the present invention, a condition of hybridization for isolating a
DNA encoding a polypeptide functionally equivalent to the human EZH2 protein can be routinely selected by a person skilled in the art. For example, hybridization may be performed by conducting pre-hybridization at 68 degrees C for 30 min or longer using "Rapid-hyb buffer" (Amersham LIFE SCIENCE), adding a labeled probe, and warming at 68 degrees C for 1 hour or longer. The following washing step can be conducted, for example, in a low stringent condition. An exemplary low stringent condition may include 42 degrees C, 2x SSC, 0.1% SDS, preferably 50 degrees C, 2x SSC, 0.1% SDS. High stringency conditions are often preferably used. An exemplary high stringency condition may include washing 3 times in 2x SSC, 0.01% SDS at room temperature for 20 min, then washing 3 times in 1x SSC, 0.1% SDS at 37 degrees C for 20 min, and washing twice in 1x SSC, 0.1% SDS at 50 degrees C for 20 min. However, several factors, such as temperature and salt concentration, can influence the stringency of hybridization and one skilled in the art can suitably select the factors to achieve the requisite stringency.

In general, modifications of one, two, or more amino acids in a protein will not influence the function of the protein. In fact, mutated or modified proteins (i.e., peptides composed of an amino acid sequence in which one, two, or several amino acid residues have been modified through substitution, deletion, insertion and/or addition) have been known to retain the original biological activity (Mark et al., Proc Natl Acad Sci USA 81: 5662-6 (1984); ZoUer and Smith, Nucleic Acids Res 10:6487-500 (1982); Dalbadie-McFarland et al., Proc Natl Acad Sci USA 79: 6409-13 (1982)). Accordingly, one of skill in the art will recognize that individual additions, deletions, insertions, or substitutions to an amino acid sequence that alter a single amino acid or a small percentage of amino acids or those considered to be a "conservative modification" wherein the alteration of a protein results in a protein with similar functions, are acceptable in the context of the instant invention. Thus, in one embodiment, the peptides of the present invention may have an amino acid sequence wherein one, two or even more amino acids are added, inserted, deleted, and/or substituted in the human EZH2 sequence.

So long as the activity of the protein is maintained, the number of amino acid mutations is not particularly limited. However, it is generally preferred to alter 5% or less of the amino acid sequence. Accordingly, in a preferred embodiment, the number of amino acids to be mutated in such a mutant is generally 30 amino acids or less, preferably 20 amino acids or less, more preferably 10 amino acids or less, more preferably 5 or 6 amino acids or less, and even more preferably 3 or 4 amino acids or less.

An amino acid residue to be mutated is preferably mutated into a different amino acid in which the properties of the amino acid side-chain are conserved (a process
known as conservative amino acid substitution). Examples of properties of amino acid side chains are hydrophobic amino acids (A, I, L, M, F, P, W, Y, V), hydrophilic amino acids (R, D, N, C, E, Q, G, H, K, S, T), and side chains having the following functional groups or characteristics in common: an aliphatic side-chain (G, A, V, L, I, P); a hydroxyl group containing side-chain (S, T, Y); a sulfur atom containing side-chain (C, M); a carboxylic acid and amide containing side-chain (D, N, E, Q); a base containing side-chain (R, K, H); and an aromatic containing side-chain (H, F, Y, W). Conservative substitution tables providing functionally similar amino acids are well known in the art. For example, the following eight groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Glycine (G);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
7) Serine (S), Threonine (T); and
8) Cysteine (C), Methionine (M) (see, e.g., Creighton, Proteins 1984).

Such conservatively modified polypeptides are included in the present EZH2 protein. However, the present invention is not restricted thereto and the EZH2 protein includes non-conservative modifications so long as they retain at least one biological activity of the EZH2 protein. Furthermore, the modified proteins do not exclude polymorphic variants, interspecies homologues, and those encoded by alleles of these proteins.

Moreover, the EZH2 gene to be used in the present invention encompasses polynucleotides that encode functional equivalents of the EZH2 protein. In addition to hybridization, a gene amplification method, for example, the polymerase chain reaction (PCR) method, can be utilized to isolate a polynucleotide encoding a polypeptide functionally equivalent to the protein, using a primer synthesized based on the sequence above information. Polynucleotides and polypeptides that are functionally equivalent to the human gene and protein, respectively, normally have a high homology to the originating nucleotide or amino acid sequence of. "High homology" typically refers to a homology of 40% or higher, preferably 60% or higher, more preferably 80% or higher, even more preferably 90% to 95% or higher. The homology of a particular polynucleotide or polypeptide can be determined by following the algorithm in "Wilbur and Lipman, Proc Natl Acad Sci USA 80: 726-30 (1983)."

When the functional equivalents of the EZH2 protein retain the methyltransferase activity of the EZH2 protein, such functional equivalents preferably maintain the SET domain of the EZH2 protein. Accordingly, the functional equivalents of the EZH2
protein may be polypeptides including the SET domain of the EZH2 protein. For example, the SET domain of the EZH2 protein is located in the amino acid position 613-726 of SEQ ID NO: 2, 604-717 of SEQ ID NO: 4, 562-675 of SEQ ID NO: 6, 618-731 of SEQ ID NO: 8, and 574-687 of SEQ ID NO: 10, respectively. The amino acid sequence of SET domain of EZH2 protein is shown in SEQ ID NO: 31. Accordingly, examples of functional equivalents of the EZH2 protein include polypeptides having the amino acid sequence of SEQ ID NO: 31 and having a methyltransferase activity of the EZH2 protein.

II. Diagnosing cancer

II-1. Method for diagnosing cancer or a predisposition for developing cancer

The expression of the EZH2 gene was found to be specifically elevated in cancer tissues.

Accordingly, the EZH2 gene as well as its transcription and translation products find diagnostic utility as a marker for cancer overexpressing EZH2 gene, and by measuring the expression of the EZH2 gene in a subject-derived biological sample such as a cell sample and a tissue sample, cancer can be diagnosed or detected. Such diagnosis or detection may be performed by comparing the expression level of EZH2 gene between a subject-derived sample and a normal sample. More particularly, the present invention provides a method for detecting or diagnosing cancer and/or a predisposition for developing cancer in a subject by determining the expression level of the EZH2 gene in the subject-derived biological sample. Preferred cancers to be diagnosed or detected by the present method include AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma.

Alternatively, the present invention provides a method for detecting or identifying cancer cells in a subject-derived biological sample such as a tissue sample, the method including the step of determining the expression level of the EZH2 gene in a subject-derived biological sample, wherein an increase in said expression level as compared to a normal control level indicates the presence or suspicion of cancer cells in the tissue.

Such result may be combined with additional information to assist a doctor, nurse, or other practitioner to diagnose that a subject suffers from the disease or is predisposed to developing the disease. Alternatively, the present invention may provide a doctor with useful information to diagnose that the subject suffers from the disease. For example, according to the present invention, when the suspicion or doubt of the presence of cancer cells in the tissue obtained from a subject is indicated, clinical decisions would be made by a doctor with consideration of this observation and another aspect including the pathological finding of the tissue, levels of known tumor marker(s) in blood, or clinical course of the subject, etc. For example, some well-
known diagnostic lung cancer markers in blood include ACT, BFP, CA19-9, CA50, CA72-4, CA130, CA602, CEA, IAP, KMO-1, SCC, SLX, SP1, Span-1, STN, TPA, and cytokeratin 19 fragment. Some well-known bladder cancer markers in blood include NMP22, BFP and TPA. Alternatively, diagnostic esophageal tumor markers in blood such as CEA, DUPAN-2, IAP, NSE, SCC, SLX and Span-1 are also well known. Some well-known diagnostic colorectal cancer markers in blood include CA72-4, STN, CA19-9, CEA, and NCC-ST-439; liver cancer markers in blood include AFP and PIVKA-2; pancreatic cancer marker in blood include CA19-9, Span-1, SLX and CEA; testicular cancer markers include AFP and BFP; acute myeloid leukemia markers in blood include ACT and SOD; and osteosarcoma markers in blood include ICTP, NTx, DPD and BAP. Namely, in a particular embodiment, according to the present invention, an intermediate result for examining the condition of a subject may also be provided.

[0071] In another embodiment, the present invention provides a method for detecting a diagnostic marker of cancer, the method including the step of detecting the expression level of the EZH2 gene in a subject-derived biological sample as a diagnostic marker of cancer. Preferable cancers to be diagnosed by the present method include AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma.

[0072] In the context of the present invention, the term "diagnosing" is intended to encompass predictions and likelihood analysis. The present method is intended to be used clinically in making decisions concerning treatment modalities, including therapeutic intervention, diagnostic criteria such as disease stages, and disease monitoring and surveillance for cancer. According to the present invention, an intermediate result for examining the condition of a subject may also be provided. Such intermediate result may be combined with additional information to assist a doctor, nurse, or other practitioner to determine that a subject suffers from the disease. Alternatively, the present invention may be used to detect cancerous cells in a subject-derived tissue, and provide a doctor with useful information to diagnose that the subject suffers from the disease.

[0073] A subject to be diagnosed by the present method is preferably a mammal. Exemplary mammals include, but are not limited to, human, non-human primate, mouse, rat, dog, cat, horse, and cow. It is preferred to collect a biological sample from the subject to be diagnosed to perform the diagnosis. Any biological material can be used as the biological sample for the determination so long as it can include the objective transcription or translation product of the EZH2 gene due to cancer. The biological samples include, but are not limited to, bodily tissues and fluids, such as biopsy, saliva, sputum, blood, serum, plasma, pleural effusion and urine.
[0074] Preferably, the biological sample contains a cell population including an epithelial cell, more preferably an epithelial cell derived from tissue suspected to be cancerous. Further, if necessary, the cells may be purified from the obtained bodily tissues and fluids, and then used as the biological sample. Alternatively, biological sample may be a tissue sample collected from an area suspected to be cancerous. Preferably, the tissue sample may be a lung tissue sample for lung cancer, bladder tissue sample for bladder cancer, esophageal tissue sample for esophageal cancer, colorectal tissue sample for colorectal cancer, renal tissue sample for renal cell carcinoma, myeloid tissue sample for AML and CML, osseous tissue sample for osteosarcoma, and bile duct tissue sample for cholangiocellular carcinoma.

[0075] According to the present invention, the expression level of the EZH2 gene is determined in a subject-derived biological sample. The expression level can be determined at the transcription product (i.e., mRNA) level, using methods known in the art. For example, the mRNA of the EZH2 gene may be quantified using probes by hybridization methods (e.g., Northern hybridization). The detection may be carried out on a filter, a chip or an array. The use of an array is preferable for detecting the expression level of a plurality of genes (e.g., various cancer specific genes) including the EZH2 gene. Those skilled in the art can prepare such probes utilizing the sequence information of the EZH2 gene. For example, the cDNA of the EZH2 gene may be used as the probes. If necessary, the probe may be labeled with a suitable label, such as dyes and isotopes, and the expression level of the gene may be detected as the intensity of the hybridized labels.

[0076] Furthermore, the transcription product of the EZH2 gene may be quantified using primers by amplification-based detection methods (e.g., RT-PCR). Such primers can also be prepared based on the available sequence information of the gene. For example, the primers used in the Example may be employed for the detection by RT-PCR, but the present invention is not restricted thereto.

Specifically, a probe or primer used for the present method hybridizes under stringent, moderately stringent, or low stringent conditions to the mRNA of the EZH2 gene. As used herein, the phrase "stringent (hybridization) conditions" refers to conditions under which a probe or primer will hybridize to its target sequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different under different circumstances. Specific hybridization of longer sequences is observed at higher temperatures than shorter sequences. Generally, the temperature of a stringent condition is selected to be about 5 degrees C lower than the thermal melting point (Tm) for a specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at
equilibrium. Since the target sequences are generally present at excess, at Tm, 50% of the probes are occupied at equilibrium. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30 degrees C for short probes or primers (e.g., 10 to 50 nucleotides) and at least about 60 degrees C for longer probes or primers. Stringent conditions may also be achieved with the addition of destabilizing agents, such as formamide.

A probe or primer of the present invention is typically a substantially purified oligonucleotide. The oligonucleotide typically includes a region of nucleotide sequence that hybridizes under stringent conditions to at least about 2000, 1000, 500, 400, 350, 300, 250, 200, 150, 100, 50, or 25 bases, consecutive sense strand nucleotide sequence of a nucleic acid including a EZH2 sequence, or an anti sense strand nucleotide sequence of a nucleic acid including a EZH2 sequence, or of a naturally occurring mutant of these sequences. In particular, for example, in a preferred embodiment, an oligonucleotide having 5-50 bases in length can be used as a primer for amplifying the gene EZH2es, to be detected. More preferably, mRNA or cDNA of a gene can be detected with oligonucleotide probe or primer of a specific size, generally 15-30 bases in length. In preferred embodiments, length of the oligonucleotide probe or primer can be selected from 15-25 bases. Assay procedures, devices, or reagents for the detection of gene by using such oligonucleotide probe or primer are well known (e.g. oligonucleotide microarray or PCR). In these assays, probes or primers can also include tag or linker sequences. Further, probes or primers can be modified with detectable label or affinity ligand to be captured. Alternatively, in hybridization based detection procedures, a polynucleotide having a few hundreds (e.g., about 100-200) bases to a few kilo (e.g., about 1000-2000) bases in length can also be used for a probe (e.g., northern blotting assay or cDNA microarray analysis).

Alternatively, the translation product (i.e., protein) of the EZH2 gene may be detected for the diagnosis or detection of the present invention. For example, the quantity of the EZH2 protein may be determined. A method for determining the quantity of the protein as the translation product includes immunoassay methods that use an antibody specifically recognizing the protein. The antibody may be monoclonal or polyclonal. Furthermore, any antibody fragments or modified antibodies (e.g., chimeric antibody, scFv, Fab, F(ab’)2, Fv, etc.) may be used for the detection, so long as they retain the binding ability to the EZH2 protein. Methods to prepare these kinds of antibodies for the detection of proteins are well known in the art, and any method may be employed in the present invention to prepare such antibodies and equivalents thereof.

As another method to detect the expression level of the EZH2 gene based on its
transcription product, the intensity of staining may be observed via immunohistochemical analysis using an antibody against the EZH2 protein. Namely, the observation of strong staining indicates increased presence of the protein and at the same time high expression level of the EZH2 gene.

Furthermore, the translation product may be detected based on its biological activity. Specifically, the EZH2 protein was demonstrated herein to be involved in the growth of cancer cells. Thus, the cancer cell growth promoting ability of the EZH2 protein may be used as an index of the EZH2 protein existing in the biological sample. Herein, cell growth promoting ability is also referred to as "cell proliferative activity" or "cell proliferation enhancing activity".

Moreover, in addition to the expression level of the EZH2 gene, the expression level of other cancer-associated genes, for example, genes known to be differentially expressed in AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma may also be determined to improve the accuracy of the diagnosis.

In the method of the present invention, the expression level of the EZH2 gene in a subject-derived biological sample is compared with the control level.

In the context of the present invention, the phrase "control level" refers to the expression level of the EZH2 gene detected in a control sample and encompasses both a normal control level and a cancer control level. The phrase "normal control level" refers to a level of the EZH2 gene expression detected in a normal healthy individual or in a population of individuals known not to be suffering from cancer. A normal individual is one with no clinical symptom of cancer. A normal control level can be determined using a normal cell obtained from a non-cancerous tissue. A "normal control level" may also be the expression level of the EZH2 gene detected in a normal healthy tissue or cell of an individual or population known not to be suffering from cancer. On the other hand, the phrase "cancer control level" refers to an expression level of the EZH2 gene detected in the cancerous tissue or cell of an individual or population suffering from cancer.

An increase in the expression level of the EZH2 gene detected in a sample as compared to a normal control level indicates that the subject (from which the sample has been obtained) suffers from or is at risk of developing cancer such as AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma.

Alternatively, the expression level of the EZH2 gene in a sample can be compared to a cancer control level of the EZH2 gene. A similarity between the expression level of a sample and the cancer control level indicates that the subject (from which the sample has been obtained) suffers from or is at risk of developing cancer.
Herein, gene expression levels are deemed to be "altered" when the gene expression increases by, for example, 10%, 25%, or 50% from, or at least 0.1 fold, at least 0.2 fold, at least 0.5 fold, at least 2 fold, at least 5 fold, or at least 10 fold or more compared to a control level. Accordingly, the expression level of cancer marker genes including the EZH2 gene in a biological sample can be considered to be increased if it increases from the normal control level of the corresponding cancer marker gene by, for example, 10% or more, 25% or more, or 50% or more; or increases to more than 1.1 fold, more than 1.5 fold, more than 2.0 fold, more than 5.0 fold, more than 10.0 fold, or more.

The control level may be determined at the same time with a test biological sample by using a sample(s) previously collected and stored from a subject/subjects whose disease state (cancerous or non-cancerous) is/are known. Alternatively, the control level may be determined by a statistical method based on the results obtained by analyzing previously determined expression level(s) of the EZH2 gene in samples from subjects whose disease state are known. Furthermore, the control level can be a database of expression patterns from previously tested cells. Moreover, according to an aspect of the present invention, the expression level of the EZH2 gene in a biological sample may be compared to multiple control levels, which control levels are determined from multiple reference samples. It is preferred to use a control level determined from a reference sample derived from a tissue type similar to that of the patient-derived biological sample. Moreover, it is preferred, to use the standard value of the expression levels of the EZH2 gene in a population with a known disease state. The standard value may be obtained by any method known in the art. For example, a range of mean +/- 2 S.D. or mean +/- 3 S.D. may be used as standard value.

When the expression level of the EZH2 gene is increased compared to the normal control level or is similar to the cancerous control level, the subject may be diagnosed to be suffering from or at a risk of developing cancer. Furthermore, in case where the expression levels of multiple cancer-related genes are compared, a similarity in the gene expression pattern between the sample and the reference that is cancerous indicates that the subject is suffering from or at a risk of developing cancer.

Difference between the expression levels of a test biological sample and the control level can be normalized to the expression level of control nucleic acids, e.g., housekeeping genes. Genes whose expression levels are known not to differ depending on the cancerous or non-cancerous state of the cell. Exemplary control genes include, but are not limited to, beta-actin, glyceraldehyde 3-phosphate dehydrogenase, and ribosomal protein PI.

Furthermore, the present invention provides the use of the EZH2 gene as a cancerous marker. The EZH2 gene is particularly useful for AML, bladder cancer, breast cancer,
cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma and renal cell carcinoma as a cancerous marker. For example, it can be determined whether a biological sample contains cancerous cells, especially lung cancerous cells, bladder cancerous cells, esophageal cancerous cells, colorectal cancerous cells, renal cell carcinoma cells, myeloid tissue tumor cells, osteosarcoma cells or cholangiocellular carcinoma cells, by detecting the expression level of the EZH2 gene as a cancerous marker. Specifically, increasing the expression level of the EZH2 gene in a biological sample as compared to a normal control level indicates that the biological sample contains cancerous cells. The expression level of the EZH2 gene can be determined by detecting the transcription or translation products of the gene as described above.

[0089] In the present invention, it is revealed that EZH2 is not only a useful diagnostic marker, but also suitable target for cancer therapy. Therefore, cancer treatment targeting EZH2 can be achieved by the present invention. In the present invention, the cancer treatment targeting EZH2 refers to suppression or inhibition of EZH2 activity and/or expression in the cancer cells. Any anti- EZH2 agents may be used for the cancer treatment targeting EZH2. In the present invention, the anti- EZH2 agents include following substance as active ingredient:

- (a) a double-stranded molecule of the present invention,
- (b) DNA encoding thereof, or
- (c) a vector encoding thereof.

[0090] Accordingly, in a preferred embodiment, the present invention provides a method of (i) diagnosing whether a subject has the cancer to be treated, and/or (ii) selecting a subject for cancer treatment, which method includes the steps of:

- a) determining the expression level of EZH2 in cancer cells or tissue(s) obtained from a subject who is suspected to have the cancer to be treated;
- b) comparing the expression level of EZH2 with a normal control level;
- c) diagnosing the subject as having the cancer to be treated, if the expression level of EZH2 is increased as compared to the normal control level; and
- d) selecting the subject for cancer treatment, if the subject is diagnosed as having the cancer to be treated, in step c).

[0091] Alternatively, such a method includes the steps of:

- a) determining the expression level of EZH2 in cancer cells or tissue(s) obtained from a subject who is suspected to have the cancer to be treated;
- b) comparing the expression level of EZH2 with a cancerous control level;
- c) diagnosing the subject as having the cancer to be treated, if the expression level of EZH2 is similar or equivalent to the cancerous control level; and
- d) selecting the subject for cancer treatment, if the subject is diagnosed as having the cancer to be treated, in step c).


The cancer includes, but is not limited to, AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma. Accordingly, prior to the administration of the double-stranded molecule of the present invention or a vector encoding the double-stranded molecule as active ingredient, it is preferable to confirm whether the expression level of EZH2 in the cancer cells or tissues to be treated is enhanced as compared with normal cells of the same organ. Thus, in one embodiment, the present invention provides a method for treating a cancer (over)expressing EZH2, which method may include the steps of:

i) determining the expression level of EZH2 in cancer cells or tissue(s) obtained from a subject with the cancer to be treated;

ii) comparing the expression level of EZH2 with normal control; and

iii) administrating at least one component selected from the group consisting of

(a) a double-stranded molecule of the present invention,

(b) DNA encoding thereof, and

(c) a vector encoding thereof,

to a subject with a cancer overexpressing EZH2 compared with normal control. Alternatively, the present invention also provides a pharmaceutical composition comprising at least one component selected from the group consisting of:

(a) a double-stranded molecule of the present invention,

(b) DNA encoding thereof, and

(c) a vector encoding thereof,

for use in administrating to a subject having a cancer overexpressing EZH2. In other words, the present invention further provides a method for identifying a subject to be treated with:

(a) a double-stranded molecule of the present invention,

(b) DNA encoding thereof, or

(c) a vector encoding thereof,

which method may include the step of determining an expression level of EZH2 in subject-derived cancer cells or tissue(s), wherein an increase of the level compared to a normal control level of the gene indicates that the subject has cancer which may be treated with a double-stranded molecule of the present invention, a DNA encoding thereof, or a vector encoding thereof.

The method of treating a cancer of the present invention will be described in more detail below.

A subject to be treated by the present method is preferably a mammal. Exemplary mammals include, but are not limited to, e.g., human, non-human primate, mouse, rat, dog, cat, horse, and cow.
According to the present invention, the expression level of EZH2 in cancer cells or tissues obtained from a subject is determined. The expression level can be determined at the transcription (nucleic acid) product level, using methods known in the art. For example, hybridization methods (e.g., Northern hybridization), a chip or an array, probes, RT-PCR can be used to determine the transcription product level of EZH2.

Alternatively, the translation product may be detected for the treatment of the present invention. For example, the quantity of observed protein (SEQ ID NO: 2, 4, 6, 8 and 10) may be determined.

As another method to detect the expression level of EZH2 gene based on its translation product, the intensity of staining may be measured via immunohistochemical analysis using an antibody against the EZH2 protein. Namely, in this measurement, strong staining indicates increased presence/level of the protein and, at the same time, high expression level of EZH2 gene.

Methods for detecting or measuring the EZH2 polypeptide and/or polynucleotide encoding thereof can be exemplified as described above.

II-2. Methods for determining or assessing the prognosis of cancer

The present invention relates, in part, to the discovery that EZH2 expression is significantly associated with poorer prognosis of subjects with lung cancer, e.g. NSCLC. Thus, the present invention provides a method for determining or assessing the prognosis of a subject with lung cancer, by detecting the expression level of the EZH2 gene in a biological sample collected from a subject with lung cancer; comparing the detected expression level to a control level; and determining an increased expression level of EZH2 in comparison to the normal control level as indicative of poor prognosis (poor survival).

On the other hand, in colorectal cancer, EZH2 expression is significantly associated with good prognosis. Thus, the present invention also provides a method for determining or assessing the prognosis of a subject with a colorectal cancer, by detecting the expression level of the EZH2 gene in a biological sample collected from a subject with colorectal cancer; comparing the detected expression level to a control level; and determining an increased expression level of EZH2 in comparison to the normal control level as indicative of good prognosis (good survival).

In other embodiments, determining a similar or increased expression level of EZH2 gene in comparison to a cancerous control level is indicative of a poor prognosis in lung cancer. On the other hand, determining a similar or increased expression level of EZH2 gene in comparison to a cancerous control level is indicative of a good prognosis in colorectal cancer.

Herein, the term "prognosis" refers to a forecast as to the probable outcome of the disease as well as the prospect of recovery from the disease as indicated by the nature
and symptoms of the case. Accordingly, a less favorable, negative, or poor prognosis is defined by a lower post-treatment survival term or survival rate. Conversely, a positive, favorable, or good prognosis is defined by an elevated post-treatment survival term or survival rate.

The terms "assessing the prognosis" refer to the ability of predicting, forecasting or correlating a given detection or measurement with a future outcome of cancer of the subject (e.g., malignancy, likelihood of curing cancer, survival, and the like). For example, a determination of the expression level of EZH2 gene over time enables a predicting of an outcome for the subject (e.g., increase or decrease in malignancy, increase or decrease in grade of a cancer, likelihood of curing cancer, survival, and the like).

In the context of the present invention, the phrase "assessing (or determining) the prognosis" is intended to encompass predictions and likelihood analysis of cancer progression, particularly cancer recurrence, metastatic spread and disease relapse. The present method for determining or assessing prognosis is intended to be used clinically in making decisions concerning treatment modalities, including therapeutic intervention, diagnostic criteria such as disease staging, and disease monitoring and surveillance for metastasis or recurrence of neoplastic disease.

The subject-derived biological sample used for the method may be any sample derived from the subject to be assessed so long as the expression level of the EZH2 gene can be detected in the sample. The subject-derived biological sample may be any sample derived from a subject, e.g., a subject known to have cancer. Preferably, the biological sample is a cancerous lung tissue sample for lung cancer or a cancerous colorectal tissue sample for colorectal cancer. Furthermore, the biological sample may include bodily fluids such as saliva, sputum, blood, serum, plasma, pleural effusion or urine. Moreover, the sample may be cells purified from a tissue. The biological samples may be obtained from a subject at various time points, including before, during, and/or after a treatment. For example, a lung cancer cell(s) or colorectal cancer cell(s) obtained from a subject to be assessed is a preferable biological sample.

According to the present invention, it was shown that the higher the expression level of the EZH2 gene measured in the subject-derived biological sample, the poorer the prognosis for post-treatment remission, recovery, and/or survival and the higher the likelihood of poor clinical outcome in lung cancer. On the other hand, it was shown that the higher the expression level of the EZH2 gene measured in the subject-derived biological sample, the better the prognosis for post-treatment remission, recovery, and/or survival and the higher the likelihood of good clinical outcome in colorectal cancer. Thus, according to the present methods, the "control level" used for comparison may be, for example, the expression level of the EZH2 gene detected before any kind of
treatment in an individual, or a population of individuals who showed good or positive prognosis of cancer after the treatment, which herein will be referred to as "good prognosis control level". Alternatively, the expression level of the EZH2 gene detected in a noncancerous tissue sample can be also used as "good prognosis control level" in lung cancer. Alternatively, the "control level" may be the expression level of the EZH2 gene detected before any kind of treatment in an individual, or a population of individuals who showed poor or negative prognosis of cancer after the treatment, which herein will be referred to as "poor prognosis control level". Alternatively, the expression level of the EZH2 gene detected in a noncancerous tissue sample can be also used as "poor prognosis control level" in colorectal cancer. The "control level" is a single expression pattern derived from a single reference population or from a plurality of expression patterns. Thus, the control level may be determined based on the expression level of the EZH2 gene detected before any kind of treatment of cancer in a subject, or a population of the subjects whose disease state (good or poor prognosis) are known. It is preferred, to use the standard value of the expression levels of the EZH2 gene in a patient group with a known disease state. The standard value may be obtained by any method known in the art. For example, a range of mean +/- 2 S.D. or mean +/- 3 S.D. may be used as standard value.

The control level may be determined at the same time with the test biological sample by using a sample(s) previously collected and stored before any kind of treatment from cancer subject(s) (control or control group) whose disease state (good prognosis or poor prognosis) are known.

Alternatively, the control level may be determined by a statistical method based on the results obtained by analyzing the expression level of the EZH2 gene in samples previously collected and stored from a control group. Furthermore, the control level can be a database of expression patterns from previously tested cells.

Moreover, according to an aspect of the present invention, the expression level of the EZH2 gene in a biological sample may be compared to multiple control levels, which control levels are determined from multiple reference samples. It is preferred to use a control level determined from a reference sample derived from a tissue type similar to that of the patient-derived biological sample.

According to the present invention, a similarity in the expression level of the EZH2 gene to a good prognosis control level indicates a more favorable prognosis of the subject and an increase in the expression level to the good prognosis control level indicates less favorable, poorer prognosis for post-treatment remission, recovery, survival, and/or clinical outcome. On the other hand, in lung cancer, a decrease in the expression level of the EZH2 to the poor prognosis control level indicates a more favorable prognosis of the patient and a similarity in the expression level to the poor
prognosis control level indicates less favorable, poorer prognosis for post-treatment remission, recovery, survival, and/or clinical outcome. In colorectal cancer, an increase in the expression level of the EZH2 to the poor prognosis control level indicates a more favorable prognosis of the patient and a similarity in the expression level to the poor prognosis control level indicates less favorable, poorer prognosis for post-treatment remission, recovery, survival, and/or clinical outcome. For example, a lung cancer cell(s) or colorectal cancer cell(s) obtained from a subject who showed good, or poor prognosis of cancer after treatment is a preferable biological sample for good, or poor prognosis control level, respectively.

In general, cancer progression may be evaluated within 5 years. Accordingly, for example, a subject with less favorable, negative, or poor prognosis includes a subject who shows recurrence, metastatic spread or disease relapse of cancer within 5 years after the treatment (e.g., surgical resection of a cancerous tissue). A cancerous tissue derived from such patient may be used as a control sample for poor prognosis in the present invention.

Alternatively, a subject with positive, favorable, or good prognosis includes a subject who does not show recurrence, metastatic spread and disease relapse of cancer within 5 years after the treatment. A cancerous tissue derived from such patient may be used as a control sample for good prognosis in the present invention.

The expression level of the EZH2 gene in a subject-derived biological sample can be considered altered when the expression level differs from the control level by more than 1.0, 1.5, 2.0, 5.0, 10.0, or more fold.

The difference in the expression level between the test biological sample and the control level can be normalized to a control, e.g., housekeeping gene. For example, polynucleotides whose expression levels are known not to differ between the cancerous and non-cancerous cells, including those coding for beta-actin, glyceraldehyde 3-phosphate dehydrogenase, and ribosomal protein PI, may be used to normalize the expression level of the EZH2 gene.

The expression level may be determined by detecting the gene transcript in the patient-derived biological sample using techniques well known in the art. The gene transcripts detected by the present method include both the transcription and translation products, such as mRNA and protein.

For instance, the transcription product of the EZH2 gene can be detected by hybridization, e.g., Northern blot hybridization analyses, that use an EZH2 gene probe to the gene transcript. The detection may be carried out on a chip or an array. The use of an array is preferable for detecting the expression level of a plurality of genes including the EZH2 gene. As another example, amplification-based detection methods, such as reverse-transcription based polymerase chain reaction (RT-PCR) which use
primers specific to the EZH2 gene may be employed for the detection (see "EXAMPLES"). The EZH2 gene-specific probe or primers may be designed and prepared using conventional techniques by referring to the whole sequence of the EZH2 gene (e.g., SEQ ID NO: 1, 3, 5, 7 or 9).

For example, the primers used in the "EXAMPLES" may be employed for the detection by RT-PCR, but the present invention is not restricted thereto. Specifically, a probe or primer used for the present method hybridizes under stringent, moderately stringent, or low stringent conditions to the mRNA of the EZH2 gene. As used herein, the phrase "stringent (hybridization) conditions" refers to conditions under which a probe or primer will hybridize to its target sequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different under different circumstances. Specific hybridization of longer sequences is observed at higher temperatures than shorter sequences. Generally, the temperature of a stringent condition is selected to be about 5 degree Centigrade lower than the thermal melting point (Tm) for a specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at equilibrium. Since the target sequences are generally present at excess, at Tm, 50% of the probes are occupied at equilibrium. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30 degrees Centigrade for short probes or primers (e.g., 10 to 50 nucleotides) and at least about 60 degrees Centigrade for longer probes or primers. Stringent conditions may also be achieved with the addition of destabilizing agents, such as formamide.

Alternatively, the translation product may be detected for the assessment of the present invention. For example, the quantity of the EZH2 protein may be determined. A method for determining the quantity of the protein as the translation product includes immunoassay methods that use an antibody specifically recognizing the EZH2 protein. The antibody may be monoclonal or polyclonal. Furthermore, any fragment or modification (e.g., chimeric antibody, scFv, Fab, F(ab')2, Fv, etc.) of the antibody may be used for the detection, so long as the fragment retains the binding ability to the EZH2 protein. Methods to prepare these kinds of antibodies for the detection of proteins are well known in the art, and any method may be employed in the present invention to prepare such antibodies and equivalents thereof.

As another method to detect the expression level of the EZH2 gene based on its translation product, the intensity of staining may be observed via immunohistochemical analysis using an antibody against EZH2 protein. Namely, the observation of
strong staining indicates increased presence of the EZH2 protein and at the same time high expression level of the EZH2 gene.

Furthermore, herein, the EZH2 protein has been demonstrated to have a cell proliferating activity. Therefore, the expression level of the EZH2 gene can be determined using such cell proliferating activity as an index. For example, cells which express EZH2 are prepared and cultured in the presence of a biological sample, and then by detecting the extent of proliferation in a predetermined time period, or by measuring the cell cycle or the colony forming ability the cell proliferating activity of the biological sample can be determined.

Moreover, in addition to the expression level of the EZH2 gene, the expression level of other cancer-associated genes, for example, genes known to be differentially expressed in lung cancer (e.g., NSCLC) or colorectal cancer may also be determined to improve the accuracy of the assessment.

Alternatively, according to the present invention, an intermediate result may also be provided in addition to other test results for assessing the prognosis of a subject. Such intermediate result may assist a doctor, nurse, or other practitioner to assess, determine, or estimate the prognosis of a subject. Additional information that may be considered, in combination with the intermediate result obtained by the present invention, to assess prognosis includes clinical symptoms and physical conditions of a subject.

In other words, the expression level of the EZH2 gene is useful prognostic marker for assessing, predicting or determining the prognosis of a subject suffering from lung cancer (e.g., NSCLC) or colorectal cancer. Therefore, the present invention also provides a method for detecting prognostic marker for assessing, predicting or determining the prognosis of a subject suffering from lung cancer including NSCLC or colorectal cancer, which comprises steps of:

a) detecting or determining an expression level of a EZH2 gene in a subject-derived biological sample, and

b) correlating the expression level detected or determined in step a) with the prognosis of the subject.

The subject to be assessed for the prognosis of cancer according to the method is preferably a mammal and includes human, non-human primate, mouse, rat, dog, cat, horse, and cow.

III. Kits and reagents

The present invention provides kits for detecting or diagnosing cancer, or determining or assessing the prognosis of cancer. Preferably, the cancer to be detected or diagnosing by the present kit is selected from the group consisting of AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma. The kits of the
present invention preferably include reagents described below.

[0115] The present invention also provides reagents for detecting or diagnosing cancer, or determining or assessing the prognosis of cancer, i.e., reagents that can detect the transcription or translation product of the EZH2 gene. Examples of such reagents include those capable of:

(a) detecting an mRNA of the EZH2 gene;
(b) detecting an EZH2 protein; or
(c) detecting a biological activity of the EZH2 protein,
in a subject-derived biological sample.

[0116] Suitable reagents include nucleic acids that specifically bind to or identify a transcription product of the EZH2 gene. For example, a nucleic acid that specifically binds to or identifies a transcription product of the EZH2 gene includes, for example, oligonucleotides (e.g., probes and primers) having a sequence that is complementary to a portion of the EZH2 gene transcription product. Such oligonucleotides are exemplified by primers and probes that are specific to the mRNA of the gene of interest and may be prepared based on methods well known in the art. Alternatively, antibodies can be exemplified as reagents for detecting the translation product of the gene. The probes, primers, and antibodies described above under the item of 11-1. Method for diagnosing cancer or a predisposition for developing cancer can be mentioned as suitable examples of such reagents. These reagents may be used for the above-described diagnosis or detection of cancer, particularly AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma. Also, above-mentioned reagents may be used for the determining or assessing the prognosis of cancer. In this case, the reagents may be preferably used for lung cancer (e.g., NSCLC) and colorectal cancer. The assay format for using the reagents may be Northern hybridization or sandwich ELISA, both of which are well-known in the art.

[0117] The detection reagents may be packaged together in the form of a kit. For example, the detection reagents may be packaged in separate containers. Furthermore, the detection reagents may be packaged with other reagents necessary for the detection. For example, a kit may include a nucleic acid or antibody (either bound to a solid matrix or packaged separately with reagents for binding them to the matrix) as the detection reagent, a control reagent (positive and/or negative), and/or a detectable label. A kit of the present invention may further include other materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes. These reagents and such may be retained in a container with a label. Suitable containers include bottles, vials, and test tubes. The containers may be formed from a variety of materials, such as glass or plastic. Instructions (e.g., written, tape, VCR, CD-
Although the present kit is suited for the detection and diagnosis of cancer, for example, AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma, it may also be useful in assessing the prognosis of cancer and/or monitoring the efficacy of a cancer therapy, for example, lung cancer (e.g., NSCLC) and colorectal cancer.

According to an aspect of the present invention, the kit of the present invention for diagnosing cancer may further include either of positive or negative controls sample, or both. The positive control sample of the present invention may be established AML cell lines, bladder cancer cell lines, breast cancer cell lines, cholangiocellular carcinoma cell lines, CML cell lines, esophageal cancer cell lines, lung cancer cell lines, osteosarcoma cell lines, colorectal cancer cell lines and renal cell carcinoma cell lines.

Alternatively, the EZH2 positive samples may also be a clinical cancerous tissue sample collected from an AML, bladder cancer, breast cancer, CML esophageal cancer, lung cancer, osteosarcoma, colorectal cancer or renal cancer patient.

In a preferred embodiment, such lung cancer tissue may be a clinical NSCLC tissue(s) obtained from a lung cancer patient(s). In a more preferred embodiment, such NSCLC tissue(s) may be a clinical lung adenocarcinoma tissue(s), lung squamous cell carcinoma tissue(s), or large cell carcinoma tissue(s) obtained from a lung cancer patient(s).

Alternatively, positive control samples may be prepared by determined a cut-off value and preparing a sample containing an amount of an EZH2 mRNA or protein more than the cut-off value. Herein, the phrase "cut-off value" refers to the value dividing between a normal range and a cancerous range. For example, one skilled in the art may be determine a cut-off value using a receiver operating characteristic (ROC) curve. The present kit may include an EZH2 standard sample providing a cut-off value amount of an EZH2 mRNA or polypeptide. On the contrary, negative control samples may be prepared from non-cancerous cell lines or non-cancerous tissues such as normal blood tissues, bladder tissues, breast tissues, bile duct tissues, esophageal tissues, lung tissues, bone tissues, colorectal tissues, renal tissues.

Likewise, the kit of the present invention for assessing the prognosis of cancer may further include either of a good prognosis control sample or a poor prognosis control sample, or both. As described in II-2. Methods for determining or assessing the prognosis of cancer, a good control may be an individual or a population of individuals who showed good or positive prognosis of cancer, after the treatment. Meanwhile, a poor control may be an individual or a population of individuals who showed poor or
negative prognosis of cancer, after the treatment.

[0123] In a preferred embodiment, a good or positive prognosis control sample may also be a clinical lung cancer tissue(s) obtained from a lung cancer patient(s) who showed good or positive prognosis of lung cancer, or a clinical colorectal cancer tissue(s) obtained from a colorectal cancer patient(s) who showed good or positive prognosis of colorectal cancer, after treatment.

[0124] In a preferred embodiment, such lung cancer tissue may be an NSCLC tissue(s) obtained from a lung cancer patient(s). In a more preferred embodiment, such NSCLC tissue may be a lung adenocarcinoma (ADC) tissue(s), a lung squamous cell carcinoma (SCC) tissue(s), and/or a large cell carcinoma tissue(s).

[0125] Alternatively, a good prognosis control sample may be prepared by determined a cut-off value and preparing a sample containing an amount of an EZH2 mRNA or protein less than the cut-off value for lung cancer, or more than the cut-off value for colorectal cancer. Herein, the phrase "cut-off value" refers to the value dividing between a good prognosis range and a poor prognosis range. For example, one skilled in the art may be determine a cut-off value using a receiver operating characteristic (ROC) curve. The present kit may include an EZH2 standard sample providing a cut-off value amount of an EZH2 mRNA or polypeptide.

[0126] On the contrary, a poor or negative prognosis control sample may be a clinical lung cancer tissue(s) obtained from a lung cancer patient(s) who showed poor or negative prognosis of lung cancer, or a clinical colorectal cancer tissue(s) obtained from a colorectal cancer patient(s) who showed poor or negative prognosis of colorectal cancer after the treatment.

In a preferred embodiment, such lung cancer tissue may be an NSCLC tissue(s) obtained from a lung cancer patient(s). In a more preferred embodiment, such NSCLC tissue may be a lung adenocarcinoma (ADC) tissue(s), a lung squamous cell carcinoma (SCC) tissue(s), and/or a large cell carcinoma tissue(s).

[0127] Alternatively, a poor prognosis control sample may be prepared by determined a cut-off value and preparing a sample containing an amount of an EZH2 mRNA or protein more than the cut-off value for lung cancer, or less than the cut-off value for colorectal cancer.

[0128] As an aspect of the present invention, the reagents for diagnosing or detecting cancer, or determining or assessing the prognosis of cancer may be immobilized on a solid matrix, such as a porous strip, to form at least one site for detecting cancer. The measurement or detection region of the porous strip may include a plurality of sites, each containing a detection reagent (e.g., nucleic acid). A test strip may also contain sites for negative and/or positive controls. Alternatively, control sites may be located on a separate strip from the test strip. Optionally, the different detection sites may contain
different amounts of immobilized detection reagents (e.g., nucleic acid), i.e., a higher amount in the first detection site and lesser amounts in subsequent sites. Upon the addition of test biological sample, the number of sites displaying a detectable signal provides a quantitative indication of the expression level of the EZH2 gene in the sample. The detection sites may be configured in any suitably detectable shape and are typically in the shape of a bar or dot spanning the width of a test strip.

IV. Screening methods

Using the EZH2 gene, a polypeptide encoded by the gene or fragment thereof, or a transcriptional regulatory region of the gene, it is possible to screen substances that alter the expression of the gene or the biological activity of a polypeptide encoded by the gene. Such substances may be used as pharmaceuticals for treating or preventing cancer, in particular, AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma. Thus, the present invention provides methods of screening for candidate substances for either or both of treating and preventing cancer using the EZH2 gene, the EZH2 polypeptide or functional equivalents thereof, or a transcriptional regulatory region of the EZH2 gene.

A substance isolated by the screening method of the present invention is a substance that is expected to inhibit the expression of the EZH2 gene, or the activity of the translation product of the gene, and thus, is a candidate for either or both of treating and preventing cancer, for example, AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma. Namely, the substances screened through the present methods are deemed to have a clinical benefit and can be further tested for its ability to inhibit cancer cell growth in animal models or test subjects.

In the context of the present invention, substances to be identified through the present screening methods may be any substance or composition including several substances. Furthermore, a test substance exposed to a cell or protein in the screening methods of the present invention may be a single substance or a combination of substances. When a combination of substances is used in the methods, the substances may be contacted sequentially or simultaneously.

Any test substances, for example, cell extracts, cell culture supernatants, products of fermenting microorganism, extracts from marine organism, plant extracts, purified or crude proteins, peptides, non-peptide compounds, synthetic micromolecular compounds (including nucleic acid constructs, such as antisense RNA, siRNA, Ribozymes, etc.) and natural compounds can be used in the screening methods of the present invention. Test substances useful in the screenings described herein can also be antibodies that specifically bind to a protein of interest or a partial peptide thereof that
lacks the biological activity of the original proteins in vivo.

[0133] Test substances of the present invention can be also obtained using any of the numerous approaches in combinatorial library methods known in the art, including:

1. biological libraries,
2. spatially addressable parallel solid phase or solution phase libraries,
3. synthetic library methods requiring deconvolution,
4. the "one-bead one-compound" library method and
5. synthetic library methods using affinity chromatography selection.


Although the construction of test substance libraries is well known in the art, herein below, additional guidance in identifying test substances and construction libraries of such substances for the present screening methods are provided.

[0135] A. Molecular Modeling:

Construction of test substance libraries is facilitated by knowledge of the molecular structure of compounds known to have the properties sought, and/or the molecular structure of EZH2 protein. One approach to preliminary screening of test substances suitable for further evaluation utilizes computer modeling of the interaction between the test substance and its target.

[0136] Computer modeling technology allows for the visualization of the three-dimensional atomic structure of a selected molecule and the rational design of new compounds that will interact with the molecule. The three-dimensional construct typically depends on data from x-ray crystallographic analysis or NMR imaging of the selected molecule. The molecular dynamics require force field data. The computer graphics systems
enable prediction of how a new compound will link to the target molecule and allow experimental manipulation of the structures of the compound and target molecule to perfect binding specificity. Prediction of what the molecule-compound interaction will be when small changes are made in one or both requires molecular mechanics software and computationally intensive computers, usually coupled with user-friendly, menu-driven interfaces between the molecular design program and the user.

[0137] An example of the molecular modeling system described generally above includes the CHARMM and QUANTA programs, Polygen Corporation, Waltham, Mass. CHARMM performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modeling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.


Once a putative inhibitor has been identified, combinatorial chemistry techniques can be employed to construct any number of variants based on the chemical structure of the identified putative inhibitor, as detailed below. The resulting library of putative inhibitors may be screened using the methods of the present invention to identify test substances suited to either or both of treating and preventing cancer.

[0140] B. Combinatorial Chemical Synthesis:

Combinatorial libraries of test substances may be produced as part of a rational drug design program involving knowledge of core structures existing in known inhibitors. This approach allows the library to be maintained at a reasonable size, facilitating high throughput screening. Alternatively, simple, particularly short, polymeric molecular libraries may be constructed by simply synthesizing all permutations of the molecular family making up the library. An example of this latter approach would be a library of all peptides six amino acids in length. Such a peptide library could include every 6 amino acid sequence permutation. This type of library is termed a linear combinatorial
chemical library.


Devices for the preparation of combinatorial libraries are commercially available (see, e.g., 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA). In addition, numerous combinatorial libraries are themselves commercially available (see, e.g., ComGenex, Princeton, N.J., Tripos, Inc., St. Louis, MO, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, etc.).

C. Other Candidates:

Another approach uses recombinant bacteriophage to produce libraries. Using the "phage method" (Scott & Smith, Science 1990, 249: 386-90; Cwirla et al., Proc Natl Acad Sci USA 1990, 87: 6378-82; Devlin et al., Science 1990, 249: 404-6), very large libraries can be constructed (e.g., $10^6$-$10^8$ chemical entities). A second approach uses primarily chemical methods, of which the Geysen method (Geysen et al., Molecular

Aptamers are macromolecules composed of nucleic acid that bind tightly to a specific molecular target. Tuerk and Gold (Science. 249:505-510 (1990)) disclose SELEX (Systematic Evolution of Ligands by Exponential Enrichment) method for selection of aptamers. In the SELEX method, a large library of nucleic acid molecules (e.g., 10^15 different molecules) can be used for screening.

A compound in which a part of the structure of the compound screened by any of the present screening methods is converted by addition, deletion and/or replacement, is included in the substances obtained by the screening methods of the present invention. Furthermore, when the screened test substance is a protein, for obtaining a DNA encoding the protein, either the whole amino acid sequence of the protein may be determined to deduce the nucleic acid sequence coding for the protein, or partial amino acid sequence of the obtained protein may be analyzed to prepare an oligo DNA as a probe based on the sequence, and screen cDNA libraries with the probe to obtain a DNA encoding the protein. The obtained DNA finds use in preparing the test substance which is a candidate drug for cancer therapy.

IV-1. Protein based screening methods

According to the present invention, the expression of the EZH2 gene is crucial for the growth and/or survival of cancer cells.

Accordingly, substances that suppress the function of the EZH2 polypeptide would be presumed to inhibit either or both of the growth and survival of cancer cells, and therefore find use in either or both of treating and preventing cancer. Thus, the present invention provides methods of screening for a candidate substance for either or both of treating and preventing cancer, using the EZH2 polypeptide or the functional equivalent thereof. Further, the present invention also provides methods of screening for a candidate substance for inhibiting either of both of the growth and survival of cancer cells, using the EZH2 polypeptide or the functional equivalent.

In addition to the EZH2 polypeptide, fragments of the EZH2 polypeptides may be used for the present screening, so long as it retains at least one biological activity of the naturally occurring EZH2 polypeptide. Thus, fragments of the EZH2 polypeptides are included in the functional equivalents of the EZH2 polypeptide defined in the section "1. Polynucleotide and polypeptide ". The EZH2 polypeptides or functional equivalents thereof may be further linked to other substances, so long as the EZH2 polypeptides
and functional equivalent retain at least one of biological activities of the EZH2 polypeptide. Usable substances include: peptides, lipids, sugar and sugar chains, acetyl groups, natural and synthetic polymers, etc. These kinds of modifications may be performed to confer additional functions or to stabilize the polypeptide and fragments.

The EZH2 polypeptides or functional equivalents thereof used for the present method may be obtained from nature as naturally occurring proteins via conventional purification methods or through chemical synthesis based on the selected amino acid sequence. For example, conventional peptide synthesis methods that can be adopted for the synthesis include:

1) Peptide Synthesis, Interscience, New York, 1966;
6) W099/67288; and

Alternatively, the proteins may be obtained through any known genetic engineering methods for producing polypeptides (e.g., Morrison J., J Bacteriology 1977, 132: 349-51; Clark-Curtiss & Curtiss, Methods in Enzymology (eds. Wu et al.) 1983, 101: 347-62). For example, first, a suitable vector including a polynucleotide encoding the objective protein in an expressible form (e.g., downstream of a regulatory sequence including a promoter) is prepared, transformed into a suitable host cell, and then the host cell is cultured to produce the protein. More specifically, a gene encoding the EZH2 polypeptide or functional equivalent thereof is expressed in host (e.g., animal) cells and such by inserting the gene into a vector for expressing foreign genes, such as pSV2neo, pcDNA I, pcDNA3.1, pCAGGS, or pCD8. A promoter may be used for the expression. Any commonly used promoters may be employed including, for example, the SV40 early promoter (Rigby in Williamson (ed.), Genetic Engineering, vol. 3. Academic Press, London, 1982, 83-141), the EF-alpha promoter (Kim et al., Gene 1990, 91:217-23), the CAG promoter (Niwa et al., Gene 1991, 108:193), the RSV LTR promoter (Cullen, Methods in Enzymology 1987, 152:684-704), the SR alpha promoter (Takebe et al., Mol Cell Biol 1988, 8:466), the CMV immediate early promoter (Seed et al., Proc Natl Acad Sci USA 1987, 84:3385-9), the SV40 late promoter (Gheysen et al., J Mol Appl Genet 1982, 1:385-94), the Adenovirus late promoter (Kaufman et al., Mol Cell Biol 1989, 9:946), the HSV TK promoter, and such. The introduction of the vector into host cells to express the gene encoding the
EZH2 polypeptide or functional equivalent thereof can be performed according to any methods, for example, the electroporation method (Chu et al., Nucleic Acids Res 1987, 15:1311-26), the calcium phosphate method (Chen et al., Mol Cell Biol 1987, 7:2745-52), the DEAE dextran method (Lopata et al., Nucleic Acids Res 1984, 12:5707-17; Sussman et al., Mol Cell Biol 1985, 4:1641-3), the Lipofectin method (Derijard B, Cell 1994, 7:1025-37; Lamb et al., Nature Genetics 1993, 5:22-30; Rabindran et al., Science 1993, 259:230-4), and such.

The EZH2 polypeptides or functional equivalents thereof may be expressed as a fusion protein including a recognition site (epitope) of a monoclonal antibody by introducing the epitope of the monoclonal antibody, whose specificity has been revealed, to the N- or C- terminus of the polypeptide. Alternatively, a commercially available epitope-antibody system may be used (Experimental Medicine 13: 85-90 (1995)). Vectors which are capable of expressing a fusion protein with, for example, beta-galactosidase, maltose binding protein, glutathione S-transferase, green fluorescence protein (GFP), and so on, by the use of its multiple cloning sites are commercially available.

A fusion protein, prepared by introducing only small epitopes composed of several to a dozen amino acids so as not to change the property of the original polypeptide by the fusion, is also provided herein. Epitopes, such as polyhistidine (His-tag), influenza aggregate HA, human c-myc, FLAG, Vesicular stomatitis virus glycoprotein (VSV-GP), T7 gene 10 protein (T7-tag), human simple herpes virus glycoprotein (HSV-tag), E-tag (an epitope on monoclonal phage) and such, and antibodies recognizing them may be used as the epitope-antibody system for detecting the binding activity between the polypeptides (Experimental Medicine 13: 85-90 (1995)).

The EZH2 polypeptides or functional equivalents thereof may also be produced in vitro adopting an in vitro translation system.

The EZH2 polypeptides or functional equivalents thereof to be used in the screening method of the present invention can be a purified polypeptide, a soluble protein, or a fusion protein fused with other polypeptides.

IV-1.1. Identifying substances that bind to the EZH2 polypeptide

A substance that binds to a protein is likely to alter the expression of the gene coding for the protein or the biological activity of the protein. Thus, as an aspect, the present invention provides a method of screening for a candidate substance for either or both of treating and preventing cancer, which includes steps of:

a) contacting a test substance with an EZH2 polypeptide or functional equivalent thereof;

b) detecting binding (or binding activity) between the EZH polypeptide or functional equivalent and the test substance; and
c) selecting a test substance that binds to the EZH2 polypeptide or functional equivalent as a candidate substance for either or both of treating and preventing cancer.

According to the present invention, the therapeutic effect of the test substance on inhibiting the cell growth or a candidate substance for either or both of treating and preventing cancer may be evaluated. Therefore, the present invention also provides a method of screening for a candidate substance for inhibiting the cell growth or a candidate substance for either or both of treating and preventing cancer, using the EZH2 polypeptide or functional equivalent thereof including the steps as follows:

a) contacting a test substance with an EZH2 polypeptide or functional equivalent thereof;

b) detecting the binding level (or binding activity) between the EZH2 polypeptide or functional equivalent and the test substance; and

c) correlating the binding level (or binding activity) of b) with the therapeutic effect of the test substance.

In the context of the present invention, the therapeutic effect may be correlated with the binding level to the EZH2 polypeptide or functional fragment thereof. For example, when the test substance binds to the EZH2 polypeptide or functional fragment thereof, the test substance may identified or selected as the candidate substance having the requisite therapeutic effect. Alternatively, when the test substance does not bind to an EZH2 polypeptide or functional fragment thereof, the test substance may identified as the substance having no significant therapeutic effect.

In the present invention, it is revealed that suppressing the expression of the EZH2 gene reduces cancer cell growth. Thus, by screening for candidate substances that binds to EZH2 polypeptide or functional equivalent thereof, candidate substances that have the potential to treat or prevent cancers can be identified. Potential of these candidate substances to treat and/or prevent cancers may be evaluated by second and/or further screening to identify therapeutic agent for cancers.

The binding of a test substance to the EZH2 polypeptide or functional equivalent thereof may be, for example, detected by immunoprecipitation using an antibody against the polypeptide. Therefore, for the purpose for such detection, it is preferred that the EZH2 polypeptide or functional equivalent thereof used for the screening contains an antibody recognition site. The antibody used for the screening may be one that recognizes an antigenic region (e.g., epitope) of the EZH2 polypeptide. Preparation methods for such antibodies are well known in the art, and any method may be employed in the present invention to prepare such antibodies and equivalents thereof.

Alternatively, the EZH2 polypeptide or functional equivalent thereof may be expressed as a fusion protein including a recognition site (epitope) of a monoclonal
antibody at its N- or C-terminus. The specificity of the antibody has been revealed, to
the N- or C-terminus of the polypeptide. A commercially available epitope-antibody
system can be used (Experimental Medicine 1995, 13:85-90). Vectors which can
express a fusion protein with, for example, beta-galactosidase, maltose binding protein,
glutathione S-transferase, green fluorescence protein (GFP), and such by the use of its
multiple cloning sites are commercially available and can be used for the present
invention. Furthermore, fusion proteins containing much smaller epitopes to be
detected by immunoprecipitation with an antibody against the epitopes are also known
in the art (Experimental Medicine 1995, 13:85-90). Such epitopes, composed of
several to a dozen amino acids so as not to change the property of the EZH2
polypeptide or functional equivalent thereof, can also be used in the present invention.
Examples include polyhistidine (His-tag), influenza aggregate HA, human c-myc,
FLAG, Vesicular stomatitis virus glycoprotein (VSV-GP), T7 gene 10 protein
(T7-tag), human simple herpes virus glycoprotein (HSV-tag), E-tag (an epitope on
monoclonal phage), and such and monoclonal antibodies recognizing them can be used
as the epitope-antibody system for screening proteins binding to the EZH2 polypeptide
or functional equivalent thereof (Experimental Medicine 13:85-90 (1995)).

Glutathione S-transferase (GST) is also well-known as the counterpart of the fusion
protein to be detected by immunoprecipitation. When GST is used as the protein to be
fused with the EZH2 polypeptide or functional equivalent thereof to form a fusion
protein, the fusion protein can be detected either with an antibody against GST or a
substance specifically binding to GST, i.e., such as glutathione (e.g., glutathione-
Sepharose 4B).

In immunoprecipitation, an immune complex is formed by adding an antibody
(recognizing the EZH2 polypeptide or functional equivalent thereof itself, or an
epitope tagged to the EZH2 polypeptide or functional equivalent) to the reaction
mixture of the EZH2 polypeptide and the test substance. If the test substance has the
ability to bind the polypeptide, then the formed immune complex will consist of the
EZH2 polypeptide or functional equivalent thereof, the test substance, and the
antibody. On the contrary, if the test substance is devoid of such ability, then the
formed immune complex only consists of the EZH2 polypeptide or functional
equivalent thereof and the antibody. Therefore, the binding ability of a test substance
to the EZH2 polypeptide or functional equivalent can be examined by, for example,
measuring the size of the formed immune complex. Any method for detecting the size
of a compound can be used, including chromatography, electrophoresis, and such. For
example, when mouse IgG antibody is used for the detection, Protein A or Protein G
sepharose can be used for quantitating the formed immune complex.

For more details on immunoprecipitation see, for example, Harlow et al., Antibodies,
Cold Spring Harbor Laboratory publications, New York, 1988, 511-52. SDS-PAGE is commonly used for analysis of immunoprecipitated proteins and the bound protein can be analyzed by the molecular weight of the protein using gels with an appropriate concentration. Detection may be achieved using conventional staining method, such as Coomassie staining or silver staining, or, for proteins that is difficult to detect, the detection sensitivity for the protein can be improved by culturing cells in culture medium containing radioactive isotope, $^{35}$S-methionine or $^{35}$S-cysteine, labeling proteins in the cells, and detecting the proteins. The target protein can be purified directly from the SDS-polyacrylamide gel and its sequence can be determined, when the molecular weight of a protein has been revealed.

Furthermore, the EZH2 polypeptide or functional equivalent thereof used for the screening of substances that bind thereto may be bound to a carrier. Example of carriers that may be used for binding the polypeptides include insoluble polysaccharides, such as agarose, cellulose and dextran; and synthetic resins, such as polyacrylamide, polystyrene and silicon; preferably commercially available beads and plates (e.g., multi-well plates, biosensor chip, etc.) prepared from the above materials may be used. When using beads, they may be filled into a column. Alternatively, the use of magnetic beads is also known in the art, and enables to readily isolate polypeptides and substances bound on the beads via magnetism.

The binding of a polypeptide to a carrier may be conducted according to routine methods, such as chemical bonding and physical adsorption. Alternatively, a polypeptide may be bound to a carrier via antibodies specifically recognizing the protein. Moreover, binding of a polypeptide to a carrier can also be conducted by means of interacting molecules, such as the combination of avidin and biotin.

Screening using such carrier-bound EZH2 polypeptide or functional equivalent thereof include, for example, contacting a test substance to the carrier-bound polypeptide, incubating the mixture, washing the carrier, and detecting and/or measuring the substance bound to the carrier. The binding may be carried out in buffer, for example, but are not limited to, phosphate buffer and Tris buffer, as long as the buffer does not inhibit the binding.

When such carrier-bound EZH2 polypeptide or functional equivalent thereof, and a composition (e.g., cell extracts, cell lysates, etc.) are used as the test substance in a screening method, such method is generally called affinity chromatography. For example, the EZH2 polypeptide or functional equivalent thereof may be immobilized on a carrier of an affinity column, and a test substance, containing a substance capable of binding to the polypeptides, is applied to the column. After loading the test substance, the column is washed, and then the substance bound to the polypeptide is eluted with an appropriate buffer.
A biosensor using the surface plasmon resonance phenomenon may be used as a
mean for detecting or quantifying the bound substrate in the present invention. When
such a biosensor is used, the interaction between the EZH2 polypeptide or functional
equivalent thereof and a test substance can be observed real-time as a surface plasmon
resonance signal, using only a minute amount of the polypeptide and without labeling
(for example, BIAcore, Pharmacia). Therefore, it is possible to evaluate the binding
between the polypeptide and test substance using a biosensor such as BIAcore.

Methods of screening for molecules that bind to a specific protein among synthetic
chemical compounds, or molecules in natural substance banks or a random phage
peptide display library by exposing the specific protein immobilized on a carrier to the
molecules, and methods of high-throughput screening based on combinatorial
chemistry techniques (Wrighton et al., Science 1996, 273:458-64; Verdone, Nature
1996, 384:11-3) to isolate not only proteins but chemical compounds are also well-
known to those skilled in the art. These methods can also be used for screening
substances (including agonist and antagonist) that bind to the EZH2 polypeptide or
functional equivalent thereof.

When the test substance is a protein, for example, West-Western blotting analysis
(Skolnik et al., Cell 1991, 65:83-90) can be used for the present method. Specifically, a
protein binding to the EZH2 polypeptide or functional equivalent thereof can be
obtained by preparing first a cDNA library from cells, tissues, organs, or cultured cells
(e.g., PC cell lines) expected to express at least one protein binding to the EZH2
polypeptide or functional equivalent thereof using a phage vector (e.g., ZAP), ex-
pressing the proteins encoded by the vectors of the cDNA library on LB-agarose,
fixing the expressed proteins on a filter, reacting the purified and labeled EZH2
polypeptide or functional equivalent thereof with the above filter, and detecting the
plaques expressing proteins to which the EZH2 polypeptide or functional equivalent
thereof has bound according to the label of the EZH2 polypeptide or functional
equivalent.

Labeling substances such as radioisotope (e.g., $^3$H, $^{14}$C, $^{32}$P, $^{35}$P, $^{32}$S, $^{125}$I, $^{131}$I),
enzymes (e.g., alkaline phosphatase, horseradish peroxidase, beta-galactosidase, beta-
glucosidase), fluorescent substances (e.g., fluorescein isothiocyanate (FITC),
rhodamine) and biotin/avidin, may be used for the labeling of EZH2 polypeptide or
functional equivalent thereof in the present method. When the protein is labeled with
radioisotope, the detection or measurement can be carried out by liquid scintillation.
Alternatively, when the protein is labeled with an enzyme, it can be detected or
measured by adding a substrate of the enzyme to detect the enzymatic change of the
substrate, such as generation of color, with absorbtiometer. Further, in case where a
fluorescent substance is used as the label, the bound protein may be detected or
measured using fluorophotometer.

Moreover, the EZH2 polypeptide or functional equivalent thereof bound to the protein can be detected or measured by utilizing an antibody that specifically binds to the EZH2 polypeptide or functional equivalent thereof, or a peptide or polypeptide (for example, GST) that is fused to the EZH2 polypeptide or functional equivalent. In case of using an antibody in the present screening, the antibody is preferably labeled with one of the labeling substances mentioned above, and detected or measured based on the labeling substance. Alternatively, the antibody against the EZH2 polypeptide or functional equivalent thereof may be used as a primary antibody to be detected with a secondary antibody that is labeled with a labeling substance. Furthermore, the antibody bound to the EZH2 polypeptide or functional equivalent thereof in the present screening may be detected or measured using protein G or protein A column.

Antibodies to be used in the present screening methods can be prepared using techniques well known in the art. Antigens to prepared antibodies may be derived from any animal species, but preferably is derived from a mammal such as a human, mouse, rabbit, or rat, more preferably from a human. The EZH2 polypeptide or functional equivalent thereof used as the antigen can be recombinantly produced or isolated from natural sources. The polypeptides to be used as an immunization antigen may be a complete protein or a partial peptide derived from the complete EZH2 polypeptide or functional equivalent thereof.

Any mammalian animal may be immunized with the antigen; however, the compatibility with parental cells used for cell fusion is preferably taken into account. In general, animals of the order Rodentia, Lagomorpha or Primate are used. Animals of the Rodentia order include, for example, mice, rats and hamsters. Animals of Lagomorpha order include, for example, hares, pikas, and rabbits. Animals of Primate order include, for example, monkeys of Catarrhini (old world monkey) such as Macaca fascicularis, rhesus monkeys, sacred baboons and chimpanzees.

Methods for immunizing animals with antigens are well known in the art. Intraperitoneal injection or subcutaneous injection of antigens is a standard method for immunizing mammals. More specifically, antigens may be diluted and suspended in an appropriate amount of phosphate buffered saline (PBS), physiological saline, etc. If desired, the antigen suspension may be mixed with an appropriate amount of a standard adjuvant, such as Freund's complete adjuvant, made into emulsion, and then administered to mammalian animals. Preferably, it is followed by several administrations of the antigen mixed with an appropriately amount of Freund's incomplete adjuvant every 4 to 21 days. An appropriate carrier may also be used for immunization. After immunization as above, the serum is examined by a standard method for an increase in the amount of desired antibodies.
Polyclonal antibodies may be prepared by collecting blood from the immunized mammal examined for the increase of desired antibodies in the serum, and by separating serum from the blood by any conventional method. Polyclonal antibodies include serum containing the polyclonal antibodies, as well as the fraction containing the polyclonal antibodies isolated from the serum. Immunoglobulin G or M can be prepared from a fraction which recognizes only the objective polypeptide using, for example, an affinity column coupled with the polypeptide, and further purifying this fraction using protein A or protein G column.

To prepare monoclonal antibodies, immune cells are collected from the mammal immunized with the antigen and checked for the increased level of desired antibodies in the serum as described above, and are subjected to cell fusion. The immune cells used for cell fusion are preferably obtained from spleen. Other preferred parental cells to be fused with the above immunocyte include, for example, myeloma cells of mammalians, and more preferably myeloma cells having an acquired property for the selection of fused cells by drugs.

The above immunocyte and myeloma cells can be fused according to known methods, for example, the method of Milstein et al., (Galfre and Milstein, Methods Enzymol 73: 3-46 (1981)).

Resulting hybridomas obtained by the cell fusion may be selected by cultivating them in a standard selection medium, such as HAT medium (hypoxanthine, aminopterin, and thymidine containing medium). The cell culture is typically continued in the HAT medium for several days to several weeks, the time being sufficient to allow all the other cells, with the exception of the desired hybridoma, to die. Then, the standard limiting dilution is performed to screen and clone a hybridoma cell producing the desired antibody.

In addition to the above method, in which a non-human animal is immunized with an antigen for preparing hybridoma, human lymphocytes, such as those infected by the EB virus, may be immunized with an antigen, cells expressing such antigen, or their lysates in vitro. Then, the immunized lymphocytes are fused with human-derived myeloma cells that are capable of indefinitely dividing, such as U266, to yield a hybridoma producing a desired human antibody that is able to bind to the antigen (Unexamined Published Japanese Patent Application No. (JP-A) Sho 63-17688).

The obtained hybridomas may be subsequently transplanted into the abdominal cavity of a mouse and the ascites may be extracted. The obtained monoclonal antibodies can be purified by, for example, ammonium sulfate precipitation, a protein A or protein G column, DEAE ion exchange chromatography, or an affinity column carrying an objective antigen.

Antibodies against the EZH2 polypeptide can be used not only in the present
screening method, but also for the detection of the polypeptides as cancer markers in biological samples as described in "II. Diagnosing cancer". They may further serve as candidates for agonists and antagonists of the polypeptides of interest. In addition, such antibodies, serving as candidates for antagonists, can be applied to the antibody treatment for cancer including AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma.

Monoclonal antibodies thus obtained can be also recombinantly prepared using genetic engineering techniques (see, for example, Borrebaeck and Larrick, Therapeutic Monoclonal Antibodies, published in the United Kingdom by MacMillan Publishers LTD (1990)). For example, a DNA encoding an antibody may be cloned from an immune cell, such as a hybridoma or an immunized lymphocyte producing the antibody, inserted into an appropriate vector, and introduced into host cells to prepare a recombinant antibody. Such recombinant antibody can also be used in the context of the present screening.

Furthermore, antibodies used in the screening and so on may be fragments of antibodies or modified antibodies, so long as they retain the original binding activity. For instance, the antibody fragment may be an Fab, F(ab')2, Fv, or single chain Fv (scFv), in which Fv fragments from H and L chains are ligated by an appropriate linker (Huston et al., Proc Natl Acad Sci USA 85: 5879-83 (1988)). More specifically, an antibody fragment may be generated by treating an antibody with an enzyme, such as papain or pepsin. Alternatively, a gene encoding an antibody fragment may be constructed, inserted into an expression vector, and expressed in an appropriate host cell (see, for example, Co et al., J Immunol 152: 2968-76 (1994); Better and Horwitz, Methods Enzymol 178: 476-96 (1989); Pluckthun and Skerra, Methods Enzymol 178: 497-515 (1989); Lamoyi, Methods Enzymol 121: 652-63 (1986); Rousseaux et al., Methods Enzymol 121: 663-9 (1986); Bird and Walker, Trends Biotechnol 9: 132-7 (1991)).

An antibody may be modified by conjugation with a variety of molecules, such as polyethylene glycol (PEG). Modified antibodies can be obtained through chemically modification of an antibody. These modification methods are conventional in the field. Antibodies obtained as above may be purified to homogeneity. For example, the separation and purification of the antibody can be performed according to separation and purification methods used for general proteins. For example, the antibody may be separated and isolated by appropriately selected and combined column chromatographies, such as affinity chromatography, filter, ultrafiltration, salting-out, dialysis, SDS polyacrylamide gel electrophoresis, isoelectric focusing, and others (Antibodies: A Laboratory Manual. Ed Harlow and David Lane, Cold Spring Harbor...
Laboratory (1988)); however, the present invention is not limited thereto. A protein A column and protein G column can be used as the affinity column. Exemplary protein A columns to be used include, for example, Hyper D, POROS, and Sepharose F.F. (Pharmacia).

Exemplary chromatography, with the exception of affinity, includes, for example, ion-exchange chromatography, hydrophobic chromatography, gel filtration, reverse-phase chromatography, adsorption chromatography, and the like (Strategies for Protein Purification and Characterization: A Laboratory Course Manual. Ed Daniel R. Marshak et al., Cold Spring Harbor Laboratory Press (1996)). The chromatographic procedures can be carried out by liquid-phase chromatography, such as HPLC and FPLC.

Alternatively, in another embodiment of the screening method of the present invention, two-hybrid system utilizing cells may be used ("MATCHMAKER Two-Hybrid system", "Mammalian MATCHMAKER Two-Hybrid Assay Kit", "MATCHMAKER one-Hybrid system" (Clontech); "HybriZAP Two-Hybrid Vector System" (Stratagene); the references "Dalton et al., Cell 1992, 68:597-612" and "Fields et al., Trends Genet 1994, 10:286-92"). In two-hybrid system, the EZH2 polypeptide or functional equivalent thereof is fused to the SRF-binding region or GAL4-binding region and expressed in yeast cells. A cDNA library is prepared from cells expected to express at least one protein binding to the EZH2 polypeptide or functional equivalent thereof such that the library, when expressed, is fused to the VP16 or GAL4 transcriptional activation region. The cDNA library is then introduced into the above yeast cells and the cDNA derived from the library is isolated from the positive clones detected (when a protein binding to the EZH2 polypeptide or functional equivalent thereof is expressed in the yeast cells, the binding of the two activates a reporter gene, making positive clones detectable). A protein encoded by the cDNA can be prepared by introducing the cDNA isolated above to E. coli and expressing the protein.

As a reporter gene, for example, Ade2 gene, lacZ gene, CAT gene, luciferase gene and such can be used in addition to the HIS3 gene.

The substance isolated by this screening is a candidate for agonists or antagonists of the EZH2 polypeptide. The term "agonist" refers to molecules that activate the function of the polypeptide by binding thereto. On the other hand, the term "antagonist" refers to molecules that inhibit the function of the polypeptide by binding thereto. Moreover, an substance isolated by this screening as an antagonist is a candidate that inhibits the in vivo interaction of the EZH2 polypeptide with molecules (including nucleic acids (RNAs and DNAs) and proteins).

IV-1-2. Identifying substances by detecting biological activity of the polypeptides

In the present invention, the EZH2 gene highly overexpressed in various cancers
including AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma. Further, the suppression of the EZH2 gene by small interfering RNA (siRNA) resulted in growth inhibition and/or cell death of cancer cells. Accordingly, the EZH2 polypeptide is involved in cancer cell survival, and thus, substances that inhibit a biological activity of the EZH2 polypeptide may become candidate substances for cancer therapy.

Thus, the present invention also provides a method for screening for a candidate substance for either or both of treating and preventing cancer using the EZH2 polypeptide or functional equivalent thereof including the steps as follows:

a) contacting a test substance with an EZH2 polypeptide or functional equivalent thereof;

b) detecting the biological activity of the EZH2 polypeptide or functional equivalent of the step (a); and

c) selecting the test substance that reduces the biological activity of the EZH2 polypeptide or functional equivalent as compared to the biological activity in the absence of the test substance as a candidate substance for either or both of treating and preventing cancer.

According to the present invention, the therapeutic effect of the test substance on inhibiting the cell growth or a candidate substance for either or both of treating and preventing cancer may be evaluated. Therefore, the present invention also provides a method of screening for a candidate substance for inhibiting the cancer cell growth or a candidate substance for either or both of treating and preventing cancer, using the EZH2 polypeptide or functional equivalent thereof, including the steps as follows:

a) contacting a test substance with an EZH2 polypeptide or a functional equivalent thereof;

b) detecting the biological activity of the EZH2 polypeptide or functional equivalent of step (a); and

c) correlating the biological activity of (b) with the therapeutic effect of the test substance.

Alternatively, in some embodiments, the present invention provides a method for evaluating or estimating a therapeutic effect of a test substance in either or both of the treatment and prevention of cancer and/or in the inhibition of the growth of a cancer cells, the method including steps of:

a) contacting a test substance with an EZH2 polypeptide or functional equivalent thereof;

b) detecting the biological activity of the EZH2 polypeptide or functional equivalent of step (a); and
(c) correlating the potential therapeutic effect and the test substance, wherein the potential therapeutic effect is shown, when a substance suppresses the biological activity of the EZH2 polypeptide or functional equivalent thereof as compared to the biological activity of the EZH2 polypeptide or functional equivalent detected in the absence of the test substance.

In the present invention, the therapeutic effect may be correlated with the biological activity of an EZH2 polypeptide or functional equivalent thereof. For example, when the test substance suppresses or inhibits the biological activity of an EZH2 polypeptide or functional equivalent thereof as compared to a level detected in the absence of the test substance, the test substance may identified or selected as the candidate substance having the therapeutic effect. Alternatively, when the test substance does not suppress or inhibit the biological activity of the EZH2 polypeptide or functional equivalent thereof as compared to a level detected in the absence of the test substance, the test substance may identified as the substance having no significant therapeutic effect.

The method of the present invention will be described in more detail below.

Any polypeptides can be used as functional equivalent of the EZH2 polypeptide for the screening method of the present invention so long as they retain a biological activity of the EZH2 polypeptide. For the EZH2 polypeptide and functional equivalents thereof, see the section of "I. Polynucleotide and polypeptide". Examples of the biological activities of the EZH2 polypeptide include, but are not limited to, cell proliferation promoting activity and methyltransferase activity (e.g., histone methyltransferase activity).

The EZH2 polypeptide or functional equivalent thereof may be expressed endogenously or exogenously by cells. Methods for preparing such polypeptides are described above.

In the present invention, it is revealed that suppressing the expression of EZH2 gene reduces cancer cell growth. Thus, by screening for candidate substances that suppresses the biological activity of the EZH2 polypeptide or functional equivalent thereof, candidate substances that have the potential for either of both of treating and preventing cancers can be identified. Potential of these candidate substances for either or both of treating and preventing cancers may be evaluated by second and further screening to identify therapeutic substances for cancers. For example, when a substance binding to the EZH2 polypeptide inhibits described above activities of the cancer, it may be concluded that such substance has the EZH2 specific therapeutic effect.

Any substances can be used for the screening so long it inhibits, suppresses or reduces a biological activity of the EZH2 polypeptide. In the context of the present invention, the phrase "inhibit, suppress or reduce a biological activity" encompasses at
least 10% suppression of the biological activity of the EZH2 polypeptide in comparison with in the absence of the substance, more preferably at least 25%, 50% or 75% suppression and most preferably at 90% suppression. Such suppression can serve an index in the present screening method.

According to the present invention, the EZH2 polypeptide has been demonstrated to be required for the growth or viability of bladder cancer cells, lung cancer cells and colorectal cancer cells. The biological activities of the EZH2 polypeptide that can be used as an index for the screening include such cell growth promoting activity of the human EZH2 polypeptide. Herein, cell growth promoting activity is also referred to as "cell proliferative activity" or "cell proliferation enhancing activity"

When the biological activity to be detected in the present method is cell growth promoting activity, it can be detected, for example, by preparing cells which express the EZH2 polypeptide or functional equivalent thereof, culturing the cells in the presence of a test substance, and determining the speed of cell proliferation, cell proliferation rate, the cell cycle or the colony forming activity. In some embodiments, cells expressing EZH2 gene are isolated and cultured cells exogenously or endogenously expressing EZH2 gene in vitro.

More specifically, the method includes the steps of:
(a) contacting a test substance with a cell overexpressing the EZH2 gene;
(b) measuring a cell growth promoting activity; and
(c) selecting a test substance that reduces the cell growth promoting activity in the comparison with the cell growth promoting activity in the absence of the test substance as a candidate substance for either or both of treating and preventing cancer.

In preferable embodiments, the method of the present invention may further include the step of:
(d) selecting a test substance that have no effect to the cells no or little expressing EZH2 gene.

When the cell growth promoting activity is evaluated, control cells that do not express the EZH2 polypeptide are used. Accordingly, the present invention also provides a method of screening for a candidate substance for inhibiting the cell growth or a candidate substance for either or both of treating and preventing cancer, using the EZH2 polypeptide or functional equivalent thereof including the steps as follows:
(a) culturing cells which express an EZH2 polypeptide or a functional equivalent thereof in the presence or absence of a test substance, and control cells that do not express an EZH2 polypeptide or functional equivalent thereof in the presence of the test substance;
(b) detecting a biological activity (e.g., cell growth activity) of the cells which express the EZH2 polypeptide or functional equivalent and the control cells; and
c) selecting the test substance that inhibits the biological activity of the cells which express the EZH2 polypeptide or functional equivalent as compared to the biological activity detected in the absence of the test substance and that does not inhibit the biological activity of the control cells.

When the biological activity to be detected in the present screening method is methyltransferase activity, the methyltransferase activity can be determined by contacting the EZH2 polypeptide or functional equivalent with a substrate (e.g., histone H3 fragment including Lys-27 or histone H2B fragment including Lys-120) and a co-factor (e.g., S-adenosyl-L-methionine) under conditions suitable for methylation of the substrate and detecting the methylation level of the substrate.

In the present invention, the screening methods using methyltransferase activity encompass the following methods of [1] to [7]:

[1] A method of screening for a candidate substance for either or both of treating and preventing cancer, or inhibiting cancer cell growth, including the steps of:

(a) contacting an EZH2 polypeptide or functional equivalent thereof with a substrate and a cofactor in the presence of a test substance;

(b) detecting the methylation level of the substrate of step (a); and

(c) selecting the test substance that suppress the methylation level of the substrate as compared to the methylation level detected in the absence of the test substance;

[2] The method of [1], wherein the substrate is a histone or fragment thereof including at least one methylation region for the EZH2 polypeptide;

[3] The method of [2], wherein the substrate is a histone H3 or a fragment thereof including at least one methylation region for the EZH2 polypeptide, or a histone H2B or a fragment thereof including at least one methylation region for the EZH2 polypeptide;

[4] The method of [3], wherein the methylation region is lysine 27 of histone H3 or lysine 120 of histone H2B;

[5] The method of any one of [1] to [4], wherein the cofactor is an S-adenosylmethyonine;

[6] The method of any one of [1] to [5], wherein the step (a) is conducted in the presence of an enhancing agent for the methylation; and

[7] The method of [6], wherein the enhancing agent for the methylation is S-adenosyl homocysteine hydrolase (SAHH).

In the present invention, methyltransferase activity of an EZH2 polypeptide or functional equivalent thereof can be determined by methods known in the art. For example, an EZH2 polypeptide or functional equivalent thereof and a substrate can be incubated with a labeled methyl donor, under a suitable assay condition. In one embodiment, the EZH2 polypeptide and the substrate are isolated from cells expressing
EZH2 and the substrate, or chemically synthesized to be contacted with a test substance in vitro.

Preferably, substrates to be methylated may be histones or fragments thereof, more preferably histone H3 or histone H2B or fragments thereof. Herein, histone fragment, histone H3 fragment and histone H2B fragment are also referred to as "histone peptide", "histone H3 peptide" and "histone H2B peptide", respectively. In preferred embodiments, the histone fragment, histone H3 fragment or histone H2B fragment to be used in the screening method of the present invention includes at least one methylation site for the EZH2 polypeptide (e.g., Lys-27 of histone H3 or Lys-120 of histone H2B). Preferably, such fragments are a peptide consisting of at least 10 amino acids. More preferably, such fragments consist of 15 or more amino acids, 20 or more amino acids, 25 or more amino acids, 30 or more amino acids, 40 or more amino acids, or 50 or more amino acids. Accordingly, histone H3 fragments including Lys-27 or histone H2B fragments including Lys-120 that consist of at least 10, 15, 20, 25, 30, 40 or 50 amino acids are preferred substrates for the screening method of the present invention.

For example, a histone H3 peptide or histone H2B peptide, and S-adenosyl-[methyl-\(^{14}\)C]-L-methionine, or S-adenosyl-[methyl-\(^{3}\)H]-L-methionine preferably can be used as such substrate and methyl donor, respectively. Transfer of the radiolabel to a histone H3 peptide or histone H2B peptide can be detected, for example, by SDS-PAGE electrophoresis and fluorography. Alternatively, following the reaction, the histone H3 peptides or histone H2B peptides can be separated from the methyl donor by filtration, and the amount of radiolabel retained on the filter quantitated by scintillation counting. Other suitable labels that can be attached to methyl donors, such as chromogenic and fluorescent labels, and methods of detecting transfer of these labels to histones and histone peptides, are known in the art.

Alternatively, the methyltransferase activity of the EZH2 polypeptide or functional equivalent thereof can be determined using an unlabeled methyl donor (e.g., S-adenosyl-L-methionine) and reagents that selectively recognize methylated histones or histone peptides. For example, after incubation of the EZH2 polypeptide or functional equivalent thereof, a substrate and a methyl donor, under the condition capable of methylation of the substrate, the methylated substrate can be detected by immunological method. Any immunological techniques using an antibody that recognizes a methylated substrate can be used for the detection. For example, an antibody against a methylated histone is commercially available (abeam Ltd.). ELISA or Immunoblotting with an antibody that recognizes a methylated histone can be used for the present invention.

In the present invention, an enhancing agent for the methylation of a substance can
be used. SAHH or functional equivalent thereof are one of the preferable enhancing agents for the methylation. As the agent enhances the methylation of the substance, the methyltransferase activity can be determined with higher sensitivity thereby. The EZH2 polypeptide or functional equivalent can be contacted with a substrate and a cofactor under the existence of a such enhancing agent. In some embodiments, cells expressing EZH2 gene are isolated and cultured cells exogenously or endogenously expressing EZH2 gene in vitro. Furthermore, the screening method of the present invention can be performed by preparing cells which express the EZH2 polypeptide or functional equivalent, culturing the cells in the presence of a test substance, and determining methylation level of a histone, for example, by using the antibody specific binding to a methylation region.

More specifically, the method can include the steps of:
(a) contacting a test substance with a cell expressing the EZH2 gene;
(b) detecting a methylation level of histone H3 or histone H2B in the cell of step (a); and
(c) selecting a test substance that reduces the methylation level detected in step (b) in the comparison with the methylation level detected in the absence of the test substance.

Alternatively, in some embodiments, the present invention also provides a method for evaluating or estimating a therapeutic effect of a test substance on treating or preventing cancer, the method including steps of:
(a) contacting a test substance with cells expressing the EZH2 gene under the condition capable of methylation of a histone H3 or histone H2B;
(b) detecting the methylation level of the histone H3 or histone H2B; and
(c) correlating the potential therapeutic effect and the test substance, wherein the potential therapeutic effect is shown, when a test substance decreases the methylation level of the histone H3 or histone H2B as compared to the methylation level detected in the absence of the test substance as the candidate substance.

IV-2. Nucleotide based screening methods
IV-2-1. Screening methods using the EZH2 gene
As discussed in detail above, by controlling the expression level of the EZH2 gene, one can control the onset and progression of cancer. Thus, substances that may be used in either or both of the treatment and prevention of cancers can be identified through screenings that use the expression level of the EZH2 gene as an index. In the context of the present invention, such screening may include, for example, the following steps:
a) contacting a test substance with a cell expressing an EZH2 gene;
b) detecting the expression level of the EZH2 gene;
c) comparing the expression level with the expression level detected in the absence of the test substance; and
d) selecting a test substance that reduces the expression level as compared to the expression level in the absence of the test substance as a candidate substance for either or both of treating and preventing cancer.

According to the present invention, the therapeutic effect of the test substance for inhibiting the cell growth or a candidate substance for either or both of treating and preventing cancer may be evaluated. Therefore, the present invention also provides a method for screening for a candidate substance that suppresses the proliferation of cancer cells, and a method for screening for a candidate substance for either or both of treating and preventing cancer.

In the context of the present invention, such screening may include, for example, the following steps:

a) contacting a test substance with a cell expressing an EZH2 gene;

b) detecting the expression level of the EZH2 gene; and

c) correlating the expression level of b) with the therapeutic effect of the test substance.

In the context of the present invention, the therapeutic effect may be correlated with the expression level of the EZH2 gene. For example, when the test substance reduces the expression level of the EZH2 gene as compared to a level detected in the absence of the test substance, the test substance may identified or selected as the candidate substance having the therapeutic effect. Alternatively, when the test substance does not reduce the expression level of the EZH2 gene as compared to a level detected in the absence of the test substance, the test substance may identified as the substance having no significant therapeutic effect.

Herein, it was revealed that suppressing the expression of EZH2 gene reduces cancer cell growth. Thus, by screening for candidate substance that reduces the expression level of EZH2 gene, candidate substance that have the potential to treat or prevent cancers can be identified. Potential of these candidate substance to treat or prevent cancers may be evaluated by second and/or further screening to identify therapeutic agent for cancers.

A substance that inhibits the expression of the EZH2 gene can be identified by contacting a cell expressing the EZH2 gene with a test substance and then determining the expression level of the EZH2 gene. Naturally, the identification may also be performed using a population of cells that express the gene in place of a single cell. A decreased expression level detected in the presence of a test substance as compared to the expression level in the absence of the test substance indicates the test substance as being an inhibitor of the EZH2 gene, suggesting the possibility that the test substance is useful for inhibiting cancer, thus the test substance to be used for the treatment or prevention of cancer.
The expression level of a gene can be estimated by methods well known to one skilled in the art. The expression level of the EZH2 gene can be, for example, determined following the method described above under the item of 11-1. Method for diagnosing cancer or a predisposition for developing cancer.

The cell or the cell population used for such identification may be any cell or any population of cells so long as it expresses the EZH2 gene. For example, the cell or population may be or contain a cancerous cell, for example, lung cancer cell, esophageal cancer cell, colorectal cancer cell, renal cell carcinoma cell, bladder cancer cell, AML cell, CML cell, osteosarcoma cell or cholangiocellular carcinoma cell. Alternatively, the cell or population may be or contain an immortalized cell derived from a carcinoma cell, including lung cancer cell, esophageal cancer cell, colorectal cancer cell, renal cell carcinoma cell, bladder cancer cell, AML cell, CML cell, osteosarcoma cell or cholangiocellular carcinoma cell. Cells expressing the EZH2 gene include, for example, cell lines established from cancers (e.g., lung, colorectal, bladder cancer cell lines such as H1780, H1373, LC319, A549, PC-14, SK-MES-1, H2170, H520, H1703, RERF-LCA1, LX1, SBC3, SBC5, DMS273, DMS114, SW780, RT4, SNU475, Huh7 etc.). Furthermore, the cell or population may be or contain a cell which has been transfected with the EZH2 gene. In some embodiments, cells expressing EZH2 gene are isolated and cultured cells exogenously or endogenously expressing EZH2 gene in vitro.

The present method allows screening of various test substances mentioned above and is particularly suited for screening functional nucleic acid molecules including antisense RNA, siRNA, and such.

Screening methods using transcriptional regulatory region of EZH2 gene

According to another aspect, the present invention provides a method which includes the following steps of:

a) contacting a test substance with a cell into which a vector, including a transcriptional regulatory region of an EZH2 gene and a reporter gene that is expressed under the control of the transcriptional regulatory region, has been introduced;

b) detecting the expression level or activity of said reporter gene;

c) comparing the expression level or activity with the expression level or activity detected in the absence of the substance; and

d) selecting the substance that reduces the expression or activity of said reporter gene as a candidate substance for treating or preventing cancer.

According to the present invention, the therapeutic effect of the test substance for inhibiting the cell growth or a candidate substance for treating and/or preventing cancer may be evaluated. Therefore, the present invention also provides a method for screening for a candidate substance that suppresses the proliferation of cancer cells,
and a method for screening for a candidate substance for treating or preventing cancer.

[0213] According to another aspect, the present invention provides a method which includes the following steps of:

a) contacting a test substance with a cell into which a vector, composed of a transcriptional regulatory region of an EZH2 gene and a reporter gene that is expressed under the control of the transcriptional regulatory region, has been introduced;

b) detecting the expression level or activity of said reporter gene; and

c) correlating the expression level or activity of b) with the therapeutic effect of the test substance.

[0214] In the present invention, the therapeutic effect may be correlated with the expression or activity of said reporter gene. For example, when the test substance reduces the expression or activity of said reporter gene as compared to a level detected in the absence of the test substance, the test substance may be identified as the candidate substance having the therapeutic effect. Alternatively, when the test substance does not reduce the expression or activity of said reporter gene as compared to a level detected in the absence of the test substance, the test substance may be identified as the substance having no significant therapeutic effect.

Herein, it was revealed that suppressing the expression of EZH2 gene reduces cell growth. Thus, by screening for test substances that reduce the expression or activity of the reporter gene, candidate substances that have the potential to treat or prevent cancers can be identified. Potential of these candidate substances to treat or prevent cancers may be evaluated by second and/or further screening to identify therapeutic agent for cancers.

[0215] Suitable reporter genes and host cells are well known in the art. The reporter construct required for the screening can be prepared using the transcriptional regulatory region of the EZH2 gene, which can be obtained as a nucleotide segment containing the transcriptional regulatory region from a genome library based on the nucleotide sequence information of the gene.

[0216] The transcriptional regulatory region may be, for example, the promoter sequence of the EZH2 gene. The reporter construct required for the screening can be prepared by connecting reporter gene sequence to the transcriptional regulatory region of EZH2 gene. The transcriptional regulatory region of EZH2 gene herein is the region from start codon to at least 500 bp upstream, preferably 1,000 bp, more preferably 5,000 or 10,000 bp upstream. A nucleotide segment containing the transcriptional regulatory region can be isolated from a genome library or can be propagated by PCR. Methods for identifying a transcriptional regulatory region, and also assay protocol are well known (Molecular Cloning third edition chapter 17, 2001, Cold Springs Harbor Laboratory Press).
When a cell transfected with a reporter gene that is operably linked to the regulatory sequence (e.g., promoter sequence) of the EZH2 gene is used, a substance can be identified as inhibiting or enhancing the expression of the EZH2 gene through detecting the expression level of the reporter gene product.

[0217] Illustrative reporter genes include, but are not limited to, luciferase, green fluorescence protein (GFP), Discosoma sp. Red Fluorescent Protein (DsRed), Chorlamphenicol Acetyltransferase (CAT), lacZ and beta-glucuronidase (GUS), and host cell is COS7, HEK293, HeLa, Ade2 gene, HIS3 gene, and others well-known in the art. Methods for detection of the expression of these genes are well known in the art.

[0218] A vector containing a reporter construct may be infected to host cells and the expression or activity of the reporter gene is detected by method well known in the art (e.g., using luminometer, absorption spectrometer, flow cytometer and so on). In some embodiments, cells of the present invention are isolated and cultured cells into which a vector, composed of the transcriptional regulatory region of the EZH2 gene and a reporter gene that is expressed under the control of the transcriptional regulatory region, has been introduced in vitro. In the context of the instant invention, the phrase "reduces the expression or activity" encompasses at least 10% reduction of the expression or activity of the reporter gene in comparison with in absence of the compound, more preferably at least 25%, 50% or 75% reduction and most preferably at 95% reduction.

[0219] V. Double stranded molecule:

As demonstrated herein, the expression of the EZH2 gene in cancer cell lines was inhibited by dsRNAs against the EZH2 gene, and consequently, the growth of those cancer cell lines were suppressed. Therefore the present invention provides isolated double-stranded molecules that are capable of inhibiting the expression of the EZZH2 gene as well as the cell growth when introduced into a cell expressing the gene. The double-stranded molecules of the present invention are useful for inhibiting cancer cell growth relating to the overexpression of the EZH2 gene, therefore, they may provide new methods for treating cancers. For example, the double-stranded molecules of the present invention are suitable for treating cancers such as AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma, in which the overexpression of the EZH2 gene was observed.

The target sequence of the double-stranded molecule against the EZH2 gene includes, for example, a nucleotide sequence corresponding to a target sequence selected from among SEQ ID NOs: 13 and 16.

[0220] Specifically, the present invention provides the following double-stranded molecules [1] to [18]:
[I] An isolated double-stranded molecule that, when introduced into a cell, inhibits in vivo expression of an EZH2 gene and cell proliferation, wherein the double-stranded molecule contains a sense strand and an antisense strand complementary thereto, hybridized to each other to form the double-stranded molecule, wherein the sense strand contains a nucleotide sequence corresponding to a part of EZH2 gene sequence;
[2] The double-stranded molecule of [1], wherein the double-stranded molecule acts on mRNA of EZH2 gene, matching a target sequence selected from among SEQ ID NOs: 13 and 16;
[3] The double-stranded molecule of [1], wherein the sense strand contains a nucleotide sequence corresponding to a target sequence selected from among SEQ ID NOs: 13 and 16;
[4] The double-stranded molecule of any one of [1] to [3], wherein the sense strand hybridizes with antisense strand at the target sequence to form the double-stranded molecule having a length of less than about 100 nucleotides pairs in length;
[5] The double-stranded molecule of any one of [1] to [4], wherein the sense strand hybridizes with antisense strand at the target sequence to form the double-stranded molecule having a length of less than about 75 nucleotide pairs in length;
[6] The double-stranded molecule of [5], wherein the sense strand hybridizes with antisense strand at the target sequence to form the double-stranded molecule having a length of less than about 50 nucleotide pairs in length;
[7] The double-stranded molecule of [6] wherein the sense strand hybridizes with antisense strand at the target sequence to form the double-stranded molecule having a length of less than about 25 nucleotide pairs in length;
[8] The double-stranded molecule of [7], wherein the sense strand hybridize with antisense strand at the target sequence to form the double-stranded molecule having a length of between about 19 and about 25 nucleotide pairs in length;
[9] The double-stranded molecule of any one of [1] to [8], composed of a single polynucleotide having both the sense and antisense strands linked by an intervening single-strand;
[10] The double-stranded molecule of [9], having the general formula 5’-[A]-[B]-[A’]-3’ or 5’-[A’]-[B]-[A]-3’, wherein [A] is the sense strand containing a nucleotide sequence corresponding to a target sequence selected from among SEQ ID NOs: 13 and 16., [B] is the intervening single-strand composed of 3 to 23 nucleotides, and [A’] is the antisense strand containing a sequence complementary to the target sequence of [A];
[II] The double-stranded molecule of any one of [1] to [10], composed of RNA;
[12] The double-stranded molecule of any one of [1] to [10], composed of both DNA and RNA;
The double-stranded molecule of [12], wherein the molecule is a hybrid of a DNA polynucleotide and an RNA polynucleotide;

The double-stranded molecule of [13] wherein the sense and the antisense strands are composed of DNA and RNA, respectively;

The double-stranded molecule of [12], wherein the molecule is a chimera of DNA and RNA;

The double-stranded molecule of [15], wherein a region flanking to the 3'-end of the antisense strand, or both of a region flanking to the 5'-end of the sense strand and a region flanking to the 3'-end of the antisense strand are RNA;

The double-stranded molecule of [16], wherein the flanking region is composed of 9 to 13 nucleotides; and

The double-stranded molecule of any one of [1] to [17], wherein the molecule contains one or two 3' overhang(s).

The double-stranded molecule of the present invention will be described in more detail below.

Methods for designing double-stranded molecules having the ability to inhibit target gene expression in cells are known (See, for example, US Patent No. 6,506,559, herein incorporated by reference in its entirety). For example, a computer program for designing siRNAs is available from the Ambion website (www.ambion.com/techlib/misc/siRNA_finder.html).

The computer program selects target nucleotide sequences for double-stranded molecules based on the following protocol.

Selection of Target Sites:

1. Beginning with the AUG start codon of the transcript, scan downstream for AA di-nucleotide sequences. Record the occurrence of each AA and the 3' adjacent 19 nucleotides as potential siRNA target sites. Tuschl et al. recommend to avoid designing siRNA to the 5' and 3'untranslated regions (UTRs) and regions near the start codon (within 75 bases) as these may be richer in regulatory protein binding sites, and UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex.

2. Compare the potential target sites to the appropriate genome database (human, mouse, rat, etc.) and eliminate from consideration any target sequences with significant homology to other coding sequences. Basically, BLAST, which can be found on the NCBI server at: www.ncbi.nlm.nih.gov/BLAST/, is used (Altschul SF et al., Nucleic Acids Res 1997 Sep 1. 25(17): 3389-402).

3. Select qualifying target sequences for synthesis. Selecting several target sequences along the length of the gene to evaluate is typical.

Any other algorithms developed for designing siRNA may be also used for designing
target sequences of the double-stranded molecules of the present invention. In the present invention, nucleotide sequences shown in SEQ ID NOs: 13 and 16 are demonstrated to be suitable for target sequences of the double-stranded molecules of the present invention. Double-stranded molecules targeting the above-mentioned target sequences were respectively examined and it was confirmed that they possessed ability to suppress the growth of cells expressing the EZH2 gene. Therefore, the present invention provides double-stranded molecules targeting the nucleotide sequence selected from the group consisting of SEQ ID NOs: 13 and 16.

The double-stranded molecule of the present invention may be directed to a single target EZH2 gene sequence or may be directed to a plurality of target EZH2 gene sequences.

A double-stranded molecule of the present invention targeting the EZH2 gene includes isolated polynucleotides that contain any of the target sequence selected from the EZH2 gene sequence and/or complementary sequences to the target sequence. Examples of polynucleotides targeting an EZH2 gene include those containing the sequence corresponding to a target sequence of SEQ ID NO: 13 or 16 and/or complementary sequences to these nucleotide sequences.

In an embodiment, a double-stranded molecule is composed of two polynucleotides, one polynucleotide has a sequence corresponding to a target sequence, i.e., sense strand, and another polypeptide has a complementary sequence to the target sequence, i.e., antisense strand. The sense strand polynucleotide and the antisense strand polynucleotide hybridize to each other to form double-stranded molecule. Examples of such double-stranded molecules include dsRNA and dsD/R-NA. In an another embodiment, a double-stranded molecule is composed of a polynucleotide that has both a sequence corresponding to a target sequence, i.e., sense strand, and a complementary sequence to the target sequence, i.e., antisense strand. Generally, the sense strand and the antisense strand are linked by a intervening strand, and hybridize to each other to form a hairpin loop structure. Examples of such double-stranded molecule include shRNA and shD/R-NA. In preferred embodiments, double-stranded molecules targeting the EZH2 gene may have a sequence selected from among SEQ ID NOs: 13 and 16 as a target sequence. Accordingly, preferable examples of the double-stranded molecule of the present invention include polynucleotides that hybridize to each other at a sequence corresponding to SEQ ID NOs: 13 and 16 and a complementary sequence thereto, and a polynucleotide that has a sequence corresponding to a target sequence of SEQ ID NOs: 13 and 16 and a complementary sequence thereto.

In one embodiment of the present invention, the double-stranded molecule of the present invention comprises a stem-loop structure, composed of the sense and
antisense strands. The sense and antisense strands may be joined by a loop. Accordingly, the present invention also provides the double-stranded molecule comprising a single polynucleotide containing both the sense strand and the antisense strand linked or flanked by an intervening single-strand.

However, the present invention is not limited to these examples, and minor modifications in the aforementioned nucleic acid sequences are acceptable so long as the modified molecule retains the ability to suppress the expression of an EZH2 gene. Herein, the phrase "minor modification" as used in connection with a nucleic acid sequence indicates one, two or several substitution, deletion, addition or insertion of nucleotide(s) to the sequence. In the context of the present invention, the term "several" as applied to nucleotide substitutions, deletions, additions and/or insertions may mean 3 to 7, preferably 3 to 5, more preferably 3 or 4, even more preferably 3 nucleic acid residues.

According to the present invention, a double-stranded molecule of the present invention can be tested for its ability using the methods utilized in the Examples. In the Examples herein below, double-stranded molecules composed of sense strands of some portions of mRNA of the EZH2 gene and antisense strands complementary thereto were tested in vitro for their ability to decrease production of an EZH2 gene product in various cancer cell lines (e.g., using SW780, RT4, A549, LC319 and SBC-5) according to standard methods. For example, reduction in the EZH2 gene product in cells transfected with the candidate double-stranded molecule compared to that in cells transfected no oligonucleotide or control siRNA (e.g., siRNA against EGFP) can be detected by, e.g., RT-PCR using primers for an EZH2 mRNA mentioned under Example: "Quantitative Real-time PCR". Candidate target sequences which decrease the production of the EZH2 gene product in vitro cell-based assays can then be tested for their inhibitory effects on cell growth. Target sequences which inhibit cell growth in vitro cell-based assay may then be tested for their in vivo ability using animals with cancer, e.g., nude mouse xenograft models, to confirm decreased production of the EZH2 product and decreased cancer cell growth.

As used herein, the term "complementary" refers to Watson-Crick or Hoogsteen base pairing between nucleotides units of a polynucleotide, and the term "binding" means the physical or chemical interaction between two polynucleotides. When the polynucleotide includes modified nucleotides and/or non-phosphodiester linkages, these polynucleotides may also bind each other as same manner. Generally, complementary polynucleotide sequences hybridize under appropriate conditions to form stable duplexes containing few or no mismatches. Furthermore, the isolated double-stranded molecule of the present invention can form double-stranded molecule or hairpin loop structure by the hybridization of the sense strand and antisense strand. In a preferred
embodiment, such double-stranded molecules contain no more than 1 mismatch for every 10 matches. In an especially preferred embodiment, where the strands of the duplex are fully complementary, such double-stranded molecules contain no mismatches.

[0230] The polynucleotide is preferably less than 2000 nucleotides in length. For example, the polynucleotide less than 500, 200, 100, 75, 50, or 25 nucleotides in length. The isolated polynucleotides of the present invention are useful for forming double-stranded molecules against the EZH2 gene or preparing template DNAs encoding the double-stranded molecules. When the polynucleotides are used for forming double-stranded molecules, the polynucleotide may be longer than 19 nucleotides, preferably longer than 21 nucleotides, and more preferably has a length of between about 19 and 25 nucleotides. Accordingly, the present invention provides the double-stranded molecules comprising a sense strand and an antisense strand, wherein the sense strand comprises a nucleotide sequence corresponding to a target sequence. In preferable embodiments, the sense strand hybridizes with antisense strand at the target sequence to form the double-stranded molecule having between 19 and 25 nucleotide pairs in length.

[0231] The double-stranded molecule serves as a guide for identifying homologous sequences in mRNA for the RISC complex, when the double-stranded molecule is introduced into cells. The identified target RNA is cleaved and degraded by the nuclease activity of Dicer, through which the double-stranded molecule eventually decreases or inhibits production (expression) of the polypeptide encoded by the RNA. Thus, a double-stranded molecule of the present invention can be defined by its ability to generate a single-strand that specifically hybridizes to the mRNA of the EZH2 gene under stringent conditions.

[0232] The double-stranded molecules of the present invention may contain one or more modified nucleotides and/or non-phosphodiester linkages. Chemical modifications well known in the art are capable of increasing stability, availability, and/or cell uptake of the double-stranded molecule. The skilled person will be aware of other types of chemical modification which may be incorporated into the present molecules (WO03/070744; WO2005/045037). In one embodiment, modifications can be used to provide improved resistance to degradation or improved uptake. Examples of such modifications include, but are not limited to, phosphorothioate linkages, 2'-0-methyl ribonucleotides (especially on the sense strand of a double-stranded molecule), 2'-deoxy-fluoro ribonucleotides, 2'-deoxy ribonucleotides, "universal base" nucleotides, 5'-C- methyl nucleotides, and inverted deoxybasic residue incorporation (US20060122137).

[0233] In another embodiment, modifications can be used to enhance the stability or to
increase targeting efficiency of the double-stranded molecule. Examples of such modifications include, but are not limited to, chemical cross linking between the two complementary strands of a double-stranded molecule, chemical modification of a 3' or 5' terminus of a strand of a double-stranded molecule, sugar modifications, nucleobase modifications and/or backbone modifications, 2'-fluoro modified ribonucleotides and 2'-deoxy ribonucleotides (WO2004/029212). In another embodiment, modifications can be used to increase or decrease affinity for the complementary nucleotides in the target mRNA and/or in the complementary double-stranded molecule strand (WO2005/044976). For example, an unmodified pyrimidine nucleotide can be substituted for a 2-thio, 5-alkynyl, 5-methyl, or 5-propynyl pyrimidine. Additionally, an unmodified purine can be substituted with a 7-deaza, 7-alkyl, or 7-alkenyl purine. In another embodiment, when the double-stranded molecule is a double-stranded molecule with a 3' overhang, the 3'-terminal nucleotide overhanging nucleotides may be replaced with deoxyribonucleotides (Elbashir SM et al., Genes Dev 2001 Jan 15, 15(2): 188-200). For further details, published documents such as US20060234970 are available. The present invention is not limited to these examples and any known chemical modifications may be employed for the double-stranded molecules of the present invention so long as the resulting molecule retains the ability to inhibit the expression of the target gene.

Furthermore, the double-stranded molecules of the present invention may include both DNA and RNA, e.g., dsD/R-NA or shD/R-NA. Specifically, a hybrid polynucleotide of a DNA strand and an RNA strand or a DNA-RNA chimera polynucleotide shows increased stability. Mixing of DNA and RNA, i.e., a hybrid type double-stranded molecule composed of a DNA strand (polynucleotide) and an RNA strand (polynucleotide), a chimera type double-stranded molecule containing both DNA and RNA on either or both of the single strands (polynucleotides), or the like may be formed for enhancing stability of the double-stranded molecule.

The hybrid of a DNA strand and an RNA strand may be either where the sense strand is DNA and the antisense strand is RNA, or the opposite so long as it can inhibit expression of the target gene when introduced into a cell expressing the gene. Preferably, the sense strand polynucleotide is DNA and the antisense strand polynucleotide is RNA. Also, the chimera type double-stranded molecule may be either where both of the sense and antisense strands are composed of DNA and RNA, or where any one of the sense and antisense strands is composed of DNA and RNA so long as it has an activity to inhibit expression of the target gene when introduced into a cell expressing the gene. In order to enhance stability of the double-stranded molecule, the molecule preferably contains as much DNA as possible, whereas to induce inhibition of the target gene expression, the molecule is required to be RNA within a range to induce
sufficient inhibition of the expression.

[0236] As a preferred example of the chimera type double-stranded molecule, an upstream partial region (i.e., a region flanking to the target sequence or complementary sequence thereof within the sense or antisense strands) of the double-stranded molecule is RNA. Preferably, the upstream partial region indicates the 5' side (5'-end) of the sense strand and the 3' side (3'-end) of the antisense strand. Alternatively, regions flanking to 5'-end of sense strand and/or 3'-end of antisense strand are referred to upstream partial region. That is, in preferable embodiments, a region flanking to the 3'-end of the antisense strand, or both of a region flanking to the 5'-end of sense strand and a region flanking to the 3'-end of antisense strand are composed of RNA. For instance, the chimera or hybrid type double-stranded molecule of the present invention include following combinations.

- sense strand:
  5'-[—DNA— ]-3'
  3'-(RNA)-[DNA]-5'
- antisense strand,
  sense strand:
  5'-(RNA)-[DNA]-3'
  3'-(RNA)-[DNA]-5'
  antisense strand, and
  sense strand:
  5'-(RNA)-[DNA]-3'
  3'-(RNA—)-5'
  antisense strand.

[0237] The upstream partial region preferably is a domain composed of 9 to 13 nucleotides counted from the terminus of the target sequence or complementary sequence thereto within the sense or antisense strands of the double-stranded molecules. Moreover, preferred examples of such chimera type double-stranded molecules include those having a strand length of 19 to 21 nucleotides in which at least the upstream half region (5' side region for the sense strand and 3' side region for the antisense strand) of the double-stranded molecule is RNA and the other half is DNA. In such a chimera type double-stranded molecule, the effect to inhibit expression of the target gene is much higher when the entire antisense strand is RNA (US20050004064).

[0238] In the present invention, the double-stranded molecule may form a hairpin, such as a short hairpin RNA (shRNA) and short hairpin consisting of DNA and RNA (shD/R-NA). The shRNA or shD/R-NA is a sequence of RNA or mixture of RNA and DNA making a tight hairpin turn that can be used to silence gene expression via RNA interference. The shRNA or shD/R-NA includes a sense strand containing a sequence
corresponding to the target sequence and an antisense containing a complementary sequence corresponding to the target sequence on a single strand wherein the sequences are separated by a loop sequence. Generally, the hairpin structure is cleaved by the cellular machinery into dsRNA or dsD/R-NA, which is then bound to the RNA-induced silencing complex (RISC). This complex binds to and cleaves mRNAs which match the target sequence of the dsRNA or dsD/R-NA.

0239 A loop sequence composed of an arbitrary nucleotide sequence can be located between the sense and antisense sequence in order to form the hairpin loop structure. Thus, the present invention also provides a double-stranded molecule having the general formula 5'-[A]-[B]-[A']-3' or 5'-[A']-[B]-[A]-3', wherein [A] is the sense strand containing a sequence corresponding to a target sequence, [B] is an intervening single-strand and [A'] is the antisense strand containing a complementary sequence to the target sequence of [A]. The target sequence may be selected from among, for example, nucleotide sequences of SEQ ID NOs: 13 and 16.

0240 The present invention is not limited to these examples, and the target sequence in [A] may be modified sequences from these examples so long as the double-stranded molecule retains the ability to suppress the expression of the targeted EZH2 gene. The region [A] hybridizes to [A'] to form a loop composed of the region [B]. The intervening single-stranded portion [B], i.e., loop sequence may be preferably 3 to 23 nucleotides in length. The loop sequence, for example, can be selected from among the following sequences (www.ambion.com/techlib/tb/tb_506.html). Furthermore, loop sequence consisting of 23 nucleotides also provides active siRNA (Jacque JM et al., Nature 2002 Jul 25, 418(6896): 435-8, Epub 2002 Jun 26):


0241 Examples of preferred double-stranded molecules of the present invention having hairpin loop structure are shown below. In the following structure, the loop sequence can be selected from among AUG, CCC, UUCG, CCACC, CTGAG, AAGCUU, CCACACC, and UUCAAGAGA; however, the present invention is not limited thereto:

CUACCAUGUUUACACUA-[B]-UAGUUUGAAAACAUGGUUAG (for target sequence of SEQ ID NO: 13);
GACAGAAGGGAAAGUGU-[B]-ACACUUUCCCUCUUCUGUC (for target sequence of SEQ ID NO: 16).
Furthermore, in order to enhance the inhibition activity of the double-stranded molecules, several nucleotide can be added to 3’ end of the sense strand and/or antisense strand of the target sequence, as 3’ overhangs. The preferred examples of nucleotides consisting a 3’ overhang include "r" and "u", but are not limited to. The number of nucleotides to be added is at least 2, generally 2 to 10, preferably 2 to 5. The added nucleotides form (a) single strand(s) at the 3’ end of the sense strand and/or antisense strand of the double-stranded molecule. In cases where double-stranded molecules consists of a single polynucleotide to form a hairpin loop structure, a 3’ overhang sequence may be added to the 3’ end of the single polynucleotide.

The method for preparing the double-stranded molecule is not particularly limited though it is preferable to use a chemical synthetic method known in the art. According to the chemical synthesis method, sense and antisense single-stranded polynucleotides are separately synthesized and then annealed together via an appropriate method to obtain a double-stranded molecule. Specific example for the annealing includes wherein the synthesized single-stranded polynucleotides are mixed in a molar ratio of preferably at least about 3:7, more preferably about 4:6, and most preferably substantially equimolar amount (i.e., a molar ratio of about 5:5). Next, the mixture is heated to a temperature at which double-stranded molecules dissociate and then is gradually cooled down. The annealed double-stranded polynucleotide can be purified by usually employed methods known in the art. Examples of purification methods include methods utilizing agarose gel electrophoresis. Remaining single-stranded polynucleotides may be optionally removed by, e.g., degradation with appropriate enzyme.

Alternatively, the double-stranded molecules may be transcribed intracellularly by cloning its coding sequence into a vector containing a regulatory sequence that directs the expression of the double-stranded molecule in an adequate cell (e.g., a RNA poly III transcription unit from the small nuclear RNA (snRNA) U6 or the human H1 RNA promoter) adjacent to the coding sequence. The regulatory sequences flanking the coding sequences of double-stranded molecule may be identical or different, such that their expression can be modulated independently, or in a temporal or spatial manner. Details of vectors which are capable of producing the double-stranded molecules will be described bellow.

Vectors containing a double-stranded molecule of the present invention:

Also included in the present invention are vectors containing one or more of the double-stranded molecules described herein, and a cell containing such a vector.

Specifically, the present invention provides the following vector of [1] to [11].

[1] A vector, encoding a double-stranded molecule that, when introduced into a cell, inhibits in vivo expression of EZH2 gene and cell proliferation, such molecules
composed of a sense strand and an antisense strand complementary thereto, hybridized to each other to form the double-stranded molecule.

[2] The vector of [1], encoding the double-stranded molecule acts on mRNA of EZH2, matching a target sequence selected from among SEQ ID NOs: 13 and 16;

[3] The vector of [1], wherein the sense strand contains a nucleotide sequence corresponding to a target sequence selected from among SEQ ID NOs: 13 and 16;

[4] The vector of any one of [1] to [3], encoding the double-stranded molecule, wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having a length of less than about 100 nucleotide pairs in length;

[5] The vector of [4], encoding the double-stranded molecule, wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having a length of less than about 75 nucleotide pairs in length;

[6] The vector of [5], encoding the double-stranded molecule, wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having a length of less than about 50 nucleotide pairs in length;

[7] The vector of [6] encoding the double-stranded molecule, wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having a length of less than about 25 nucleotide pairs in length;

[8] The vector of [7], encoding the double-stranded molecule, wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having between about 19 and about 25 nucleotide pair in length;

[9] The vector of any one of [1] to [8], wherein the double-stranded molecule is composed of a single polynucleotide having both the sense and antisense strands linked by an intervening single-strand;

[10] The vector of [9], encoding the double-stranded molecule having the general formula 5'-[A]-[B]-[A']-3' or 5'-[A']-[B]-[A]-3', wherein [A] is the sense strand containing a sequence corresponding to a target sequence selected from among SEQ ID NOs: 13 and 16, [B] is the intervening single-strand composed of 3 to 23 nucleotides, and [A'] is the antisense strand containing a sequence complementary to the target sequence of [A]; and

[11] The vector of any one of [1] to [10], wherein the double-stranded molecule contains one or two 3' overhang(s).

[0246] A vector of the present invention preferably encodes a double-stranded molecule of
the present invention in an expressible form. Herein, the phrase "in an expressible form" indicates that the vector, when introduced into a cell, will express the molecule. In a preferred embodiment, the vector includes regulatory elements necessary for expression of the double-stranded molecule. Accordingly, in one embodiment, the expression vector encodes the nucleic acid sequences of the present invention and is adapted for expression of said nucleic acid sequences. Such vectors of the present invention may be used for producing the present double-stranded molecules, or directly as an active ingredient for treating cancer.

Vectors of the present invention can be produced, for example, by cloning the sequence encoding the double-stranded molecule into an expression vector so that regulatory sequences are operatively-linked to the coding sequences of the double-stranded molecule in a manner to allow expression (by transcription of the DNA molecule) of both strands (Lee NS et al., Nat Biotechnol 2002 May, 20(5): 500-5). For example, RNA molecule that is the antisense strand to mRNA is transcribed by a first promoter (e.g., a promoter sequence flanking to the 3' end of the cloned DNA) and RNA molecule that is the sense strand to the mRNA is transcribed by a second promoter (e.g., a promoter sequence flanking to the 5' end of the cloned DNA). After transcribed, the sense and antisense strands hybridize to each other in vivo to generate a double-stranded molecule constructs for silencing of the gene. Alternatively, two vectors constructs respectively encoding the sense and antisense strands of the double-stranded molecule are utilized to respectively express the sense and antisense strands and then forming a double-stranded molecule construct. Furthermore, the cloned sequence may encode a construct having a secondary structure (e.g., hairpin); namely, a single transcript of a vector contains both the sense and complementary antisense sequences of the target gene.

The vectors of the present invention may also be equipped so to achieve stable insertion into the genome of the target cell (see, e.g., Thomas KR & Capecchi MR, Cell 1987, 51: 503-12 for a description of homologous recombination cassette vectors). See, e.g., Wolff et al., Science 1990, 247: 1465-8; US Patent Nos. 5,580,859; 5,589,466; 5,804,566; 5,739,118; 5,736,524; 5,679,647; and WO 98/04720. Examples of DNA-based delivery technologies include "naked DNA", facilitated (bupivacaine, polymers, peptide-mediated) delivery, cationic lipid complexes, and particle-mediated ("gene gun") or pressure-mediated delivery (see, e.g., US Patent No. 5,922,687).

The vectors of the present invention include, for example, viral or bacterial vectors. Examples of expression vectors include attenuated viral hosts, such as vaccinia or fowlpox (see, e.g., US Patent No. 4,722,848). This approach involves the use of vaccinia virus, e.g., as a vector to express nucleotide sequences that encode the double-stranded molecule. Upon introduction into a cell expressing the target gene, the re-
combinant vaccinia virus expresses the double-stranded molecule and thereby suppresses the proliferation of the cell. Another example of useable vector includes Bacille Calmette Guerin (BCG). BCG vectors are described in Stover et al., Nature 1991, 351: 456-60. A wide variety of other vectors are useful for therapeutic administration and production of the double-stranded molecules; examples include adeno and adeno-associated virus vectors, retroviral vectors, Salmonella typhi vectors, detoxified anthrax toxin vectors, and the like. See, e.g., Shata et al., Mol Med Today 2000, 6: 66-71; Shedlock et al., J Leukoc Biol 2000, 68: 793-806; and Hipp et al., In Vivo 2000, 14: 571-85.

VII. Methods of treating cancer using double-stranded molecules:

The present invention provides methods for inhibiting cancer cell growth, e.g., bladder cancer, cervical cancer, osteosarcoma, lung cancer, soft tissue tumor, breast cancer, chronic myelogenous leukemia (CML), esophageal cancer and gastric cancer cell growth, by inducing dysfunction of an EZH2 gene via inhibiting the expression of EZH2 gene. The EZH2 gene expression can be inhibited by any of the aforementioned double-stranded molecules of the present invention which specifically target of the EZH2 gene or the vectors of the present invention that can express any of the double-stranded molecules.

Such ability of the present double-stranded molecules and vectors to inhibit cell growth of cancerous cell indicates that they can be used for methods for treating cancer. Thus, the present invention provides methods to treat patients with cancer associated EZH2 overexpression, for example, AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma, by administering a double-stranded molecule against an EZH2 gene or a vector expressing the molecule without adverse effect because that the gene is hardly expressed in normal organs.

Specifically, the present invention provides the following methods of [1] to [22]:

[1] A method of either or both of treating and preventing cancer, or inhibiting cancer cell growth in a subject comprising administering to a subject a pharmaceutically effective amount of a double-stranded molecule against an EZH2 gene or a vector encoding the double-stranded molecule, wherein the double-stranded molecule, when introduced into a cell, inhibits in vivo expression of an EZH2 gene as well as cell proliferation, the molecule comprising a sense strand and an antisense strand complementary thereto, the strands hybridized to each other to form the double-stranded molecule;

[2] The method of [1], wherein the double-stranded molecule acts at mRNA which matches a target sequence selected from among SEQ ID NOS: 13 and 16;

[3] The method of [1], wherein the sense strand contains the nucleotide sequence cor-
responding to a target sequence selected from among SEQ ID NOs: 13 and 16;
[4] The method of any one of [1] to [3], wherein the cancer is selected from the group consisting of AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma;
[5] The method of any one of [1] to [4], wherein the cancer is lung cancer, bladder cancer or colorectal cancer;
[6] The method of any one of [1] to [5], wherein plural kinds of the double-stranded molecules are administered;
[7] The method of any one of [1] to [6], wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having less than about 100 nucleotide pairs in lengths;
[8] The method of [7], wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having less than about 75 nucleotide pairs in lengths;
[9] The method of [8], wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having less than about 50 nucleotide pairs in lengths;
[10] The method of [9], wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having less than about 25 nucleotides pairs in lengths;
[11] The method of [10], wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having between about 19 and about 25 nucleotide pairs in length;
[12] The method of any one of [1] to [11], wherein the double-stranded molecule is composed of a single polynucleotide containing both the sense strand and the antisense strand linked by an intervening single-strand;
[13] The method of [12], wherein the double-stranded molecule has the general formula 5’-[A]-[B]-[A’]-3’ or 5’-[A’]-[B]-[A]-3’, wherein [A] is the sense strand containing a sequence corresponding to a target sequence selected from among SEQ ID NOs: 13 and 16, [B] is the intervening single strand composed of 3 to 23 nucleotides, and [A’] is the antisense strand containing a sequence complementary to the target sequence of [A];
[14] The method of [1] to [13], wherein the double-stranded molecule is an RNA;
[15] The method of [1] to [13], wherein the double-stranded molecule contains both DNA and RNA;
[16] The method of [14], wherein the double-stranded molecule is a hybrid of a DNA polynucleotide and an RNA polynucleotide;
[17] The method of [15] wherein the sense and antisense strand polynucleotides are composed of DNA and RNA, respectively;
[18] The method of [15], wherein the double-stranded molecule is a chimera of DNA and RNA;
[19] The method of [18], wherein a region flanking to the 3'-end of the antisense strand, or both of a region flanking to the 5'-end of sense strand and a region flanking to the 3'-end of antisense strand are composed of RNA;
[20] The method of [19], wherein the flanking region is composed of 9 to 13 nucleotides;
[21] The method of any one of [1] to [20], wherein the double-stranded molecule contains one or two 3' overhang(s); and
[22] The method of any one of [1] to [21], wherein the double-stranded molecule is contained in a composition which includes, in addition to the molecule, a transfection-enhancing agent and pharmaceutically acceptable carrier.

The method of the present invention will be described in more detail below.

The growth of cells expressing an EZH2 gene may be inhibited by contacting the cells with a double-stranded molecule against the EZH2 gene, a vector expressing the molecule or a composition containing the same. The cell may be further contacted with a transfection agent. Suitable transfection agents are known in the art. The phrase "inhibition of cell growth" indicates that the cell proliferates at a lower rate or has decreased viability as compared to a cell not exposed to the molecule. Cell growth may be measured by methods known in the art, e.g., using the MTT cell proliferation assay.

The growth of any kind of cell may be suppressed according to the present method so long as the cell expresses or over-expresses the target gene of the double-stranded molecule of the present invention. Exemplary cells include bladder cancer cells, colorectal cancer cells and lung cancer cells.

Thus, patients suffering from or at risk of developing disease related to EZH2 may be treated by administering at least one of the present double-stranded molecules, at least one vector expressing at least one of the molecules or at least one composition containing at least one of the molecules. For example, subjects with AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma may be treated according to the present methods. The type of cancer may be identified by standard methods according to the particular type of tumor to be diagnosed. AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma may be diagnosed, for example, with tumor markers, such as CEA as colorectal cancer and lung cancer marker, CYFRA and pro-GRP as lung cancer marker, and TPA as bladder cancer
marker, or with Chest X-Ray and/or Sputum Cytology. More preferably, patients treated by the methods of the present invention are selected by detecting the expression of an EZH2 gene in a biological sample from the patient by conventional methods such as RT-PCR or immunoassay. Preferably, before the treatment of the present invention, the biopsy specimen from the subject is confirmed for an EZH2 gene over-expression by methods known in the art, for example, immunohistochemical analysis or RT-PCR.

According to the present method to inhibit cell growth and thereby treating cancer, when administering plural kinds of the double-stranded molecules (or vectors expressing or compositions containing the same), each of the molecules may have different structures but acts at mRNA which matches the same target sequence. Alternatively plural kinds of the double-stranded molecules may acts at mRNA which matches different target sequence of same gene or acts at mRNA which matches different target sequence of different gene. For example, the method may utilize double-stranded molecules directed to one, two or more target sequence of an EZH2 gene. Alternatively, the method may utilize the double-stranded molecules directed to target sequences of an EZH2 gene and other genes.

For inhibiting cell growth, a double-stranded molecule of present invention may be directly introduced into the cells in a form to achieve binding of the molecule with corresponding mRNA transcripts. Alternatively, as described above, a DNA encoding the double-stranded molecule may be introduced into cells by means of a vector. For introducing the double-stranded molecules and vectors into the cells, transfection-enhancing agent, such as FuGENE (Roche diagnostics), Lipofectamine 2000 (Invitrogen), Oligofectamine (Invitrogen), and Nucleofector (Wako pure Chemical), may be employed.

A treatment is deemed "efficacious" if it leads to clinical benefit such as, reduction in expression of an EZH2 gene, or a decrease in size, prevalence, or metastatic potential of the cancer in the subject. When the treatment is applied prophylactically, "efficacious" means that it retards or prevents cancers from forming or prevents or alleviates a clinical symptom of cancer. Efficaciousness is determined in association with any known method for diagnosing or treating the particular tumor type.

It is understood that the double-stranded molecule of the present invention degrades the EZH2 mRNA in substoichiometric amounts. Without wishing to be bound by any theory, it is believed that the double-stranded molecule of the present invention causes degradation of the target mRNA in a catalytic manner. Thus, compared to standard cancer therapies, significantly less a double-stranded molecule needs to be delivered at or near the site of cancer to exert therapeutic effect.

One skilled in the art can readily determine an effective amount of the double-stranded molecule of the present invention to be administered to a given subject, by
taking into account factors such as body weight, age, sex, type of disease, symptoms and other conditions of the subject; the route of administration; and whether the administration is regional or systemic. Generally, an effective amount of the double-stranded molecule of the present invention is an intercellular concentration at or near the cancer site of from about 1 nanomolar (nM) to about 100 nM, preferably from about 2 nM to about 50 nM, more preferably from about 2.5 nM to about 10 nM. It is contemplated that greater or smaller amounts of the double-stranded molecule can be administered. The precise dosage required for a particular circumstance may be readily and routinely determined by one of skill in the art.

The present methods can be used to inhibit the growth or metastasis of cancer expressing EZH2; for example, AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma. In particular, a double-stranded molecule containing a target sequence of EZH2 (e.g., SEQ ID NO: 13 and 16) is particularly preferred for the treatment of AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma.

For treating cancer, the double-stranded molecule of the present invention can also be administered to a subject in combination with a pharmaceutical agent different from the double-stranded molecule. Alternatively, the double-stranded molecule of the present invention can be administered to a subject in combination with another therapeutic method designed to treat cancer. For example, the double-stranded molecule of the present invention can be administered in combination with therapeutic methods currently employed for treating cancer or preventing cancer metastasis (e.g., radiation therapy, surgery and treatment using chemotherapeutic agents, such as cisplatin, carboplatin, cyclophosphamide, 5-fluorouracil, adriamycin, daunorubicin or tamoxifen).

In the present methods, the double-stranded molecule can be administered to the subject either as a naked double-stranded molecule, in conjunction with a delivery substance, or as a recombinant plasmid or viral vector which expresses the double-stranded molecule.

Suitable delivery substances for administration in conjunction with the double-stranded molecule include the Mirus Transit TKO lipophilic substance; lipofectin; lipofectamine; cellfectin; or polycations (e.g., polylysine), or liposomes. Preferred examples of delivery substance are liposomes.

Liposomes can aid in the delivery of the double-stranded molecule to a particular tissue, such as lung tumor tissue, and can also increase the blood half-life of the double-stranded molecule. Liposomes suitable for use in the present invention are
formed from standard vesicle-forming lipids, which generally include neutral or negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of factors such as the desired liposome size and half-life of the liposomes in the blood stream. A variety of methods are known for preparing liposomes, for example as described in Szoka et al., Ann Rev Biophys Bioeng 1980, 9: 467; and US Pat. Nos. 4,235,871; 4,501,728; 4,837,028; and 5,019,369, the entire disclosures of which are herein incorporated by reference.

Preferably, the liposomes encapsulating the double-stranded molecule of the present invention includes a ligand molecule that can deliver the liposome to the cancer site. Ligands which bind to receptors prevalent in tumor or vascular endothelial cells, such as monoclonal antibodies that bind to tumor antigens or endothelial cell surface antigens, are preferred.

Particularly preferably, the liposomes encapsulating the double-stranded molecule of the present invention are modified so as to avoid clearance by the mononuclear macrophage and reticuloendothelial systems, for example, by having opsonization-inhibiting moieties bound to the surface of the structure. In one embodiment, a liposome may include both opsonization-inhibiting moieties and a ligand.

Opsonization-inhibiting moieties for use in preparing liposomes are typically large hydrophilic polymers that are bound to the liposome membrane. As used herein, an opsonization-inhibiting moiety is "bound" to a liposome membrane when it is chemically or physically attached to the membrane, e.g., by the intercalation of a lipid-soluble anchor into the membrane itself, or by binding directly to active groups of membrane lipids. These opsonization-inhibiting hydrophilic polymers form a protective surface layer which significantly decreases the uptake of the liposomes by the macrophage-monocyte system ("MMS") and reticuloendothelial system ("RES"); e.g., as described in US Pat. No. 4,920,016, the entire disclosure of which is herein incorporated by reference. Liposomes modified with opsonization-inhibition moieties thus remain in the circulation much longer than unmodified liposomes. For this reason, such liposomes are sometimes called "stealth" liposomes.

Stealth liposomes are known to accumulate in tissues fed by porous or "leaky" microvasculature. Thus, target tissue characterized by such microvasculature defects, for example, solid tumors, will efficiently accumulate these liposomes; see Gabizon et al., Proc Natl Acad Sci USA 1988, 18: 6949-53. In addition, the reduced uptake by the RES lowers the toxicity of stealth liposomes by preventing significant accumulation in liver and spleen. Thus, liposomes modified with opsonization-inhibiting moieties can deliver the double-stranded molecule of the present invention to tumor cells.

Opsonization-inhibiting moieties suitable for modifying liposomes are preferably water-soluble polymers with a molecular weight from about 500 to about 40,000
daltons, and more preferably from about 2,000 to about 20,000 daltons. Such polymers include polyethylene glycol (PEG) or polypropylene glycol (PPG) derivatives; e.g., methoxy PEG or PPG, and PEG or PPG stearate; synthetic polymers such as polyacrylamide or poly N-vinyl pyrrolidone; linear, branched, or dendrimeric polyamidoamines; polyacrylic acids; polyalcohols, e.g., polyvinylalcohol and polyxyritol to which carboxylic or amino groups are chemically linked, as well as gangliosides, such as ganglioside GM1. Copolymers of PEG, methoxy PEG, or methoxy PPG, or derivatives thereof, are also suitable. In addition, the opsonization-inhibiting polymer can be a block copolymer of PEG and either a polyamino acid, polysaccharide, polyamidoamine, polyethyleneamine, or polynucleotide. The opsonization-inhibiting polymers can also be natural polysaccharides containing amino acids or carboxylic acids, e.g., galacturonic acid, glucuronic acid, mannuronic acid, hyaluronic acid, pectic acid, neuraminic acid, alginic acid, carrageenan; aminated polysaccharides or oligosaccharides (linear or branched); or carboxylated polysaccharides or oligosaccharides, e.g., reacted with derivatives of carbonic acids with resultant linking of carboxylic groups.

Preferably, the opsonization-inhibiting moiety is a PEG, PPG, or derivatives thereof. Liposomes modified with PEG or PEG-derivatives are sometimes called "PEGylated liposomes".

The opsonization-inhibiting moiety can be bound to the liposome membrane by any one of numerous well-known techniques. For example, an N-hydroxysuccinimide ester of PEG can be bound to a phosphatidyl-ethanolamine lipid-soluble anchor, and then bound to a membrane. Similarly, a dextran polymer can be derivatized with a stearylamine lipid-soluble anchor via reductive amination using Na(CN)BH3 and a solvent mixture such as tetrahydrofuran and water in a 30:12 ratio at 60 degrees C.

Vectors expressing a double-stranded molecule of the present invention are discussed above. Such vectors expressing at least one double-stranded molecule of the present invention can also be administered directly or in conjunction with a suitable delivery substance, including the Mirus Transit LT1 lipophilic substance; lipofectin; lipofectamine; cellfectin; polycations (e.g., polylysine) or liposomes. Methods for delivering recombinant viral vectors, which express a double-stranded molecule of the present invention, to an area of cancer in a patient are within the skill of the art.

The double-stranded molecule of the present invention can be administered to the subject by any means suitable for delivering the double-stranded molecule into cancer sites. For example, the double-stranded molecule can be administered by gene gun, electroporation, or by other suitable parenteral or enteral administration routes.

Suitable enteral administration routes include oral, rectal, or intranasal delivery.

Suitable parenteral administration routes include intravascular administration (e.g.,
intravenous bolus injection, intravenous infusion, intra-arterial bolus injection, intra-arterial infusion and catheter instillation into the vasculature; peri- and intra-tissue injection (e.g., peri-tumoral and intra-tumoral injection); subcutaneous injection or deposition including subcutaneous infusion (such as by osmotic pumps); direct application to the area at or near the site of cancer, for example by a catheter or other placement device (e.g., a suppository or an implant including a porous, non-porous, or gelatinous material); and inhalation. It is preferred that injections or infusions of the double-stranded molecule or vector be given at or near the site of cancer.

The double-stranded molecule of the present invention can be administered in a single dose or in multiple doses. Where the administration of the double-stranded molecule of the present invention is by infusion, the infusion can be a single sustained dose or can be delivered by multiple infusions. Preferably, the double-stranded molecule is injected directly into the tissue at or near the site of cancer. Multiple injections of the double-stranded molecule into the tissue at or near the site of cancer are particularly preferred.

One skilled in the art can also readily determine an appropriate dosage regimen for administering the double-stranded molecule of the present invention to a given subject. For example, the double-stranded molecule can be administered to the subject once, for example, as a single injection or deposition at or near the cancer site. Alternatively, the double-stranded molecule can be administered once or twice daily to a subject for a period of from about three to about twenty-eight days, more preferably from about seven to about ten days. In a preferred dosage regimen, the double-stranded molecule is injected at or near the site of cancer once a day for seven days. Where a dosage regimen includes multiple administrations, it is understood that the effective amount of a double-stranded molecule administered to the subject can include the total amount of a double-stranded molecule administered over the entire dosage regimen.

VIII. Compositions containing double-stranded molecules:

In addition to the above, the present invention also provides pharmaceutical compositions that include at least one of the double-stranded molecules of the present invention or the vectors coding for the molecules.

In the context of the present invention, the term "composition" is used to refer to a product including that include the specified ingredients in the specified amounts, as well as any product that results, directly or indirectly, from combination of the specified ingredients in the specified amounts. Such terms, when used in relation to the modifier "pharmaceutical" (as in "pharmaceutical composition"), are intended to encompass products including a product that includes the active ingredient(s), and any inert ingredient(s) that make up the carrier, as well as any product that results, directly or indirectly, from combination, complexation or aggregation of any two or more of
the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients. Accordingly, in the context of the present invention, the term "pharmaceutical composition" refers to any product made by admixing a molecule or compound of the present invention and a pharmaceutically or physiologically acceptable carrier.

[0277] The phrase "pharmaceutically acceptable carrier" or "physiologically acceptable carrier", as used herein, means a pharmaceutically or physiologically acceptable material, composition, substance or vehicle, including but not limited to, a liquid or solid filler, diluent, excipient, solvent or encapsulating material.

[0278] The term "active ingredient" herein refers to a substance in composition that is biologically or physiologically active. Particularly, in the context of pharmaceutical composition, the term "active ingredient" refers to a substance that shows an objective pharmacological effect. For example, in case of pharmaceutical compositions for use in the treatment or prevention of cancer, active ingredients in the agents or compositions may lead to at least one biological or physiologically action on cancer cells and/or tissues directly or indirectly. Preferably, such action may include reducing or inhibiting cancer cell growth, damaging or killing cancer cells and/or tissues, and so on. Before being formulated, the "active ingredient" may also be referred to as "bulk", "drug substance" or "technical product".

[0279] Specifically, the present invention provides the following compositions [1] to [22]:

[1] A composition for either or both of treating and preventing cancer, or inhibiting cancer cell growth, wherein the cancer cell and the cancer expresses an EZH2 gene, including a pharmaceutically effective amount of an isolated double-stranded molecule against the EZH2 gene or pharmaceutically acceptable salt thereof, or a vector encoding the double-stranded molecule, which molecule is composed of a sense strand and an antisense strand complementary thereto, hybridized to each other to form the double-stranded molecule, wherein the double-stranded molecule, when introduced into a cell, inhibits in vivo expression of the EZH2 gene as well as cell proliferation, and pharmaceutically acceptable carrier;

[2] The composition of [1], wherein the double-stranded molecule acts at mRNA which matches a target sequence selected from among SEQ ID NOs: 13 and 16;

[3] The composition of [1], wherein the double-stranded molecule, wherein the sense strand contains a nucleotide sequence corresponding to a target sequence selected from among SEQ ID NOs: 13 and 16;

[4] The composition of any one of [1] to [3], wherein the cancer is selected from the group consisting of AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma;
[5] The composition of any one of [1] to [4], wherein the cancer is lung cancer, colorectal cancer or bladder cancer;
[6] The composition of any one of [1] to [5], wherein the composition contains plural kinds of the double-stranded molecules;
[7] The composition of any one of [1] to [6], wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having less than about 100 nucleotide pairs in length;
[8] The composition of [7], wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having less than about 75 nucleotide pairs in length;
[9] The composition of [8], wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having less than about 50 nucleotide pairs in length;
[10] The composition of [9], wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having less than about 25 nucleotide pairs in length;
[11] The composition of [10], wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having between about 19 and about 25 nucleotide pairs in length;
[12] The composition of any one of [1] to [11], wherein the double-stranded molecule is composed of a single polynucleotide containing the sense strand and the antisense strand linked by an intervening single-strand;
[13] The composition of [12], wherein the double-stranded molecule has the general formula 5'-[A]-[B]-[A']-3' or 5'-[A']-[B]-[A]-3', wherein [A] is the sense strand sequence containing a nucleotide sequence corresponding to a target sequence selected from among SEQ ID NOs: 13 and 16, [B] is the intervening single-strand consisting of 3 to 23 nucleotides, and [A'] is the antisense strand containing a nucleotide sequence complementary to the target sequence of [A];
[14] The composition of any one of [1] to [13], wherein the double-stranded molecule is an RNA;
[15] The composition of any one of [1] to [13], wherein the double-stranded molecule is DNA and/or RNA;
[16] The composition of [15], wherein the double-stranded molecule is a hybrid of a DNA polynucleotide and an RNA polynucleotide;
[17] The composition of [16], wherein the sense and antisense strand polynucleotides are composed of DNA and RNA, respectively;
[18] The composition of [16], wherein the double-stranded molecule is a chimera of DNA and RNA;
[19] The composition of [18], wherein a region flanking to the 3’-end of the antisense strand, or both of a region flanking to the 5’-end of sense strand and a region flanking to the 3’-end of antisense strand are composed of RNA;
[20] The composition of [19], wherein the flanking region is composed of 9 to 13 nucleotides;
[21] The composition of any one of [1] to [20], wherein the double-stranded molecule contains one or two 3’ overhang(s); and
[22] The composition of any one of [1] to [21], wherein the composition includes a transfection-enhancing agent.

Additional details of the compositions of the present invention are described below. Compositions of the present invention preferably formulated as pharmaceutical compositions, according to techniques known in the art. Compositions of the present invention are characterized as being at least sterile and pyrogen-free. As used herein, "pharmaceutical compositions" include formulations for human and veterinary use.

In the context of the present invention, suitable pharmaceutical formulations of the present invention include those suitable for oral, rectal, nasal, topical (including buccal, sub-lingual, and transdermal), vaginal or parenteral (including intramuscular, subcutaneous and intravenous) administration, or for administration by inhalation or insufflation. Other formulations include implantable devices and adhesive patches that release a therapeutic agent. When desired, the above-described formulations may be adapted to give sustained release of the active ingredient.

Methods for preparing the compositions of the present invention are within the skill in the art, for example as described in Remington's Pharmaceutical Science, 17th ed., Mack Publishing Company, Easton, Pa. (1985), the entire disclosure of which is herein incorporated by reference.

The compositions of the present invention contain at least one of the double-stranded molecules of the present invention or vectors encoding them (e.g., 0.1 to 90% by weight), or pharmaceutically acceptable salts of the molecules, mixed with a pharmaceutically acceptable carrier. Preferred pharmaceutically acceptable carrier are water, buffered water, normal saline, 0.4% saline, 0.3% glycine, hyaluronic acid and the like.

According to the present invention, the composition may contain plural kinds of the double-stranded molecules, each of the molecules may be directed to different target sequences of EZH2, or target sequences of EZH2 and other genes. For example, the composition may contain double-stranded molecules directed to one, two or more target sequences of EZH2. Alternatively, for example, the composition may contain double-stranded molecules directed to a target sequence of EZH2 and double-stranded molecules directed to target sequences of other genes.

Furthermore, the present composition may contain a vector coding for one or plural
double-stranded molecules. For example, the vector may encode one, two or several kinds of the double-stranded molecules of the present invention. Alternatively, the present composition may contain plural kinds of vectors, each of the vectors coding for a different double-stranded molecule.

Moreover, the double-stranded molecules of the present invention may be contained as liposomes encapsulating the molecules in the present composition. See under the item of "VII. Methods of treating cancer using double-stranded molecules " for details of liposomes.

Compositions of the present invention may also include conventional pharmaceutical excipients and/or additives. Suitable pharmaceutical excipients include stabilizers, antioxidants, osmolality adjusting agents, buffers, and pH adjusting agents. Suitable additives include physiologically biocompatible buffers (e.g., tromethamine hydrochloride), additions of chelants (such as, for example, DTPA or DTPA-bisamide) or calcium chelate complexes (for example calcium DTPA, CaNaDTPA-bisamide), or, optionally, additions of calcium or sodium salts (for example, calcium chloride, calcium ascorbate, calcium gluconate or calcium lactate). Compositions of the present invention can be packaged for use in liquid form, or can be lyophilized.

For solid compositions, conventional nontoxic solid carriers can be used; for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like.

For example, solid compositions for oral administration can include any of carriers and excipients listed above and 10-95%, preferably 25-75%, of one or more double-stranded molecule of the present invention. Compositions for aerosol (inhalational) administration can include 0.01-20% by weight, preferably 1-10% by weight, of one or more double-stranded molecule of the present invention encapsulated in a liposome as described above, and propellant. For intranasal delivery, compositions may include carriers such as lecithin.

In addition to the above, the present composition may contain other pharmaceutical active ingredients so long as they do not inhibit the in vivo function of the double-stranded molecules of the present invention. For example, the composition may contain chemotherapeutic agents conventionally used for treating cancers. The pharmaceutical compositions may also contain other active ingredients such as antimicrobial agents, immunosuppressants or preservatives. Furthermore, it should be understood that, in addition to the ingredients particularly mentioned above, the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question; for example, those suitable for oral administration may include flavoring agents.
In another embodiment, the present invention also provides the use of the double-stranded molecules of the present invention or a vector encoding the double-stranded molecule in manufacturing a pharmaceutical composition for treating a cancer characterized by the expression of EZH2 gene. For example, the present invention relates to a use of double-stranded molecule inhibiting the expression of an EZH2 gene in a cell, which molecule includes a sense strand and an antisense strand complementary thereto, hybridized to each other to form the double-stranded molecule and targets to a nucleotide sequence selected from among SEQ ID NOs: 13 and 16, or a vector encoding the double-stranded molecule for manufacturing a pharmaceutical composition for treating cancer expressing EZH2 gene, such as AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma.

Alternatively, the present invention further provides the double-stranded nucleic acid molecules of the present invention or a vector(s) encoding the double-stranded nucleic acid molecule for use in treating a cancer expressing the EZH2 gene.

Alternatively, the present invention further provides a method or process for manufacturing a pharmaceutical composition for treating cancer characterized by the expression of EZH2 gene, wherein the method or process includes a step for formulating a pharmaceutically or physiologically acceptable carrier with a double-stranded nucleic acid molecule inhibiting the expression of EZH2 gene in a cell, which over-expresses the gene, which molecule includes a sense strand and an antisense strand complementary thereto, hybridized to each other to form the double-stranded molecule and targets to a nucleotide sequence selected from among SEQ ID NOs: 13 and 16 or a vector encoding the double-stranded nucleic acid molecule as active ingredients.

In another embodiment, the present invention also provides a method or process for manufacturing a pharmaceutical composition for treating cancer characterized by the expression of EZH2 gene, wherein the method or process includes a step for admixing an active ingredient with a pharmaceutically or physiologically acceptable carrier, wherein the active ingredient is a double-stranded nucleic acid molecule inhibiting the expression of EZH2 gene in a cell, which over-expresses the gene, which molecule includes a sense strand and an antisense strand complementary thereto, hybridized to each other to form the double-stranded molecule and targets to a nucleotide sequence selected from among SEQ ID NOs: 13 and 16 or a vector encoding the double-stranded nucleic acid molecule.

Hereinafter, the present invention is described in more detail with reference to the Examples. However, the following materials, methods and examples only illustrate aspects of the invention and in no way are intended to limit the scope of the present invention. As such, methods and materials similar or equivalent to those described
herein can be used in the practice or testing of the present invention.

Examples

[0293] Materials and Methods

Bladder tissue samples and RNA preparation.

Bladder tissue sampling and RNA preparation were described previously (Wallard MJ, et al. Br J Cancer. 2006; 94: 569-77.). Briefly, 119 surgical specimens of primary urothelial carcinoma were collected, either at cystectomy or transurethral resection of bladder tumor (TURBT), and snap frozen in liquid nitrogen. 25 specimens of normal bladder urothelial tissue were collected from areas of macroscopically normal bladder urothelium in patients with no evidence of malignancy. Vimentin is primarily expressed in mesenchymally derived cells, and was used as a stromal marker. Uroplakin is a marker of urothelial differentiation and is preserved in up to 90% of epithelially derived tumors (Olsburgh J, et al. The Journal of pathology. 2003; 199: 41-9.). Use of tissues for this study was approved by Cambridge shire Local Research Ethics Committee (Ref 03/018). For immunohistochemistry, the whole tissue section and tissue microarray were purchased from BioChain (Hayward, CA).

[0294] Lung tissue samples for tissue microarray.

Primary non-SCLC (NSCLC) tissue samples, and corresponding normal tissue adjacent to resection margins, were obtained from patients having no anticancer treatment before tumor resection with informed consent (Kato T, et al. Cancer Res. 2005; 65: 5638-46., Kikuchi T, et al. Oncogene. 2003; 22: 2192-205., Taniwaki M, et al. Int J Oncol. 2006; 29: 567-75.). All tumors were staged on the basis of the pathologic tumor-node-metastasis classification of the International Union Against Cancer. Formalin-fixed primary lung tumors and adjacent normal lung tissue samples used for immunostaining on tissue microarrays had been obtained from 292 patients undergoing curative surgery at Saitama Cancer Center (Saitama, Japan) (Ishikawa N, et al. Clin Cancer Res. 2004; 10: 8363-70., Ishikawa N, et al. Cancer Res. 2007; 67: 11601-11.). To be eligible for this study, tumor samples were selected from patients who fulfilled all of the following criteria: (a) patients suffered primary NSCLC with a histologically confirmed stage (only pT1 to pT3, pNO to pN2, and pMO); (b) patients had undergone curative surgery, but did not receive any preoperative treatment; (c) among them, NSCLC patients with positive lymph node metastasis (pN1, pN2) had been treated with platinum-based adjuvant chemotherapies after surgical resection, whereas patients with pNO did not receive adjuvant chemotherapies; and (d) clinical follow-up data were available. This study and the use of all clinical materials mentioned were approved by the relevant institutional ethics committees.

[0295] Colorectal tissue samples for immunohistochemistry.
For Japanese cases, 172 primary human colorectal adenocarcinomas were studied by immunohistochemistry. All tumors were obtained from patients who had undergone endoscopic resection or surgery at Nagasaki University Hospital, Japan between 2000 and 2004. Of the 172 patients with colorectal carcinoma, there were 91 men and 81 women. Median age was 63.5 years (range, 16-90). Each tumor was assigned a histological type according to the World Health Organisation classification: well differentiated adenocarcinoma, moderately differentiated adenocarcinoma, poorly differentiated adenocarcinoma, and mucinous adenocarcinoma (Kusaba T, et al. J Clin Pathol. 2005; 58: 833-8).

Quantitative real-time PCR.

Specific primers for human GAPDH (housekeeping gene) and EZH2 were designed (primer sequences in Table 1). PCR reactions were performed using the LightCycler™ 480 System (Roche Applied Science, Mannheim, Germany) following the manufacturer's protocol.

<table>
<thead>
<tr>
<th>Primer sequences for quantitative RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene name</strong></td>
</tr>
<tr>
<td><strong>GAPDH (housekeeping gene) - f</strong></td>
</tr>
<tr>
<td><strong>GAPDH (housekeeping gene) - r</strong></td>
</tr>
<tr>
<td><strong>β2M (housekeeping gene) - f</strong></td>
</tr>
<tr>
<td><strong>β2M (housekeeping gene) - r</strong></td>
</tr>
<tr>
<td><strong>EZH2 - f</strong></td>
</tr>
<tr>
<td><strong>EZH2 - r</strong></td>
</tr>
</tbody>
</table>

siRNA transfection and cell growth assay.

siRNA oligonucleotide duplexes were purchased from SIGMA Genosys for targeting the human EZH2 transcripts. siEGFP and siNegative control (siNC, B-Bridge), which consists of three different oligonucleotide duplexes, were used as control siRNAs. The siRNA sequences are described in Table 2. siRNA duplexes (100 nM final concentration) were transfected into lung and bladder cancer cell lines with Lipofectamine 2000 (Invitrogen) for 72 h, and cell growth was examined using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) (Hamamoto R, et al. Nat Cell Biol. 2004; 6: 731-40.).
### Table 2

<table>
<thead>
<tr>
<th>siRNA name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>siEGFP</td>
<td>Sense: 5' GCAGCACGACUUCUUCAAG 3' (SEQ ID NO: 23)</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5' CUUGAAGAAGUGUCUGCGUC 3' (SEQ ID NO: 24)</td>
</tr>
<tr>
<td></td>
<td>Sense: 5' AUCCGCGCGUAUAGACU 3' (SEQ ID NO: 25)</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5' UACGUACUAUGGCGGUAU 3' (SEQ ID NO: 26)</td>
</tr>
<tr>
<td>iNegative control</td>
<td>Sense: 5' CGUAAUACGCUAGCGGUAA 3' (SEQ ID NO: 28)</td>
</tr>
<tr>
<td>(Cocktail)</td>
<td>Antisense: 5' AUAUCGCGCGUAUAGCGGU 3' (SEQ ID NO: 29)</td>
</tr>
<tr>
<td>Target#1</td>
<td>Antisense: 5' ACCGUUAUAGGCGCAUAA 3' (SEQ ID NO: 30)</td>
</tr>
<tr>
<td>Target#2</td>
<td>Sense: 5' CUAAACAUUUUAACACUA 3' (SEQ ID NO: 11)</td>
</tr>
<tr>
<td>Target#3</td>
<td>Antisense: 5' UAGUUGUAAACUGUAGGUG 3' (SEQ ID NO: 12)</td>
</tr>
<tr>
<td></td>
<td>Target: 5' CTAAACCATTGTATTACAATA 3' (SEQ ID NO: 13)</td>
</tr>
<tr>
<td>siEZH2#1</td>
<td>Sense: 5' GACAAGAGGGGAAAGUGU 3' (SEQ ID NO: 14)</td>
</tr>
<tr>
<td>siEZH2#2</td>
<td>Antisense: 5' ACACUUUUCUUUUCUGUC 3' (SEQ ID NO: 15)</td>
</tr>
<tr>
<td></td>
<td>Target: 5' ACACCTTTCCCTTCTTGTCA 3' (SEQ ID NO: 16)</td>
</tr>
</tbody>
</table>

[0300] Western blot analysis.

Whole cell lysates were prepared from the cells with RIPA-like buffer, and total protein (10 micro-g) was transferred to nitrocellulose membrane (Cho HS, et al. Cancer Res. 2011; 71: 1-6.). The membrane was probed with anti-EZH2 antibody (141.2, Santa Cruz Biotechnology, Santa Cruz, CA). ACTB (1-19, Santa Cruz Biotechnology) was used to ensure equal loading and transfer of proteins. Protein bands were detected by incubating with horseradish peroxidase-conjugated antibodies (GE Healthcare, Little Chalfont, UK) and visualizing with Enhanced Chemiluminescence (GE Healthcare).

[0301] Immunohistochemical staining and tissue microarray.

for sampling was selected based on visual alignment with the corresponding H&E-stained section on a slide. Three, four or five tissue cores (diameter, 0.6 mm; depth, 3–4 mm) taken from a donor tumor block were placed into a recipient paraffin block with a tissue microarrayer (Beecher Instruments, Sun Prairie, WI). A core of normal tissue was punched from each case, and 5-micro-m sections of the resulting microarray block were used for immunohistochemical analysis. Three independent investigators semiquantitatively assessed EZH2 positivity without prior knowledge of clinicopathologic data. Because the intensity of staining within each tumor tissue core was mostly homogeneous, the intensity of EZH2 staining was semiquantitatively evaluated using the following criteria: negative (no appreciable staining in tumor cells) and positive (brown staining appreciable in the nucleus of tumor cells). Cases were accepted as positive only if all reviewers independently defined them as such.

[0302] Statistical analysis.

Student’s t-test or Mann-Whitney’s U-test was used to analyze the difference between two independent subgroups. Survival curves were calculated from the date of surgery to either the time of death relating to only cancer, or to the last follow-up observation. Kaplan-Meier curves were calculated for each relevant variable and for EZH2 expression; differences in survival times among patient subgroups were analyzed using the log-rank test. Univariate and multivariate analyses were done with the Cox proportional hazard regression model to determine associations between clinicopathological variables and cancer-related mortality. First, associations between death and possible prognostic factors including age, gender, histology, pT classification and pN classification were analyzed, taking into consideration one factor at a time. Second, multivariate Cox analysis was applied on backward (stepwise) procedures that always forced strong EZH2 expression into the model, along with any and all variables that satisfied an entry level of P < 0.05. As the model continued to add factors, independent factors did not exceed an exit level of P < 0.05.

[0303] Results

EZH2 expression is up-regulated in clinical bladder cancer tissues in both British and Japanese cases yet is hardly detectable in normal tissues.

By examining the level of histone lysine methyltransferase gene products in a small subset of British clinical bladder cancer samples, we found significant overexpression of EZH2 in the cancer samples compared with non-cancerous samples (data not shown). So, 119 bladder cancer samples and 25 normal control samples (British) were analyzed, and a significant elevation of EZH2 expression levels in tumor cells compared with normal cells was confirmed (Fig. 1A; P < 0.0001, Mann-Whitney U test). Importantly, quantitative real-time PCR analysis showed that expression levels of EZH2 in bladder tumor tissues are notably higher than those in any other normal
tissues (Fig.1C). Subclassification of tumors according to tumor grade and stage identified no significant difference in their expression levels (Fig. 8). Then, the expression patterns of EZH2 were analyzed in a number of clinical samples derived from Japanese bladder cancer subjects by cDNA microarray (Fig. IB), and significant over-expression was identified again (P < 0.0001, Mann-Whitney U test). To evaluate protein expression levels of EZH2 in bladder tissues, immunohistochemical analysis using an anti-EZH2 antibody was performed, and strong staining was observed in the nucleus of malignant cells, but weak or absent staining in non-neoplastic tissues (Fig. ID, E). Tissue microarray analysis indicated that EZH2 was positively stained in 14 out of 29 bladder cancer cases (48.3%), whereas no staining was observed in normal bladder tissues (Table 3). Subclassification of tumors according to tumor grade identified Grade III tumors showed significantly higher EZH2 protein expression than Grade I and Grade II tumors, implying that EZH2 expression is more elevated in advanced bladder tumors (Table 3). Importantly, specific EZH2 signals were not detected in normal heart, lung, liver, colon, kidney and bladder tissues (Fig. IF), consistent with the real-time PCR result.
**Clinicopathological characteristics of bladder tissues on the tissue microarray**

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Age</th>
<th>Sex</th>
<th>Histology</th>
<th>Grade</th>
<th>Stage (TNM)</th>
<th>EZH2 expression**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>71</td>
<td>M</td>
<td>Normal</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>59</td>
<td>M</td>
<td>Normal</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>65</td>
<td>M</td>
<td>Chronic cystitis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>51</td>
<td>F</td>
<td>Chronic cystitis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>71</td>
<td>M</td>
<td>Squamous cell carcinoma</td>
<td>I</td>
<td>T1N0M0</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>M</td>
<td>Squamous cell carcinoma</td>
<td>I</td>
<td>T2NOM0</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>76</td>
<td>M</td>
<td>Adenocarcinoma</td>
<td>II</td>
<td>T2NOM0</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>M</td>
<td>Adenocarcinoma</td>
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<tr>
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<tr>
<td>11</td>
<td>27</td>
<td>M</td>
<td>Transitional cell carcinoma</td>
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<td>TisNOMO</td>
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<tr>
<td>12</td>
<td>50</td>
<td>M</td>
<td>Transitional cell carcinoma</td>
<td>I</td>
<td>T1N0M0</td>
<td>+</td>
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<tr>
<td>13</td>
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<td>F</td>
<td>Transitional cell carcinoma</td>
<td>I</td>
<td>T1N0M0</td>
<td>+</td>
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<td>Transitional cell carcinoma</td>
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*All tissue samples were purchased from BioChain|

**(-) negative expression ; (+) low expression, (++) high expression**

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**Clinicopathological characteristics of colorectal tissues on the tissue microarray**

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EZH2 expression is significantly high in lung cancer tissues, and correlates with poor prognosis in NSCLC.

In addition to bladder tissues, expression levels of EZH2 in lung tissues were measured. cDNA microarray experiments showed that EZH2 expression was similarly elevated in lung tumor tissues compared with corresponding non-neoplastic tissues (Fig. 2A; P < 0.0001, Mann-Whitney U test). Then, EZH2 protein expression levels in lung tissue were examined by immunohistochemistry and strong EZH2 staining was observed in cancer tissues but no significant staining in non-neoplastic tissues (Fig. 2B). To analyze the significance of EZH2 protein expression in lung cancer tissues in
more detail, immunohistochemical analysis was conducted on a tissue microarray containing tissue sections from 292 NSCLC patients, who had undergone surgical resection. EZH2 stained positively in 135 out of 292 cases (46.2%) and negatively in 157 cases (53.8%; Fig. 2C,D). Subsequently, the association of EZH2 expression with clinical outcomes was analyzed, and it was found that expression of EZH2 in NSCLC patients was significantly associated with male gender (p = 0.0002, Fisher’s exact test; Table 5), non-ADC histology (p < 0.0001), smoking status (smoker; p < 0.0001), pN factor (N1 + N2; p = 0.0269) and tumor-specific 5-year survival after the resection of primary tumors (p = 0.0239 by log-rank test; Fig. 2D). Univariate analysis revealed associations between poor prognosis in patients with NSCLC and several factors including EZH2 expression, age, gender, histologic type (non-ADC versus ADC), pT stage (tumor size, T1 versus T2 + T3) and pN stage (node status, N0 versus N1+N2, Table 6). On the contrary, multivariate analysis revealed that EZH2 status did not show statistical significance as an independent prognostic factor for surgically treated NSCLC patients enrolled in this study, whereas age, pT and pN factors did (Table 6). This result might be due to the preponderance of EZH2 expression up to the pN factor.
# Table 5

## Association between EZH2-positive in NSCLCs and characteristics of patients

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### Smoking status

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ADC, adenocarcinoma; SCC, squamous-cell carcinoma; NS, not significant
Others, large-cell carcinoma (LCC) plus adenosquamous-cell carcinoma (ASC)
*P < 0.05 (Fisher's exact test)
** ADC versus non-ADC
The association between EZH2 overexpression and clinical outcomes in colorectal cancer.

Then, the expression profile of EZH2 in normal and cancer cell lines was examined. Quantitative real-time PCR analysis revealed that EZH2 expression levels in cancer cells were significantly higher than those in normal human cells, and intriguingly, expression levels of EZH2 in colorectal cancer cell lines are remarkably high (Fig. 3A). Therefore, EZH2 expression levels in colorectal clinical tissues were examined by immunohistochemistry and it was observed that elevated expression of EZH2 was restricted to cancer tissues (Fig. 3B). Tissue microarray analysis showed that overexpression of EZH2 protein was observed in 55 out of 72 colorectal carcinoma cases (76.4%; Table 4). To clarify the association between EZH2 overexpression and clinical outcomes in colorectal cancer, additional immunohistochemical analysis using a number of Japanese cases, which had been operated at Nagasaki University Hospital, was performed (Fig. 3C, D). EZH2 stained positively in 158 out of 172 cases (91.4%) and negatively in 14 cases (8.6%; Table 8). Next, the association of EZH2 expression with clinical outcomes was examined, and it was found that expression of EZH2 in colorectal cancer patients was significantly associated with P-factor negative cases (P = 0.019, Fisher's exact test; Table 8). M-factor negative (P = 0.011) and also tumor-specific 5-year survival after resection of primary tumors (P = 0.014 by log-rank test;
Figure 3D). Univariate analysis of associations between patient prognosis and several factors including EZH2 expression, age, gender, serum CEA level, serum CA19-9 level, pT factor, pM factor and M factor. All those factors except age and gender were associated with poor prognosis (Table 9). In addition, multivariate analysis revealed that EZH2 status and M factor for colorectal cancer did show statistical significance as an independent prognostic factor for the surgically treated colorectal cancer patients enrolled in this study. These results imply that EZH2 protein is markedly over-expressed at the protein level in colorectal cancer, and EZH2 overexpression indicates a good prognosis, in contrast to the poor prognosis associated with EZH2 overexpression in lung cancer.

[Table 7] Expression profile of EZH2 analyzed by cDNA microarray*

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<th>Ratio (Tumor / Normal)</th>
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<td>CML</td>
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</table>

*The signal intensity of EZH2 in tumor tissues was compared with corresponding nonneoplastic tissues derived from the same patient.
### Association between EZH2-positivity in colorectal cancer and patients' characteristics (n = 172)

<table>
<thead>
<tr>
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<th>P value positive vs negative</th>
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NS, not significant

*P < 0.05 (Fisher's exact test)
EZH2 is required for cancer cell proliferation.

In order to examine whether elevated expression of EZH2 plays a critical role in the proliferation of cancer cells, two independent siRNA oligonucleotide duplexes to specifically suppress the expression of EZH2 (siEZH2#1, #2) were prepared, and each of them was transfected into cancer cells. Knockdown of EZH2 in cancer cells was confirmed by real-time PCR and Western blotting as shown (Fig. 4A). Cell growth assays were performed on cancer cells transfected with these siRNAs, and significant growth suppression was observed in two bladder cancer cell lines (SW780 and RT4), two lung cancer cell lines (A549 and SBC5) and one colorectal cancer cell lines (HCT116) (Fig. 4B). When these EZH2 siRNAs were transfected into the normal cell line CCD-18C0 cells expressing undetectable levels of EZH2 (Fig. 9), they had no effect on CCD-18C0 cells (Fig. 4B). This implies that suppression of cancer cell growth by treatment with these specific siRNAs does not deploy a mechanism used by normal cells, and thus directly acts on a growth mechanism specific to cancer cells. To further assess the mechanism of growth suppression induced by the siRNA, BrdU and 7-AAD staining were performed and cell cycle status was examined (Fig. 4C). The proportion of cancer cells at the G1 phase was significantly higher in the cells treated with siEZH2 than those treated with control siRNAs, while that at the S phase was remarkably decreased (Fig. 4C). The data revealed that EZH2 plays a crucial role in

---

**Table 9**

Cox’s proportional hazards model analysis of prognostic factors in patients with colorectal cancer

<table>
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*P < 0.05 (Fisher’s exact test)
Gl/S transition of cancer cells.

[0315] EZH2 methylates histone H2BK120.

To clarify the functions of EZH2 in human carcinogenesis in more detail, in vitro methyltransferase assays using various candidate substrates were conducted and it was finally found that EZH2 can methylate histone H2B (Fig. 5A). Next, LC-MS/MS analysis of histone H2B after in vitro methyltransferase reaction was performed and it was found that lysine 120 on histone H2B is mono-methylated by EZH2 (Fig. 5B, C). Then, an anti-mono-methylated rabbit polyclonal antibody was generated using a K120-methylated peptide as an antigen. According to the Western blot analysis to examine the quality of the antibody (Fig. 5D), the specific signal recognized by the antibody was increased in a dose-dependent manner of EZH2 and the signal was clearly diminished when using lysine 120-substituted histone H2B as a substrate. This result implied that the antibody specifically recognized mono-methylated lysine 120 on histone H2B. In order to examine the methylation status of H2BK120 in cells, immunocytochemical analysis of HeLa cells 24 h after transfection with an EZH2 expression vector was performed. As shown in Fig. 5E, the cells overexpressing EZH2 also showed high methylation levels of histone H2B, indicating that EZH2 methylates lysine 120 of histone H2B in vivo. Taken together, it was found that lysine 120 of histone H2B, which is the novel substrate of EZH2, was mono-methylated both in vitro and in vivo. Importantly, methylation levels of H2BK120 in cancer cells were higher than those in non-cancerous cells (Fig. 6), and methylation status is likely to be correlated with expression levels of EZH2.

[0316] As histone H2B at lysine 120 was reported to be ubiquitinated and the ubiquitination enhanced the transcription through the methylation of histone H3 at lysine 4 (Kim J, et al. Cell. 2009; 137: 459-71.), next the relationship between methylation and ubiquitination of lysine 120 on histone H2B. Interestingly, ubiquitination levels of histone H2B at lysine 120 in SBC5 cells were significantly increased after treatment with siEZH2 (Fig. 7A), implying that methylation may antagonize ubiquitination of lysine 120 on histone H2B. Consistent with this, immunocytochemical analysis revealed that HeLa cells overexpressing EZH2 dominantly reduced the ubiquitination of H2BK120 (Fig 7B). These data indicate that EZH2-dependent methylation of histone H2B at lysine 120 competitively antagonizes the ubiquitination of H2BK120.

[0317] Discussion

EZH2 is a polycomb group (PcG) protein homologous to Drosophila enhancer of zeste, a histone methyltransferase associated with transcriptional repression. EZH2 has a SET domain that is typical of histone methyltransferases, and catalyses the addition of methyl groups to histone H3 at lysine 27 (H3K27). This Polycomb group protein is conserved during evolution and is required for early mouse development (O'Carroll D,
EZH2 acts as the catalytic subunit of the PRC2 core complex, which also contains the two PcG proteins SUZ12 and EED. The PRC2 complex is responsible for repressing the large number of genes which are essential for development and differentiation (Boyer LA, et al. Nature. 2006; 441: 349-53., Bracken AP, et al., Genes Dev. 2006; 20: 1123-36., Lee TI, et al. Cell. 2006; 125: 301-13.). PcG proteins specify positional information such as antero-posterior patterning, through activating or repressing the stable state of Hox gene expression (Gould A. Curr Opin Genet Dev. 1997; 7: 488-94., Simon JA, Tamkun JW. Curr Opin Genet Dev. 2002; 12: 210-8.). In addition to these well-established functions in embryonic development, a series of studies suggest that PcG proteins may influence both Hox-dependent and independent downstream pathways that control cell proliferation. The complex has been reported to interact with retinoblastoma (Rb) protein, resulting in cell cycle alteration. (Dahiya A, et al., Mol Cell. 2001; 8: 557-69.) Moreover, a deficiency in PcG group gene bmi-1 resulted in a marked increase in tumor suppressors p16INK4a and p19ARF levels, and impaired progression into the S phase of the cell cycle (Issaeva I, et al. Biol. 2007; 27: 1889-903.). In this study, we found that EZH2 can play a crucial role in the growth regulation of cancer cells, especially at the G1/S transition.

Cancer-related death is on the rise in most countries. While molecular-targeting agents such as cetuximab and bevacizumab have been developed and proven to be efficacious, their adverse effects and limited application for some patients make it important to continue to search for novel molecular-targeting agents (Hurwitz H, et al. N Engl J Med. 2004; 350: 2335-42., Pirker R, et al. Lancet. 2009; 373: 1525-31., Sandler A, et al. N Engl J Med. 2006; 355: 2542-50.). Recent tumor genomics data can be used to develop rationally selected drugs that target proteins expressed exclusively or at particularly high levels in tumor compared with essential normal adult cells. Specific pharmaceutical targeting of such proteins may result in a new generation of highly active drugs having minimal collateral host toxicity (Gautschi O, et al., Clin Cancer Res. 2008; 14: 1639-48.). In this study, it was demonstrated that in various cancer tissues, expression levels of EZH2 were significantly high at both RNA and protein levels whereas EZH2 expression in various normal tissues was hardly detected (see also Supporting Information Fig. 10 and Table 10). Indeed, other researchers reported the involvement of EZH2 in progression of human cancer as well (Varambally S, et al. Nature. 2002; 419: 624-9.). Although knockdown of EZH2 expression by specific siRNAs significantly suppressed the growth of cancer cells, no growth suppression was observed in the normal cell CCD-I8C0 cells. Actually, development of methyltransferase inhibitors has started recently (Ragno R, Simeoni S, et al. J Med Chem. 2007; 50: 1241-53., Spannhoff A, et al. J Med Chem. 2007; 50:
Among all types of cancer, lung cancer is the leading cause of death from cancer in both the United States and Japan, with the median survival of advanced NSCLC patients treated with standard chemotherapy only 8 months (Jemal A, et al. Cancer statistics, 2008. CA Cancer J Clin. 2008; 58: 71-96., Sawabata N, et al. Nihon Kokyuki Gakkai Zasshi. 48: 333-44.). The data demonstrated herein, demonstrates that EZH2 could also be a good marker, enabling us to predict the prognosis of NSCLC patients; and to conduct a more intensive follow-up according to EZH2 expression status of resected specimens. In contrast, EZH2 overexpression indicated a good prognosis in colorectal cancer. In this case, EZH2 was overexpressed in the majority of cancer cases (91.4%), and the number of EZH2-negative cases was small (n = 14). Therefore, while indicative of cancer it appears to be hard to use EZH2 expression as a prognostic marker in colorectal cancer. It is also possible that EZH2 has a different function in these two cancer tissues. For instance, most of the colorectal cancers are adenocarcinomas, and highly-differentiated and moderately-differentiated adenocarcinomas dominate around 90% of all colorectal cancer cases (Akasu T, et al. Ann Surg Oncol. 2008; 15: 2668-76., Ponz de Leon M, Di Gregorio C. Dig Liver Dis. 2001; 33: 372-88.). Indeed, this ratio is similar to the positive ratio of EZH2 expression in this study. There is the possibility that overexpressed EZH2 might be a factor to maintain the differentiation of tumor tissues, and in consequence, the depletion of EZH2 expression could cause increased malignancy and a bad outcome for cancer patients. Large-scale validation of the present results, and further functional analyses of this protein in human carcinogenesis, may assist in comprehending the various modes of EZH2 expression in different cancer types, and develop anti-cancer therapy targeting
EZH2 with due attention.

Industrial Applicability

[0320] The gene-expression analysis of cancers described herein using the genome-wide cDNA microarray has identified specific genes as a target for cancer prevention and therapy. Based on the differentially expression of EZH2 gene, the present invention provides a molecular diagnostic marker for diagnosing or detecting cancers as well as assessing the prognosis, in particular, AML, bladder cancer, breast cancer cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma and renal cell carcinoma.

[0321] The data provided herein add to a comprehensive understanding of cancers, facilitate development of novel diagnostic strategies, and provide clues for identification of a molecular target for therapeutic drugs and preventative agents. Such information contributes to a more profound understanding of tumorigenesis, and provides indicators for developing novel strategies for diagnosis, treatment, and ultimately prevention of cancers.

As demonstrated herein, cell growth is suppressed by double-stranded molecules that specifically target the EZH2 gene. Thus, these novel double-stranded molecules are useful as anti-cancer pharmaceuticals.

[0322] The expression of the EZH2 gene is markedly elevated in cancer, specifically AML, bladder cancer, breast cancer cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma and renal cell carcinoma, as compared to normal organs. Accordingly, this gene can be conveniently used as a diagnostic marker for cancer, in particular, AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma and renal cell carcinoma, and the proteins encoded thereby find utility in diagnostic assays for cancer.

Furthermore, the methods described herein are also useful in diagnosis of cancer, including AML, bladder cancer, breast cancer cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma and renal cell carcinoma.

Moreover, the present invention provides new therapeutic approaches for treating cancer including AML, bladder cancer, breast cancer cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma and renal cell carcinoma. The EZH2 gene is a useful target for the development of anti-cancer pharmaceuticals.

[0323] All patents, patent applications, and publications cited herein are incorporated by reference in their entirety.

Furthermore, while the invention has been described in detail and with reference to specific embodiments thereof, it is to be understood that the foregoing description is exemplary and explanatory in nature and is intended to illustrate the invention and its
preferred embodiments. Through routine experimentation, one skilled in the art will readily recognize that various changes and modifications can be made therein without departing from the spirit and scope of the invention. Thus, the invention is intended to be defined not by the above description, but by the following claims and their equivalents.


Claims

[Claim 1] A method for detecting or diagnosing cancer or a predisposition for developing the cancer in a subject, comprising a step of determining an expression level of an EZH2 gene in a subject-derived biological sample, wherein an increase in said expression level as compared to a normal control level of said gene indicates that said subject suffers from or is at a risk of developing cancer, wherein said expression level is determined by any method selected from a group consisting of:
(a) detecting an mRNA of an EZH2 gene;
(b) detecting a protein encoded by an EZH2 gene; and
(c) detecting a biological activity of a protein encoded by an EZH2 gene.

[Claim 2] The method of claim 1, wherein said expression level is at least 10% greater than the normal control level.

[Claim 3] The method of claim 1 or 2, wherein the subject-derived biological sample is a biopsy specimen, saliva, sputum, blood, serum, plasma, pleural effusion or urine sample.

[Claim 4] The method of any one of claims 1 to 3, wherein the biological activity is cell proliferative activity or histone methyltransferase activity.

[Claim 5] A method for assessing prognosis of a subject with cancer, wherein the method comprises steps of:
(a) detecting an expression level of EZH2 gene in a subject-derived biological sample;
(b) comparing the detected expression level to a control level; and
(c) determining prognosis of the subject based on the comparison of (b).

[Claim 6] The method of claim 5, wherein the control level is a good prognosis control level and an increase of the expression level compared to the control level indicates poor prognosis when the cancer is lung cancer.

[Claim 7] The method of claim 5, wherein the control level is a poor prognosis control level and an increase of the expression level compared to the control level indicates good prognosis when the cancer is colorectal cancer.

[Claim 8] The method of claim 6 or 7, wherein the increase is at least 10% greater than said control level.

[Claim 9] A method of screening for a candidate substance for either or both of treating and preventing cancer, wherein said method comprises steps...
of:
(a) contacting a test substance with an EZH2 polypeptide or functional equivalent thereof;
(b) detecting binding between the EZH2 polypeptide or functional equivalent and the test substance; and
(c) selecting a test substance that binds to the EZH2 polypeptide or functional equivalent as a candidate substance for either or both of treating and preventing cancer.

[Claim 10] A method of screening for a candidate substance for either or both of treating and preventing cancer, wherein said method comprises steps of:
(a) contacting a test substance with an EZH2 polypeptide or functional equivalent thereof;
(b) detecting a biological activity of the EZH2 polypeptide or functional equivalent;
(c) comparing the biological activity of the EZH2 polypeptide or functional equivalent with the biological activity detected in the absence of the test substance; and
(d) selecting a test substance that suppresses the biological activity of the EZH2 polypeptide or functional equivalent as a candidate substance for either or both of treating and preventing cancer.

[Claim 11] The method of claim 10, wherein the biological activity is cell proliferative activity or methyltransferase activity.

[Claim 12] A method of screening for a candidate substance for either or both of treating and preventing cancer, which comprises steps of:
(a) contacting a test substance with a cell expressing an EZH2 gene;
(b) detecting expression level of the EZH2 gene;
(c) comparing the expression level with the expression level detected in the absence of the test substance; and
(d) selecting a test substance that reduces the expression level of the EZH2 gene as a candidate substance for either or both of treating and preventing cancer.

[Claim 13] A method of screening for a candidate substance for either or both of treating and preventing cancer, wherein said method comprises steps of:
(a) contacting a test substance with a cell introduced with a vector that comprises a transcriptional regulatory region of an EZH2 gene and a reporter gene expressed under control of the transcriptional regulatory
region;
(b) measuring an expression level or activity of said reporter gene;
(c) comparing the expression level or activity with the expression level or activity detected in the absence of the test substance; and
(d) selecting a test substance that reduces the expression level or activity as a candidate substance for treating and/or preventing cancer.

[Claim 14] A double-stranded molecule that, when introduced into a cell expressing an EZH2 gene, inhibits expression of the gene, wherein the double-stranded molecule comprises a sense strand and an antisense strand, wherein the sense strand comprises a nucleotide sequence corresponding to a target sequence selected from the group consisting of SEQ ID NOs: 13 and 16, and the antisense strand comprises a nucleotide sequence complementary to the target sequence of the sense strand so that the sense and antisense strands hybridize to each other to form the double-stranded molecule.

[Claim 15] The double-stranded molecule of claim 14, wherein the sense strand hybridizes with antisense strand at the target sequence to form the double-stranded molecule having between 19 and 25 nucleotide pair in length.

[Claim 16] The double-stranded molecule of claim 14 or 15, wherein said double-stranded molecule is a single polynucleotide construct comprising the sense strand and the antisense strand linked via a single-strand.

[Claim 17] The double-stranded molecule of claim 16, which has a general formula 5'-[A]-[B]-[A']-3' or 5'-[A']-[B]-[A]-3', wherein [A] is a sense strand comprising a nucleotide sequence corresponding to a target sequence selected from the group consisting of SEQ ID NO: 13 and 16, [B] is a single-strand and consists of 3 to 23 nucleotides, and [A'] is an antisense strand comprising a nucleotide sequence complementary to the target sequence of [A].

[Claim 18] A vector encoding the double-stranded molecule of any one of claims 14 to 17.

[Claim 19] Vectors comprising each of a combination of polynucleotide comprising a sense strand nucleic acid and an antisense strand nucleic acid, wherein said sense strand nucleic acid comprises a nucleotide sequence corresponding to SEQ ID NO: 13 or 16, and said antisense strand nucleic acid consists of a sequence complementary to the sense strand, wherein the transcripts of said sense strand and said antisense strand hybridize to each other to form a double-stranded molecule, and
wherein said vectors, when introduced into a cell expressing EZH2 gene, inhibit the cell proliferation.

[Claim 20] A method of either of both of treating and preventing cancer in a subject, comprising administering to said subject a pharmaceutically effective amount of a double-stranded molecule against an EZH2 gene or a vector encoding said double-stranded molecule, wherein the double-stranded molecule, when introduced into a cell expressing EZH2 gene, inhibits the expression of the EZH2 gene.

[Claim 21] The method of claim 20, wherein the double-stranded molecule is that of any one of claims 14 to 17.

[Claim 22] The method of claim 21, wherein the vector is that of claim 18 or 19.

[Claim 23] The method of any one of claims 1 to 4, 9 to 13, 20 and 22, wherein the cancer is selected from the group consisting of AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma.

[Claim 24] A composition for either or both of treating and preventing cancer, which comprises a pharmaceutically effective amount of a double-stranded molecule against an EZH2 gene or a vector encoding said double-stranded molecule, when introduced into a cell expressing EZH2 gene, inhibits the expression of the EZH2 gene, and a pharmaceutically acceptable carrier.

[Claim 25] The composition of claim 24, wherein the double-stranded molecule is that of any one of claims 14 to 17.

[Claim 26] The composition of claim 25, wherein the vector is that of claim 18 or 19.

[Claim 27] The composition of any one of claims 24 to 26, wherein the cancer is selected from the group consisting of AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma.

[Claim 28] A kit for diagnosing or detecting cancer, or assessing or determining prognosis of a subject with cancer comprising a reagent for detecting a transcription or translation product of an EZH2 gene.

[Claim 29] The kit of claim 28, wherein the reagent comprises a nucleic acid that binds to a transcription product of the EZH2 gene or an antibody that binds to a translation product of the EZH2 gene.

[Claim 30] A reagent for diagnosing or detecting cancer, or assessing or determining prognosis of a subject with cancer, comprising a nucleic acid that binds to a transcription product of an EZH2 gene or an antibody
that binds to a translation product of the EZH2 gene.

[Claim 31] The kit of claim 28 or 29, or the reagent of claim 30, wherein the cancer is selected from the group consisting of AML, bladder cancer, breast cancer, cholangiocellular carcinoma, CML, esophageal cancer, lung cancer, osteosarcoma, colorectal cancer and renal cell carcinoma.
Fig. 1A-C

A

Median value
1. Normal: 0.034
2. Tumor: 0.444
Ratio (T/N) = 13.06

British case

Relative EZH2 expression

U = 158.0
Z = -7.012
P < 0.0001

Normal bladder (n = 25)
Bladder tumor (n = 119)

B

Japanese case

Signal intensity of EZH2 [x 10^3]

U = 2.0
Z = -7.065
P < 0.0001

Normal bladder (n = 34)
Bladder tumor (n = 34)

C

Relative EZH2 expression levels

Heart, Liver, Lung, Kidney, BN-1A, BN-1B, BN-12A, BT-23, BT-50, BT-53, BT-72, BT-90

Normal tissue
Bladder tumor
[Fig. 1D-F]

**D**  
**Bladder cancer**  
(transitional cell carcinoma)  
61/M, TCC, T2N0M0, Grade III  

Normal bladder  

![Image of normal bladder at x100 and x200 magnification.]

**E**  
**Adenocarcinoma**  
50/M, T2N0M0, Grade II  

**Squamous cell carcinoma**  
71/M, T1N0M0, Grade I  

** Transitional cell carcinoma**  
61/M, TCC, T1N0M0, Grade III  

![Image showing different types of carcinoma at x100 and x200 magnification.]

**F**  
**Heart**  

**Lung**  

**Liver**  

**Colon**  

**Kidney**  

**Bladder**  

![Images of different organs at x100 magnification.]
**Fig. 2A-C**

**A**

Lung cancer

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- **U = 8**
- **Z = 4.334**
- **P < 0.0001**

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**B**

- Lung squamous cell carcinoma
- Lung adenocarcinoma
- Normal lung

**C**

- EZH2 positive
- EZH2 negative
- Normal lung
[Fig. 2D]

Lung cancer mortality

Survival rate (%)

EZH2 negative
(n=157)

EZH2 positive
(n=135)

P = 0.0239

Postoperative days

0 500 1000 1500 2000 2500 3000 3500
A

![Graph showing relative EZH2 expression in various cell lines and cancer types.]

B

**Colorectal cancer (adenocarcinoma)**

![Microscopic images of colorectal cancer and normal colon with magnifications x100 and x400.].

**Normal colon**
**C**

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<th>EZH2 positive</th>
<th>EZH2 negative</th>
<th>Normal colon</th>
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×100

×200

**D**

Colorectal cancer mortality

Survival rate (×100%)

- EZH2 positive (n=158)
- EZH2 negative (n=14)

\[ P = 0.014 \]

Postoperative days
[Fig. 4C-D]

C

A549

SW780

Percentage of cells

![Graph showing cell cycle distribution for A549 and SW780 cells with siEGFP, siNC, and siEZH2 treatments.]

D

A549-siEGFP

A549-siEZH2

BrdU

DNA content

![Graphs showing BrdU incorporation and DNA content for A549 cells with siEGFP and siEZH2 treatments.]

SW780-siEGFP

SW780-siEZH2

BrdU

DNA content

![Graphs showing BrdU incorporation and DNA content for SW780 cells with siEGFP and siEZH2 treatments.]
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### Graphs

- **RMS error 892 ppm**
  - **Mass (Da)**
  - **Error (Da)**

- **RMS error 892 ppm**
  - **Mass (Da)**
  - **Error (Da)**
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**IB:**
- H2BK120Me1B
- H2B
- EZH2
- ACTB
[Fig. 8]

A

![Box plot of relative EZH2 expression across different stages of bladder cancer: Normal (n = 25), pTa + pT1 (n = 83), pT2 (n = 26), pT3 + pT4 (n = 7). The NS (not significant) label indicates the comparison between Normal and pTa + pT1 stages.](image)

B

![Box plot of relative EZH2 expression across different grades of bladder cancer: Normal (n = 25), G1 (n = 11), G2 (n = 58), G3 (n = 49). The NS (not significant) label indicates the comparison between Normal and G1 stages.](image)

[Fig. 9]

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### INTERNATIONAL SEARCH REPORT

**International application No.**

PCT/JP2012/002721

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**A. CLASSIFICATION OF SUBJECT MATTER**

Int.Cl. C12Q1 / 68 (2006.01)i, A61K3 / 1 / 7105 (2006.01)i, A61K4 / 8 / 00 (2006.01)i, C12N / 5 / 09 (2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

---

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl. C12Q1 / 68, A61K3 / 1 / 7105, A61K4 / 8 / 00, C12N / 5 / 09

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

- Published examined utility model applications of Japan 1922-1996
- Published unexamined utility model applications of Japan 1971-2012
- Registered utility model specifications of Japan 1996-2012
- Published registered utility model applications of Japan 1994-2012

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

CA/MEDLINE/ EMBASE/BIOS 1S (ΣTN), DDBJ/ UmProt, JSTPlus / JMEDPlus/ JST7 bit u (JDreaml 1 )

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**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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<td>US 2010/0286237 A1 (The Government of the USA as Represented by the Secretary, Department of Health &amp; Human Services) 2010.11.11, Claim 1,2,10,11,13, 15, 18 &amp; WO 2008/101118 A2</td>
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☑️ Further documents are listed in the continuation of Box C.  

☐ See patent family annex.

* Special categories of cited documents:
  
  "A" document defining the general state of the art which is not considered to be of particular relevance
  
  "E" earlier application or patent but published on or after the international filing date
  
  "L" 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)
  
  "O" document referring to an oral disclosure, use, exhibition or other means
  
  "P" document published prior to the international filing date but later than the priority date claimed

  "T" 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
  
  "X" 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
  
  "Y" 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

**Date of the actual completion of the international search**

26. 06. 2012

**Date of mailing of the international search report**

03. 07. 2012

**Name and mailing address of the ISA/JP**

Japan Patent Office

3-4-3, Kasumigaseki, Chiyoda-ku, Tokyo 100-8915, Japan

**Authorized officer**

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Form PCT/ISA/210 (second sheet) (July 2009)
<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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</thead>
<tbody>
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<td>A</td>
<td>US 2010/0311815 A1 (The Regents of the University of Michigan) 2010.12.09, claim 7,12,13 (No Family)</td>
<td>9-19, 24-31</td>
</tr>
</tbody>
</table>
Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of 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. ✓ Claims Nos.: 1-8, 20-23
   because they relate to subject matter not required to be searched by this Authority, namely:
   - The subject matter of claims 1-8 relates to diagnostic methods practiced on the human body, and the subject matter of claims 20-23 relates to methods for treatment of the human body, which does not require an international search by the International Searching Authority in accordance with PCT Article 17(2)(a)(i) and Rule 39.1(iv).

2. ✓ Claims Nos.:  
   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:

3. ✓ Claims 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 claims 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 claims 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.