Abstract:
The present invention provides a method for detecting colon cancer and the kit thereof, wherein the expression or methylation of the TBX5 gene is used as a marker for the presence and prognosis of colon cancer. The invention also provides a method for inhibiting the development of colon cancer cells by expressing the TBX5 gene in the cancer cells.
MARKER FOR COLON CANCER AND METHOD
FOR DETECTING COLON CANCER

Field

The subject matter disclosed generally relates to markers for colon cancer, and methods for detecting colon cancer.

Background

The following prior art publications are noted:
8. Yu J, Cheng YY, Tao Q, Cheung KF, Lam CN, Geng H, Tian LW, Wong YP, Tong JH, Ying JM, Jin H, To KF, Chan FK, Sung JJ. Methylation of protocadherin 10, a novel tumor suppressor, is associated with poor prognosis in patients with gastric

Summary

The applicants have identified a novel preferentially methylated gene, *T-box transcription factor 5 (TBX5)* (also known as *T-box 5*), in human colon cancer.

In a first embodiment, there is disclosed a method for detecting colon cancer in a biological sample. The method may comprise the step of: detecting methylation of a patient sample sequence of at least 15 consecutive base pairs, within a contiguous sequence at least 95% similar to the region consisting of SEQ. ID. NO: 1: wherein significant methylation level is indicative of cancer presence in the sample.

In alternative embodiments, the target sequence may be at least 50 base pairs long and contain a plurality of CpG base pairs. The method may further comprise comparing the methylation level of the patient sample DNA with methylation level of non-cancerous cells to thereby detect the presence of colon cancer. The determining may comprise treating the sample with a reagent that differentially modifies methylated and unmethylated DNA. The reagent may comprise a restriction enzyme that preferentially cleaves methylated DNA or that preferentially cleaves unmethylated DNA. The
determining may comprise treating the sample with sodium bisulphate. The determining may comprise the steps of: amplifying DNA using primers flanking a CpG-containing genomic sequence, the genomic sequence may be contained within SEQ ID NO: 1. The amplifying may use the polymerase chain reaction.

In alternative embodiments; the target sequence may be at least 25 base pairs long. The sample may comprise colon tissue. The detecting may comprise amplifying the region. The amplifying may use the polymerase chain reaction. The detecting may comprise using a primer selected from the group consisting of alternative primer sequences.

In another embodiment, there is disclosed a composition for suppressing colon cancer in a patient. The composition may comprise a biologically acceptable expression vector for expressing a portion of the TBX5 gene in the patient's cells.

In alternative embodiments, the vector may be suitable to direct expression of TBX5 protein in patient's cells.

In another embodiment, there is disclosed a method for inhibiting colon cancer in a subject. The method may comprise exposing the cells of the subject to the compositions disclosed herein.

In alternative embodiments, the vector may be delivered by viral transduction.

In another embodiment, there is disclosed a method for assessing the progress of colon cancer in a subject. The method may comprise the steps of: detecting methylation of a patient sample sequence of at least 10 consecutive base pairs, within a contiguous sequence at least 95% similar to the region consisting SEQ. ID. NO: 1; comparing the results to a reference; and using the results to determine the progress of the colon cancer in the subject.

In an embodiment there is disclosed a method for detecting colon cancer in a biological sample, said method comprising the step of: detecting methylation of a patient sample comprising a target sequence of at least 15 consecutive base pairs of genomic DNA,
within a contiguous sequence at least 95% similar to SEQ. ID. NO: 1; wherein significant methylation level is indicative of cancer presence in the sample.

In an alternative embodiment the target sequence is at least 50 base pairs long and contains a plurality of CpG base pairs.

In an alternative embodiment the method may further comprise comparing the methylation level of the target sequence in a subject with the methylation level of the target sequence in non-cancerous cells.

In an alternative embodiment the determining comprises treating the sample with a reagent that differentially modifies methylated and unmethylated DNA.

In an alternative embodiment the method further comprises treating the DNA with a reagent that preferentially cleaves methylated DNA.

In an alternative embodiment the determining comprises treating the sample with sodium bisulphate.

In an alternative embodiment the determination is performed by combined bisulfite restriction analysis (COBRA).

In an alternative embodiment the sample is a blood sample or a stool sample.

In an alternative embodiment the determining comprises the steps of: a) amplifying DNA from the sample with primers flanking CpG-containing genomic sequence, wherein the genomic sequence is comprised within SEQ. ID. NO: 1; and comparing the methylation level of the amplified portion of the genomic sequence in the sample with the methylation level of the genomic sequence in a non-cancerous sample, to thereby detect the presence of colon cancer.

In an alternative embodiment the reagent that preferentially cleaves methylated DNA is a restriction enzyme.
In an alternative embodiment the amplifying uses the polymerase chain reaction.

In an alternative embodiment the detecting uses a primer or probe selected from the group consisting of: SEQ. ID. NO: 4, SEQ. ID. NO: 5, SEQ. ID. NO: 8, SEQ. ID. NO: 9, SEQ. ID. NO: 10, and SEQ. ID. NO: 11.

In an alternative embodiment there is disclosed a method for inhibiting the development of colon cancer cells, the method comprising the step of: 1 expressing a biologically effective portion of SEQ. ID. 15 in the cancer cells to thereby inhibit the growth of the cells.

In an alternative embodiment the expressing comprises demethylating a DNA sequence in the said colon cancer cells with at least 95% sequence similarity over at least 15 contiguous base pairs to SEQ. ID. NO: 1.

In an alternative embodiment the expressing comprises introducing into said cells an isolated DNA molecule comprising a TBX5 coding sequence operatively linked to promoter.

In an alternative embodiment there is disclosed an isolated nucleic acid molecule comprising a sequence with at least 95% sequence similarity over at least 15 contiguous base pairs to SEQ. ID. NO: 1.

In an alternative embodiment the isolated nucleic acid molecule further comprises a vector.

In an alternative embodiment of the isolated nucleic acid molecule the sequence is demethylated

In an alternative embodiment there is disclosed a method for detecting colon cancer in a biological sample, the method comprising the steps of: amplifying a region of the DNA in the sample with at least 95% similarity over at least 15 contiguous base pairs to a portion of SEQ. ID. NO: 1; cleaving the amplified DNA with a reagent that preferentially cleaves methylated DNA; and comparing the cleavage products to the cleavage products an
isolated DNA molecule to thereby detect colon cancer in the sample.

In an alternative embodiment there is disclosed a kit for detecting colon cancer in a biological sample, the kit comprising: two primers suitable to amplify a region of the DNA in the sample with at least 95% similarity over at least 15 contiguous base pairs to a portion of SEQ. ID. NO: 1.

In an embodiment there is disclosed a method for detecting colon cancer in a biological sample, said method comprising the step of: detecting the level in the sample of an RNA sequence having at least 95% sequence similarity to a region of at least 15 contiguous base pairs of SEQ. ID. NO: 15, wherein a significantly lower amount of the said sequence in the sample relative to a non-cancerous control is indicative of presence of colon cancer in the biological sample.

In embodiments the sequence similarities may be greater than 90%, or greater than 95%, or greater than 99%, and may extend over a region of 15, 20, 25, 30, 40, 50 or more bases.

In an alternative embodiment there is disclosed a kit for detecting colon cancer in a biological sample, the kit comprising: a primer or a probe suitable to detecting the level in the sample of an RNA sequence having at least 95% sequence similarity to a region of at least 15 contiguous base pairs of SEQ. ID. NO: 15.

Features and advantages of the subject matter hereof will become more apparent in light of the following detailed description of selected embodiments, as illustrated in the accompanying figures. As will be realized, the subject matter disclosed and claimed is capable of modifications in various respects, all without departing from the scope of the claims. Accordingly, the drawings and the description are to be regarded as illustrative in nature, and not as restrictive and the full scope of the subject matter is set forth in the claims.

Brief Description of the Drawings

FIG.1A is the structure of the promoter region of the TBX5 gene of an embodiment.
FIG. 1B is an illustration of the methylation levels of the TBX5 promoter in an embodiment.

FIG. 1C is a BGS analysis of the methylation of the TBX5 promoter in an embodiment.

FIG. 1D is a TBX mRNA expression pattern after treatment with demethylating agent in an embodiment.

FIG. 2 shows TBX5 effects on tumour cell viability and clonogenicity in an embodiment.

FIG. 3 shows TBX5 effects on apoptosis of SW620 cells and migration rates of SW620 cells in an embodiment.

FIG. 4 shows the relationships between methylation of the TBX5 gene, the occurrence of colon cancer, and patient survival in an embodiment.

FIG. 5 shows sequences of portions of human TBX5 according to an embodiment.

FIG. 6 shows primer sequences according to embodiments.

FIG. 7 is the human TBX5 mRNA sequence.

FIG. 8 is a series of primers and primer pairs according to an embodiment.

Detailed Description of Embodiments

Terms

In this disclosure the following terms have the meanings set forth below:

In this disclosure the term "or" is generally employed in its sense including "and/or" unless the content clearly dictates otherwise.
In this disclosure, the term "biomarker" or "marker" means a substance such as a gene, a measurement of a variable related to a disease that may serve as an indicator or predictor of that disease. Biomarkers or markers are parameters from which the presence or risk of a disease can be inferred, rather than being a measure of the disease itself.

In this disclosure, the terms "nucleic acid", "nucleic acid sequence," and the like mean polynucleotides, which may be gDNA, cDNA or RNA and which may be single-stranded or double-stranded. The term also includes peptide nucleic acid (PNA), or any chemically DNA-like or RNA-like material. "cDNA" refers to copy DNA made from mRNA that is naturally occurring in a cell. "gDNA" refers to genomic DNA. Combinations of the same are also possible (i.e., a recombinant nucleic acid that is part gDNA and part cDNA).

In this disclosure the terms "operably associated" and "operably linked," mean functionally coupled nucleic acid sequences.

In this disclosure the terms "stringent hybridization conditions" and "high stringency" refer to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different 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) and will be readily understood by those skilled in the art. Generally, stringent conditions are selected to be about 5-10 °C. lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m 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 T_m, 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 background, preferably 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5
xSSC, and 1% SDS, incubating at 42 °C, or, 5 xSSC, 1% SDS, incubating at 65 °C, with wash in 0.2xSSC, and 0.1% SDS at 65.6°C.

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in lxSSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional guidelines for determining hybridization parameters are provided in numerous references, e.g., Current Protocols in Molecular Biology, ed. Ausubel, et al.

For PCR, a temperature of about 36°C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C, depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can range from about 50°C to about 65°C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90°C-95°C for 30 sec-2 min., an annealing phase lasting 30 sec-2 min., and an extension phase of about 72°C for 1-2 min. Protocols and guidelines for low and high stringency amplification reactions are well known in the art and are provided, e.g., in Innis et al. (1990) PCR Protocols, A Guide to Methods and Applications, Academic Press, Inc. N.Y.).

In this disclosure the term "polypeptide" means a polypeptide encoded by a nucleic acid molecule.

In this disclosure the terms "gene expression" and "protein expression" mean and includes any information pertaining to the amount of gene transcript or protein present in
a sample, as well as information about the rate at which genes, RNA or proteins are being expressed or are accumulating or being degraded (e.g., reporter gene data, data from nuclear runoff experiments, pulse-chase data etc.). Certain kinds of data might be viewed as relating to both gene and protein expression. For example, protein levels in a cell are reflective of the level of protein as well as the level of transcription, and such data is intended to be included by the phrase "gene or protein expression information." Such information may be given in the form of amounts per cell, amounts relative to a control gene or protein, in unitless measures, etc.; the term "information" is not to be limited to any particular means of representation and is intended to mean any representation that provides relevant information. The term "expression levels" refers to a quantity reflected in or derivable from the gene or protein expression data, whether the data is directed to gene transcript accumulation or protein accumulation or protein synthesis rates, etc.

In this disclosure the term "polypeptide" means a molecule comprised of two or more amino acids, preferably more than three. Its exact size will depend upon many factors.

In this disclosure the term "oligonucleotide" means a molecule comprised of two or more nucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide. In particular embodiments an oligonucleotide may have a length of about 10 nucleotides to 100 nucleotides or any integer therebetween. In embodiments oligonucleotides may be about 10 to 30 nucleotides long, or may be between about 20 and 25 nucleotides long. In embodiments an oligonucleotide may be greater than about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides long for specificity. In certain embodiments oligonucleotides shorter than these lengths may be suitable.

In this disclosure the term "primer" means an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA or RNA polymerase and at a suitable temperature and pH. The primer may be either single-
stranded or double-stranded and must be sufficiently long to prime the synthesis of the
desired extension product in the presence of the inducing agent. The exact length of the
primer will depend upon many factors, including temperature, source of primer and the
method used. For example, for diagnostic applications, depending on the complexity of
the target sequence, the oligonucleotide primer typically contains at least or more than
about 10, or 15, or 20, or 25 or more nucleotides, although it may contain fewer
nucleotides or more nucleotides. The factors involved in determining the appropriate
length of primer are readily known to one of ordinary skill in the art.

In this disclosure the term "primer pair", means a pair of primers which hybridize to
opposite strands a target DNA molecule, to regions of the target DNA which flank a
nucleotide sequence to be amplified.

In this disclosure the term "primer site", means the area of the target DNA to which a
primer hybridizes.

In this disclosure, the nucleic acids, polynucleotides, proteins, and polypeptides described
and claimed refer to all forms of nucleic acid and amino acid sequences, including but not
limited to genomic nucleic acids, pre-mRNA, mRNA, polypeptides, polypeptides,
polymorphic variants, alleles, mutants, and interspecies homologs that:

(1) have or encode an amino acid sequence that has greater than about 60% amino acid
sequence identity, 65%, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%,
95%, 96%, 97%, 98% or 99% or greater amino acid sequence identity, preferably over a
region of at least about 25, 50, 100, 200, 500, 1000, or more amino acids, to a
polypeptide encoded by a referenced nucleic acid or an amino acid sequence described
herein;

(2) specifically bind to or encode polypeptides that specifically bind to antibodies, e.g.,
polyclonal antibodies, raised against an immunogen comprising a referenced amino acid
sequence, immunogenic fragments thereof, and conservatively modified variants thereof;
(3) specifically hybridize under stringent hybridization conditions to a disclosed nucleic acid sequence or to a nucleic acid sequence encoding a disclosed amino acid sequence, and conservatively modified variants thereof,

(4) have a nucleic acid sequence that has greater than about 95%, preferably greater than about 96%, 97%, 98%, 99%, or higher nucleotide sequence identity, preferably over a region of at least about 15, 25, 50, 100, 200, 500, 1000, or more nucleotides, to a reference nucleic acid sequence.

A polynucleotide or polypeptide sequence is typically from a mammal including, but not limited to, primate, e.g., human; rodent, e.g., rat, mouse, hamster; cow, pig, horse, sheep, or any mammal. In particular embodiments the polynucleotide and polypeptide sequences disclosed are from humans. The nucleic acids and proteins of the invention include both naturally occurring or recombinant molecules.

In this disclosure the term "biological sample" or "sample" includes sections of tissues such as biopsy and autopsy samples, and frozen sections taken for histologic purposes, or processed forms of any of such samples. Biological samples include blood and blood fractions or products (e.g., serum, plasma, platelets, red blood cells, and the like), sputum or saliva, lymph and tongue tissue, cultured cells, e.g., primary cultures, explants, and transformed cells, stool, urine, stomach biopsy tissue etc. A biological sample is typically obtained from a eukaryotic organism, which may be a mammal, may be a primate and may be a human subject.

In this disclosure the term "biopsy" refers to the process of removing a tissue sample for diagnostic or prognostic evaluation, and to the tissue specimen itself. Any biopsy technique known in the art can be applied to the diagnostic and prognostic methods of the present invention. The biopsy technique applied will depend on the tissue type to be evaluated (e.g., tongue, colon, prostate, kidney, bladder, lymph node, liver, bone marrow, blood cell, stomach tissue, etc.) among other factors. Representative biopsy techniques include, but are not limited to, excisional biopsy, incisional biopsy, needle biopsy, surgical biopsy, and bone marrow biopsy and may comprise colonoscopy. A wide range
of biopsy techniques are well known to those skilled in the art who will choose between them and implement them with minimal experimentation.

In this disclosure the term "isolated" nucleic acid molecule means a nucleic acid molecule that is separated from other nucleic acid molecules that are usually associated with the isolated nucleic acid molecule. Thus, an "isolated" nucleic acid molecule includes, without limitation, a nucleic acid molecule that is free of sequences that naturally flank one or both ends of the nucleic acid in the genome of the organism from which the isolated nucleic acid is derived (e.g., a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease digestion). Such an isolated nucleic acid molecule is generally introduced into a vector (e.g., a cloning vector, or an expression vector) for convenience of manipulation or to generate a fusion nucleic acid molecule. In addition, an isolated nucleic acid molecule can include an engineered nucleic acid molecule such as a recombinant or a synthetic nucleic acid molecule. A nucleic acid molecule existing among hundreds to millions of other nucleic acid molecules within, for example, a nucleic acid library (e.g., a cDNA, or genomic library) or a portion of a gel (e.g., agarose, or polyacrylamine) containing restriction-digested genomic DNA is not to be considered an isolated nucleic acid.

In this disclosure a "cell" may be isolated, may be comprised in a group of cells, may be in culture, or may be comprised in a living subject and may be a mammalian cell and may be a human cell. Similarly "tissue" may comprise any number of cells and may be comprised in a living subject more may be isolated therefrom.

In this disclosure "cancer" means and includes any malignancy, or malignant cell division or malignant tumour, or any condition comprising uncontrolled or inappropriate cell proliferation and includes without limitation any disease characterized by uncontrolled or inappropriate cell proliferation.

In this disclosure the terms "colon cancer" and "colorectal cancer" have the same meaning and mean a cancer of the colon, or rectum and include cells characteristic of
colorectal cancer. Without limitation, colon cancers may be adenocarcinomas, leiomyosarcomas, lymphomas, melanomas, and neuroendocrine tumors.

In this disclosure the term "colon cancer cell" or "colorectal cancer cell" means a cell characteristic of colon cancer, and includes cells which are precancerous.

In this disclosure the term "precancerous" means a cell which is in the early stages of conversion to a cancer cell or which is predisposed for conversion to a cancer cell. Such cells may show one or more phenotypic traits characteristic of the cancerous cell.

In this disclosure the term "purified," or "substantially purified" means nucleic acids or polypeptides separated from their natural environment so that they are at least about 75%, 80, 85, 90 or 95% of total nucleic acid or polypeptide or organic chemicals in a given sample. Protein purity is assessed herein by SDS-PAGE and silver staining. Nucleic acid purity is assessed by agarose gel and EtBr staining.

In this disclosure the term "detection" means any process of observing a marker, or a change in a marker (such as for example the change in the methylation state of the marker, or the level of expression of nucleic acid or protein sequences), in a biological sample, whether or not the marker or the change in the marker is actually detected. In other words, the act of probing a sample for a marker or a change in the marker, is a "detection" even if the marker is determined to be not present or below the level of sensitivity. Detection may be a quantitative, semi-quantitative or non-quantitative observation and may be based on a comparison with one or more control samples. It will be understood that detecting a colon cancer as disclosed herein includes detecting precancerous cells that are beginning to or will, or have an increased predisposition to develop into colon cancer cells. Detecting a colon cancer also includes detecting a likely probability of mortality or a likely prognosis for the condition.

In this disclosure the term "expression vector" means a replicable DNA construct used to express DNA which encodes a desired protein or RNA sequence and which includes a transcriptional unit comprising an assembly of (1) genetic element(s) having a regulatory role in gene expression, for example, promoters, operators, or enhancers, operatively
linked to (2) a DNA sequence encoding a desired protein (in this case, an TBX5 protein) which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences. The choice of promoter and other regulatory elements generally varies according to the intended host cell. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome or in the form of viral sequences which may or may not integrate into the chromosomes. A wide range of expression vectors will be readily recognised and used by those skilled in the art.

In this disclosure the terms "homology", "identity" and "similarity" mean sequence similarity between two peptides or between two nucleic acid molecules. They can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When an equivalent position in the compared sequences is occupied by the same base or amino acid, then the molecules are identical at that position; when the equivalent site occupied by the same or a similar amino acid residue (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous (similar) at that position. Expression as a percentage of homology/similarity or identity refers to a function of the number of identical or similar amino acids at positions shared by the compared sequences. A sequence which is "unrelated or "non-homologous" shares less than 40% identity, preferably less than 25% identity with a sequence of the present invention. In comparing two sequences, the absence of residues (amino acids or nucleic acids) or presence of extra residues also decreases the identity and homology/similarity. In particular embodiments two or more sequences or subsequences may be considered substantially or significantly homologous, similar or identical if their sequences are about 60% identical, or are about 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher over a specified region, when compared and aligned for maximum correspondence over a comparison window or designated region, as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection such as provided on-line by the National Center for Biotechnology Information (NCBI). This
definition also refers to, or may be applied to, the compliment of a test sequence. Thus, to the extent the context allows, for instance where a nucleotide sequence may be expected to naturally occur in a DNA duplex, or may naturally occur in the form of either or both of the complementary strands, then a nucleotide sequence that is complimentary to a specified target sequence or its variants, is itself deemed "similar" to the target sequence and a reference to a "similar" nucleic acid sequence includes both the single strand sequence, its complimentary sequence, the double stranded complex of the strands, sequences able to encode the same or similar polypeptide products, and any permissible variants to any of the foregoing. Circumstances where similarity must be limited to an analysis of the sequence of a single nucleic acid strand may include for example the detection and quantification of the expression of a specific RNA sequence or coding sequence within a cell. The definition also includes sequences that have deletions and/or additions, as well as those that have substitutions. In embodiments identity or similarity may exist over a region that is at least about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 10, 21, 22, 23, 24, 25 or more amino acids or nucleotides in length, or over a region that is more than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or more than about 100 amino acids or nucleotides in length.

In this disclosure the term "methylation-sensitive PCR" (i.e., MSP) means a polymerase chain reaction in which amplification of the compound-converted template sequence is performed. Two sets of primers are designed for use in MSP. Each set of primers comprises a forward primer and a reverse primer. One set of primers, called methylation-specific primers (see below), will amplify the compound-converted template sequence if C bases in CpG dinucleotides within the vimentin DNA are methylated. Another set of primers, called unmethylation-specific primers (see below), will amplify the compound-converted template sequences if C bases in CpG dinucleotides within the vimentin DNA are not methylated.

In this disclosure the terms "inhibit" and "suppress" where used with reference to cancer cells or the growth or development thereof, mean and include any effects that result in or comprise slowing or preventing growth or cell division of the cells, killing the cells, disabling the cells, and in any way reducing the viability, rate of division or longevity of
the cells and includes any metabolic changes which change the characteristics of the cells in ways more characteristic of benign rather than malignant cell populations.

In this disclosure "Antibody" refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. Typically, the antigen-binding region of an antibody will be most critical in specificity and affinity of binding. Antibodies can be polyclonal or monoclonal, derived from serum, a hybridoma or recombinantly cloned, and can also be chimeric, primatized, or humanized. Antibodies exist, e.g., as intact immunoglobulins or as a number of well-characterized fragments which can be produced by digestion with various peptidases. The term antibody, as used herein, includes both complete antibodies and also antibody fragments either produced by the modification of whole antibodies, or synthesized de novo using recombinant DNA methodologies (e.g., single chain Fv) or those identified using phage display libraries.

In this disclosure the term "specifically (or selectively) binds" to an antibody or "specifically (or selectively) immunoreactive with," when referring to a protein or peptide, refers to a binding reaction that is determinative of the presence of the protein, often in a heterogeneous population of proteins and other biologies. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times the background and more typically more than 10 to 100 times background. Specific binding to an antibody under such conditions requires an antibody that is selected for its specificity for a particular protein. For example, polyclonal antibodies can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with the selected antigen and not with other proteins. This selection may be achieved by subtracting out antibodies that cross-react with other molecules. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with
a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein.

In this disclosure the term "amplify", means a process whereby multiple copies are made of one particular locus of a nucleic acid, such as genomic DNA or cDNA. Amplification can be accomplished using any one of a number of known means, including but not limited to the polymerase chain reaction (PCR), transcription based amplification and strand displacement amplification (SDA).

In this disclosure the term "polymerase chain reaction" or "PCR", means, a technique in which cycles of denaturation, annealing with primer, and extension with DNA polymerase are used to amplify the number of copies of a target DNA sequence by approximately $10^6$ times or more. The polymerase chain reaction process for amplifying nucleic acid is covered by U.S. Pat. Nos. 4,683,195 and 4,683,202.

In this disclosure the term "conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described
sequence with respect to the expression product, but not with respect to actual probe sequences.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

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)).

In this disclosure a "label" or a "detectable moiety" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, chemical, or other physical means. For example, useful labels include $^{32}$P, fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins which can be made detectable, e.g., by incorporating a radiolabel into the peptide or used to detect antibodies specifically reactive with the peptide.

In this disclosure the term "recombinant" when used with reference, e.g., to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within
the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

Exclusion of certain sequences:

It will be understood that in particular embodiments individual examples of sequences, probes, primers, polypeptides or the like may be excluded.

Detection of Nucleic Acids and Polypeptides:

A range of methods for the detection of specific nucleic acid sequences and polypeptides and their application will be readily apparent to those skilled in the art.

Nucleic acid molecules and polypeptides can be detected using a number of different methods. Methods for detecting nucleic acids include, for example, PCR and nucleic acid hybridizations (e.g., Southern blot, Northern blot, or in situ hybridizations). Specifically, oligonucleotides (e.g., oligonucleotide primers) capable of amplifying a target nucleic acid can be used in a PCR reaction. PCR methods generally include the steps of obtaining a sample, isolating nucleic acid (e.g., DNA, RNA, or both) from the sample, and contacting the nucleic acid with one or more oligonucleotide primers that hybridize(s) with specificity to the template nucleic acid under conditions such that amplification of the template nucleic acid occurs. In the presence of a template nucleic acid, an amplification product is produced. Conditions for amplification of a nucleic acid and detection of an amplification product are known to those of skill in the art. A range of modifications to the basic technique of PCR also have been developed, including but not limited to anchor PCR, RACE PCR, RT-PCR, and ligation chain reaction (LCR). A pair of primers in an amplification reaction must anneal to opposite strands of the template nucleic acid, and should be an appropriate distance from one another such that the polymerase can effectively polymerize across the region and such that the amplification product can be readily detected using, for example, electrophoresis. Oligonucleotide primers can be designed using, for example, a computer program such as OLIGO (Molecular Biology Insights Inc., Cascade, Colo.) to assist in designing primers that have similar melting temperatures. Typically, oligonucleotide primers are 10 to 30 or 40 or 50
nucleotides in length (e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides in length), but can be longer or shorter if appropriate amplification conditions are used.

In this disclosure the term "standard amplification conditions" refers to the basic components of an amplification reaction mix, and cycling conditions that include multiple cycles of denaturing the template nucleic acid, annealing the oligonucleotide primers to the template nucleic acid, and extension of the primers by the polymerase to produce an amplification product.

Detection of an amplification product or a hybridization complex is usually accomplished using detectable labels. The term "label" with regard to a nucleic acid is intended to encompass direct labeling of a nucleic acid by coupling (i.e., physically linking) a detectable substance to the nucleic acid, as well as indirect labeling of the nucleic acid by reactivity with another reagent that is directly labeled with a detectable substance. Detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include $^{125}$I, $^{131}$I, $^{35}$S or $^3$H. An example of indirect labeling includes end-labeling a nucleic acid with biotin such that it can be detected with fluorescently labeled streptavidin.

Specific polypeptide sequences may be detected using polyclonal or monoclonal antibodies which can be prepared in conventional ways as will be readily understood and applied by those skilled in the art. Those skilled in the art will readily identify and
prepare and raise antibodies to desirable polypeptide sequences to implement the subject matter disclosed and claimed.

The term "probe" with regard to nucleic acid sequences is used in its ordinary sense to mean a selected nucleic acid sequence that will hybridise under specified conditions to a target sequence and may be used to detect the presence of such target sequence. It will be understood by those skilled in the art that in some instances probes may be also be useable as primers, and primers may useable as probes.

**Methylation:**

In this disclosure, DNA "methylation" refers to the addition of a methyl group to the 5 position of cytosine (C), typically (but not necessarily) in the context of CpG (a cytosine followed by a guanine) dinucleotides. As used herein, "an increased methylation level" or "a significant methylation level" refers to the presence of at least one methylated C nucleotide in a DNA sequence where the corresponding C is not methylated in a normal control sample (such as a DNA sample extracted from a non-cancerous cell or tissue sample, or a DNA sample that has been treated to the methylation on DNA residues), in some embodiments at least 2, 3, 4, 5, 6, 7, 8, 9, 10 or more Cs may be methylated at locations where the Cs are unmethylated in a control DNA sample.

In embodiments, DNA methylation alterations can be detected using a number of different methods. Methods for detecting DNA methylation include, for example, methylation-sensitive restriction endonucleases (MSREs) assay by either southern or polymerase chain reaction (PCR) analysis, methylation specific or methylation sensitive-PCR (MS-PCR), methylation-sensitive single nucleotide primer extension (Ms-SnuPE), high resolution melting (HRM) analysis, bisulifte sequencing, pyrosequencing, methylation-specific single-strand conformation analysis (MS-SSCA), combined bisulifte restriction analysis (COBRA), methylation-specific denaturing gradient gel electrophoresis (MS-DGGE), methylation-specific melting curve analysis (MS-MCA), methylation-specific denaturing high-performance liquid chromatography (MS-DHPLC), methylation-specific microarray (MSO). These assays can be either PCR analysis,
quantitative analysis with fluorescence labelling or southern blot analysis. In
embodiments the degree of methylation of a sequence may be determined using a
methylation sensitive DNA cleaving reagent which may be a restriction enzyme and for
example may be AatII, AcII, Agel, Ascl, Asp718, Aval, BrpI, BceAI, BmgBI,
BsaAI, BsaHI, BsiEI, BsiWI, BsmBI, BspDI, BsrFI, BssHII, BstBI, BstUI, Clal, Eagl,
Eagl-HF™, Faul, Fsel, Fspl, Haell, Hgal, Hhal, HinPII, Hpall, Hpy99I, HpyCH4IV,
KasI, MluI, NarI, NgoMIV, NotI, NotI-HF™, Nrul, Nt.BsmAI, PaeR7I, PspXI, PvuI,
RsrII, SacII, Sail, Sall-HF™, Sfol, SgrAI, Smal, SnaBI or TspMI.

Articles of Manufacture

This disclosure encompasses articles of manufacture (e.g., kits) that contain one or more
nucleic acid molecules, or one or more vectors that encode a nucleic acid molecule. Such
nucleic acid molecules are formulated for administration as described herein, and can be
packaged appropriately for the intended route of administration. For example, a nucleic
acid molecule or a vector encoding a nucleic acid molecule can be contained within or
accompanied by a pharmaceutically acceptable carrier.

Kits of according to embodiments can include additional reagents (e.g., buffers, co-
factors, or enzymes). Pharmaceutical compositions according to embodiments can
include instructions for administering the composition to an individual. Kits may also
contain a control sample or a series of control samples that can be assayed and compared
to the biological sample. Each component of a kit may be enclosed within an individual
container and all of the various containers are within a single package.

Compositions and Delivery of Compositions to Target Cells

In certain embodiments there are disclosed compositions for delivery to target cells. It
will be understood that the compositions used in particular embodiments may be used in
combination with suitable pharmaceutically acceptable carriers or excipients and may be
used in any suitable dosage forms. Those skilled in the art will readily identify, select
from, and use the foregoing to suit the circumstances in question. Where a cell to be
treated is comprised in the body of a subject the methods disclosed may be implemented
and the compositions disclosed may be delivered to the cell in any conventional ways including without limitation the delivery of the tetrose or prodrug, orally, parentally, enterally, intramuscularly, subcutaneously, intravenously, or by inhalation and may be delivered in combination with suitable carriers or excipients, in suitable dosage forms including without limitation tablets, capsules, subdermal pumps or other routes useful to achieve an effect. Alternative delivery methods may include osmotic pumps, implantable infusion systems, intravenous drug delivery systems, and refillable implantable drug delivery systems. Delivery by inhalation may comprise delivery using nebulizers, metered dose inhalers, powder inhalers, all of which are familiar to those skilled in the art. Suitable methods, compositions and routes of delivery will be readily recognised and implemented by those skilled in the art.

**Embodiments**

In embodiments there are disclosed TBX5 genomic sequences, which may be TBX5 promoter sequences, and whose methylation may be correlated with the presence of colon cancer, and which may be useable as a diagnostic or prognostic marker for colon cancer. In embodiments it may be possible to prevent or inhibit the progression of colon cancer by demethylating the diagnostic sequences. The embodiments and examples are described with reference to FIGs. 1 through 8 and are further illustrated by examples presented herein. Further explanation of particular terms used in describing the embodiments is provided above and the use of such terms below incorporates all such additional explanation, and also includes all further explanation presented in the Examples.

The TBX5 promoter region comprised in or comprising a marker or markers of an embodiment is shown in FIG. 1A, and FIG. 5A and is generally designated SEQ. ID. NO: 1 and may comprise basepairs -605 through -902 relative to the transcription start site of the human TBX5 gene. This region includes CpG nucleotide pairs which may be methylated. The applicants have found that methylation of the TBX5 promoter sequences in a subject may be associated with the presence of colon cancer, and may be associated with poor prognosis for such cancer and may be associated with decreased life
expectancy. Broadly, it was found that the TBX5 gene promoter was partly or highly methylated in colon cancer tissues compared with adjacent non-tumor tissues, but was not methylated in normal controls. It will be understood that in alternative embodiments particular portions of SEQ. ID. NO: 1 may be used as markers, and may be as short or as long as 15, 20, 25, 30, 35, 40, 45, 50 or more or fewer base pairs. It will be understood that the complement of SEQ. ID. NO: 1 can be used instead of SEQ. ID. NO: 1 itself and that any reference herein indicating SEQ. ID. NO: 1 includes its complementary sequence.

In an alternative embodiment there is disclosed a method for detecting colon cancer in a biological sample. The method may comprise the step of detecting methylation of a patient sample comprising a target sequence of at least 15 consecutive base pairs of genomic DNA, within a contiguous sequence at least 95% similar to SEQ. ID. NO: 1 (corresponding to bases -902bp to -605bp of the human TBX5 gene); wherein a significant methylation level is indicative of cancer presence in the sample. It will be appreciated that in variants of the embodiment, the level of similarity in the target sequence may be greater than about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or may be 100% and that the target sequence may be greater than about 15, 20, 25, 30, 35, 40, 45, 50 or more base pairs long. In embodiments the region of similarity used as a marker may comprise one or more CpG base pairs and may comprise a plurality of CpG base pairs. As explained herein the detecting may be accomplished in a range of ways all readily understood by those skilled in the art. Similarly as set out herein methylation, or significant methylation may be partial or complete.

In embodiments the method may further comprise comparing the methylation level of the target sequence in a subject with the methylation level of the target sequence in non-cancerous cells, or with unmethylated target sequence. In embodiments the determining may comprise treating the sample with a reagent that differentially modifies methylated and unmethylated DNA. The reagent that differentially modifies may convert unmethylated cytosine to uracil whereas methylated cytosine remains unchanged. In embodiments, a restriction enzyme may preferentially cleave the unchanged CpG sites and in alternative embodiments may preferentially cleave changed CpG sites. In embodiments the determining may comprise treating the sample with sodium bisulphate
and may be performed by combined bisulfite restriction analysis (COBRA). In embodiments the biological sample may be a blood sample or may be a stool sample.

In embodiments the determining may comprise the steps of amplifying DNA from the sample with primers selective for a CpG-containing genomic sequence, wherein the genomic sequence is, or is comprised within, SEQ. ID. NO: 1; and comparing the methylation level of the amplified portion of the genomic sequence in the sample with the methylation level of the genomic sequence in a non-cancerous sample or a non methylated sample to thereby detect the presence of colon cancer. In embodiments the reagent that preferentially cleaves unmethylated DNA may be a restriction enzyme. In embodiments, amplifying the DNA may use the polymerase chain reaction.

In embodiments the step of detecting may comprise amplifying and may comprise using a primer or probe selected from the group consisting of: SEQ. IDS. 4, 5, 8, 9, 10 and 11.

In further embodiments there are disclosed kits for detecting colon cancer, the kits comprising primers disclosed in the other embodiments. Such kits may also comprise isolation buffers, reagents, standard and control samples and instructions on the use of the foregoing.

It can be understood that the kits are used to perform the methods as disclosed herein and therefore may comprise agents used in the methods. For example, where the kits are directed to detection of methylation of a biological sample comprising a target sequence of at least 15 consecutive base pairs of genomic DNA, within a contiguous sequence at least 95% similar to SEQ. ID. NO: 1, the kits may comprise a primer pair or probe selected from the group consisting of: SEQ. IDS. 4, 5, 8, 9, 10 and 11.

In a further embodiment there is disclosed a method for detecting colon cancer in a biological sample, the method comprising the step of: detecting the level in the sample of an RNA sequence having at least 95% sequence similarity to a region of at least 15 contiguous base pairs of an mRNA transcript of the TBX5 gene (SEQ. ID. NO: 15, FIG. 7) wherein a significantly lower amount of the said sequence in the sample relative to a non-cancerous control sample is indicative of the presence of colon cancer in the
biological sample. In embodiments the similarity may be assessed over a region at least about 15, 20, 25, 30, 35, 40, 45, 50 or more base pairs long. In embodiments the sample may comprise colon tissue, the detecting may comprise amplifying the region, and the sample may be a blood or stool sample. In embodiments the transcript may be detected using suitable amplification primers and in embodiments suitable primers may have sequences selected from the group consisting of SEQ. ID. NOS. 2, 3, and l6 through 29, or primer pairs A though G, or the primer pair consisting of SEQ. ID. NOS. 2 and 3.

In an alternative embodiment, accordingly, the kits for performing the method of detecting colon cancer in a biological sample as disclosed herein, comprise: a primer or a probe suitable to detecting the level in the sample of an RNA sequence having at least 95% sequence similarity to a region of at least 15 contiguous base pairs of SEQ. ID. NO: 15. In a preferable embodiment, the primer or probe is selected from the group consisting of SEQ. ID. NOS. 2, 3, and l6 through 29, or primer pairs A though G, or the primer pair consisting of SEQ. ID. NOS. 2 and 3.

Further Alternative Embodiments

In embodiments it was found that restoration of TBX5 expression in the colorectal cancer cells significantly inhibited colony formation and cell proliferation and induced apoptosis. Ectopic expression of TBX5 in colon cancer cells decreased migration ability. Thus, it is apparent that expressing the TBX5 sequences in colon cancer cells may inhibit growth and development of the cells and of tumors comprising such cells. In embodiments the restoration of TBX5 expression may be achieved in cell lines CaC02 and SW620, but those skilled in the art will recognise that such restoration of expression can be readily achieved in a range of other cell lines and in cells in vivo, and in a subject, and in a human subject and will readily identify and implement methods, vectors and strategies to achieve such restored expression. Those skilled in the art will recognise that restored expression may utilise exogenous introduction of a TBX5 transcription unit (whether natural or artificially constructed), or may utilise reactivation of the endogenous TBX5
gene, or may utilise the introduction of a TBX5 gene from a different organism or that has been modified or that is associated with heterologous sequences suitable to drive the expression of the TBX5 coding sequences or protein or portions thereof.

Thus in embodiments there is disclosed a method for inhibiting the development of colon cancer cells, the method comprising the step of expressing a biologically effective portion of the TBX5 gene in the cancer cells to thereby inhibit the growth of the cells. It will be understood that "a biologically effective portion" of the TBX5 gene may be a nucleic acid or a protein and may be a DNA or RNA sequence. It will be further appreciated that such sequences or portions of the gene may contain a region more than about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or up to 100% similar to the amino acid sequence of the protein encoded by the TBX5 gene and presented in FIG. 5C and designated SEQ. ID. NO: 12 or may have the same levels of similarity to the TBX5 mRNA presented in FIG. 7 and designated SEQ. ID. NO: 15, or may have such levels of similarity over a biologically effective region of such sequences that excludes one or more portions thereof that are not necessary for such sequence to achieve the desired biological effect.

In further embodiments such expressing may be achieved in whole or in part by demethylating the DNA sequence in the said colon cancer cells with sequence similarity greater than about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% over at least 15, 20, 25, 30, 35, 40, 45, 50 or more contiguous base pairs to SEQ. ID. NO: 1. It will be understood that demethylating may be achieved in a variety of ways readily understood by those skilled in the art and may be achieved by exposing the cells to reagents such as those set out in the Examples. A range of alternatives will be readily apparent to those skilled in the art.

In alternative embodiments expressing a sequence in colon cancer cells may comprise introducing into the cells an isolated DNA molecule comprising a TBX5 coding sequence (SEQ. ID. 15, FIG 7) operatively linked to a promoter, or comprised in a suitable expression vector, or exposing the cells to the compositions of the other embodiments disclosed herein. In embodiments the colon cancer cells may be cells of a human subject
or patient and may be in the body of said subject or patient. A range of promoters and vectors suitable to drive expression of the TBX sequences in mammalian, human, and colorectal cells will be readily identified and implemented by those skilled in the art. The expressed sequence may comprise sequences disclosed in other embodiments and may comprise a region with more than about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or up to 100% similarity to a portion of the sequence of the TBX5 gene presented as SEQ. ID. NO: 15 or may encode a protein identical or having the same levels of similarity to the TBX5 protein encoded by such nucleic acid sequences and presented as SEQ. ID. NO: 12. In embodiments the introduced TBX5 DNA may be a complete TBX5 gene including a demethylated copy of its endogenous promoter. In an embodiment illustrated in FIG. 2, exogenous expression of TBX5 may be achieved by transfection of target cells with pcDNA3.1-7BX5.

In a further alternative embodiments there is disclosed an isolated nucleic acid molecule comprising a sequence with at least 95% sequence similarity over at least 15 contiguous base pairs to SEQ. ID. NO: 1. In embodiments the isolated nucleic acid molecule may further comprise a suitable vector, which may be a plasmid or a virus, and may comprise added primer sequences or tags. In embodiments the isolated nucleic acid sequence may be demethylated. It will be apparent that further embodiments may comprise sequences that are more or less similar, for example more than about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% similar, or may display such similarity over longer or shorter sequences such as about, or more than about 15, 20, 25, 30, 35, 40, 45 and 50 base pairs, as set out in alternative embodiments and in the definitions.

In further alternative embodiments there is disclosed a method for detecting colon cancer in a biological sample, the method comprising the steps of amplifying a region of the DNA in the sample with at least 95% similarity over at least 15 contiguous base pairs to a portion of SEQ. ID. NO: 1; cleaving the amplified DNA with a reagent that preferentially cleaves unmethylated DNA; and comparing the cleavage products to the cleavage products of the isolated DNA molecule to thereby detect colon cancer in the sample. In embodiments the level of sequence similarity to SEQ. ID. NO: 1, and the length of the region of similarity may vary as in the other embodiments.
In embodiments there are disclosed primers for detecting DNA sequences at least 95% similar over 15 or more bases with SEQ. ID. NO: 1.

In embodiments, the materials and methods disclosed may be applied to assess the progress of a cancer, its prognosis, or other factors relating to the cancer. All of which will be readily understood by those skilled in the art.

EXAMPLES

The following are examples that illustrate materials, methods, and procedures for practicing the subject matter of the embodiments disclosed. It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

Figure legends

Figure 1. (A) A typical CpG island (CGI) spans the promoter region of TBX5 exon 1. Each vertical bar represents a single CpG site. The transcription start site (TSS) is indicated by a curved arrow. A region for combined bisulfite restriction analysis (COBRA) and bisulfite genomic sequencing (BGS) is shown. BstUl digestive sites are indicated. (B) TBX5 was frequently silenced or downregulated in colon cancer cell lines, but readily expressed in normal colon tissues by semi-quantitative RT-PCR. Methylation of TBX5 was determined by COBRA. The undigested fragment (upper band) represents the unmethylated DNA (U). The digested fragments represent the methylated DNA (M). (C) Detailed BGS analysis confirmed the methylation status of the TBX5 in colon cancer cell lines and in normal colon tissues. Five to six colonies of cloned BGS-PCR products from each bisulfite-treated DNA sample were sequenced and each is shown as an individual row, representing a single allele of the promoter CpG island analyzed. One circle indicates one CpG site. Filled circle, methylated; Open circle, unmethylated. (D) The mRNA expression of TBX5 was restored after treatment with demethylation agent 5-Aza-2'-deoxycytidine (5-Aza).
Figure 2. (A) TBX5 inhibited tumor cell viability. SW620 and CaC02 cell lines were transiently transfected with pcDNA3.1 (Vector), pcDNA3.1-7BX5 (TBX5) or green fluorescent protein (GFP) plasmid for 48 hr. (A1) The transfection efficiency was estimated by GFP under a fluorescence microscope; (A2) Ectopic expression of TBX5 in SW620 and CaC02 cell lines was evidenced by RT-PCR. (A3) Ectopic expression of TBX5 in SW620 and CaC02 significantly suppressed cell viability. (B) TBX5 suppressed tumor cell clonogenicity. (B1) The effect of ectopic TBX5 expression on cancer cell growth was further investigated by colony formation assay. Cells were transfected with pcDNA3.1-7BX5 or control vector, and selected with G418 for 10-14 days, and stained with Gentian Violet. (B2) Ectopic expression of TBX5 in SW620 and CaC02 was determined by RT-PCR. (B3) Quantitative analysis of colony formation. Values are expressed as the mean ± SD from three independent experiments *P<0.05, **P<0.001.

Figure 3. (A) TBX5 induced apoptosis of SW620 cells. Apoptosis was measured by flow cytometry analysis of Annexin V-FITC double-labeled pcDNA3.1-7BX5 (TBX5) or empty vector (Vector) transfected SW620 cells. (A1) Flow cytometry profile represents Annexin-V-FITC staining in x axis and propidium iodide in y axis. Dual staining of cells with annexin-V-FITC and propidium iodide enabled categorization of cells into four regions. Region Q1 shows the necrotic cells, Q2 shows the late apoptotic cells, Q3 shows the live cells, and Q4 shows the early apoptotic cells. (A2) The experiment was repeated three times and data represent the average of the early apoptotic and late apoptotic cells. *P<0.05, **P<0.001. (A3) Protein expressions of apoptotic related genes were determined by Western blot, β-actin was used as internal control. (B) TBX5 reduces the migration rates of SW620 cells. Representative image of cell migration of SW620 stable cell lines in scratch wound healing assay. pcDNA3.1-7BX5 or empty vector stably transfected SW620 cells grew up to 90% confluence and were wounded. Photographs were taken at 0, 48 and 96 hr, respectively, after the wound was made.
Figure 4. (A) TBX5 was frequently methylated in primary colon cancers. (A1) Representative gel images of TBX5 methylation in primary colon cancers and paired adjacent non-tumor tissues by COBRA. M, methylated; U, unmethylated. (A2) Detailed BGS analysis confirmed dense methylation in tumor tissues but less in paired normal tissues. Filled circle, methylated; Open circle, unmethylated. (B) Kaplan-Meier survival curves show that colon cancer patients with TBX5 methylation had poorer survival than those without TBX5 methylation. This difference is statistically significant based on log-rank test (P<0.001).

Materials and Methods

Tumor cell lines Eight colon cancer cell lines (DLD-1, HT-29, LOVO, SW480, SW620, CaCO2, LSI 80, CL14) were obtained from obtained from the American Type Culture Collection (Manassas, VA). They were cultured in RPMI 1640 medium (Gibco BRL, Rockville, MD) supplemented with 10%-20% fetal bovine serum (Gibco BRL) and incubated in 5% CO₂ at 37°C. Culture media were renewed every two or three days.

Primary tumor and normal tissue samples A total of 80 colorectal cancers were obtained at the time of operation. This included 49 males and 31 females. The median age of patients was 69 years (range, 36-91 years). Tumor was staged according to the TNM staging system. Twenty of their corresponding adjacent non-cancerous tissues, which were at least 5 cm away from the tumor edge, were obtained from colon cancer patients at the time of surgery. In addition, 15 age- and gender-matched normal colon mucosae from healthy subjects were collected as normal control. The specimens were snap-frozen in liquid nitrogen and stored at -80°C for molecular analyses. All subjects were given informed consent for obtaining the study materials. The study protocol was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong.

RNA extraction, semi-quantitative RT-PCR and real-time PCR analyses Total RNA was extracted from cell pellets and tissues using TRIzol Reagent according to the manufacturer's instruction (Molecular Research Center, Inc., Cincinnati, OH). cDNA was synthesized from 2 µg total RNA using Transcriptor Reverse Transcriptase (Roche
Applied Sciences, Indianapolis, IN). For semi-quantitative RT-PCR, a 151 bp fragment of the TBX5 gene was amplified for 36 cycles using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) as previously reported (Reference 8), with β-actin as internal control. The primer sequences of TBX5 are: forward 5'-CTCAAGCTCACCAACAACCA-3' (SEQ. ID. NO: 2) and reverse 5'-CAGGAAAGACGTGAGTGCAG-3' (SEQ. ID. NO: 3). Real-time PCR was performed using SYBR Green master mixture on HT7900 system according to the manufactures' instructions (Applied Biosystems).

DNA extraction and sodium bisulfite modification Genomic DNA was extracted from colon cancer cell lines and colon tissues using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) and treated with sodium bisulfite using a Zymo DNA Modification kit (Zymo Research, Hornby, Canada). Sodium bisulfite leads to deamination of unmethylated cytosines to uracils without modifying methylated sites. This allows their differentiation by restriction digestion or sequencing.

Combined bisulfite restriction analysis (COBRA) The methylation level of the promoter region of TBX5 gene was determined by COBRA, a semi-quantitative methylation assay. Hot start PCR amplification with 1.5 µl of bisulfite-treated DNA gives a PCR product of 298 bp, spanning promoter region -902bp to -603bp relative to the transcription start site (TSS) of TBX5 transcript variant 1. The primer sequences are forward 5'-TATTGTAGTTTGGTTGAGAGAAAGGA-3' (SEQ. ID. NO: 4) and reverse 5'-CTAAATCTAAACTTACCCTCCACTTC-3' (SEQ. ID. NO: 5). This region contained 19 CpG dinucleotides and 3 BstUI restriction sites (Fig. 1A). PCR products were digested with BstUI (New England Biolabs, Ipswich, MA) at 60°C overnight (New England BioLabs). BstUI cleaved the sequence 5'-CGCG-3' (SEQ. ID. NO: 6) which was retained in the bisulfite-treated methylated DNA, but not the unmethylated DNA. The DNA digests were separated in 10 % non-denaturing polyacrylamide gels and stained with ethidium bromide.

Cloned bisulfite genomic sequencing Cloned bisulfite sequencing was performed to identify the methylation status of 19 CG dinucleotide sites (Fig. 1A). The
above PCR products for COBRA from three colon cell-lines, one colon tumor and its adjacent non-cancer tissue and two normal colon tissues were cloned into pCR2.1-TOPO vector (Invitrogen, Carsbad, CA). Ten colonies were chosen randomly for plasmid DNA extraction with Qiaprep Spin Mini kit (Qiagen, Valencia, CA), and were sequenced. Sequencing analysis was performed by SeqScape software (Applied Biosystems, Foster City, CA).

Demethylation with 5-aza-2'-deoxycytidine (5-Aza) Cells were seeded at a density of 1x10^6 cells/mL. After 24 hours, cells were treated with 2µM of the DNA demethylating agent 5-Aza (Sigma-Aldrich, St Louis, MO) for 5 days. 5-Aza was replenished every day. Controlled cells were treated with an equivalent concentration of vehicle (DMSO). Cells were then harvested for DNA and RNA extractions.

DNA mutation analysis Genetic deletion and mutation analyses of TBX5 coding exons were performed by DNA direct sequencing. PCR products were purified and sequenced according to the recommendation of the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequence homologies were analyzed using BLAST program of the National Centre for Biological Information (NCBI).

Construction of TBX5 expression vector Mammalian expression vector pcDNA3.1-TBX5 encoding the full-length open reading frame of human TBX5 gene was constructed. Briefly, RNA from human tissue (Ambion, Austin, TX) was transcribed into cDNA. Sequence corresponding to the open reading frame clone of TBX5 was amplified and verified by DNA sequencing. PCR amplified inserts were subcloned into the pcDNA3.1 TOPO TA expression vector (Invitrogen). Plasmids used for transfection were isolated using EndoFree Plasmid Maxi Kit (Qiagen).

Western blot analysis Total protein was extracted and protein concentration was measured by the DC protein assay method of Bradford (Bio-Rad, Hercules, CA). Thirty micrograms of protein from each sample were used for Western blotting. Bands were quantified by scanning densitometry.
Cell viability assay  Cell viability was determined by [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI). Briefly, cells (4.5x10^7 well) were seeded in 96-well plates and TBX5-expressing vector (pcDNA3.1-7BX5) or empty vector (pcDNA3.1) or green fluorescent protein (GFP) plasmid (1.6 µg/each). The transient transfection efficiency was estimated by GFP under a fluorescence microscope. After 48 hr of transfection, 20 µl of reaction solution was added to cultured cells in 100 µl culture medium and incubated at 37°C for 1.5 h. The optical density was measured at a wavelength of 490 nm using a VICTOR^3TM multilabel counter (Perkin Elmer, Fremont, CA).

Colony formation assay  Cells (2x10^5/well) were plated in a 12-well plate and transfected with pcDNA3.1-7BX5, empty vector or GFP plasmid using Lipofectamine™ 2000 (Invitrogen). After 48 h of transfection, cells were collected and plated in a six well plate and selected with 0.6 mg/mL of G418 (Calbiochem, Darmstadt, Germany) for 10 to 14 days, and then with 0.15 mg/mL of G418 for an additional 1 week to establish stable clones. Surviving colonies (> 50 cells/colony) were counted after fixed with methanol/acetone (1:1) and stained with 5% Gentian Violet (ICM Pharma, Singapore) (Reference 8). All the experiments were performed in triplicate wells in three independent experiments.

Annexin V apoptosis assay  Apoptosis was determined by dual staining with Annexin V:FITC and propidium iodide (Invitrogen, Carlsbad, CA). Briefly, pcDNA3.1-7BX5 or empty vector transfected cells (5x10^5 cells/well) were harvested at 48 hours post-transfection. Annexin V:FITC and propidium iodide (PI) were added to the cellular suspension according to the manufacturer’s instructions, and sample fluorescence of 10,000 cells was analyzed using FACSCalibur System (Becton Dickinson Pharmingen, San Jose, CA). The relative proportion of Annexin V-positive cells was determined using the ModFitLT software (Becton Dickinson, San Diego, CA) and counted as apoptotic cells. All the experiments were performed three times independently.

Wound-healing assay  Cell migration was assessed using a scratch wound assay. Briefly, pcDNA3.1-7BX5 or empty vector stably transfected SW620 cells were cultured
in six-well plates (5x10^5 cells/well). When the cells grew up to 90% confluence, a single wound was made in the center of cell monolayer using a P-200 pipette tip. At 0, 48, 96 hr of incubation, the wound closure areas were visualized under phase-contrast microscope with a magnification × 100, and the migrated cells were counted. The experiments were performed in triplicate.

Statistical analysis Data were expressed as mean ± standard deviation (SD). Non-parametric data between two groups was computed by Chi-square test or Fisher Exact test. Multiple group comparisons were made by one-way ANOVA after Bonferroni's correction or Krusal-Wallis test where appropriate. The difference for two different groups was determined by Mann-Whitney U test. The Fisher's Exact Test was used for comparison of patient characteristics by methylation status, and distributions of methylation and covariates by vital status. Patients' age (at entry of follow-up) by vital status was compared using i-test. Relative risks (RRs) of death associated with TBX5 methylation and other predictor variables were estimated from univariate Cox proportional hazards model first. Multivariate Cox models were also constructed to estimate the RR for TBX5 methylation. Overall survival in relation to methylation status was evaluated by Kaplan-Meier survival curve and log-rank test. All analyses were performed using SAS for Windows, version 9, software (SAS Institute, Inc., Cary, North Carolina). P-value < 0.05 was taken as statistical significance.

Results

Identification of TBX5 as a novel gene down-regulated or silenced in colon cancer cells

To identify candidate cancer genes with promoter methylation, we performed methylation sensitive arbitrary primed PCR. A preferentially methylated CpG island of TBX5 promoter was identified that epigenetically silences TBX5 expression in human cancer. We then examined the mRNA expression of TBX5 in 8 colon cancer cell lines. RT-PCR showed that TBX5 transcript was silenced or reduced in 7 (88%) cell lines except LSI 80 (Fig. IB). In contrast, TBX5 expression was readily detected in normal colon tissues (Fig. IB), suggesting an aberrant gene silencing of TBX5 in colon cancers.
Promoter methylation of TBX5 was correlated with transcriptional silencing.

To elucidate whether silencing of TBX5 is due to the epigenetic inactivation, TBX5 methylation status was examined by COBRA. Seven cell lines with decreased or silenced TBX5 expression displayed methylated promoter including DLD-1, HT-29, LOVO, SW480, SW620, CaC02 and CL14, whilst, no methylation was detected in the normal colon tissues (Fig. 1B). We further validated the COBRA results by cloned bisulfite genomic sequencing (BGS) (Fig. 1C). The BGS results were consistent with those of COBRA in which dense methylation was found in methylated cell lines (SW620, HT-29), but not in unmethylated cell line (LSI 80) and normal colon tissues (Fig. 1C).

Demethylation treatment with 5-Aza restored TBX5 expression.

To further reveal whether methylation directly mediates TBX5 silencing, we randomly treated 4 methylated cell lines which showed silence or downregulation of TBX5 with 5-Aza. This treatment resulted in the restoration of TBX5 expression in all cell lines examined (Fig. ID), inferring promoter methylation directly contributed to the TBX5 silencing.

Genetic deletion and mutation of TBX5 were not detected in colon cancer cell lines.

Further genetic deletion and mutation analyses of TBX5 coding exons by DNA direct sequencing did not show any homozygous deletion or mutation in 8 colon cancer cell lines and 20 primary colon cancers, suggesting genetic alteration does not contribute to the silence or downregulation of TBX5 gene in colon cancer.

Ectopic expression of TBX5 suppressed colon cancer cell growth.

The frequent silencing of TBX5 mediated by promoter methylation in colon cancer cells but not in normal colon tissue implicated that TBX5 may play a role in tumor growth. To test this speculation, we examined the growth inhibitory effect through ectopic expression of TBX5 in SW620 and CaC02 which showed no TBX5 expression. Re-expression of TBX5 in the transient transfected SW620 and CaC02 was confirmed by RT-PCR (Fig. 2A1 and A2), which caused a significant decrease in cell viability in both SW620 (P<0.05) and CaC02 (P<0.05) (Fig. 2A3). The suppressive effect on cancer cell
proliferation was further confirmed by colony formation assay. A significant reduction of colony numbers was observed in cells stably transfected with pcDNA3.1-7BX5, compared to empty vector in monolayer culture (down to 54-65% of vector controls, P<0.001) (Fig. 2B). Thus, TBX5 exhibits growth inhibitory ability in tumor cells and functions as a potential tumor suppressor.

**TBX5 induced apoptosis**
We examined the contribution of apoptosis to the observed growth inhibition of TBX5-transfected cells. Apoptosis was investigated using two-color fluorescence-activated cell sorting analysis. Our results indicated an increase in the numbers of both early apoptotic cells (12.33 ± 0.70% vs. 9.97 ± 1.02%, P<0.05) and late apoptotic cells (16.2 ± 1.48% vs. 10.9 ± 0.95%, P<0.01) in 7BX5-transfected SW620 cells than those vector-transfected SW620 cells (Fig. 3A1 and 3A2). Induction of apoptosis was further confirmed by analysis the expression of apoptosis-related proteins. As shown in Fig. 3A3, re-expression of TBX5 enhanced the protein level of the active form of caspase-3, caspase-7 and nuclear enzyme poly (ADP-ribose) polymerase (PARP) in the stably transfected SW620 cells.

**TBX5 reduced the migration rate of SW620 cells**
We tested TBX5 for another tumor-suppressor activity, namely the ability to inhibit cell migration by wound healing assay. As shown in Fig. 3B, a decrease of the cell migration ability in wound closure was observed in SW620 cells transfected with TBX5 as compared to cells transfected with empty vector, as measured after 48 and 96 hr of wound healing, indicating the migration of SW620 cells was inhibited by TBX5.

**Frequent TBX5 methylation in primary colon cancers**
We next examined the TBX5 methylation status in 80 primary colon cancers, 20 their adjacent non-tumor tissues and 15 normal colon tissues. Frequent methylation was detected in primary colon cancers (52/80, 65%), but less in paired non-tumor tissues (6/20, 30%) (Fig. 4A1) (P=0.005). Further detailed BGS methylation analysis showed densely methylated promoter alleles in tumor, which are rare in paired non-tumor tissue
(Fig. 4A2). Fifteen normal colon tissues showed no methylation, indicating that TBX5 methylation was tumor-specific.

TBX5 methylation was associated with poor survival of colon cancer patients. The association of TBX5 methylation status and clinicopathologic characteristics including clinical outcome was analyzed in 80 colon cancer patients with known survival data (median survival time is 54.4 months, range from 2.3 to 65.6 months). There was no correlation between TBX5 methylation and clinicopathologic features of colon cancer patients such as age, gender, tumor staging, lymph node metastasis, distant metastasis and overall TNM staging. This is shown in Table 1.
Table 1. Clinicopathologic features of TBX5 methylation in colon cancer

<table>
<thead>
<tr>
<th>Variable</th>
<th>Methylated (n = 52)</th>
<th>%</th>
<th>Non-methylated (n = 28)</th>
<th>%</th>
<th>P value</th>
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<td>63.3</td>
<td>F 21</td>
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<td>18</td>
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<td>33.3</td>
<td>T2 1</td>
<td>50.0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>T3 42</td>
<td>62.7</td>
<td>T4 8</td>
<td>8.0</td>
<td>25</td>
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<tr>
<td>Lymph node status</td>
<td>Negative 22</td>
<td>62.9</td>
<td>Positive 30</td>
<td>66.7</td>
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<tr>
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<td>60.0</td>
<td>Yes 16</td>
<td>80.0</td>
<td>24</td>
</tr>
<tr>
<td>TNM stage</td>
<td>I 1</td>
<td>33.3</td>
<td>II 18</td>
<td>64.3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>III 17</td>
<td>58.6</td>
<td>III 16</td>
<td>80.0</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>IV 16</td>
<td>80.0</td>
<td></td>
<td>5</td>
<td>4</td>
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</table>

An analysis of characteristics of colon cancer patients related to the survival status showed that TBX5 was significantly associated with death. This is shown in Table 2:

Table 2. TBX5 methylation relative to survival status

<table>
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<tr>
<th>TBX5 methylation</th>
<th>Alive (n = 43)</th>
<th>%</th>
<th>Dead (n = 37)</th>
<th>%</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Yes</td>
<td>20</td>
<td>38.5</td>
<td>32</td>
<td>61.5</td>
<td>.0003</td>
</tr>
<tr>
<td>No</td>
<td>23</td>
<td>82.1</td>
<td>5</td>
<td>17.9</td>
<td></td>
</tr>
</tbody>
</table>
In univariate Cox regression analysis, $TBX5$ methylation was associated with a significantly increased risk of cancer-related death (RR, 6.21; 95% CI, 2.39-16.2; P=0.0002). After the adjustment for potential confounding factors (TNM staging), multivariate Cox regression analysis showed that $TBX5$ methylation was a predictor of poorer survival of colon cancer patients (RR, 5.42; 95% CI, 2.06-14.4; P=0.0007). As shown in the Kaplan-Meier survival curves, colon cancer patients with $TBX5$ methylation had significantly shorter survival than others (P<0.001, log-rank test) (Fig. 4B).

The embodiments and examples presented herein are illustrative of the general nature of the subject matter claimed and are not limiting. It will be understood by those skilled in the art how these embodiments can be readily modified and/or adapted for various applications and in various ways without departing from the spirit and scope of the subject matter disclosed claimed. The claims hereof are to be understood to include without limitation all alternative embodiments and equivalents of the subject matter hereof. Phrases, words and terms employed herein are illustrative and are not limiting. Where permissible by law, all references cited herein are incorporated by reference in their entirety. It will be appreciated that any aspects of the different embodiments disclosed herein may be combined in a range of possible alternative embodiments, and alternative combinations of features, all of which varied combinations of features are to be understood to form a part of the subject matter claimed. Particular embodiments may alternatively comprise or consist of or exclude any one or more of the elements disclosed.
CLAIMS

1. A method for detecting colon cancer in a biological sample, said method comprising the step of:

   detecting methylation of a patient sample comprising a target genomic DNA sequence at least 95% similar over 15 contiguous base pairs to a region of SEQ. ID. NO: 1;

   wherein significant methylation level is indicative of cancer presence in the sample.

2. The method according to claim 1 wherein the target sequence is at least 50 base pairs long and contains a plurality of CpG base pairs.

3. The method according to claim 1 further comprising comparing the methylation level of the target sequence in a subject with the methylation level of the target sequence in non-cancerous cells.

4. The method according to claim 1, wherein said determining comprises treating the sample with a reagent that differentially modifies methylated and unmethylated DNA.

5. The method according to claim 4, further comprising treating the DNA with a reagent that preferentially cleaves methylated DNA.

6. The method according to claim 1 wherein said determining comprises treating the sample with sodium bisulphate.

7. The method according to claim 3, wherein the determination is performed by combined bisulfite restriction analysis (COBRA).

8. The method according to claim 1 wherein the sample is a blood sample or a stool sample.

9. The method according to claim 1 wherein said determining comprises the steps of:
a) amplifying DNA from the sample with primers flanking a CpG-containing genomic sequence, wherein the genomic sequence is comprised within SEQ. ID. NO: 1; and

b) comparing the methylation level of the amplified portion of the genomic sequence in the sample with the methylation level of the genomic sequence in a non-cancerous sample to thereby detect the presence of colon cancer.

10. The method according to claim 5, wherein the reagent that preferentially cleaves methylated DNA is a restriction enzyme.

11. The method according to claim 9, wherein said amplifying uses the polymerase chain reaction.

12. The method according to claim 1 wherein said detecting uses a primer or probe selected from the group consisting of: SEQ. ID. NOs. 4, 5, 8, 9, 10, and 11.

13. A method for inhibiting the development of colon cancer cells, the method comprising the step of:

expressing a biologically effective portion of SEQ. ID. 15 in the cancer cells to thereby inhibit the growth of the cells.

14. The method according to claim 13 wherein said expressing comprises demethylating a DNA sequence in the said colon cancer cells with at least 95% sequence similarity over at least 15 contiguous base pairs to SEQ. ID. NO: 1.

15. The method according to claim 13 wherein said expressing comprises introducing into said cells an isolated DNA molecule comprising a TBX5 coding sequence operatively linked to promoter.

16. An isolated nucleic acid molecule comprising a sequence with at least 95% sequence similarity over at least 15 contiguous base pairs to SEQ. ID. NO: 1.
17. The isolated nucleic acid molecule according to claim 16 further comprising a vector.

18. A method for detecting colon cancer in a biological sample, said method comprising the step of:

detecting the level in the sample of an RNA sequence having at least 95% sequence similarity to a region of at least 15 contiguous base pairs of SEQ. ID. NO: 15,

wherein a significantly lower amount of the said sequence in the sample relative to a non-cancerous control is indicative of presence of colon cancer in the biological sample.

19. A method for detecting colon cancer in a biological sample, the method comprising the steps of:

amplifying a region of the DNA in the sample with at least 95% similarity over at least 15 contiguous base pairs to a portion of SEQ. ID. NO: 1;

cleaving the amplified DNA with a reagent that preferentially cleaves methylated DNA; and

comparing the cleavage products to the cleavage products of the isolated DNA molecule according to claim 16

to thereby detect colon cancer in the sample.

20. A kit for detecting colon cancer in a biological sample, the kit comprising:

two primers suitable to amplify a region of the DNA in the sample with at least 95% similarity over at least 15 contiguous base pairs to a portion of SEQ. ID. NO: 1.

21. The kit according to claim 20 wherein said primers are selected from the group consisting of: SEQ. ID. NOs. 4, 5, 8, 9, 10, and 11.
22. The kit according to claim 20, further comprising a reagent for detecting methylation level of the region of the DNA.

23. The kit according to claim 22, wherein said reagent is a reagent that preferentially cleaves methylated DNA.

24. A kit for detecting colon cancer in a biological sample, said kit comprising a primer or probe suitable to detecting the level in the sample of an RNA sequence having at least 95% sequence similarity to a region of at least 15 contiguous base pairs of SEQ. ID. NO: 15.

25. The kit according to claim 24 wherein said primer or probe is selected from the group consisting of: SEQ. ID. NOS: 2, 3, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 and 29, or primer pairs A though G as shown in figure 8.

26. The kit according to any of claims 20 to 25, further comprising a control sample.

27. The method according to any of claims 20 to 25, wherein the biological sample is a blood sample or a stool sample.
FIG. 5

A) (SEQ. ID. NO: 1) Sequence of promoter region of the human TBX 5 gene (-902 to -605 bp from the transcription start site) UCSC database: chr12:114,846,852-114,847,149 cactgcagctctgtgagagaagagcggggggtgttctctctggaagcagaaaggggtgctggcagctgctctggagctg
tgtggccggtctggaaagctgccccgtctctcccggcgggctgactcttgctccgccgceactctgctgctccg
ctccacattttgctcgtctgcagttcatcagaaaccacctggagccagcgtcctggagaaaagccccctgggggttgggacgcg
C) (SEQ. ID. NO: 12) Human TBX5 amino acid sequence, NCBI accession number: NP_000183
MADADEGFGLAHTPLEPDALKDLPCSKPESALGAPSKPSSPQAAFTQQGMEGIKVFLHERELWLKFHEVGTEMIIKAGRRMFPSYKVKVTCGLNPKTGYITLMDIVPADDHRYKFDNKNKWSVTGKAEPAMPGLYVHPDSPATGAIHWMRQLVSFQKIKLTNNHLDPFGHIIILNSMHKYQPRLPVHKADENNFSGKNTAFCTHVFPPETAFIAVTSY
QNHKITQLKIEENNPFAKGRGSDDMELHRMSRMQSKKEYPVPPVPRSTVRQKVASHN
SPFSSESRALSTSSNLGSQYQCENGVSGBPSQDLLPPPNYLPLQEHSQYHYICTKRKEEECTTDHPYKKPYPMTSMSPSEDSFRYSSYPQQGLGASYRTESAQRQACMYAS
SAPPSEPVPSPLEDISCNTWPSMPSYSSCTVTTVQMDRLPYQHFSAHFTSGPLVPR
LAGMANHGSQPLGEGMFQHQSTSVAHPQVVRQCQPQPTGQLSPGTLQPPEFLYSH
GVPRTLSPHQYSVHSVHGVMVPEWSDNS
FIG. 6

Forward transcript primer 5’-CTCAAGCTCACCAACAACCA-3’ (SEQ. ID. NO: 2)

Reverse transcript primer 5’-CAGGAAAGACGTGAGTGCAG-3’ (SEQ. ID. NO: 3).

Promoter forward primer 5’TATTGTAGTTGGTTGAAGAAAGGA-3’ (SEQ. ID. NO: 4)

Promoter reverse primer 5’-CTAAATCTAAACTTACCCCCCACTTC-3’ (SEQ. ID. NO: 5).

BstUI cleavage sequence 5’-CGCG-3’ (SEQ. ID. NO: 6)

Sense primer 5’-GGTTGTGTTTTTTTTGAAGTAAA-3’ (SEQ. ID. NO: 8)

Sense primer 5’-GTGTAGTTGGTTTGGGAGTGTG-3’ (SEQ. ID. NO: 9)

Sense primer 5’-GTGTGTGTGTGTGTGTGTGT-3’ (SEQ. ID. NO: 10)

Antisense primer 5’-ACAACAAAAATATACAAAAAAACATC-3’ (SEQ. ID. NO: 11)
FIG. 7

(SEQ. ID. NO:15): Homo sapiens T-box 5 (TBX5), transcript variant 1, mRNA sequence
UCSC database: chr12:114791736-114846247
catgcttatgcaagagactcagtcgccccgcacacactgatttctccatacatgaggtctgctggtgacactccacacctctgct
taagatgttcctctctctctctctctctctaagaaagctagctctgtgctcttatgtgttatgtgtatatagccctagccctactctccctccgg
gagaggttggtgttgtgttgtgttgtgtgttgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtg...
FIG. 7 (continued)

aacagacccaaataaaagagaacaggtaagagtggagtggagtttaagttacgtagagaggtggtagtttttgtaagtgaagtcttcccatsggtgattgtgccttttaagtttggaggggagggtgagggttaagttacttgtgcctttaatttttgtagccttaaaatacttgctttaaectaagtttaaaaaaaaaa
FIG. 8
Primer pair A)
Sense primer (+254): 5’-TAGGCGAAGACGGAGAGGAAAA-3’ (SEQ. ID. NO: 16)
Anti-sense primer (+472): 5’-GCTCAAGTTGTTGTGTTGTTG-3’ (SEQ. ID. NO: 17)

Primer pair B)
Sense primer (+453): 5’-ACACAAACACACCTTGGCAG-3’ (SEQ. ID. NO: 18)
Anti-sense primer (+642): 5’-GCAAGGGTTCTGCTCTCAACC-3’ (SEQ. ID. NO: 19)

Primer pair C)
Sense primer (+891): 5’-TCATAACCAAGGCTGAGGAG-3’ (SEQ. ID. NO: 20)
Anti-sense primer (+1024): 5’-GCCCGTCACAGACCCATTTAT-3’ (SEQ. ID. NO: 21)

Primer pair D)
Sense primer (+1504): 5’-AGCTCTTCACCTCCTCATCCA-3’ (SEQ. ID. NO: 22)
Anti-sense primer (+1714): 5’-TTCACTGCGATGTCTCAACC-3’ (SEQ. ID. NO: 23)

Primer pair E)
Sense primer (+2494): 5’-CATCAACCAGCCACATTCAC-3’ (SEQ. ID. NO: 24)
Anti-sense primer (+2696): 5’-AGGGACTCGGTCCCATTAAG-3’ (SEQ. ID. NO: 25)

Primer pair F)
Sense primer (+2863): 5’-TTCAGTTTGTGCCTCGCTTC-3’ (SEQ. ID. NO: 26)
Anti-sense primer (+3019): 5’-CCACGTGTAGCGACAGACA-3’ (SEQ. ID. NO: 27)

Primer pair G)
Sense primer (+3317): 5’-CCGCTATGCAACACAAAACCTG-3’ (SEQ. ID. NO: 28)
Anti-sense primer (+3489): 5’-AGCTCCTATGCTGGGTCTTC-3’ (SEQ. ID. NO: 29)
INTERNATIONAL SEARCH REPORT

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/68 (2006.01)

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)

IPC: C12Q

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

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

IVEN, CNKI, CNABS, CPRSABS, CNTXT, EPTXT, USTXT, WOTXT, IPTXT, ISI web of Knowledge, EMBASE, NCBI: colon cancer, methylation, DNA, TBX5, T-box 5, CpG detect+, diagnosis+, sequence search on SEQ ID NOs: 1-15

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<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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<td>WO2009069984 A2(GENO-N), 4 Jun. 2009(04.06.2009), see claims 1-8, description page 25 lines 1-26, page 10 lines 20-26, the sequence list</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

11 Jul. 2011 (11.07.2011)

Date of mailing of the international search report


Name and mailing address of the ISA/CN

The State Intellectual Property Office, the P.R.China 6 Xitucheng Rd., Jimen Bridge, Haidian District, Beijing, China 100088 Facsimile No. 86-10-62019451

Authorized officer

WU, Li

Telephone No. (86-10) 62411046

Form PCT/ISA/210 (second sheet) (July 2009)
## INTERNATIONAL SEARCH REPORT

### Box No. 1  Nucleotide and/or amino acid sequence(s) (Continuation of item item.1c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of:

   a. a sequence listing filed or furnished
      - □ on paper
      - ◼ in electronic form
   
   b. time of filing or furnishing
      - ◼ contained in the application as filed
      - □ filed together with the application in electronic form
      - □ furnished subsequently to this Authority for the purposes of search

2. □ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

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Form PCT/ISA/210 (continuation of first sheet (1)) (July 2009)
**INTERNATIONAL SEARCH REPORT**

**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.: 13-15
   because they relate to subject matter not required to be searched by this Authority, namely:
   Claims 13-15 are directed to a method of treatment of human/animal body by therapy (Rules 39.1 (iv) PCT), however, the search has been carried out and based on the alleged effects of the compositions.

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 an additional fee, this Authority did not invite payment of additional fee.

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.
## INTERNATIONAL SEARCH REPORT

### Information on patent family members

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