RUNX3 GENE SHOWING ANTI-TUMOR ACTIVITY AND USE THEREOF

FIELD OF THE INVENTION

The present invention relates to the RUNX3 gene and uses thereof as an anticancer agent. More specifically, the present invention relates to characterization of the RUNX3 gene, its expression and tumor suppressor activity, and its use in the development of cancer diagnosis methods and anticancer agents.

BACKGROUND OF THE INVENTION

Tumors are assemblies of cells that show excessive autonomous proliferation, and are classified into malignant tumors, which may result in death, and benign tumors; however, it can be difficult to discriminate between these types. Cancers are genetic diseases attributed to the mutation of genes such as oncogenes and tumor suppressor genes, and their causes can be found at a cellular level. Oncogene products constitute a signaling network within cells, and are involved in regulating signal transduction systems for cell division and differentiation. When their regulation becomes abnormal, cells do not differentiate further, but rather divide infinitely; that is, they become tumorigenic. If a way could be found to enable such an abnormal signal transduction pathway to be converted into a normal one, anticancer agents could be developed that would obtain excellent therapeutic effects without side effects.

At present, cancers are for the most part treated in three ways: with surgical therapy, chemical therapy, and radiation therapy. In practice, combinations of these therapies or further combination with laser therapy are prevalent. However, chemical therapy is preferred to other therapies when the pain accompanying therapy and metastatic conditions are taken into account. Numerous anticancer agents have been developed, most of which are based on the selective killing of cells that are actively dividing. However, these anticancer agents suffer from the disadvantage of also killing some normal cells, such as immune cells and hair root cells, with concomitant significant side effects; they are thus not able to be used for long periods of time. Therefore, there remains a need for novel anticancer agents. A therapeutic agent based on the causes of cancer might be expected to be highly effective and to have few or no accompanying side effects.

Abnormal activation of oncogenes induces cell proliferation, and is one cause of cancer. In contrast, tumor suppressor genes function to prevent abnormal cell proliferation or to trigger programmed cell death (apoptosis). Often, tumor suppressor genes trigger apoptosis to kill the cells with abnormally activated oncogenes thus preventing the formation of cancerous cells. Where tumor suppressor genes show normal activity, cells with abnormally activated oncogenes cannot progress toward cancer, but are annihilated. Therefore, to become cancerous cells, cells must have inactivated tumor suppressor genes as well as activated

oncogenes.

One of the mechanisms by which tumor suppressor genes are inactivated is by hyper-methylation of CpG islands (Jones and Laird, Nature Genet. Vol. 21, 163-167, 1999). Methylation of CpG islands is performed by DNA methyltransferase. After significant methylation, DNA binding proteins such as methyl cytosine binding protein 2 (MECP2) bind to the methylated cytosine of the DNA, which recruits histone deacetylase (HDAC) to repress gene expression. In detail, HDAC removes the acetyl groups associated with histones, and the chromosomal DNA in the vicinity of the deacetylated histones becomes dense, which leads to repression of gene transcription. If gene expression is repressed by DNA methylation, DNA methyltransferase or HDAC inhibitors may be useful for inducing gene expression. Tumor suppressor genes whose expression is repressed by DNA methylation are exemplified by RB1, TP53, VHL, CDKN2A, CDKN2B, MLH1, and APC (Jones and Laird, Nature Genet. Vol. 21, 163-167, 1999).

As mentioned previously, histone acetylation and deacetylation are known to play important roles in regulating DNA transcription in eukaryotic cells (Grunstein M., Nature, 389, 349-352, 1997). Some naturally occurring compounds have been found to prevent cells from progressing toward cancer by inhibiting HDAC. Exemplified by trapoxin, trichostatin A, and depudecin, HDAC inhibitors have been studied for their ability to reverse the transformation of cancerous cells. Of the HDAC inhibitors, depudecin is the best characterized as to its anti-angiogenic activity in vivo and in vitro. In addition,

the HDAC inhibitors have been studied with regard to cellular responses, including cell cycle interruption, alteration of gene expression patterns, and induction of apoptosis.

The TGF-β signal transduction system is well known for its tumor suppressor activity. The binding of TGF-β to TGF-β receptors causes the activation of the receptors, which in turn activate Smad proteins by phosphorylation. Once activated, Smad proteins move into the nucleus and regulate gene expression in cooperation with other transcription factors, thereby suppressing cell division or inducing apoptosis (Massague et al., Cell, 103 (2):295-309, 2000). Runx3 is one of the transcription factors that physically interact with Smad proteins (Hanai et al., J. Biol. Chem. 274; 31577-31582, 1999). Deletion or mutation of TGF-β receptors or Smad genes is observed in cells of various types of cancer. The tumor suppressor activity of TGF-β receptors was also demonstrated by an experiment in a cell strain lacking the TGF-β receptor. When the cell was transformed to express the TGF-β receptor, cell proliferation was reduced and tumorigenesis was decreased in an assay in nude mice (Chang et al., Cancer Res., 57 (14):2856-2859, 1997). The TGF-β signal transduction system is well characterized as to the repression of cell proliferation, which is achieved by promoting the expression of the CDK inhibitor protein p21. However, the mechanism by which TGF-β induces apoptosis remains to be clearly elucidated.

PEBP2 (polyoma virus enhancer binding protein 2)is composed of two submits, α and β. There are three genes which

encode the α subunit.: RUNX1/PEBP2α B/CBFA2/AML1, RUNX2/PEBP2α A/CBFA2/AML2, and RUNX3/PEBP2α C/CBFA3/AML2 (Bae and Ito, Histol. Histopathol, 14(4):1213-1221, 1999). The RUNX1, RUNX2, and RUNX3 genes show homology of about 60-70% in amino acid sequence among them. They are highly conserved evolutionarily, with homology of about 95% between mouse and human.

Regarded as an important causative gene in leukemia, the RUNX1 gene becomes associated with other genes by chromosome translocation to cause acute myeloid leukemia or acute lymphoid leukemia in humans (Miyoshi et al., EMBO J., 12:2715-2721, 1993; Romana et al., Blood, 85:3662-3670, 1995; Okuda et al., Blood, 91:3134-3143, 1998; Okuda et al., Cell, 84:321-330, 1996). The RUNX2 gene plays a crucial role in osteogenesis, and its disruption has been implicated in causing cleidocranial dysplasia (Komori et al., Cell, 89:755-764, 1997; Lee et al., Nat. Genet., 15:307-310, 1997; Mundlos et al., Cell, 89:773-779, 1997; Otto et al., Cell, 89:756-771, 1997). Also, it has been reported that the RUNX2 gene shows oncogenic activity in the formation of T-cell lymphoma (Stewart et al., Proc. Natl. Acad. Sci. U. S. A., 94(16):8646-8651, 1997).

The RUNX3 gene was identified by the present inventors several years ago as a member of the PEBP2 family (Bae et al., Gene, 159(2):245-248, 1995; Levanon et al., Genomics, 23(2):425-532, 1994). However, diseases associated with the activation or inactivation of the RUNX3 gene, other than what we describe in this invention, hasn't been reported.

SUMMARY OF THE INVENTION

Leading to the present invention, the thorough and intensive research on diseases associated with the activation of the RUNX3 gene, conducted by the present inventors, resulted in the finding that RUNX3 gene products have tumor suppressor activity. RUNX3 is indispensable for TGF-β-induced apoptosis, and inactivation of the RUNX3 gene by DNA methylation at loci near RUNX3 exon 1 is closely correlated with cancer development. In the present invention, the RUNX3 gene is characterized as to its expression and tumor suppressor activity, offering the possibility of developing cancer diagnosis methods and anticancer agents from it.

The objectives of the present invention are to elucidate the tumor suppressor activity of the RUNX3 gene and its mechanism, and to describe a diagnostic method and a therapeutic agent for cancer based on the tumor suppressor activity of RUNX3.

To achieve the above objectives, the present invention provides a cell strain having a sense RUNX3 cDNA, in which the RUNX3 cDNA is over-expressed, and a cell strain having an antisense RUNX3 cDNA, in which the expression of the RUNX3 gene is inhibited are provided.

Also, the present invention provides a RUNX3 cDNA with tumor suppressor activity and its corresponding protein.

In the present invention, the RUNX3 gene is shown to play a crucial role in TGF-β-dependent apoptosis, using cell

strains that either overexpress RUNX3 cDNA or selectively inhibit the expression of the RUNX3 gene.

Additionally, it is shown that the suppression of RUNX3 gene expression in various cell lines can be attributed to DNA methylation in the vicinity of exon 1 of the RUNX3 gene.

Further, the present invention provides a pharmaceutical composition including the RUNX3 gene or its protein.

Finally, the present invention provides uses of the RUNX3 gene and its proteins in the development of anticancer agents and diagnostic methods.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1a is a cDNA nucleotide sequence of the human RUNX3 gene with arrows denoting the primers used for RT-PCR analysis and bold letters denoting the translation initiation codon (ATG) and translation stop codon (TGA).

Fig. 1b continues the cDNA nucleotide sequence of the human RUNX3 gene from Fig. 1a.

Fig. 1c is an amino acid sequence deduced from the sequence of the cDNA of the human RUNX3 gene shown in Fig. 1a.

Fig. 2a is a nucleotide sequence showing CpG islands present in exon 1 of the human RUNX3 gene with a site used as a probe in genomic DNA Southern blot analysis denoted by underlining and a translation initiation codon (ATG) denoted by bold letters.

Fig. 2b continues the nucleotide sequence of the CpG islands present in exon 1 of the human RUNX3 gene from Fig. 2a.

Fig. 3 is an autoradiogram resulting from Northern blotting analysis for determining whether SNU16-Rx3-AS-c11 and SNU16-Rx3-AS-c12, both having an antisense RUNX3 gene, and MKN28-Rx3, having a sense RUNX3 gene, express the antisense and sense RUNX3 genes.

Fig. 4a is a graph in which counts of viable SNU16-Rx3-AS-c11 cells are plotted with regard to time, along with those of the control SNU16 cells, after treatment with 1 ng/ml of TGF-β to analyze TGF-β-induced cell death.

Fig. 4b is a photograph showing the results of the electrophoresis of the genomic DNAs obtained from SNU16, SNU16-Rx3-AS-c11, and SNU16-Rx3-AS-c12 after treatment with 1 ng/ml of TGF-β1 for 6 hours so as to determine whether RUNX3 is involved in the TGF-β-induced programmed cell death pathway.

Fig. 5a is a graph showing that the size of the tumors formed in nude mice were measured periodically for over 98 days after the subcutaneous injection of SNU16-Rx3-AS-c11 and SNU16-Rx3-AS-c12, both having restricted RUNX3 expression due to the over-expression of the antisense RUNX3 gene therein; and control-SNU16 which expresses the RUNX3 gene normally.

Fig. 5b is a graph in which tumors formed in nude mice were measured for size periodically for over 37 days after the subcutaneous injection of control MKN28, which does not express the RUNX3 gene, and MKN28-Rx3, in which a RUNX3 cDNA is expressed.

Fig. 5c shows a comparison of cancerous masses excised from the mice sacrificed on the final day of the experiment.

Fig. 6a is a schematic view showing the structure of the

RUNX3 cDNA with arrows denoting the RT-PCR primer positions, and a striped box denoting the Runt domain, which plays an important role in binding of the RUNX3 protein to DNA.

Fig. 6b shows the results of electrophoresis of RT-PCR products obtained from total RNAs of 15 gastric cancer cell lines by use of pairs of PS-N primers (PS-N), PS-C primers (PS-C), human PEBP2β/CBFB cDNA primers (PEBP2β), and human β-actin cDNA primers (β-actin), and the results of Southern blotting (PS-C ST). For the Southern blot analysis, RT-PCR products obtained by use of PS-C primers were hybridized with RUNX3 cDNA as a probe.

Fig. 6c shows the results of electrophoresis of RT-PCR products obtained from total RNAs of 17 lung cancer cell lines by use of pairs of PS-N primers (PS-N), PS-C primers (PS-C), and human β-actin cDNA primers (β-actin), to analyze the production of RUNX3 mRNA.

Fig. 7a is a schematic illustration showing restriction enzyme sites in the vicinity of RUNX3 exon 1 and the CpG frequency within typical CpG islands, with thick bars denoting the position of a DNA fragment used as a probe in genomic Southern blot analysis.

Fig. 7b is an autoradiogram resulting from Southern blotting analysis for DNA methylation in the vicinity of RUNX3 exon 1 in various gastric cancer cell lines by use of DNA probes which can detect parts of the CpG islands. The genomic DNAs were isolated from the cancer cell lines and treated with SmaI (upper), which can not digest methylated DNA, and BamHI (lower), which can digest DNA irrespective of DNA methylation.

Fig. 8 shows the results of electrophoresis of RT-PCR products obtained from total RNAs of NUGC3, MKN28, and MKN74 after treatment with 5-aza-2-deoxycytidine (AZA,300 nM) or trichostatin A (TSA, 1 mM), separately or in combination. The results indicate that the treatment with the DNA methyltransferase inhibitors or HDAC inhibitors allows the RUNX3 gene to be expressed.

DETAILED DESCRIPTION OF THE INVENTION

In the present invention, the role of RUNX3 in cells is elucidated. For this purpose, a sense RUNX3 cDNA or an antisense RUNX3 cDNA is introduced into cells to establish strains over-expressing sense or antisense RUNX3 RNA, respectively.

Therefore, in one aspect of the present invention, cell lines are provided that harbor a sense RUNX3 cDNA or an antisense RUNX3 cDNA to excessively express the RUNX3 protein and to selectively suppress the expression of the RUNX3 gene, respectively.

The human RUNX3 gene cloned by the present inventors in 1995 (Bae et al., Gene, 159 (2):245-248, 1995; cDNA nucleotide sequence GenBank Accession No. Z35278) was used to construct a plasmid vector capable of RUNX3 expression. The cDNA of the RUNX3 gene has the nucleotide sequence SEQ. ID. NO: 1. This cDNA has an open reading frame ranging from the translation start codon to the translation stop codon, and encodes a polypeptide consisting of 415 amino acids whose putative amino

acid sequence is SEQ. ID. NO: 2 (see Figs. 1a to 1c). A DNA fragment comprising CpG islands in the vicinity of exon 1 of the RUNX3 gene has the nucleotide sequence SEQ. ID. NO: 3 (see Figs. 2a and 2b).

A 2,244 bp DNA fragment comprising a coding region of the RUNX3 gene cDNA SEQ. ID. NO: 1 (see Fig. 1a) was inserted in the sense direction into a pEF-BOS vector (Mizushima et al., Nucleic Acids Res., 18(17):5322, 1990) to construct a recombinant plasmid vector, named pEF-BOS-Rx3, which expresses a sense RUNX3 gene. Separately, the RUNX3 cDNA was inserted in the antisense orientation into a pEF-BOS vector to construct a recombinant vector, named pEF-BOS-Rx3-AS.

After the plasmid vector pEF-BOS-Rx3 was co-transfected with another vector bearing a neo resistance selection marker into various cancer cell strains, stable transfectants over-expressing the sense RUNX3 RNA were selected. In a preferred embodiment of the present invention, the pEF-BOS-Rx3 plasmid and a plasmid with a neo selection marker were co-transfected into the MKN28 strain (which does not express a RUNX3 gene) using Lipofectamine (Gibco-BRL), and the selection of transfectants was achieved by means of G418 resistance. The MKN28 strain that shows over-expression of the sense RUNX3 gene was named MKN28-Rx3 and deposited with the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) under the deposition No. KCTC 0933BP on Jan. 9, 2001.

The strains that express the antisense RUNX3 RNA were established by co-transfecting the plasmid vector pEF-BOS-Rx3-

AS and a plasmid with a selection marker into cells of the SNU16 gastric cancer cell line (Kim et al., J. Natl. Cancer Inst., 8(13):938-943, 1991) which has an inactivated p53 gene and a normal TGF-β gene system (Park et al., Proc. Natl. Acad. Sci. U. S. A., 91 (10):8772-8776, 1994). Stable transfectants were obtained in the same manner as above. Two transfectants which expressed the antisense RUNX3 RNA in excess were named SNU16-Rx3-AS-c11 and SNU16-Rx3-AS-c12, and deposited with the Korean Collection for Type Culture of KRIBB under the deposition Nos. KCTC 0934BP and KCTC 0935BP on Jan. 9, 2001.

To determine whether the transfectants MKN28-Rx3, SNU16-Rx3-AS-c11, and SNU16-Rx3-AS-c12 actually express the sense RUNX3 gene or the antisense RUNX3 gene, the RUNX3 cDNA of SEQ. ID. NO: 1 was used as a DNA probe for Northern blotting analysis. A RUNX3 transcript from the MKN28-Rx3 strain was detected as a single band at a position almost identical to that of 18S RNA, verifying the expression of RUNX3 mRNA in the cell. In contrast, antisense RUNX3 RNAs of various sizes were detected from SNU16-Rx3-AS-c11 and SNU16-Rx3-AS-c12 cells. In the control SUN16 strain, the expression of the endogenous RUNX3 gene was hardly detected by Northern blotting analysis (see Fig. 3).

In another aspect of the present invention, a RUNX3 gene and its product, which show tumor suppressor activity, are provided.

In the present invention, the function of the RUNX3 gene is elucidated by using the MKN28-Rx3 strain, which expresses the RUNX3 gene, and the SNU16-Rx-AS-11c and the SNU16-Rx-AS-

12c strains, whose RUNX3 gene expression is selectively interrupted.

To analyze the role of RUNX3 in the TGF-β-induced programmed cell death (apoptosis), the SNU16-Rx3-AS-c11 and SNU16-Rx3-AS-c12 strains in which RUNX3 expression is selectively inactivated were treated with TGF-β1; normal SNU16 cells were used as a control. Counts of viable cells revealed that TGF-β1 induces apoptosis in the control strain, but not in the mutant strains. In detail, treatment with TGF-β1 killed all SNU16 cells within 2 days. However, treatment with TGF-β1 did not kill the SNU16-Rx3-AS-c11 and SNU16-Rx3-AS-c12 cells (see Fig. 4a). Accordingly, these results show that the RUNX3 gene is involved in the TGF-β1-induced cell death in SNU16 cells.

In the present invention, inventors also determine whether the mechanism of the RUNX3-dependent TGF-β1-induced cell death in SNU16 cells treated with TGF-β1 is via the apoptosis pathway or a necrosis pathway. Electrophoresis of DNA from cells killed through apoptosis yields specific apoptotic DNA bands. These bands are apparent in the DNA from SNU16 cells treated with TGF-β1 (see Fig. 4b Lanes 1 and 2). However, no apoptotic bands were observed in the DNA of the SNU16-Rx3-AS-c11 and SNU16-Rx3-AS-c12 cells (see Fig. 4b, Lanes 3 to 6). Therefore, TGF-β1 stimuli kill SNU16 cells through an apoptotic pathway, and RUNX3 is indispensable for this process.

It is known that TGF-β1-induced apoptosis is a very important mechanism for suppressing the progression of normal

cells toward cancer, and that most of the factors involved in the TGF-β pathway have tumor suppressor activity (Chang et al., Cancer Res., 57(14):2856-2859, 1997). To examine the anticancer activity of RUNX3, a tumorigenesis assay was performed using nude mice. For the tumor suppressor activity assay, the cell lines are introduced into nude mice by subcutaneous injection, after which the cancerous masses formed are periodically measured for their external sizes. After the mice are sacrificed on the final day, the cancerous masses are excised for comparison.

The tumors formed in the nude mice grew at a higher rate in the mice injected with the cells expressing SNU16-Rx3-AS-c11 or the SNU16-Rx3-AS-c12 than those in the mice injected with the control SNU16 cells (see Fig. 5a). In detail, the cancerous masses were measured on the final day. Those of the experimental group into which SNU16-Rx3-AS-c11 and SNU16-Rx3-AS-c12 had been injected were 1,600 mm^3 in volume, while those of the control group were about 800 mm^3. The cancerous masses were excised from the mice 98 days after the injection and compared to one another. The cancerous masses from the experimental group were remarkably larger than those from the control group (see Fig. 5c).

The tumorigenicity of control MKN28 cells and MKN28-Rx3 cells in which the sense RUNX3 RNA is expressed was also assayed, using nude mice in the manner described above for the SNU16 cells. Significantly reduced tumorigenesis was observed in the mice injected with MKN28-Rx3 cells compared to that of the mice injected with control MKN28 cells (see Fig. 5b). In

detail, the cancerous masses of the control group were 600 mm^3 in volume on the final day, in contrast to those of the cancerous masses of the MKN28-Rx3-injected experimental group, which were found to be as small as 250 mm^3. This represents a reduction of tumor growth of 58%. The cancerous masses were excised from the mice 30 days after the injection and compared to one another(Fig. 5c).

These results demonstrate that the RUNX3 gene has tumor suppressor activity.

In the present invention, the RUNX3 gene was analyzed for its expression pattern in various cancer cell lines. Total RNAs were isolated from 15 gastric cancer cell lines and 17 lung gastric cancer cell lines and used to determine whether these cell lines produce RUNX3 mRNAs, by use of RT-PCR. The gastric cancer cell lines used in this RT-PCR were SNU1, SNU5, SNU719, NUGC3, MKN1, MKN7, MKN28, MKN45, MKN74, AGS, KatoIII, RF1, RF48, and AZ521. The lung cancer cell lines were NCI-H522, 86-2, A549, LK79, LK87, LCSC#1, LCSC#2, NCI-H23, NCI-H226, NCI-460, NCI-H322, Sq-19, NCI-H1915, NCI-H630, Hs 888Lu, Lu65, and LX-1. These cell lines were obtained from the Korean Research Institute of Bioscience and Biotechnology (KRIBB).

RT-PCR was conducted using two primer pairs, named PS-N and PS-C (Fig. 1a). Among the gastric cancer cell lines, SNU5, SNU16, SNU719, MKN1, MKN45, RF1, RF48, MKN7, and AZ521 were found to produce RT-PCR products with expected sizes (1,080 bp with the PS-N primer pair and 870 bp with the PS-C primer

pair), although MKN7 produced RT-PCR products in very small quantities (Fig. 6b). In contrast, no RT-PCR products were obtained from the SNU1, NUGC3, MKN28, MKN74, AGS, and KatoIII cell lines when using either the PS-N primer pair or the PS-C primer pair.

In the case of the lung cancer cell lines, the expression of the RUNX3 gene was found to be suppressed significantly in the HS888Lu cell line and completely in NCI-H226, NCI-460, NCI-H630, and Lu65 cell lines (see Fig. 6c).

Southern blotting analysis was used to verify that the RT-PCR products were amplified from the cDNA of the RUNX3 gene (see Fig. 6b, PS-3 ST). Additionally, the amplification of PEBP2β (see Fig. 6b PEBP2β) and β-actin cDNAs (see Fig. 6b, β-actin) from identical first cDNA strands was observed, indicating that the lack of amplification of RUNX3 cDNAs in the gastric cancer cell lines SNU1, NUGC3, MKN28, MKN74, AGS, KatoIII, NCI-H226, NCI-460, NCI-H630, and Lu65 can be attributed to a lack of expression of the RUNX3 gene in those cell lines. Likewise, the low levels of the RT-PCR products in the MKN7 and Hs888Lu cell lines result from the low expression levels of the RUNX3 gene in the cell lines.

The above results show that the RUNX3 gene is remarkably or completely suppressed in about 47% of the gastric cancer cell lines and about 30% of the lung cancer cell lines. Therefore, the suppression of the RUNX3 gene expression can be an important diagnostic index in about 47% of gastric cancer cells and about 30% of lung cancer cells (in about 37% of the total number of gastric and lung cancer cell lines we

examined).

Mutation analysis of the RUNX3 gene in cancer cell lines was performed using all exons of the RUNX3 gene, amplified from various cancer cell lines. No point mutation affecting the amino acid sequence was found. These results led to the conclusion that cancer cell lines in which the expression of the RUNX3 gene was not detected contain a normal RUNX3 gene, but do not express it. To determine whether the suppression of the RUNX3 gene expression can be attributed to DNA methylation, the correlation between the expression of the RUNX3 gene and the hypermethylation of CpG islands at loci near RUNX3 exon 1 was analyzed through genomic DNA Southern blotting using restriction enzymes sensitive to DNA methylation. Genomic DNAs isolated from cancer cell lines were separately treated with restriction enzymes which are unable to cut methylated DNA and enzymes that are able to cut DNA irrespective of methylation.

In one preferred embodiment of the present invention, SmaI, which cannot digest methylated DNA, and BamH1, which can digest DNA irrespective of its methylation, were used. The DNA was specifically protected from SmaI digestion in the gastric cancer cell lines whose RUNX3 gene expression was not detected, that is, the SNU1, NUGC3, MKN28, MKN74, AGS, and KatoIII cell lines. The MKN7 cell line in which the expression level was very low showed a partially methylated pattern (see Fig. 7b). These results suggest that the expression of the RUNX3 gene is closely correlated with DNA

methylation at loci near RUNX3 exon 1 in cancer cell lines.

To examine further whether DNA methylation causes RUNX3 gene silencing, the influence of a DNA methyltransferase inhibitor and a histone deacetylase inhibitor on the reactivation of RUNX3 gene expression was analyzed. 5-aza-3-deoxycytidine (AZA, 300 nM) and trichostatin A (TSA, 1 mM) were used as DNA methylase and histone deacetylase inhibitors, respectively. Upon treatment with either the DNA methyltransferase inhibitor or the histone deacetylase inhibitor, the expression of the RUNX3 gene was induced (see Fig. 8). These results demonstrate that the hyper-methylation of CpG islands at loci near exon 1 of the RUNX3 gene is responsible for the suppression of RUNX3 gene expression in about 37% of the cancer cells. This result also indicates that agents capable of promoting the reactivation of the RUNX3 gene can be developed from DNA methyltransferase inhibitors or histone deacetylase inhibitors and used as anticancer agents.

According to the present invention, therefore, the product of the RUNX3 gene is involved in TGF-β1-dependent apoptosis and shows tumor suppressor activity. The expression of the RUNX3 gene is suppressed in a substantial portion of the cancer cells by the hyper-methylation of CpG islands at loci near exon 1 of the gene.

In a further aspect of the present invention, expression vectors are provided that are capable of expressing RUNX3 cDNA,

and pharmaceutical compositions are provided that comprise proteins of the genes as pharmaceutically effective ingredients.

The pharmaceutical compositions of the present invention can be used for the diagnosis, prophylaxis, and treatment of the diseases attributed to abnormal TGF-β-dependent apoptosis, including gastric and lung cancers.

As the pharmaceutically effective ingredients, RUNX3 cDNA, RUNX3 RNA, or proteins produced therefrom may be used. Expression vectors capable of expressing the RUNX3 gene are preferable because they can produce RUNX3 proteins continuously if they are injected. Where a pharmaceutical composition comprising such an expression vector is injected into a bio-tissue via a parenteral route, the bio-tissue can produce RUNX3. That is, the RUNX3 gene of the present invention or an expression vector anchoring the RUNX3 gene can be used as a pharmaceutically effective ingredient of a pharmaceutical composition for use in gene therapy.

Administration of the RUNX3 cDNA of the present invention and/or the RUNX3 RNA or the RUNX3 protein produced therefrom may take an oral or a parenteral route, with preference for parenteral injection.

The expression vector comprising the RUNX3 gene or the RUNX3 protein produced therefrom may be administered in various oral or parenteral dosage forms. For the formulation of the pharmaceutically useful ingredient, diluents, expedients, and/or carriers, such as fillers, thickeners, binders, wetting agents, disintegrating agents, surfactants,

etc., may be used. Solid formulations for oral administration may be formed into pills, tablets, powders, granules, capsules, etc. These solid formulations may comprise at least one expedient, such as starch, calcium carbonate, sucrose, lactose, gelatin, etc. In addition to simple expedients, lubricants such as magnesium stearate and talc may be used. Liquid formulations for oral administration may be exemplified by suspensions, internal solutions, emulsions, syrups, etc. These liquid formulations may comprise various expedients, for example, wetting agents, sweeteners, odorants, and preservatives, as well as simple diluents such as water, liquid paraffin, etc. Examples of formulations for parenteral administration include sterile aqueous solutions, non-aqueous solutions, suspensions, emulsions, freeze-dried agents, and suppositories. For the formation of non-aqueous solutions or suspensions, vegetable oils such as polypropylene glycol, polyethylene glycol, and olive oil, and an injectable ester such as ethyloleate may be used. As bases of suppositories, witepsol, microgol, Tween 61, cacao oil, lauringe, and glycerogelatin may be used. In order to help the absorption of the pharmaceutically useful ingredient into cells, liposomal dosage forms may be used.

The therapeutically effective dose of the pharmaceutically effective ingredients is within the range of 0.002 to 1 mg/kg of body weight and preferably within the range of 0.02 to 0.2 mg/kg of body weight for RUNX3 cDNA, within the range of 0.001 to 0.5 mg/kg of body weight and preferably within the range of 0.01 to 0.1 mg/kg of body

weight for RUNX3 RNA, and within the range of 0.04 to 4 mg/kg and preferably within the range of 0.4 to 1 mg/kg of body weight for RUNX3 protein.

In accordance with a further aspect of the present invention, uses of the RUNX3 gene and its proteins in the development of anticancer agents and the diagnosis of cancers are provided.

As described above, the RUNX3 gene of the present invention shows excellent tumor suppressor activity and is indispensable for TGF-β-dependent apoptosis. The expression of RUNX3 is suppressed in about 47% of gastric cancer cell lines and about 30% of lung cancer cell lines (in about 37% of the total number of gastric and lung cancer cell lines). In addition, the suppression of RUNX3 gene expression is attributed to abnormal DNA methylation of CpG islands located at loci near exon 1 of the RUNX3 gene. Further, treatment with a DNA methylase inhibitor or a histone deacetylase inhibitor can induce the expression of the RUNX3 gene. Therefore, the RUNX3 gene of the present invention and proteins produced therefrom can be utilized in developing not only anticancer agents, but also diagnostic methods for detecting cancers, in which abnormal DNA methylation of CpG islands at the loci near exon 1 of the RUNX3 gene is measured.

Based on the facts presented in the present invention, pharmaceutical compositions comprising materials capable of regulating the methylation of a nucleotide sequence that includes CpG islands near RUNX3 exon 1 can be provided for the

prophylaxis and treatment of cancers as pharmaceutically effective ingredients.

A method for diagnosing cancers characterized by detecting the methylation of the nucleotide sequence containing CpG islands present near exon 1 of the RUNX3 gene comprises the steps of:

isolating genomic DNA from a tumor tissue of a subject;

digesting the genomic DNA with a restriction enzyme sensitive to DNA methylation;

separating the genomic DNA digests on an agarose gel by electrophoresis and transferring them onto a membrane;

hybridizing the genomic DNA digests with a radiolabeled DNA probe for the RUNX3 gene; and

exposing the membrane to a film to detect the expression of the RUNX3 gene.

In accordance with another embodiment of the present invention, a method for diagnosing cancers comprises the steps of:

Isolating genomic DNA from blood or a tumor tissue of a subject;

digesting the genomic DNA with a restriction enzyme sensitive to DNA methylation;

performing a polymerase chain reaction (PCR) with the genomic DNA digests serving as templates and with parts of the nucleotide sequence of SEQ. ID. NO: 3 serving as primers;

separating the PCR products on an agarose gel by electrophoresis to detect the amplification of a DNA sequence of interest.

The diagnostic method using PCR is based on the principle that PCR products are obtained if the DNA region of interest is methylated; otherwise, the products are not obtained.

In accordance with a still further aspect of the present invention, a biological microchip for use in cancer diagnosis is provided, which comprises a part of the RUNX3 cDNA SEQ. ID. NO: 1 or proteins produced therefrom.

Generally, a biological microchip for the diagnosis of diseases, which is based on principles of molecular biology and electronic engineering, has tens to tens of thousands of DNAs or proteins integrated on a small chip. Thanks to the high level of integration, the biological microchip can be used to search for at least hundreds of genes or proteins within a short period of time. Therefore, the biological chip can be used to perform a search for novel genes or to diagnose diseases very rapidly and simply, compared to conventional bioengineering techniques. Biological chips can be classified into oligonucleotide chips and cDNA chips according to the genetic materials integrated thereon, and they can be fabricated according to use, for example, genes to be searched, diseases to be diagnosed, etc. Microchips using parts of the RUNX3 cDNA nucleotide sequence or antibodies against proteins produced from the RUNX3 cDNA fragments can be used for the diagnosis of various cancers, including gastric and lung cancers.

EXAMPLES

A better understanding of the present invention may be obtained in light of the following examples which are set forth to illustrate, but are not to be construed to limit, the present invention.

EXAMPLE 1: Establishment of the SNU16-Rx3-AS and MKN28-Rx3 Cell Strains

To elucidate the function of the RUNX3 gene in vivo, cell strains capable of over-expressing a sense RUNX3 gene and an antisense RUNX3 gene were established as follows.

1-1 Construction of expression plasmid vectors containing sense and antisense RUNX3 genes

The human RUNX3 gene cloned by the present inventors in 1995 (Bae et al., Gene, 159 (2):245-248, 1995; cDNA nucleotide sequence GenBank Accession No. Z35278) was used to construct plasmid vectors capable of expressing RUNX3. The cDNA of the RUNX3 gene has the nucleotide sequence SEQ. ID. NO: 1 with an open reading frame ranging from the translation start codon to the translation stop codon, which encodes a polypeptide consisting of 415 amino acids whose putative amino acid sequence is SEQ. ID. NO: 2 (Figs. 1a to 1c). A DNA fragment comprising CpG islands present in the vicinity of exon 1 of the RUNX3 gene has the nucleotide sequence SEQ. ID. NO: 3 (Figs. 2a and 2b).

A 2,244 bp BamHI DNA fragment (Fig. 1a) comprising a coding region out of the cDNA of the RUNX3 gene SEQ. ID. NO: 1 was inserted in the sense direction into a pEF-BOS vector (Mizushima et al., Nucleic Acids Res., 18(17):5322, 1990) at an XbaI site to construct a recombinant plasmid vector, named pEF-BOS-Rx3, which expresses a sense RUNX3 gene.

Separately, the RUNX3 cDNA was inserted in the antisense direction into a pEF-BOS vector at an XbaI site to construct a recombinant vector, named pEF-BOS-Rx3-AS.

1-2 Stable transfection

The plasmid vector pEF-BOS-Rx3, established in Example 1-1, and pcDNA3.1, which contains a neo selection marker, were co-transfected into the MKN28 strain by the Lipofectamine (Gibco BRL) method as specified by the manufacturer. MKN28 does not endogenously express RUNX3. Stable transfectants that harbored pEF-BOS-Rx3 (sense RUNX3 RNA) were selected in the presence of G418. The MKN28 strain overexpressing sense RUNX3 RNA was named MKN28-Rx3 and deposited with the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) under the deposition No. KCTC 0933BP on Jan. 9, 2001.

The strains capable of expressing the antisense RUNX3 gene were established by co-transfecting the plasmid vector pEF-BOS-Rx3-AS along with pcDNA3.1, which contains a selection marker, into the SNU16 strain, a gastric cancer cell line (Kim et al., J. Natl. Cancer Inst., 8(13):938-943, 1991) that has an inactivated p53 gene and a normal TGF-β signaling system

(Park et al., Proc. Natl. Acad. Sci. U. S. A., 91 (10):8772-8776, 1994). Stable transfectants were obtained in the same manner as above. Two transfectants that excessively express the antisense RUNX3 gene to selectively inhibit the expression of the RUNX3 gene were named SNU16-Rx3-AS-c11 and SNU16-Rx3-AS-c12, and deposited with the Korean Collection for Type Culture of KRIBB under the deposition Nos. KCTC 0934BP and KCTC 0935BP on Jan. 9, 2001.

For comparison, pcDNA3.1 was transfected alone into SUN16 and MKN28, which were cultured in the presence of G418. The clones obtained were used as controls, named control-SNU16 and control-MKN28.

1-3 Northern blot analysis

To determine whether the transfectants MKN28-Rx3, SNU16-Rx3-AS-c11, and SNU16-Rx3-AS-c12, established in Example 1-2, express the sense RUNX3 gene or the antisense RUNX3 gene in large quantities, Northern blotting analysis was conducted.

Total cellular RNA was prepared from each cell strain according to a standard method (Sambrook et al., Molecular Cloning, a laboratory manual, 2nd Ed., 1988, Cold Spring Harbor Laboratory). Five micrograms of each RNA preparation was separated on a 1.2% formaldehyde/agarose gel by electrophoresis and transferred onto a Hybond-N^+ nylon membrane (Amersham). The RUNX3 cDNA of SEQ. ID. NO: 1 (Bae et al., Gene, 159 (2):245-248, 1995) was labeled with [α-^{32}P]dCTP for use as a probe for blotting analysis. Hybridization was conducted by incubating the Hybond-N^+ nylon membrane and the

radiolabeled RUNX3 DNA probe at 65°C for 16 hours in 5x Denhardt's solution comprising 5x SSPE, 0.5% SDS and 100 mg/ml of salmon sperm. Afterwards, the nylon membrane was washed twice with 2x SSPE/0.1% SDS solution at room temperature for 15 min and twice with 0.1x SSPE/0.1% SDS solution at 65°C for 15 min. The membrane was exposed to Kodak XAR-5 film for 16 hours at -70°C to yield an autoradiogram.

On the autoradiogram obtained from the MKN28-Rx3 strain, a single band was observed at a position close to that of 18S RNA, verifying the expression of the RUNX3 cDNA in the strain. In contrast, SNU16-Rx3-AS-c11 and SNU16-Rx3-AS-c12 cells, which harbor the antisense RUNX3 cDNA, were found to give antisense RUNX3 gene transcripts of various sizes as detected by multiple bands on the autoradiogram. In the control-SUN16 strain, the expression of the endogenous RUNX3 gene was not detected by Northern blotting analysis (see Fig. 3).

EXAMPLE 2: Cell Death Effect of TGF-β and Role of the RUNX3 Gene In TGF-β-Induced Cell Death

2-1 Cell death effect of TGF-β

SNU16 cells, which normally express the RUNX3 gene, and SNU16-Rx3-AS-c11 and SNU16-Rx3-AS-c12 cells in which an antisense RUNX3 cDNA is over-expressed to selectively inhibit the expression of the normal RUNX3 gene, were individually treated with TGF-β1 (1 ng/ml), after which viable cells were counted over a period of time. The results are shown in Fig. 4a.

As seen in Fig. 4a, the TGF-β1 treatment induced cell death in SNU16 cells. Almost all SNU16 cells were killed within 2 days after treatment with TGF-β1 at a concentration of 1 ng/ml. However, the SNU16-Rx3-AS-c11 cells, in which the antisense RUNX3 gene was excessively expressed to selectively inhibit the expression of the RUNX3 gene, were not killed after treatment with TGF-β1. These results suggest that the RUNX3 gene is involved in TGF-β1-induced cell death in SNU16 cells.

2-2 Role of RUNX3 gene in TGF-β induced cell death.

Cells dying through the programmed cell death pathway reveal a distinct pattern of DNA bands when electrophoresed in an agarose gel. After being treated with 1 ng/ml TGF-β1 for 6 hours, the control-SNU16, SNU16-Rx3-AS-c11, and SNU16-Rx3-AS-c12 strains were lysed to isolate genomic DNAs according to a standard method (Sambrook et al., 1988, Molecular Cloning: a laboratory manual, 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA). 0.2 μg of each of the genomic DNA preparations was electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide under UV light. The results are given in Fig. 4b.

As seen in this figure, apparent programmed cell death-specific apoptotic DNA bands were formed from the SNU16 cells treated with TGF-β1 (fig 4b, lanes 1 and 2). No apoptotic bands were observed in the DNA from SNU16-Rx3-AS-c11 and SNU16-Rx3-AS-c12 cells, both of which over-express the antisense RNX3 gene, and therefore have a selective reduction

in RUNX3 expression (fig 4b, lanes 3 to 6).

We concluded from these results that SNU16 cells are killed by TGF-β1 stimuli through an apoptotic pathway and that RUNX3 is required for the process.

EXAMPLE 3: Suppressive Activity of RUNX3 Against Tumorigenicity

It is known that TGF-β1-induced apoptosis is a very important mechanism for suppressing the progression of normal cells toward cancer and that most of the factors in the TGF-β1 pathway have tumor suppressor activity. The RUNX3 gene was assayed for tumor suppressor activity using nude mice, with the expectation that an anticancer effect would be seen.

To this end, the SNU16-Rx3-AS-c11 and SNU16-Rx3-AS-c12 strains, in which RUNX3 gene expression is selectively inhibited and the control-SUN16 strain were suspended in 0.85% phosphate buffered saline (PBS) at a density of $3x10^7$ cells/ml, and 0.3 ml of each suspension was subcutaneously injected into groups of nude mice. Separately, the MKN28-Rx3 strain that shows over-expression of the sense RUNX3 RNA and the control-MKN28 strain were suspended in 0.85% PBS at a density of $5x10^7$ cells/ml, and 0.3 ml of each suspension was subcutaneously injected into other groups of nude mice. Each strain clone was administered to 9 nude mice, which constituted one group. The tumorigenicity of MKN28-Rx3 and control MKN-28 strains was observed for 37 days after the injection. The tumorigenicity of SNU16-Rx3-AS-c11, SNU16-Rx3-AS-c12, and control-SNU16 cells

was monitored for 98 days. During these time periods, the cancerous masses that formed were periodically measured for their external sizes. On the final days, the mice were sacrificed to excise the cancerous masses for comparison.

It was observed that tumors developed at higher rates in the mice into which the SNU16-Rx3-AS-c11 or the SNU16-Rx3-AS-c12 cells were injected than the mice into which the control SNU16 cells were injected, as shown in Fig. 5a. In detail, cancerous masses formed in the control SNU16 cell-injected group were measured to be 800 mm^3 in volume at the final day. In contrast, cancerous masses of the groups into which SNU16-Rx3-AS-c11 and SNU16-Rx3-AS-c12 were injected were measured at 1,600 mm^3 in volume, twice as large as those of the control group. Ninety-eight days after the injection, the cancerous masses were excised from the mice and compared to one another. The cancerous masses from the experimental group were remarkably larger than those from the control group, as seen in Fig. 5c.

MKN28-Rx3 cells, in which the sense RUNX3 gene is expressed, and control MKN28 cells were also assayed for tumor suppressor activity using nude mice in the same manner as was used for the SNU16 cells. Mice injected with MKN28-Rx3 cells exhibited significantly reduced tumorigenesis compared to the mice injected with the control MKN28 cells, as shown in Fig. 5b. In detail, the cancerous masses of the control group were measured at 600 mm^3 in volume on the final day. In contrast, the cancerous masses of the MKN28-Rx3-injected experimental group were found to be as small as 250 mm^3, demonstrating that

the tumor growth was reduced by 58%. Thirty days after the injection, the cancerous masses were excised from the mice and compared to one another. The cancerous masses excised from the MKN28-Rx3-injected experimental group were macroscopically smaller than those excised from the control group (Fig. 5c).

Taken together, these results demonstrate that the RUNX3 gene has tumor suppressor activity.

EXAMPLE 4: Analysis of the Expression Pattern of the RUNX3 Gene in Cancer Cells

4-1 RT-PCR analysis

The expression pattern of the RUNX3 gene was analyzed in various cancer cell lines by RT-PCR. Total RNAs were isolated from 15 gastric cancer cell lines and 17 lung cancer cell lines and used to determine whether these cell lines produce RUNX3 mRNAs. The gastric cancer cells used in this analysis were SNU1, SNU5, SNU719, NUGC3, MKN1, MKN7, MKN28, MKN45, MKN74, AGS, KatoIII, RF1, RF48, and AZ521. The lung cancer cell lines were NCI-H522, 86-2, A549, LK79, LK87, LCSC#1, LCSC#2, NCI-H23, NCI-H226, NCI-460, NCI-H322, Sq-19, NCI-H1915, NCI-H630, Hs 888L u, Lu65, and LX-1. All were obtained from the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB).

Total RNA was isolated from each cancer cell line, according to a standardized single-step guanidium method (Sambrook et al., Molecular Cloning: a laboratory manual, 2nd Ed., 1988, Cold Spring Harbor Laboratory). Using a

Superscript kit (Gibco BRL) according to the manufacturer's protocol, reverse transcription was performed to prepare cDNA from the isolated total RNA with oligo-dTs serving as primers. Based on the cDNA, the RUNX3 gene was amplified by RT-PCR to determine the production of RUNX3 mRNA. For this RT-PCR, two pairs of PCR primers, PS-N and PS-C, were synthesized, which were composed of a set of the sense primer PS-NA SEQ. ID. NO: 4 and the antisense primer PS-NB SEQ. ID. NO: 5 and a set of the sense primer PS-CA SEQ. ID. NO: 6 and the antisense primer PS-CB SEQ. ID. NO: 7. The nucleotide sequences of these two pairs of PCR primers were designed on the basis of the cDNA nucleotide sequence of the RUNX3 gene SEQ. ID. NO: 1 (Fig. 5a).

For comparison, PEBP2β/CBFB cDNA and β-actin cDNA were used as controls in the RT-PCR. To amplify the controls, a primer pair for PEBP2β/CBFB, consisting of the sense primer h-β5 SEQ. ID. NO: 8 and the antisense primer h-β3 SEQ. ID. NO:9, and a primer pair for β-actin, consisting of the sense primer h-actin 5 SEQ. ID. NO: 10 and the antisense primer h-actin 3 SEQ. ID. NO: 11, were synthesized. Nucleotide sequences of these primer pairs were designed on the basis of human PEBP2β cDNA and β-actin cDNA nucleotide sequences.

In a thermal cycler, such as "Model 9600" manufactured by Perkin-Elmer Cetus, RT-PCR was started with a 95°C pre-denaturation of 50 μl of each PCR solution for 2 min and carried out with 30 cycles of denaturing at 95°C for 15 sec, annealing at 50°C for 1 min, and extending at 72°C for 1 min. After completion of the RT-PCR, 5 μl of the RT-PCR product was electrophoresed on a 1.2% agarose gel and dyed with ethidium

bromide for observation under UV light.

Among the gastric cancer cell lines, SNU5, SNU16, SNU719, MKN1, MKN45, RF1, RF48, MKN7, and AZ521 were found to produce RT-PCR products with the expected sizes (1,080 bp with the PS-N primer pair and 870 bp with the PS-C primer pair). However, MKN7 produced RT-PCR products in very small quantities, as shown in Fig. 6b. No RT-PCR products were obtained from the SNU1, NUGC3, MKN28, MKN74, AGS, and KatoIII cell lines when using either the PS-N primer pair or the PS-C primer pair.

Among the 17 lung cancer cell lines, expression of the RUNX3 gene was suppressed significantly in the HS888Lu cell line and completely in the NCI-H226, NCI-460, NCI-H630, and Lu65 cell lines, as shown in Fig. 6c.

4-2 DNA Southern blot analysis

Southern blotting analysis was conducted to verify the amplification of the RT-PCR products from the cDNA of the RUNX3 gene. The RT-PCR products were run on a 1.5% agarose gel under an electric field and the separated DNA fragments were transferred onto a Hybond-N^+ nylon membrane.

Using the 2,244 bp BamHI fragment of the RUNX3 cDNA as a probe, hybridization was conducted in the same manner as in the Northern blotting analysis of Example 1-3, followed by autoradiography on XAR-5 films. The results are given in Fig. 6b.

As apparent from the autoradiogram, the RT-PCR products were amplified from the RUNX3 cDNA (PS-3 ST). In addition, the amplification of PEBP2β and β-actin cDNAs from identical

first cDNA strands was observed, indicating that the lack of amplification of RUNX3 cDNAs in the gastric cancer cell lines SNU1, NUGC3, MKN28, MKN74, AGS, KatoIII, NCI-H226, and lung cancer cell lines, NCI-460, NCI-H630, and Lu65 can be attributed to a lack of expression of the RUNX3 gene in those cell lines. Likewise, the low levels of the RT-PCR products in the MKN7 and Hs888Lu cell lines result from the low expression levels of the RUNX3 gene in those cell lines.

From the above results, it is apparent that the RUNX3 gene is remarkably or completely suppressed in about 47% of the gastric cancer cell lines and about 30% of the lung cancer cell lines. Because TGF-β-induced apoptosis does not occur and the tumorigenicity of cancer cells is increased in the substantial absence of RUNX3, the suppression of RUNX3 gene expression can be an important diagnostic index in about 47% of gastric cancer cells and about 30% of lung cancer cells (in about 37% of the total cancer cells examined).

EXAMPLE 5: Analysis of DNA Methylation Around Exon 1 of the RUNX3 Gene In Cancer Cell Lines

5-1 Analysis of DNA methylation by Southern blotting analysis of genomic DNA

To determine whether the suppression of RUNX3 gene expression was caused by DNA methylation, correlation between the expression of the RUNX3 gene and the hypermethylation of CpG islands at loci near RUNX3 exon 1 was analyzed through genomic DNA Southern blotting using restriction enzymes

sensitive to DNA methylation.

Genomic DNAs were isolated from cancer cell lines according to a standardized SDS/protease K method (Sambrook et al., 1989). The isolated genomic DNAs were treated with a restriction enzyme that is unable to cut methylated DNA (SmaI), and separately, with one that is able to cut DNA irrespective of methylation (BamHI). The restriction enzyme digests were electrophoresed on a 1.5% agarose gel and then transferred onto a Hybond-N^+ membrane. A genomic DNA NheI/MluI fragment comprising CpG islands of the RUNX3 gene was used as a DNA probe (Fig. 7a), and hybridization was carried out in the same manner as in the Northern blotting analysis of Example 1-3, followed by autoradiography on XAR-5 films. The genomic DNA was isolated from human genome DNA libraries with the RUNX3 cDNA of SEQ. ID. NO: 1 serving as a probe.

The SmaI digestion was observed to be greatly reduced specifically in those gastric cancer cell lines whose RUNX3 gene expression was not detected, that is, in the SNU1, NUGC3, MKN28, MKN74, AGS, and KatoIII cell lines, as shown in Fig. 7b. The MKN7 cell line in which the expression level was significantly low showed a partially methylated pattern (Fig. 7b). These results indicate that the expression of the RUNX3 gene is closely correlated with the level of DNA methylation at loci near RUNX3 exon 1 in cancer cell lines. Since RUNX3 gene-specific bands are detected in all of the cell lines despite differences in methylation, it can be concluded that some cancer cell lines in which the expression of the RUNX3 gene is not detected under normal conditions have the normal

RUNX3 gene, but do not express it due to abnormal methylation.

5-2 Reactivation of the RUNX3 gene

To determine whether the DNA methylation observed in Example 5-1 is the direct cause of the inactivation of the RUNX3 gene, the influence of a DNA methyltransferase inhibitor and a histone deacetylase inhibitor on the reactivation of the RUNX3 gene expression was analyzed.

The NUGC3, MKN28, and MKN74 cell lines (selected randomly from the cell lines showing no RUNX3 gene expression by RT-PCR analysis) were treated with 5-aza-3-deoxycytidine (AZA, 300 nM), which inhibits DNA methyltransferase, and trichostatin A (TSA, 1 mM), which inhibits histone deacetylase, alone or in combination. After 3 days of the treatment, total RNA was isolated from each cell line in accordance with the same standardized single-step guanidium method used in Example 4-1, and then analyzed for the expression of the RUNX3 gene by RT-PCR using the PS-N primer pair of SEQ. ID. NOS: 4 and 5.

Upon treatment with the DNA methyltransferase inhibitor or the histone deacetylase inhibitor, the expression of the RUNX3 gene was observed, as seen in Fig. 8. These results reveal that hyper-methylation suppresses the expression of the RUNX3 gene in about 37% of the gastric and lung cancer cells owing to the hyper-methylation of CpG islands at loci near the exon 1 of the RUNX3 gene.

The expression plasmid capable of expressing the sense RUNX3 gene, constructed in Example 1-1, was assayed for acute toxicity as follows.

EXAMPLE 6: Acute Toxicity Test upon Parenteral Administration of pEF-BOS-Rx3 to Rat

An acute toxicity test was conducted using rats of a specific pathogen-free SD lineage at 6 weeks of age. pEF-BOS-Rx3 (prepared as described in Example 1-1) was suspended in 1 ml of a physiological saline solution and administered to the anterior tibialis of two rats at a dose of 1 mg/kg by intramuscular injection. After the injection, the rats were observed for death, clinical traits, and body weight changes, and serological and serobiochemical analyses were conducted. In addition, the rats were sacrificed in order to observe the abdominal and thoracic organs. In neither animal were specifically noteworthy clinical traits found. No animals died from the administration of the test material, and no changes were observed in the body weight. The serological assay, serobiochemical assay, and autopsy test were all normal. Accordingly, pEF-BOS-Rx3 was identified as safe, with an LD_{50} of at least 1 mg/kg upon parenteral injection.

INDUSTRIAL APPLICABILITY

As described previously herein, the RUNX3 gene of the present invention shows excellent tumor suppressor activity and is indispensably involved in TGF-β-dependent programmed cell death (apoptosis). Additionally, the suppression of RUNX3 gene expression is attributed to abnormal DNA

methylation of CpG islands located near exon 1 of the RUNX3 gene. Accordingly, the RUNX3 gene and its products (RNA and proteins) can be utilized for the development of gene therapy for cancers. Measurement of the abnormal DNA methylation in the vicinity of exon 1 of the RUNX3 gene is useful for diagnosis of cancers. In addition, chemicals capable of regulating the abnormal DNA methylation and its consequences (for examples DNA methyltransferase inhibitors and HDAC inhibitors) can be used as anticancer agents because of their ability to promote the expression of the RUNX3 gene.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : BAE, Suk-Chul
Department of Biochemistry, College of Medicine, Chungbuk National University,
S48 Gaeshin-dong, Heungduk-ku, Cheongju-si, Chungbuk 361-763
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: **MKN28-Rx3**	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **KCTC 0933BP**
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: [x] a scientific description [ ] a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **January 09 2001.**	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s): Bae K.S. BAE, Kyung Sook, Director Date: **January 26 2001**

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

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TO : BAE, Suk-Chul
Department of Biochemistry, College of Medicine, Chungbuk National University,
S48 Gaeshin-dong, Heungduk-ku, Cheongju-si, Chungbuk 361-763
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: **SNU16-Rx3-AS-c11**	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **KCTC 0934BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

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BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

Issued pursuant to Rule 7

TO : BAE, Suk-Chul
Department of Biochemistry, College of Medicine, Chungbuk National University,
S48 Gaeshin-dong, Heungduk-ku, Cheongju-si, Chungbuk 361-763
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: **SNU16-Rx3-AS-c12**	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **KCTC 0935BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

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WHAT IS CLAIMED IS:

1. A human RUNX3 gene with tumor suppressor activity, composed of a cDNA nucleotide sequence SEQ. ID. NO: 1.

2. An expression plasmid, which has the RUNX3 gene of claim 1 inserted in the sense direction thereto and allows a sense RUNX3 gene to be expressed.

3. An expression plasmid, which has the RUNX3 gene of claim 1 inserted in the antisense direction thereto and allows an antisense RUNX3 gene to be expressed.

4. A cell strain, which harbors the expression plasmid of claim 3 therein and shows the over-expression of the RUNX3 gene of claim 1.

5. A cell strain as set forth in claim 5, wherein the cell strain is MKN28-Rx3 (Deposition No. KCTC0933BP) which is established by transfecting the expression plasmid of claim 3 into the MKN28 strain.

6. A cell strain that harbors the expression vector of claim 4 and is selectively interrupted in expressing a RUNX3 gene.

7. The cell strain as set forth in claim 7, wherein the cell strain which selectively inhibnits the expression of the RUNX3 gene, is SNU16-Rx3-AS-c11 (Deposition NO: KCTC 0934BP) established by transfecting the expression plasmid of claim 4 into the SNU16 strain.

8. The cell strain as set forth in claim 7, wherein the cell strain which selectively inhibits the expression of the RUNX3 gene, is SNU16-Rx3-AS-c12 (Deposition NO: KCTC 0935BP) established by transfecting the expression plasmid of claim 4 into the SNU16 strain.

9. A pharmaceutical composition as an anticancer agent, comprising the expression plasmid of claim 3.

10. A pharmaceutical composition as an anticancer agent, comprising the RUNX3 gene of claim 1.

11. A pharmaceutical composition as an anticancer agent, comprising the RUNX3 cDNA having the nucleotide sequence SEQ. ID. NO: 1.

12. A pharmaceutical composition as an anticancer agent, comprising the RUNX3 RNA transcribed from the RUNX3 gene of claim 1.

13. A pharmaceutical composition as an anticancer agent, comprising the RUNX3 protein of claim 2.

14. A pharmaceutical composition as an anticancer agent, comprising a material capable of regulating the histone deacethylase activity and methylation of a nucleotide sequence SEQ. ID. NO: 3, containing CpG islands in the vicinity of the exon 1 of an RUNX3 gene.

15. A method for diagnosing cancers, characterized by detecting the methylation of a nucleotide sequence SEQ. ID. NO: 3, containing CpG islands present near exon 1 of the RUNX3 gene, comprising the steps of:

isolating genomic DNA from a tumor tissue of a subject;

digesting the genomic DNA with a restriction enzyme sensitive to DNA methylation;

separating the genomic DNA digests on an agarose gel by electrophoresis and transferring them onto a membrane;

hybridizing the genomic DNA digests with a radiolabeled DNA probe for a RUNX3 gene; and

exposing the membrane to a film to detect the expression of the RUNX3 gene.

16. A method for diagnosing cancers, characterized by detecting the methylation of a nucleotide sequence SEQ. ID. NO: 3, containing CpG islands present near exon 1 of the RUNX3 gene, comprising the steps of:

Isolating genomic DNA from the blood or tumor tissue of a subject;

digesting the genomic DNA with a restriction enzyme

sensitive to DNA methylation;

performing a polymerase chain reaction (PCR) with the genomic DNA digests serving as templates and with parts of the nucleotide sequence of SEQ. ID. NO: 3 serving as primers;

separating the PCR products on an agarose gel by electrophoresis to detect the amplification of a DNA sequence of interest.

17. A biological microchip for the diagnosis of cancers, using parts of the RUNX3 cDNA SEQ. ID. NO: 1 or an antibody against a RUNX3 protein produced from the cDNA.