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(54) **USE OF IN1-GHRELIN FOR THE DIAGNOSIS OF PROSTATE CANCER**

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(57) **ABSTRACT**

The present invention relates to a diagnostic and/or prognostic method for the diagnosis and/or prognosis of cancer in a patient. Particularly, the present invention relates to diagnostic and/or prognostic methods for the diagnosis and/or prognosis of cancer which comprise quantifying the expression levels of In1-ghrelin in a plasma sample isolated from an individual; comparing the obtained levels with the expression levels of In1-ghrelin in a reference sample; and assigning the patients having expression levels of In1-ghrelin that are greater than and statistically significant with respect to the levels of the reference sample to the group of individuals at risk of having cancer. The present invention also provides a diagnostic method for the diagnosis of bladder and/or colon cancer which comprises quantifying the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in tissue samples. The present invention also relates to an siRNA useful in a cancer treatment method.

Specification includes a Sequence Listing.

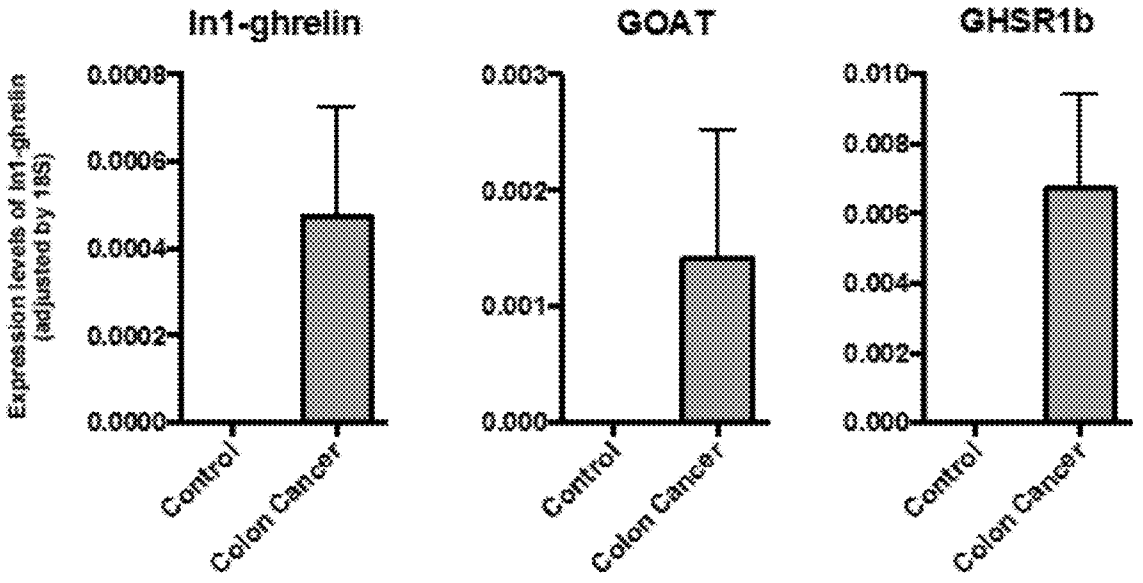


Figure 1

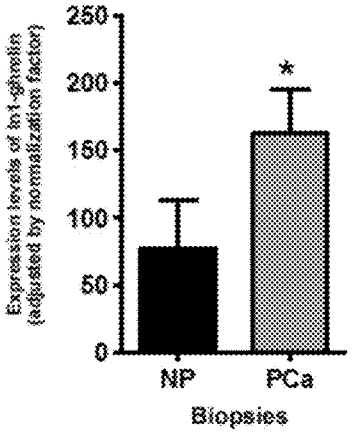


Figure 2

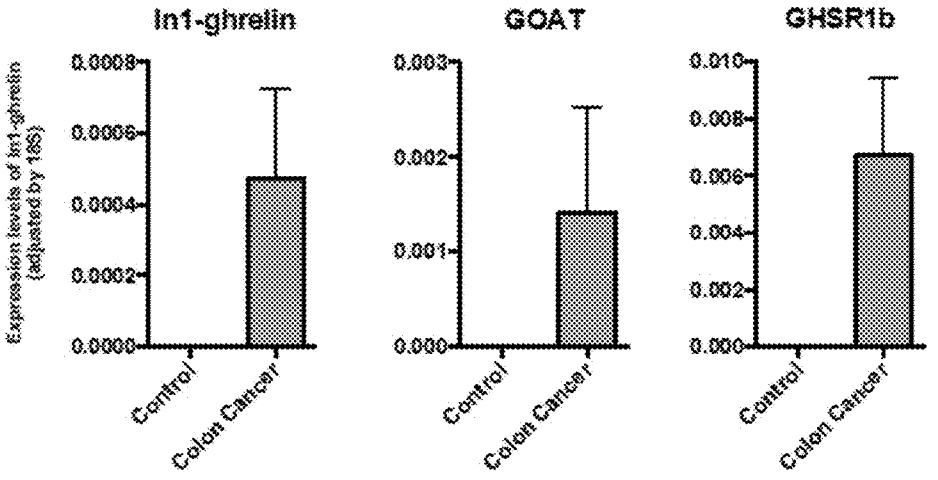


Figure 3

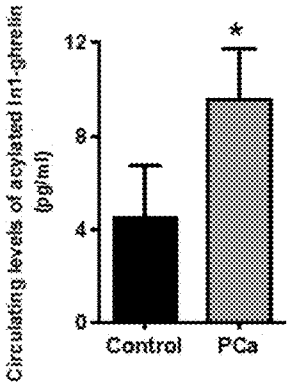


Figure 4

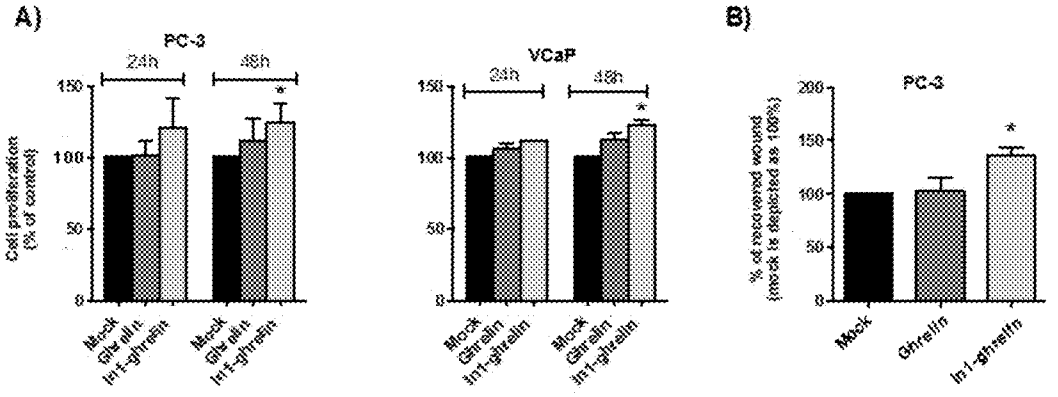


Figure 5

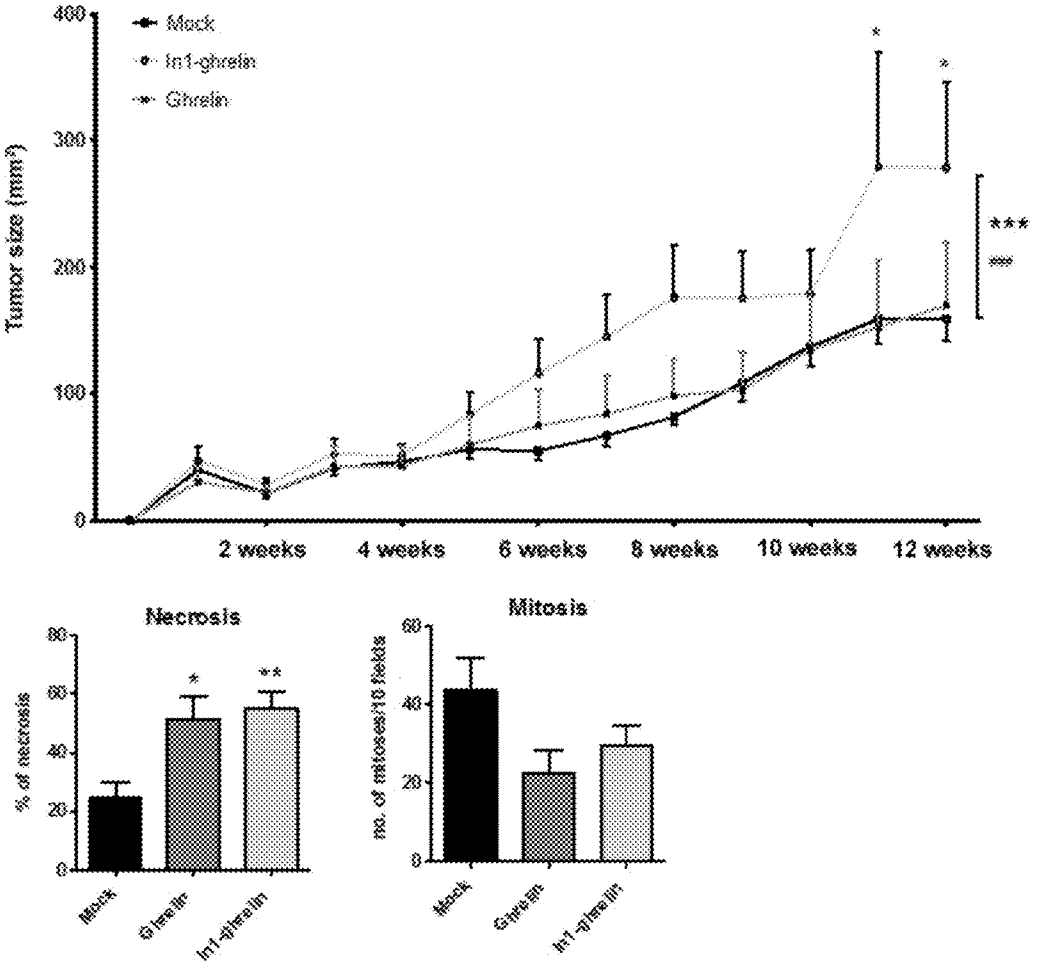

Mock
In1-ghrelin
Ghrelin
Tumor size (mm³)
400
300
200
100
0
2 weeks
4 weeks
6 weeks
8 weeks
10 weeks
12 weeks
*
*
***
Necrosis
% of necrosis
80
60
40
20
0
*
**
Mock
Ghrelin
In1-ghrelin
Mitosis
no. of mitoses/10 fields
60
40
20
0
Mock
Ghrelin
In1-ghrelin

Figure 6

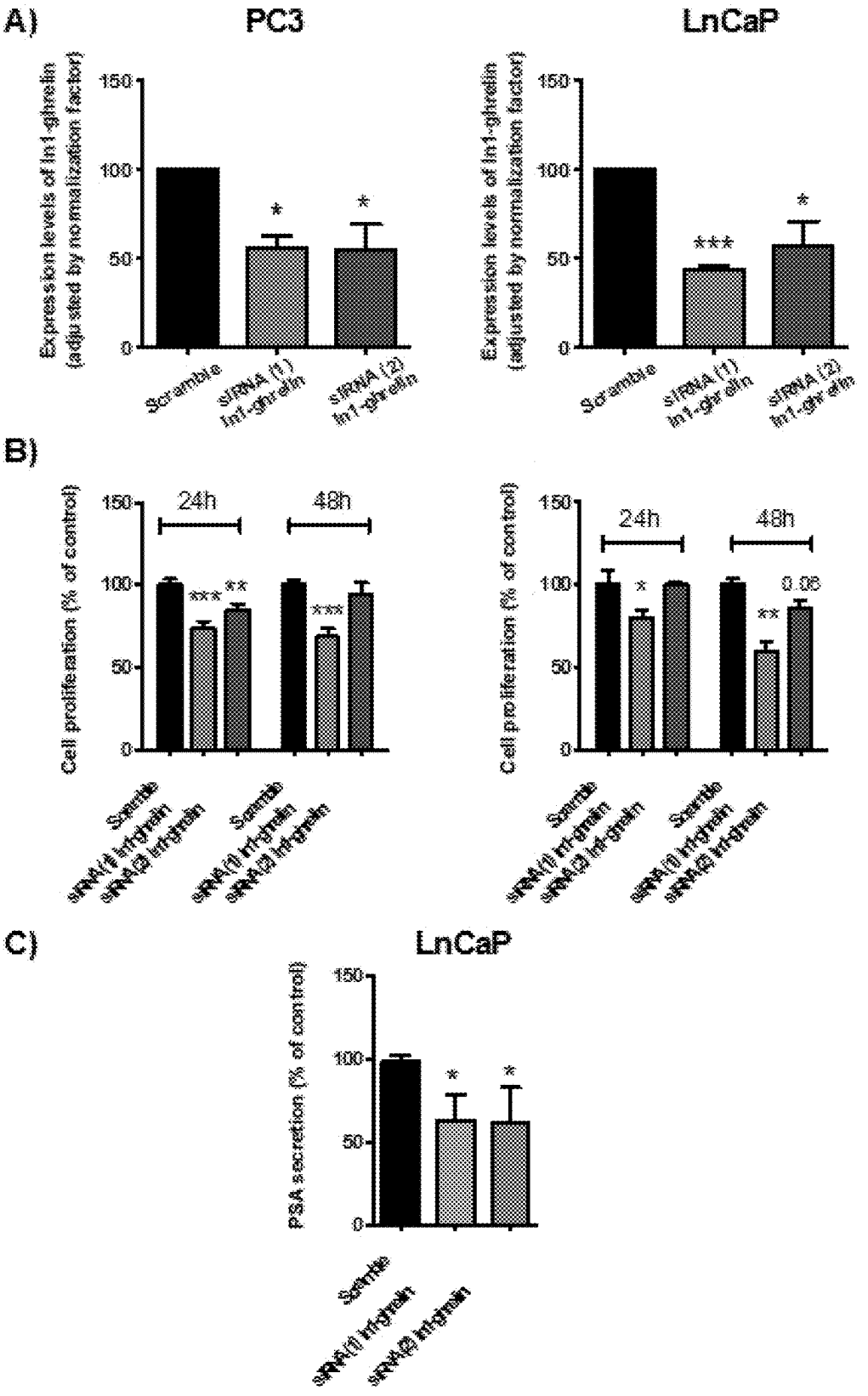
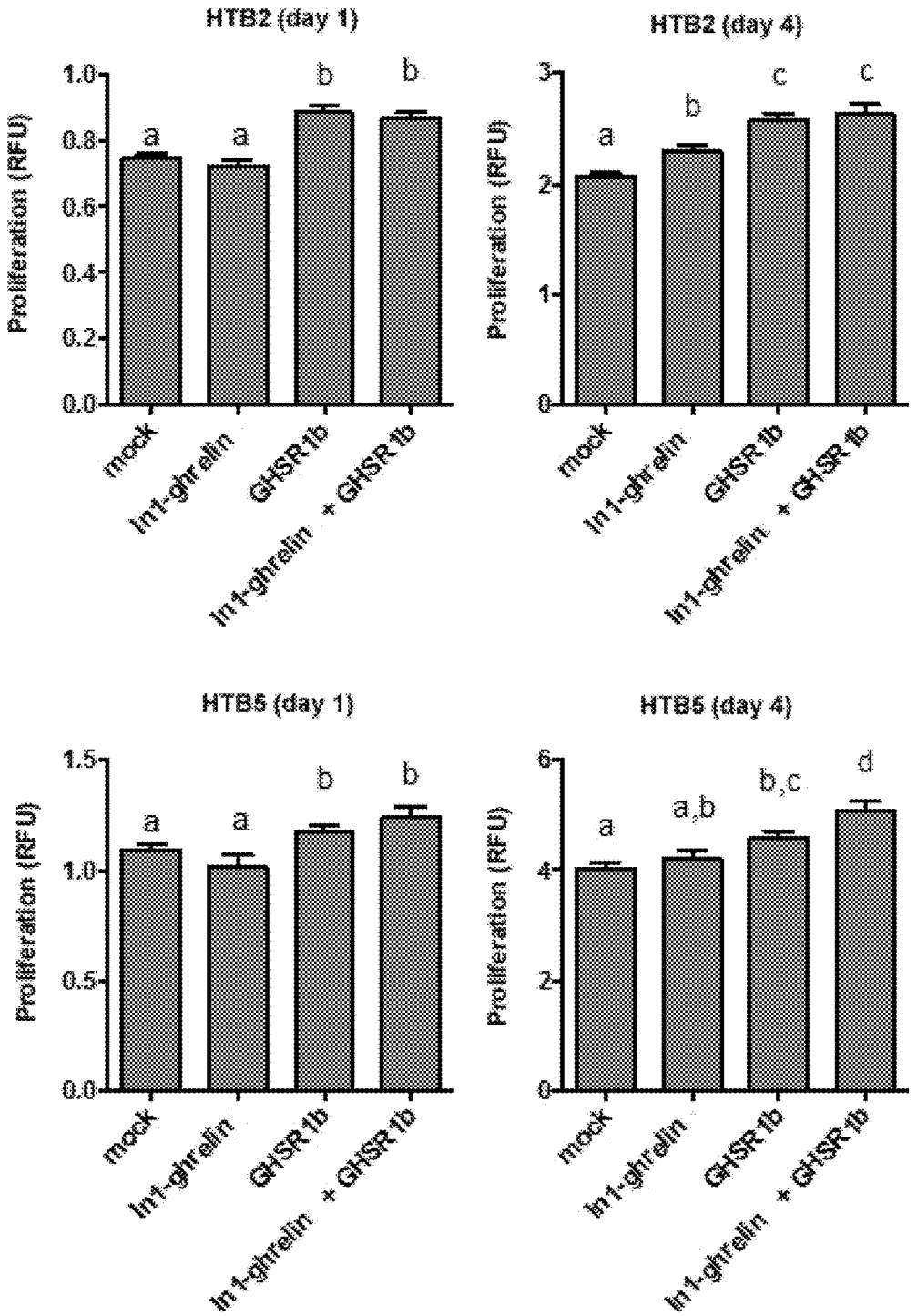

A)
PC3
LnCaP
Expression levels of ln1-ghrelin (adjusted by normalization factor)
150
100
50
0
*
***
Scramble
siRNA (1) ln1-ghrelin
siRNA (2) ln1-ghrelin
B)
Cell proliferation (% of control)
24h
48h
**
0.06
C)
PSA secretion (% of control)

Figure 7


HTB2 (day 1)
Proliferation (RFU)
1.0
0.8
0.6
0.4
0.2
0.0
a
a
b
b
mock
ln1-ghrelin
GHSR1b
ln1-ghrelin + GHSR1b
HTB2 (day 4)
Proliferation (RFU)
3
2
1
0
a
b
c
c
mock
ln1-ghrelin
GHSR1b
ln1-ghrelin + GHSR1b
HTB5 (day 1)
Proliferation (RFU)
1.5
1.0
0.5
0.0
a
a
b
b
mock
ln1-ghrelin
GHSR1b
ln1-ghrelin + GHSR1b
HTB5 (day 4)
Proliferation (RFU)
6
4
2
0
a
a,b
b,c
d
mock
ln1-ghrelin
GHSR1b
ln1-ghrelin + GHSR1b

USE OF IN1-GHRELIN FOR THE DIAGNOSIS OF PROSTATE CANCER

FIELD OF THE ART

[0001] The present invention is comprised within the field of biomedicine and provides, in particularly, a non-invasive diagnostic method for the diagnosis of cancer which comprises quantifying the expression levels of In1-ghrelin in plasma, serum, blood and/or urine. The present invention also provides a diagnostic method for the diagnosis of bladder and/or colon cancer which comprises quantifying the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in tissue samples. Furthermore, the present invention provides small interfering RNAs (siRNAs) for use in a cancer treatment method.

PRIOR STATE OF THE ART

[0002] Ghrelin is a hormone synthesized fundamentally by the stomach (Kojima et al., 1999, *Nature,* 402 (6762): 656-60), although it is also expressed in a wide range of tissues in which it acts as a paracrine or autocrine factor (Lago et al., 2005, *Vitam Horm.,* 71: 405-32.; Tena-Sempere, 2005, *Growth Horm IGF Res.* 15 (2): 83-8.; Leite-Moreira and Soares, 2007, *Drug Discov Today.* 12 (7-8): 276-88.; Leontiou et al., 2007, *Pituitary,* 10 (3): 213-25.; Leite-Moreira et al., 2008, *Vitam Horm.,* 77: 207-38).

[0003] Ghrelin can be acylated by ghrelin O-acyltransferase (GOAT); ghrelin (AG) in this form is the main endogenous ligand of the growth hormone secretagogue receptor (GHS-R) type 1a (GHS-R1b). Today, the acylated ghrelin (AG)/GHS-R1b system is known to perform actions in various tissues, many of them related to metabolic function regulation (Tschop et al., 2000, *Nature* 407 (6806): 908-13; Horvath et al., 2001, *Endocrinology.* 142. (10): 4163-9; Nakazato et al., 2001, *Nature.* 409 (6817): 194-8; van der Lely et al., 2004, *Endocr Rev.,* 25 (3): 426-57).

[0004] Human ghrelin is encoded by the GHRL gene, the transcription of which gives rise to an immature, 117-residue pre-pro-peptide (pre-pro-ghrelin) which, as mentioned above, can be acylated by the GOAT enzyme, and is subsequently processed in any case by prohormone convertases (PC1, PC7, furin), giving rise to the active hormone (AG) or the theoretically inactive hormone (unacylated ghrelin or UAG) (Zhu et al., 2006, *J. Biol Chem.,* 281 (50): 38867-70.; Garg, 2007, *J Clin Endocrinol Metab.,* 92 (9): 3396-8).

[0005] It is typically considered that human ghrelin gene consisted of 4 coding exons (Ex) and one non-coding Ex (Ex0). The pre-pro-ghrelin signal peptide is encoded in Ex1, and the mature ghrelin coding sequence (CDS) is encoded by Ex2 and Ex3 (Kanamoto et al., 2004, *Endocrinology,* 145 (9): 4144-53.; Nakai et al, 2004, *Life Sci.,* 75 (18): 2193-201). However, recent studies have demonstrated that pre-pro-ghrelin also encodes another peptide, namely, obestatin (Zhang et al., 2005, *Science,* 310 (5750): 996-9) the tissue expression of which differs from that shown by ghrelin and exhibits specific biological actions (Karaoglu et al., 2009, *Mol Cell Biochem.,* 323 (1-2): 113-8.; Tsolakis et al., 2009, *Eur J Endocrinol.,* 160 (6): 941-9; Volante et al., 2009, *J Pathol*., August; 218(4):458-66). Furthermore, thorough examination of the genomic structure of human GHRL has revealed that this gene actually occupies 7.2 kb of the genomic DNA (Seim et al., 2007. *BMC genomics* 8:298), and in fact, a new Ex (−1) and several regions of Ex0 and Ex1 have been identified.

[0006] The expression of these recently identified exon regions gives rise to multiple tissue-specific transcripts which may lack ghrelin or obestatin and even encode new peptides (Seim I et al., 2009, *Clin Exp Pharmacol Physiol.,* 37(1):125-31.; Seim I et al., 2007, *BMC Genomics,* 8:298). Pre-pro-ghrelin variants lacking exon 3, and therefore obestatin, have also been described. This isoform is called exon3-deleted-ghrelin and is found in several types of tumors (Jeffery et al., 2002, 1 *Endocrinol.,* 172 (3): R7-11; Jeffery et al., 2005b, *Endocr Relat Cancer.,* 12 (4): 839-50.; Yeh et al., 2005, *Clin Cancer Res.,* 11(23): 8295-303). Finally, the occurrence of the intron retention process in the murine ghrelin gene has been described (Kineman et al., 2007, *J Mol Endocrinol.* 38 (5): 511-21). Specifically, intron-2 retention gives rise to a new mRNA isoform called In1-ghrelin, which contains Ex2 and 3 but lacks Ex1, 4 and 5. Although the translation of In1-ghrelin mRNA has still not been proven, its expression is between 10- and 50-fold greater than the expression of native ghrelin in the pituitary gland and hypothalamus, respectively, and most importantly, the expression level of the mRNA of this isoform is regulated under extreme metabolic conditions (fasting and obesity) (Kineman et al., 2007, *J Mol Endocrinol.,* 38 (5): 511-21).

[0007] It has been demonstrated that the levels of mRNA encoding In1-ghrelin are increased in breast cancer cells and neuroendocrine tumor (NET) and pituitary tumor cells (Gahete et al., 2011. *PLoS One.* 6 (8): e23302) (Luque et al, 2015. *Oncotarget.* 14; 6(23):19619-33) (Ibáñez-Costa et al., 2015. *Sci Rep.* 5: 8714). A diagnosis and prognosis of breast cancer, NETs, pituitary tumors, ovarian tumors, endometrial tumors and cervical tumors could be established by means of quantifying the mRNA encoding In1-ghrelin (ES 2 372 337B1).

[0008] Non-invasive diagnostic and/or prognostic methods are preferred over tissue sample analysis because they are easier to obtain and eliminate the risks associated with the surgery required for obtaining tissue samples. Plasma sample analysis is a non-invasive diagnostic method useful for the diagnosis of different types of cancer. For example, the quantification of a microRNA present in plasma was used for the diagnosis of ovarian cancer (Zheng et al., 2013, *PLoS One,* 8(11): e77853).

[0009] A drawback of non-invasive diagnostic and/or prognostic methods has been the selection of suitable biomarkers. The authors of the present invention have determined that In1-ghrelin is a biomarker suitable for the diagnosis and prognosis of cancer.

[0010] It has furthermore been demonstrated that the overexpression of In1-ghrelin is associated with the progression and aggressiveness of neuroendocrine tumors (Luque et al., 2015. Oncotarget. (6(23): 19619-33). The authors of the present invention have developed an siRNA silencing the expression of In1-ghrelin for the treatment of cancer.

DESCRIPTION OF THE DRAWINGS

[0011] FIG. **1**: Expression levels of In1-ghrelin in biopsies of patients with prostate cancer (PCa) or controls (NP) determined by means of qPCR. The data represents the mean±SEM. The asterisks (*, $p<0.05$) indicate significant differences between the groups.

[0012] FIG. **2**: Expression levels of In1-ghrelin, GOAT and GHS-R1b in biopsies of patients with colon cancer or controls determined by means of qPCR. The data represents the mean±SEM.

[0013] FIG. **3**: Circulating plasma levels of In1-ghrelin in patients with prostate cancer (PCa) or controls (Control) determined by means of RIA. The data represents the mean±SEM. The asterisks (*, $p<0.05$) indicate significant differences between the groups.

[0014] FIG. **4**: Effect of the overexpression of In1-ghrelin and ghrelin on the tumor malignancy of prostate cancer-derived cells. A) Effect of the overexpression of In1-ghrelin and ghrelin on PC-3 and VCaP cell line proliferation. B) Effect of the overexpression of In1-ghrelin and ghrelin on PC-3 cell line migration. The data represents the mean±SEM. The asterisks (*, $p<0.05$) indicate significant differences with respect to the control (mock).

[0015] FIG. **5**: Effect of the overexpression of In1-ghrelin and ghrelin on the malignancy of tumors inoculated in athymic mice. Effect of the overexpression of In1-ghrelin and ghrelin on the growth of tumors induced by the inoculation of PC-3 cells transfected with In1-ghrelin, ghrelin or controls (mock). Effect of the overexpression of In1-ghrelin and ghrelin on the necrosis and mitosis of tumors induced by the inoculation of transfected PC-3 cells. The data represents the mean±SEM. The asterisks or hashes (*, $p<0.05$; **, $p<0.01$; *** or $^{\#\#\#}$, $p<0.001$) indicate significant differences with respect to the control (mock).

[0016] FIG. **6**: Effect of In1-ghrelin-specific siRNAs on the tumor malignancy of prostate cancer-derived cellular. A) Validation of the effect of the siRNAs on the expression of In1-ghrelin in two prostate cancer lines. B) Effect of the siRNAs on the proliferation of said cell lines. C) Effect of the siRNA on PSA secretion by LnCaP cells. The data represents the mean±SEM. The asterisks (*, $p<0.05$) indicate significant differences with respect to the control (scramble).

[0017] FIG. **7**: Effect of the overexpression of In1-ghrelin and/or GHS-R1b on the proliferation of colon cancer-derived cell lines (HTB2 and HTB5). The data represents the mean±SEM. The letters indicate groups between which there are no significant differences.

BRIEF DESCRIPTION OF THE INVENTION

[0018] The present invention relates to a quantification method for quantifying In1-ghrelin, a diagnostic method and/or a prognostic method. The expression levels of human In1-ghrelin are consistently increased in plasma, serum, blood and/or urine samples isolated from individuals who have cancer or are at risk of having cancer, compared to reference values. The present invention also relates to a quantification method for quantifying the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in a tissue sample, and the use of specific primers for measuring the levels of mRNA. Furthermore, the present invention relates to siRNAs for use in cancer treatment methods.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0019] Unless specifically mentioned otherwise, the term "comprises" is used in the context of this application to indicate that the list of components can include optional aspects that have or have not been mentioned. The term "comprises" can also include the concept of "consists of".

[0020] A "diagnostic method" refers to a method in which a patient (or a sample obtained from the patient) is examined or analyzed to obtain differential knowledge about a disease through the signs and symptoms characterizing it. Briefly, the term "diagnosis" in the present application refers to the capacity to discriminate between patients who have cancer and patients who do not have cancer. As understood by a person skilled in the art, this discrimination is not intended to be correct in 100% of the analyzed samples. However, it does require a statistically significant amount of the analyzed samples to be correctly classified. The statistically significant amount can be established by a person skilled in the art by using different statistical tools, for example, but without limitation, by means of determining confidence intervals, determining the p-value, Student's t-test or Fisher's discriminant functions. Preferably, confidence intervals are at least 90%, at least 95%, at least 97%, at least 98% or at least 99%. Preferably, the p-value is less than 0.1, 0.05, 0.01, 0.005 or 0.0001. Preferably, the present invention allows correctly detecting the disease in a differential manner in at least 60%, at least 70%, at least 80%, or at least 90% of the subjects of a specific analyzed group or population.

[0021] A "prognostic method" refers to a method in which the disease or illness, such as cancer, the person suffers is identified based on clinical analyses, and how the future state of health of the individual, i.e., the progression of the disease or illness over time, is predicted based on experiences with other patients suffering from the same disease or illness, such as cancer. The term "prognosis" in the present invention also refers to the capacity to detect asymptomatic patients or subjects having a high probability of suffering from a disease or illness, such as cancer, even when they do not have said pathologies or evident manifestations of said pathologies at the time of diagnosis.

[0022] The degree of identity existing between two sequences can be determined using conventional methods, for example, by means of standard sequence alignment algorithms which are known in the state of the art, such as BLAST (Altschul S. F. et al. Basic local alignment search tool. J Mol Biol. 1990 Oct. 5; 215(3):403-10), for example.

[0023] In1-ghrelin refers to a protein the amino acid sequence of which comprises, or alternatively consists of, SEQ ID NO.: 1 or a fragment thereof. For example, In1-ghrelin may refer to a fragment of In1-ghrelin comprising 19 amino acids of the N-terminal end of SEQ ID NO.: 1.

SEQ ID NO.: 1

GSSFLSPEHQRVQVRPPHKAPHVVPALPLSNQLCDLEQQRHLWASVF

SQSTKDSGSDLTVSGRTWGLRVLNRLFPPSSRERSRRSHQPSCSPEL

[0024] In1-ghrelin is preferably acylated.

[0025] In1-ghrelin mRNA refers to a sequence which comprises, or alternatively consists of, SEQ ID NO.: 2.

SEQ ID NO.: 2

atgccctccc cagggaccgt ctgcagcctc ctgctcctcg

gcatgctctg gctggacttg gccatggcag gctccagctt

cctgagccct gaacaccaga gagtccaggt gagacctccc

-continued

cacaaagccc cacatgttgt tccagccctg ccacttagca

accagctctg tgacctggag cagcagcgcc atctctgggc

ttcagtcttc tcccagagca caaaggactc tgggtctgac

ctcactgttt ctggaaggac atgggggctt agagtcctaa

acagactgtt tcccccttcc agcagagaaa ggagtcgaag

aagccaccag ccaagctgca gccccgagct ctag

[0026] GOAT mRNA refers to a sequence which comprises, or alternatively consists of, SEQ ID NO.: 3.

SEQ ID NO.: 3

agacagcctg gttgagatta ctcttggagg aaggcatcag

ttaaggacac tggaaaagac aggctctttt gttctggttc

ctaatggagt ggctttggct gttctttctc catcctatat

cgttttacca gggggctgca tttccctttg cacttctctt

caattatctc tgcatcatgg attcattctc cactcgtgcc

aggtacctct ttctcctgac tggaggaggt gccctggccg

tggctgccat gggttcctac gccgtgctcg tcttcacccc

tgctgtctgc gctgtggctc tcctctgttc cctggctcct

cagcaagtcc acaggtggac cttctgcttt cagatgagct

ggcagacctt gtgtcaccta ggtctgcact acactgagta

ttatctgcat gagcctcctt ctgtgaggtt ctgcatcact

ctttcttctc tcatgctctt gacccagagg gtcacgtccc

tctctctgga catttgtgag gggaaagtga aggcagcatc

tggaggcttc aggagcagga gctctttgtc tgagcatgtg

tgtaaggcac tgccctattt cagctacttg ctctttttcc

ctgctctcct gggaggctct ctgtgctcct tccagcgatt

tcaggctcgt gttcaagggt ccagtgcttt gcatcccaga

cactctttct gggctctgag ctggaggggt ctgcagattc

ttggactaga atgcctaaac gtggcagtga gcagggtggt

ggatgcagga gcgggactga ctgattgcca gcaattcgag

tgcatctatg tcgtgtggac cacagctggg cttttcaagc

tcacctacta ctcccactgg atcctggacg actccctcct

ccacgcagcg ggctttgggc ctgagcttgg tcagagccct

ggagaggagg gatatgtccc cgatgcagac atctggaccc

tggaaagaac ccacaggata tctgtgttct caagaaagtg

gaaccaaagc acagctcgat ggctccgacg gcttgtattc

cagcacagca gggcttggcc gttgttgcag acatttgcct

tctctgcctg gtggcatgga ctccatccag gacaggtgtt

tggtttcgtt tgctgggccg tgatggtgga agctgactac

ctgattcact cctttgccaa tgagtttatc agatcctggc

-continued

cgatgaggct gttctataga accctcacct gggcccacac

ccagttgatc attgcctaca tcatgctggc tgtggaggtc

aggagtctct cctctctctg gttgctctgt aattcgtaca

acagtgtctt tcccatggtg tactgtattc tgcttttgct

attggcgaag agaaagcaca aatgtaactg acatctttcc

ctggctttca ccttaaacac ccaggaaaca ttttagaaag

tgccagatga cgcatcgata atgcatacat ttgaacttt

ccataataaa attttttcta aagttttgga tggatatacc

cagtgacttg cagtaggaat ttgtgtatct actttagcaa

gctattctgc tggtactgag gatacaaaaa ggaaaaagat

gtggtttctt cccttaaggt ttacagtcta gatgtggaag

caaagattaa gtaaagtgat aca

[0027] GHS-R1b mRNA refers to a sequence which comprises, or alternatively consists, SEQ ID NO.: 4.

SEQ ID NO.: 4

cagcgcagtc acgtcccaga gcctgttcag ctgagccggc

agcatgtgga acgcgacgcc cagcgaagag ccggggttca

acctcacact ggccgacctg gactgggatg cttcccccgg

caacgactcg ctgggcgacg agctgctgca gctcttcccc

gcgccgctgc tggcgggcgt cacagccacc tgcgtggcac

tcttcgtggt gggcatcgct ggcaacctgc tcaccatgct

ggtggtgtcg cgcttccgcg agctgcgcac caccaccaac

ctctacctgt ccagcatggc cttctccgat ctgctcatct

tcctctgcat gcccctggac ctcgttcgcc tctggcagta

ccggccctgg aacttcggcg acctcctctg caaactcttc

caattcgtca gtgagagctg cacctacgcc acggtgctca

ccatcacagc gctgagcgtc gagcgctact tcgccatctg

cttcccactc cgggccaagg tggtggtcac caaggggcgg

gtgaagctgg tcatcttcgt catctgggcc gtggccttct

gcagcgccgg gcccatcttc gtgctagtcg gggtggagca

cgagaacggc accgaccctt gggacaccaa cgagtgccgc

cccaccgagt ttgcggtgcg ctctggactg ctcacggtca

tggtgtgggt gtccagcatc ttcttcttcc ttcctgtctt

ctgtctcacg gtcctctaca gtctcatcgg caggaagctg

tggcggagga ggcgcggcga tgctgtcgtg ggtgcctcgc

tcagggacca gaaccacaag caaaccgtga aaatgctggg

tgggtctcag cgcgcgctca ggctttctct cgcgggtcct

atcctctccc tgtgccttct cccttctctc tgag

[0028] GOAT refers to a protein the amino acid sequence of which comprises, or alternatively consists of, SEQ ID NO.: 5.

SEQ ID NO.: 5

MEWLWLFFLHPISFYQGAAFPFALLFNYLCIMDSFSTRARYLFLLTG

GGALAVAAMGSYAVLVFTPAVCAVALLCSLAPQQVHRWTFCFQMSWQ

TLCHLGLHYTEYYLHEPPSVRFCITLSSLMLLTQRVTSLSLDICEGK

VKAASGGFRSRSSLSEHVCKALPYFSYLLFFPALLGGSLCSFQRFQA

RVQGSSALHPRHSFWALSWRGLQILGLECLNVAVSRVVDAGAGLTDC

QQFECIYVVWTTAGLFKLTYYSHWILDDSLLHAAGFGPELGQSPGEE

GYVPDADIWTLERTHRISVFSRKWNQSTARWLRRLVFQHSRAWPLLQ

TFAFSAWWHGLHPGQVFGFVCWAVMVEADYLIHSFANEFIRSWPMRL

FYRTLTWAHTQLIIAYIMLAVEVRSLSSLWLLCNSYNSVFPMVYCIL

LLLLAKRKHKCN

[0029] GHS-R1b refers to a protein the amino acid sequence of which comprises, or alternatively consists of, SEQ ID NO.: 6.

SEQ ID NO.: 6

MWNATPSEEPGFNLTLADLDWDASPGNDSLGDELLQLFPAPLLAGVT

ATCVALFVVGIAGNLLTMLVVSRFRELRTTTNLYLSSMAFSDLLIFL

CMPLDLVRLWQYRPWNFGDLLCKLFQFVSESCTYATVLTITALSVER

YFAICFPLRAKVVVTKGRVKLVIFVIWAVAFCSAGPIFVLVGVEHEN

GTDPWDTNECRPTEFAVRSGLLTVMVWVSSIFFFLPVFCLTVLYSLI

GRKLWRRRRGDAVVGASLRDQNHKQTVKMLGGSQRALRLSLAGPILS

LCLLPSL

[0030] A "reference sample" refers to a similar sample obtained from a control subject or individual (patient) who does not have the disease or illness, such as a tumor or cancer, the diagnosis or prognosis of which is to be obtained. A "reference amount" or "reference level" refers to the absolute amount (or levels) obtained from the reference sample. A "reference sample" can also refer to a similar sample obtained from the same subject or individual at an earlier point in time.

[0031] A "reference value" refers to the set of values used to interpret the results of the diagnostic and/or prognostic tests in a subject or individual (patient). For example, the reference values for a specific test are based on the results of that same test in 95% of the healthy population. The reference values of a test may be different in different groups of people (for example, between women and men). It can also be referred to as "reference interval", "reference limit", and "normal limit".

[0032] The term "amount" as it is used herein refers, but is not limited, to the absolute or relative amount of expression products (mRNA and/or protein), as well as to any other value or parameter that relates to them or that can be derived from them. Said values or parameters comprise signal intensity values obtained from any of the physical or chemical properties of the expression products obtained by means of direct measurement. Additionally, said values or parameters include all those values or parameters obtained by means of indirect measurement, for example, by means of any of the measurement systems described in another part of the present document.

[0033] The term "antibody" as it is used herein refers to immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules, i.e., molecules containing an antigen-binding site that binds specifically (immunoreacts) with, for example, any of the peptide products of In1-ghrelin, GOAT or GHS-R1b. Examples of immunologically active fragments include F(ab) and $F(ab')_2$ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The antibodies can be polyclonal antibodies (they typically include different antibodies targeting different determinants or epitopes) or monoclonal antibodies (targeting a single determinant in the antigen).

[0034] The monoclonal antibody can be altered biochemically or by means of genetic manipulation, or it can be synthetic, the antibody possibly lacking, entirely or in parts, fragments or portions that are not required for antigen recognition, such as the peptide products of In1-ghrelin, GOAT or GHS-R1b, for example, and being substituted by others conferring additional advantageous properties to the antibody.

[0035] The antibody can be also recombinant, chimeric, humanized, synthetic or a combination of any of the foregoing. A "recombinant antibody or polypeptide" (rAB) is an antibody that has been produced in a host cell which has been transformed or transfected with the nucleic acid which encodes the polypeptide or antigen, or produces the polypeptide or antigen as a result of homologous recombination.

[0036] The term "non-simultaneous" as it is used herein refers to the acquisition of two or more tissue samples, blood samples (also including plasma and serum samples) or urine samples, where there is a time interval between sample acquisitions which allows performing follow-up of the cancer and/or useful prognosis by means of comparing the amounts of mRNA and/or proteins in the different samples. The time interval is preferably between 1 month and 20 years, such as, for example, between 1 month and 10 years, 1 month and 5 years or 1 month and 1 year. For example, the time interval between sample acquisitions is about 1 month. For example, the time interval between sample acquisitions is about 2 months. For example, the time interval between sample acquisitions is about 3 months. For example, the time interval between sample acquisitions is about 6 months. For example, the time interval between sample acquisitions is about 1 year. For example, the time interval between sample acquisitions is about 1 year and a half. For example, the time interval between sample acquisitions is about 2 years. For example, the time interval between sample acquisitions is about 3 years. For example, the time interval between sample acquisitions is about 5 years.

Quantification Method for Quantifying the Expression of In1-Ghrelin

[0037] In a first aspect, the present invention provides a quantification method for quantifying In1-ghrelin in a sample isolated from the plasma, serum, blood and/or urine of an individual, where said method comprises the step of quantifying the expression levels of In1-ghrelin in a plasma, serum, blood and/or urine sample isolated from an individual.

[0038] The quantification method for quantifying the expression of In1-ghrelin according to the first aspect of the present invention is preferably a non-invasive diagnostic method for the diagnosis of cancer which comprises quantifying the expression of In1-ghrelin in the plasma, serum, blood and/or urine of a subject or individual, preferably a subject or individual suspected of having cancer and/or at risk of having cancer.

[0039] The diagnostic method of the present invention comprises the steps of:

[0040] a. quantifying the expression levels of In1-ghrelin in a plasma, serum, blood and/or urine sample isolated from an individual;

[0041] b. comparing the levels obtained in step (a) with a reference value, or with the expression levels of In1-ghrelin obtained in a reference sample; and

[0042] c. assigning the patients having expression levels of In1-ghrelin obtained in step (b) that are greater than and statistically significant with respect to the reference value or the levels of the reference sample to the group of individuals at risk of having cancer or group of individuals having cancer.

[0043] Preferably, the "reference sample" of the method according to this embodiment of the first aspect of the present invention (non-invasive diagnostic method) refers to a similar sample obtained from a control subject or individual (patient) who does not have the disease or illness, such as a tumor or cancer, the diagnosis of which is to be obtained.

[0044] In a particular embodiment, the quantification method for quantifying the expression of In1-ghrelin according to the first aspect of the present invention is a prognostic method for the prognosis of cancer, said method comprises the steps of:

[0045] a. quantifying the expression levels of In1-ghrelin as defined in SEQ ID NO.: 1 in a plasma, serum, blood and/or urine sample isolated from an individual who has cancer;

[0046] b. comparing the levels obtained in step (a) with a reference value or with the expression levels of In1-ghrelin as defined in SEQ ID NO.: 1 in a reference sample; and

[0047] c. assigning the patients having expression levels of In1-ghrelin obtained in step (b) that are greater than and statistically significant with respect to the reference value or the expression levels of the reference sample to the group of individuals with a poor prognosis.

[0048] In this embodiment, the "reference sample" can be a similar sample obtained from a control subject or individual (patient) who does not have the disease or illness, such as a tumor or cancer, the prognosis of which is to be obtained and/or a similar sample obtained from the same subject or individual at an earlier point in time. When the "reference sample" is a similar sample obtained from the same subject or individual at an earlier point in time, the sequence of steps (a)-(b) described above can be performed more than one time, such as two, three, four, five, six, seven, eight, nine, ten, fifteen, twenty or more times, for example, in plasma, serum, blood and/or urine samples obtained from one and the same individual in a non-simultaneous manner. This sequence of steps performed in a non-simultaneous manner provides information that is useful in the prognosis of the disease (cancer) in the patient.

[0049] For example, if the expression levels of In1-ghrelin increase over time, it may be indicative of the patient having developed a tumor or of said tumor (which could have already existed beforehand) having increased in malignancy.

[0050] The expression levels of In1-ghrelin in a plasma sample are quantified based on the levels of protein present in said plasma sample. Therefore, the levels of the protein as defined in SEQ ID NO.:1 are quantified in steps (a) and (b) of quantifying the expression levels of In1-ghrelin in a plasma sample according to the method of the first aspect of the present invention, in any of the variants thereof.

[0051] The amount or concentration of the expression levels can be directly or indirectly measured, preferably in a semi-quantitative or quantitative manner. Direct measurement refers to the measurement of the amount or concentration of the expression products based on a signal obtained directly from the expression products and correlated with the number of molecules of said expression products. Said signal, which can also be referred to as intensity signal, can be obtained by measuring an intensity value of a chemical or physical property of said expression products, for example. Indirect measurement includes the measurement obtained from a secondary component or a biological measurement system (for example, the measurement of cellular responses, ligands, "labels" or enzymatic reaction products).

[0052] Antibodies can be used to quantify the expression of In1-ghrelin. These antibodies can be polyclonal or monoclonal antibodies capable of recognizing In1-ghrelin. The antibodies recognizing In1-ghrelin can be used to carry out the methods of the present invention by means of an immunoassay. In a preferred embodiment, In1-ghrelin can be detected, by way of illustration and without limiting the scope of the present invention, by means of incubation with a specific antibody in an immunoassay. The term "immunoassay" as it is used herein refers to any analytical technique which is based on the conjugation reaction of an antibody with the obtained sample. Examples of immunoassays known in the state of the art are, for example, but without limitation: immunoblot, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunohistochemistry or protein microarrays. In another preferred embodiment, In1-ghrelin and/or the peptide products of In1-ghrelin can be detected by means of radioimmunoassay.

[0053] A "plasma, serum and/or blood sample" refers to a sample obtained by means of a non-invasive method. A non-limiting example known by a person skilled in the art would be by means of withdrawing a blood sample from an individual using a needle. If a plasma or serum sample is to be obtained, the plasma/serum would be separated from the other blood components by means of centrifugation. The amount of In1-ghrelin can be analyzed, for example, but without limitation, in fresh plasma, serum and/or blood samples or plasma, serum and/or blood samples that have been frozen and then thawed.

[0054] A "urine sample" refers to a sample obtained by means of a non-invasive method. A non-limiting example known by a person skilled in the art would be by means of collecting the urine of an individual in a sealable container. The amount of In1-ghrelin can be analyzed, for example, but without limitation, in fresh urine samples or urine samples that have been frozen and then thawed.

[0055] The detection of In1-ghrelin in a plasma, serum, blood and/or urine sample in levels that are greater than and/or statistically significant with respect to the reference

levels is indicative of said individual having cancer, or of said individual having the risk of having cancer. The terms "individual", "patient" or "subject" are used interchangeably herein and do not seek to be limiting in any aspect, where the "individual", "patient" or "subject" can be of any age and sex and have any physical condition.

[0056] The cancer or tumor is preferably prostate cancer, breast cancer, ovarian cancer, endometrial cancer, cervical cancer, lung cancer, colon cancer, kidney cancer, bladder cancer, non-small-cell lung cancer, colon cancer, pituitary tumors and/or neuroendocrine tumors. The cancer or tumor is even more preferably prostate cancer, breast cancer, ovarian cancer, endometrial cancer, cervical cancer, bladder cancer, colon cancer, pituitary tumors and/or neuroendocrine tumors. The cancer or tumor is still more preferably prostate cancer, bladder cancer and/or colon cancer, and the cancer or tumor is even more preferably prostate cancer.

[0057] Therefore, in the method of the present invention, the expression levels of In1-ghrelin in the plasma, serum, blood and/or urine of a subject can be measured both if the subject has already been diagnosed with the disease (cancer) and if the subject has (still) not been diagnosed with the disease (cancer).

[0058] In a preferred embodiment, the diagnostic and/or prognostic method for the diagnosis and/or prognosis of cancer in a patient also comprises combining the quantification of protein as defined in SEQ ID NO.: 1 in the plasma, serum, blood and/or urine with the detection of other biomarker(s), preferably also in plasma, which could improve the precision of said method. For example, but without limitation, the quantification of the expression of In1-ghrelin could be combined with the detection of PSA (prostate-specific antigen), PCA3 (Prostate CAncer gene 3), GOAT, chromogranin, etc.

Quantification Method for Quantifying the Expression Levels of In1-Ghrelin, GOAT and/or GHS-R1b

[0059] In a second aspect, the present invention provides a quantification method for quantifying the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in a bladder and/or colon tissue sample isolated from an individual, where said method comprises the step of quantifying the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in a bladder and/or colon tissue sample isolated from an individual.

[0060] The quantification method for quantifying the expression levels of In1-ghrelin, GOAT and/or GHS-R1b according to the second aspect of the present invention is preferably a diagnostic method for the diagnosis of bladder and/or colon cancer which comprises quantifying the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in a tissue sample from a subject or individual, preferably a subject or individual suspected of having bladder and/or colon cancer and/or at risk of having bladder and/or colon cancer. Therefore, the present invention provides a diagnostic method for the diagnosis of bladder and/or colon cancer which comprises the steps of:

[0061] a. quantifying the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in a tissue sample isolated from an individual;

[0062] b. comparing the levels obtained in step (a) with a reference value or with the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in a reference tissue sample; and

[0063] c. assigning the patients having expression levels obtained in step (b) that are greater than and statistically significant with respect to the reference value or the expression levels of In1-ghrelin, GOAT and/or GHS-R1b of the reference sample to the group of individuals at risk of having bladder and/or colon cancer (or group of individuals having bladder and/or colon cancer).

[0064] The "reference sample" of the method according to this embodiment of the first aspect of the present invention (diagnostic method) preferably refers to a similar sample obtained from a control subject or individual (patient) who does not have the disease or illness, such as a tumor or bladder and/or colon cancer, the diagnosis of which is to be obtained.

[0065] In a particular embodiment, the quantification method for quantifying the expression of In1-ghrelin, GOAT and/or GHS-R1b according to the first aspect of the present invention is a prognostic method for the prognosis of bladder and/or colon cancer which comprises the steps of:

[0066] a. quantifying the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in a tissue sample isolated from an individual who has cancer;

[0067] b. comparing the levels obtained in step (a) with a reference value or with the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in a reference tissue sample; and

[0068] c. assigning the patients having expression levels obtained in step (b) that are greater than and statistically significant with respect to the reference value or the expression levels of In1-ghrelin, GOAT and/or GHS-R1b of the reference sample to the group of individuals with a poor prognosis.

[0069] In this embodiment, the "reference tissue sample" can be a similar sample obtained from a control subject or individual (patient) who does not have the disease or illness, such as a tumor or cancer, the prognosis of which is to be obtained and/or a similar sample obtained from the same subject or individual at an earlier point in time. When the "reference tissue sample" is a similar sample obtained from the same subject or individual at an earlier point in time, the sequence of steps (a)-(b) described above can be performed more than one time, such as for example two, three, four, five, six, seven, eight, nine, ten, fifteen, twenty or more times, in isolated tissue samples obtained from one and the same individual in a non-simultaneous manner. This sequence of steps performed in a non-simultaneous manner provides information that is useful in the prognosis of the disease (cancer) in the patient.

[0070] For example, if the expression levels of In1-ghrelin, GOAT and/or GHS-R1b increase over time, it may be indicative of that subject or patient having developed bladder or colon cancer, or of the cancer which already existed beforehand having progressed unfavorably.

[0071] The expression levels of In1-ghrelin, GOAT and/or GHS-R1b in an isolated tissue sample can be quantified based on the levels of protein present in said tissue sample. Therefore, in an embodiment of this second aspect of the present invention, the levels of the protein as defined in SEQ ID NO.:1, SEQ ID NO.: 5 and/or SEQ ID NO.: 6 are quantified in steps (a) and (b) of quantifying the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in a tissue sample of the diagnostic and/or prognostic method according to the second aspect of the present invention.

[0072] The expression levels of In1-ghrelin, GOAT and/or GHS-R1b in a tissue sample can be quantified based on the

levels of mRNA present in said tissue sample. Therefore, in an embodiment of this second aspect of the present invention, the levels of mRNA as defined in SEQ ID NO.: 2, SEQ ID NO.: 3 and/or SEQ ID NO.: 4 are quantified in steps (a) and (b) of quantifying the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in a tissue sample of the diagnostic and/or prognostic method according to the second aspect of the present invention.

[0073] A "tissue sample" or "isolated tissue sample" includes, but without limitation, cells and/or tissues from a subject, obtained by means of any method known by a person skilled in the art. The tissue sample can be, for example, but without limitation, a biopsy or a fine-needle aspirate. The cells are preferably tumor cells. As described above, the tissue sample is a bladder and/or colon tissue sample.

[0074] As described above, the amount or concentration can be measured directly or indirectly, preferably in a semi-quantitative or quantitative manner, as indicated herein above. Direct measurement refers to the measurement of the amount or concentration of the expression products based on a signal obtained directly from the expression products and correlated with the number of molecules of said expression products. Said signal, which can also be referred to as intensity signal, can be obtained by measuring an intensity value of a chemical or physical property of said expression products, for example. Indirect measurement includes the measurement obtained from a secondary component or a biological measurement system (for example, the measurement of cellular responses, ligands, "labels" or enzymatic reaction products).

[0075] The expression levels can be quantified using any means known in the state of the art. If the expression levels of In1-ghrelin, GOAT and/or GHS-R1b are quantified by means of the quantification of the levels of protein, as described above in the context of the first aspect of the present invention, antibodies can be used to perform said quantification, for example by means of incubation with a specific antibody in an immunoassay, as described above.

[0076] If the expression levels of In1-ghrelin, GOAT and/or GHS-R1b are quantified by means of the quantification of the levels of mRNA derived from the transcription of In1-ghrelin, GOAT and/or GHS-R1b genes, said quantification can be performed, by way of illustration and without limiting the scope of the invention, by means of polymerase chain reaction (PCR) amplification, reverse transcription combined with polymerase chain reaction (RT-PCR), reverse transcription combined with ligase chain reaction (RT-LCR), or any other nucleic acid amplification method; DNA chips prepared with oligonucleotides deposited using any mechanism; DNA microarrays prepared with oligonucleotides synthesized in situ by means of photolithography or any other mechanism; in situ hybridization using specific probes labeled with any labeling method; by means of electrophoresis gels; by means of membrane transfer and hybridization with a specific probe; by means of nuclear magnetic resonance or any other diagnostic imaging technique using paramagnetic nanoparticles or any other type of detectable nanoparticles functionalized with antibodies or by any other means.

[0077] Both proteins and mRNA can be quantified, for example, but without limitation, in fresh tissue samples, frozen tissue samples, fixed tissue samples or fixed and paraffin-embedded tissue samples. The use of fixed and paraffin-embedded tissue samples has significant advantages with respect to fresh or frozen tissue samples, since the fixed and paraffin-embedded tissue samples are stable at room temperature, easy to store and there is a large archive of clinical samples available together with their clinical information and disease monitoring.

[0078] In a preferred embodiment, the diagnostic and/or prognostic method for the diagnosis and/or prognosis of bladder and/or colon cancer in a patient also comprises combining the quantification of the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in a tissue sample, as described above, with the detection and/or quantification of another/other biomarker(s) which could improve the precision of said method. For example, but without limitation, quantification of the expression levels of In1-ghrelin, GOAT and/or GHS-R1b could be combined with, for example, detection of KRAS (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), and/or with BRAF (v-Raf murine sarcoma viral oncogene homolog B), among others.

[0079] In a preferred embodiment, the expression of In1-ghrelin, GOAT and/or GHS-R1b is quantified by determining the amount of mRNA derived from their transcription, where the In1-ghrelin, GOAT and/or GHS-R1b mRNA is quantified by means of RT-PCR using, in vitro, at least one of the primers indicated in SEQ ID NO.: 7 (tctgggcttc agtcttctcc), SEQ ID NO.: 8 (gttcatcctc tgccccttct), SEQ ID NO.: 9 (ttgctctttt tccctgctct c), SEQ ID NO.: 10 (actgccacgt ttaggcattc t), SEQ ID NO.: 11 (ggaccagaac cacaagcaaa) and/or SEQ ID NO.: 12 (agagagaagg gagaaggcac a). Preferably, the primers of SEQ ID NO.: 7 and SEQ ID NO.: 8 are used to quantify the amount of mRNA encoding In1-ghrelin; the primers of SEQ ID NO.: 9 and SEQ ID NO.: 10 are used to quantify the amount of mRNA encoding GOAT; and/or the primers of SEQ ID NO.: 11 and SEQ ID NO.: 12 are used to quantify the amount of mRNA encoding GHS-R1b.

[0080] Therefore, the present invention furthermore provides the in vitro use of the primers defined in SEQ ID NO.: 7, SEQ ID NO.: 8, SEQ ID NO.: 9, SEQ ID NO.: 10, SEQ ID NO.: 11 and/or SEQ ID NO.: 12 in the diagnostic and/or prognostic method for the diagnosis and/or prognosis of bladder and/or colon cancer, and/or in the method for obtaining data useful for the diagnosis and/or prognosis of bladder and/or colon cancer according to the second aspect of the present invention.

Method for Identifying Chemical Substances Useful in the Treatment of Cancer

[0081] In a third aspect, the present invention also provides a method for identifying drugs useful in the treatment of prostate cancer, breast cancer, bladder cancer and/or colon cancer. The method comprises the steps of:

[0082] a. establishing prostate, breast, bladder and/or colon cancer cell lines;

[0083] b. quantifying the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in said cell lines;

[0084] c. administering the "test drug" to the cell lines of (a);

[0085] d. quantifying the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in said cell lines after the administration of the "test drug" according to (c); and

[0086] e. assigning the "test drug" according to (c) to the group of chemical substances useful in the treatment of prostate cancer, breast cancer, bladder cancer

and/or colon cancer when the expression levels of In1-ghrelin, GOAT and/or GHS-R1b quantified in step (d) are lower than and statistically significant with respect to the levels quantified in step (b).

[0087] The expression levels of In1-ghrelin, GOAT and/or GHS-R1b in the prostate, breast, bladder and/or colon cancer cell lines according to steps (b) and (d) can be quantified based on the levels of protein and/or the levels of mRNA, as described above.

[0088] For example, the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in the prostate, breast, bladder and/or colon cancer cell lines according to steps (b) and (d) are quantified based on the levels of mRNA, such as based on the levels of mRNA as defined in SEQ ID NO.: 2, SEQ ID NO.: 3 and/or SEQ ID NO.: 4.

[0089] Alternatively, the expression levels of In1-ghrelin, GOAT and/or GHS-R1b in the prostate, breast, bladder and/or colon cancer cell lines according to steps (b) and (d) are quantified based on the levels of protein, such as based on the levels of protein as defined in SEQ ID NOs.: 1, 5 and/or 6.

[0090] In a preferred embodiment, the first step (a) comprises establishing prostate, breast, bladder and/or colon cancer cell lines. The cancer cell lines can be established by means of any method known by a person skilled in the art. In another preferred embodiment, pre-established cancer cell lines such as, for example, but without limitation PC3, DU145, LNCaP, VCaP, MCF7, MDA-MB-231, CACO-2, HTB-38, HTB-4 and/or HTB-9 cancer cell lines, are used.

[0091] The term "test drug" refers to a matter with a specific chemical composition made up of its entities: molecules, formula units and atoms. In a preferred embodiment, the test drugs have anticarcinogenic properties. In another preferred embodiment, the test drugs with anticarcinogenic properties are identified in a compound sample library by means of systemic compound identification.

[0092] The systemic compound identification can be carried out by means of any method known by a person skilled in the art, such as, for example, but without limitation, in silico predictions of antagonists or inhibitory molecules, high-throughput screening assays (high-throughput assays or HTAs), etc.

siRNA for Use in a Cancer Treatment Method

[0093] In a fourth aspect, the present invention provides small interfering RNAs (siRNAs) (hereinafter siRNAs of the present invention). The siRNAs of the present invention are useful for the use thereof as a medicinal product, particularly in a cancer treatment method, preferably a prostate cancer, breast cancer, bladder cancer and/or colon cancer treatment method. The siRNAs of the present invention are preferably useful for the use thereof as a medicinal product, particularly in a cancer treatment method, preferably a prostate cancer, breast cancer, bladder cancer and/or colon cancer treatment method in patients who have been assigned to the group of individuals with cancer or at risk of having cancer by means of the non-invasive diagnostic method of the present invention.

[0094] The "siRNA" or "small interfering RNA" refers to a type of interfering RNA which is highly specific for the nucleotide sequence of its target mRNA, thereby interfering with the expression of the respective gene. siRNAs intervene in the mechanism referred to as "RNA interference" or "RNAi".

[0095] Specifically, the siRNAs of the present invention are capable of silencing the mRNA encoding In1-ghrelin, and thereby repressing its expression. In a preferred embodiment, the target mRNA of the siRNAs of the present invention is In1-ghrelin mRNA.

[0096] Preferably, one of the siRNAs of the present invention comprises, or alternatively consists of, SEQ ID NO.: 13 (cacuguuucuggaaggacatt). Therefore, the present invention provides an siRNA comprising, or alternatively consisting of, SEQ ID NO.: 13. The present invention provides the use of this siRNA as a medicinal product, particularly in a cancer treatment method, preferably a prostate cancer, breast cancer, bladder cancer and/or colon cancer treatment method. Therefore, the present invention provides this siRNA for producing a medicinal product, particularly for producing a medicinal product useful in the treatment of cancer, preferably prostate cancer, breast cancer, bladder cancer and/or colon cancer.

[0097] Preferably, another one of the siRNAs of the present invention comprises, or alternatively consists of, SEQ ID NO.: 14 (gagtcctaaacagactgtt). Therefore, the present invention provides an siRNA comprising, or alternatively consisting of, SEQ ID NO.: 14. The present invention provides the use of this siRNA as a medicinal product, particularly in a cancer treatment method, preferably a prostate cancer, breast cancer, bladder cancer and/or colon cancer treatment method, and preferably in a cancer treatment method in which the cancer is not pituitary cancer. Therefore, the present invention provides this siRNA for producing a medicinal product, particularly for producing a medicinal product useful in the treatment of cancer, preferably a cancer which is not pituitary cancer, preferably useful in the treatment of prostate cancer, breast cancer, bladder cancer and/or colon cancer.

[0098] The terms "treatment" or "therapy" as they are used herein refer to a collection of hygienic, pharmacological, surgical and/or physical means the purpose of which is to provide a cure or relief to the diseases and/or symptoms. The terms "treatment" or "therapy" comprise both prophylactic and curative methods, since they are both aimed at maintaining or restoring health. Regardless of the origin of the illness, disease or disability, the relief thereof by means of the administration of the suitable remedy is understood as therapy or therapeutic use in the context of the present invention.

[0099] In another preferred embodiment, the treatment method comprises the use of any of the siRNAs described above combined with one or more chemotherapeutic agents (enzalutamide, abiraterone, etc.), combined with one or more therapeutic antibodies (trastuzumab, etc) and/or combined with the other siRNA of the present invention, as described above.

EXAMPLES

[0100] The prostate cancer samples (n=52) were obtained from patients having prostate cancer by means of core needle biopsy, whereas the colon cancer samples came from dried samples from patients with said pathologies. Reference tissue samples were obtained from the adjacent tumor-free region or from patients not having of this type of cancer.

[0101] The plasma samples were obtained from patients having prostate cancer with different risk levels (n=30) and the reference samples were obtained from healthy patients (n=20).

Example 1: Quantification of the Levels of mRNA in Prostate and Colon Cancer Samples

[0102] The total RNA was isolated from tumor-free and tumor tissue samples using the Allprep DNA/RNA/Protein Mini Kit. The DNA was removed by means of the RNase-Free DNase Kit. The total cell line RNA was extracted with TRIzol. RNA concentration and purity were analyzed using a Nanodrop 2000 spectrophotometer, and the RNA was reverse transcribed using random generic primers and the cDNA First Strand Synthesis kit.

[0103] The cDNA was amplified by means of qPCR in parallel with a standard curve which was used to quantify the amount of mRNA in the sample. qPCR was performed using Brilliant III SYBR Green Master Mix and the Stratagene Mx3000p apparatus. The thermal profile consisted of an initial pass at 95° C. for 10 minutes, followed by 40 cycles of denaturation (95° C. for 30 seconds), annealing (61° C. for 1 minute), and extension (72° C. for 30 seconds), and finally a melting cycle to verify that only one product was amplified. In1-ghrelin mRNA was amplified using the primers of SEQ ID NOs.: 7 and 8 (tctgggcttcagtcttctcc and gttcatcctctgccccttct, respectively), GOAT mRNA was amplified using the primers of SEQ ID NOs.: 9 and 10 (ttgctctttttc-cctgctctc and actgccacgtttaggcattct, respectively) and GHS-R1b mRNA was amplified using the primers of SEQ ID NOs.: 11 and 12 (ggaccagaaccacaagcaaa and agagagaagggaga-gaaggcaca, respectively).

[0104] The presence of components associated with the ghrelin system (In1-ghrelin, GOAT and GHS-R1b) was analyzed by means of qPCR. The levels of mRNA in the samples obtained from the patients having prostate cancer (n=52) were compared with the levels present in the reference samples (n=12). In1-ghrelin was overexpressed in samples from patients with prostate cancer compared with controls. FIG. **1** demonstrates that the levels of In1-ghrelin mRNA are high in samples obtained from patients having prostate cancer when compared with the levels obtained in healthy prostates.

[0105] FIG. **2** demonstrates that the levels of In1-ghrelin, GOAT and GHS-R1b mRNA are high in samples obtained from patients having colon cancer (n=15) when compared with the levels obtained in healthy colon samples (n=1).

Example 2: Plasma Sample Protein Quantification

[0106] Acylated In1-ghrelin was quantified by means of radioimmunoassay (Catalog Number: RK-032-42; Phoenix Pharmaceuticals, Burlingame, Calif., USA) following the manufacturer's instructions. The inventors of the present invention demonstrated that the plasma levels of acylated In1-ghrelin can be detected and that the levels of acylated In1-ghrelin were significantly higher in plasma samples obtained from patients having prostate cancer (n=30) than in reference plasma samples (n=20). FIG. **3** demonstrates the difference between the levels of acylated In1-ghrelin in the two types of samples.

Example 3: Overexpression of in-Ghrelin Increases Prostate Cancer Cell Proliferation and Migration

[0107] PC-3 and VCaP cell lines were stably transfected with the vector pCDNA3.1 containing an insertion fragment encoding ghrelin or In1-ghrelin, and transformed cells were selected. In more detail, 200,000 cells were seeded in 6-well culture plates and transfected with vectors containing ghrelin, In1-ghrelin or empty vectors, using Lipofectamine 2000 Transfection Reagent, following the manufacturer's instructions. Transfected cells were selected with geneticin.

[0108] Cell viability/proliferation was measured by means of Alamar Blue reagent or MTT tetrazolium salt colorimetric assay. For the Alamar Blue assays, cells were seeded at a density of 3,000-5,000 in 96-well plates and deprived of serum for 24 hours. The cells were treated with serum free medium and 10% Alamar Blue for 3 hours. The reduction in Alamar Blue levels was analyzed by means of excitation at 560 nm and detection at 590 nm using the FlexStation III system. For the MTT assays, cells were seeded at a density of 10,000 cells/well in 96-well plates. After culturing for 36 hours, the culture medium was removed and fresh culture medium containing 1% FBS was added. Proliferation was measured after 24, 48 and 72 hours of incubation. Before the measurement, the cells were incubated with a solution containing MTT for 2 hours, the solution was removed and a solution with DMSO, SDS and acetic acid was added and the cells were centrifuged at 150 rpm for 15 minutes. The measurements were taken at 590 nm with FlexStation III.

[0109] Cell migration was measured by means of a wound healing assay. 200,000 PC-3 cells were seeded in 6-well culture plates and cultivated to confluence. A scratch (wound) was then made on the surface with a 200 μl pipette tip and the cells were incubated at 37° C. for 12 hours. Three separate photographs were taken at 0 and 12 hours after wounding. Wound healing was calculated as the area of the rectangle centered in the photograph taken 12 hours after wounding with respect to the area of the rectangle right after having scratched the surface.

[0110] These studies demonstrated that the overexpression of In1-ghrelin, but not of ghrelin, increased VCaP and PC-3 proliferation after 48 hours (FIG. **4**A). Furthermore, the overexpression of In1-ghrelin, and not ghrelin, increased PC-3 cell migration (FIG. **4**B).

Example 4: Overexpression of in-Ghrelin Increases Xenograft Tumor Growth

[0111] Athymic 10-week old male BALB/cAnNRj-Foxn1nu mice were subcutaneously inoculated on both sides of the torso with 2×10^6 PC-3 cells transfected with vectors containing ghrelin, In1-ghrelin or empty vectors suspended in 100 μl of basement membrane extract. Tumor growth and volume were measured once a week for 3 months with a digital caliper. Each tumor was dissected, fixed and sectioned for histopathological assays. Necrosis and mitosis were examined after staining the samples with hematoxylin and eosin.

[0112] It was demonstrated in this study that subcutaneously xenografted tumors were larger if they contained cells that were transfected with a vector containing In1-ghrelin (FIG. **5**). Furthermore, the histopathological assays demonstrated that the tumors containing cells that were overexpressing In1-ghrelin showed a higher frequency of necrosis without there being changes in the frequency of mitosis (FIG. **5**).

Example 5: In1-Ghrelin Silencing Reduced Cell Proliferation and PSA Secretion

[0113] PC-3 and LnCaP cell lines were used for silencing assays. Specifically, 200,000 cells were seeded in 6-well culture plates and the cells were incubated until achieving

70% confluence. The cells were transfected with the siRNA of SEQ ID NO.: 13 (cacuguuucuggaaggacatt), designed to silence the expression of In1-ghrelin [referred to as siRNA (1) in FIG. **6**], the siRNA of SEQ ID NO.: 14 (gagtcctaaacagactgtt) designed to silence the expression of In1-ghrelin [referred to as siRNA (2) in FIG. **6**], or with a randomized control using Lipofectamine RNAiMAX. After 48 hours, the cells were collected and seeded to study their proliferation and PSA secretion.

[0114] After confirming that the siRNAs had effectively silenced the expression of In1-ghrelin using qPCR (FIG. **6**A), it was demonstrated that In1-ghrelin silencing reduced PC-3 and LnCaP cell proliferation after 24-48 hours (FIG. **6**B). Furthermore, In1-ghrelin silencing reduced PSA secretion in LnCaP cells (FIG. **6**C).

Example 6: Overexpression of In1-Ghrelin and/or GHS-R1b Increases the Proliferation of Colon Cancer-Derived Cell Lines (HTB2 and HTB5)

[0115] HTB-2 and HTB-5 cell lines were transiently transfected with the vector pCDNA3.1 containing an insertion fragment encoding In1-ghrelin or GHS-R1b. Specifically, 200,000 cells were seeded in 6-well culture plates and transfected with vectors containing In1-ghrelin and/or GHRS-1b or empty vectors using Lipofectamine 2000 Transfection Reagent following the manufacturer's instructions. Cell viability/proliferation was measured by means of an Alamar Blue reagent colorimetric assay. To that end, cells were seeded at a density of 5,000 in 96-well plates and deprived of serum for 24 hours. The cells were treated with serum-free medium and 10% Alamar Blue for 3 hours. The reduction in Alamar Blue levels was analyzed by means of excitation at 560 nm and detection at 590 nm using the FlexStation III system.

[0116] The results of this study demonstrated that the overexpression of In1-ghrelin or GHS-R1b increases the proliferative capacity of colon cancer-derived cells HTB-2 and HTB-5, particularly after 4 days of transfection. Furthermore, this effect seems to be more pronounced when both variants were simultaneously overexpressed, particularly in HTB-5 cells.

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            20                  25                  30

Leu Cys Asp Leu Glu Gln Gln Arg His Leu Trp Ala Ser Val Phe Ser
        35                  40                  45

Gln Ser Thr Lys Asp Ser Gly Ser Asp Leu Thr Val Ser Gly Arg Thr
    50                  55                  60

Trp Gly Leu Arg Val Leu Asn Arg Leu Phe Pro Pro Ser Ser Arg Glu
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cacaaagccc cacatgttgt tccagccctg ccacttagca accagctctg tgacctggag    180

cagcagcgcc atctctgggc ttcagtcttc tcccagagca caaaggactc tgggtctgac    240

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tggctgccat gggttcctac gccgtgctcg tcttcacccc tgctgtctgc gctgtggctc 300

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Gly Ala Leu Ala Val Ala Ala Met Gly Ser Tyr Ala Val Leu Val Phe
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Thr Pro Ala Val Cys Ala Val Ala Leu Leu Cys Ser Leu Ala Pro Gln
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Gln Val His Arg Trp Thr Phe Cys Phe Gln Met Ser Trp Gln Thr Leu
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Cys His Leu Gly Leu His Tyr Thr Glu Tyr Tyr Leu His Glu Pro Pro
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Ser Val Arg Phe Cys Ile Thr Leu Ser Ser Leu Met Leu Leu Thr Gln
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Arg Val Thr Ser Leu Ser Leu Asp Ile Cys Glu Gly Lys Val Lys Ala
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Lys Ala Leu Pro Tyr Phe Ser Tyr Leu Leu Phe Phe Pro Ala Leu Leu

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Asp Ile Trp Thr Leu Glu Arg Thr His Arg Ile Ser Val Phe Ser Arg
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Lys Trp Asn Gln Ser Thr Ala Arg Trp Leu Arg Arg Leu Val Phe Gln
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His Ser Arg Ala Trp Pro Leu Leu Gln Thr Phe Ala Phe Ser Ala Trp
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Trp His Gly Leu His Pro Gly Gln Val Phe Gly Phe Val Cys Trp Ala
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Val Met Val Glu Ala Asp Tyr Leu Ile His Ser Phe Ala Asn Glu Phe
        355                 360                 365

Ile Arg Ser Trp Pro Met Arg Leu Phe Tyr Arg Thr Leu Thr Trp Ala
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His Thr Gln Leu Ile Ile Ala Tyr Ile Met Leu Ala Val Glu Val Arg
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Ser Leu Ser Ser Leu Trp Leu Leu Cys Asn Ser Tyr Asn Ser Val Phe
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Glu Leu Leu Gln Leu Phe Pro Ala Pro Leu Leu Ala Gly Val Thr Ala
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Trp Asp Thr Asn Glu Cys Arg Pro Thr Glu Phe Ala Val Arg Ser Gly
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Leu Leu Thr Val Met Val Trp Val Ser Ser Ile Phe Phe Phe Leu Pro
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225                 230                 235                 240

Arg Arg Arg Arg Gly Asp Ala Val Val Gly Ala Ser Leu Arg Asp Gln
                245                 250                 255

Asn His Lys Gln Thr Val Lys Met Leu Gly Gly Ser Gln Arg Ala Leu
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1. A diagnostic method for the diagnosis of cancer in a patient comprising the steps of:

a. quantifying the expression levels of In1-ghrelin as defined in SEQ ID NO.: 1 in a plasma, serum, blood and/or urine sample isolated from an individual suspected of having cancer;

b. comparing the levels obtained in step (a) with a reference value or with the expression levels of In1-ghrelin as defined in SEQ ID NO.: 1 in a reference sample; and

c. assigning the individuals having expression levels of In1-ghrelin obtained in step (b) that are greater than and statistically significant with respect to the reference value or the expression levels of the reference sample to the group of individuals at risk of having cancer.

2. A prognostic method for the prognosis of cancer comprising the steps of:

a. quantifying the expression levels of In1-ghrelin as defined in SEQ ID NO.: 1 in a plasma, serum, blood and/or urine sample isolated from an individual who has cancer;

b. comparing the levels obtained in step (a) with a reference value or with the expression levels of In1-ghrelin as defined in SEQ ID NO.: 1 in a reference sample; and

c. assigning the individuals having expression levels of In1-ghrelin obtained in step (b) that are greater than and statistically significant with respect to the reference value or the expression levels of the reference sample to the group of individuals with a poor prognosis.

3. The method according to any of claims **1** to **2**, characterized in that the quantification of the expression levels of In1-ghrelin as defined in SEQ ID NO.: 1 in a plasma, serum, blood and/or urine sample isolated from an individual is performed by means of an immunoassay, where the immunoassay is an immunoblot, an enzyme-linked immunosorbent assay, radioimmunoassay, immunohistochemistry or protein microarrays.

4. The method according to any of claims **1** to **3**, characterized in that the cancer is prostate and/or colon cancer.

* * * * *