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(54) Title: METHODS FOR DIAGNOSING BENIGN VERSUS MALIGNANT THYROID LESIONS

(57) Abstract: The invention relates to methods for determining whether a thyroid nodule is malignant or benign. In particular, the present invention relates to a method for determining whether a thyroid nodule in a subject is benign or malignant said method comprising the steps of: providing a thyroid nodule sample previously collected from the said subject; and determining the level of expression of helicase-like transcription factor (HLTF) protein or a fragment thereof in a thyroid tissue sample from the subject, wherein said level of expression is indicative whether the nodule is benign or malignant.



Methods for diagnosing benign versus malignant thyroid lesions

Field of the invention

The invention relates to methods for determining whether a thyroid nodule is malignant or benign.

5 Background of the invention

Thyroid cancer is one of the most common endocrine malignancies and its most frequent clinical presentation is as a thyroid nodule, either solitary or within a multinodular goiter. Approximately 5% to 10% of adults have palpable thyroid nodules and 30% to 50% have nodules identified by ultrasound. Although the majority of these are benign, approximately
10 5% to 7% of thyroid nodules are malignant.

It is particularly challenging to distinguish between thyroid adenomas and the different carcinomas. Surgery is usually required to obtain a definitive tissue sample. However, because only 5% to 7% of the clinically identified nodules prove to be malignant, the indeterminate findings subject most patients to unnecessary surgery, and potential risks.
15 Complications from a thyroidectomy are rare (1-3%), but the procedure is expensive and there are lifelong consequences (e.g., thyroid hormone replacement and calcium deficiency treatment).

Therefore, in order to reduce the number of unnecessary operations there is a need for a diagnostic test that is more accurate than conventional methods.

20 It is an object of the present invention to overcome or ameliorate at least one of the disadvantages of the prior art, or to provide a useful alternative.

Summary of the invention

The present invention presents a method for classifying a thyroid nodule sample as an adenoma or a carcinoma comprising the step of identifying the level of expression of
25 HLTF in said sample.

In particular the present invention provides a method for determining whether a thyroid nodule in a subject is benign or malignant said method comprising the steps of:

providing a thyroid nodule sample previously collected from the said subject;

determining the level of expression of helicase-like transcription factor (HLTF) protein or a
30 fragment thereof in a thyroid tissue sample from the subject, wherein said level of expression is indicative whether the nodule is benign or malignant.

The present invention concerns an in vitro method for the prediction or for the diagnosis of a specific thyroid disease in a subject comprising the step determining the subcellular (also referred herein as intracellular) localization and/or abundance of HLTF protein or a fragment thereof in a thyroid sample from said subject.

- 5 The present invention also provides a method of identifying the stage of a thyroid tumor in a subject comprising: providing a thyroid tumor sample previously collected from the said subject; and quantifying and/or determining the level of expression of helicase-like transcription factor (HLTF) protein or a fragment thereof in a thyroid tissue sample from the subject, wherein said level of expression is indicative of the stage of the tumor.

10 **Description of the figure**

Figure 1 is a schematic representation of the exon/intro structure of the HLTF gene and two variants thereof.

- Figure 2 represents the sequence alignment between wild type HLTF (SEQ ID NO 2, bottom sequence in Figure 2) with the truncated sequence (SEQ ID NO 1 (top sequence))
15 of the HLTF cDNA from FTC-133 cell lines.

Figure 3 represents the amino acid sequence (SEQ ID NO 3) of the HLTF cDNA from FTC-133 cell lines.

- Figure 4 represents photographs showing the results of immunohistochemical expression of HLTF with the ART2 antiserum directed against HLTF on adenomas (A : 25X ; B : 50X),
20 on papillary carcinomas (C : 25X ; D : 50X), on follicular carcinomas (E : 25X) and on anaplastic carcinomas (F : 25X). In adenomas HLTF was found in the nucleus and in the cytoplasm but with a nuclear predominance. In papillary carcinomas HLTF was found mostly in the cytoplasm. In follicular and anaplastic carcinomas, the staining was weak but evenly distributed over the nucleus and the cytoplasm.

- 25 Figure 5 represents a graph showing the comparison of the intensity of the staining with the ART2 antiserum between different types of tumors. The Kruskal-Wallis test detected a significant difference between the 4 tumor types ($p = 0.0005$) and post hoc test revealed a significant difference between adenomas and anaplastic carcinomas ($p = 0.03$), papillary and follicular carcinomas ($p = 0.02$) and papillary and anaplastic carcinomas ($p = 0.002$).
30 The significance threshold associated with the Kruskal Wallis multiple comparison test is located in the top right corner of the graph. The horizontal arrows represent the post hoc comparison of the two respective tumor types.

Figure 6 represents a graph showing the comparison of the surface stained with the ART2 antiserum for the different types of tumors. The Kruskal-Wallis test detected a significant difference between the 4 tumor types ($p=10^{-6}$) and post hoc test revealed a significant difference between adenomas and follicular carcinomas ($p=0.02$) between adenomas and anaplastic carcinomas ($p=0.03$), between the papillary and follicular carcinomas ($p=0.000003$) and between papillary and anaplastic carcinomas ($p=0.000006$). The significant threshold associated with the Kruskal Wallis multiple comparison test is located in the top right corner of the graph. The horizontal arrows represent the post hoc comparison of the two respective tumor types.

Figure 7 represents a graph showing the comparison of the percentage of nuclei positive to the ART2 antiserum between different types of tumors. A significant difference was detected among the 4 tumor types ($p=10^{-6}$). The difference between the adenoma and papillary carcinoma is significant according to the post hoc test ($p=0.000004$). The Kruskal-Wallis test significant threshold is in the top right corner of the graph. The horizontal arrow represents the comparison between adenoma and papillary carcinoma.

Detailed description of the invention

The present invention will now be further described. In the following passages, different aspects of the invention are defined in more detail. Each aspect so defined may be combined with any other aspect or aspects unless clearly indicated to the contrary. In particular, any feature indicated as being preferred or advantageous may be combined with any other feature or features indicated as being preferred or advantageous.

Determination of the expression level of the HLTF protein in a thyroid sample from a subject provides a tool to screen for, diagnose, and also classify the thyroid sample as benign or malignant. The present invention allows determining or diagnosing whether subjects are afflicted with a benign or malignant thyroid condition.

When describing the method of the present invention, the terms used are to be construed in accordance with the following definitions, unless a context dictates otherwise.

As used herein the term "comprising" should not be interpreted as being restricted to the means listed thereafter; i.e. it does not exclude other elements or steps.

Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases "in one embodiment" or "in an embodiment" in various places

throughout this specification are not necessarily all referring to the same embodiment, but may. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner, as would be apparent to a person skilled in the art from this disclosure, in one or more embodiments. Furthermore, while some embodiments
5 described herein include some but not other features included in other embodiments, combinations of features of different embodiments are meant to be within the scope of the invention, and form different embodiments, as would be understood by those in the art. For example, in the following claims, any of the claimed embodiments can be used in any combination.

- 10 As used in the specification and the appended claims, the singular forms "a", "an," and "the" include plural referents unless the context clearly dictates otherwise. By way of example, "a method" means one method or more than one method.

The term "and/or" as used in the present specification and in the claims implies that the phrases before and after this term are to be considered either as alternatives or in
15 combination.

The term "subject" is interchangeably referred as "patient" and encompasses mammals such as humans, as well as animals other than humans (such as cats and dogs), swine (pigs, hogs, and wild boars), poultry, ruminants (such as cattle, oxen, sheep, giraffes, deer, goats, bison, and camels), and horses.

- 20 The present invention provides a method for determining whether a thyroid nodule in a subject is benign or malignant said method comprising the steps of: (a) providing a thyroid nodule sample previously collected from the said subject; (b) quantifying the level of expression of helicase-like transcription factor (HLTF) protein or a fragment thereof in a thyroid tissue sample from the subject, and (c) identifying whether the level of expression
25 and/or the intracellular location in said sample correlates with a benign or malignant condition.

As used herein the term "nodule" can be interchangeably referred as "lesion" and refers to any suspect, or pathological or traumatic discontinuity of tissue or loss of function of a part thereof, or to small lump, swelling or collection of tissue of the thyroid gland.

- 30 In an embodiment of the present method, the thyroid nodule is classified as a benign nodule. Preferably the benign nodule is an adenoma. Said adenoma can be selected from the group comprising follicular adenoma, adenomatoid nodule, Hurthle cell adenoma, lymphocytic thyroiditis nodule, hyperplastic nodule, papillary adenoma, thyroiditis nodule and multinodular goiter.

In an embodiment of the present method, the thyroid nodule is classified as a malignant thyroid nodule. Preferably said malignant thyroid nodule is a carcinoma. For example said carcinoma can be selected from the group comprising papillary thyroid carcinoma, follicular variant of papillary thyroid carcinoma, follicular carcinoma, and anaplastic thyroid carcinoma.

In a embodiment, the present invention concerns a method for determining whether a thyroid nodule in a subject is an adenoma or a papillary carcinoma said method comprising the steps of:

providing a thyroid nodule sample previously collected from the said subject;

- 10 determining the level of expression of helicase-like transcription factor (HLTF) protein or a fragment thereof in a thyroid tissue sample from the subject, wherein said level of expression is indicative whether the nodule is an adenoma or a papillary carcinoma.

One embodiment of the present invention concerns a method wherein the HLTF protein expression level is determined using antibodies raised against HLTF or part thereof.

- 15 Preferably, said HLTF protein expression level is determined using polyclonal antibodies raised against HLTF or part thereof. A monoclonal antibody raised against HLTF or part thereof could similarly be used. A peptide or a nucleic acid (aptamer) or a phage expressing a peptide with specific affinity for HLTF or part thereof could similarly be used. Combined antibody and PCR techniques can also be used to determine the HLTF protein
20 expression level.

In an embodiment, the HLTF protein level of expression using an analytical method comprising enzyme-linked immunosorbent assay (ELISA), immunoblot, immunohistochemistry, mass spectrometry or a combination thereof.

- 25 Preferably, the level of HLTF protein expression is determined by performing an immunohistochemistry procedure. Another visualization procedure on histological sections could also be used such as immunofluorescence. The level of HLTF protein expression can also be determined by a quantitative image analysis procedure.

- The present inventors have found that the HLTF protein was differentially expressed and/or that the cell sub-localization of said protein was different in benign and malignant
30 thyroid conditions.

In a preferred embodiment, the level of HLTF protein expression is determined by subcellular localization of the HLTF protein.

In a preferred embodiment, the HLTF expression is determined by the staining intensity of the HLTF protein.

More preferably, the HLTF expression is determined by the staining intensity and/or subcellular localization of an antibody-HLTF complex.

- 5 The present method allows the determination of the benign or malignant character of thyroid nodule sample, preferably by comparing the subcellular localization and intensity of the HLTF protein, and identifying whether the subcellular localization and/or intensity of the HLTF protein correlates with a malignant or benign condition.

For example, the identification step (c) comprises the comparison of the HLTF protein
10 level of expression in said sample with the HLTF protein level of expression in a reference sample obtained from healthy thyroid tissue of the patient.

For example, the identification step (c) comprises determining if the percentage of nucleus expressing HLTF protein is below 24% , and identifying the sample as being malignant.

In an embodiment, the method according to the invention comprises the step of
15 determining if the number (%) of nucleus expressing HLTF protein is below 24%.

The method also comprises the step of measuring if the percentage of nucleus expressing HLTF protein is below 23%, for example below 22%, 21%, 20%, 15%, 14%, 13%, preferably below 12%, more preferably 10%, for example below 9%, below 8%, more preferably below 7%, and classifying the nodule sample as being malignant.

20 Fine-needle aspiration biopsy (FNAB) can be used to obtain the thyroid nodule samples. This is particularly useful in pre-surgery determination, to avoid cumbersome surgery procedure in case of benign nodule. The sample can also be obtained by surgery on the thyroid, to confirm whether the lesion was benign or malignant. This has the advantage to help the practitioner define a post surgery treatment of the patient.

25 The sample can, of course, be subjected to a variety of well-known post-collection preparative and storage techniques (e. g. fixation, storage, freezing, lysis, homogenization, ultrafiltration, concentration, evaporation, centrifugation, etc.) prior to determining the level of protein expression in the sample.

In an embodiment, the present method comprises the steps of determining the
30 immunohistochemical level of expression of the HLTF protein and determining according thereto whether the nodule sample is benign or malignant.

In an embodiment, the present method comprises the steps of determining immunohistochemical level of expression and/or the subcellular localization of the HLTF

protein or a fragment thereof in a cell of said sample thereby classifying the thyroid nodule sample in the subject according to the HLTF localization and/or abundance.

If the nodule is classified as being from a malignant lesion, the nodule can be further classified as being from papillary thyroid carcinoma, follicular variant of papillary thyroid carcinoma, follicular carcinoma, or anaplastic thyroid carcinoma. Therefore, utilizing the methods of the present invention, one of skill in the art can diagnose a benign or malignant lesion in a subject, as well as the type of benign or malignant lesion in said subject.

The present inventors have found that the HLTF protein expression diminishes as the tumor becomes more aggressive and the cells more dedifferentiated.

The present invention therefore also provides a method of identifying the stage of a thyroid tumor in a subject comprising: providing a thyroid tumor sample previously collected from the said subject; and quantifying/ determining the level of expression of helicase-like transcription factor (HLTF) protein or a fragment thereof in a thyroid tissue sample from the subject, wherein said level of expression is indicative of the stage of the tumor.

Those skilled in the art will immediately recognize the many other effects and advantages of the present method and the numerous possibilities for end uses of the present invention from the detailed description and examples provided below.

Example

Materials and Methods

Antibodies

The HLTF protein possesses a DNA-binding domain (DBD), 7 helicase domains and a RING domain (GenBank # AJ418064). Two initial variants have been characterized HLTFMet1 and Met123 which result from alternative translation initiation site in the same reading frame and differ in their amino-terminus. Two truncated variant of the Met1 and Met23 variant which differ in their carboxyl terminus have been characterized in HeLa cells.

Figure 1 shows the structural difference between the wild type Met1 HLTF protein and the two truncated variants thereof. The wild type protein of 115kDa, is characterized by its DNA-binding domain, its 7 helicase domains and its RING domain. The truncated HLTFΔA variant of 83kDa lost the RING domain and the last 3 helicase domains, while the truncated variant HLTFΔB of 95kDa has lost the last 3 helicase domains. The vertical

arrows indicate the two sites of translation initiation (Met1 and Met123 codons). Two human HLTF variants were expressed from the same open reading frame and differed only in the translation start site (Met1 or Met123).

The rabbit antiserum (ART2) specific for the HLTFMet1 variant was raised against a peptide having the following sequence VIPPDDFLTSDEEVD (SEQ ID NO 4) in the amino-terminal sequence that is missing in the shorter Met123 variant (residues 42-56) as previously described by Debauve et al. (2006). Mol. Cancer, 5: 23.). The antiserum also recognizes the HLTFΔA and the HLTFΔB variants.

Cell lines

3 cell lines derived from human thyroid cancer (B-CPAP: papillary carcinoma, FTC-133: follicular carcinoma and 8505C: anaplastic carcinoma) were used. Two other cell lines are also used as negative and positive control:

RKO, deriving from colorectal cancer wherein the HLTF promoter is hypermethylated and the HLTF protein thus not expressed.

TE671, deriving from human rhabdomyosarcoma cells transfected with an expression vector (pCI-neo Mammalian Expression Vector) encoding the wild type HLTF protein. The pCI-neo vector was prepared by inserting cDNA encoding for wild type HLTF protein fused with 6 histidine in its amino terminal region.

The FTC-133 cell lines were maintained in Dulbecco's Modified Essential Medium (DMEM) DMEM-F12 medium, and 8505C and B-CPAP cell lines in a RPMI 1640. RKO cell lines were maintained in a Mc Coy's 5A and TE671 cell lines in a DMEM medium. Each medium (Lonza) contained 10% fetal calf serum (FCS) (GIBCO Invitrogen) and 1% of fungicides and antibiotics and (PPA The cell culture company).

Immunoprecipitation/western blot of the cell lines and sequencing of the carcinoma cell line HLTF

The expression of HLTF by immunoprecipitation and Western blot was studied in 3 cell lines derived from human thyroid cancer (B-CPAP: papillary carcinoma, FTC-133: follicular carcinoma and 8505C: anaplastic carcinoma). The cell lines were suspended in isotonic PBS. The cell suspension were then centrifuged at 3,000 g for 3 min at 4°C, and the pellets were resuspended 4°C and lysed in BugBuster Protein Extraction Reagent (Novagen, Darmstadt, Germany). the protein concentrations were measured using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Then, the proteins were resuspended in lysis buffer (4µl solution Nu Page LDS Sample Buffer 4X (Invitrogen), and 1 µl of reducing agent (Fermentas) and were heated for 5 min at 95°C and separated by SDS-8% PAGE. After separation, the proteins were electro-transferred from the gel onto a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech) using an electrophoretic transfer cell (Trans-Blot SD Semi-Dry, Bio-Rad) at 24 Volts for 30 min. Nonspecific protein-binding sites on the membranes were blocked for 1h at room temperature using PBS, 5% ECL Advance Blocking Reagent (Amersham PharmaciaBiotech) and 0.2% Tween 20. Membranes were then incubated 10 min at 4°C in ART2 primary antibody diluted 300X in PBS –Tween 20 0,1% - BSA 1%. After incubation, the membranes were washed three times in washing buffer (1X PBS and 0.2% Tween 20) and incubated 10min at room temperature with HRP-conjugated goat anti-rabbit antibodies (Amersham Pharmacia Biotech) diluted 300 times in PBS -Tween 0,1% - BSA 1%. Finally, after 5 min incubation in the presence of a revealing solution (LumiLight, Roche), immunoreactive bands were visualized by exposure of the membrane to a sensitive film (Hyperfilm ECL, Amersham Pharmacia Biotech). Biotinylated molecular weight markers were analyzed in parallel for internal calibration (Biorad Laboratories). The film is developed in an automatic processor (Durst RCP 20 Automatic color Processor, Germany). As expected no band was detected for the RKO negative control (data not shown). A band at 119kDa was observed for the B-CPAP: FTC-133, and 8505C cell lines as well as for the positive control TE671 cells. A faint band at 107kDa was observed for 8505C cell lines which might be the HLTFMet1ΔB variant. A faint band at 124kDa was observed for the FTC-133 cell lines. In order to have more information on the HLTF detected FTC-133 cell lines, an RNA ligase mediated Rapid amplification of cDNA ends (3'RLM-RACE) was performed on the total mRNA extracted from the FTC-133 cell lines. cDNA of interest was amplified using a forward primer specific to the exon 19 of the HLTF gene. Intern PCR was performed using a reverse primer was specific to the exon 20 of the HLTF gene. The product of the 3'RLM-RACE were amplified in a plasmid (CloneJET1.2/blunt) using a ligase and the plasmid transformed in bacteria C600. The plasmid DNA was then extracted using a DNA purification kit and the sequence of interest was sequenced and aligned against wild type HLTF in databases using the Basic Local Alignment Search Tool (NCBI Blast: Nucleotide Sequence). As can be seen in Figure 2, part of the sequence obtained correspond with 98% homology to exon 20, 21, 22, 23 and 24 of wild type HLTF cDNA. A thymine was found to be inserted in exon 23 creating a shift of the reading frame with an early STOP codon. The protein coded by this sequence contained the 880 first amino acids of the wild type HLTFMet1 protein and the peptide FGLFHGPKEKS (SEQ ID NO 5) from the shifted reading frame (Figure 3). Its theoretical

molecular weight calculated using the program Compute pI/Mw of Expasy was about 101kDa. It can be seen that the mutant HLTF from the FTC-133 cell lines has lost part of the helicase V carboxy terminal end and has lost the complete last helicase domain, most probably rendering it ineffective at repairing DNA.

5 Immunohistochemistry

The experiment was based on a series 20 patients presenting thyroid adenoma, 20 patients presenting thyroid papillary carcinoma, 12 patients presenting thyroid follicular carcinoma and 8 patients presenting thyroid anaplastic carcinoma. All tumor samples were fixed in 10% formaldehyde during 24h, dehydrated and embedded in paraffin.

10 Immunohistochemistry was performed on of 5µm -thick sections mounted on silane-coated glass slides. Tissue sections were exposed to microwave in a 0.01 M citrate buffer (pH 6.2) for 2X5 min at 900W. The sections were then incubated in a solution of 0.06% hydrogen peroxide for 4 min, rinsed in phosphate-buffered saline (PBS: 0.04M Na₂HPO₄, 0.01 M KH₂PO₄ anhydrous, 0.12 M NaCl, pH 7.4), and exposed during 5 min to solutions
15 containing avidin (1 mg/ml in PBS) and biotin (1 mg/ml in PBS). After washing with PBS, the sections were incubated for 15 min with 0.5% casein in PBS and exposed respectively at room temperature to solutions of the specific primary anti-HLTFMet1 ART2 antiserum (polyclonal rabbit anti-human IgG, the specific biotinylated secondary antibody (polyclonal goat anti-rabbit IgG), and the avidin-biotin-peroxidase complex (ABC kit from
20 DakoCytomation, Glostrup, Denmark). Incubation steps were alternated with washing steps to remove unbound proteins. The enzymatic activity transformed substrates (diaminobenzidine and H₂O₂) in a brown precipitate which located the HLTF protein on the section. After rinsing, sections were counterstained with luxol fast blue and mounted with a synthetic medium. The ART2 antiserum has been characterized for its specificity
25 towards HLTFMet1 as described in Debauve et al 2006. Moreover, an analysis on the ART2 antiserum specificity by immunohistochemistry in female rat mammary gland was realized. Section series of female rat mammary gland have been made 40 days after dimethylbenzanthracen (DMBA) intragastrical injection. Consecutive sections have been incubated with ART2 antiserum alone (A) or combined with 20µM immunogen peptide in
30 final volume of PBS (B), and incubated overnight at 4°C. The final revelation step was performed with a TSA fluorescein system. The results obtained showed that ART2 serum was specific to HLTFMet1. The specificity of ART2 serum was also confirmed by immunocytology by competition with the peptide in immunofluorescence on HS683 cell (cell line derived from a human glioblastoma kindly provided by Dr. F. Lefranc, Toxicology

Laboratory, ULB). The antibody were pre-incubated or not with 20 μ M of corresponding immunogen peptides prior to use in immunofluorescence.

Computer-assisted microscopy

After the immunohistochemical steps, the levels of HLTF protein expression were quantitatively determined using a computer-assisted KS 400 imaging system (Carl Zeiss Vision, Hallbergmoos, Germany) connected to a Zeiss Axioplan microscope. For each section, 10 fields were scanned at 20X enlarging and a picture was recorded for each of them by the ProgRes CapturePro 2.1 program. The morphometry analysis was focused on beforehand defined tumoral region where two variables were measured: the labeling index (LI) i.e. the percentage of immunopositive tissue areas, and the mean optical density (MOD) i.e. the staining intensity of positive cells and the mean was calculated for each section.

Optical microscopy

All sections were observed on 10 different fields at 40X enlarging to count positive nuclei in a gate of 0,084mm². The mean was calculated for each section. Then, two values were retained; means of positive nuclei for each tumoral type.

Statistical analysis

Independent groups of quantitative data were compared using the nonparametric Kruskal- Wallis tests. In the case of significant Kruskal-Wallis tests, post-hoc tests (Dunn procedure) were used to compare pairs of groups (to avoid multiple comparison effects).

All statistical analyses were carried out with Statistica software (Statsoft, Tulsa, USA)

Results

The immunohistochemical expression of HLTF was measured on the samples obtained from the 20 cases of adenoma, 20 cases of papillary carcinoma, 12 cases of follicular carcinoma and 8 cases of anaplastic carcinoma. Detection was based on a chemical reaction in which the peroxidase will catalyze the oxidation of its substrate, diaminobenzidine, into a brown precipitate. The colored precipitate localized the antigenic site of the HLTF protein on the histological sections.

The results of the immunodetection are shown in Figure 4. In adenomas HLTF is localized in both the nucleus and the cytoplasm of cells, but with significant higher staining intensity at the nuclear level. In papillary carcinomas, the immunostaining is exclusively cytoplasmic. For follicular and anaplastic carcinomas, the HLTF protein is homogeneously present in both compartments with an apparent lower intensity of labeling (Figure 4).

For each clinical case, a counting of the labeled nuclei and of the total nuclei over 10 different fields covering a surface area of 0.084 mm², at a magnification of 40X. The average percentage of labeled nuclei was then calculated for each case. Positive nuclei were characterized by a medium to strong staining, and blue nuclei or weakly stained
5 were considered as negative.

For each slide, 10 fields were scanned at a magnification of 20X and a color photograph for each of them was recorded by the Image acquisition software Progres CapturePro 2.1. After defining the tumor area for each selected field, morphometric analysis was performed using the software KS 400. Two parameters were measured, namely, the MOD
10 for the staining intensity and the LI for the percentage of immunopositives cells. An average was calculated for each clinical case's two parameters and a median for each tumor type. The intensity of staining by the anti-HLTF was compared between the 4 tumor types: the adenomas, papillary, follicular and anaplastic carcinomas. A significant difference was detected (MOD p-value = 0.0005, Kruskal-Wallis). Two-two comparison
15 detected a significant difference between adenomas and anaplastic carcinomas (p-value = 0.03; post hoc test), between papillary and follicular carcinomas (p-value = 0.02, post hoc test), and between papillary and anaplastic carcinoma (p-value = 0.002, post hoc test). The results are shown in Figure 5. The intensity of the HLTF staining (MOD HLTF) was higher in papillary carcinomas and in adenomas and gradually decreased in follicular and
20 anaplastic carcinomas.

A comparison of the areas stained by the anti-HLTF ART2 antiserum for adenoma, and papillary, follicular and anaplastic carcinoma was performed. A significant difference was observed (LI: p-value = 10⁻⁶; Kruskal-Wallis). A comparison of the tumors was performed and significant differences were observed between adenomas and follicular carcinomas
25 (p-value = 0.02, post hoc test) between the adenomas and anaplastic carcinomas (p-value = 0.05, post hoc test) between papillary and follicular carcinomas (p-value = 0.000003; post hoc test) and between the papillary and anaplastic carcinomas (p-value = 0.000006; post hoc test). The results are shown in Figure 6. The percentage of cells expressing HLTF (LI HLTF) was high in papillary carcinoma and decreased gradually in the
30 adenomas, and in anaplastic and follicular carcinoma.

The percentage of immunopositive nuclei was also compared between the 4 tumors and a significant difference was measured (% nuclei+ p -value = 10⁻⁶ ; Kruskal-Wallis). The post-hoc test detected only a significant difference between the adenomas and the papillary carcinomas (p-value = 0.000004 ; post hoc test). The results are shown in Figure

7. It can be seen that the adenomas median is clearly higher than the papillary carcinomas median.

Papillary carcinomas represent more than 80% of the carcinomas, and give the most difficult differential diagnosis problem because of their highest occurrence. The present
5 inventors have shown that the HLTF protein is differentially expressed in tumors such as adenomas, papillary, follicular and anaplastic carcinomas of the thyroid gland and more in particular in adenomas, and papillary carcinomas, thereby providing an efficient diagnostic tool.

The HLTF expression was also measured by immunocyto-histochemistry and Western
10 blot for three established cell line derived from human thyroid cancer. The expression of HLTF was in particular measured on a line derived from human papillary carcinoma (B-CPAP), human follicular carcinoma (FTC-133), and human anaplastic carcinoma (8505C). In the 3 cancer cell lines, HLTF was detected by immunofluorescence exclusively in the nucleus of cells. A protein of apparent molecular weight to the HLTF wild type form was
15 detected by Western blot in cell lines derived from follicular and anaplastic carcinomas, while a truncated variant was detected in the anaplastic cells. HLTF mRNA from follicular carcinoma cells was amplified by 3'RLM – RACE and the nucleotide sequence showed the expression of a truncated mutant protein.

The expression of HLTF was studied in 3 cell lines derived from human thyroid cancer (B-
20 CPAP: papillary carcinoma, FTC-133: follicular carcinoma and 8505C: anaplastic carcinoma). Surprisingly, the results (not shown) have demonstrated that HLTF is only expressed in the nucleus of these established cancer cell lines, while in vitro immunohistochemistry on biological thyroid samples showed that the HLTF was differentially expressed at the sub cellular level. In addition the level of expression was
25 high in the established follicular and anaplastic carcinoma cell lines, while the level of expression was low in the biopsy samples of follicular and anaplastic carcinomas.

Analysis of the LI and MOD parameters for the HLTF staining of the biopsy samples provided an efficient tool to discriminate between the adenomas and the other carcinomas. For instance, the transition from adenoma to papillary carcinoma is
30 characterized by a shuttle of HLTF from the nucleus (positive in the adenomas) into the cytoplasm (positive in the papillary carcinoma), in addition loss of tumor differentiation observed in follicular and anaplastic carcinomas is mainly associated with a decrease of HLTF expression.

Immunohistochemical study of HLTF expression according to an embodiment of the present invention allows an early determination of the type of thyroid nodule. It can be used in preoperative stage to guide the therapeutic decision (surgery or monitoring of the patient).

- 5 The present invention allows avoiding surgeries of thyroid benign pathology, whereas currently the "return" operation is very low with about 90% of patients being operated for a benign pathology. These surgeries can now be avoided using the method according to the present invention to determine or classify the thyroid nodule beforehand.

Claims

1. A method for determining whether a thyroid nodule in a subject is benign or malignant said method comprising the steps of:
 - 5 providing a thyroid nodule sample previously collected from the said subject;
determining the level of expression of helicase-like transcription factor (HLTF) protein or a fragment thereof in a thyroid tissue sample from the subject, wherein said level of expression is indicative whether the nodule is benign or malignant.
- 10 2. The method according to claim 1, wherein the thyroid nodule is classified as a benign nodule.
3. The method according to claim 2, wherein the benign nodule is an adenoma.
4. The method according to claim 1, wherein the thyroid nodule is classified as a malignant thyroid nodule.
- 15 5. The method according to claim 4, wherein the malignant thyroid nodule is a carcinoma.
6. The method according to any one of claims 1 to 5, wherein the HLTF protein expression level is determined using monoclonal or polyclonal antibodies raised against HLTF or part thereof, or using a peptide or a nucleic acid or a phage expressing a peptide with specific affinity for HLTF.
- 20 7. The method according to any one of claims 1 to 6 wherein the HLTF protein expression level is determined using polyclonal antibodies raised against HLTF or part thereof.
8. The method according to any one of claims 1 to 7, wherein the level of HLTF protein expression is determined by performing an immunohistochemistry procedure.
- 25 9. The method according to any one of claims 1 to 7, wherein the level of HLTF protein expression is determined by performing an immunofluorescence procedure.
10. The method according to any one of claim 1 to 9, wherein the level of HLTF protein expression is determined by a quantitative image analysis procedure.
11. The method according to any one of claims 1 to 10, wherein the level of HLTF protein
30 expression is determined by subcellular localization of the HLTF protein.

12. The method according to any one of claims 1 to 11, wherein the HLTF expression is determined by the staining intensity of the HLTF protein.
13. The method according to any one of claims 1 to 12, wherein the HLTF expression is determined by the staining intensity and/or subcellular localization of an antibody-
5 HLTF complex.
14. The method according to any one of claims 1 to 13, wherein a percentage of nucleus expressing HLTF protein below 24% is indicative of a malignant nodule.
15. A method of identifying the stage of a thyroid tumor in a subject comprising: providing a thyroid tumor sample previously collected from the said subject; and quantifying/
10 determining the level of expression of helicase-like transcription factor (HLTF) protein or a fragment thereof in a thyroid tissue sample from the subject, wherein said level of expression is indicative of the stage of the tumor.

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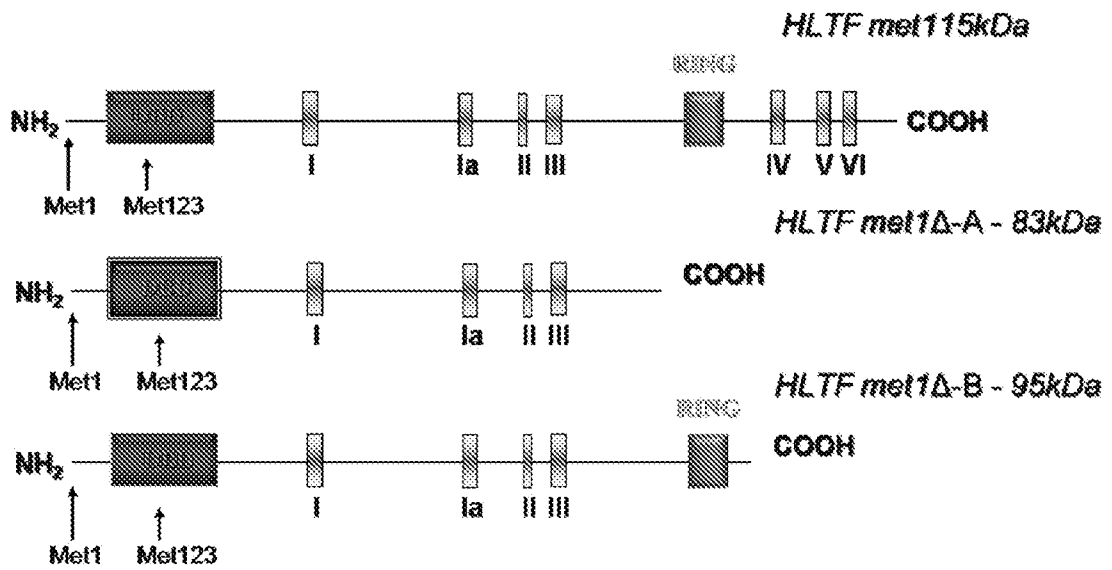


FIG. 1

FIG. 2

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10	20	30	40	50	60
MSWMFKRDPV	WKYLQIVQYG	VHGNFPRLSY	PTFFPRFEFQ	DVIPPDDFLT	SDEEVDSVLF
70	80	90	100	110	120
GSLEGHVVGL	RYVTGVVNNN	EMVALQRDPN	NPYDKNAIKV	NNVNGNQVGH	LKKELAGALA
130	140	150	160	170	180
YINDNKLAQI	EGVVPFGANN	AFTMPLHMTF	WGKEENRKAV	SDQLKKHGFK	LGPAPKTLGF
190	200	210	220	230	240
NLESGWGSGR	AGPSYSMPVH	AAVQMTTEQL	KTEFDKLFED	LKEDDKTHEM	EPAEAIETPL
250	260	270	280	290	300
LPHQKQALAW	MVSRENSKEL	PPFWBQRNDL	YYNTITNFSE	KLRPENVRGG	ILADDMGLGK
310	320	330	340	350	360
TLTAIAVILT	NFHDGRPLPI	ERVKKNLLKK	EYNVNDDSMK	LGGNNTSEKA	DGLSKDASRC
370	380	390	400	410	420
SEQPSISDIK	EKSKFRMSL	SSSRPKRRKT	AVQYIESSDS	EEIETSELPQ	KMKGKLKNVQ
430	440	450	460	470	480
SETKGRAKAG	SSKVIEDVAF	ACALTSSVPT	TNKKMLKKGA	CAVEGSKKTD	VEERPPTLI
490	500	510	520	530	540
ICPLSVLSNW	IDQFGQHIKS	DVHLNFYVYY	GPDRIREPAL	LSKQDIVLTT	YNILTHDYGT
550	560	570	580	590	600
KGDSPLHSIR	WLRVILDEGH	AIRNPNAQQT	KAVLDLESER	RWVLTGTPIQ	NSLKDLWSSL
610	620	630	640	650	660
SFLKLKPFID	REWWHRTIQR	PVTMGDEGGL	ERLQSLIKNI	TLRRTKTSKI	KGKPVLELPE
670	680	690	700	710	720
RKVFIQHITL	SDEERNIYQS	VKNEGRATIG	RYFNEGTVLA	HYADVLGLLL	RLRQIOCHTY
730	740	750	760	770	780
LLTNAVSSNG	PSGNDTPEEL	RKKLIRKMKL	ILSSGSDEEC	AICLDSLTVP	VITHCAHVFC
790	800	810	820	830	840
KPCICQVIQN	EQPHAKCPLC	RNDIHEDNLL	ECFPEELARD	SEKKSMEWT	SSSKINALMH
850	860	870	880	890	
ALTDLRKKNF	NIKSLVVSQF	TTFLSLIEIP	LKASGFVFTR	SLFHGFKEK	

FIG. 3

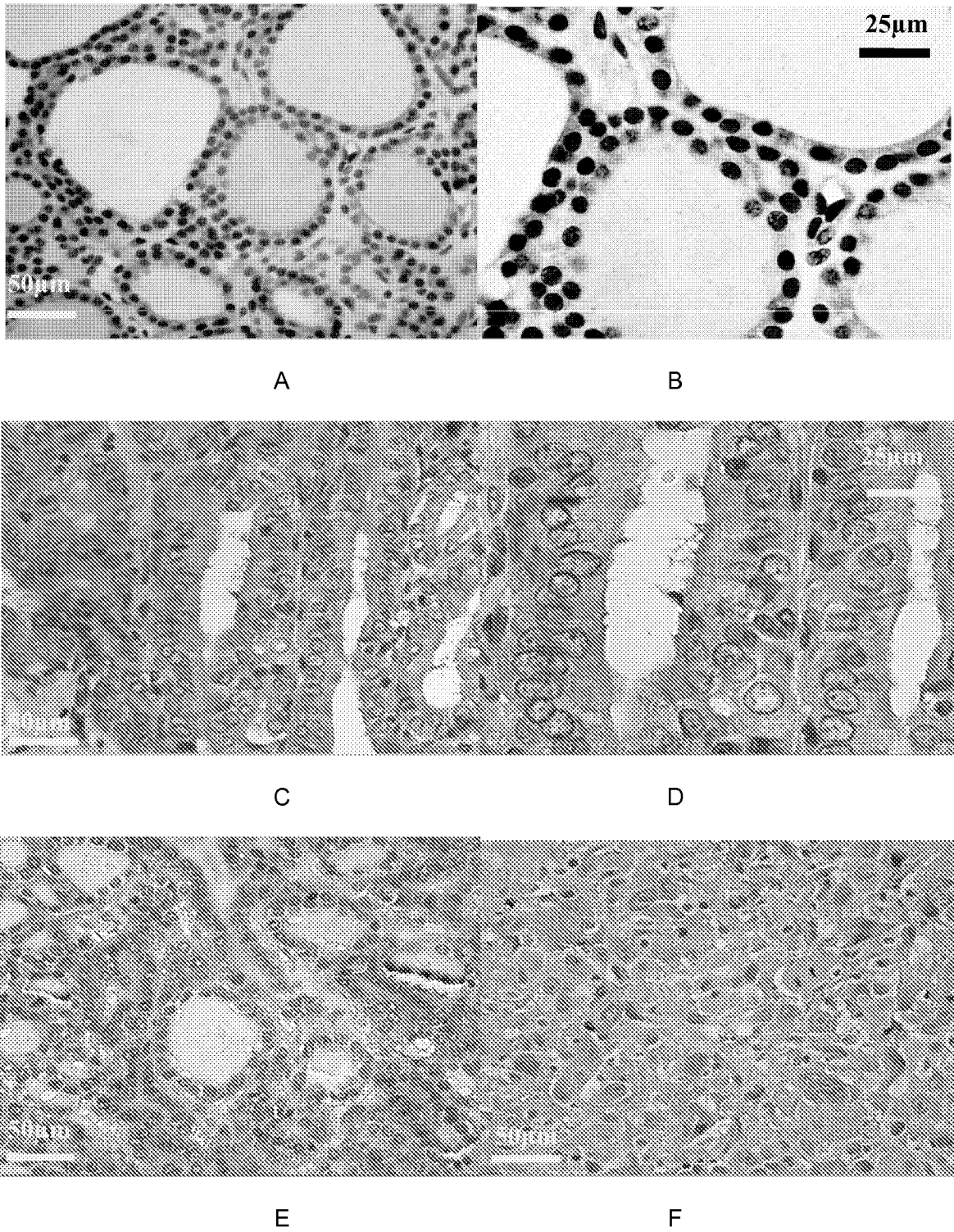


FIG. 4

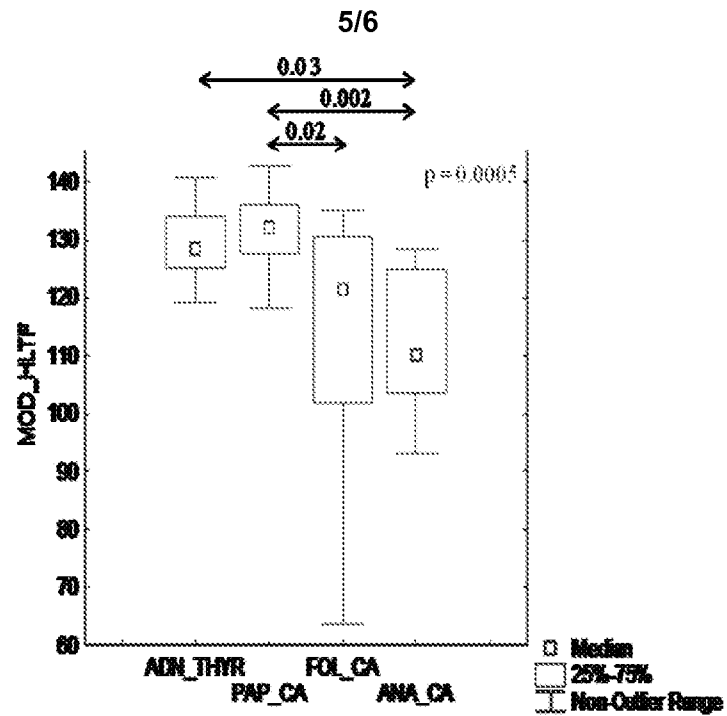


FIG.5

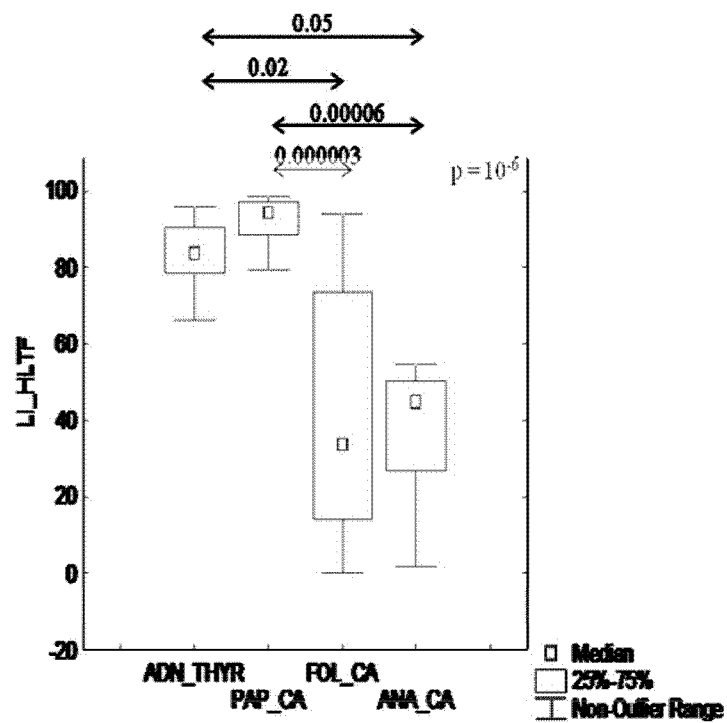


FIG.6

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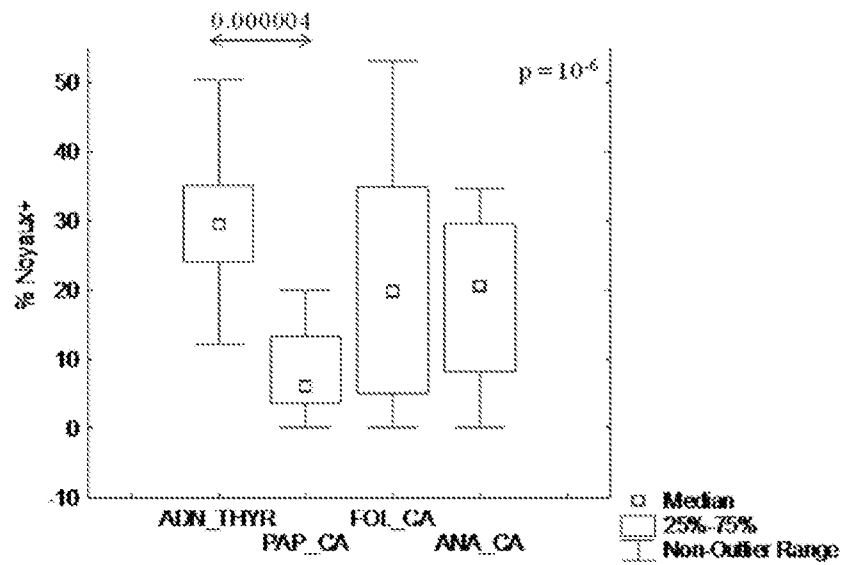


FIG.7

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/057744

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/574 G01N33/68 C12Q1/68

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N C12Q

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2008/130887 A1 (UNIV COLORADO [US]; HUNSUCKER STEPHEN W [US]; DUNCAN MARK W [AU]; HAUG) 30 October 2008 (2008-10-30) abstract; claims	1-15
A	CHEVILLARD SYLVIE ET AL: "Gene expression - profiling of differentiated thyroid neoplasms: diagnostic and clinical implications" CLINICAL CANCER RESEARCH, THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, US, vol. 10, no. 19, 1 October 2004 (2004-10-01), pages 6586-6597, XP002444872 ISSN: 1078-0432 abstract tables 2, 3	1-15

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

☒ See patent family annex.

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Date of the actual completion of the international search

22 October 2009

Date of mailing of the international search report

30/10/2009

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/057744

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 1 862 178 A1 (UNIV MONS HAINAUT [BE]; UNIV BRUXELLES [BE]) 5 December 2007 (2007-12-05) abstract; claims -----	1-15
A	DEBAUVE G ET AL: "The Helicase-Like Transcription Factor and its implication in cancer progression" CMLS CELLULAR AND MOLECULAR LIFE SCIENCES, BIRKHÄUSER-VERLAG, BA, vol. 65, no. 4, 24 November 2007 (2007-11-24), pages 591-604, XP019583888 ISSN: 1420-9071 abstract -----	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2009/057744

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 2008130887	A1	30-10-2008	NONE	
EP 1862178	A1	05-12-2007	NONE	