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(54) Title: A GAMMACARBOXYGLUTAMATE-RICH PROTEIN, METHODS AND ASSAYS FOR ITS DETECTION, PURIFICATION AND QUANTIFICATION AND USES THEREOF

(57) Abstract: The present invention refers to a gammacarboxylglutamate -rich protein that shows in vivo a high capacity to bind calcium through specific gamma carboxylated glutamic acid residues (Gla). It includes a description of the referred protein, purification procedures, protein detection and quantification tools and methods.. This invention also refers to a kit for the detection and quantification of said protein in samples. This kit includes the use of one or more antibodies produced against the homologous sequence of the target species to be analyzed, and thus, methods for the production of such antibodies are disclosed as well. In another aspect of the invention, the methods and tools described hereby are used as biomarkers for evaluation of presence or risk to develop certain diseases. In another aspect of this invention, available complete GRP cDNA and gene sequences obtained from several species also enable the in vitro production of antigens, the quantification of GRP expression, the screening of GRP polymorphisms to access the predisposition for certain diseases and the screening for GRP mutations.



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AMENDED CLAIMS

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1. A biomarker for biological analysis (GRP) consisting of:

- a) a novel vitamin K-dependent protein rich in gammacarboxyglutamate residues (GRP) comprising between 1 to 16 of gammacarboxyglutamate residues (Gla), in substitution of the glutamic acid residues (Glu), according to the species, providing a GRP fully or partially gammacarboxylated;
- b) cDNA and gene sequences (including promoter and 5' and 3' flanking regions) for the protein from animal origin, including human or other mammalian species, or fish, or amphibian, or reptile origin.

2. The biomarker according to claim 1 wherein it includes the following specific motifs:

- a) a signal peptide including 26-27 amino acids;
- b) a propeptide domain containing a gamma-carboxylase recognition site, and an AXXF motif; and
- c) a proven Gla-containing mature protein with 65-74 amino acids and containing between 14 and 16 Gla residues, depending on the species.

3. A method for producing the biomarker of claims 1-2 using in vitro recombinant techniques wherein it includes the expression by the same cell of the 4 enzymes required, and comprises the following steps:

- a) constructing a cDNA which encodes in frame the:

- (1) GRP open reading frame;
 - (2) gamma-carboxylase open reading frame;
 - (3) vitamin K oxidoreductase;
 - (4) PACE/furin open reading frame;
 - b) cloning the corresponding cDNA into viral expression vectors;
 - c) transforming mammalian or insect cells; and expressing the four cDNAs simultaneously in the referred host systems;
 - d) isolation and purification of the protein.
4. A method for isolating and purifying the native biomarker of claims 1 to 2, comprising the following steps:
- a) introducing the sample into an extraction solution of guanidine 1 to 6 M; urea 1 to 4 M or a combination of both;
 - b) applying a demineralization step procedure for extracting GRP from calcified tissues samples
 - c) applying a dialysis step;
 - d) a dissolution in a denaturant solution or a solution with pH in the acidic range (1-7);
 - e) applying an isolation step;
 - f) applying a purification step.
5. The method according to previous claim wherein samples are from animal origin, more specifically mammalian origin including human, or fish or amphibian, or reptile origin and wherein samples are tissues, fluid samples, organs, whole organisms and or cell cultures.

6.The method according to claims 4-5 wherein the isolation step is performed by incubating the extracted solution with hydroxyapatite or other calcium mineral phase or surface followed by a demineralization step procedure.

7.The antibodies required to detect a biomarker as described in claims 1-2 including:

- a) the hybridomas for producing a IgG antibody comprising the homologous amino acid sequence against a protein containing at least those amino acid residues that can specifically recognize and bind the epitopes contained or part of a sequence with regions A and/or B accordingly to the sequences identified in Table 1; wherein said hydridoma is formed by fusion of cells from a mouse myeloma and spleen cells from a mouse previously immunized with a peptide homologous to a sequence with regions A and or B as described in Table 1.
- b) a polyclonal antibody of class IgG, comprising a polyclonal antibody directed against an epitope in the biomarker protein residues 56-74 from rat or human according to the sequence identified in Table 1 region B wherein said antibody is produced by an animal previously immunized with a peptide homologous to rat or human biomarker protein residues 56-74.

8.A method for detecting and/or quantifying the biomarker described in claims 1-2, comprising the following steps:

- a) obtaining the sample, from a group consisting of blood, serum or plasma sample or other source sample or extract obtained from, for example, biological fluids, cell cultures (from mammalian or non-mammalian origin), biopsies, organs, tissues or even a complete organism;
- b) incubation of the targeted samples with at least one of the antibodies of claim 7;
- c) measuring the amount of the biomarker in the samples using molecular techniques.

9. The method according to previous claim wherein the amount of the protein in the sample is measured using an immunoassay, wherein the immunoassay is an immunoprecipitation assay, a competitive immunoassay, an ELISA immunoassay, a radioimmunoassay (RIA), a western-blot based assay, or a dot-blot based assay.

10. The method according to claims 8-9 wherein the immunoassay utilizes a detectable label to measure the protein selected from the group consisting of radioisotopes, enzymes, fluorescent molecules, chemiluminescent molecules, bioluminescent molecules and colloidal metals.

11. A method for analysing the presence of GRP polymorphisms and mutations comprising the following steps:

- a) extraction of genomic human DNA, selected from normal or diseased populations or from populations thought to be possible targets for presence of mutations in GRP gene based on the observed phenotypes, from human fetus cells extracted after amniocentesis or from umbilical cord blood;
- b) amplification by PCR of specific GRP fragments that are coding, non-coding and/or regulatory regions, including exons, introns, promoters and flanking DNA regions, both 5' and 3', respectively;
- c) sequencing of those amplified fragments;
- d) comparison with the wild type sequences of claim 1-2 for identification of mutations, multiple deletions, insertions, substitutions, and/or SNPs.

12. A diagnostic kit for detecting and/or quantifying a biomarker of claim 1 and 2 in a sample of blood, serum or plasma, cell cultures, biological fluids, cell or other biological extracts, biopsies, organs, tissues or even a complete organism and comprising the following:

- a) mRNA probes for the biomarker described in claims 1-2 comprising any region of the species-specific cDNA with more than 25 bp of the sequence;

b) species-specific primers or probes for detecting and quantifying the mRNA of the biomarker described in claims 1-2;

c) one or more monoclonal antibodies directed comprising at least one antibody against an epitope in the biomarker protein residues 1-53 or 54-75 or combinations thereof as described in claim 8;

d) one or more polyclonal antibody directed against an epitope in the biomarker human or rat protein residues 56-74 according to the sequence identified in Table 1 wherein said antibody is produced by an animal previously immunized with a peptide homologous to human biomarker protein residues 56-74 as described in claim 8.

13. The use of the kit of previous claim, for detecting and evaluating the status of the biomarker described in claims 1-2 produced in physiological conditions or experimental systems using cell/organ cultures or whole organisms and including levels, sites of expression, types of isoforms and degree of postranslation modifications such as including gammacarboxylation.

14. The use of the kit of claim 12 for monitoring, detecting or determining the risk for abnormal calcification of tissues in a vertebrate, in samples from said vertebrate, wherein a variation on the level/status of the biomarker as compared to that found in a control, indicates that said vertebrate is at increased risk for abnormal

calcification of tissues associated with coronary atherosclerosis, vascular calcification angiogenesis, diseases of bone and cartilage including joints, a disease of the vascular system, diabetes mellitus, or ectopic calcification in tumour development, or in skin, thereby monitoring or detecting coronary atherosclerosis, vascular calcification angiogenesis, diseases of bone and cartilage including joints, a disease of the vascular system, diabetes mellitus, ectopic calcification in the skin or in tumor development.

15. Use of the biomarker of claims 1 to 2 for detect vertebrate pathological conditions selected from the group of coronary atherosclerosis, vascular calcification angiogenesis, diseases of bone and cartilage including joints, a disease of the vascular system, diabetes mellitus, ectopic calcification in tumor development, or in skin.

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Lisbon, 13th May, 2010

Dear Sirs,

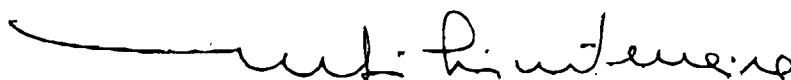
Re: International Application No. PCT/ PT2009/000046

Applicant: UNIVERSIDADE DO ALGARVE

Statement under Article 19(1) PCT

Please find enclosed a new set of claims for the above-mentioned patent application as well the Letter reflecting the changes made to the claims.

Yours sincerely,



Maria Silvina Ferreira
Patent Attorney