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(54) Title: NEW ASSAY FOR DETECTING OR PREDICTING THORACIC AORTIC ANEURYSMS

(57) Abstract: The invention relates to a method for diagnosing the cause or the predisposition for thoracic aortic aneurysms and/or other arterial aneurysm by testing a sample of a subject for a mutation in the SMAD3 gene and/or SMAD3 protein wherein said mutation is chosen from the group of 1157C>T, 1080C>T, 1039-1040 delAT, (on protein level Arg287Trp, Thr261Ile, a premature protein termination at position 309, respectively) and combinations thereof.

Title: New assay for detecting or predicting Thoracic Aortic Aneurysms

FIELD OF THE INVENTION

The present invention relates to the field of molecular diagnostics, more specifically to diagnostics on basis of changes in human genes. More particularly, the invention relates to the diagnosis of aneurysms or the predisposition to develop aneurysms.

BACKGROUND TO THE INVENTION

A thoracic aortic aneurysm (TAA) is a widening (bulging) of part of the wall of the aorta, the body's largest artery. Approximately 25 percent of aortic aneurysms occur in the chest, and the rest involve the abdominal aorta. Thoracic aortic aneurysms are serious health risks because they can burst or rupture. A ruptured aneurysm can cause severe internal bleeding, which can rapidly lead to shock or death.

Thoracic aneurysms affect approximately 15,000 people in the United States each year. Some patients may have aortic aneurysm extending to the abdominal aorta or may have aneurysms in other arteries of the body. Only about 10 percent of patients who get to the hospital with a ruptured TAA survive in contrast to the 95 % that survive after elective surgery. For this reason, it is crucial to diagnose and treat aneurysms early, in order to prevent their rupture.

Many diseases and conditions, such as atherosclerosis (hardening of the arteries), hypertension, genetic conditions (such as Marfan Syndrome, Ehler-Danlos disorder, polychondritis, scleroderma, osteogenesis imperfecta, polycystic kidney disease, and Turner Syndrome) and injury, can cause the aorta to dilate (widen) or dissect (tear), placing the patient at increased risk for future life-threatening events.

About 20% of the thoracic aorta aneurysms may be part of a recognizable multisystem disorder such as Marfan syndrome, and Loeys-Dietz syndrome. These hereditable syndromic forms of TAA are characterized by cardiovascular, skeletal, cutaneous and craniofacial abnormalities. Marfan syndrome is caused by mutations in Fibrillin-1 gene and mutations in TGFBR1 and TGFBR2 genes cause Loeys-Dietz syndrome. All three genes are involved in the TGF-beta signalling pathway which plays a central role in the pathogenesis of both syndromic and non-syndromic thoracic aneurysms (Jones, J.A. et al., 2009, J. Vasc. Res. 46:119-137).

SUMMARY OF THE INVENTION

The current inventors now have found that mutations in the SMAD3 gene can be used in a diagnostic assay for detecting a hereditary form of TAA. The clinical presentation of these patients is very similar to Marfan syndrome and Loeys-Dietz syndrome and familial TAA. A distinctive feature of SMAD3-related disease is the presence of early onset osteoarthritis and other joint abnormalities.

Therefore the invention comprises a method for diagnosing the cause of a thoracic aortic aneurysm or any other aneurysm or the predisposition for developing a (thoracic) aortic aneurysm or any other aneurysm comprising:

a. taking a sample from a subject;
b. assaying said sample for the presence of a mutation in the SMAD3 gene or protein; and
c. reaching the diagnosis on basis of the results of said assay.

Preferably in said method the mutation in the SMAD3 protein is chosen from the group consisting of Arg287Trp, Thr261Ile, a premature protein termination at position 309, and combinations thereof. Alternatively, the mutation in the SMAD3 gene is chosen from the group consisting of 1157C>T, 1080C>T, 1039-1040 delAT, and combinations thereof.

In such a method according to the invention the sample is preferably a blood, saliva or other tissue sample.

In another embodiment the invention comprises a method for diagnosing the cause of a thoracic aortic aneurysm or any other aneurysm, or the predisposition for developing a thoracic aortic aneurysm or any other aneurysm comprising:

a. taking a sample from a subject;
b. assaying said sample for the presence of a mutation in the SMAD3 gene or protein, the fibrillin-1 gene and the TGFBR1 and TGFBR2 genes; and
c. reaching the diagnosis on basis of the results of said assay.

In a further embodiment, the invention comprises a method for treatment of a subject suffering from a thoracic aortic aneurysm or having a predisposition for developing a thoracic aortic aneurysm, wherein said subject has a mutation in the SMAD3 gene, but does not have a mutation in the fibrillin-1 gene and the TGFBR1 and TGFBR2 genes, by administration of an angiotensin II type 1 receptor blocker, preferably losartan. Preferably, in such a method next to the angiotensin II type 1 receptor blocker an inhibitor of matrix metalloproteases, preferably doxycycline, is administered.

Also the invention relates to an angiotensin II type 1 receptor blocker, preferably losartan, or the combination of said angiotensin II type 1 receptor blocker and doxycycline, for use in the treatment of a subject suffering from a thoracic aortic aneurysm or having a predisposition for developing a thoracic aortic aneurysm, wherein said subject has a mutation in the SMAD3 gene but does not have a mutation in the fibrillin-1 gene and the TGFBR1 and TGFBR2 genes.

LEGEND TO THE FIGURES

Figure 1 shows a schematic representation of the SMAD3 gene and the corresponding protein domains. Three mutations clustering in exon 6 (1039-1040 delAT, 1080C>T and 1157C>T) are indicated. The predicted abnormal SMAD3 protein as consequence of the 1039-1040 deletion is shown and the strong aminoacid conservation around the mutated amino acids Thr261 and Arg287, indicating the importance of these regions for normal protein function is illustrated. A tridimensional image of the SMAD3-SMAD4 heterotrimer is shown and the positions of the mutations are indicated.
Figure 2 shows the cDNA nucleotide sequence of the SMAD3 gene (A) and the protein that is encoded thereby (B). These sequences are also available form GenBank under the accession no. NM_005902.3 and NP_005893.1, respectively. Sequences containing the mutations of fig. 1 are underlined. Please note that only isoform 1 is shown, more isoforms are known.

DETAILED DESCRIPTION

A "complement" or "complementary sequence" is a sequence of nucleotides which forms a hydrogen-bonded duplex with another sequence of nucleotides according to Watson-Crick base-paring rules. For example, the complementary base sequence for 5'-AAGGCT-3' is 3'-TTCCGA-5'.

"Expression" refers to the transcription of a gene into structural RNA (rRNA, tRNA) or messenger RNA (mRNA) and, if applicable, subsequent translation into a protein.

Polynucleotides are "heterologous" to one another if they do not naturally occur together in the same organism. A polynucleotide is heterologous to an organism if it does not naturally occur in its particular form and arrangement in that organism.

Polynucleotides have "homologous" sequences if the sequence of nucleotides in the two sequences is similar when aligned for maximum correspondence as described herein. Sequence comparison between two or

more polynucleotides is generally performed by comparing portions of the two sequences over a comparison window to identify and compare local regions of sequence similarity. The comparison window is generally from about 20 to 200 contiguous nucleotides. The "percentage of sequence homology" for polynucleotides, such as 50, 60, 70, 80, 90, 95, 98, 99 or 100 percent sequence homology may be determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may include additions or deletions (i.e. gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by: (a) determining the number of positions at which the identical nucleic acid base occurs in both sequences to yield the number of matched positions; (b) dividing the number of matched positions by the total number of positions in the window of comparison; and (c) multiplying the result by 100 to yield the percentage of sequence homology. Optimal alignment of sequences for comparison may be conducted by computerized implementations of known algorithms, or by inspection. Readily available sequence comparison and multiple sequence alignment algorithms are, respectively, the Basic Local Alignment Search Tool (BLAST) (Altschul, S.F. et al. 1990. J. Mol. Biol. 215:403; Altschul, S.F. et al. 1997. Nucleic Acid Res. 25:3389-3402) and ClustalW programs both available on the internet. Other suitable programs include GAP, BESTFIT and FASTA in the Wisconsin Genetics Software Package (Genetics Computer Group (GCG), Madison, WI, USA).

As used herein, "substantially complementary" means that two nucleic acid sequences have at least about 90%, preferably about 93%, more preferably about 95%, and most preferably about 98%, sequence complementarity to each other. This means that the primers and probes must exhibit sufficient complementarity to their template and target nucleic acid, respectively, to hybridise under stringent conditions. Therefore, the primer sequences proposed in this specification need not reflect the exact sequence of

the binding region on the template and degenerate primers can be used. A substantially complementary primer sequence is one that has sufficient sequence complementarity to the amplification template to result in primer binding and second-strand synthesis.

The term "hybrid" refers to a double-stranded nucleic acid molecule, or duplex, formed by hydrogen bonding between complementary nucleotides. The terms "hybridise" or "anneal" refer to the process by which single strands of nucleic acid sequences form double-helical segments through hydrogen bonding between complementary nucleotides.

The term "oligonucleotide" refers to a short sequence of nucleotide monomers (usually 6 to 100 nucleotides) joined by phosphorous linkages (e.g., phosphodiester, alkyl and aryl-phosphate, phosphorothioate), or non-phosphorous linkages (e.g., peptide, sulfamate and others). An oligonucleotide may contain modified nucleotides having modified bases (e.g., 5-methyl cytosine) and modified sugar groups (e.g., 2'-O-methyl ribosyl, 2'-O-methoxyethyl ribosyl, 2'-fluoro ribosyl, 2'-amino ribosyl, and the like). Oligonucleotides may be naturally-occurring or synthetic molecules of double- and single-stranded DNA and double- and single-stranded RNA with circular, branched or linear shapes and optionally including domains capable of forming stable secondary structures (e.g., stem-and-loop and loop-stem-loop structures).

The term "primer" as used herein refers to an oligonucleotide which is capable of annealing to the amplification target nucleotide sequence allowing a DNA polymerase to attach thereby serving as a point of initiation of DNA synthesis when placed under conditions in which synthesis of primer extension product which is complementary to a nucleic acid strand is induced, i.e., in the presence of nucleotides and an agent for polymerization such as DNA polymerase and at a suitable temperature and pH. The (amplification) primer is preferably single stranded for maximum efficiency in amplification. Preferably, the primer is an oligodeoxy ribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact lengths of the primers will depend on many factors, including temperature and source of primer. A "pair of bi-directional primers" as used herein refers to one forward and one reverse primer as commonly used in the art of DNA amplification such as in PCR amplification.

The term "probe" refers to a single-stranded oligonucleotide sequence that will recognize and form a hydrogen-bonded duplex with a complementary sequence in a target nucleic acid sequence analyte or its cDNA derivative.

The terms "stringency" or "stringent hybridization conditions" refer to hybridization conditions that affect the stability of hybrids, e.g., temperature, salt concentration, pH, formamide concentration and the like. These conditions are empirically optimised to maximize specific binding and minimize non-specific binding of primer or probe to its target nucleic acid sequence. The terms as used include reference to conditions under which a probe or primer will hybridise to its target sequence, to a detectably greater degree than other sequences (e.g. at least 2-fold over background). Stringent conditions are sequence dependent and will be different in different circumstances. Longer sequences hybridise specifically at higher temperatures. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridises to a perfectly matched probe or primer (i.e. 100% complementarity). Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na^+ ion, typically about 0.01 to 1.0 M Na^+ ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes or primers (e.g. 10 to 50 nucleotides) and at least about 60°C for long probes or primers (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringent conditions or "conditions of reduced stringency" include hybridization with a buffer solution of 30% formamide, 1 M NaCl, 1% SDS at 37°C and a wash in 2x SSC at 40°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1x SSC at 60°C. Hybridization procedures are well known in the art and are described in e.g. Ausubel et al, Current Protocols in Molecular Biology, John Wiley & Sons Inc., 1994.

“Polymorphisms” are allelic variants that occur in a population. The polymorphism can be a single nucleotide difference present at a locus, or can be an insertion or deletion of one or a few nucleotides. As such, a single nucleotide polymorphism (“SNP”) is characterized by the presence in a population of one or two, of four nucleotides (i. e., adenosine, cytosine,

guanosine or thymidine) at a particular locus in a genome such as the human genome. Accordingly, it will be recognized that, while the methods of the invention are exemplified primarily by the detection of SNPs, the disclosed methods or others known in the art similarly can be used to identify other polymorphisms in the exemplified genetic region. Generally the occurrence of a polymorphism is a result of a mutation in the nucleic acid sequence, and in the present invention the terms mutation and polymorphism (or SNP) are used interchangeably.

As used herein, the term "infer" or "inferring" means drawing a conclusion about the occurrence of or predisposition for thoracic aortic aneurysms of a subject using a process of analyzing individually or in combination nucleotide occurrence(s) of one or more SNP(s) of the invention in a nucleic acid sample of the subject. As disclosed herein, the nucleotide occurrence(s) can be identified directly by examining nucleic acid molecules of the SMAD3 gene, or indirectly by examining the polypeptide encoded by said gene, when the polymorphism is associated with an amino acid change in the encoded polypeptide.

It was found by the present inventors that several mutations/polymorphisms in the SMAD3 gene were linked with a hereditary, syndromic form of TAA that can not be classified within any known syndrome. Further, it has appeared in the subjects that have been tested that none of the other mutations that are known to be related to TAA (such as the mutations of the fibrillin-1 gene and the mutations of the TGFBR1 and TGFBR2 genes) were present, indicating that the presently found mutations are indicative for a different form of TAA.

The SMAD3 gene and the closely related SMAD2 gene have been linked to the TGF-ß1 pathway, which pathway has been indicated to play a major role in TAA and other arterial aneurysms. Smad family members have been identified as essential intracellular signaling components of the transforming growth factor-ß (TGF-ß) superfamily. Smad2 and Smad3 are structurally

highly similar and mediate TGF-ß signals (Nakao, A. et al., 1997, EMBO J., 16:5353-5362; Kalinina, N. et al., 2004, Arterioscl. Thromb. Vasc. Biol. 24:1391).

The TGF-ß signaling pathway has been shown to be involved in the currently known hereditary syndromes in which thoracic aortic aneurysms are one of the symptoms, such as Marfan syndrome and Loeys-Dietz syndrome. Yet, these syndromes only can explain for about 20% of the thoracic aortic aneurysms. It has now been found that mutations in another gene involved the TGF-ß pathway (the SMAD3 gene) are linked to the occurrence of a hereditary thoracic aortic aneurysm syndrome that is unrelated to Marfan and Loeys-Dietz, i.e. the subjects suffering from this syndrome do not show the characterizing mutations in the fibrillin-1 gene or the TGFBR1 or TGFBR2 genes, that are indicative of respectively the Marfan and the Loeys-Dietz syndrome.

As is shown in the examples, subjects suffering from this newly characterized syndrome often showed other (congenital) heart diseases, such as mitral valve abnormalities and atrial fibrillation. However, none of the subjects suffered from dyslipideamia or homocystinuria. Other abnormalities, such as skeletal (osteoarthritis, osteochondritis dissecans, joint laxity, *pes planus*, scoliosis), skin/integument (multiples hernias) and craniofacial (hypertelorism, abnormal palate/uvula and dental malocclusion) abnormalities, however, did occur in several individual cases. No ophthalmologic abnormalities were reported. Also spontaneous pneumothorax appeared to be absent. All families had a strong history of sudden deaths occurring at young ages and aortic dissections/ruptures occurred at smaller aorta diameters than expected.

In the subjects suffering from this syndrome three different mutations/polymorphisms were found, all residing in the SMAD3 gene. The three mutations in the gene sequence all cause a mutation in the SMAD3

protein. Two of them are point mutations giving rise to a difference in one amino acid: 1080C>T (a mutation on nucleotide at base 1080 replacing a C by a T) leads to Thr261Ile (replacement of threonine at amino acid 261 by an isoleucine), while 1157C>T caused Arg287Trp. A third mutation relates to a deletion of two nucleotides (AT) at positions 1039 and 1040. This leads to a frame shift in the coding sequence causing a premature protein termination at position 309. All these mutations reside in exon 6 of the SMAD3 gene, i.e. in the sequence coding for the MH2 domain (see Fig. 1). The MH2 domain of Smad proteins contains a positively charged groove next to the L3 loop that is believed to interact with a conserved region of the cytoplasmic domain of the type I TGF-ß receptor.

In more detail the following sequences were found:

Normal: gtggagctgacacggagacacatcg
V E L T R R H I

Mutated: gtggagctgaca**t**ggagacacatcg (1157C>T)

Normal: gtggatggcttcaccgacccctcc
V D G F T D P S

Mutated: gtggatggcttca**t**cgacccctcc (1080C>T)

Normal: cgcgtcggggagacattccacgcctcg
R V G E T F H A S

Mutated: cgcgtcggggagac--tccacgcctcg (1039-1040 delAT)

Figure 2 contains the full nucleotide and amino acid sequence of the SMAD3 gene and protein.

In the control experiments in healthy persons, the indicated mutations were absent. Accordingly, the presence of one or more of the above mentioned mutations is indicative for the presence of, or the predisposition for the

development of thoracic aortic aneurysms. Further, this allows for the development of a diagnostic assay which can be used in addition to the already commercially available assays for detection of Marfan syndrome and Loeys-Dietz syndrome.

For such a diagnostic assay a sample of the subject suspected of having a TAA or having a predisposition for developing TAA has to be taken and the presence or absence of one or more of the above mentioned mutations/polymorphisms should be detected.

The sample taken from the subject may comprise any sample form, such as blood or biopsy material, as long as it contains a cell from which proteins or DNA of the subject can be analysed. Said cell may be any body cell, but preferably will be a blood cell and/or an epithelial cell. All such samples may be used as a sample in a method of the present invention.

Methods of obtaining and preparing such samples for use in the method of the invention are known to those skilled in the art or will be apparent from the present disclosure. For general methods concerning DNA analysis and manipulations see e.g. Molecular Cloning: A Laboratory Manual, 2nd Ed., Vol. 1-3, eds. Sambrook et al. Cold Spring Harbor Laboratory Press (1989) or Current Protocols in Molecular Biology, eds. Ausubel et al., Greene Publishing and Wiley-Interscience, New York (1987) and periodic updates thereof.

The test cell component (either the nucleic acid or the protein encoded by the nucleic acid) may be detected directly *in situ* or it may be isolated from other cell components by common methods known to those of skill in the art before contacting with the reagent (see for example, "Current Protocols in Molecular Biology", Ausubel et al. 1995. 4th edition, John Wiley and Sons; "A Laboratoty Guide to RNA: Isolation, analysis, and synthesis", Krieg (ed.), 1996, Wiley-Liss)

Detection methods include such analyses as Southern and Northern blot analyses, RNase protection, immunoassays, in situ hybridization, PCR (Mullis 1987, U.S. Pat. No. 4,683,195, 4,683,202, en 4,800,159), LCR (Barany

1991, Proc. Natl. Acad. Sci. USA 88:189-193; EP Application No., 320,308), 3SR (Guatelli *et al.*, 1990, Proc. Natl. Acad. Sci. USA 87:1874-1878), SDA (U.S. Pat. Nos. 5,270,184, en 5,455,166), TAS (Kwoh *et al.*, Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi *et al.*, 1988, Bio/Technology 6:1197), Rolling Circle Amplication (RCA) or other methods for the amplification of DNA. In an alternative method RNA may be detected by such methods as NASBA (L. Malek *et al.*, 1994, Meth. Molec. Biol. 28, Ch. 36, Isaac PG, ed., Humana Press, Inc., Totowa, N.J.) or TMA.

Nucleic acid probes, primers and antibodies can be detectably labeled, for instance, with a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator, an enzyme or a biologically relevant binding structure such as biotin or digoxygenin. Those of ordinary skill in the art will know of other suitable labels for binding to the reagents or will be able to ascertain such, using routine experimentation.

Other methods for detection include such analyses as can be performed with nucleic acid arrays (See i.a. Chee *et al.*, 1996, Science 274(5287):610-614). For example, DNA arrays may be used for the detection of nucleic acids according to the invention. Such arrays comprise oligonucleotides with sequences capable of hybridizing under stringent conditions to the nucleic acid cell component of which the level is detected in a method of the present invention.

Methods for detecting a nucleotide change can utilize one or more oligonucleotide probes or primers, including, for example, an amplification primer pair that selectively hybridize to a target polynucleotide, which contains one or more mutations. Oligonucleotide probes useful in practicing a method of the invention can include, for example, an oligonucleotide that is complementary to and spans a portion of the target polynucleotide, including the position of the mutation/SNP, wherein the presence of a specific nucleotide at the position (i. e., the SNP) is detected by the presence or absence of

selective hybridization of the probe. Such a method can further include contacting the target polynucleotide and hybridized oligonucleotide with an endonuclease, and detecting the presence or absence of a cleavage product of the probe, depending on whether the nucleotide occurrence at the SNP site is complementary to the corresponding nucleotide of the probe. A pair of probes that specifically hybridize upstream and adjacent and downstream and adjacent to the site of the SNP, wherein one of the probes includes a nucleotide complementary to a nucleotide occurrence of the SNP, also can be used in an oligonucleotide ligation assay, wherein the presence or absence of a ligation product is indicative of the nucleotide occurrence at the SNP site. An oligonucleotide also can be useful as a primer, for example, for a primer extension reaction, wherein the product (or absence of a product) of the extension reaction is indicative of the nucleotide occurrence. In addition, a primer pair useful for amplifying a portion of the target polynucleotide including the SNP site can be useful, wherein the amplification product is examined to determine the nucleotide occurrence at the SNP site.

Exemplary primer pairs are provided below for the individual exons of the SMAD3 gene. Also indicated is the PCR enzyme set that preferably is used in the amplification reaction.

Exon	Forward primer	Reverse primer	PCR Enzyme
1.1	GGATCTGCGCATCAAAGCTAG	GGCTGGGCTCGACGGGAC	Fast Start Taq
1.2	CCGAGAGTTGAGGCGAAG	GCACCCACACAGTCTCAC	Fast Start Taq
2	GCTCTGATCTCCTGGACCTC	CAAGTGCACCGCAGACCCTCG	Regular Taq
3	GTCATCACCTCTCCCCGGCTC	GTCACGCTGCTCCTCTATGC	Regular Taq
4	CTCCTACAGCCACGGATGC	CCAGCTGCTAATCAGTTAAG	Regular Taq
5	CATAATCATAAGAGACCTCTG	GAGCTGGGCTGATGGTAGG	Fast Start Taq
6	CTTTACACACAAGGCTGATGG	CAAGGTTCCCTTCAGAGGCTG	LA Taq
6	TCTGAAATGCGGGGAAATGG	CAGCTAAGGATGGACGCAAGG	
7	GCTGTTCTGCCTCCTTTGC	CACCTCCAGATTGACAACGC	Regular Taq
8	GTTCAAGGGGAGGGACTGG	GGTGCCAGCAAACATCGTTC	Regular Taq
9.1	GTTACTGGTACCGCTTCTAGG	GCCATTTCTTGCCCTTCAAAG	Regular Taq
9.2	CACGAGCAAACCCAGAGGTG	CCTCCCAATCAGTATGTTCTG	Regular Taq

Where, as in the present invention, the particular nucleotide occurrence of an SNP or mutation is such that the nucleotide occurrence or absence results in an amino acid change in an encoded polypeptide, the nucleotide occurrence or absence can be identified indirectly by detecting the particular amino acid in the polypeptide. The method for determining the amino acid will depend, for example, on the structure of the polypeptide or on the position of the amino acid in the polypeptide. Where the polypeptide contains only a single occurrence of an amino acid encoded by the particular SNP, the polypeptide can be examined for the presence or absence of the amino acid. For example, the polypeptide can be treated with one or more enzymes and a peptide fragment containing the amino acid position of interest can be examined, for example, by sequencing the peptide, or by detecting a particular migration of the peptide following electrophoresis. Where the particular amino acid comprises an epitope of the polypeptide, the specific binding, or absence thereof, of an antibody or antibody fragment that is specific for the epitope can be detected. Other methods for detecting a particular amino acid in a polypeptide or peptide fragment thereof are well known and can be selected based, for example, on convenience or availability of equipment such as a mass spectrometer, capillary electrophoresis system, magnetic resonance imaging

equipment, and the like. Where the polymorphism, as in one case of the polymorphisms of the present invention, results in a truncated polypeptide, the presence of the truncated polypeptide and hence the presence of the polymorphism can e.g. be detected by migration following electrophoresis.

In order to amplify DNA with a small number of mismatches to one or more of the amplification primers, an amplification reaction may be performed under conditions of reduced stringency (e.g. a PCR amplification using an annealing temperature of 38°C, or the presence of 3.5 mM MgCl2). The person skilled in the art will be able to select conditions of suitable stringency.

The primers herein are selected to be "substantially" complementary (i.e. at least 65%, more preferably at least 80% perfectly complementary) to their target regions present on the different strands of each specific sequence to be amplified. It is possible to use primer sequences containing e.g. inositol residues or ambiguous bases or even primers that contain one or more mismatches when compared to the target sequence. In general, sequences that exhibit at least 65%, more preferably at least 80% homology with the target DNA oligonucleotide sequences, are considered suitable for use in a method of the present invention. Sequence mismatches are also not critical when using low stringency hybridization conditions.

The detection of the amplification products can in principle be accomplished by any suitable method known in the art. The detection fragments may be directly stained or labelled with radioactive labels, antibodies, luminescent dyes, fluorescent dyes, or enzyme reagents. Direct DNA stains include for example intercalating dyes such as acridine orange, ethidium bromide, ethidium monoazide or Hoechst dyes.

Alternatively, the DNA fragments may be detected by incorporation of labelled dNTP bases into the synthesized DNA fragments. Detection labels which may be associated with nucleotide bases include e.g. fluorescein, cyanine dye or BrdUrd.

When using a probe-based detection system, a suitable detection procedure for use in the present invention may for example comprise an enzyme immunoassay (EIA) format (Jacobs et al., 1997, J. Clin. Microbiol. 35, 791-795). For performing a detection by manner of the EIA procedure, either the forward or the reverse primer used in the amplification reaction may comprise a capturing group, such as a biotin group for

immobilization of target DNA PCR amplicons on e.g. a streptavidin coated microtiter plate wells for subsequent EIA detection of target DNA -amplicons (see below). The skilled person will understand that other groups for immobilization of target DNA PCR amplicons in an EIA format may be employed.

Probes useful for the detection of the target DNA as disclosed herein preferably bind only to at least a part of the DNA sequence region as amplified by the DNA amplification procedure. Those of skill in the art can prepare suitable probes for detection based on the nucleotide sequence of the target DNA without undue experimentation as set out herein. Also the complementary sequences of the target DNA may suitably be used as detection probes in a method of the invention, provided that such a complementary strand is amplified in the amplification reaction employed.

Suitable detection procedures for use herein may for example comprise immobilization of the amplicons and probing the DNA sequences thereof by e.g. southern blotting. Other formats may comprise an EIA format as described above. To facilitate the detection of binding, the specific amplicon detection probes may comprise a label moiety such as a fluorophore, a chromophore, an enzyme or a radio-label, so as to facilitate monitoring of binding of the probes to the reaction product of the amplification reaction. Such labels are well-known to those skilled in the art and include, for example, fluorescein isothiocyanate (FITC), β-galactosidase, horseradish peroxidase, streptavidin, biotin, digoxigenin, 35S or 125I. Other examples will be apparent to those skilled in the art.

Detection may also be performed by a so called reverse line blot (RLB) assay, such as for instance described by Van den Brule et al. (2002, J. Clin. Microbiol. 40, 779-787). For this purpose RLB probes are preferably synthesized with a 5'-amino group for subsequent immobilization on e.g. carboxyl-coated nylon membranes. The advantage of an RLB format is the ease of the system and its speed, thus allowing for high throughput sample processing.

Any suitable method for screening the nucleic acids for the presence or absence of polymorphisms is considered to be part of the instant invention. Such methods include, but are not limited to: DNA sequencing, restriction fragment length polymorphism (RFLP) analysis, amplified fragment length polymorphism (AFLP) analysis; heteroduplex analysis, single strand conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), real time PCR analysis (e.g. Taqman®), temperature gradient gel

electrophoresis (TGGE), primer extension, allele-specific hybridization, and INVADER® genetic analysis assays, cleavase fragment length polymorphism (CFLP) analysis, sequence-characterized amplified region (SCAR) analysis and cleaved amplified polymorphic sequence (CAPS) analysis

The use of nucleic acid probes for the detection of specific DNA sequences is well known in the art. Mostly these procedures comprise the hybridization of the target DNA with the probe followed by post-hybridization washings. Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the Tm can be approximated from the equation of Meinkoth and Wahl, Anal. Biochem., 138: 267-284 (1984): Tm = 81.5 °C + 16.6 (log M) + 0.41 (% GC)-0.61 (% form)-500/L; where M is the molarity of monovalent cations, % GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The Tm is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. Tm is reduced by about 1°C for each 1 % of mismatching; thus, the hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with > 90% identity are sought, the Tm can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4 °C lower than the thermal melting point (Tm); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10 °C lower than the thermal melting point (Tm); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (Tm). Using the equation, hybridization and wash compositions, and desired Tm, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a Tm of less than 45°C (aqueous solution) or 32°C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology—Hvbridization with Nucleic Acid

Probes, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier. New York (1993); and Current Protocols in Molecular Biology, Chapter 2, Ausubel, et al., Eds., *supra*.

The development of primers and probes useful for the detection of polymorphic positions in a nucleic acid is within the realm of ordinary skill (see for instance Sambrook, J., Russell D.W., Sambrook, J. (2001) Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, Plainview, N.Y.).

By using standard DNA technology it is possible to produce probes and primers that directly or indirectly hybridize to the DNA samples to be tested or cDNA produced from RNA by reverse transcription, and which can be used in assays for the detection of the SNPs. Nucleic acid amplification techniques allow the amplification of fragments of nucleic acids, which may be present in very low amounts.

In order to develop nucleic acid-based detection methods, the SNP-specific sequences must be determined for which primers or probes may then be developed. To detect the SNPs by nucleic acid amplification and/or probe hybridization, the nucleic acid may be isolated from any raw sample material, optionally reverse transcribed into cDNA and directly cloned and/or sequenced. DNA and RNA isolation kits are commercially available from for instance QIAGEN GmbH, Hilden, Germany, or Roche Diagnostics, a division of F. Hoffmann-La Roche Ltd, Basel, Switzerland.

A sample useful for practicing a method of the invention can be any biological sample of a subject that contains nucleic acid molecules, including portions of the gene sequences to be examined, or corresponding encoded polypeptides, depending on the particular method. As such, the sample can be a cell, tissue or organ sample, or can be a sample of a biological fluid such as semen, saliva, blood, and the like. A nucleic acid sample useful for practicing a method of the invention will depend, in part, on whether the SNPs to be identified are in coding regions or in non-coding regions. Thus, where at least one of the SNPs to be identified is in a noncoding region, the nucleic acid sample generally is a deoxyribonucleic acid (DNA) sample, particularly

genomic DNA or an amplification product thereof. However, where heteronuclear ribonucleic acid (RNA), which includes unspliced mRNA precursor RNA molecules, is available, a cDNA or amplification product thereof can be used. Where each of the SNPs is present in a coding region of the gene, the nucleic acid sample can be DNA or RNA, or products derived therefrom, for example, amplification products. Furthermore, it will be recognized that the polymorphisms/mutations of the present invention reside in coding regions of the SMAD3 gene and result in polypeptides containing different amino acids at the positions corresponding to the polymorphisms due to non-degenerate codon changes. As such, in another aspect, the methods of the invention can be practiced using a sample containing polypeptides of the subject.

The DNA, or alternatively, the cDNA may be PCR amplified by using for instance Pfu and Taq DNA polymerases and amplification primers specific for the target DNA sequences. Also complete commercially available systems may be used for PCR (e.g. available form various suppliers such as Roche Diagnostics). A suitable method may for instance include mixing into a suitable aqueous buffering system (e.g. a commercially available PCR buffer) a suitable amount of total DNA as a template (e.g. 1 to 5 µg), a suitable amount (e.g. 10 pmol) of a pair of bi-directional amplification primers, a suitable amount of dNTPs and the DNA polymerase, denaturing the nucleic acids by boiling for 1 min, and performing a cycling reaction of around 10-50 alternating cycles of stringent primer hybridization, strand elongation and denaturing, at suitable temperatures to obtain DNA copies of the DNA template as amplification product. The amount of copies produced upon a certain number of cycles correlates directly to the amount of target DNA in the DNA template.

The skilled person is well aware of the available quantitative PCR methods presently available from commercial suppliers to quantify the amount of target DNA in the template. The term “hybridization signal” as used herein

inter alia refers to the amount of amplification product produced upon a certain number of cycles and thus to the amount of target DNA available as template in the reaction.

In order to amplify a nucleic acid with a small number of mismatches to one or more of the amplification primers, an amplification reaction may be performed under conditions of reduced stringency (e.g. a PCR amplification using an annealing temperature of 38°C, or the presence of 3.5 mM MgCl2). The person skilled in the art will be able to select conditions of suitable stringency.

The detection of the amplification products can in principle be accomplished by any suitable method known in the art. The amplified fragments may be directly stained or labeled with radioactive labels, antibodies, luminescent dyes, fluorescent dyes, or enzyme reagents. Direct DNA stains include for example intercalating dyes such as acridine orange, ethidium bromide, ethidium monoazide or Hoechst dyes.

Alternatively, the DNA or RNA fragments may be detected by incorporation of labeled dNTP bases into the synthesized fragments. Detection labels which may be associated with nucleotide bases include e.g. fluorescein, cyanine dye, digoxigenin (DIG) or bromodeoxyuridine (BrdUrd).

In a quantitative PCR method, the reaction is preferably performed by using an oligonucleotide primer that contains one or more 'locked' nucleic acid (LNA®) monomers, or by using LNA® fluorescent probes. LNA® technology involves an oligonucleotide (probe or primer that contains one or more LNA® monomers [2'-O, 4'-C-methylene-ß-D-ribofuranosyl-modified] (e.g. Petersen and Wengel, 2003. TRENDS in Biotechnology Vol.21(2):74-81). In an LNA monomer, the ribose sugar moiety of the nucleotide is modified, while the base itself is unaltered. The result is a covalent bridge that 'locks' the ribose in the N-type (3'-endo) conformation, which enhances base stacking and phosphate backbone pre-organisation. This provides the oligonucleotide with improved affinity for complementary DNA or RNA sequences and therefore a higher Tm.

When using LNA® primers, the detection of the double stranded amplification products may for instance be performed by using a double-stranded DNA stain, such as SYBR Green® [Molecular Probes, Inc.] (see for instance Ponchel et al. 2003, BMC Biotechnology 3:18).

Other methods of analysing the nuclei acid suitably comprise the use of a primer extension assay; a Taqman® PCR; a differential hybridization assay; an assay which detects allele-specific enzyme cleavage; and/or allele-specific PCR.

Once a diagnosis has been established, the subjects that suffer from or have a predisposition for developing a thoracic aortic aneurysm may suitably be treated with losartan. Losartan has a proven efficacy for preventing aneurysms in Marfan syndrome by its antagonistic effects on TGF-ß (Matt, P. et al., 2008, J. Thorac. Cardiovasc. Surg. 135:389-394). Also other antihypertensive drugs, such as atenolol, may be used.
It is further proposed to use these antihypertensive drugs, preferably losartan, in a combination therapy with metalloproteinase inhibitors, like doxycycline (Chung, A.W. et al., 2008, Circ. Res. 102:e73-85; Yang, H.H. et al., 2010, Acquired Cardiovasc. Dis., in press).
Furthermore, patients with SMAD3-related disease should have strict surveillance of the entire arterial tree, and prophylactic, early surgical intervention, as proposed for LDS patients (Williams JA, et al., Ann Thorac Surg 2007;83(2):S757-63; discussion S85-90).

EXAMPLE 1

Mutations in SMAD3 identified in a new syndromic form of Thoracic Aortic Aneurysms and Dissections with early-onset Osteoarthritis

Materials and Methods

Clinical studies

We identified a family spanning four generations with 11 patients presenting with aneurysms of the aorta or other large arteries compatible with autosomal dominant inheritance (fig pedigree). A total of 19 first-degree (nonconsanguineous) relatives and 6 unrelated spouses were enrolled. Five family members died before or during the study. Sixteen family members had an extensive cardiologic examination including transthoracic echocardiogram, electrocardiogram, and CT or MRI scan of the thorax and abdomen. One family member declined cardiologic examination. The diameters at the aortic root, sinus of Valsalva, the proximal ascending aorta, aortic arch, the descending aorta, the abdominal aorta and other large arteries (e.g. splenic artery, iliac artery) were measured at the maximum systolic dimensions. Cross-sectional echocardiography images were obtained in the parasternal long-axis orientation and plotted against nomograms derived from normal individuals' measurements (Roman et al 1989). The images of both CT and MRI were plotted against normograms derived from normal individuals' measurements corrected for Body Surface Area (BSA) . The BSA was calculated by the DuBois and DuBois formula (BSA (m^2)= 0.007184 x Height (cm)$^{0.725}$ x Weight (kg)$^{0.425}$) The individuals were identified as affected if they had a BSA corrected aortic diameter greater than 95% confidence interval or an aneurysm of one of the other arteries. Also patients who were operated for a thoracic aortic aneurysm or dissection or who died from an aneurysm or dissection as confirmed by autopsy were considered affected. Thirteen family members had an extensive physical examination with particular notice for other features of MFS, based on the Ghent nosology for MFS, and Loeys Dietz syndrome and vascular type Ehlers-Danlos syndrome.

A skeletal survey with X-rays of the feet, knees, hips, total spine, and hands was performed in 11 family members. The definitions for osteoarthritis (OA) originally described by Kellgren and Lawrence were used (Kellgren JH, et al. Annals of the rheumatic diseases 1957;16(4):494-502).

Molecular studies

Genomic DNA was isolated from peripheral blood using the Puregene DNA purification kit (Gentra Systems) using standard procedures. DNA samples from deceased patients was obtained from stored tissue material (frozen or paraffin embedded tissue). The genome wide search was conducted using DNA from 12 members of the family including three spouses. The Affymetrix GeneChip Mapping 250K *Nsp* Array containing 262,264 SNP (Single Nucleotide Polymorphism) markers was used. Samples were processed according to the manufacturer's instructions (Affymetrix GeneChip Mapping Assay). Affymetrix GCOS v1.4, and GTYPE software v4.1 were used.

Microsatellite markers

Microsatellite markers mapping to the identified genomic regions were selected. Polymerase chain reaction (PCR) products were run on an ABI Prism 3130*xl* genetic sequencer (Applied Biosystems) and analyzed using the GeneMapper software v.3.0 (Applied Biosystems). DNAs from 25 available family members were included.

Sequencing analysis

Bidirectional sequencing of the coding region and the exon-intron boundaries of candidate genes was undertaken using PCR primers designed by Primer3 software. PCR products were purified and sequenced using BigDye Terminator chemistry v3.1 on an ABI Prism 3130*xl* genetic analyzer (Applied Biosystems). Sequences were aligned and compared with consensus sequences obtained from the human genome databases using the Applied Biosystems software package SeqScape v2.5.

Linkage analysis and loci identification

The statistical package EasyLinkage Plus v5.08 (ref) designed to perform automated linkage analyses using large-scale SNP data, was used to perform all analyses. Allegro v1.2c software (incorporated in the EasyLinkage Plus v5.08 package) was used to perform fully automated single point and multipoint linkage analysis.

LOD scores were obtained using a dominant model of inheritance, with a penetrance of 90% and a disease allele frequency of 1:1000. A phenocopy rate of 1% was considered. Allele frequencies of genotyped SNPs were set to codominant. Map order and genetic inter-SNPs distances were taken from the Affymetrix website.

Since closely spaced SNP markers were used for the linkage analysis, the genome analyses were performed with predefined spacing of 0.2 to 0.1 centiMorgan (cM), in blocks of 90 and 100 SNPs. Then, single chromosomes showing positive linkage signals were independently analyzed under the same conditions and haplotypes were constructed.

Pathology and Immunohistochemistry

Paraffin embedded tissues from four patients who died from TAA were available (IV-3 from family 1 and II-7, III-2 and III-3 from family 2). Fragments from the ascending aorta taken during surgical procedure were available from two patients (IV-4 and IV-9). Control aortas from three age-matched donors were available as well. All samples were histologically examined after Hematoxylin-Eosin, Verhoeff-van Gieson (elastin), Alcian blue, Masson's trichrome (collagen) staining using standard techniques.

For immunohistochemistry, sections (7 µM) were deparaffinised followed by antigen retrieval using microwave treatment in 0.01 M sodium citrate solution. Endogenous peroxidase activity blocking and immunoincubation were performed as described before , using antibodies against phosphorylated-Smad3 (ab52903), Smad3 (ab28379), TGF-ß1 (ab53169), and CTGF (ab5097) all obtained from Abcam and phosporylated-Smad2 (3108) from Cell Signaling

Technology. Antibodies against collagen type III, IV, and smooth muscle actin (SMA) were used.

Results

A new TAA locus found by genome wide linkage analysis (GWLA)

Because no mutations were found in any of the obvious candidate genes, such as *FBN1, TGFBR1, TGFBR2* and *ACTA2,* we performed a genome wide search using 250k SNP arrays.

The linkage analysis revealed two genomic regions on chromosome 4 and 15. We then tested 20 microsatellite markers mapping to chromosome 4 (100-132 cM) and chromosome 15 (35-75 cM). We could include DNA samples from all 11 patients with aneurysms. Haplotype analysis allowed the exclusion of the chromosome 4q23-q28.3 locus because one patient was not sharing the same haplotype (data not shown).

A significant maximum LOD score of 3.6 (D15S131-D15S1026) was obtained. Further fine mapping in the area determined the borders of the maximum candidate region between markers D15S155 until D15S980 (60.4-73.2 Mb) containing 157 genes (NCBI build 37.1).

Mutations in SMAD3

We selected two positional candidate genes, *SMAD3* and *SMAD6* (*mothers against decapentaplegic homolog 3* and *6*), for further sequence analysis. Their roles in the TGFB signalling pathway make them excellent candidates. Sequence analysis of the four exons of *SMAD6* did not reveal any putative pathogenic mutation. Interestingly, we found a heterozygous novel base change c.1157C>T, in exon 6 of the *SMAD3* gene (NM_005902.3), that segregates perfectly with the disease in the family . This DNA variant leads to an aminoacid change Arginine (charged polar, basic) to Tryptophan (non polar) at position 287 of the in the SMAD3 protein (p.Arg287Trp, NP_005893.1).

The 1157C>T variant was not present in 544 control chromosomes (Dutch origin) and affected an evolutionary conserved aminoacid1The Arginine287 shows a complete homology with all other SMADs proteins (Fig. 1) within the MH2 domain of the SMAD3 protein.

To evaluate the frequency of SMAD3 mutations in patients with aneurysms, we sequenced all coding exons in a group of 99 cases with TAA and Marfan-like features that were negative for FBN1, and TGFBR1&2 mutations. We found two novel SMAD3 mutations. The first nucleotide change c.1080C>T, leads to the replacement of Threonine for Isoleucine (p.Thr261Ile) affecting a highly conserved aminoacid (Fig. 2). The second mutation is a deletion of two nucleotides (c.1039-1040 delAT) leading to a frameshift in the protein sequence (p.Thr247fs) and a premature protein termination at position 309 (family 3). This heterozygous deletion in exon 6 was present in two affected siblings and not found in the healthy mother. No DNA was available from other family members. None of these variants were found in 544 control chromosomes. Both normal and mutated alleles were present at cDNA level, indicating that the abnormal RNA is not subjected to nonsense RNA decay.

A new aneurysm syndrome with cardiovascular, skeletal, cutaneous and craniofacial involvement

All families had a strong history of sudden deaths occurring at young ages. Seven patients died from dissections of the aorta; four had a DeBakey type I, three a DeBakey type II, and two a DeBakey type III dissection. Aortic dissections occurred at smaller aorta diameters than expected; two patients (family 1, patient III-2 and III-12) presented aortic dissections with a maximal ascending aortic dimension of 4.0 cm and 4.5 cm, respectively.

Cardiovascular abnormalities were found in 88% of the patients. Twelve patients had an aneurysm of the aorta at the level of the sinus of Valsalva with a mean age at diagnosis of 42 years, ranging from 26 to 67. SMAD3 mutation carriers also presented aneurysms and/or dilatation of other arteries mainly

involving the lienalis, iliac and mesenteric arteries (six patients) and pulmonary artery (two patients). In half of the patients arterial tortuosity of the thoracic and abdominal arteries was present. Seven patients had surgical interventions for aortic aneurysms and/or dissections at maximum aortic diameter between 4 and 5.3 cm, from which three died within 5 years. Mean age at surgery was 44 years (31-57 years). None of the patients showed dyslipidemia or homocystinuria. One patient had hypertension from the age of 31 years for which he received medication.

Other (congenital) heart diseases were frequently found in patients such as persistent ductus arteriosus (PDA), atrial septal defect (ASD), pulmonary valve stenosis and atrial fibrillation. Mitral valve abnormalities ranging from mild valve prolaps to severe regurgitation requiring valve replacement were reported in 17% of the patients (3 out of 18). In addition, five patients (26%) had borderline to moderate left ventricular hypertrophy which was mainly concentric.

Skeletal and connective tissue abnormalities

Joint abnormalities

One of the most striking features was that all SMAD3 mutation carriers had radiological proven OA of one or more joints mainly involving the spine and knees. Half of the patients had two or more joints affected. OA was detected as early as 28 years and the mean age at diagnosis was 42 years. Hand/wrist OA only involved the scaphotrapezotrapezoidal (STT), carpometacarpal (CMC) and metacarpophalangeal (MCP) joints in contrast to the most commonly affected hand joints in OA (distal and proximal interphalangeal joints).

Twelve patients (86 %) had intervertebral disc degeneration, mainly involving the cervical and lumbar discs. In addition, many affected family members had osteochondritis dissecans (OD) and/or meniscal lesions not preceded by major injury or trauma at young ages. All patients with OD were operated before the age of 40 years, the youngest patient being 10 years old.

Cutaneous findings

Umbilical and/or inguinal hernias were a frequent finding (nine patients with age range 1 to 50 years). Cutaneous findings including velvety skin (69%) and striae (54%) were present in a majority of the patients.
Other recurrent findings included easy bruising, atrophic scars, and pelvic floor prolaps.

Other features

Craniofacial abnormalities included hypertelorism, abnormal palate/uvula and dental malocclusion. One patient was operated for a cleft palate. No craniosynostosis, malar hypoplasia, and retrognathia were found.
Ophthalmologic examination in 10 patients revealed no abnormalities.

Pathology

Histological examinations of the aorta were performed postoperatively (two patients) or postmortem (four patients). Disorganization and degeneration of the tunica media with fragmentation and loss of elastic fibers was observed in all cases with a variable range of severity. Deposition of mucoid material and a marked excess of collagen III and IV in the tunica media of the aortic wall was observed. We observed increased signal intensity in the cytoplasmatic staining of CTGF and TGF-ß1 in two patients from whom aorta fragments were collected during aortic surgery. Phosphorylated Smad2 (pSmad2) was localized to the nucleus of the vascular smooth cells and its expression was higher in the patients regarding both the signal intensity and number of positive cells. Interestingly, both non-phosphorylated and phosphorylated Smad3 revealed increased nuclear (pSmad3 and Smad3) and cytoplasmatic immunostaining in the patients with SMAD3 mutations. Aorta tissues isolated during autopsy were not included is these experiments because of proteolysis

and rapidly postmortem dephosphorylation of proteins that could influence the results.

Claims

1. Method for diagnosing the cause of a thoracic aortic aneurysm and/or other arterial aneurysm or the predisposition for developing a thoracic aortic aneurysm and/or other arterial aneurysm comprising:
   a. taking a sample from a subject;
   b. assaying said sample for the presence of a mutation in the SMAD3 gene or protein; and
   c. reaching the diagnosis on basis of the results of said assay.

2. Method according to claim 1, wherein the mutation in the SMAD3 protein is chosen from the group consisting of Arg287Trp, Thr261Ile, a premature protein termination at position 309, and combinations thereof.

3. Method according to claim 1, wherein the mutation in the SMAD3 protein is chosen from the group consisting of 1157C>T, 1080C>T, 1039-1040 delAT, and combinations thereof.

4. Method according to any of claims 1-3, wherein the sample is a tissue sample or a blood sample.

5. Method for diagnosing the cause of a thoracic aortic aneurysm and/or other arterial aneurysm or the predisposition for developing a thoracic aortic aneurysm and/or other arterial aneurysm comprising:
   a. taking a sample from a subject;
   b. assaying said sample for the presence of a mutation in the SMAD3 gene or protein, the fibrillin-1 gene and the TGFBR1 and TGFBR2 genes; andreaching the diagnosis on basis of the results

of said assay

6. Method for treatment of a subject suffering from a thoracic aortic aneurysm and/or other arterial aneurysm or having a predisposition for developing a thoracic aortic aneurysm and/or other arterial aneurysm, wherein said subject has a mutation in the SMAD3 gene, but does not have a mutation in the fibrillin-1 gene and the TGFBR1 and TGFBR2 genesby administration of an angiotensin II type 1 receptor blocker, preferably losartan.

7. Method according to claim 6, wherein next to the angiotensin II type 1 receptor blocker a matrix metalloproteases inhibitor, preferably doxycycline, is administered.

8. An angiotensin II type 1 receptor blocker, preferably losartan, or the combination of said angiotensin II type 1 receptor blocker and doxycyline, for use in the treatment of a subject suffering from a thoracic aortic aneurysm and/or other arterial aneurysm or having a predisposition for developing a thoracic aortic aneurysm and/or other arterial aneurysm, wherein said subject has a mutation in the SMAD3 gene, but does not have a mutation in the fibrillin-1 gene and the TGFBR1 and TGFBR2 genes

Fig 1.

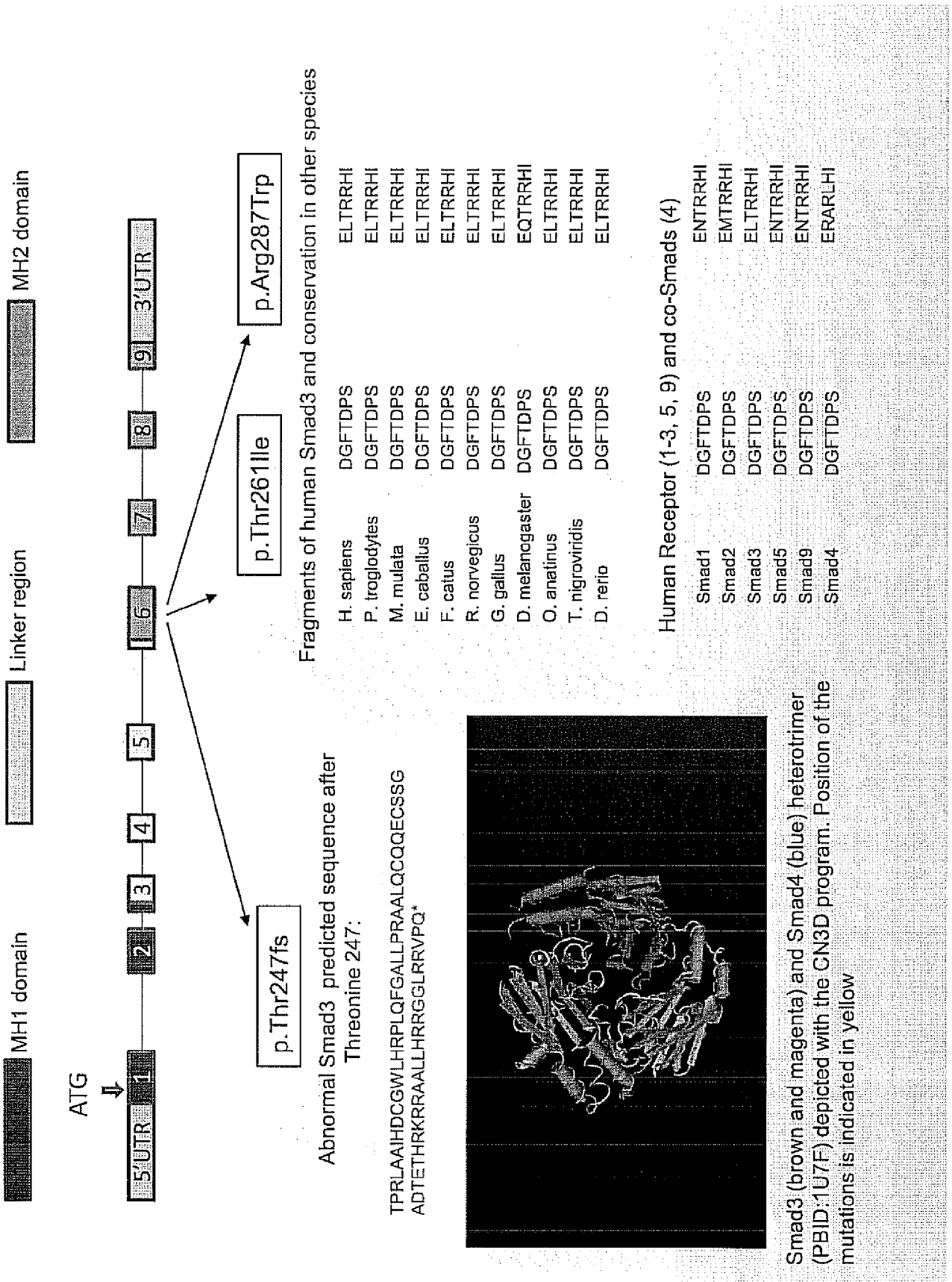

MH1 domain
Linker region
MH2 domain
ATG
5'UTR
1
2
3
4
5
6
7
8
9
3'UTR
p.Thr247fs
p.Thr261Ile
p.Arg287Trp
Abnormal Smad3 predicted sequence after Threonine 247:
TPRLAAIHDCGWLHRPLQFGALLPRAALQCQQECSSG
ADTETHRKRRAALLHRRGGLRRVPQ*
Fragments of human Smad3 and conservation in other species
H. sapiens DGFTDPS ELTRRHI
P. troglodytes DGFTDPS ELTRRHI
M. mulata DGFTDPS ELTRRHI
E. caballus DGFTDPS ELTRRHI
F. catus DGFTDPS ELTRRHI
R. norvegicus DGFTDPS ELTRRHI
G. gallus DGFTDPS ELTRRHI
D. melanogaster DGFTDPS EQTRRHI
O. anatinus DGFTDPS ELTRRHI
T. nigroviridis DGFTDPS ELTRRHI
D. rerio DGFTDPS ELTRRHI
Human Receptor (1-3, 5, 9) and co-Smads (4)
Smad1 DGFTDPS ENTRRHI
Smad2 DGFTDPS EMTRRHI
Smad3 DGFTDPS ELTRRHI
Smad5 DGFTDPS ENTRRHI
Smad9 DGFTDPS ENTRRHI
Smad4 DGFTDPS ERARLHI
Smad3 (brown and magenta) and Smad4 (blue) heterotrimer (PBID:1U7F) depicted with the CN3D program. Position of the mutations is indicated in yellow

Fig. 2A

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   1 gcggccgccg cctccgcccc gcgttcgggg ccttcccgac cctgcactgc tgccgtccgc
  61 ccgcccggcc gctcttctct tcgccgtggg agccgctccg ggcgcagggc cgcgcgccga
 121 gccccgcagg ctgcagcgcc gcggcccggc ccggcgcccc ggcaacttcg ccgagagttg
 181 aggcgaagtt tgggcgaccg cggcaggccc cggccgagct cccctctgcg cccccggcgt
 241 cccgtcgagc ccagccccgc cgggggcgct cctcgccgcc cgcgcgccct ccccagccat
 301 gtcgtccatc ctgcctttca ctcccccgat cgtgaagcgc ctgctgggct ggaagaaggg
 361 cgagcagaac gggcaggagg agaaatggtg cgagaaggcg gtcaagagcc tggtcaagaa
 421 actcaagaag acggggcagc tggacgagct ggagaaggcc atcaccacgc agaacgtcaa
 481 caccaagtgc atcaccatcc ccaggtccct ggatggccgg ttgcaggtgt cccatcggaa
 541 ggggctccct catgtcatct actgccgcct gtggcgatgg ccagacctgc acagccacca
 601 cgagctacgg gccatggagc tgtgtgagtt cgccttcaat atgaagaagg acgaggtctg
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 721 acgccacaca gagatcccgg ccgagttccc cccactggac gactacagcc attccatccc
 781 cgaaaacact aacttccccg caggcatcga gccccagagc aatattccag agaccccacc
 841 ccctggctac ctgagtgaag atggagaaac cagtgaccac cagatgaacc acagcatgga
 901 cgcaggttct ccaaacctat ccccgaatcc gatgtcccca gcacataata acttggacct
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1021 ccagcgcgtc ggggagacat tccacgcctc gcagccatcc atgactgtgg atggcttcac
1081 cgacccctcc aattcggagc gcttctgcct agggctgctc tccaatgtca acaggaatgc
1141 agcagtggag ctgacacgga gacacatcgg aagaggcgtg cggctctact acatcggagg
1201 ggaggtcttc gcagagtgcc tcagtgacag cgctatttct gtccagtctc ccaactgtaa
1261 ccagcgctat ggctggcacc cggccaccgt ctgcaagatc ccaccaggat gcaacctgaa
1321 gatcttcaac aaccaggagt tcgctgccct cctggcccag tcggtcaacc agggctttga
1381 ggctgtctac cagttgaccc gaatgtgcac catccgcatg agcttcgtca aaggctgggg
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1621 catgcaggag gtggagaaaa ttggaactct actcaaccca ttgttgtcaa ggaagaagaa
1681 atctttctcc ctcaactgaa ggggtgcacc cacctgtttt ctgaaacaca cgagcaaacc
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Fig. 2B

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INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2010/050244

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

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)
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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATAATMADJA MARIA ET AL: "Angiotensin II Receptor Antagonism and Transforming Growth Factor Beta and SMAD Signalling in Thoracic Aortic Aneurysm: Implications for Aneurysm Progression" CIRCULATION, LIPPINCOTT WILLIAMS & WILKINS, US, vol. 118, no. 18, Suppl. 2, 1 October 2008 (2008-10-01), page S857, XP009138614 ISSN: 0009-7322	1-5
Y	the whole document	6-8

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[X] 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	Date of mailing of the international search report
15 September 2010	04/10/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Costa Roldán, Nuria

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GOMEZ DELPHINE ET AL: "Syndromic and non-syndromic aneurysms of the human ascending aorta share activation of the Smad2 pathway." THE JOURNAL OF PATHOLOGY MAY 2009 LNKD-PUBMED:19224541, vol. 218, no. 1, May 2009 (2009-05), pages 131-142, XP002600645 ISSN: 1096-9896 page 131 - page 132	5-8
Y	CARTA LUCA ET AL: "p38 MAPK is an early determinant of promiscuous Smad2/3 signaling in the aortas of fibrillin-1 (Fbn1)-null mice." THE JOURNAL OF BIOLOGICAL CHEMISTRY 27 FEB 2009 LNKD- PUBMED:19109253, vol. 284, no. 9, 27 February 2009 (2009-02-27), pages 5630-5636, XP002600646 ISSN: 0021-9258 * abstract page 5635	5-8
A	WESSELS M W ET AL: "Autosomal dominant inheritance of cardiac valves anomalies in two families: Extended spectrum of left-ventricular outflow tract obstruction" AMERICAN JOURNAL OF MEDICAL GENETICS, PART A 200902 US LNKD- DOI:10.1002/AJMG.A.32594, vol. 149, no. 2, February 2009 (2009-02), pages 216-225, XP002600647 * abstract	1-8