(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 March 2015 (19.03.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/036405 A1

(51) **International Patent Classification**:
G01N 33/574 (2006.01)

(21) **International Application Number**:
PCT/EP2014/069219

(22) **International Filing Date**:
9 September 2014 (09.09.2014)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
13306237.2 10 September 2013 (10.09.2013) EP

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

WO 2015/036405 A1

(54) **Title**: METHODS FOR DIAGNOSING AND TREATING BASAL CELL CARCINOMA

(57) **Abstract**: The present invention relates to a method for determining whether a subject is at risk of having a basal cell carcinoma comprising the steps consisting of detecting an ACTRT1 deficiency in the subject's epidermis and concluding that the subject is at risk of having a basal cell carcinoma when an ACTRT1 deficiency in the subject's epidermis is detected.

METHODS FOR DIAGNOSING AND TREATING BASAL CELL CARCINOMA

FIELD OF THE INVENTION:

The present invention relates to methods and kits for whether a subject is at risk of having a basal cell carcinoma. The present invention also relates to methods and pharmaceutical compositions for the treatment of basal cell carcinoma.

BACKGROUND OF THE INVENTION:

Basal cell carcinoma (BCC) is the most common form of skin cancer and the most common form of cancer of any type in Caucasians. It develops in the basal germinative cell layer of the epidermis, often on sun-exposed areas of the skin. Although BCC rarely spreads (i.e., metastasizes) to other parts of the body, it can be very destructive and disfiguring. BCC may cause local tissue destruction that may lead to disfigurement or functional impairment of surrounding non-cancerous tissue. Disfigurement may be a particular concern of BCC patients because many BCC tumors occur on the sun-exposed—and, therefore, also typically otherwise exposed—skin of the head and neck. Larger tumors, tumors that have been present for long periods of time, and tumors that have recurred after initial therapy may be biologically more aggressive and especially difficult to cure. While the mortality rate of BCC is relatively low, its increasing incidence and prolonged morbidity means that the disease can be very costly to treat. In 1996, mutations in the tumor suppressor gene PATCHED (PTCH1) have been found to be associated to the nevoid basal cell carcinoma (Hahn et al, 1996, J. Biol. Chem. 271, 12125; Johnson et al, 1996, Science 272, 1668; W097/43414). Nonetheless, no single biomarker is sufficiently specific to provide adequate clinical utility for the predisposition for basal cell carcinoma in an individual subject. Therefore, there is a need for identifying other factors that provide a more accurate prediction of basal cell carcinoma. Thus, the invention aims to provide a novel method for determining whether a subject is at risk to basal cell carcinoma using new genetic biomarkers

SUMMARY OF THE INVENTION:

The present invention relates to a method for determining whether a subject is at risk of having a basal cell carcinoma comprising the steps consisting of detecting an ACTRT1 deficiency in the subject's epidermis and concluding that the subject is at risk of having a basal cell carcinoma when an ACTRT1 deficiency in the subject's epidermis is detected.

DETAILED DESCRIPTION OF THE INVENTION:

Cutaneous basal cell carcinomas (BCCs) are a major feature of X-linked Bazex-Dupré-Christol syndrome (BDCS). Two BDCS families carried an insertion in the *ACTRT1* gene (c.739_740InsA) resulting in a truncated Arp-T1 protein. Specific epidermal and adnexal Arp-T1 expression was lost in BCCs from BDCS patients and 34 of 40 unrelated sporadic BCCs. One family carried a germline variation in CNE12, a putative non-coding RNA. Its expression was restricted to the epidermis but absent from the skin of patients with a CNE12 variant. Arp-T1 overexpression resulted in G2/M-phase arrest, hence it may regulate cell cycle. In BDCS patients, Hedgehog target genes were overexpressed in skin and tumors. Since Arp-T1 binds to *GLI1* promoter, it appears as a key Hedgehog signaling regulator. *ACTRT1* is an uncommon example of X-linked tumor suppressor gene which inhibits the Hedgehog signaling pathway. The results may have significant therapeutic implications for the most common human cancers, BCCs.

The present invention relates to a method for determining whether a subject is at risk of having a basal cell carcinoma comprising the steps consisting of detecting an ACTRT1 deficiency in the subject's epidermis and concluding that the subject is at risk of having a basal cell carcinoma when an ACTRT1 deficiency in the subject's epidermis is detected.

A "subject" in the context of the present invention can be a male or female. A subject can also be one who has not been previously diagnosed as having a basal cell carcinoma. In one embodiment of the invention, the subject having or being at risk of having a basal cell carcinoma may be a substantially healthy subject, which means that the subject has not been previously diagnosed or identified as having or suffering from a basal cell carcinoma. In another embodiment, said subject may also be one that is asymptomatic for a basal cell carcinoma. As used herein, an "asymptomatic" subject refers to a subject that does not exhibit the traditional symptoms of a basal cell carcinoma. In another embodiment of the invention, said subject may be one that is at risk of having a basal cell carcinoma, as defined by clinical indicia such as ultraviolet (UV) light exposure, living closer to the equator or at a higher elevation, a family history of basal cell carcinomas...

"Risk" in the context of the present invention, relates to the probability that an event will occur over a specific time period, as in the conversion to a basal cell carcinoma, and can

mean a subject's "absolute" risk or "relative" risk. Absolute risk can be measured with reference to either actual observation post-measurement for the relevant time cohort, or with reference to index values developed from statistically valid cohorts that have been followed for the relevant time period. Relative risk refers to the ratio of absolute risks of a subject compared either to the absolute risks of low risk cohorts or an average population risk, which can vary by how clinical risk factors are assessed. Odds ratios, the proportion of positive events to negative events for a given test result, are also commonly used (odds are according to the formula p/(l-p) where p is the probability of event and (1- p) is the probability of no event) to no- conversion. Alternative continuous measures which may be assessed in the context of the present invention include time to basal cell carcinoma conversion and therapeutic geographic atrophy form of basal cell carcinoma conversion risk reduction ratios.

"Determining whether a subject is at risk of having a basal cell carcinoma" in the context of the present invention encompasses making a prediction of the probability, odds, or likelihood that basal cell carcinoma may occur. Risk evaluation can also comprise prediction of future clinical parameters, traditional laboratory risk factor values, or other indices of basal cell carcinoma, such as excessive ultraviolet (UV) light exposure, living closer to the equator or at a higher elevation, a family history of basal cell carcinoma... The methods of the present invention may be used to make continuous or categorical measurements of the risk of conversion to a basal cell carcinoma thus diagnosing and defining the risk spectrum of a category of subjects defined as being at risk for a basal cell carcinoma. In the categorical scenario, the invention can be used to discriminate between normal and other subject cohorts at higher risk for a basal cell carcinoma.

As used herein, the term "ACTRT1" has its general meaning in the art and refers to the Actin-Related Protein T1.

In the context of the invention, the term "ACTRT1 deficiency" denotes that the epidermal cells of the subject or a part thereof have an ACTRT1 dysfunction, a low or a null expression of ACTRT1. Said deficiency may typically result from a mutation in the ACTRT1 so that the pre-ARNm is degraded through the NMD (non sense mediated decay) system. Said deficiency may also typically result from a mutation so that the protein is misfolded and degraded through the proteasome. Said deficiency may also result from a loss of function mutation leading to a dysfunction of the protein. For example said mutation is,c.739_740InsA

that leads to a truncated protein. Said deficiency may also result from an epigenetic control of gene expression (e.g. methylation) so that the gene is less expressed in the cells of the subject. Said deficiency may also result from a repression of the ACTRT1 gene induce by a particular signalling pathway. Said deficiency may also result from a mutation in a nucleotide sequence that control the expression of ACTRT1. Typically said sequence may be a conserved non coding element, such as CNE12 as described in EXAMPLE.

In one embodiment, the first step consists in detecting the mutation that is responsible for the ACTRT1 deficiency. One skilled in the art can easily identify a mutation in ACTRT1 gene or in a conserved non coding element, such as CNE12. Typically the mutation may be detected by analyzing nucleic acid molecule. In the context of the invention, nucleic acid molecules include mRNA, genomic DNA and cDNA derived from mRNA. DNA or RNA can be single stranded or double stranded. These may be utilized for detection by amplification and/or hybridization with a probe, for instance. The nucleic acid sample may be obtained from any cell source or tissue biopsy. Non-limiting examples of cell sources available include without limitation blood cells, buccal cells, epithelial cells, fibroblasts, or any cells present in a tissue obtained by biopsy. Cells may also be obtained from body fluids, such as blood, plasma, serum, lymph, etc. DNA may be extracted using any methods known in the art, such as described in Sambrook et al., 1989. RNA may also be isolated, for instance from tissue biopsy, using standard methods well known to the one skilled in the art such as guanidium thiocyanate-phenol-chloroform extraction. Mutations may be detected in a RNA or DNA sample, preferably after amplification. For instance, the isolated RNA may be subjected to coupled reverse transcription and amplification, such as reverse transcription and amplification by polymerase chain reaction (RT-PCR), using specific oligonucleotide primers that are specific for a mutated site or that enable amplification of a region containing the mutated site. According to a first alternative, conditions for primer annealing may be chosen to ensure specific reverse transcription (where appropriate) and amplification; so that the appearance of an amplification product be a diagnostic of the presence of a particular mutation. Otherwise, RNA may be reverse-transcribed and amplified, or DNA may be amplified, after which a mutated site may be detected in the amplified sequence by hybridization with a suitable probe or by direct sequencing, or any other appropriate method known in the art. For instance, a cDNA obtained from RNA may be cloned and sequenced to identify a mutation in ACTRT1 sequence. Actually numerous strategies for genotype analysis are available (Antonarakis et al., 1989 ; Cooper et al., 1991 ; Grompe, 1993). Briefly, the

nucleic acid molecule may be tested for the presence or absence of a restriction site. When a base substitution mutation creates or abolishes the recognition site of a restriction enzyme, this allows a simple direct PCR test for the mutation. Further strategies include, but are not limited to, direct sequencing, restriction fragment length polymorphism (RFLP) analysis; hybridization with allele-specific oligonucleotides (ASO) that are short synthetic probes which hybridize only to a perfectly matched sequence under suitably stringent hybridization conditions; allele-specific PCR; PCR using mutagenic primers; ligase-PCR, HOT cleavage; denaturing gradient gel electrophoresis (DGGE), temperature denaturing gradient gel electrophoresis (TGGE), single-stranded conformational polymorphism (SSCP) and denaturing high performance liquid chromatography (Kuklin et al., 1997). Direct sequencing may be accomplished by any method, including without limitation chemical sequencing, using the Maxam-Gilbert method ; by enzymatic sequencing, using the Sanger method ; mass spectrometry sequencing ; sequencing using a chip-based technology; and real-time quantitative PCR. Preferably, DNA from a subject is first subjected to amplification by polymerase chain reaction (PCR) using specific amplification primers. However several other methods are available, allowing DNA to be studied independently of PCR, such as the rolling circle amplification (RCA), the InvaderTMassay, or oligonucleotide ligation assay (OLA). OLA may be used for revealing base substitution mutations. According to this method, two oligonucleotides are constructed that hybridize to adjacent sequences in the target nucleic acid, with the join sited at the position of the mutation. DNA ligase will covalently join the two oligonucleotides only if they are perfectly hybridized. Therefore, useful nucleic acid molecules, in particular oligonucleotide probes or primers, according to the present invention include those which specifically hybridize the regions where the mutations are located. Oligonucleotide probes or primers may contain at least 10, 15, 20 or 30 nucleotides. Their length may be shorter than 400, 300, 200 or 100 nucleotides.

The mutation may be also detected at a protein level (e.g. for loss of function mutation) according to any appropriate method known in the art. In particular a biological sample, such as a tissue biopsy, obtained from a subject may be contacted with antibodies specific of a mutated form of ACTRT1 protein, i.e. antibodies that are capable of distinguishing between a mutated form of ACTRT1 and the wild-type protein, to determine the presence or absence of a ACTRT1 specified by the antibody. The antibodies may be monoclonal or polyclonal antibodies, single chain or double chain, chimeric antibodies, humanized antibodies, or portions of an immunoglobulin molecule, including those portions

known in the art as antigen binding fragments Fab, Fab', F(ab')2 and F(v). They can also be immunoconjugated, e.g. with a toxin, or labelled antibodies. Whereas polyclonal antibodies may be used, monoclonal antibodies are preferred for they are more reproducible in the long run. Procedures for raising "polyclonal antibodies" are also well known. Alternatively, binding agents other than antibodies may be used for the purpose of the invention. These may be for instance aptamers, which are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random sequence library.

In one embodiment, the first step consists in determining the expression level of ACTRT1 gene in the biological sample obtained from the subject. Typically, said biological sample is a blood sample or a PBMC sample or is a tissue sample resulting from a biopsy (e.g. an endoscopical biopsy performed in the colon of the subject). In one embodiment, the first step consist in i) determining the expression level of ACTRT1 gene, ii) comparing the level determined at i) with a predetermined reference value and iii) concluding that the subject has a ACTRT1 deficiency when the expression level determined at i) is lower than the predetermined reference value. Typically the predetermined reference value is the expression level determined in a healthy population of subject (e.g. the mean expression).

One skilled in the art may easily select the appropriate method for determining the expression level of the gene.

Typically, the expression level of a gene may be determined by determining the quantity of mRNA. Methods for determining the quantity of mRNA are well known in the art. For example the nucleic acid contained in the samples (e.g., cell or tissue prepared from the patient) is first extracted according to standard methods, for example using lytic enzymes or chemical solutions or extracted by nucleic-acid-binding resins following the manufacturer's instructions. The extracted mRNA is then detected by hybridization (e. g., Northern blot analysis, in situ hybridization) and/or amplification (e.g., RT-PCR).

Other methods of Amplification include ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid sequence based amplification (NASBA).

Nucleic acids having at least 10 nucleotides and exhibiting sequence complementarity or homology to the mRNA of interest herein find utility as hybridization probes or

amplification primers. It is understood that such nucleic acids need not be identical, but are typically at least about 80% identical to the homologous region of comparable size, more preferably 85% identical and even more preferably 90-95% identical. In certain embodiments, it will be advantageous to use nucleic acids in combination with appropriate means, such as a detectable label, for detecting hybridization.

Typically, the nucleic acid probes include one or more labels, for example to permit detection of a target nucleic acid molecule using the disclosed probes. In various applications, such as in situ hybridization procedures, a nucleic acid probe includes a label (e.g., a detectable label). A "detectable label" is a molecule or material that can be used to produce a detectable signal that indicates the presence or concentration of the probe (particularly the bound or hybridized probe) in a sample. Thus, a labeled nucleic acid molecule provides an indicator of the presence or concentration of a target nucleic acid sequence (e.g., genomic target nucleic acid sequence) (to which the labeled uniquely specific nucleic acid molecule is bound or hybridized) in a sample. A label associated with one or more nucleic acid molecules (such as a probe generated by the disclosed methods) can be detected either directly or indirectly. A label can be detected by any known or yet to be discovered mechanism including absorption, emission and/ or scattering of a photon (including radio frequency, microwave frequency, infrared frequency, visible frequency and ultra-violet frequency photons). Detectable labels include colored, fluorescent, phosphorescent and luminescent molecules and materials, catalysts (such as enzymes) that convert one substance into another substance to provide a detectable difference (such as by converting a colorless substance into a colored substance or vice versa, or by producing a precipitate or increasing sample turbidity), haptens that can be detected by antibody binding interactions, and paramagnetic and magnetic molecules or materials.

Particular examples of detectable labels include fluorescent molecules (or fluorochromes). Numerous fluorochromes are known to those of skill in the art, and can be selected, for example from Life Technologies (formerly Invitrogen), e.g., see, The Handbook—A Guide to Fluorescent Probes and Labeling Technologies). Examples of particular fluorophores that can be attached (for example, chemically conjugated) to a nucleic acid molecule (such as a uniquely specific binding region) are provided in U.S. Pat. No. 5,866, 366 to Nazarenko et al., such as 4-acetamido-4'-isothiocyanatostilbene-2,2' disulfonic acid, acridine and derivatives such as acridine and acridine isothiocyanate, 5-(2'-aminoethyl) aminonaphthalene-1-sulfonic acid (EDANS), 4-amino -N- [3 vinylsulfonyl)phenyl]naphthalimide-3,5 disulfonate (Lucifer Yellow VS), N-(4-anilino-1-

naphthyl)maleimide, antl1ranilamide, Brilliant Yellow, coumarin and derivatives such as coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcouluarin (Coumarin 151); cyanosine; 4',6-diarninidino-2-phenylindole (DAPI); 5',5"dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red); 7 -diethylamino -3 - (4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfor1ic acid; 5-[dimethylamino] naphthalene-1-sulfonyl chloride (DNS, dansyl chloride); 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives such as eosin and eosin isothiocyanate; erythrosin and derivatives such as erythrosin B and erythrosin isothiocyanate; ethidium; fluorescein and derivatives such as 5-carboxyfluorescein (FAM), 5-(4,6dicl1lorotriazin-2-yDarninofluorescein (DTAF), 2'7'dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), fluorescein, fluorescein isothiocyanate (FITC), and QFITC Q(RITC); 2',7'-difluorofluorescein (OREGON GREEN®); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthaldialdehyde; pyrene and derivatives such as pyrene, pyrene butyrate and succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron Brilliant Red 3B-A); rhodamine and derivatives such as 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride, rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, rhodamine green, sulforhodamine B, sulforhodamine 101 and sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin; rosolic acid and terbium chelate derivatives. Other suitable fluorophores include thiol-reactive europium chelates which emit at approximately 617 mn (Heyduk and Heyduk, Analyt. Biochem. 248:216-27, 1997; J. Biol. Chem. 274:3315-22, 1999), as well as GFP, LissamineTM, diethylaminocoumarin, fluorescein chlorotriazinyl, naphthofluorescein, 4,7-dichlororhodamine and xanthene (as described in U.S. Pat. No. 5,800,996 to Lee et al.) and derivatives thereof. Other fluorophores known to those skilled in the art can also be used, for example those available from Life Technologies (Invitrogen; Molecular Probes (Eugene, Oreg.)) and including the ALEXA FLUOR® series of dyes (for example, as described in U.S. Pat. Nos. 5,696,157, 6, 130, 101 and 6,716,979), the BODIPY series of dyes (dipyrrometheneboron difluoride dyes, for example as described in U.S. Pat. Nos. 4,774,339, 5,187,288, 5,248,782, 5,274,113, 5,338,854, 5,451,663 and 5,433,896),

Cascade Blue (an amine reactive derivative of the sulfonated pyrene described in U.S. Pat. No. 5,132,432) and Marina Blue (U.S. Pat. No. 5,830,912).

In addition to the fluorochromes described above, a fluorescent label can be a fluorescent nanoparticle, such as a semiconductor nanocrystal, e.g., a QUANTUM DOTTM (obtained, for example, from Life Technologies (QuantumDot Corp, Invitrogen Nanocrystal Technologies, Eugene, Oreg.); see also, U.S. Pat. Nos. 6,815,064; 6,682,596; and 6,649, 138). Semiconductor nanocrystals are microscopic particles having size-dependent optical and/or electrical properties. When semiconductor nanocrystals are illuminated with a primary energy source, a secondary emission of energy occurs of a frequency that corresponds to the handgap of the semiconductor material used in the semiconductor nanocrystal. This emission can he detected as colored light of a specific wavelength or fluorescence. Semiconductor nanocrystals with different spectral characteristics are described in e.g., U.S. Pat. No. 6,602,671. Semiconductor nanocrystals that can he coupled to a variety of biological molecules (including dNTPs and/or nucleic acids) or substrates by techniques described in, for example, Bruchez et al., Science 281 :20132016, 1998; Chan et al., Science 281:2016-2018, 1998; and U.S. Pat. No. 6,274,323. Formation of semiconductor nanocrystals of various compositions are disclosed in, e.g., U.S. Pat. Nos. 6,927, 069; 6,914,256; 6,855,202; 6,709,929; 6,689,338; 6,500,622; 6,306,736; 6,225,198; 6,207,392; 6,114,038; 6,048,616; 5,990,479; 5,690,807; 5,571,018; 5,505,928; 5,262,357 and in U.S. Patent Puhlication No. 2003/0165951 as well as PCT Puhlication No. 99/26299 (puhlished May 27, 1999). Separate populations of semiconductor nanocrystals can he produced that are identifiable based on their different spectral characteristics. For example, semiconductor nanocrystals can he produced that emit light of different colors hased on their composition, size or size and composition. For example, quantum dots that emit light at different wavelengths based on size (565 mn, 655 mn, 705 mn, or 800 mn emission wavelengths), which are suitable as fluorescent labels in the probes disclosed herein are available from Life Technologies (Carlshad, Calif.).

Additional labels include, for example, radioisotopes (such as 3 H), metal chelates such as DOTA and DPTA chelates of radioactive or paramagnetic metal ions like Gd3+, and liposomes.

Detectable labels that can he used with nucleic acid molecules also include enzymes, for example horseradish peroxidase, alkaline phosphatase, acid phosphatase, glucose oxidase, beta-galactosidase, beta-glucuronidase, or beta-lactamase.

Alternatively, an enzyme can he used in a metallographic detection scheme. For example, silver in situ hyhridization (SISH) procedures involve metallographic detection schemes for identification and localization of a hybridized genomic target nucleic acid sequence. Metallographic detection methods include using an enzyme, such as alkaline phosphatase, in combination with a water-soluble metal ion and a redox-inactive substrate of the enzyme. The substrate is converted to a redox-active agent by the enzyme, and the redoxactive agent reduces the metal ion, causing it to form a detectable precipitate. (See, for example, U.S. Patent Application Puhlication No. 2005/0100976, PCT Publication No. 2005/ 003777 and U.S. Patent Application Publication No. 2004/ 0265922). Metallographic detection methods also include using an oxido-reductase enzyme (such as horseradish peroxidase) along with a water soluble metal ion, an oxidizing agent and a reducing agent, again to form a detectable precipitate. (See, for example, U.S. Pat. No. 6,670,113).

Probes made using the disclosed methods can be used for nucleic acid detection, such as ISH procedures (for example, fluorescence in situ hybridization (FISH), chromogenic in situ hybridization (CISH) and silver in situ hybridization (SISH)) or comparative genomic hybridization (CGH).

In situ hybridization (ISH) involves contacting a sample containing target nucleic acid sequence (e.g., genomic target nucleic acid sequence) in the context of a metaphase or interphase chromosome preparation (such as a cell or tissue sample mounted on a slide) with a labeled probe specifically hybridizable or specific for the target nucleic acid sequence (e.g., genomic target nucleic acid sequence). The slides are optionally pretreated, e.g., to remove paraffin or other materials that can interfere with uniform hybridization. The sample and the probe are both treated, for example by heating to denature the double stranded nucleic acids. The probe (formulated in a suitable hybridization buffer) and the sample are combined, under conditions and for sufficient time to permit hybridization to occur (typically to reach equilibrium). The chromosome preparation is washed to remove excess probe, and detection of specific labeling of the chromosome target is performed using standard techniques.

For example, a biotinylated probe can be detected using fluorescein-labeled avidin or avidin-alkaline phosphatase. For fluorochrome detection, the fluorochrome can be detected directly, or the samples can be incubated, for example, with fluorescein isothiocyanate (FITC)-conjugated avidin. Amplification of the FITC signal can be effected, if necessary, by incubation with biotin-conjugated goat antiavidin antibodies, washing and a second incubation with FITC-conjugated avidin. For detection by enzyme activity, samples can be incubated, for example, with streptavidin, washed, incubated with biotin-conjugated alkaline

phosphatase, washed again and pre-equilibrated (e.g., in alkaline phosphatase (AP) buffer). For a general description of in situ hybridization procedures, see, e.g., U.S. Pat. No. 4,888,278.

Numerous procedures for FISH, CISH, and SISH are known in the art. For example, procedures for performing FISH are described in U.S. Pat. Nos. 5,447,841; 5,472,842; and 5,427,932; and for example, in Pir1kel et al., Proc. Natl. Acad. Sci. 83:2934-2938, 1986; Pinkel et al., Proc. Natl. Acad. Sci. 85:9138-9142, 1988; and Lichter et al., Proc. Natl. Acad. Sci. 85:9664-9668, 1988. CISH is described in, e.g., Tanner et al., Am. .1. Pathol. 157:1467-1472, 2000 and U.S. Pat. No. 6,942,970. Additional detection methods are provided in U.S. Pat. No. 6,280,929.

Numerous reagents and detection schemes can be employed in conjunction with FISH, CISH, and SISH procedures to improve sensitivity, resolution, or other desirable properties. As discussed above probes labeled with fluorophores (including fluorescent dyes and QUANTUM DOTS®) can be directly optically detected when performing FISH. Alternatively, the probe can be labeled with a nonfluorescent molecule, such as a hapten (such as the following non-limiting examples: biotin, digoxigenin, DNP, and various oxazoles, pyrrazoles, thiazoles, nitroaryls, benzofurazans, triterpenes, ureas, thioureas, rotenones, coumarin, courmarin-based compounds, Podophyllotoxin, Podophyllotoxin-based compounds, and combinations thereof), ligand or other indirectly detectable moiety. Probes labeled with such non-fluorescent molecules (and the target nucleic acid sequences to which they bind) can then be detected by contacting the sample (e.g., the cell or tissue sample to which the probe is bound) with a labeled detection reagent, such as an antibody (or receptor, or other specific binding partner) specific for the chosen hapten or ligand. The detection reagent can be labeled with a fluorophore (e.g., QUANTUM DOT®) or with another indirectly detectable moiety, or can be contacted with one or more additional specific binding agents (e.g., secondary or specific antibodies), which can be labeled with a fluorophore.

In other examples, the probe, or specific binding agent (such as an antibody, e.g., a primary antibody, receptor or other binding agent) is labeled with an enzyme that is capable of converting a fluorogenic or chromogenic composition into a detectable fluorescent, colored or otherwise detectable signal (e.g., as in deposition of detectable metal particles in SISH). As indicated above, the enzyme can be attached directly or indirectly via a linker to the relevant probe or detection reagent. Examples of suitable reagents (e.g., binding reagents) and chemistries (e.g., linker and attachment chemistries) are described in U.S. Patent Application Publication Nos. 2006/0246524; 2006/0246523, and 2007/ 01 17153.

It will he appreciated by those of skill in the art that by appropriately selecting labelled probe-specific binding agent pairs, multiplex detection schemes can he produced to facilitate detection of multiple target nucleic acid sequences (e.g., genomic target nucleic acid sequences) in a single assay (e.g., on a single cell or tissue sample or on more than one cell or tissue sample). For example, a first probe that corresponds to a first target sequence can he labelled with a first hapten, such as biotin, while a second probe that corresponds to a second target sequence can be labelled with a second hapten, such as DNP. Following exposure of the sample to the probes, the bound probes can he detected by contacting the sample with a first specific binding agent (in this case avidin labelled with a first fluorophore, for example, a first spectrally distinct QUANTUM DOT®, e.g., that emits at 585 mn) and a second specific binding agent (in this case an anti-DNP antibody, or antibody fragment, labelled with a second fluorophore (for example, a second spectrally distinct QUANTUM DOT®, e.g., that emits at 705 mn). Additional probes/binding agent pairs can he added to the multiplex detection scheme using other spectrally distinct fluorophores. Numerous variations of direct, and indirect (one step, two step or more) can he envisioned, all of which are suitable in the context of the disclosed probes and assays.

Probes typically comprise single-stranded nucleic acids of between 10 to 1000 nucleotides in length, for instance of between 10 and 800, more preferably of between 15 and 700, typically of between 20 and 500. Primers typically are shorter single-stranded nucleic acids, of between 10 to 25 nucleotides in length, designed to perfectly or almost perfectly match a nucleic acid of interest, to be amplified. The probes and primers are “specific” to the nucleic acids they hybridize to, i.e. they preferably hybridize under high stringency hybridization conditions (corresponding to the highest melting temperature Tm, e.g., 50 % formamide, 5x or 6x SCC. SCC is a 0.15 M NaCl, 0.015 M Na-citrate).

The nucleic acid primers or probes used in the above amplification and detection method may be assembled as a kit. Such a kit includes consensus primers and molecular probes. A preferred kit also includes the components necessary to determine if amplification has occurred. The kit may also include, for example, PCR buffers and enzymes; positive control sequences, reaction control primers; and instructions for amplifying and detecting the specific sequences.

In a particular embodiment, the methods of the invention comprise the steps of providing total RNAs extracted from cumulus cells and subjecting the RNAs to amplification and hybridization to specific probes, more particularly by means of a quantitative or semi-quantitative RT-PCR.

In another preferred embodiment, the expression level is determined by DNA chip analysis. Such DNA chip or nucleic acid microarray consists of different nucleic acid probes that are chemically attached to a substrate, which can be a microchip, a glass slide or a microsphere-sized bead. A microchip may be constituted of polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, or nitrocellulose. Probes comprise nucleic acids such as cDNAs or oligonucleotides that may be about 10 to about 60 base pairs. To determine the expression level, a sample from a test subject, optionally first subjected to a reverse transcription, is labelled and contacted with the microarray in hybridization conditions, leading to the formation of complexes between target nucleic acids that are complementary to probe sequences attached to the microarray surface. The labelled hybridized complexes are then detected and can be quantified or semi-quantified. Labelling may be achieved by various methods, e.g. by using radioactive or fluorescent labelling. Many variants of the microarray hybridization technology are available to the man skilled in the art (see e.g. the review by Hoheisel, Nature Reviews, Genetics, 2006, 7:200-210).

Expression level of a gene may be expressed as absolute expression level or normalized expression level. Typically, expression levels are normalized by correcting the absolute expression level of a gene by comparing its expression to the expression of a gene that is not a relevant, e.g., a housekeeping gene that is constitutively expressed. Suitable genes for normalization include housekeeping genes such as the actin gene ACTB, ribosomal 18S gene, GUSB, PGK1 and TFRC. This normalization allows the comparison of the expression level in one sample, e.g., a patient sample, to another sample, or between samples from different sources.

Other methods for determining the expression level of a gene include the determination of the quantity of proteins encoded by said genes.

Such methods comprise contacting the sample with a binding partner capable of selectively interacting with a marker protein present in the sample. The binding partner is generally an antibody that may be polyclonal or monoclonal, preferably monoclonal. The binding partner may also be an aptamer.

The presence of the protein can be detected using standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination tests; enzyme-labeled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; immunoelectrophoresis; immunoprecipitation,

etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the antigen and the antibody or antibodies reacted therewith.

The aforementioned assays generally involve separation of unbound protein in a liquid phase from a solid phase support to which antigen-antibody complexes are bound. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with an antibody against the protein to be tested. A biological sample containing or suspected of containing the marker protein is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate (s) can be washed to remove unbound moieties and a detectably labeled secondary binding molecule added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate washed and the presence of the secondary binding molecule detected using methods well known in the art.

Alternatively an immunohistochemistry (IHC) method may be preferred. IHC specifically provides a method of detecting targets in a sample or tissue specimen in situ. The overall cellular integrity of the sample is maintained in IHC, thus allowing detection of both the presence and location of the targets of interest. Typically a sample is fixed with formalin, embedded in paraffin and cut into sections for staining and subsequent inspection by light microscopy. Current methods of IHC use either direct labeling or secondary antibody-based or hapten-based labeling. Examples of known IHC systems include, for example, EnVision(TM) (DakoCytomation), Powervision(R) (Immunovision, Springdale, AZ), the NBA(TM) kit (Zymed Laboratories Inc., South San Francisco, CA), HistoFine(R) (Nichirei Corp, Tokyo, Japan).

In particular embodiment, a tissue section (e.g. a sample comprising cumulus cells) may be mounted on a slide or other support after incubation with antibodies directed against the proteins encoded by the genes of interest. Then, microscopic inspections in the sample mounted on a suitable solid support may be performed. For the production of photomicrographs, sections comprising samples may be mounted on a glass slide or other planar support, to highlight by selective staining the presence of the proteins of interest.

Therefore IHC samples may include, for instance: (a) preparations comprising cumulus cells (b) fixed and embedded said cells and (c) detecting the proteins of interest in said cells samples. In some embodiments, an IHC staining procedure may comprise steps such as: cutting and trimming tissue, fixation, dehydration, paraffin infiltration, cutting in thin sections, mounting onto glass slides, baking, deparaffination, rehydration, antigen retrieval, blocking steps, applying primary antibodies, washing, applying secondary antibodies (optionally coupled to a suitable detectable label), washing, counter staining, and microscopic examination.

A further object of the invention relates to an inhibitor of the hedgehog signalling pathway for use in the prevention or treatment of basal cell carcinoma in a subject in need thereof.

In a particular embodiment, the subject has an ACTRT1 deficiency (as above defined) in its epidermis.

A further object relates to an inhibitor of the hedgehog signalling pathway for use in the prevention or treatment of Bazex-Dupré-Christol syndrome

As used herein, the term "inhibitor of the hedgehog signalling pathway" includes any compound or agent that prevents signal transduction in the hedgehog signaling pathway, and specifically includes any compound that inhibits hedgehog from binding with its receptor. Exemplary inhibitors include, but are not limited to, the Cyclopamine analogs cyclopamine-4-ene-3-one, and Sigma Chemical Product Code J 4145 (see Williams et al., PNAS USA 100, 4616-4621, 2003, herein incorporated by reference). Additional analogs include Cur61414, 5El mab, HIP, Frzb, Cerberus, WIF-I, Xnr-3, Gremlin, Follistatin or a derivative, fragment, variant, mimetic, homologue or analogue thereof, Ptc, Cos2, PKA, and an agent of the cAMP signal transduction pathway. References that describe additional inhibitors include: U.S. Pat. Pub. 20050112125; Chen et al., Proc. Nat. Acad. Sci. 2002, 99:22, 14071-14076; Taipale et al., Nature 2002, 418, 892-897; Taipale et al., Nature 2000, 406, 1005-1009; U.S. Pat. Pub. 20050222087; U.S. Pat. Pub. 20050085519; U.S. Pat. Pub. 20040127474; U.S. Pat. Pub. 20040110663; U.S. Pat. Pub. 20040038876; and U.S. Pat. Pub. 20030166543; Int. Pat. Pub. WO2009132032 and WO2006078283 all of which are herein incorporated by reference in their entirities, and particularly for the hedgehog signaling agents taught therein.

The inhibitor of the sonic hedgehog signaling pathway may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form pharmaceutical compositions. "Pharmaceutically" or "pharmaceutically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

Preferably, the pharmaceutical compositions contain vehicles, which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Solutions comprising compounds of the invention as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The sonic hedgehog polypeptide can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric

hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetables oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active polypeptides in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

EXAMPLE 1: Inactivation of Arp-T1 causes aberrant Hedgehog activation in Bazex-Dupré-Christol syndrome and basal cell carcinoma.

Methods:

Patients and samples: A total of 48 patients from 13 BDCSs families and most of their unaffected relatives underwent complete clinical examination. All affected individuals

had two or more of the following signs on clinical examination or personal history: hypotrichosis, facial milia, follicular atrophoderma and basal cell carcinomas. DNA was extracted from peripheral blood lymphocytes obtained after informed consent by conventional phenol-chloroform method. Skin tumor tissue was obtained from 13 patients who underwent surgical excision of biopsy-confirmed BCCs. Forty additional paraffin-embedded skin tumor samples were obtained from the Necker hospital pathology department specimen collection.

Linkage analysis, targeted sequencing of genes in the candidate region and CNEs sequencing: Linkage analysis was performed using fluorescent polymorphic microsatellite markers (DXS1212, DXS8057, DXS8009, DXS1047, DXS994, DXS8072, GDB 185506 (HPRT), DXS1062 and DXS1192). After amplification and using GeneScan 400D ROX (PE Applied Biosystems, Foster City, CA) as a standard ladder, PCR products were analyzed using Genotyper™ and GeneScan™ softwares on an automatic sequencer (ABI PRISM 3100™ system). Single nucleotide polymorphism (SNP) markers rs 62619090 and rs 41304510 were analyzed by direct sequencing. The whole *ACTRT1* gene and selected CNE sequences were PCR-amplified using primers listed in Table S1. Both DNA strands were sequenced using the Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Sequence variation of *ACTRT1* was numbered with Adenine of the ATG initiation codon as the first nucleotide (GenBank accession NM_138289). To search for deletions/duplications around *ACTRT1*, we designed a high-density tiling-path comparative genomic hybridization (CGH) array using probes covering the entire 7.5 cM candidate region.

RNA extraction, RT-PCR and-real time PCR: Total RNA was isolated from cells or skin biopsies by RNeasy® Plus Minikit, (Qiagen GmbH, Hilden, Germany) and processed with the DNAse (Roche Diagnostics Gmbh, Mannheim, Germany) to eliminate genome DNA contamination. cDNA was synthesized by the priming of total RNA with random hexamer using High capacity cDNA Reverse Transcription kit (PE Applied Biosystems, Foster City, CA, USA). Reverse transcript (RT)-real time PCR was carried out using Fast SYBR Green PCR Master Mix (PE Applied Biosystems) on ABI prism 7000 (PE Applied Biosystems) in triplicates. The mRNA levels of *GLI1*, *PTCH1* and *Cyclin B1* were normalized to those of human actin gene. The sequences of primers were obtained from previous publications. PCR conditions were 95 °C for 20 seconds followed by 40 cycles of 95 °C for 3 seconds, 60 °C for 30 seconds. Dissociation analysis for each primer pair and reaction was performed to verify specific amplification.

***ACTRT1* cloning, expression and immunoblotting analysis:** The human cDNA containing the entire open reading frame of wild-type and mutant *ACTRT1* were amplified

from a control individual and the patient carrying the c.739-740InsA mutation respectively. cDNAs were subsequently cloned into the pcDNA3.1 expression vector, and resequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA). HEK293T cells were transiently transfected using jetPRIME™ (Polyplus-transfection Inc, New York, U.S.A) with either mutant or wild-type *ACTRT1* constructs. 24h after, cells were lysed in EBC buffer (50 mmol L^{-1} Tris-HCl pH 8, 170 mmol L^{-1} NaCl, 0·5% NP-40, 50 mmol L^{-1} NaF) with protease inhibitors (Roche Diagnostics Gmbh, Mannheim, Germany). Western blotting was performed using rabbit anti-Arp-T1 (Sigma Aldrich, St Louis, MO, USA), mouse anti-HDAC2 , mouse anti-HSP90 and rabbit anti-calreticulin antibodies (Millipore Corporation, Billerica, MA, USA). Bound antibodies were visualized with horseradish-peroxidase-conjugated antibodies against rabbit or mouse IgG (Santa Cruz Biotechnology, Heidelberg, Germany) by using the Enhanced Chemiluminescence (ECL Plus™ Western Blotting Detection System ; GE healthcare, Buckinghamshire, UK).

***In situ* hybridization:** For probes synthesis, a 348 pb PCR fragment of CNE12 sequence was amplified from human genomic DNA. A T7 extension was added to both primers to generate the control sense probe and the antisense probe, respectively. Sense and antisense templates were obtained using DIG RNA labeling kit (T7 RNA polymerase, Roche Diagnostics GmbH, Mannheim, Germany). Skin sections were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at five micrometer. Paraffin sections were mounted on SuperFrost®Plus slides (Thermo scientific, Vantaa, Finland), air-dried for 1-2 h at RT, melted in the oven at 60°C for 45 min, deparaffinized in xylene and hydrated through decreasing ethanol concentrations into PBS. Sections were hybridized with digoxygenin-labeled probe at 70°C overnight. In a second time sections were hybridized with alkaline-phosphatase-conjugated anti-Digoxygenin antibody (Roche Diagnostics) at 1:2000. Finally, slides were incubated in NBT-BCIP solution (Thermo scientific) to develop the dark-blue NBT-formazan precipitate, and mounted in Faramount Aqueous Mounting Medium (Dako, Glostrup, Denmark). Acquisition was performed with a motorized microscope system (Olympus IX81), with a DP70 camera (Olympus), and with the Cell^{P} software (Olympus).

Immuno-electron microscopy: Skin sections were washed with Dulbecco's Phosphate Buffered Saline 1X (DPBS1X), fixed for two hours at room temperature in formaldehyde 4% and glutaraldehyde 0.1%, rinsed twice with DPBS1X, then afterward embedded in sucrose 2.3M and frozen in liquid nitrogen. Cryosections were made using an ultracryomicrotome (Reichert Ultracut S., Leica, Wetzler, Austria), and ultrathin sections

were mounted on formvar-coated nickel grids. Skin sections were incubated for 15 minutes with Phosphate Buffered Saline 1X (PBS1X) - 15% glycine, 5 minutes with PBS1X - 15% glycine - 0.1% bovine serum albumin (BSA); and 20 minutes with DPBS1X - 15% glycine - 0.1% BSA - 10% normal goat serum followed by 2 hours of incubation with the rabbit anti - Arp-T1 (1/50 ; Sigma Aldrich, St Louis, MO, USA), After extensive rinsing in PBS1X - 15% glycine - 0.1% BSA, sections were incubated for 1 hour with either gold-labeled secondary goat anti-rabbit antibody conjugated with 10 nM gold particle (GAR 10 or GAM 10; British Biocell, Cardiff, U.K., http://www. british-biocell.co.uk). Sections were then washed for 30 minutes with PBS1X - 15% glycine, stained with 4% uranyl acetate in 2% methylcellulose for 10 minutes and air-dried. Section examinations were performed with a JEOL 1011 Transmission Electronic Microscope (TEM). Acquisitions were recorded with an ORIUS SC1000 CCD camera (GATAN) and processed with the Digital Micrograph software GATAN. Image acquisition and image analysis were performed on the Cochin Imaging Facility (Paris, France).

Cell proliferation assays: 10 cm dishes of HEK293T cells were transiently transfected with 10µg of the wild–type *ACTRT1* plasmid or mutant *ACTRT1* carrying the c.739_740InsA mutation or empty vector plasmid using jetPRIME™ reagent (Polyplus-transfection Inc, New York, U.S.A) following the manufacture protocol. 24h after transfection, 50000 cells of each transfection were plated in triplicates in a 96-well plates, and then maintained in culture in completed growth media containing 10% fetal bovine serum. After 24h and 48h, the cells were counted using the CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT ; Promega, Madison, WI, USA). The number of cells, based on the average count of the three wells was compared. Three independent experiments were performed.

Flow cytometry: 10 cm dishes of HEK293T cells were transiently transfected with 10µg of the wild–type *ACTRT1* plasmid or mutant *ACTRT1* carrying the c.739_740InsA mutation or empty vector plasmid using jetPRIME™ reagent (Polyplus-transfection Inc, New York, U.S.A) following the manufacture protocol. 24h after transfection, cells were synchronized through serum starvation for 12h, BrdU was incorporated during 2h, 6h and 24h followed by Flow cytometry assay. Staining of cells, we performed using APC BrdU Flow kit (BD biosciences, San Diego, CA, USA).

Luciferase hedgehog reporter assays: For the Gli Reporter Assay, the HEK293Tcells were seeded into six-well plates (35mm, 3.10^5 cells/well) and incubated with Smo agonist (SAG). Cells were cotransfected by using jetPRIMETM reagent (Polyplus-

transfection Inc, New York, U.S.A) with wild-type or 739_740InsA mutated *ACTRT1* plasmids together with 0.2 μg of a 8x3'Gli-BSδ51LucII plasmid carrying the firefly luciferase gene under the control of a *GLI* promoter. The pRenilla vector (0.1 μg) carrying the Renilla luciferase gene under the control of a ubiquitous promoter was used for normalizing transfection efficiency. The total amount of transfected DNA was kept constant by adding empty vector. The same experiments were performed used a mutant 8x3'Gli-mutS4-BSδ51LucII plasmid mutated in the regulatory region of *GLI1*. 24h after transfection, luciferase activity was determined with the Dual luciferase kit (Promega, Madison, WI, USA).

Chromatin immunoprecipitation assay: The chromatin immunoprecipitation (ChIP) assay was carried out using EZ-Magna ChIP™ A/G kit (Millipore Corporation, Billerica, MA, USA). Briefly, tissues were treated with 1% formaldehyde for 10 min. The cross-linked chromatin was then prepared and sonicated to an average size of 300 - 500 bp. The DNA fragments were immunoprecipitated with polyclonal anti-Arp-T1 antibody or IgG isotype control antibody at room temperature. After reversal of cross-linking, the immunoprecipitated chromatin was amplified by PCR amplification of specific regions of the *GLI1* genomic locus. The amplified DNA products were analyzed by agarose gel electrophoresis.

Results and discussion:

Cutaneous basal cell carcinoma (BCC) is the most common cancer in Western Countries. It rarely metastases but may cause significant destruction of surrounding tissues. Most BCCs are sporadic but some forms are inherited. Bazex-Dupré-Christol syndrome (BDCS) is an X-linked dominant predisposition to BCCs with typical skin manifestations, suggestive of a primary hair follicle anomaly[1,2,3,4]. We have mapped the BDCS gene to Xq24-q27 in three large pedigrees[5]. Studying three additional families, we found genetic homogeneity of the disease (Zmax=15 at $\theta = 0$) and recombinants allowed to narrow down the genetic interval to 7.5 cM (DXS8057-rs62619090). The encompassed genes (36) were sequenced and an identical single nucleotide insertion in the Actin-Related Protein T1 (Arp-T1) gene (*ACTRT1*) was identified in 2/6 families (c.547_548 InsA, Family C and D). This insertion is predicted to cause a frame shift and a premature termination codon at position 199 (p.M183NfsX17). It was found to segregate with the disease in the two families and was absent from available databases (dbSNPs and 1000 genomes) and 600 control chromosomes.

ACTRT1 expression in HEK293T cells showed that the mutant cDNA encodes a 25 kDa truncated protein. Yet, no mutation of the coding region of *ACTRT1* was found in the four other families linked to the same interval. No additional exons in *ACTRT1* were found by 5'-3' RACE-PCR and no rearrangements in the candidate region were identified in the remaining 4/6 patients by high-density tiling-path comparative genomic hybridization array.

Because the *ACTRT1* gene is located in a large 2.5 Mb gene desert, we performed *in silico* search for highly conserved non-coding elements[6] (CNEs), since these elements are known to control expression of neighboring genes[7,8,9,10]. A total of 17 CNEs were identified. Sanger sequencing detected a g.127372937A>T variation in CNE12 in two Turkish patients (Family E and F; CNE12 chrX: 127371674-127374249). This variation segregated with the disease and was absent from dbSNP, the 1000 genome databases and from 261 Turkish individuals (404 X chromosomes). This unusually long CNE (2.5 kb) is conserved in mammals (61% identity with the mouse sequence) and teleost fish. *In silico* predicted secondary structure of CNE12 suggested that it may be transcribed[6,11]. RT-PCR and *in situ* hybridization (ISH) experiments showed that CNE12 was indeed expressed in epidermis and epidermal appendages but not in the dermis. Interestingly, ISH of skin sections showed complete abolition of the specific ncRNA in the two male patients carrying the CNE12 variation, demonstrating the drastic impact of the g.127372937A>T variation on its stability or expression. Conversely, ISH analysis detected CNE12 non-coding RNAs (ncRNAs) in a patient carrying the c.547-548 InsA *ACTRT1* mutation. Considering that a subset of transcribed ncRNAs act as a novel class of enhancers (eRNAs), we hypothesized that CNE12 could act as an enhancer in addition to being transcribed. CNE12 was subcloned in a pGL4.23 luciferase reporter plasmid. Interestingly, wild-type CNE12 had an increased enhancer activity in HaCat keratinocyte cell line, while the CNE12 construct harbouring the A>T mutation was inactive.

Sequence conservation between species is not a consistent indicator of regulatory elements[12]. Because no mutation in the 17 CNEs surrounding the *ACTRT1* locus was found in the remaining two families, we carried out systematic array-based capture and high throughput sequencing of the complete 7.5 Mb candidate region in the remaining probands. Owing to the number of variants detected, a specific genome browser and filter was used to detect candidate variants. The *ACTRT1* mutation and CNE12 variant were confirmed and no mutation in the coding sequences of the other 36 genes located in the 7.5 Mb interval was identified, suggesting that other mutations may involve hitherto unknown *ACTRT1* regulatory sequences. Two variations were selected (A2: g.125959394C>G variation and B2:

127968123T>C variation) as candidates and subcloned in pGL4.23. Interestingly, both wild-type sequences had enhancer activity in HaCAT cells but this activity was reduced when the variant constructs were used. Similarly, when used as probes, sequences A2 and B2 stained positively on control skin biopsies, but no staining was detected on skin biopsies from the corresponding patients. Cross tests were positive, demonstrating that absence of ISH staining was patient and mutation-specific.

While epigenetic marks (H3K4me1, H3K36me3 and H3K27ac) usually identify enhancer elements in the genome, no chromatin signature in the vicinity of *ACTRT1* was found in an atlas of 43,000 candidate active human enhancers[13]. In order to identify a putative chromatin signature at the *ACTRT1* locus, chromatin immunoprecipitation (ChIP) followed by quantitative targeted PCR[14] was performed on proteins extracted from normal human epidermis. Indeed, an enhancer signature was found for A2, B2 and CNE12 sequences, with an enrichment in H3K27Ac, H3K4Me1. Our results provide genetic and functional evidence that BDCS is caused by loss-of-function mutations altering either the coding region of the *ACTRT1* gene or eRNA enhancers in its transcribed non coding vicinity.

Immuno-histochemical (IHC) analyses in six control skin sections detected Arp-T1 in tissues involved in BDCS (epidermal layers, hair follicles, sebaceous glands and eccrine sweat glands) but not in dermal connective tissue. Interestingly, IHC failed to detect any specific staining in BCC tumors of 9/9 BDCS patients (at least one patient of each BDCS family) and found only a mild signal in unaffected epidermis. Furthermore, Arp-T1 was also undetectable in BCCs from 34/40 unrelated sporadic BCC cases in whom neither germ line nor somatic tumoral *ACTRT1* mutations had been found. Conversely, Arp-T1 was normally detected in BCCs from two patients with Gorlin syndrome carrying a germline *PTCH1* mutation and one Xeroderma Pigmentosum patient. These results emphasize genetic heterogeneity of skin tumors and suggest that sporadic BCCs may quasi-consistently exhibit loss of function at the *ACTRT1* locus.

Arp-T1 belongs to the Actin-Related Proteins (Arps) family. Nuclear Arps are essential elements of the macromolecular machinery that controls chromatin remodeling, dynamic changes in DNA structure, transcription and repair[15,16,17,18,19,20,21,22]. Ultra-thin sections of normal skin processed for transmission electronic microscopy analyses detected Arp-T1 in both nucleus and cytoplasm. Subcellular protein fractionation assays showed that Arp-T1 binds chromatin, while the truncated Arp-T1 protein was absent in the chromatin-bound fraction. Arp-T1 chromatin-binding in controls but not BDCS is consistent with its putative involvement in chromatin remodeling and its alteration in the disease.

Since the Hedgehog signaling pathway is activated in more than 70% of BCC cases[23,24], we hypothesized that links may exist between Arp-T1 and the Hedgehog pathway. Studying expression of Hedgehog target genes, we found that *GLI1, GLI2* and *PTCH1* were overexpressed both in non tumoral skin and BCCs derived from BDCS patients carrying either an *ACTRT1* c.547_548 InsA mutation or a CNE12 variant. Transactivation assay showed that Arp-T1 but not the mutant protein can inhibit the Hedgehog pathway. Consistently, stable expression of *ACTRT1* significantly reduced the aberrant activation of Hedgehog signaling pathway in MDA-MB231 cells. ChIP assays on control skin revealed that Arp-T1 can bind two regions upstream of the *GLI1* transcription initiation site, supporting the view that Arp-T1 can directly bind the regulatory sequences of Hedgehog signaling target genes. In keeping with these results, recent studies have shown that *GLI1* transcriptional activity and Hedgehog signaling are controlled by chromatin regulators, such as Brg1 and Snf5, components of the mammalian *SWI/SNF* chromatin remodeling complex, required for signal-induced transcription of Hedgehog signaling target genes *via* binding to *GLI1* regulatory regions. Moreover, loss of Snf5 leads to aberrant activation of the Hedgehog signaling pathway in human rhabdoid tumors[25,26]. Taken together, our results suggest that transcriptional regulation of *GLI1* may be pivotal not only for tumor suppressor activity of Snf5 and Brg1, but for Arp-T1 as well.

Germline mutations in the *ACTRT1* gene and its non-coding surrounding elements in BCCs represent a hitherto unreported mechanism of inherited predisposition to skin tumors. This study highlights the impact of intergenic mutations in human diseases. Elucidating the disease mechanism in BDCS, a rare inherited condition, has thus shed light on a most common human cancer, BCC. Developing novel Hedgehog signaling inhibitors targeting Arp-T1 will hopefully help improving treatment of this frequent, potentially devastating condition.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A method for determining whether a subject is at risk of having a basal cell carcinoma comprising the steps consisting of detecting an ACTRT1 deficiency in the subject's epidermis and concluding that the subject is at risk of having a basal cell carcinoma when an ACTRT1 deficiency in the subject's epidermis is detected.

2. A method for the prevention or treatment of basal cell carcinoma in a subject in need thereof comprising administering the subject with a therapeutically effective amount of an inhibitor of the hedgehog signalling pathway.

3. A method for prevention or treatment of Bazex-Dupré-Christol syndrome in a subject in need thereof comprising administering the subject with a therapeutically effective amount of an inhibitor of the hedgehog signalling pathway.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/069219

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/574
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)
G01N

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 practicable, search terms used)

EPO-Internal, 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	JAMES KIM ET AL: "Itraconazole, a Commonly Used Antifungal that Inhibits Hedgehog Pathway Activity and Cancer Growth", CANCER CELL, CELL PRESS, US, vol. 17, no. 4, 13 April 2010 (2010-04-13) , pages 388-399, XP002637909, ISSN: 1535-6108, DOI: 10.1016/J.CCR.2010.02.027 [retrieved on 2010-04-12]	2
A	abstract; fig. 1; fig. 7; whole document	1

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 December 2014	11/12/2014

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	Schindler-Bauer, P

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	L.J.M.T. PARREN ET AL: "Hereditary tumour syndromes featuring basal cell carcinomas", BRITISH JOURNAL OF DERMATOLOGY, vol. 165, no. 1, 3 June 2011 (2011-06-03), pages 30-34, XP055155973, ISSN: 0007-0963, DOI: 10.1111/j.1365-2133.2011.10334.x abstract; p. 31, col. 1-p. 32, col. 1, par. 1; p. 33, col. 2; p. 34, par. 1	2,3
A	DAYA-GROSJEAN L ET AL: "Sonic hedgehog signaling in basal cell carcinomas", CANCER LETTERS, NEW YORK, NY, US, vol. 225, no. 2, 28 July 2005 (2005-07-28) , pages 181-192, XP004949373, ISSN: 0304-3835, DOI: 10.1016/J.CANLET.2004.10.003 the whole document	2,3
A	TANG ET AL: "Novel Hedgehog pathway targets against basal cell carcinoma", TOXICOLOGY AND APPLIED PHARMACOLOGY, ACADEMIC PRESS, AMSTERDAM, NL, vol. 224, no. 3, 18 October 2007 (2007-10-18), pages 257-264, XP022303638, ISSN: 0041-008X, DOI: 10.1016/J.TAAP.2006.12.011 the whole document	2,3
A	JULIE K. IWASAKI ET AL: "The molecular genetics underlying basal cell carcinoma pathogenesis and links to targeted therapeutics", JOURNAL OF THE AMERICAN ACADEMY OF DERMATOLOGY, vol. 66, no. 5, 1 May 2012 (2012-05-01), pages e167-e178, XP055085189, ISSN: 0190-9622, DOI: 10.1016/j.jaad.2010.06.054 the whole document	2,3
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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.
A	STEVEN HOSEONG YANG ET AL: "Pathological responses to oncogenic Hedgehog signaling in skin are dependent on canonical Wnt/[beta]-catenin signaling", NATURE GENETICS, vol. 40, no. 9, 1 August 2008 (2008-08-01), pages 1130-1135, XP055155976, ISSN: 1061-4036, DOI: 10.1038/ng.192 the whole document	2,3
X	L.J.M.T. PARREN ET AL: "Linkage refinement of Bazex-Dupré-Christol syndrome to an 11.4-Mb interval on chromosome Xq25-27.1", BRITISH JOURNAL OF DERMATOLOGY, vol. 165, no. 1, 24 June 2011 (2011-06-24), pages 201-203, XP55156191, ISSN: 0007-0963, DOI: 10.1111/j.1365-2133.2011.10219.x the whole document	1-3
X	R N Amaria ET AL: "Vismodegib in basal cell carcinoma", Drugs of today (Barcelona, Spain : 1998), 1 July 2012 (2012-07-01), page 459, XP55156203, United States DOI: 10.1358/dot.2012.48.7.1808490 Retrieved from the Internet: URL:http://www.ncbi.nlm.nih.gov/pubmed/22844657 [retrieved on 2014-12-01]	2
Y	abstract	3
X	CIRRONE FRANK ET AL: "Vismodegib and the Hedgehog Pathway: A New Treatment for Basal Cell Carcinoma", CLINICAL THERAPEUTICS, vol. 34, no. 10, October 2012 (2012-10), pages 2039-2050, XP028945760, ISSN: 0149-2918, DOI: 10.1016/J.CLINTHERA.2012.08.011	2
Y	abstract	3

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Daniel D Von Hoff ET AL: "Inhibition of the Hedgehog Pathway in Advanced Basal-Cell Carcinoma", The New England Journal of Medicine, 17 September 2009 (2009-09-17), pages 1164-1172, XP55156198, United States DOI: 10.1056/NEJMoa0905360 Retrieved from the Internet: URL:http://content.nejm.org/cgi/content/abstract/361/12/1164	2
Y	the whole document	3

A	HOWELL B G ET AL: "Microarray profiles of human basal cell carcinoma: Insights into tumor growth and behavior", JOURNAL OF DERMATOLOGICAL SCIENCE, ELSEVIER SCIENCE PUBLISHERS, SHANNON, IE, vol. 39, no. 1, 1 July 2005 (2005-07-01), pages 39-51, XP027793055, ISSN: 0923-1811 [retrieved on 2005-07-01] abstract; table 1	1

A	EP 1 900 824 A1 (DEUTSCHES KREBSFORSCH [DE]) 19 March 2008 (2008-03-19) abstract; table 1	1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2014/069219

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 2(partially)

   Detecting a deficiency of the gene ACTRT1 is a method for determining the risk of having basal cell carcinoma

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2. claims: 1, 2(partially)

   A method for prevention or treatment of basal cell carcinoma comprising administering a not further defined inhibitor of the hedgehog signalling pathway.

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3. claim: 3

   A method for prevention or treatment of Bazex-Dupré-Christol syndrome comprising administering a not further defined inhibitor of the hedgehog signalling pathway.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2014/069219

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 1900824 A1	19-03-2008	EP 1900824 A1	19-03-2008
		EP 2074228 A2	01-07-2009
		US 2010261617 A1	14-10-2010
		WO 2008031839 A2	20-03-2008