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(54) **Title:** BIOMARKERS FOR HPV INFECTIONS, RELATED DIAGNOSTIC METHODS AND KITS

(57) **Abstract:** The present invention refers to a biomarker belonging to the group of at least one protein involved in the sumoylation pathway, variants thereof and mRNAs encoding the same, for use in assessing the risk and/or diagnosing and/or prognosing and/or monitoring the progression of an HPV infection, to an in vitro method and kit for assessing the risk and/or diagnosing and/or prognosing and/or monitoring the progression of an HPV infection.



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BIOMARKERS FOR HPV INFECTIONS, RELATED DIAGNOSTIC METHODS AND KITS

5 TECHNICAL FIELD

Human Papillomaviruses (HPVs) infect stratified epithelium and are the causative agents of cervical cancer, the second most common cause of cancer-related death in women. A critical aspect that still persists in the HPV field is the selection of very sensitive and specific HPV diagnostic assays. Authors provide evidence that proteins
10 involved in the Small Ubiquitin-like Modifier (SUMO) pathway, in particular Ubc9, are affected by HPV E6 and E7 expression and thus could play a major role in the diagnosis of oncogenic HPV infections.

The present invention refers to a biomarker consisting in a protein involved in the sumoylation pathway or an mRNA encoding the same for use in assessing the risk
15 and/or diagnosing and/or prognosing and/or monitoring the progression of an HPV oncogenic infection, to an in vitro method for assessing the risk and/or diagnosing and/or prognosing and/or monitoring the progression of an HPV oncogenic infection and to a diagnostic kit for HPV oncogenic infection.

20 BACKGROUND

Human papillomaviruses (HPVs) are a group of small, double-stranded DNA viruses known to be the primary cause of cervical cancer. Evidence is now emerging on their role in the etiology of Head and Neck and other cancers as well.

HPVs can be classified as low-risk and high-risk viruses: low-risk HPVs are
25 associated with a spectrum of benign warts, such as condyloma [22], whereas infections with high-risk HPVs, such as HPV16 (European German, GenBank Accession number AF536179) and 18 (Human papillomavirus type 18, GenBank Accession number X05015.1), are manifested by intraepithelial malignant progression caused by viral E6 and E7 [1]. High-risk E6 and E7 are considered as oncogenes that,
30 through the degradation of p53 and Rb by ubiquitin-proteasome pathway, respectively, cause perturbation of cell cycle regulation in the infected cell [1].

Robust and convincing evidence now reinforces the acceptance of cervical cancer prevention approaches. The strategy is to strongly aim and focus on persistent infection caused by HPV. Besides laboratory tests to detect HPV DNA, there are
35 other promising markers of HPV oncogenic infection. These include: i) markers of

increased HPV mRNA and protein expression; ii) markers of increased cell proliferation, such as Ki-67, MCM2, TOP2a, and p16INK4a; and iii) markers of chromosomal instability, such as a gain of chromosome arm 3q and HPV DNA integration [2].

- 5 Currently, the most promising candidate as a clinical biomarker is detection of p16INK4a (GenBank Accession number L27211). p16INK4a overexpression is a result of inactivation of the cell-cycle regulatory retinoblastoma protein (pRb) by High Risk HPV E7. [3-6]. p16INK4a detection has now multiple clinical indications, in the screening and prevention programs, either at cytology or histopathology level
- 10 [2].

The most used HPV diagnosis is still based on cytology, since the virus clearly alters the infected cells. Precisely, this phenomenon is called koilocytosis and indicates the presence of an HPV infection.

- A koilocyte is a squamous epithelial cell that has undergone a number of structural
- 15 changes, which occur as a result of infection of the cell by human papillomavirus (REF : Nucci MR, Oliva E., ed. (2009). *Gynecologic pathology: A volume in the series - Foundations in diagnostic Pathology*. Elsevier Churchill Livingstone). Such morphological changes are not necessarily of dysplastic, as those present in the Cervical Intraepithelial Neoplasia (CIN). p16INK4a (p16) has been recently proposed
- 20 as a marker for Cervical Intraepithelial Neoplasia 2 (CIN2), CIN3 or even cancer development within 3 years in HPV-positive women. Thus HPV positive p16 positive women would need immediate colposcopy (clinical intervention) to be safe (Carozzi et al. Lancet Oncology 2013, 14(2):168-76)). However, the p16 marker is not always effective in detecting CIN2.

- 25 Sumoylation is a post-translational modification where the Small Ubiquitin-like Modifier (SUMO) proteins are reversibly attached to the protein target through an ubiquitin-like pathway, with many different outcomes on protein stability, interaction and localization, DNA repair and replication, transcriptional regulation, cell cycle control, apoptosis, cell signalling and viral replication [7, 8]. Among their different
- 30 ways to exploit host cellular systems, viruses are also known to target post-translational modification systems, such as the SUMO pathway [9]. In fact, most of the DNA viruses (parvoviruses, adenoviruses, papoviruses and herpesviruses) have viral proteins that are sumoylated or interact with SUMO components. Conjugation of target proteins with SUMO requires a series of events catalyzed by the E1 (SUMO-

activating SAE1/SAE2) (e.g., GenBank Accession numbers (SAE1): NM_001145714, SUMO1-activating enzyme subunit 1 transcript variant 3; NR_027280, SUMO1-activating enzyme subunit 1 transcript variant 4; NM_005500, SUMO1-activating enzyme subunit 1 transcript variant 1; NM_001145713, SUMO1-activating enzyme subunit 1 transcript variant 2; Homo sapiens ubiquitin-like modifier activating enzyme 2 (UBA2 or SAE2), NM_005499), E2 (SUMO-conjugating Ubc9) (e.g., GenBank Accession numbers: NM_003345, Homo sapiens ubiquitin-conjugating enzyme E2I (UBE2I), transcript variant 1; NM_194259, Homo sapiens ubiquitin-conjugating enzyme E2I (UBE2I), transcript variant 2; NM_194260, Homo sapiens ubiquitin-conjugating enzyme E2I (UBE2I), transcript variant 3; NM_194261, Homo sapiens ubiquitin-conjugating enzyme E2I (UBE2I), transcript variant 4) and E3 (SUMO-ligating) enzymes family (this family comprises several members, including members of the PIAS protein family (Protein Inhibitors of Activated Stat) (e.g., PIAS1 GenBank Accession number: NM_016166; PIAS4 (PIASy) GenBank Accession number: NM_015897)); RanBP2 (Nup358), a nuclear pore protein (GenBank Accession number NM_194301); the polycomb protein, Pc2 (GenBank Accession number NM_003655); TOPORS, topoisomerase I binding protein (GenBank Accession number NG_017050), and members of the TRIM family (GenBank Accession number NG_030346)). Human tissues express four SUMO family members, SUMO1 (including GenBank Accession numbers NM_001005782, NM_003352, NM_001005781, NR_002189, NR_002190), the nearly identical SUMO2 (GenBank Accession numbers NM_001005849, NM_006937) and SUMO3 (GenBank Accession number NM_006936), all ubiquitously expressed, and SUMO4 (GenBank Accession number NG_012301), which is highly similar to SUMO2 but primarily expressed in kidney, lymph node and spleen [7, 8]. The mechanisms regulating paralog-selective sumoylation are still not well defined, although SUMO E3 enzymes are the likely candidates to regulate the preferential usage of SUMO1 over SUMO2/3 in the cells.

The authors have previously demonstrated that other viral proteins, such as the adenoviral protein Gam1, can dysregulate the SUMO pathway by interacting with cellular proteins [9-14].

Heaton PR. et al. (Virus Res. 2011 Jun;158(1-2):199-208) demonstrated that high risk HPV E6 oncoproteins are capable of targeting the sumoylation system through reducing the SUMO conjugating enzyme, Ubc9, levels.

The authors thus asked whether HPV could affect components of the SUMO pathway. Indeed, the present studies in cervical biopsies and in an in vitro experimental models show that HPV16 E6 and E7, contrary to what is shown in Heaton et al., 2011, have the ability to induce the up-regulation of enzymes involved in the sumoylation machinery, in particular Ubc9. The oncoproteins also diversely affect the two main SUMO paralogs, namely SUMO1 and SUMO2/3.

Ronen O, et al. (Head Neck. 2009 Jul;31(7):845-55) suggests that Ubc9 may play an important role in tumorigenesis and tumor progression in squamous cell carcinoma of the head and neck (SCCHN) and could potentially serve as a molecular marker of progression of this type of cancer.

Szendefi M, et al. (Obstet Gynecol. 2003 Dec;102(6):1269-77) supports the use of promyelocyte protein-containing nuclear bodies and their association to SUMO-1, as a cytodiagnostic marker that parallels cervical cancer progression.

Wang Q, et al. (Oncogene 2012, doi: 10.1038/onc.2012.250) shows that SENP1 (SUMO-specific protease 1, member of de-SUMOylation protease family expression) directly correlates with prostate cancer aggressiveness and recurrence. Modulating SENP1 level dictates colony formation of prostate cancer cell lines, tumor growth in nude mice and also prostate cancer cell migration and invasion. Silencing SENP1 level in highly metastatic prostate cancer cells perturbs their ability to metastasize to the bone and initiates secondary tumors. Mechanistically, the expression of two critical bone remodeling proteins, matrix metalloproteinase 2 (MMP2) and MMP9, is regulated by SENP1 through the HIF1 α signaling pathway. All these results show the contribution of SENP1 to the progression of prostate cancer, and suggest that SENP1 may be a prognostic marker and a therapeutic target for metastasis in prostate cancer patients.

Chen SF et al (Chin J Cancer 2011, 30(9):638-44) aimed to evaluate the contribution of Ubc9 in the chemoresistance of breast cancer. Immunohistochemistry (IHC) was used to examine the expression level of Ubc9. The proportion of Ubc9-positive cells was higher in invasive ductal carcinoma than in normal breast tissues. High Ubc9 expression was associated with poor differentiation, larger tumor size, advanced clinical stage, lymph node metastasis, basal-like phenotype, and poor clinical response to neoadjuvant chemotherapy. These data indicate that high Ubc9 expression correlates with poor response to chemotherapy and poor clinical prognosis.

WO2010051261 refers to compositions and methods for the differential detection of high risk forms of HPV from a urine sample provided by a patient. Specifically, the invention refers to primers and probes that specifically recognize and bind sequences within the E1 gene of HPV. Detection of high risk forms of HPV identify individuals at risk of developing or in the early stages of cervical carcinoma.

KR 20090102318 relates to a kit for diagnosing breast cancer using UBC9 gene or Ubc9 protein provided to measure the expression amount of Ubc9 protein and diagnose the breast cancer.

WO2011/130660 supports the use of SUMO as a marker of cancer development and target for cancer therapy, in particular for sarcoma, carcinoma, lymphoma, glioblastoma, or astrocytoma, brain cancer. No data on cervical cancer are provided.

WO 03/025543 refers to methods of diagnosing whether an epithelial tissue is an abnormal tissue by determining an expression pattern for Promyelocytic leukemia protein (PML) in the epithelial tissue; determining an expression pattern for nuclear bodies in the epithelial tissue; determining SUMO-1 co-localization and comparing the expression pattern for PML and the expression pattern for nuclear bodies with a control.

Thus, it is still needed a biomarker for HPV oncogenic infection which allows precise, sensitive and selective detection of the infection.

DESCRIPTION OF INVENTION

The authors thus envisage the use of proteins involved in the SUMO pathway, as e.g. the conjugating enzyme Ubc9, SUMO1, SUMO2/3, SAE-1, SAE-2, SUMO E3 ligases, SUMO4 and PIAS protein family, as a biomarker of HPV oncogenic infection. The present immunohistochemistry (IHC) data, as depicted in the following section, show that the detection of at least one protein belonging to the SUMO pathway, e.g. by immunohistochemical staining of cells containing viral oncoproteins, allows to predict with high accuracy the presence of Low grade lesions (Low Squamous Intraepithelial Lesions, LSILs, or Cervical Intraepithelial Neoplasia 1, CIN 1) and High grade lesions (High Squamous Intraepithelial Lesions, HSILs, or Cervical Intraepithelial Neoplasia 2/3, CIN2/3). The authors foresee that said proteins could also be useful in the detection of other HPV-related lesions, i.e. condyloma. Since the above biomarkers are proteins, this method could also be extended to other detection technologies, such as ELISA, cell suspension, cytology (Fig. 14).

Said biomarkers can be used either alone or in combination with other markers of HPV oncogenic infection, e.g. p16 (p16INK4a). Authors herein show that Ubc9 staining can spot CIN2 cases missed by p16INK4a staining (Tables 6-7). The same applies also to CIN1 and CIN3.

5 An object of the present invention is a biomarker belonging to the group of at least one:

a) protein involved in the sumoylation pathway;

b) a variant thereof;

c) mRNA encoding a) or b),

10 for use in assessing the risk and/or diagnosing and/or prognosing and/or monitoring the progression of an HPV infection.

Preferably the HPV infection is an high-risk HPV infection, more preferably causative of cervical cancer.

Alternatively the HPV infection is a low-risk HPV infection, more preferably
15 causative of condyloma.

In a preferred embodiment the biomarker as above defined, belong to the group of: Ubc9, SUMO1, SUMO2/3, SAE-1, SAE-2, SUMO E3 ligases, SUMO4 and/or PIAS protein family.

More preferably the biomarker is Ubc9, SUMO1and/or SUMO2/3. Even more
20 preferably the biomarker is Ubc9.

Another object of the invention is an in vitro method for assessing the risk and/or for diagnosing and/or for prognosing and/or for monitoring the progression of an HPV infection comprising the steps of:

a) detecting the biomarker as above defined in a test sample isolated from a subject
25 and

b) comparing with respect to an appropriate control.

In a preferred aspect, the in vitro method for monitoring the progression of an high-risk HPV infection, comprises the steps of:

a) measuring the alteration of the amount of said biomarker in said test sample and
30 b) comparing the measured amount of step a) with an appropriate control amount.

Said alteration can be an increase or a decrease in the amount of the biomarker. An increase can be correlated to an aggravation of the infection, which in case of high-risk HPV infections can lead to a cervical cancer, or to an aggravation of the cervical cancer. A decrease can be correlated to an amelioration of the infection or to recovery

of the subject.

If by comparing the measured alteration of the amount of step a) with the appropriate control amount of said biomarker, the alteration of the amount of said biomarker in the test sample corresponds to an increase, the subject of step a) may go towards an
5 aggravation of the infection, which can lead to a cervical cancer, or to an aggravation of the cervical cancer, in case of high-risk HPV infections.

If by comparing the measured alteration of the amount of step a) with the appropriate control amount of said biomarker, the alteration of the amount of said biomarker in the test sample corresponds to a decrease, the subject of step a) may go toward an
10 amelioration of the infection or to recovery.

In another preferred aspect, the in vitro method for assessing the risk and/or diagnosing and/or prognosing an HPV infection, comprises the steps of:

a) measuring the amount of said biomarker in said test sample and
b) comparing the measured amount of step a) with an appropriate control amount,
15 wherein an amount of said biomarker in the test sample higher than the control amount indicates that the subject is either at increased risk for developing or is affected by an HPV infection.

In preferred embodiments the HPV infection is an high-risk HPV infection, possibly causative of cervical cancer.

20 In alternative preferred embodiments the HPV infection is a low-risk HPV infection, possibly causative of condyloma.

In the in vitro method according to the invention, the detection of the biomarker is preferably correlated with the presence of HPV-related lesions, which are preferably condyloma, Low grade Squamous Intraepithelial Lesions (LSILs) or High grade
25 Squamous Intraepithelial Lesions (HSILs).

An amount of said biomarker in the test sample higher than the control amount can indicate that the subject of step a) presents HPV-related lesions and is either at increased risk for developing or is affected by an HPV oncogenic infection and/or cervical cancer.

30 In the in vitro methods according to the invention, the amount of other different biomarkers of HPV oncogenic infection can also be measured and compared with an appropriate control amount of the same biomarkers.

The in vitro method according to the invention preferably further comprises the measure of the amount of p16 (p16INK4a) and the comparison with an appropriate

control amount of p16. p16 (p16INK4a) amount can be the amount of the respective protein or mRNA.

A further object of the invention is a kit for assessing the risk and/or diagnosing and/or prognosing and/or monitoring the progression of an HPV infection,
5 comprising:

- means to detect and/or measure the amount and/or measure the alteration of the amount of at least one biomarker as above defined and optionally
- control means.

Control means can be used to compare the increase of amount of the biomarker to an
10 appropriate control value. The control value may be obtained for example, with reference to known standard, either from a normal subject or from normal population. The means to detect and/or measure the amount and/or measure the alteration of the amount of at least one biomarker as above defined are preferably at least one antibody, functional analogous or derivatives thereof.

15 Said antibody, functional analogous or derivatives thereof can be specific for said biomarker.

In the present invention, the detection and/or the measurement of the amount and/or the measurement of the alteration of the amount may be referred to one, two, three, four, five, six, seven, eight, etc biomarkers as above defined. Any combination of said
20 biomarkers is suitable for the purpose of the invention.

In a preferred embodiment, the kit of the invention comprises:

- a solid phase adhered antibody specific for said biomarker;
- detection means of the ligand specific-biomarker complex.

It is a further object of the invention a kit for assessing the risk and/or diagnosing
25 and/or prognosing and/or monitoring the progression of an HPV infection, comprising:

- means to detect and/or measure the amount and/or measure the alteration of the amount of at least one mRNA encoding the biomarker as above defined and optionally
- 30 - control means.

The kits according to the invention can further comprise customary auxiliaries, such as buffers, carriers, markers, etc. and/or instructions for use.

The above biomarker can detect the presence of HPV-related lesions, preferably condyloma, Low grade Squamous Intraepithelial lesions (LSILs) (Cervical

Intraepithelial Neoplasia 1 (CIN 1)) and/or High grade Squamous Intraepithelial Lesions (HSILs) (Cervical Intraepithelial Neoplasia 2/3 (CIN 2/3)).

In the present invention, the “appropriate control amount” may be the amount quantified, measured or assessed in a sample isolated from a healthy subject or from a patient affected by another infection or from a subject not affected by high-risk HPV infection and/or cervical cancer.

In the case of a method for monitoring the progression of an high-risk HPV infection, the appropriate control amount may be the amount quantified, measured or assessed in a sample isolated from the same subject at various time point e.g. before the start of a therapy, at various time point throughout the course of the therapeutic agent, etc.

The test sample of the in vitro methods according to the present invention can be a cell and/or tissue sample and/or a biological fluid.

The in vitro methods according to the present invention can be carried out e.g. in FFPE (Formalin-fixed-paraffin-embedded) samples or in a suspension colorimetric assay.

In the in vitro methods according to the present invention, step a) is preferably carried out by immunohistochemistry, cytology, ELISA, or spectrofluorometry.

High-risk HPV infections, as e.g. HPV16 and 18 infections, can lead to HPV oncogenic infections.

In the present invention, the expression “detecting” in relation to a protein or a nucleic acid, refer to any use of any method of observing, ascertaining or quantifying signals indicating the presence of the target protein or nucleic acid in a sample or the absolute or relative quantity of that target protein or nucleic acid in a sample. Methods can be combined with protein or nucleic acid labeling methods to provide a signal, for example: immunohistochemical staining, ELISA, cell suspension, cytology, fluorescence, radioactivity, colorimetry, gravimetry, X-ray diffraction or adsorption, magnetism, enzymatic activity and the like.

In the present invention, the expression “measuring the amount” can be intended as measuring the amount or concentration or level of the respective protein and/or mRNA thereof, preferably semi-quantitative or quantitative. Measurement of a protein biomarker can be performed directly or indirectly. Direct measurement refers to the amount or concentration measure of the biomarker, based on a signal obtained directly from the protein, and which is directly correlated with the number of protein molecules present in the sample. This signal - which can also be referred to as

intensity signal - can be obtained, for example, by measuring an intensity value of a chemical or physical property of the biomarker. Indirect measurements include the measurement obtained from a secondary component (e.g., a different component from the gene expression product) and a biological measurement system (e.g. the measurement of cellular responses, ligands, "tags" or enzymatic reaction products).

The term "amount", as used in the description refers but is not limited to the absolute or relative amount of proteins, and any other value or parameter associated with the same or which may result from these. Such values or parameters comprise intensity values of the signal obtained from either physical or chemical properties of the protein, obtained by direct measurement, for example, intensity values in an immunoassay, mass spectroscopy or a nuclear magnetic resonance. Additionally, these values or parameters include those obtained by indirect measurement, for example, any of the measurement systems described herein.

Methods of measuring mRNA in samples are known in the art. To measure mRNA levels, the cells in a test sample can be lysed, and the levels of mRNA in the lysates or in RNA purified or semi-purified from lysates can be measured by any variety of methods familiar to those in the art. Such methods include hybridization assays using detectably labeled DNA or RNA probes (i.e., Northern blotting) or quantitative or semi-quantitative RT-PCR methodologies using appropriate oligonucleotide primers.

Alternatively, quantitative or semi-quantitative in situ hybridization assays can be carried out using, for example, tissue sections, or unlysed cell suspensions, and detectably labeled (e.g., fluorescent, or enzyme-labeled) DNA or RNA probes. Additional methods for quantifying mRNA include RNA protection assay (RPA), cDNA and oligonucleotide microarrays, representation difference analysis (RDA), differential display, EST sequence analysis, and serial analysis of gene expression (SAGE).

The term "variant" refers to a protein substantially homologous to the protein involved in the sumoylation pathways as above defined. In general, a variant includes additions, deletions or substitutions of amino acids. The term "variant" also includes protein resulting from post-translational modifications such as, without limitation, glycosylation, phosphorylation or methylation. Examples of variants are disclosed at pp. 2 and 3. Nucleic acid encoding or translating such variants are included in the scope of protection of the invention, as well.

In the present invention, at least one biomarker as above defined may be detected in the same test sample in the in vitro methods of the invention, said biomarker being selected from the group of: Ubc9, SUMO1, SUMO2, SUMO3, SAE-1, SAE-2, SUMO E3 ligases, SUMO4, PIAS protein family and/or any other protein involved in the sumoylation pathway, preferably Ubc9, SUMO1, SUMO2, SUMO3, SAE-1 and/or SAE-2, more preferably Ubc9 and/or SUMO 2/3, even more preferably Ubc9.

E.g. Two biomarkers may be detected, said biomarkers being selected from the group of: Ubc9, SUMO1, SUMO2, SUMO3, SAE-1, SAE-2, SUMO E3 ligases, SUMO4, PIAS protein family and/or any other protein involved in the sumoylation pathway.

Any combination is suitable for the purpose of the invention, for instance detecting, e.g. Ubc9 and SUMO2, Ubc9 and SUMO1, or SUMO1 and SUMO2, etc.

In the present invention, three biomarkers may be detected in the same test sample, said biomarkers being selected from the group of: Ubc9, SUMO1, SUMO2, SUMO3, SAE-1, SAE-2, SUMO E3 ligases, SUMO4, PIAS protein family and/or any other protein involved in the sumoylation pathway. Any combination is suitable for the purpose of the invention, for instance measuring Ubc9, SUMO1 and SUMO2, or measuring Ubc9, SUMO2 and SUMO3, etc...

In the present invention, four biomarkers may be measured in the same test sample, said biomarkers being selected from the group of: Ubc9, SUMO1, SUMO2, SUMO3, SAE-1, SAE-2, SUMO E3 ligases, SUMO4, PIAS protein family or any other protein involved in the sumoylation pathway. Any combination is suitable for the purpose of the invention, for instance measuring Ubc9, SUMO1, SUMO2, and SUMO3, or measuring SUMO1, SUMO2, SUMO3, SAE-1, etc.

In the present invention, also many, i.e. five, six, seven, eight, etc biomarkers may be detected in the same test sample. Any combination is suitable for the purpose of the invention.

In the present invention, all biomarkers may be measured in the same sample, said biomarkers being selected from the group of: Ubc9, SUMO1, SUMO2, SUMO3, SAE-1, SAE-2, SUMO E3 ligases, SUMO4, PIAS protein family or any other protein involved in the sumoylation pathway.

The in vitro methods of the invention can also be used to follow the course of a HPV oncogenic infection and/or cervical cancer, or to follow the effect of a therapeutic treatment on HPV oncogenic infection and/or cervical cancer, thus e.g. monitoring the progression of an HPV oncogenic infection. In such case, the appropriate control

amount is quantified in the same subject before pharmacological treatment or at different time points.

The in vitro methods and kits of the invention can assess the risk, diagnose or prognose or monitor the progression of cancer caused by HPV, in particular by high-risk HPV, such as HPV16 and 18, as e.g. cervical cancer.

The present invention also refers to a method for prevention of cancer caused by HPV, preferably by high-risk HPV, as e.g. cervical cancer, comprising the identification or detection of HPV-related lesions, preferably CIN1 and/or CIN2/3, with at least one biomarker as above defined. Once the lesions have been identified they can be eradicated.

The biomarker as described in the present invention can be used to prevent cancer caused by HPV, preferably by high-risk HPV, as e.g. cervical cancer.

FIGURE LEGENDS

The invention will now be described by non-limiting examples referring to the following figures:

Figure 1. Scoring for Ubc9 and SUMO2. The graphs show the number of samples, expressed as percentage on total samples analyzed, that scored positive or negative for either Ubc9 or SUMO2. Some of them were not assessable (indicated as NA in figure). Specifically, 18 Formalin-Fixed, Paraffin-Embedded (FFPE) samples from Condyloma (panel A), 32 from CIN1 patients, 35 from CIN2 and CIN3 patients (panel B) were scored for Ubc9 positivity, based upon the intensity of the colouring and the percentage of cells staining positive, giving a score from basal/negative to positive (+) or very positive (++). Basal indicates a basal colouring for the antibody (normal tissue staining).

Figure 2. Ubc9 expression in CIN1 (Low Grade SIL).

Immunohistochemical analysis of Ubc9 protein expression in a cervical tissue of low grade SIL (LSIL or CIN1) from the study cases. A qualitative interpretation of the immunostaining pattern was used. (Ubc9, X 10)

Figure 3. Ubc9 expression in CIN1 (Low Grade SIL) vs CIN2/CIN3 (High Grade SIL).

Immunohistochemical analysis of Ubc9 protein expression in cervical tissues of cases from the study, Low grade (LSIL) vs High Grade (HSIL). A qualitative interpretation of the immunostaining pattern was used. (Ubc9, X 5)

Figure 4. SUMO2 and Ubc9 expression in CIN3 (High Grade SIL).

Immunohistochemical analysis of SUMO2 (panel A) and Ubc9 (panel B) protein expression in cervical tissues of High Grade SIL of cases from the study. A qualitative interpretation of the immunostaining pattern was used. (Ubc9 and SUMO2, X 10).

Figure 5. Analysis of Ubc9 expression in HPV16-transduced Human Keratinocytes (HKs).

(A) Analysis of Ubc9 expression in HPV16-transduced Human Keratinocytes (HKs). Left: Protein extracts from primary keratinocytes transduced with empty retrovirus vector or recombinant retroviruses, as indicated, were analyzed by immunoblotting using anti-Ubc9 antibody. Right: The intensities of the protein bands in twelve independent experiments were quantified using ImageJ software, and standardized to the GAPDH levels. Data are expressed as fold over the empty-transduced HKs. Bars represent means $\pm$ SEM of $n = 12$ biological replicates. * $P = 0.002$; ** $P < 0.005$ (Two-tailed Student's t test vs empty).

(B) Real-time detection of Ubc9 transcript in HPV-infected HKs. Total RNA was extracted from primary keratinocytes transduced with empty retrovirus vector or HPV16 E6E7 proteins, and Ubc9 or GAPDH mRNA levels were measured by quantitative RT-PCR. The expression of UBC9 mRNA was quantified by the comparative DDCT method. mRNA relative quantities are expressed as fold over the empty vector-transduced HKs. Bars represent means $\pm$ SEM of $n = 12$ biological replicates.

Figure 6. SUMO-1 in HPV16-transduced primary Human Keratinocytes (HKs).

(A) Analysis of overall SUMO-1 conjugation in HPV16-transduced HKs. Left: Protein extracts of primary keratinocytes transduced with empty retrovirus vector or recombinant retroviruses, as indicated, were analyzed by immunoblotting using anti-SUMO-1 antibody. Right: The intensities of the protein smears in twelve independent experiments were quantified using ImageJ software, and standardized to the GAPDH levels. Data are expressed as fold over the empty-transduced HKs. Bars represent means $\pm$ SEM of $n = 12$ biological replicates. * $P = 0.01$; (Two-tailed Student's t test vs empty).

(B) Real-time detection of SUMO-1 transcript in HPV-infected HKs. Total RNA was extracted from primary keratinocytes transduced with empty retrovirus vector or HPV16 E6E7 proteins, and SUMO-1 or GAPDH mRNA levels were measured by

quantitative RT-PCR. The expression of SUMO-1 mRNA was quantified by the comparative DDCT method. mRNA relative quantities are expressed as fold over the empty vector-transduced HKs. Bars represent means $\pm$ SEM of $n = 12$ biological replicates.

5 **Figure 7. SUMO-2/3 in HPV16-transduced primary Human Keratinocytes (HKs).**

A) Analysis of overall SUMO-2/3 conjugation in HPV16-transduced HKs. Left: Protein extracts of primary keratinocytes transduced with empty retrovirus vector or recombinant retroviruses, as indicated, were analyzed by immunoblotting using anti-SUMO-2/3 antibody. Right: The intensities of the protein smears in twelve independent experiments were quantified using ImageJ software, and standardized to the GAPDH levels. Data are expressed as fold over the empty-transduced HKs. Bars represent means $\pm$ SEM of $n = 12$ biological replicates.

B) Real-time detection of SUMO-2/3 transcript in HPV-infected HKs. Total RNA was extracted from primary keratinocytes transduced with empty retrovirus vector or E6E7 HPV16 proteins, and SUMO-2/3 or GAPDH mRNA levels were measured by quantitative RT-PCR. The expression of SUMO-2/3 mRNA was quantified by the comparative DDCT method. mRNA relative quantities are expressed as fold over the empty vector-transduced HKs. Bars represent means $\pm$ SEM of $n = 12$ biological replicates.

Figure 8. SAE1 expression in HPV16-transduced Human Keratinocytes (HKs).

A) Analysis of SAE1 expression in HPV16-transduced Human Keratinocytes (HKs). Left: Protein extracts from primary keratinocytes transduced with empty retrovirus vector or recombinant retroviruses, as indicated, were analyzed by immunoblotting using anti-SAE1 antibody. Right: The intensities of the protein bands in six independent experiments were quantified using ImageJ software, and standardized to the GAPDH levels. Data are expressed as fold over the empty-transduced HKs. Bars represent means $\pm$ SEM of $n = 6$ biological replicates. $*P < 0.05$ (Two-tailed Student's t test vs empty).

B) Real-time detection of SAE1 transcript in HPV-infected HKs. Total RNA was extracted from primary keratinocytes transduced with empty retrovirus vector or HPV16 E6E7 proteins, and SAE1 or GAPDH mRNA levels were measured by quantitative RT-PCR. The expression of SAE1 mRNA was quantified by the comparative DDCT method. mRNA relative quantities are expressed as fold over the

empty vector-transduced HKs. Bars represent means $\pm$ SEM of $n = 6$ biological replicates. $*P = 0.005$ (Two-tailed Student's t test vs empty).

Figure 9. PIASy expression in HPV16-transduced Human Keratinocytes (HKs).

Analysis of PIASy expression in HPV16-transduced Human Keratinocytes (HKs).

- 5 Left: Protein extracts from primary keratinocytes transduced with empty retrovirus vector or recombinant retroviruses, as indicated, were analyzed by immunoblotting using anti-PIASy antibody. Right: The intensities of the protein bands in eight independent experiments were quantified using ImageJ software, and standardized to the GAPDH levels. Data are expressed as fold over the empty-transduced HKs. Bars
10 represent means $\pm$ SEM of $n = 8$ biological replicates. $*P = 0.001$ (Two-tailed Student's t test vs empty).

Figure 10. Ubc9 vs p16 expression in CIN1 (Low Grade SIL).

Immunohistochemical analysis of Ubc9 (panel B) and p16 (panel A) protein expression in a cervical tissue of low grade SIL (LSIL or CIN1) from the study cases.

- 15 A qualitative interpretation of the immunostaining pattern was used. (Ubc9, p16 X 10)

Figures 11, 12 and 13. Ubc9 vs p16 expression in CIN1 (Low Grade SIL) and CIN2 (High Grade SIL).

Immunohistochemical analysis of Ubc9 (figure 11, panel B and figure 13) and p16 (figure 11, panel A and figure 12) protein expression in cervical tissues of both low
20 grade SIL (LSIL or CIN1) from the study cases (figure 11) and high grade SIL (HSIL or CIN2) (figures 12 and 13). A qualitative interpretation of the immunostaining pattern was used. (Figure 11: Ubc9, p16 X 10; Figures 12 and 13: Ubc9, p16 X 20).

Figure 14.

Cytological analysis of Ubc9 in a LSIL and a HSIL lesion (Figure 40X).

25

EXAMPLES

MATERIALS and METHODS

Cell cultures

- 30 Adult human epidermal keratinocytes were isolated from skin biopsies and grown in Keratinocyte Serum-Free Medium (KSFM, Gibco) as described previously [15]. Briefly, human keratinocytes were isolated from skin by Dispase 10U/ml (Gibco) digestion for 16 hours at 4°C to remove the epidermis, followed by a trypsinization (Trypsin 500mg/l, Versene, 30 minutes at 37°C) step, used to obtain the isolated cells.

Primary cultures were then established in KSFM containing bovine pituitary extract (BPE, 30 micrograms/ml, Gibco) and epidermal growth factor (EGF, 0.2 ng/ml, Gibco). Cells were used from passage 2 to 5 for the retroviral transduction.

Retroviral transduction

- 5 The plasmids pLXSN, pLXSN-HPV16E6, pLXSN-HPV16E7, and pLXSN-HPV16E6E7 were kindly provided by M. Tommasino (IARC, Lyon, France). Retroviral supernatants generated by transfection of the pLXSN constructs into Phoenix amphi cells were used to transduce primary human keratinocytes, as previously described[16]. Twenty-four hours after transduction, keratinocytes were
10 selected with 0.1 micrograms/ml G418 (Gibco) for 1 week.

RNA extraction, reverse transcription and qRT-PCR

- Total RNA from G418-selected keratinocytes was extracted with the RNeasy mini kit (Qiagen), following the manufacturer's instruction. Residual contaminating DNA was removed with the RNase-free DNase set (Qiagen). RNA was reversely transcribed
15 using the ImProm-II system for RT-PCR (Invitrogen). Real-time quantitative RT-qPCR for UBC9, SUMO-1 and SUMO-2/3 was performed with the ABI Prism 7900 Sequence Detection System, using the Fast SYBR Green Master Mix (Applied Biosystems) and primers 5'-ACAGTGTGCCTGTCCATCTTAG-3' (forward) (SEQ ID No.1) and 5'-TGTTTTGGCAGTAAATCGTGTA-3' (reverse) (SEQ ID No.2),
20 5'-TGTCAAAGACAGGGTGTTCCTGATAAAC-3' (forward) (SEQ ID No.3) and 5'-CCCCCGGTTTGTTCCTGATAAAC-3' (reverse) (SEQ ID No.4), and 5'-TGCCCTACATGTAACAGCTACG-3' (forward) (SEQ ID No.5) and 5'-CCTTTCTCCATTAGTGGCATG-3' (reverse) (SEQ ID No.6), respectively. Triplicates were set up for each sample, and the expression of UBC9 mRNA was
25 quantitated by the comparative DDC_i method [17]. GAPDH (primers sequences: 5'-GCCTCAAGATCATCAGCAATG-3' forward (SEQ ID No.7), and 5'-CCACGGATACCAAAGTTGTCAT-3' (SEQ ID No.8)) was used for C_t values normalization.

Western blotting

- 30 For Ubc9 detection, G418-selected keratinocytes were lysed with E1A Buffer (50mM Hepes, 250mM NaCl, 0.1% NP-40 and cocktail of protease inhibitors, Sigma Aldrich). Total proteins (30 micrograms) were resolved by 16% SDS-PAGE under reducing conditions, and electroblotted with antibodies raised against Ubc9 (Abcam, no. 21193, 1:5000 dilution), SUMO-1 (Sigma Aldrich, S8070, 1:3000 dilution),

SUMO-2/3 (Abcam, AB22654, 1:5000 dilution), SAE1 (Abcam, no. 5645, 1:1000 dilution, PIASy (Santa Cruz, sc30876, 1:1000 dilution) or GAPDH (Abcam, 1:5000 dilution). Membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized by chemiluminescent reaction using ECL Western Blotting Detection System kit (GE Healthcare) and BIOMAX-MR film (Eastman Kodak). The intensities of the protein bands were quantified using ImageJ software (Rasband W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2011), and standardized to the GAPDH levels.

10 **Statistical analysis**

Statistical significance was assessed by Student's *t* test of paired sample, using the SPSS software (SPSS for Windows, Rel. 17.0 Chicago: SPSS Inc). Values of $P < 0.05$ were considered significant.

Immunohistochemistry analysis

15 For immunohistochemistry, cervical samples were formalin-fixed and paraffin-embedded according to the established procedures. All sections were counterstained with Mayer's haematoxylin and visualized using a bright field microscope. Images were generated with a Leica DFC280 Microscope.

The following primary antibodies were used: anti-Ubc9 (Santa Cruz, SC10759) (Antigen retrieval at 99 C for 40 minutes in water bath, EDTA buffer pH 8.0, cool down to R.T. for 20 minutes), 1:1000, incubation O/N at 4 C; anti-SUMO2/3 (Abcam, AB22654) (Antigen retrieval at 99 C for 40 minutes in water bath, Sodium Citrate buffer pH 6.0, cool down to R.T. for 20 minutes), 1:1000, incubation O/N at 4 C. The Envision kit from DAKO was utilized and after the chromogenic visualization step using the 3,3'- Diaminobenzidine (DAB) chromogen, slides were counterstained with hematoxylin and coverslipped. For p16 immunostaining, the CINTec p16-INK4A Cytology kit (Dako Cytomation, now Roche mtm laboratories AG, Heidelberg, Germany) was used, which applies a p16-specific monoclonal antibody (clone E6H4).

Cytology

30 The following primary antibodies were used: anti-Ubc9 (Santa Cruz, SC10759) (Antigen retrieval at 99 C for 15 minutes in water bath, EDTA buffer pH 8.0, cool down to R.T. for 20 minutes), 1:3200, incubation O/N at 4 C. The DAKO kit (LSAB+ System-HRP) was utilized and after the chromogenic visualization step, slides were counterstained with hematoxylin and coverslipped.

Concordance Analysis

Slides from 24 cervical biopsies were stained with Ubc9 antibody and p16 antibody. Original pathological diagnosis (by hematoxylin-eosin, H-E) of the biopsies were: condyloma 4 cases; CIN1 8 cases; CIN2 6 cases; and CIN3 6 cases. Three
5 independent pathologists (Operators 1-3) reviewed the slides blinded at the original diagnosis; the diagnostic concordance was analyzed overall on all diagnostic categories (negative; CIN1, including condyloma; CIN2; CIN3), or grouping the diagnosis into three categories (Neg: negative; CIN1, including condyloma; CIN2+: CIN2 and CIN3) or into two categories (CIN2-: less than CIN2; CIN2+: CIN2 or
10 CIN3). The results were plotted into tables (Tab. 1-5). The concordance is expressed as percentage of exact agreement.

RESULTS

Immunohistochemistry analysis of cervical intraepithelial (CIN) neoplasia.

15 The authors assessed by immunohistochemistry (IHC) analysis whether there was a differential expression of E1 (SAE1/SAE2), E2 (Ubc9), SUMO1 and SUMO2/3 in different grades of cervical intraepithelial (CIN) neoplasia (also referred to as HSILs (High grade Squamous Intrapethelial Lesions) or LSILs (Low grade Squamous
20 Intraepithelial Lesions) from HPV positive patients. Typically CIN1 refers to LSILs, whereas CIN2 and 3 to HSILs. Specifically, the authors analyzed low grade SILs, high grade SILs, and condyloma. All high grade lesions showed a highly statistically significant increase of SUMO2 proteins and Ubc9 enzymes as compared to normal tissue (Figure 1 and Figures 3 and 4).

Both CIN1 and condyloma lesions scored positive for both Ubc9 and SUMO2.

25

HPV16 E6 and E7 oncoproteins affect SUMO pathway components.

If the observed effects are an authentic function of HPV 16 E6 and /or E7 oncoproteins, one should then observe similar alterations of SUMO pathway components during viral replication. Human primary keratinocytes were thus infected
30 with constructs expressing either HPV 16 E6, HPV 16 E7, and both, and mRNA and protein expression for Ubc9, SUMO1 and SUMO2/3 were assessed (figures 5-7). The present data are indicating that upon oncoprotein expression there is a statistically significant increase of protein levels of Ubc9 (Figure 5A), relevant even in the presence of E6 only. These data support and validate the IHC analysis, underscoring

also the novelty that Ubc9 can be a potential marker for HPV infection. Unlike p16INK4a, it is a E6 target. Furthermore, both SUMO1 and SUMO2/3 are affected by expression of the oncoproteins (figures 6 and 7), although SUMO1 to a greater extent (figure 6). Different results were also obtained with other proteins involved in the sumoylation pathway (figures 8 and 9). Interestingly, the SUMO E3 ligase PIASy (fig9), seems to be downregulated, in agreement with recent findings that SUMO E3 ligases play an important role in paralog-specific sumoylation.

Ubc9 is different from p16INK4a.

p16INK4a is an established HPV surrogate biomarker. In fact, there is a p16 test (CINtec p16 kits from MTM/ROCHE) used to correlate p16INK4a overexpression with oncogenic transformation of the cervical mucosa [18, 19]. p16INK4a overexpression is a result of inactivation of the cell-cycle regulatory retinoblastoma protein (pRb) by High Risk HPV E7.

The authors thus chose 24 cervix uteri biopsies with lesions ranging from condiloma acuminatum to CIN 3 (high grade SIL) and whose diagnosis was clearly and independently evaluated by an expert gynecopathologist (CC), through Haematoxylin Eosin (H-E) staining. Three different skilled pathologists (referred to as Operator 1, 2 and 3) gave their diagnosis by only reading both Ubc9 and p16INK4a staining (Tables 1-5). Clearly, Ubc9 reading was more precise than p16 in interpreting high grade SILs (CIN2+). Ubc9 is indeed more sensitive in detecting HSILs than p16. Ubc9 always scores positive for HSILs, while p16 sometimes gives false negative. Ubc9 can highlight disease gravity since it follows the lesion, i.e. follows the virus. Furthermore, Table 7 shows that in low grade SILs Ubc9 can detect the lesion where p16 fails.

Same results were obtained with the other proteins involved in the sumoylation pathway.

Table 1

Operator	All categories	Neg/CIN1/CIN2+	CIN2-/CIN2+
Ope 1	0.54	0.71	0.71
Ope 2	0.67	0.83	0.88
Ope 3	0.54	0.75	0.79

Table 2

Operator	All categories	Neg/CIN1/CIN2+	CIN2-/CIN2+
Ope 1	0.33	0.42	0.75
Ope 2	0.33	0.38	0.63
Ope 3	0.33	0.46	0.75

Tables 1-2. Concordance with histological diagnosis (hematoxylin and eosin) of three different pathologists (Ope 1-3) after Ubc9 (Table 1) and p16 (Table 2)

staining. Results are expressed for all diagnosis categories; subgrouped into three categories, namely negative, CIN1, CIN2+ (CIN2 or CIN3); and subgrouped into two categories CIN2- (less than CIN2) and CIN2+.

The data show an increase in concordance after Ubc9 staining, more evident than after p16 staining.

Table 3

Operator	All categories	Neg/CIN1/CIN2+	CIN2-/CIN2+
Ope 1 vs Ope 2	0.50	0.79	0.83
Ope 1 vs Ope 3	0.46	0.63	0.67
Ope 2 vs Ope 3	0.58	0.75	0.83

Table 4

Operator	All categories	Neg/CIN1/CIN2+	CIN2-/CIN2+
Ope 1 vs Ope 2	0.75	0.75	0.75
Ope 1 vs Ope 3	0.71	0.75	0.83
Ope 2 vs Ope 3	0.79	0.83	0.88

Tables 3-4. Reproducibility of Ubc9 and of p16 scorings .

Table 3. Data show the concordance of Ubc9 scorings. Results are expressed for all diagnosis categories; subgrouped into three categories, namely negative, CIN1, CIN2+; and subgrouped into two categories CIN2- and CIN2+. The data show better concordance grouping in CIN2- and CIN2+.

Table 4. Data show the concordance of p16 scorings. Results are expressed for all diagnosis categories, subgrouped as in Table 3.

Table 5: Concordance of Ubc9 vs p16 stainings. The data show that the two markers are different.

Operator			
Ope 1	0.29		
Ope 2	0.33		
Ope 3	0.42		

Table 6. CIN2 or worse (p16 vs Ubc9)

		Ubc9		
	CIN	2	3	Total
p16	2	22	0	22
	3	4	28	32
Total		26	28	54

Table 7. CIN1 (p16 vs Ubc9)

		Ubc9		
	CIN	1	2	Total
p16	Neg or -/+	7	0	7
	1	2	0	2
	2	0	2	2
	Total	9	2	11

5 **Tables 6-7: Data are based on Ubc9 vs p16 reading on 65 patients.**

54 patients CIN2 or worse (Table 6) and 11 CIN1 (Table 7) were immunostained with both p16 and Ubc9. The data show again that the two markers are different, that they both stain CIN2 or worse (Table 6) with high concordance. In four patients classified as CIN2 by H/E, Ubc9 staining marked the lesions as CIN2 (indicated as 2 in table), whereas p16 as CIN3 (3 in table) (Table 6). However seven CIN1 patients (classified by H/E) were clearly negative for p16 (Neg or -/+) but positive for Ubc9 marking the lesion as CIN1 (1 in table) (Table 7), indicating that Ubc9 staining can detect CIN1 lesions in instances where p16 staining fails to do so.

Overall, the present data are indicating that proteins involved in the sumoylation pathway, in particular Ubc9, are excellent markers for condyloma, CIN1 and CIN2/3 lesions. Ubc9 is indeed regulated both by E6 and E7. Although Ubc9 is known to be upregulated in cancer [20, 21], this regulation via E6 and E7 in a model that resembles the in vivo situation is not yet described. This finding is underscored by the present data reported in the cervical tissues samples, in which there is a clear increase in expression of the proteins involved in the sumoylation pathway, in particular Ubc9 enzyme, when comparing a LSIL vs a HSIL sample. This is a striking result because although viruses are notorious for their effect on SUMO pathway components [9], such a direct relationship between oncoprotein expression and enzyme activation in HPV related cancer lesions is very remarkable.

Ubc9 is a different marker, when compared to p16INK4a which is only upregulated by E7 oncoprotein expression, making it a novel diagnostic tool.

The precise diagnosis of LSILs and HSILs is the most important and determinant key

to cervical cancer prevention. Today's strategy implies both the identification and the eradication of CIN2/3 to prevent invasive cancer and to monitor LSILs towards clearance or possible progression to HSILs. The trigger to treatment is the histopathological diagnosis of CIN2/3, which the present data are indicating as very sensitive to proteins involved in the sumoylation pathway, in particular Ubc9 staining. Furthermore, Ubc9 can also detect CIN1, thus making it a valuable marker to avoid overdiagnosis and therefore over-treatments.

Cervical cancer prevention is a world-wide problem, especially increasing in developing countries. To increase the screening power, pathologists must be trained to spot out CIN2/3 diagnosis, with no doubt. Thus, proteins involved in the sumoylation pathway, in particular Ubc9, staining could be very helpful and there is clearly a market potential for this wide market. In conclusion, proteins involved in the sumoylation pathway, in particular Ubc9, could be exploited for this cancer prevention process.

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- 5

CLAIMS

1. A biomarker belonging to the group of at least one:
 - a) protein involved in the sumoylation pathway;
 - 5 b) a variant thereof;
 - c) mRNA encoding a) or b),for use in assessing the risk and/or diagnosing and/or prognosing and/or monitoring the progression of an HPV infection.
- 10 2. The biomarker for use according to claim 1 wherein the HPV infection is an high-risk HPV infection.
3. The biomarker for use according to claim 2 wherein the high-risk HPV infection is causative of cervical cancer.
- 15 4. The biomarker for use according to claim 1 wherein the HPV infection is a low-risk HPV infection.
5. The biomarker for use according to claim 4 wherein the low-risk HPV infection is causative of a condyloma.
- 20 6. The biomarker for use according to any of previous claims belonging to the group of: Ubc9, SUMO1, SUMO2/3, SAE-1, SAE-2, SUMO E3 ligases, SUMO4 and/or PIAS protein family.
- 25 7. The biomarker according to claim 6, being Ubc9, SUMO1 and/or SUMO2/3.
8. The biomarker according to claim 7, being Ubc9.
- 30 9. An in vitro method for assessing the risk and/or for diagnosing and/or for prognosing and/or for monitoring the progression of an HPV infection comprising the steps of:
 - a) detecting the biomarker as defined in any one of claims 1-8 in a test sample isolated from a subject and

b) comparing with respect to an appropriate control.

10. An in vitro method for monitoring the progression of an HPV infection according to claim 9, comprising the steps of:

- 5 a) measuring the alteration of the amount of said biomarker in said test sample and
b) comparing the measured amount of step a) with an appropriate control amount.

11. An in vitro method for assessing the risk and/or diagnosing and/or prognosing an HPV infection according to claim 9, comprising the steps of:

- 10 a) measuring the amount of said biomarker in said test sample and
b) comparing the measured amount of step a) with an appropriate control amount,
wherein an amount of said biomarker in the test sample higher than the control
amount indicates that the subject is either at increased risk for developing or is
affected by an HPV infection.

15

12. The in vitro method according to any one of claims 9-11 wherein the HPV infection is an high-risk HPV infection.

13. The in vitro method according f to claim 12 wherein the high-risk HPV infection
20 is causative of cervical cancer.

14. The in vitro method according to any one of claims 9-11 wherein the HPV infection is a low-risk HPV infection.

25 15. The in vitro method according f to claim 14 wherein the low-risk HPV infection is causative of condyloma.

16. An in vitro method according to any one of claims 9-15, wherein the detection of the biomarker is correlated with the presence of HPV-related lesions.

30

17. The in vitro method according to claim 16, wherein the HPV-related lesions are condyloma, Low grade Squamous Intraepithelial Lesions (LSILs) or High grade Squamous Intraepithelial Lesions (HSILs).

18. The in vitro method according to any one of claims 9-17, further comprising the measure of the amount of at least one further marker of HPV oncogenic infection and the comparison with an appropriate control amount thereof.

5 19. The in vitro method according to claim 18, wherein the further marker of HPV oncogenic infection is p16 (p16INK4a).

20. The in vitro method according to any one of claims 9-19 wherein the test sample isolated from the subject is a biological fluid, a cell sample or a tissue sample.

10

21. A kit for assessing the risk and/or diagnosing and/or prognosing and/or monitoring the progression of an HPV infection, comprising:

- means to detect and/or measure the amount and/or measure the alteration of the amount of at least one biomarker as defined in any one of claims 1-8 and, optionally

15 - control means.

22. The kit according to claim 21 wherein the means to detect and/or measure the amount and/or measure the alteration of the amount of said biomarker are at least one antibody, functional analogous or derivatives thereof.

20

23. A kit for assessing the risk and/or diagnosing and/or prognosing and/or monitoring the progression of an HPV infection, comprising:

- means to detect and/or measure the amount and/or measure the alteration of the amount of at least one mRNA encoding the biomarker as defined in any one of claims

25 1-8 and optionally

- control means.

30

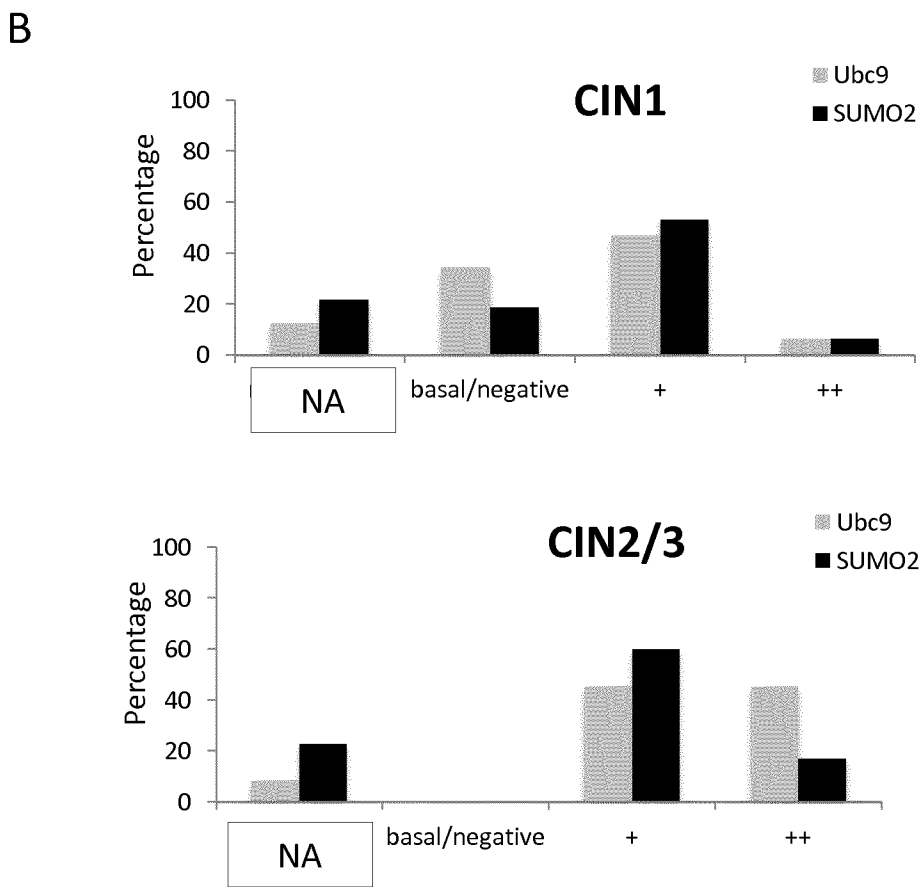
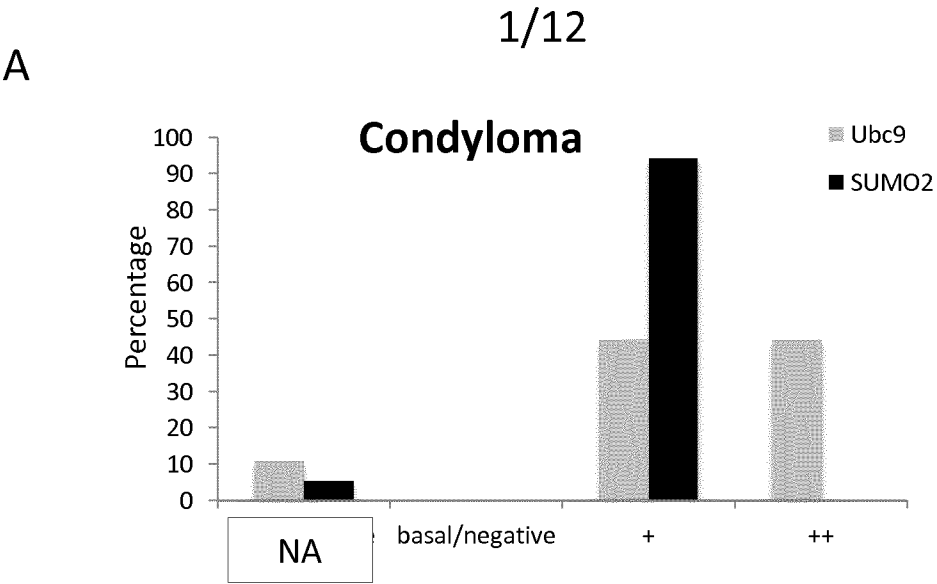


Fig. 1

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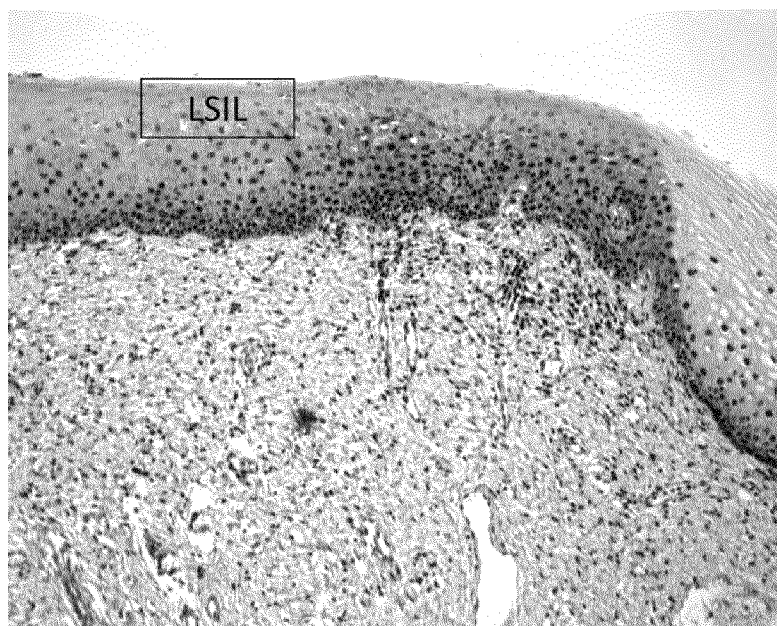


Fig. 2

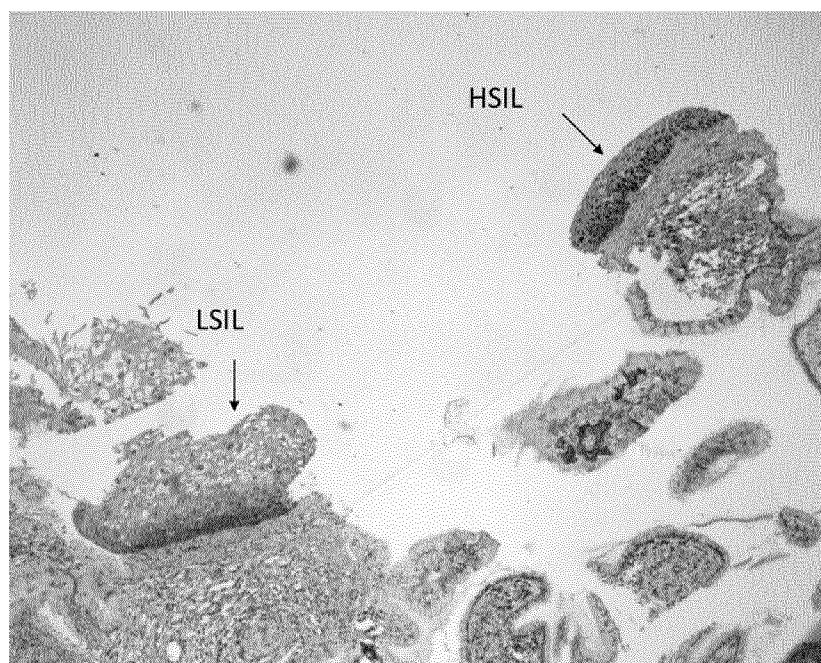
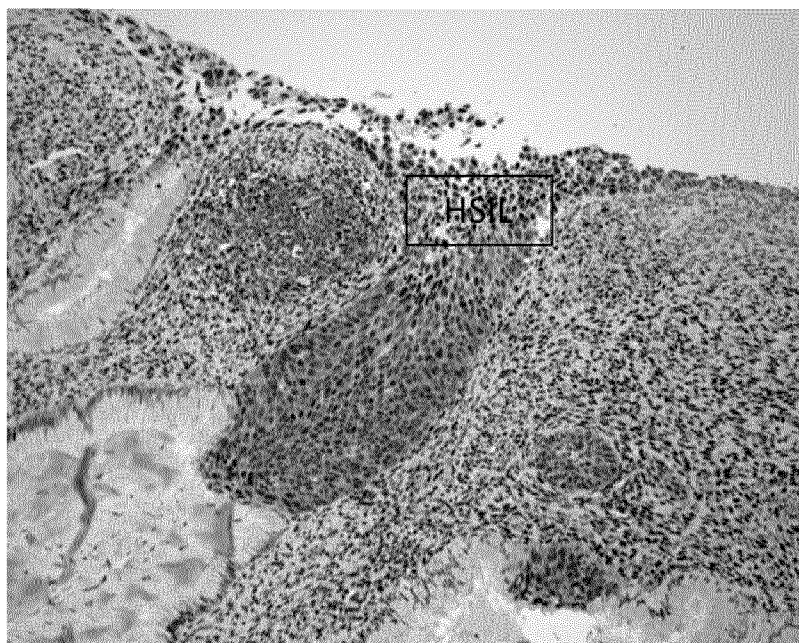


Fig. 3

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A



B

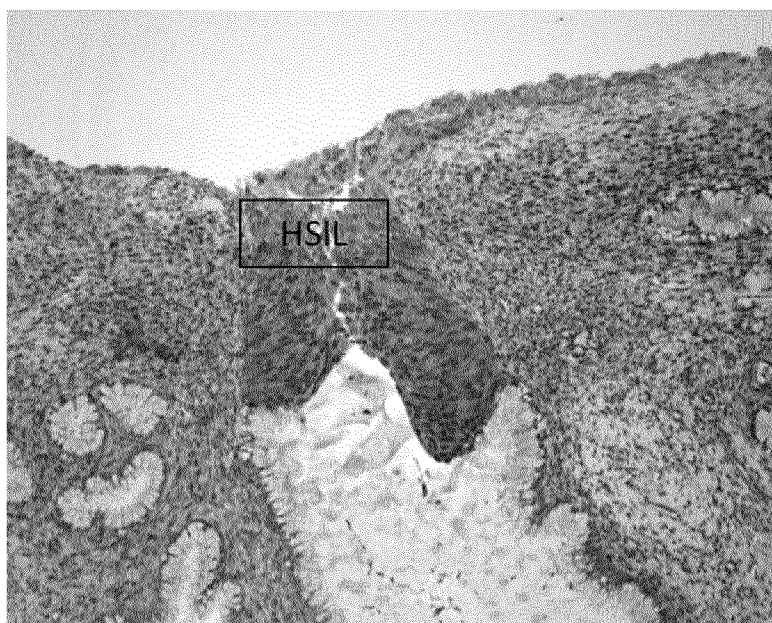
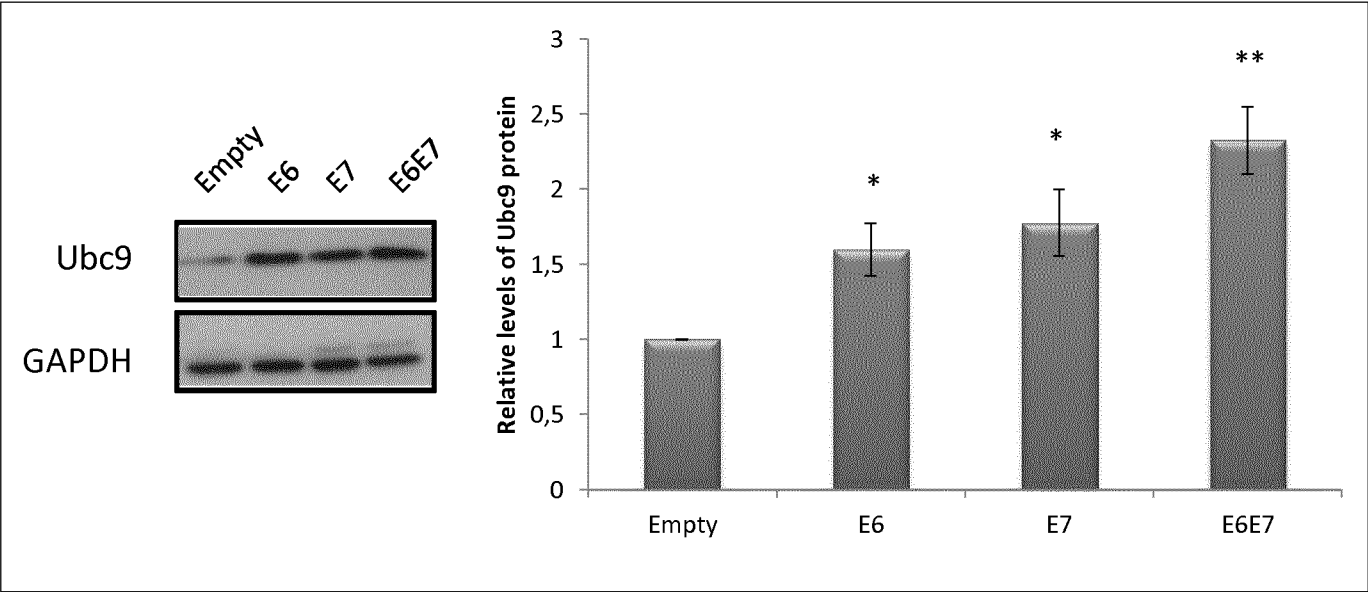


Fig. 4

A



B

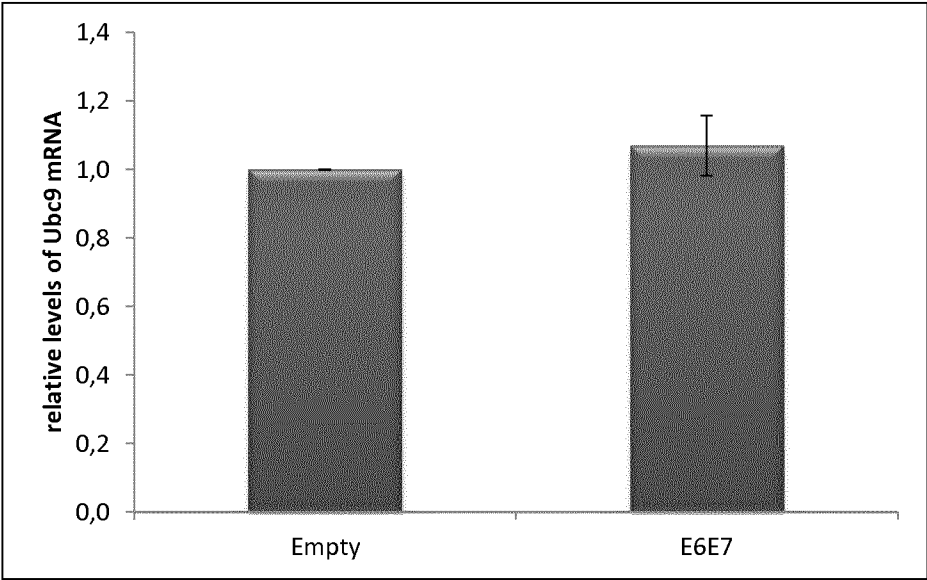
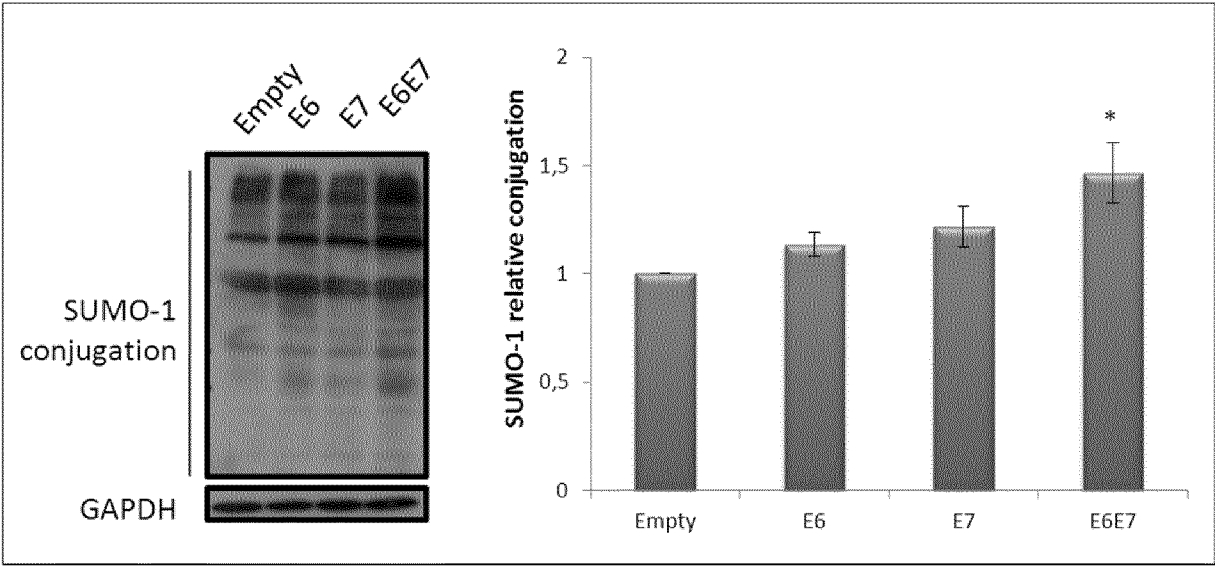


Fig. 5

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A



B

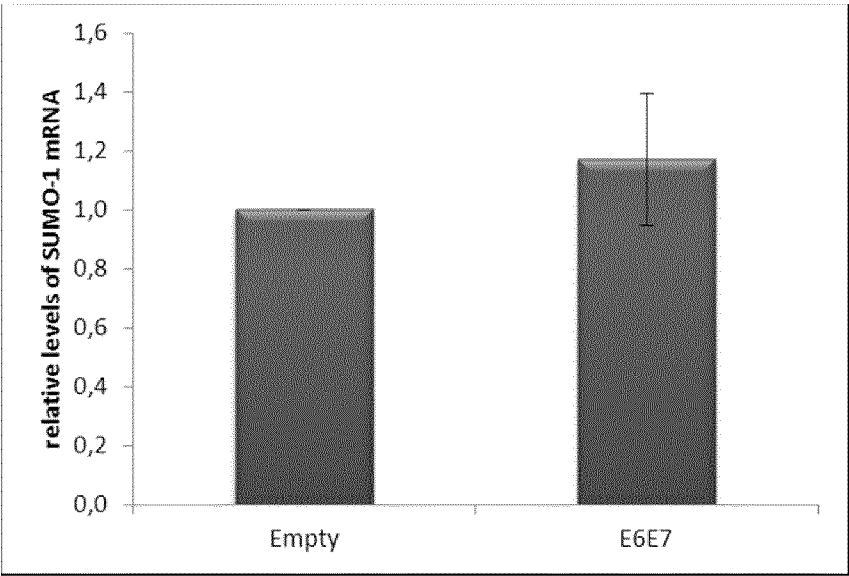


Fig. 6

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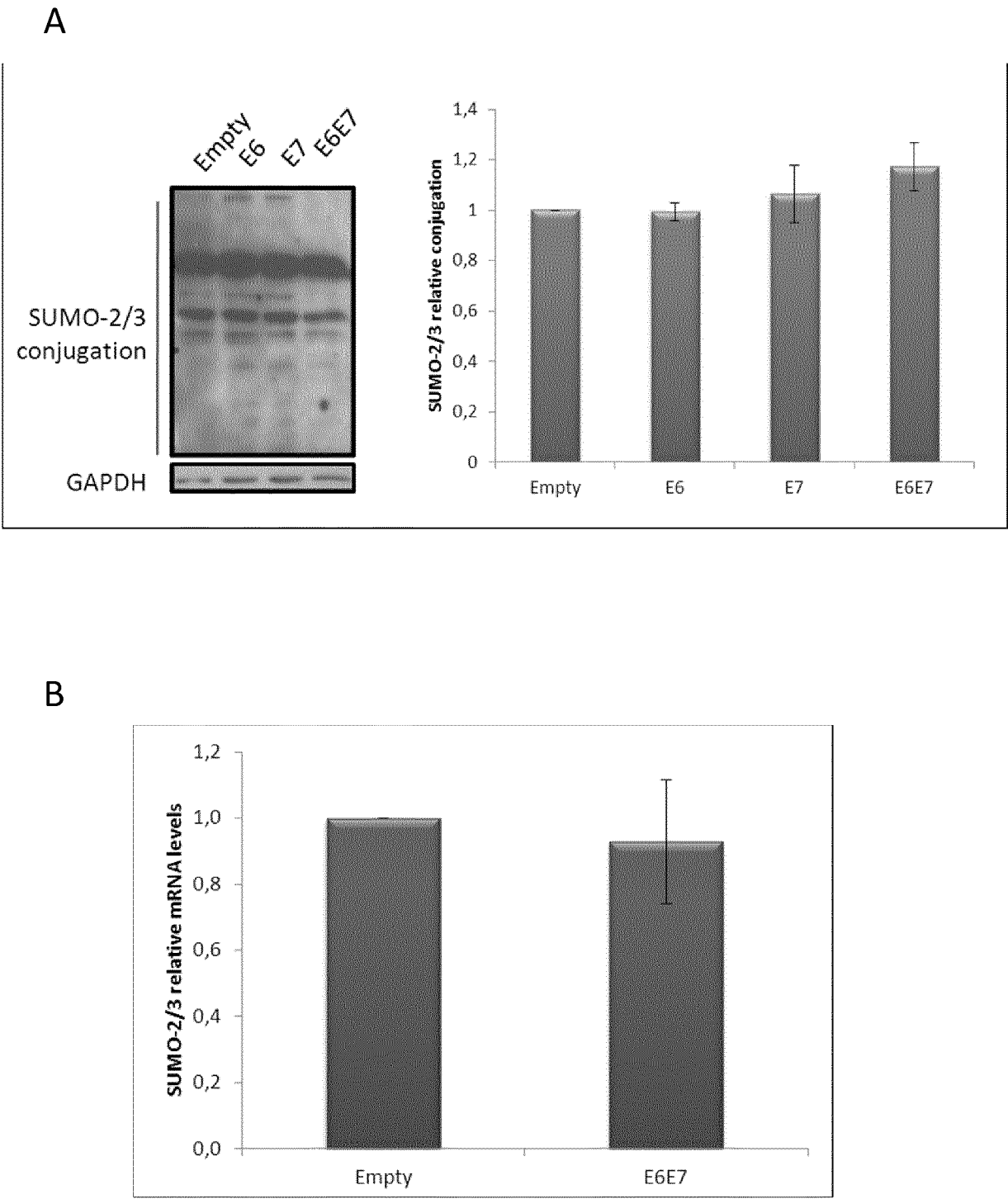
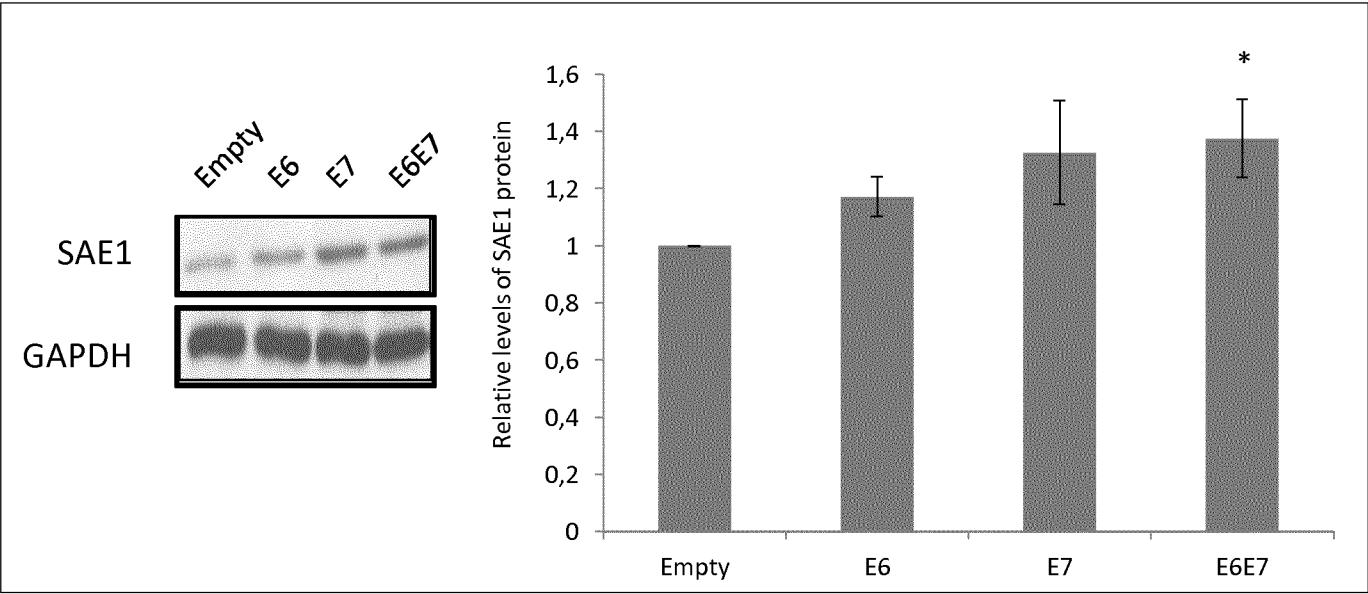


Fig. 7

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A



B

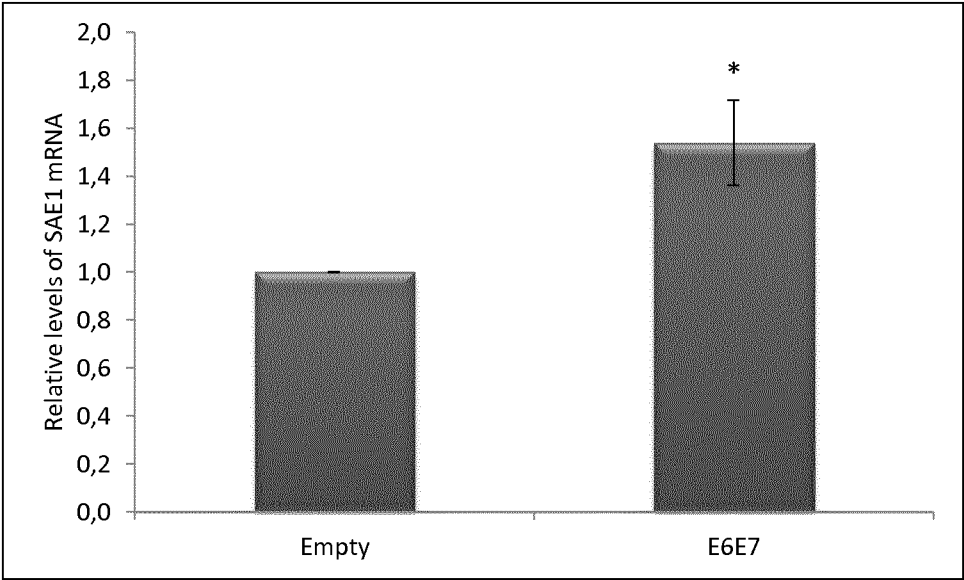


Fig. 8

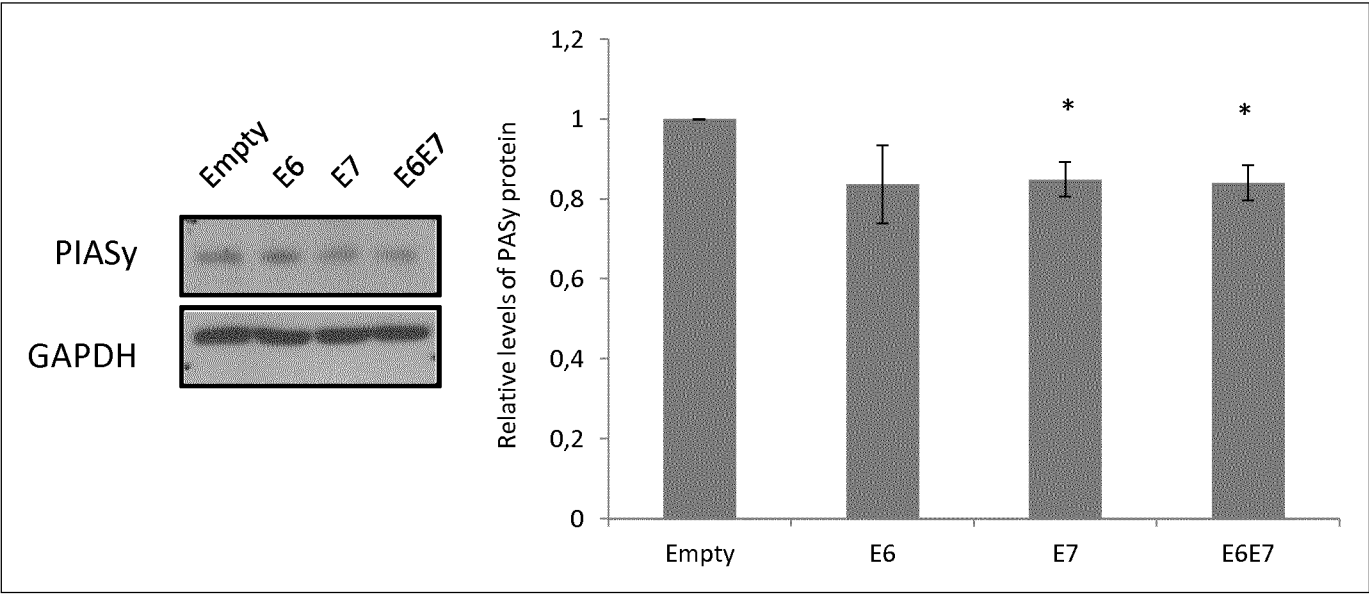
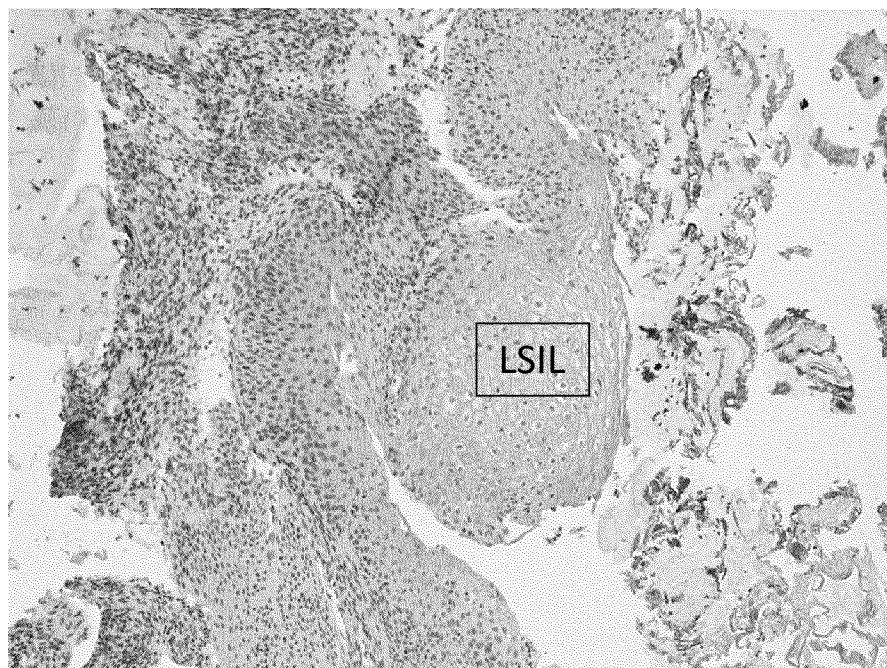


Fig. 9

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A



B

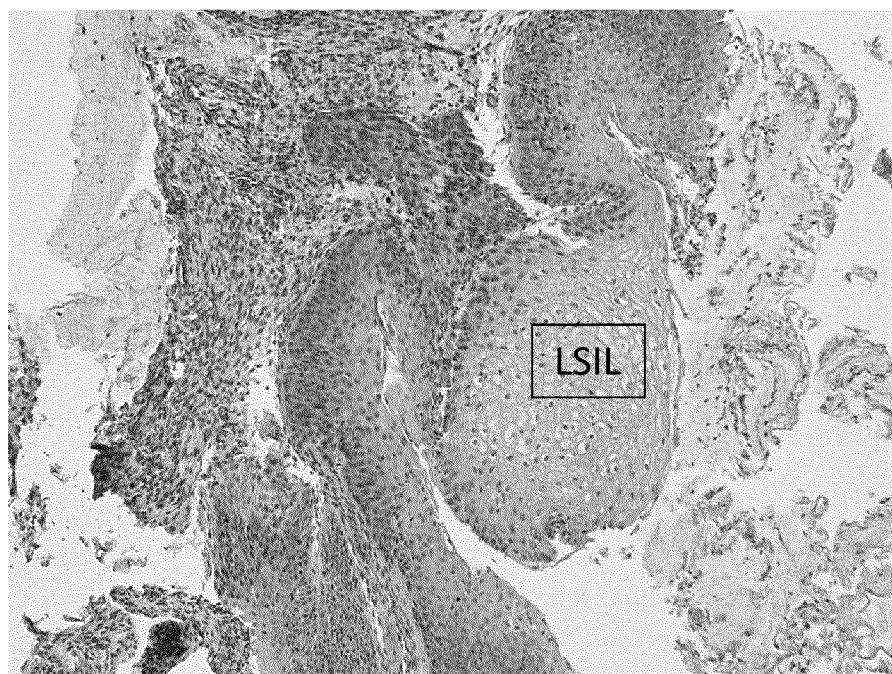
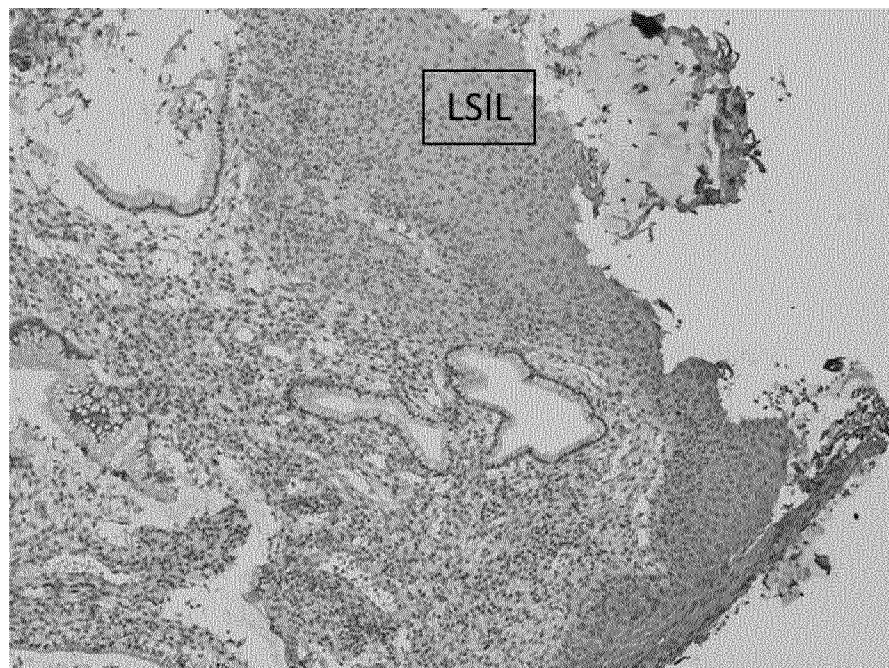


Fig. 10

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A



B

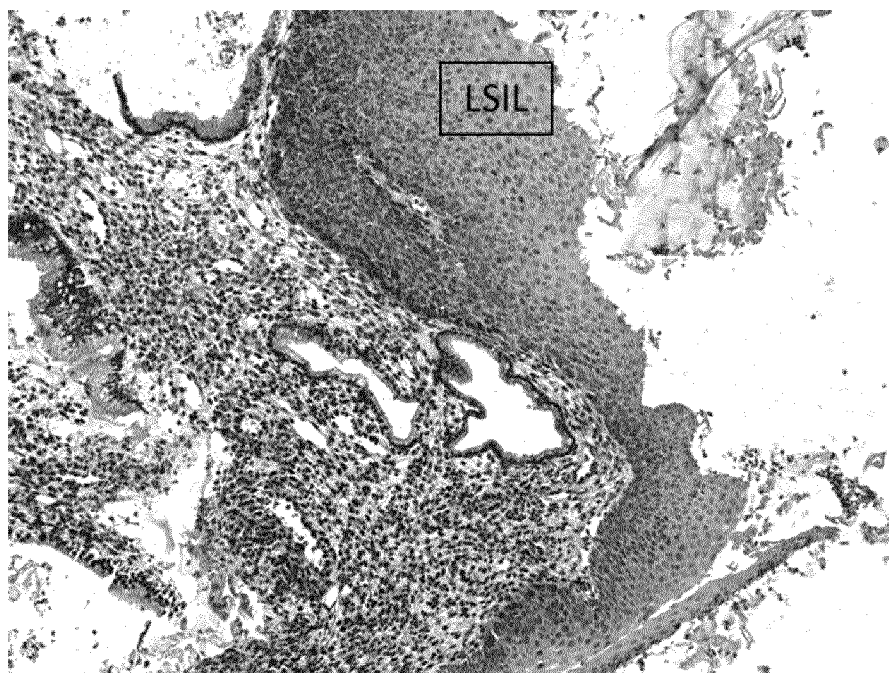


Fig. 11

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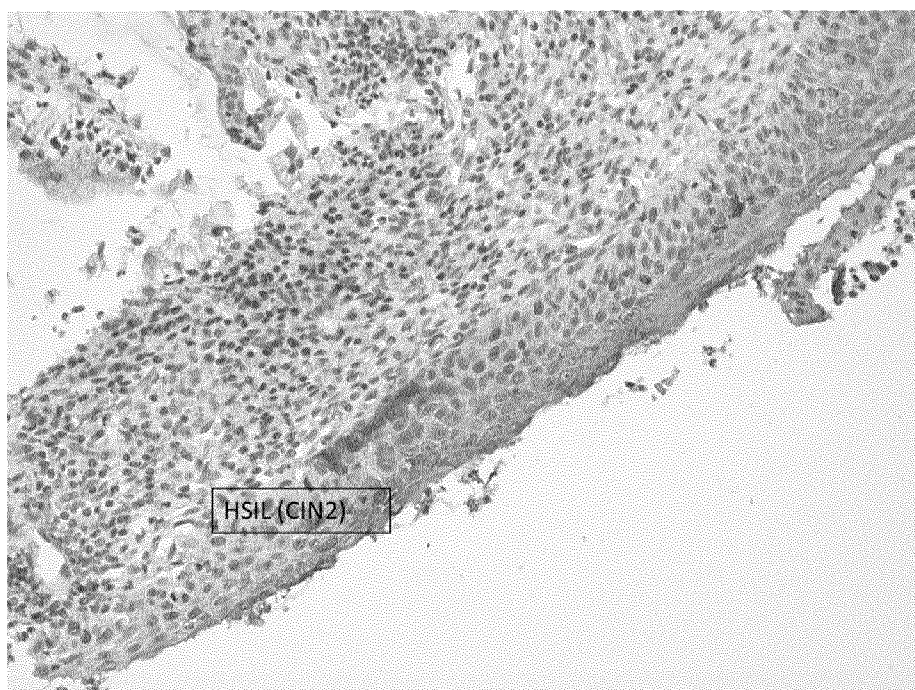


Fig. 12

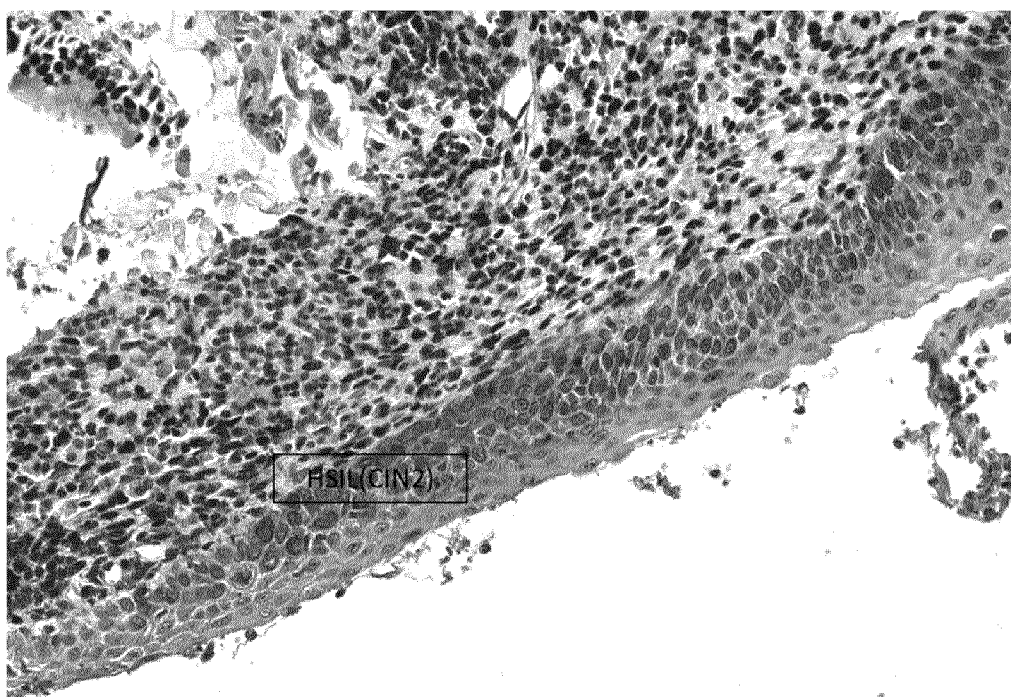
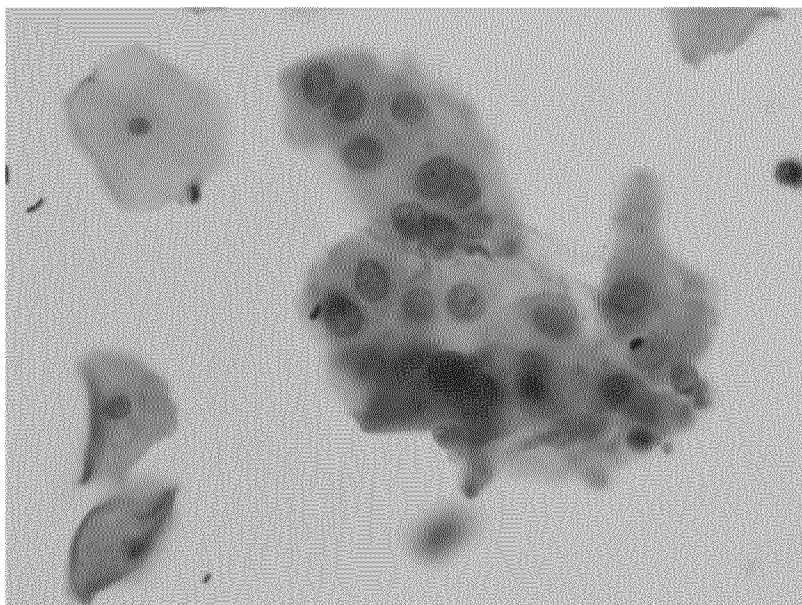
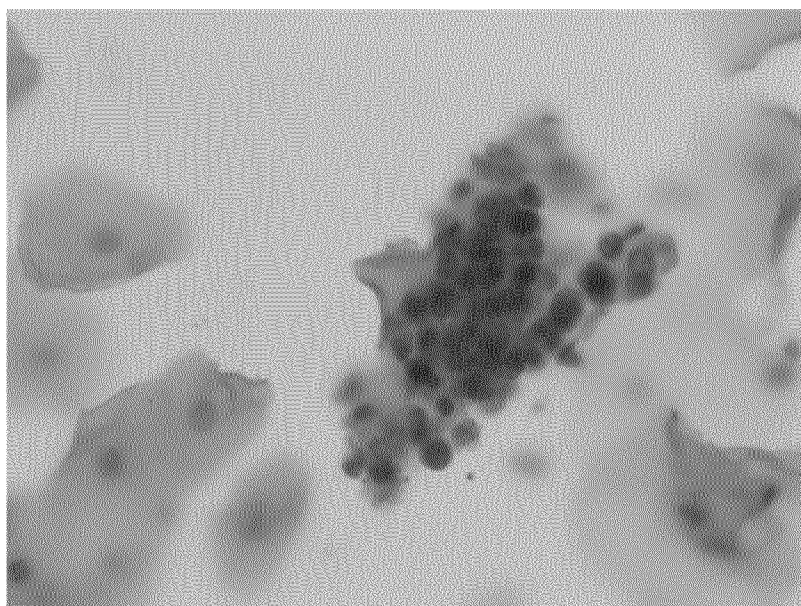


Fig. 13

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LSIL



HSIL

Fig. 14