

Endometrial cancer is the most common gynaecologic malignancy in many developed countries (Siegel et al., CA Cancer J. Clin., 64 (2014), pp. 9–29). Early stage endometrial cancer has a very good prognosis. Therefore, early detection will increase the chance of cure and avoid or reduce cumbersome and costly therapeutic intervention. As shedding of tumor cells in cervical scrapes has been demonstrated, early detection of endometrial cancer by non-invasive sampling is feasible. However, conventional cytology on cervical scrapes has a very low sensitivity for detection of endometrial cancer. Testing for molecular alterations, such as DNA methylation, associated with endometrial cancer, may provide a promising approach for early detection of endometrial cancer (de Strooper et al., J Clin Pathol. 2014;67(12):1067-71, Bakkum-Gamez et al. Gynecol Oncol. 2015;137(1):14-22). Similarly, testing for cancer specific DNA alterations in cervical scrapes for the detection of ovarian cancer has recently been proposed (Kindle et al., Sci Transl Med. 2013; 5(167):1-21).

SUMMARY

The inventors now have found a method for detecting HPV-induced invasive cancers, nonHPV-induced gynaecological and anogenital cancers, and their high-grade precancerous lesions wherein said method comprises the detection of hypermethylation in the ZIC1 and/or GHSR gene in a cell whereby such hypermethylation indicates the presence of HPV-induced precursor lesions with invasive potential, HPV-induced invasive cancers, nonHPV-induced gynaecological and anogenital cancers. Preferably, in such a method said HPV-induced high-grade precancerous lesion or HPV-induced invasive carcinoma is a high-grade premalignant cervical lesion or invasive cervical cancer, more preferably a high-risk HPV-induced invasive cancer. Preferably, said nonHPV-induced gynaecological cancer is an endometrial cancer. Preferably said nonHPV-induced anogenital cancer is a vulvar or penile cancer. In one example, the present disclosure refers to an *in vitro* method for detecting HPV-induced high-grade precancerous lesions and HPV-induced invasive cancers and non HPV-induced gynaecological and anogenital cancers, said method comprising detection of hypermethylation in the GHSR and/or ZIC1 gene in a cell whereby such hypermethylation indicates the presence of HPV-induced precursor lesions with invasive potential, HPV-induced invasive cancers and nonHPV-induced gynaecological and anogenital cancers. In one aspect, the present disclosure refers to an *in vitro* method for detecting HPV-induced high-grade precancerous lesions and HPV-induced invasive cancers and non HPV-induced endometrial cancer, ovarian cancer, vulvar cancer, vaginal cancer, anal cancer and penile cancer, said method

comprising: -detection of hypermethylation in the GHSR and/or ZIC1 gene in a cell whereby such hypermethylation indicates the presence of HPV-induced high-grade precancerous lesions or HPV-induced invasive cancers; - detection of hypermethylation in the GHSR and ZIC1 gene in a cell whereby such hypermethylation indicates the presence nonHPV-induced endometrial cancer, ovarian cancer, vulvar cancer, vaginal cancer, anal cancer and penile cancer; or - detection of hypermethylation in the ZIC1 gene in a cell whereby such hypermethylation indicates the presence of nonHPV-induced endometrial cancer, vulvar cancer or penile cancer. In another example, the present disclosure refers to an in vitro method of detecting HPV-induced high-grade precancerous lesion and HPV-induced invasive cancers and nonHPV-induced gynaecological or anogenital cancer as disclosed herein, wherein said hypermethylation is an increased

methylation of GHSR and/or ZIC1 CpG rich promoter and/or gene sequences in the test cell as compared to the comparable normal cell. In another aspect, the present disclosure refers to an *in vitro* method of detecting HPV-induced high-grade precancerous lesion and HPV-induced invasive cancers and nonHPV-induced

5 endometrial cancer, ovarian cancer, vulvar cancer, vaginal cancer, anal cancer and penile cancer as disclosed herein, wherein -said hypermethylation in the GHSR and/or ZIC1 gene that indicates the presence of HPV-induced high-grade precancerous lesions is an increased methylation of GHSR and/or ZIC1 CpG rich promoter and/or gene sequences in the test cell as compared to the comparable normal cell; or - said

10 hypermethylation in the GHSR and ZIC1 gene that indicates the presence of nonHPV-induced endometrial cancer, ovarian cancer, vulvar cancer, vaginal cancer, anal cancer and penile cancer is an increased methylation of GHSR CpG rich promoter and/or gene sequence and ZIC1 CpG rich promoter and/or gene sequences in the test cell as compared to the comparable normal cell; or - said hypermethylation in the ZIC1 gene

15 that indicates the presence of nonHPV-induced gynaecological and anogenital cancers is an increased methylation of ZIC1 CpG rich promoter and/or gene sequences in the test cell as compared to the comparable normal cell. In yet another example, the present disclosure refers to the use of GHSR and/or ZIC1 as a molecular diagnostic marker for the detection of HPV-induced high-grade precancerous lesion or HPV-

20 induced invasive carcinoma or nonHPV-induced gynaecological or anogenital cancer, preferably wherein the methylation of said marker is predictive for the occurrence of said lesion, carcinoma or cancer. In yet another aspect, the present disclosure refers to use of the promoter regions of GHSR and/or ZIC1 as a molecular diagnostic marker for the detection of HPV-induced high-grade precancerous lesion or HPV-induced invasive

25 carcinoma, preferably wherein the methylation of said marker is predictive for the occurrence of said lesion, carcinoma or cancer.

In a preferred example the hypermethylation is detected in the CpG rich sequences as indicated in Figures 1 and 2.

Disclosed herein is a method as defined above wherein said hypermethylation

30 is an increased methylation of ZIC1 and/or GHSR CpG rich promoter and /or genomic sequences in the test cell as compared to the comparable normal cell.

In a preferred example the detection of (hyper)methylation is performed by using a methylation sensitive restriction endonuclease, chosen from the group consisting of BssHII, MspI, NotI and HpaII. In another preferred example the detection of

35 (hyper)methylation is performed using nanotechnology. In an alternative preferred

example, the detection of (hyper)methylation is performed via a methylation specific PCR, which is based on bisulfite modification of DNA, followed by specific PCR reactions that target CpG rich sequences. Preferably in such a method the reagent is a nucleic acid probe or primer that binds to the nucleic acid as indicated in Figure 1 or 2,
5 and more preferably said nucleic acid probe or primer has a detectable label.

In another example the nucleic acid probe has a nucleotide sequence selected from the group consisting of:

- a) a polynucleotide sequence capable of hybridizing under stringent conditions to the sequence ZIC1 as set forth in Figure 1 or to the sequence
10 GHSR as set forth in Figure 2;
- b) a polynucleotide having at least 70% identity to the polynucleotide of a);
- c) a polynucleotide complementary to the polynucleotide of a); and
- d) a polynucleotide comprising at least 15 bases of a nucleotide of a) or b).

15 Further preferred in one example the methylation of both the ZIC1 and the GHSR gene is determined.

Also disclosed herein is the use of ZIC1 and/or GHSR as a molecular diagnostic marker for the detection of HPV-induced invasive carcinoma, nonHPV-induced gynaecological or anogenital carcinoma, and their high-grade precancerous

lesion. Preferably, in such a use the methylation of said marker is predictive for the occurrence of said lesion or carcinoma.

The disclosure also comprises a kit of parts for use in a method of detecting HPV-induced invasive carcinoma, nonHPV-induced gynaecological or anogenital

5 carcinoma and their high- grade precancerous lesion said kit comprising

-means for the detection of *ZIC1* and/or *GHSR* methylation wherein said means comprise probes and/or primers specific for the *ZIC1* nucleotide sequence of Figure 1 and/or the *GHSR* nucleotide sequence of Figure 2 and

-means for the detection of HPV infection, wherein said means comprise
 10 probes and primers specific for HPV. Thus, in one example, the present disclosure refers to a kit of parts for use in a method of detecting HPV-induced high- grade precancerous lesion or HPV-induced invasive carcinoma or nonHPV-induced gynaecological or anogenital cancer, said kit comprising means for the detection of *GHSR* and *ZIC1* methylation wherein said means comprise probes and/or primers
 15 specific for the *ZIC1* nucleotide sequence of Figure 1 and the *GHSR* nucleotide sequence of Figure 2; -means for the detection of HPV infection, wherein said means comprise probes and primers specific for HPV. In yet another aspect, the present disclosure refers to a kit of parts for use in a method of detecting HPV-induced high-
 20 grade precancerous lesion or HPV-induced invasive carcinoma or nonHPV-induced gynaecological or anogenital cancer, said kit comprising -means for the detection of *GHSR* and *ZIC1* methylation wherein said means comprise probes and/or primers specific for the promoter of *ZIC1* nucleotide sequence as present in SEQ ID NO: 7 and the promoter of *GHSR* nucleotide sequence of as present in SEQ ID NO: 8; and -means
 25 for the detection of HPV infection, wherein said means comprise probes and primers specific for HPV.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the *ZIC1* 5' regulatory region and coding sequence. Transcription start is indicated in bold italic underlined, CpG islands shaded in grey
 30 and coding sequence is in upper case.

Figure 2 shows the *GHSR* 5' regulatory region and coding sequence. Transcription start is indicated in bold italic underlined, CpG islands shaded in grey and coding sequence is in upper case.

Figure 3 shows boxplots of the levels of ZIC1 and GHSR as measured by quantitative methylation specific PCR in primary keratinocytes, hrHPV-immortalized keratinocytes and cervical cancer cell lines. On the y-axes levels of methylated DNA are presented; on the x-axes the cell lines are grouped according to degree of transformation (primary keratinocytes, early passage HPV16 and HPV18 immortalized

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Claims

1. An *in vitro* method for detecting HPV-induced high-grade precancerous lesions and HPV-induced invasive cancers and non HPV-induced endometrial cancer, ovarian cancer, vulvar cancer, vaginal cancer, anal cancer and penile cancer, said method comprising:
 - detection of hypermethylation in the GHSR and/or ZIC1 gene in a cell whereby such hypermethylation indicates the presence of HPV-induced high-grade precancerous lesions or HPV-induced invasive cancers;
 - detection of hypermethylation in the GHSR and ZIC1 gene in a cell whereby such hypermethylation indicates the presence nonHPV-induced endometrial cancer, ovarian cancer, vulvar cancer, vaginal cancer, anal cancer and penile cancer; or
 - detection of hypermethylation in the ZIC1 gene in a cell whereby such hypermethylation indicates the presence of nonHPV-induced endometrial cancer, vulvar cancer or penile cancer..
2. Method according to claim 1, wherein said HPV-induced high-grade precancerous lesion or HPV-induced invasive carcinoma is a high-grade premalignant cervical lesion or invasive cervical cancer.
3. Method according to claim 1 or 2, wherein said HPV-induced invasive cancer is a high-risk HPV-induced invasive cancer.
4. Method according to claim 1, wherein said nonHPV-induced gynaecological cancers is an endometrial cancer.
5. Method according to any one of the preceding claims, wherein said hypermethylation is detected in the CpG rich sequences as indicated in Figures 1 and 2.
6. An *in vitro* method of detecting HPV-induced high-grade precancerous lesion and HPV-induced invasive cancers and nonHPV-induced endometrial cancer, ovarian cancer, vulvar cancer, vaginal cancer,

anal cancer and penile cancer according to any of claims 1-5, wherein

- said hypermethylation in the GHSR and/or ZIC1 gene that indicates the presence of HPV-induced high-grade precancerous lesions is an increased methylation of GHSR and/or ZIC1 CpG rich promoter and/or gene sequences in the test cell as compared to the comparable normal cell; or
- said hypermethylation in the GHSR and ZIC1 gene that indicates the presence of nonHPV-induced endometrial cancer, ovarian cancer, vulvar cancer, vaginal cancer, anal cancer and penile cancer is an increased methylation of GHSR CpG rich promoter and/or gene sequence and ZIC1 CpG rich promoter and/or gene sequences in the test cell as compared to the comparable normal cell; or
- said hypermethylation in the ZIC1 gene that indicates the presence of nonHPV-induced gynaecological and anogenital cancers is an increased methylation of ZIC1 CpG rich promoter and/or gene sequences in the test cell as compared to the comparable normal cell.

7. Method according to any of claims 1-6, wherein hypermethylation is detected using a methylation sensitive restriction endonuclease, chosen from the group consisting of BssHII, MspI, NotI and HpaII.

8. Method according to any of claims 1-6 wherein the method involves lab on a chip technology.

9. Method according to claim any of claims 1-6, wherein a methylation specific PCR is performed, which is based on bisulfite modification of DNA, followed by specific PCR reactions that target CpG rich sequences.

10. Method according to claim 9, wherein the PCR reactions utilize a nucleic acid probe or primer that binds to the nucleic acid as indicated in Figure 1 or 2.

11. Method according to claim 10, wherein said nucleic acid probe or primer has a detectable label.

12. Method according to claim 9, 10 or 11, wherein the nucleic acid probe has a nucleotide sequence selected from the group consisting of:
- a) a polynucleotide sequence capable of hybridizing under stringent conditions to the sequence ZIC1 as set forth in Figure 1 or to the sequence GHSR as set forth in Figure 2;
 - b) a polynucleotide having at least 70% identity to one of the polynucleotides of a);
 - c) a polynucleotide complementary to one of the polynucleotides of a); and
 - d) a polynucleotide comprising at least 15 bases of one of the polynucleotides of a) or b).
13. Method according to any of claims 1 -12, wherein the methylation of both the GHSR and the ZIC1 gene is determined
14. Use of the promoter regions of GHSR and/or ZIC1 as a molecular diagnostic marker for the detection of HPV-induced high-grade precancerous lesion or HPV-induced invasive carcinoma, preferably wherein the methylation of said marker is predictive for the occurrence of said lesion, carcinoma or cancer.
15. Method according to any of claims 1 – 14, wherein the cell is derived from a urine sample or a cervical sample.
16. Method according to claim 15, wherein the cervical sample is a self-sample.
17. Kit of parts for use in a method of detecting HPV-induced high-grade precancerous lesion or HPV-induced invasive carcinoma or nonHPV-induced gynaecological or anogenital cancer, said kit comprising
- means for the detection of GHSR and ZIC1 methylation wherein said means comprise probes and/or primers specific for the promoter of ZIC1 nucleotide sequence as present in SEQ ID NO: 7 and the promoter of GHSR nucleotide sequence of as present in SEQ ID NO: 8; and

-means for the detection of HPV infection, wherein said means comprise probes and primers specific for HPV.