EPIGENETICS
2018, VOL. 13, NO. 7, 769–778
https://doi.org/10.1080/15592294.2018.1507197

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RESEARCH PAPER

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Host-cell DNA methylation patterns during high-risk HPV-induced carcinogenesis reveal a heterogeneous nature of cervical pre-cancer

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ABSTRACT

Cervical cancer development following a persistent infection with high-risk human papillomavirus (hrHPV) is driven by additional host-cell changes, such as altered DNA methylation. In previous studies, we have identified 12 methylated host genes associated with cervical cancer and pre-cancer (CIN2/3). This study systematically analyzed the onset and DNA methylation pattern of these genes during hrHPV-induced carcinogenesis using a longitudinal *in vitro* model of hrHPV-transformed cell lines (n = 14) and hrHPV-positive cervical scrapings (n = 113) covering various stages of cervical carcinogenesis. DNA methylation analysis was performed by quantitative methylation-specific PCR (qMSP) and relative qMSP values were used to analyze the data. The majority of genes displayed a comparable DNA methylation pattern in both cell lines and clinical specimens. DNA methylation onset occurred at early or late immortal passage, and DNA methylation levels gradually increased towards tumorigenic cells. Subsequently, we defined a so-called cancer-like methylation-high pattern based on the DNA methylation levels observed in cervical scrapings from women with cervical cancer. This cancer-like methylation-high pattern was observed in 72% (38/53) of CIN3 and 55% (11/20) of CIN2, whereas it was virtually absent in hrHPV-positive controls (1/26). In conclusion, hrHPV-induced carcinogenesis is characterized by early onset of DNA methylation, typically occurring at the pre-tumorigenic stage and with highest DNA methylation levels at the cancer stage. Host-cell DNA methylation patterns in cervical scrapings from women with CIN2 and CIN3 are heterogeneous, with a subset displaying a cancer-like methylation-high pattern, suggestive for a higher cancer risk.

ARTICLE HISTORY
Received 10 May 2018
Revised 19 July 2018
Accepted 27 July 2018

KEYWORDS
Cervical carcinogenesis; DNA methylation markers; cervical scrapings; *in vitro* model; hrHPV; quantitative methylation-specific PCR (qMSP); epigenetics

Introduction

Following a persistent infection with a high-risk (hr) type of human papillomavirus (HPV), additional genetic and epigenetic changes in the host cell genome are necessary for progression to cervical cancer [1]. Part of these host cell alterations are induced by expression of viral oncogenes E6 and E7 and include DNA methylation of tumor suppressor genes [2]. Methylation of cytosines at CpG-sites in promoter regions can lead to gene silencing. The DNA methyltransferases (DNMTs) responsible for CpG methylation can be activated by both hrHPV E6 and E7. E7 can directly bind to and activate DNMT1, whereas E6 can upregulate DNMT1 via p53 [3,4]. Conversely, silencing of E6 and E7 has been shown to reduce DNA methylation of tumor suppressor genes and to restore the transformed phenotype in cervical cancer cells [5].

Increased DNA methylation levels of several (candidate) tumor suppressor genes are associated with cervical cancer and a subset of its high-grade precursor lesions, i.e., cervical intraepithelial neoplasia grade 2 and 3 (CIN2 and CIN3) [1,6,7]. Using both targeted and genome-wide approaches,

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we previously identified 12 genes, including *ANKRD18CP, C13orf18, EPB41L3*, and *JAM3* [8,9]; *SOX1* and *ZSCAN1* [9,10]; *GHSR, SST*, and *ZIC1* [11]; and *FAM19A4, PHACTR3*, and *PRDM14* [12]. These genes were considered promising biomarkers to identify hrHPV-positive women and/or women with abnormal cytology at risk for cervical cancer [1,7–14].

So far, the majority of these genes has been evaluated individually or in small sets in separate studies. Herein, we performed a systematic analysis of 12 genes on the same cohort to compare their DNA methylation onset and patterns during cervical carcinogenesis. To this end, we analyzed a well characterized longitudinal *in vitro* model of primary keratinocytes immortalized by HVP16 or HPV18 [15], complemented with a large series of clinically annotated hrHPV-positive cervical scrapings covering various stages of cervical carcinogenesis. The consecutive passages of hrHPV-immortalized cells, which are characterized by an initial acquisition of telomerase activity, followed by a more transformed phenotype characterized by anchorage independent growth, were shown to closely mimic the progressive stages of cervical precancerous disease with respect to both genetic and epigenetic changes [2,15–17].

Results

Early onset and gradual increase in DNA methylation during hrHPV-induced transformation in vitro

DNA methylation patterns of the 12 genes (*ANKRD18CP, C13orf18, EPB41L3, FAM19A4, GHSR, JAM3, PHACTR3, PRDM14, SST, ZIC1, SOX1*, and *ZSCAN1*) were analyzed in an *in vitro* model of hrHPV-induced transformation, using HFKs, consecutive passages of hrHPV-transformed cell lines reflecting the progressive stages of cervical precancerous disease [2,15], and cervical cancer cell lines. In general, the 12 genes showed no DNA methylation in HFKs, a progressive increase in DNA methylation levels from early to late passages of hrHPV-immortalized keratinocytes, and highest levels in the cervical cancer cell lines (Figure 1). The onset of DNA methylation varied between the 12 genes from early immortal passages (*ANKRD18CP, FAM19A4, GHSR, JAM3, PRDM14, SST*, and *ZSCAN1*) to late immortal passages (*C13orf18, EPB41L3, PHACTR3, SOX1*, and *ZIC1*) (Supplementary Figure 1).

A cancer-like methylation-high pattern in a subset of cervical scrapings from women with CIN2/3

Next, DNA methylation patterns of the 12 genes in hrHPV-positive cervical scrapes, covering different underlying histology (no disease, CIN2, CIN3, and cancer) were analyzed. Alike the *in vitro* model, a progressive and significant increase in DNA methylation levels towards cervical cancer was observed for all 12 genes ($P < 0.05$, Figure 2).

The DNA methylation signature of the 12 genes depicted by predicted probabilities is shown in Figure 3. All cancer cases (n = 14) had an average predicted probability of ≥0.19, which was further considered to be a cancer-like methylation-high pattern. Using this threshold, 72% (38 of 53) of CIN3 and 55% (11 of 20) of CIN2 showed a cancer-like methylation-high pattern. The remaining CIN2 and CIN3 lesions had predicted probabilities that were similar to those detected in the far majority (25 of 26) of hrHPV-positive controls, and were considered as methylation-low.

A few differences in DNA methylation patterns were observed between genes. *ANKRD18CP* and *C13orf18* were the only 2 genes with low predicted probabilities in the single hrHPV-control with a cancer-like methylation-high pattern. Furthermore, *C13orf18* showed either very low or very high predicted probabilities, whereas the other 11 methylated genes showed a gradual range of values. *PHACTR3* had generally very low predicted probabilities in CIN2 and CIN3 lesions. This is also illustrated by unsupervised clustering, revealing a similar cluster for most genes, except for *ANKRD18CP, C13orf18*, and *PHACTR3* (Supplementary Figure 2).

Discussion

This study systematically analyzed the onset and DNA methylation pattern of 12 genes during hrHPV-induced carcinogenesis using consecutive passages of hrHPV-immortalized cells, reflecting

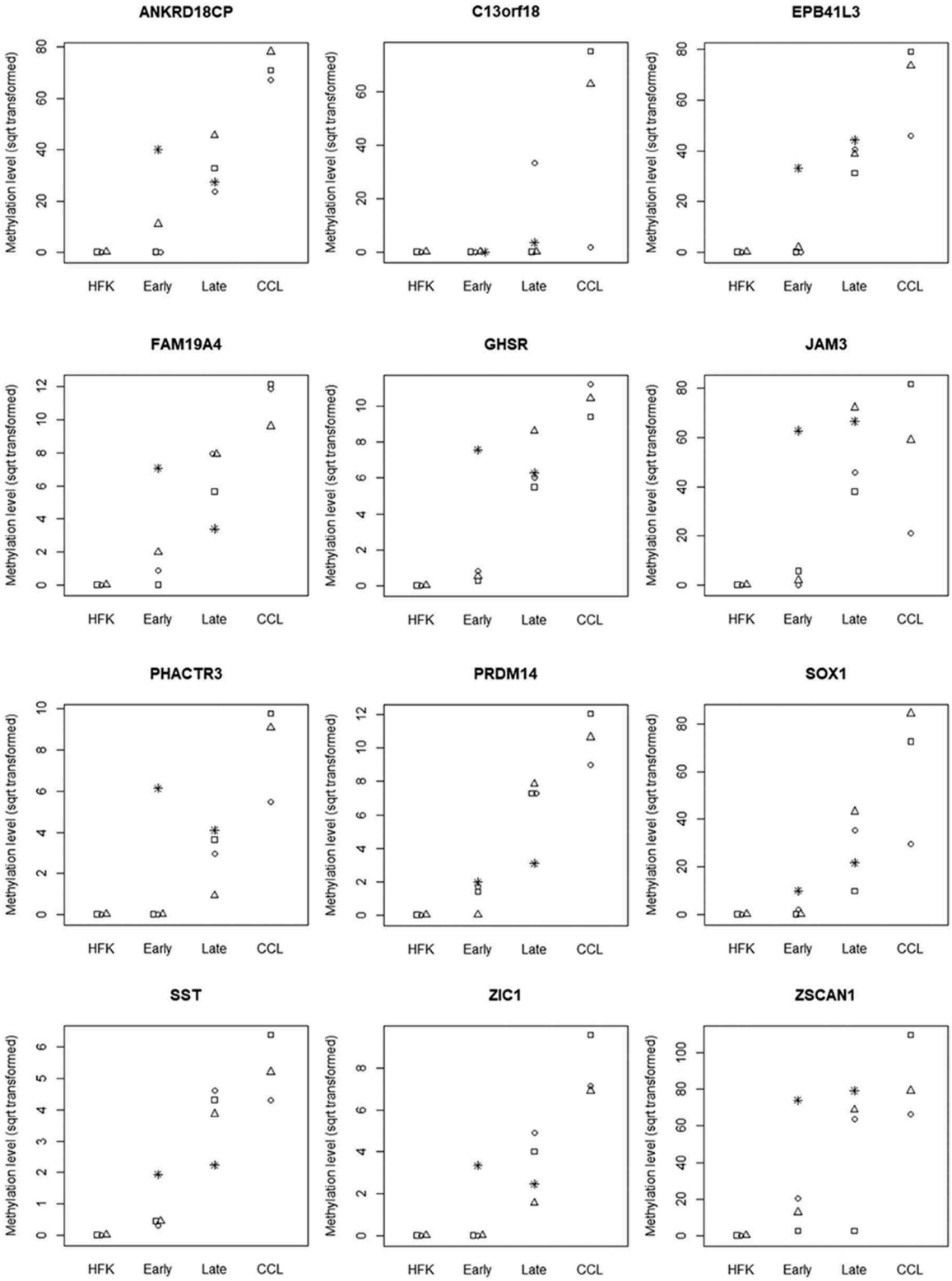

Figure 1. DNA methylation levels of 12 genes in the *in vitro* model. HFK, human foreskin keratinocytes (n = 3); Early, early immortal passages of FK16A, FK16B, FK18A and FK18B p32–p52 (n = 4); Late, late immortal passages of FK16A, FK16B, FK18A and FK18B p129-p156 (n = 4); CCL, cancer cell lines (n = 3). Each of the individual cell lines is indicated by a different symbol. Only for the FK16A, FK16B, FK18A, and FK18B in the 2nd and 3rd column the symbols refer to the early and late passages of the same cell line.

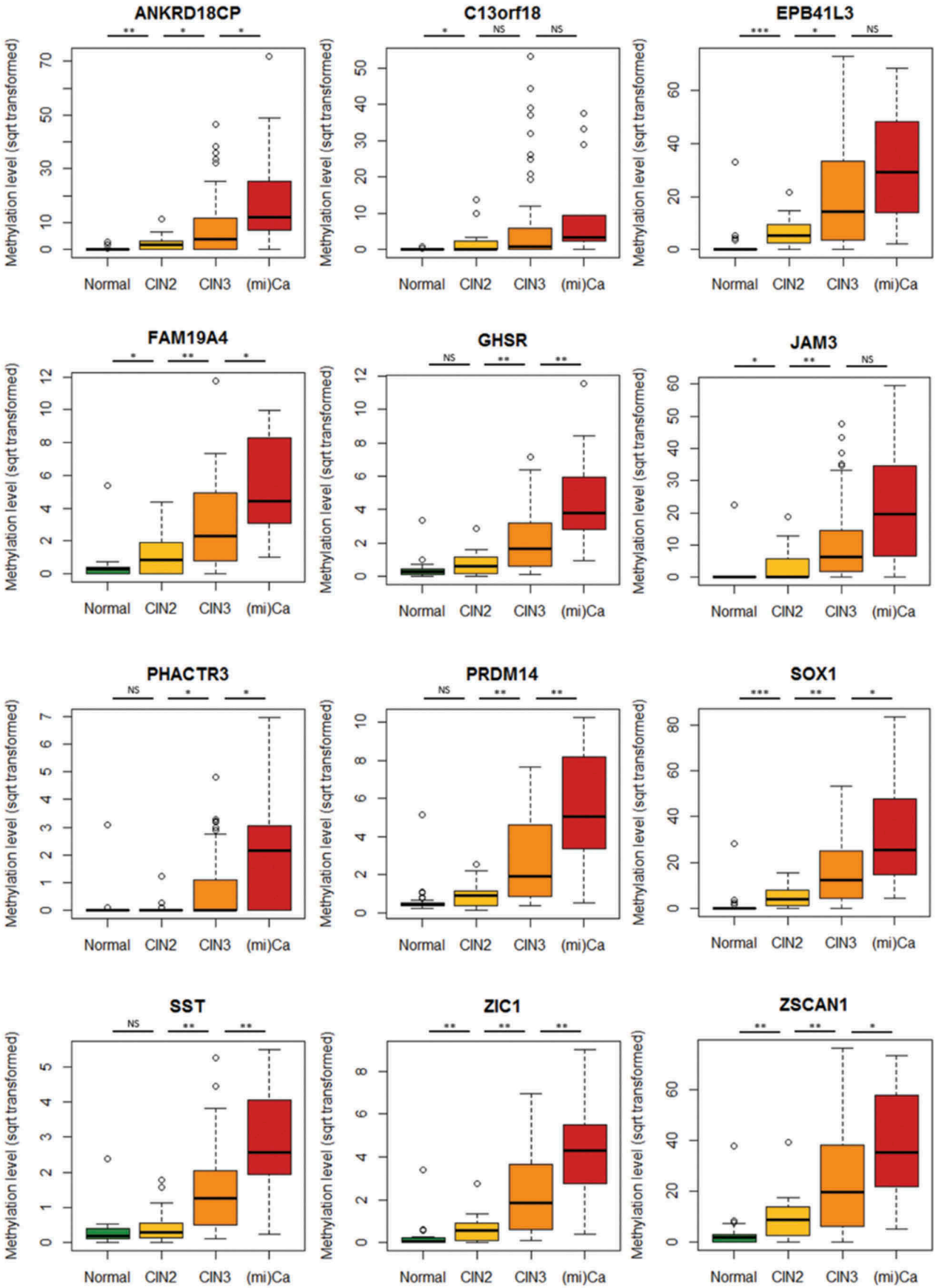

Figure 2. DNA methylation level distributions in hrHPV-positive cervical scrapings across histological subgroups for 12 genes. Normal, hrHPV-positive controls (n = 26); CIN2 and CIN3, cervical intraepithelial neoplasia grade 2 (n = 20) and 3 (n = 53); (mi)Ca, micro-invasive cervical cancer (n = 14). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

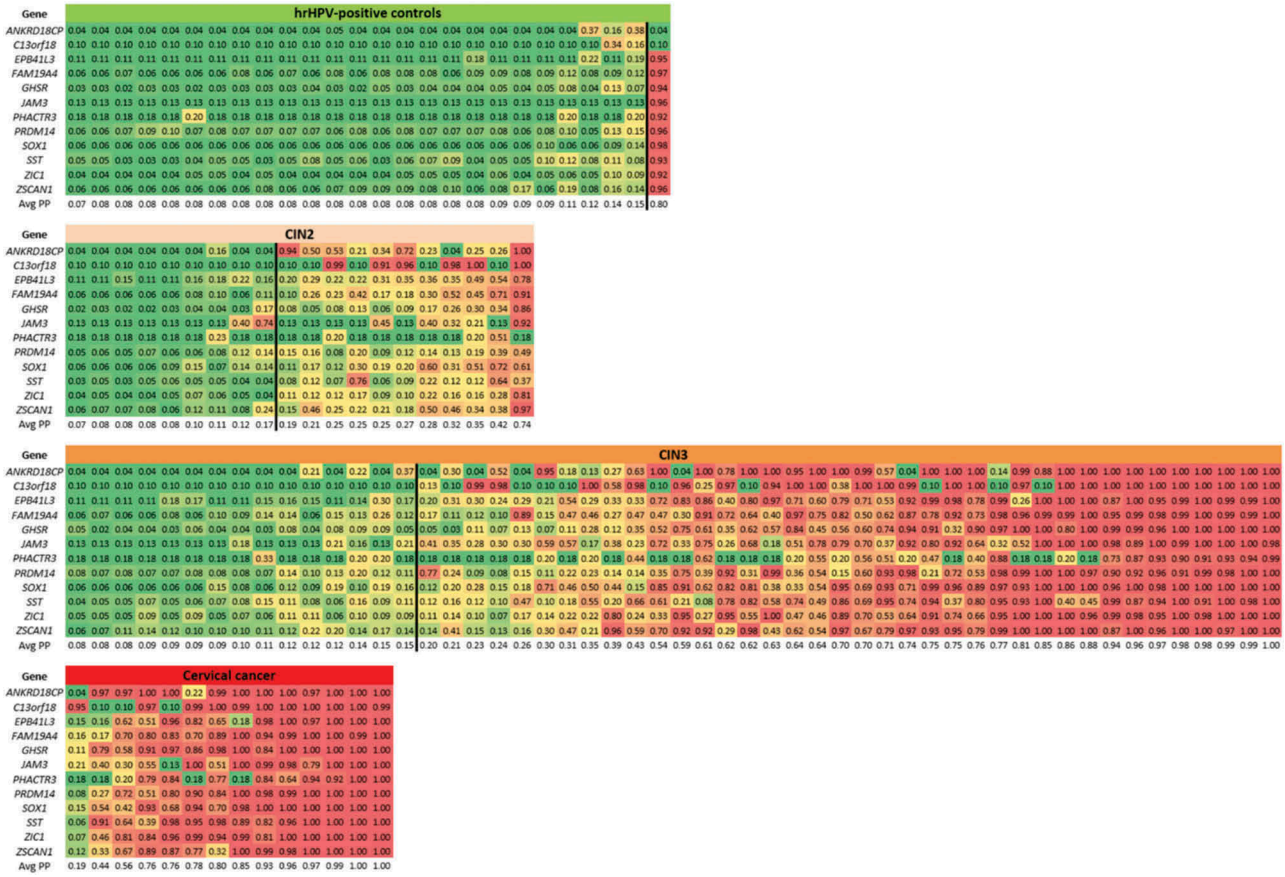

Figure 3. DNA methylation signature of 12 genes in hrHPV-positive cervical scrapings. Predicted probabilities are shown for 12 genes in the different histological subgroups and colored from green (predicted probability of 0) to red (predicted probability of 1). In each group, samples are ordered based on their average predicted probability (Avg PP). The black line indicates the cut-off for a cancer-like methylation-high pattern at ≥0.19.

the progressive stages of cervical precancerous disease, complemented with clinically annotated specimens. A progressive increase in DNA methylation levels represented a general phenomenon during hrHPV-induced carcinogenesis. All genes showed increased DNA methylation levels during hrHPV-induced transformation *in vitro* and in clinical specimens proportional to disease severity. Highest DNA methylation levels were consistently seen in cervical cancer cell lines and cervical scrapings from women with cervical cancer. Using consecutive stages of hrHPV-transformed cells, we showed that the onset of DNA methylation occurred either at the early immortal stage or late immortal stage, when cells are still pre-tumorigenic. In a series of hrHPV-positive cervical scrapings, we showed that the majority of genes displayed a comparable DNA methylation pattern with an onset of a cancer-like methylation-high pattern being detected at the CIN2/3 stage.

Interestingly, the hrHPV-positive scrapings from women with CIN2/3 displayed a heterogeneous DNA methylation pattern, in which three-quarters of the CIN3 samples and half of the CIN2 samples had a cancer-like methylation-high pattern. These results correspond to the finding that only a subset of CIN2/3 will progress to cancer over a long time period [18,19]. Previous studies on copy number changes and DNA methylation levels of only a few genes also showed a cancer-like profile in only a subset of CIN2/3 lesions [11,14,20,21]. These lesions were characterized by a preceding hrHPV infection of >5 years and considered as more advanced lesions, which have a high short-term progression risk to cancer. Conversely, the methylation-low CIN2/3 may be more likely to regress, knowing that approximately ~30% of CIN3 and ~40–54% of CIN2 regress [19,22,23].

Most of the genes evaluated in this study were reported to have a (putative) tumor suppressive

function (i.e., *C13orf18* [24], *EPB41L3* [25–29], *PRDM14* [30], *SOX1* [31,32], *SST* [33,34], *ZIC1* [35,36], and *ZSCAN1* [37,38]). The remaining genes with a currently unknown function await further study.

A few genes (*ANKRD18CP, C13orf18*, and *PHACTR3*) showed a different DNA methylation pattern compared with the majority of genes. This may in part relate to a different onset of DNA methylation during cervical carcinogenesis. DNMTs can be activated by both hrHPV E6 and E7 [3,4], and specific promoter sequences might be more prone to DNA methylation compared to others [39,40], which may relate to the binding of either the polycomb repressor complex (PRC) 1 or 2 [41]. We purposely retained these 3 genes in our analysis on DNA methylation patterns in order to obtain an unbiased representative result. In cervical scrapings, only one hrHPV-positive control showed a cancer-like methylation-high pattern with very high individual predicted probabilities for 10 of 12 methylated genes. However, no gynecological diseases were identified in the follow-up data. Other unidentified underlying abnormalities may have resulted in increased DNA methylation levels. Considering a potential effect of age, i.e., 55 year for this control, DNA methylation levels of the markers tested were not or minimally affected by age in our previous studies using large cohorts [8–11,42,43]. Therefore, solely age is very unlikely to explain the cancer-like methylation-high pattern in this hrHPV-positive control.

A limitation is that the amount of dysplastic cells in the specimens is unknown, and a potential effect of varying sample constitution cannot be fully excluded.

The genes evaluated in this study may well serve as objective molecular tools to improve cervical cancer screening, especially as triage test after primary hrHPV testing [1,7,44]. In several countries, including The Netherlands, hrHPV testing is replacing cytology as primary screening method. Compared to cytology, hrHPV testing has a higher sensitivity for CIN2+ detection [45,46]. However, its 3–5% lower specificity makes triage testing of hrHPV-positive women necessary to prevent over-referral and overtreatment. In the Dutch population-based hrHPV screening program, cytology is included as triage method. However, cytology comes with some limitations, including its subjective nature and the required repeated cytology to ensure sufficient safety in the screening program. Moreover, prior knowledge of hrHPV-positivity influences cytology reading, which may result in an increase of false-positive referrals with simultaneously higher costs for the healthcare system [47–49]. Recent clinical validation studies in screening populations have shown that DNA methylation markers provide a good alternative for cytology [14,50–54].

In conclusion, this study showed that hrHPV-induced carcinogenesis is characterized by increased DNA methylation, with onset typically occurring at the pre-tumorigenic stage and highest DNA methylation levels at the cancer stage. Host-cell DNA methylation patterns of the 12 genes are comparable and reveal the heterogeneous nature of cervical pre-cancer, with a subset of CIN2 and CIN3 lesions displaying a cancer-like methylation-high pattern, suggestive for a higher risk of progression to cervical cancer.

Materials and methods

Cell lines

Cells representing the various stages of hrHPV-induced transformation, consisted of (i) primary human foreskin keratinocytes (HFK) from three donors, (ii) HPV16- (FK16A and FK16B) and HPV18- (FK18A and FK18B) immortalized keratinocytes, including early (passages 32 to 52) and late (passage 129 to 156) passages [15], and (iii) hrHPV-positive cervical cancer cell lines SiHa, HeLa and CaSki. HFKs, FK16A, FK16B, FK18A and FK18B were obtained and cultured as described before [15]. Cervical cancer cell lines SiHa, HeLa, and CaSki (American Type Culture Collection) were cultured as described previously [55]. Cell lines were authenticated using the PowerPlex 16 System (Promega) and were negative for mycoplasma.

Clinical samples and hrHPV testing

A set of hrHPV-positive scrapings (n = 113) obtained from screening or gynecologic outpatient

populations was used, comprising scrapings from control women with normal cytology and/or without evidence of CIN2+ (n = 26) or from women who were histologically diagnosed with CIN2 (n = 20), CIN3 (n = 53) or (micro-invasive) cervical cancer [(mi)Ca; n = 14]. HrHPV was detected by GP5+/6+ PCR enzyme immunoassay [56] or HPV-Risk assay (Self-screen B.V.) [57]. This study followed the ethical guidelines of the Institutional Review Board of the VU University Medical Center and University Medical Center Groningen.

DNA isolation, modification, and qMSP

DNA extraction, quality assessment, sodium bisulfite-treatment, (multiplex) qMSP conditions, and DNA methylation ratio calculations were described previously for *ANKRD18CP, C13orf18, EPB41L3*, and *JAM3* [8,9]; *SOX1* and *ZSCAN1* [9,10]; *GHSR, SST* and *ZIC1* [11]; and *FAM19A4, PHACTR3*, and *PRDM14* [12]. Quality control was routinely performed using a methylation-independent assay for the housekeeping gene *ACTB* [13].

Data analyses

The DNA methylation ratios of the 12 genes were relatively analyzed to obtain equivalent values from each of the individual genes in the datasets of both the *in vitro* model and the cervical scrapings.

To analyze the onset of DNA methylation using the *in vitro* model representing the various stages of hrHPV-induced transformation, we set the highest DNA methylation ratio of each methylated gene at 100% and subsequently defined the time point of DNA methylation onset at the stage where DNA methylation was detected in at least 2 out of 4 cell lines above 5%.

In cervical scrapings, the Kruskal-Wallis test was first performed on square-root transformed DNA methylation ratios of each methylated gene to assess the differences in DNA methylation levels among disease categories. Following a significant result from the test, post-hoc testing was performed using Wilcoxon rank sum test. The Bonferroni correction was subsequently applied for multiple testing, with a significance level of 0.05 (two-sided).

To obtain equivalent DNA methylation values in cervical scrapings, univariable logistic regression analysis was performed on the square-root transformed DNA methylation ratios of the 12 genes. For this analysis, scrapings from women with cervical cancer were categorized as cases and scrapings from hrHPV-positive control women (i.e., with normal cytology and/or without evidence of CIN2+) as controls. Subsequently, the logistic regression models were used to calculate the predicted probability (value range 0 to 1), representing the risk for an underlying cervical cancer. By this, equivalent values for the levels of DNA methylation were obtained for each of the individual genes allowing direct comparison of the genes. The average predicted probability of the 12 methylated genes was calculated for each sample. The lowest average predicted probability in cervical cancer was used as a threshold to define a cancer-like methylation-high pattern (i.e., threshold of ≥0.19). Hierarchical clustering was performed for the 12 methylated genes using the predicted probabilities.

All statistical analyses have been performed using R version 3.1.2.

Acknowledgments

We thank Annina van Splunter for excellent technical assistance.

Disclosure statement

AGJvdZ, ES, and GBAW are inventors of patents related to the contents of the manuscript. RDMS, CJLMM, PJFS, DAMH have minority stake in Self-screen B.V., a spin-off company of VU University Medical Center. Self-screen B.V. holds patents related to this work. ES is a member of the scientific advisory board of Roche, Hologic and QCMD, and received travel reimbursements from Roche, Abbott, Hologic Inc. and QCMD. GBAW is a member of the scientific advisory board of CC Diagnostics. DAMH has been on the speaker´s bureau of Qiagen, and serves occasionally on the scientific advisory boards of Pfizer and Bristol-Meyer Squibb. CJLMM has received speakers' fee from SPMSD/Merck, served occasionally on the scientific advisory board (expert meeting) of Qiagen, SPMSD/Merck. He has been co-investigator on a Sanofi Pasteur MSD sponsored trial, of which his institute received research funding. He is part-time director of Self-screen B.V. and has a very small number of Qiagen shares. Until April 2016 he had minority stock of Diassay B. V. PJFS has been on the speaker's bureau of Roche, Abbott,

Gen-Probe, Qiagen and Seegene. He is consultant for Crucell Holland B.V.

Funding

This work was supported by the Dutch Cancer Society under Grant RUG-NKB 2009-4577; the Graduate School of Medical Sciences, University Medical Center Groningen, Groningen, the Netherlands; and the European Research Council under Grant ERC advanced 2012 - AdG, proposal 322986 (MassCare).

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References

1. Steenbergen RDM, Snijders PJF, Heideman DAM, et al. Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions. Nat Rev Cancer. 2014;14:395–405.
2. Wilting SM, Steenbergen RDM. Molecular events leading to HPV-induced high grade neoplasia. Papillomavirus Res. 2016;2:85–88.
3. Burgers WA, Blanchon L, Pradhan S, et al. Viral oncoproteins target the DNA methyltransferases. Oncogene. 2007;26:1650–1655.
4. Yeung CLA, Tsang WP, Tsang TY, et al. HPV-16 E6 upregulation of DNMT1 through repression of tumor suppressor p53. Oncol Rep. 2010;24:1599–1604.
5. Li L, Xu C, Long J, et al. E6 and E7 gene silencing results in decreased methylation of tumor suppressor genes and induces phenotype transformation of human cervical carcinoma cell lines. Oncotarget. 2015;6:23930–23943.
6. Lorincz AT. Cancer diagnostic classifiers based on quantitative DNA methylation. Expert Rev Mol Diagn. 2014;14:293–305.
7. Luttmer R, De SLMA, Steenbergen RDM, et al. Management of high-risk HPV-positive women for detection of cervical (pre)cancer. Expert Rev Mol Diagn. 2016;16:961–974.
8. Eijsink JJH, Á L, Deregowski V, et al. A four-gene methylation marker panel as triage test in high-risk human papillomavirus positive patients. Int J Cancer. 2012;130:1861–1869.
9. Boers A, Wang R, van Leeuwen RW, et al. Discovery of new methylation markers to improve screening for cervical intraepithelial neoplasia grade 2/3. Clin Epigenetics. 2016;8:29.
10. Wang R, van Leeuwen RW, Boers A, et al. Genome-wide methylome analysis using MethylCap-seq uncovers 4 hypermethylated markers with high sensitivity for both adeno- and squamous-cell cervical carcinoma. Oncotarget. 2016;7:80735–80750.
11. Verlaat W, Snijders PJF, Novianti PW, et al. Genome-wide DNA methylation profiling reveals methylation markers associated with 3q gain for detection of cervical precancer and cancer. Clin Cancer Res. 2017;23:3813–3822.
12. Steenbergen RDM, Ongenaert M, Snellenberg S, et al. Methylation-specific digital karyotyping of HPV16E6E7-expressing human keratinocytes identifies novel methylation events in cervical carcinogenesis. J Pathol. 2013;231:53–62.
13. Wisman GB, Nijhuis ER, Hoque MO, et al. Assessment of gene promoter hypermethylation for detection of cervical neoplasia. Int J Cancer. 2006;119:1908–1914.
14. Lma DS, Meijer CJLM, Berkhof J, et al. Methylation analysis of the FAM19A4 gene in cervical scrapes is highly efficient in detecting cervical carcinomas and advanced CIN2/3 lesions. Cancer Prev Res. 2014;7:1251–1257.
15. Steenbergen RD, Walboomers JM, Meijer CJLM, et al. Transition of human papillomavirus type 16 and 18 transfected human foreskin keratinocytes towards immortality: activation of telomerase and allele losses at 3p, 10p, 11q and/or 18q. Oncogene. 1996;13:1249–1257.
16. Sm W, Miok V, Jaspers A, et al. Aberrant methylation-mediated silencing of microRNAs contributes to HPV-induced anchorage independence. Oncotarget. 2016;7:43805–43819.
17. Fe H, Sm W, Rm O, et al. Sequential gene promoter methylation during HPV-induced cervical carcinogenesis. Br J Cancer. 2007;97:1457–1464.
18. Mre M, Sharples KJ, Paul C, et al. Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: a retrospective cohort study. Lancet Oncol. 2008;9:425–434.
19. Castle PE, Schiffman M, Wheeler CM, et al. Evidence for frequent regression of cervical intraepithelial neoplasia-grade 2. Obstet Gynecol. 2009;113:18–25.
20. Bierkens M, Wilting SM, van Wieringen WN, et al. Chromosomal profiles of high-grade cervical intraepithelial neoplasia relate to duration of preceding high-risk human papillomavirus infection. Int J Cancer. 2012;131:579–585.
21. Bierkens M, Hesselink AT, Meijer CJLM, et al. CADM1 and MAL promoter methylation levels in hrHPV-positive cervical scrapes increase proportional

to degree and duration of underlying cervical disease. Int J Cancer. 2013;133:1293–1300.
22. Holowaty P, Miller AB, Rohan T, et al. Natural history of dysplasia of the uterine cervix. J Natl Cancer Inst. 1999;91:252–258.
23. Ostör AG. Natural history of cervical intraepithelial neoplasia: a critical review. Int J Gynecol Pathol. 1993;12:186–192.
24. Huisman C, Wisman GBA, Kazemier HG, et al. Functional validation of putative tumor suppressor gene C13ORF18 in cervical cancer by artificial transcription factors. Mol Oncol. 2013;7:669–679.
25. Huisman C, Mgp VDW, Falahi F, et al. Prolonged re-expression of the hypermethylated gene EPB41L3 using artificial transcription factors and epigenetic drugs. Epigenetics. 2015;10:384–396.
26. Tran YK, Bögler O, Gorse KM, et al. A novel member of the NF2/ERM/4.1 superfamily with growth suppressing properties in lung cancer. Cancer Res. 1999;59:35–43.
27. Gutmann DH, Donahoe J, Perry A, et al. Loss of DAL-1, a protein 4.1-related tumor suppressor, is an important early event in the pathogenesis of meningiomas. Hum Mol Genet. 2000;9:1495–1500.
28. Wong SY, Haack H, Kissil JL, et al. Protein 4.1B suppresses prostate cancer progression and metastasis. Proc Natl Acad Sci USA. 2007;104:12784–12789.
29. Dafou D, Grun B, Sinclair J, et al. Microcell-mediated chromosome transfer identifies epb41l3 as a functional suppressor of epithelial ovarian cancers. Neoplasia. 2010;12:579–589.
30. Snellenberg S, Cillessen SAGM, Van Criekinge W, et al. Methylation-mediated repression of PRDM14 contributes to apoptosis evasion in HPV-positive cancers. Carcinogenesis. 2014;35:2611–2618.
31. Lin Y-W, Tsao C-M, Yu P-N, et al. SOX1 suppresses cell growth and invasion in cervical cancer. Gynecol Oncol. 2013;131:174–181.
32. Tsao CM, De YM, Shih YL, et al. SOX1 functions as a tumor suppressor by antagonizing the WNT/β-catenin signaling pathway in hepatocellular carcinoma. Hepatology. 2012;56:2277–2287.
33. Jin Z, Mori Y, Hamilton JP, et al. Hypermethylation of the somatostatin promoter is a common, early event in human esophageal carcinogenesis. Cancer. 2008;112:43–49.
34. Leiszter K, Sipos F, Galamb O, et al. Promoter hypermethylation-related reduced somatostatin production promotes uncontrolled cell proliferation in colorectal cancer. PLoS One. 2015;10:e0118332.
35. Gan L, Chen S, Zhong J, et al. ZIC1 is downregulated through promoter hypermethylation, and functions as a tumor suppressor gene in colorectal cancer. PLoS One. 2011;6:e16916.
36. Qiang W, Zhao Y, Yang Q, et al. ZIC1 is a putative tumor suppressor in thyroid cancer by modulating major signaling pathways and transcription factor FOXO3a. J Clin Endocrinol Metab. 2014;99:1163–1172.
37. Chen Y, Zhang Z, Yang K, et al. Myeloid zinc-finger 1 (MZF-1) suppresses prostate tumor growth through enforcing ferroportin-conducted iron egress. Oncogene. 2015;34:3839–3847.
38. Tsai SJ, Hwang JM, Hsieh SC, et al. Overexpression of myeloid zinc finger 1 suppresses matrix metalloproteinase-2 expression and reduces invasiveness of SiHa human cervical cancer cells. Biochem Biophys Res Commun. 2012;425:462–467.
39. Hoque MO, Kim MS, Ostrow KL, et al. Genome-wide promoter analysis uncovers portions of the cancer methylome. Cancer Res. 2008;68:2661–2670.
40. De Carvalho D, Sharma S, You JS, et al. DNA methylation screening identifies driver epigenetic events of cancer cell survival. Cancer Cell. 2012;21:655–667.
41. Deaton A, Bird A. CpG islands and the regulation of transcription. Genes Dev. 2011;25:1010–1022.
42. Yang N, Eijsink JJH, Á L, et al. Methylation markers for CCNA1 and C13ORF18 are strongly associated with high-grade cervical intraepithelial neoplasia and cervical cancer in cervical scrapings. Cancer Epidemiol Biomarkers Prev. 2009;18:3000–3007.
43. Luttmer R, Lma DS, Berkhof J, et al. Comparing the performance of FAM19A4 methylation analysis, cytology and HPV16/18 genotyping for the detection of cervical (pre)cancer in high-risk HPV-positive women of a gynecologic outpatient population (COMETH study). Int J Cancer. 2016;138:992–1002.
44. Cuzick J, Bergeron C, Von Knebel Doeberitz M, et al. New technologies and procedures for cervical cancer screening. Vaccine. 2012;30:F107–F116.
45. Ronco G, Dillner J, Elfström KM, et al. Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials. Lancet. 2014;383:524–532.
46. Arbyn M, Ronco G, Anttila A, et al. Evidence regarding human papillomavirus testing in secondary prevention of cervical cancer. Vaccine. 2012;30:F88–F99.
47. Bergeron C, Giorgi-Rossi P, Cas F, et al. Informed cytology for triaging HPV-positive women: substudy nested in the NTCC randomized controlled trial. J Natl Cancer Inst. 2015;107:dju423.
48. Wright TC, Stoler MH, Aslam S, et al. Knowledge of patients' human papillomavirus status at the time of cytologic review significantly affects the performance of cervical cytology in the ATHENA study. Am J Clin Pathol. 2016;146:391–398.
49. Richardson LA, El-Zein M, Ramanakumar AV, et al. HPV DNA testing with cytology triage in cervical cancer screening: influence of revealing HPV infection status. Cancer Cytopathol. 2015;123:745–754.
50. Verhoef VMJ, Bosgraaf RP, Van Kemenade FJ, et al. Triage by methylation-marker testing versus cytology in women who test HPV-positive on self-collected cervicovaginal specimens (PROHTECT-3): A randomised controlled non-inferiority trial. Lancet Oncol. 2014;15:315–322.

51. Lma DS, Verhoef VMJ, Berkhof J, et al. Validation of the FAM19A4/mir124-2 DNA methylation test for both lavage- and brush-based self-samples to detect cervical (pre) cancer in HPV-positive women. Gynecol Oncol. 2016;141:341–347.
52. Boers A, Bosgraaf RP, van Leeuwen RW, et al. DNA methylation analysis in self-sampled brush material as a triage test in hrHPV-positive women. Br J Cancer. 2014;111:1095–1101.
53. Hesselink AT, Heideman DAM, Steenbergen RDM, et al. Methylation marker analysis of self-sampled cervico-vaginal lavage specimens to triage high-risk HPV-positive women for colposcopy. Int J Cancer. 2014;135:880–886.
54. Hesselink AT, Heideman DAM, Steenbergen RDM, et al. Combined promoter methylation analysis of CADM1 and MAL: an objective triage tool for high-risk human papillomavirus DNA-positive women. Clin Cancer Res. 2011;17:2459–2465.
55. Steenbergen RDM, Kramer D, Braakhuis BJM, et al. TSLC1 gene silencing in cervical cancer cell lines and cervical neoplasia. J Natl. Cancer Inst. 2004;96:294–305.
56. Jacobs MV, Snijders PJ, Brule AJC Van Den, et al. A general primer GP5+/GP6+-mediated pcr-enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in cervical scrapings. J Clin Microbiol. 1997;35:791–795.
57. Hesselink AT, Berkhof J, Van Der Salm ML, et al. Clinical validation of the HPV-risk assay, a novel real-time PCR assay for detection of high-risk human papillomavirus DNA by targeting the E7 region. J Clin Microbiol. 2014;52:890–896.