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TUMOR MARKERS AND SIGNATURES

DNA methylation markers as triage test for the early identification of cervical lesions in a Chinese population

Na Li[1,3] | Yuanjing Hu[1] | Xinying Zhang[2] | Yixin Liu[2] | Ya He[1] | Ate G. J. van der Zee[3] | Ed Schuuring[4] | G. Bea A Wisman[3]

[1]Department of Gynecologic Oncology, Tianjin Central Hospital of Gynecology Obstetrics/Tianjin Key Laboratory of Human Development and Reproductive Regulation, Nankai University Affiliated Hospital, Tianjin, China

[2]Department of Pathology, Tianjin Central Hospital of Gynecology Obstetrics/Tianjin Key Laboratory of Human Development and Reproductive Regulation, Nankai University Affiliated Hospital, Tianjin, China

[3]Department of Gynecologic Oncology, Cancer Research Center Groningen, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

[4]Department of Pathology, Cancer Research Center Groningen, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

Correspondence
G. Bea A Wisman, Department of Gynecologic Oncology, Cancer Research Center Groningen, University of Groningen, University Medical Center Groningen, PO Box 30.001 9700 RB, Groningen, The Netherlands.
Email: g.b.a.wisman@umcg.nl

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Abstract

Objective strategies are required in cervical cancer screening. We have identified several DNA methylation markers with high sensitivity and specificity to detect cervical intraepithelial neoplasia 2 or worse (CIN2+) in Dutch women. Our study aims to analyze the diagnostic characteristics of these markers in a Chinese cohort. A total of 246 liquid-based cytology samples were included, of which 205 women underwent colposcopy due to an abnormal cytology result (atypical squamous cells of undetermined significance [ASCUS] or worse), while 227 were tested high-risk human papillomavirus (hrHPV) positive. All six individual markers (ANKRD18CP, C13ORF18, EPB41L3, JAM3, SOX1 and ZSCAN1) showed enhanced methylation levels and frequency with increasing severity of the underlying lesion ($P \leq .001$). In cytological abnormal women, sensitivity to detect CIN2+ was 79%, 76% and 72% for the three panels (C13ORF18/EBP41L3/JAM3, C13ORF18/ANKRD18CP/JAM3 and ZSCAN1/SOX1, respectively), with a specificity of 57%, 65% and 68%. For the first two panels, these diagnostic characteristics were similar to the Dutch cohort, while for ZSCAN1/SOX1 the sensitivity was higher in the Chinese cohort, but with a lower specificity (both $P < .05$). In hrHPV-positive samples, similar sensitivity and specificity for the detection of CIN2+ were found as for the abnormal cytology cohort, which were now all similar between both cohorts and non-inferior to HPV16/18 genotyping. Our analysis reveals that the diagnostic performances are highly comparable for C13ORF18/EBP41L3/JAM3 and C13ORF18/ANKRD18CP/JAM3 methylation marker panels in both Chinese and Dutch cohorts. In conclusion, methylation panels identified in a Dutch population are also applicable for triage testing in cervical cancer screening in China.

KEYWORDS
(pre)malignant cervical cancer, cervical cancer, cervical scrapings, DNA methylation markers, quantitative methylation-specific PCR (QMSP)

Abbreviations: ADC, adenocarcinoma; ASC-US, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; CIS, carcinoma in situ; hrHPV, high-risk human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; SCC, squamous cell carcinoma.

Ed Schuuring and G. Bea A. Wisman shared senior authorship.

1 | INTRODUCTION

Cervical cancer is the fourth most frequent type of cancer among women worldwide and the fourth leading cause of cancer death among women.[1] Early detection screening programs and high-risk human papillomavirus (hrHPV) vaccination have emerged as effective strategies in cervical cancer prevention; however, high incidence rates are observed in low- and middle-income countries.[2] The latest statistics show 106 430 new cervical cancer cases and 47 750 deaths every year in China.[3]

Cytology and hrHPV testing are currently used for cervical cancer screening. Although cytology as a cervical screening method has been proven to be highly successful with high specificity, a substantial number of cervical cancer cases are still being missed owing to false-negative test results caused by sampling errors and interobserver and intraobserver variability.[4,5] Due to the higher sensitivity for the detection of cervical intraepithelial neoplasia 2 or worse (CIN2+), hrHPV testing has been implemented as primary cervical cancer population-based screening program in several developed countries.[6] However, the natural history of transient hrHPV infection makes the specificity of hrHPV testing lower, and therefore an efficient triage strategy is necessary to accurately identify those women warranting colposcopy.[7-11]

Until now, there is no well-organized cervical screening system in China. A cytology-based approach is not the first choice for primary screening due to China's large population, associated complicated technology and infrastructure for cytology testing, and highly different (regional) levels of economic development.[12] The use of hrHPV testing has increased dramatically in China in recent years. However, the lack of clinical validation of the majority of hrHPV tests has led to a huge number of colposcopy referrals.[13] Therefore, there is an urgent need for a proper, objective and reproducible screening strategy for cervical cancer in China.

In order to improve the early detection of cervical cancer, we identified and validated several sensitive and specific DNA methylation markers over the last years.[14-19] A Dutch cytologically abnormal cohort was primarily used to validate the diagnostic performance of several methylation markers. Combinations of various methylation markers (C13ORF18/EBP41L3/JAM3, C13ORF18/ANKRD18CP/JAM3 and ZSCAN1/SOX1) as triage methods showed a similar sensitivity of ~75% for the detection of CIN2+ compared to hrHPV testing, but with better specificity. Furthermore, similar sensitivity and specificity to detect CIN2+ were observed in a subgroup analysis of only hrHPV-positive samples.[15,18]

The current study aims to assess the diagnostic potential (ie, sensitivity, specificity) of our previously established CIN2+ specific methylation panels in a Chinese population. The diagnostic performance was subsequently compared to data previously acquired from Dutch cohorts.[15,19]

Whats's new?

Cytology and high-risk human papillomavirus (hrHPV) testing are currently used for cervical cancer screening. However, their effective implementation and the large potential for unwanted referrals remain a challenge in China, where there is an urgent need for a proper, objective and reproducible screening strategy for cervical cancer. In this study, the authors tested three previously identified CIN2+ specific methylation marker panels, primarily validated in a Dutch cohort of women with abnormal cytology results. The promising diagnostic performance and high concordance with the previous Dutch data make these methylation panels potentially useful for triage testing in cervical cancer screening in China.

2 | METHODS

2.1 | Patients selection

In China, women with complaints or women who would like to get opportunistic cervical cancer screening could go to the gynecologic clinic directly. To test the diagnostic potential of our previously established CIN2+ specific methylation markers in a Chinese population, we selected a population that was most similar to our previously analyzed Dutch population.[19] Women who went to the gynecologic clinic from March 2017 to February 2018 in Tianjin Central Hospital of Gynecology Obstetrics due to complaints (70%) or just for opportunistic screening (27%) or for unknown reasons (3%) with either an abnormal cytology result and/or a positive hrHPV result were recruited. The residual cervical scrapings were prospectively collected and stored in PreservCyt Solution (ThinPrep, Hologic, Marlborough, MA) at room temperature after completion of analysis for cytological diagnosis and hrHPV testing. Study inclusion criteria were females who were sexually active, not pregnant, had an intact uterus and had no history of treatment for CIN or cervical cancer. Patients who had a history of cancer related to the reproductive tract, therapy for cervical lesions or a current pregnancy were excluded. The follow-up of the selected patients is shown in Figure 1.

Women were all tested with routine cytological examination as well as hrHPV genotyping using the Cobas 4800 hrHPV test (Roche Molecular Systems, Alameda, CA). Women with normal cytology and hrHPV16 or hrHPV18 positive, or women with abnormal cytology (atypical squamous cells of undetermined significance [ASCUS] or worse) independent of hrHPV status were referred for colposcopy. Cervical scrapings were cytologically classified according to the 2014 Bethesda System (TBS 2014). Cytology and hrHPV testing were repeated in women with a normal cytology with hrHPV genotype other than HPV16/18, and colposcopy referral was recommended for women with either abnormal cytology or hrHPV test results. An exit test was performed at the endpoint of 18 months. Colposcopy-directed biopsies were performed for histological analysis, according to standard procedures in China. The final diagnosis was based on the results of tissue-proven pathology that served as the gold standard. Biopsy specimens were histologically classified as normal/no CIN, CIN1, CIN2, CIN3 (including cervical carcinoma in situ [CIS]), squamous cell carcinoma (SCC), or adenocarcinoma (ADC), according to the international criteria. The standard guidelines for the management and treatment of cervical neoplasia were followed in all patients.[20] All patient recruitment and clinical information collection processes were periodically monitored.

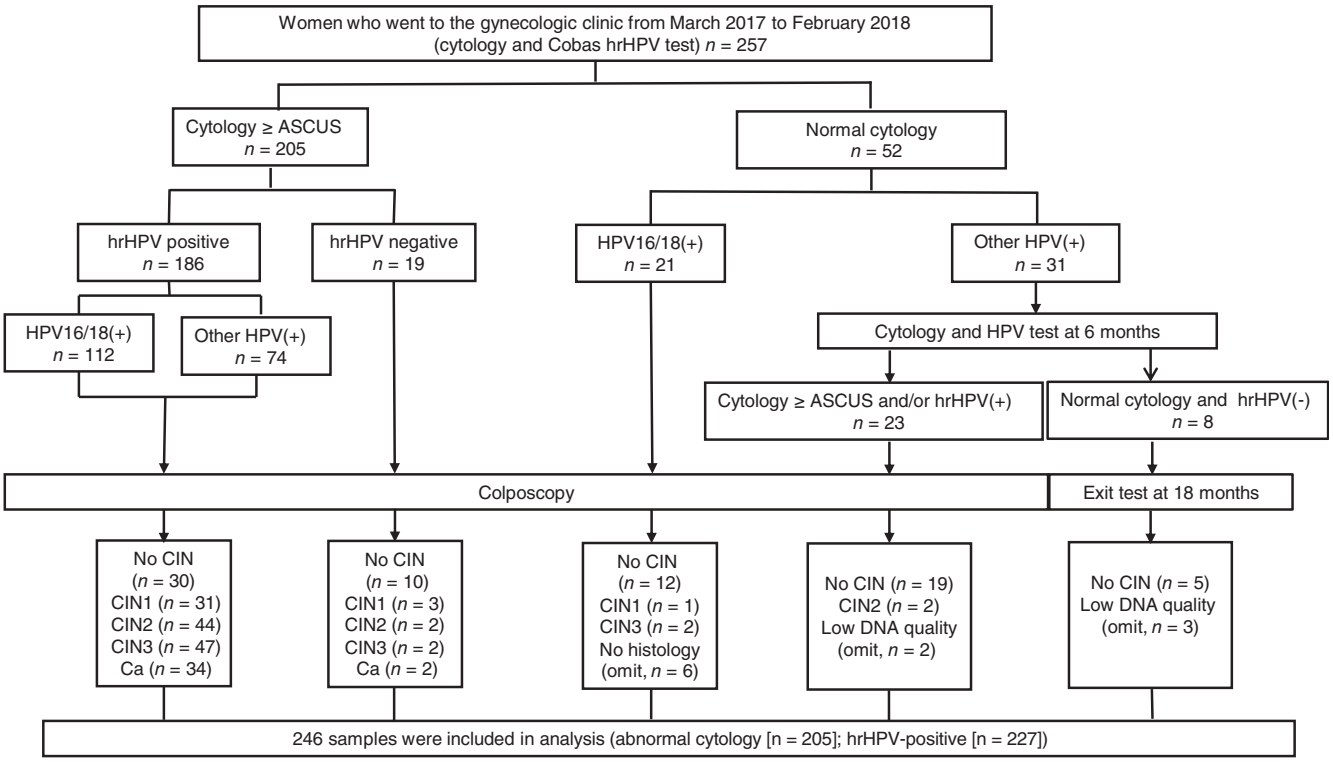

FIGURE 1 Follow-up flow chart with samples of patients selected with either abnormal cytology and/or hrHPV+ with their final histology in the Chinese cohort

2.2 | HrHPV detection

Liquid-based cytology samples were subjected to detect HPV16, HPV18 or any of 12 other hrHPV genotypes (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) DNA using the Cobas 4800 hrHPV test (Roche Molecular Systems), a fully-automated platform based on a real-time PCR technique.

2.3 | DNA isolation and bisulfite treatment

Processing of cervical scrapings and assessment of the DNA's structural integrity were as described previously.[19] Genomic DNA was extracted from the cervical exfoliated cells using the TIANamp Micro DNA Kit (Tiangen Biotech Co. Ltd, Beijing, China) following the manufacturer's recommendations. DNA concentrations and 260/280 ratios were measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc, Valencia, CA). A 260/280 ratio around 1.8 to 2.0 and the capability to produce amplicons of at least 300 base pairs (bp) using a BIOMED2 multiplex PCR[21] was required for all DNA samples. Sodium bisulfite modification of denatured genomic DNA was performed as previously reported.[22] One microgram DNA was treated with sodium bisulfite using EZ DNA Methylation-Gold kit according to manufacturer's instructions of (Zymo Research, Irvine, CA) and eluted in 100 μL to obtain 10 ng/μL. Samples were randomly distributed among DNA isolation batches and were again randomized across multiple bisulfite treatments. Leukocyte DNA from five healthy women was pooled and used as negative control for methylation, whereas in vitro methylated leukocyte DNA (IV), produced using M. SssI methyltransferase (New England Biolabs, Ipswitch, MA), served as a positive control.

2.4 | Quantitative methylation-specific PCR

Methylation analysis for six markers (ANKRD18CP, C13ORF18, EPB41L3, JAM3, SOX1 and ZSCAN1) was performed as described previously using ACTB as a methylation-independent reference.[15,18,19,22] The methylation level was assessed on a ABI PRISM 7900HT Sequence Detection system (Thermo Fisher Scientific, Waltham, MA) in a randomized fashion, blinded from clinical data using quantitative methylation-specific PCR (QMSP) in 10 μL containing 300 nM of each primer, 200 nM probe, QuantiTect Probe PCR Master Mix (Qiagen, Hilden, Germany) and 2.5 μL bisulfite-treated DNA (approximately 25 ng). Each sample was analyzed in triplicate. Serial dilutions of IV DNA enabled absolute quantification of (methylated) template. Sodium bisulfite conversion was repeated in samples with mean quantity of ACTB less than 1 ng per reaction. The relative level of methylation of the region of interest was calculated as follows: average quantity of the methylated region of interest/average quantity of the reference ACTB gene * 10000.[22] A sample was considered

methylated if at least two out of the three wells were methylation positive with a Ct below 50.[18,19] A sample was considered methylation positive for SOX1 or ZSCAN1 if the methylation level was above a threshold of 19.1 or 132, respectively,[15,23] which we applied both in the Chinese population and in the Dutch population.

2.5 | Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 22 (IBM Corporation, New York, NY). Analysis of variance (ANOVA) was used to compare age differences between groups. The Kruskal-Wallis test was used to assess whether the methylation levels changed with the severity of the underlying lesion. The sensitivity and specificity were calculated for the diagnostic evaluation. The Fisher's exact test was used to attribute differences to either sensitivity or specificity. A gene combination labeled a sample positive if at least one of the markers was positive. A *P* value below .05 was considered to be significant.

3 | RESULTS

3.1 | Sample characteristics

A flow chart of the Chinese study population is shown in Figure 1. Data of the Dutch population were obtained as described previously[15,19,23] and collected according the cytology-based cervical cancer screening program in the Netherlands (Figure S1). Clinicopathological data and available hrHPV and cytology results for cervical scrapings in both Chinese and previous Dutch cohorts are listed in Table 1. For the composition of the Chinese cohort, in total 257 scrapings were collected. Six samples without histological result and 5 samples with low DNA quality were excluded. Pathology results for the remaining 246 samples were as follows: no CIN, n = 76; CIN1, n = 35; CIN2, n = 48; CIN3, n = 51; and cervical cancer, n = 36 (ADC = 4, SCC = 30, adenosquamous = 1, clear cell carcinoma = 1), of whom most were of early stage (Table S1). Cervical cancer patients (mean age 49 years) were older than non-cancerous women (mean age 39-43 years) (*P* = .001), while there were no age differences within the rest of the histological subgroups (Table 1).

3.2 | DNA methylation is associated with severity of the underlying histological lesions in a Chinese cohort

Methylation analysis of all 246 cervical scrapings revealed that both methylation levels and frequencies of all six markers (ANKRD18CP, C13ORF18, EPB41L3, JAM3, SOX1 and ZSCAN1) increased with the severity of the underlying histological lesion ($P \leq .001$ for each marker) (Figure 2). All cervical

TABLE 1 Clinicopathological data of scrapings samples in Chinese and Dutch cohorts

	Chinese cohort Histology results						Dutch cohort Histology results					
	No CIN	CIN1	CIN2	CIN3	Cancer	Total	No CIN	CIN1	CIN2	CIN3	Cancer	Total
Number of subjects												
N	76	35	48	51	36	246	27	38	45	61	44	215
Age												
Mean age	40	39	39	43	49		39	39	36	36	42	
Range	25 to 70	22 to 66	22 to 59	18 to 63	24 to 67		26 to 60	23 to 55	21 to 60	27 to 51	29 to 60	
Cytology results												
Normal	36	1	2	2	0	41	0	0	0	0	0	0
ASCUS	23	19	28	21	10	101	9	9	2	0	0	20
LSIL	15	12	14	9	1	51	18	27	36	18	5	104
ASC-H	0	0	0	0	2	2	0	0	0	0	0	0
HSIL	2	3	4	17	10	36	0	2	7	43	35	87
Cancer	0	0	0	2	13	15	0	0	0	0	3	3
Unknown	0	0	0	0	0	0	0	0	0	0	1	1
hrHPV test												
HPV16/18 positive	21	7	31	37	31	127	7	10	24	39	27	107
Other positive	45	25	15	12	3	100	5	14	12	10	4	45
hrHPV negative	10	3	2	2	2	19	14	12	9	10	12	57
Unknown	0	0	0	0	0	0	1	2	0	2	1	6

Note: The clinicopathological data were retrieved from a Dutch cohort of 215 women as reported previously.[19]

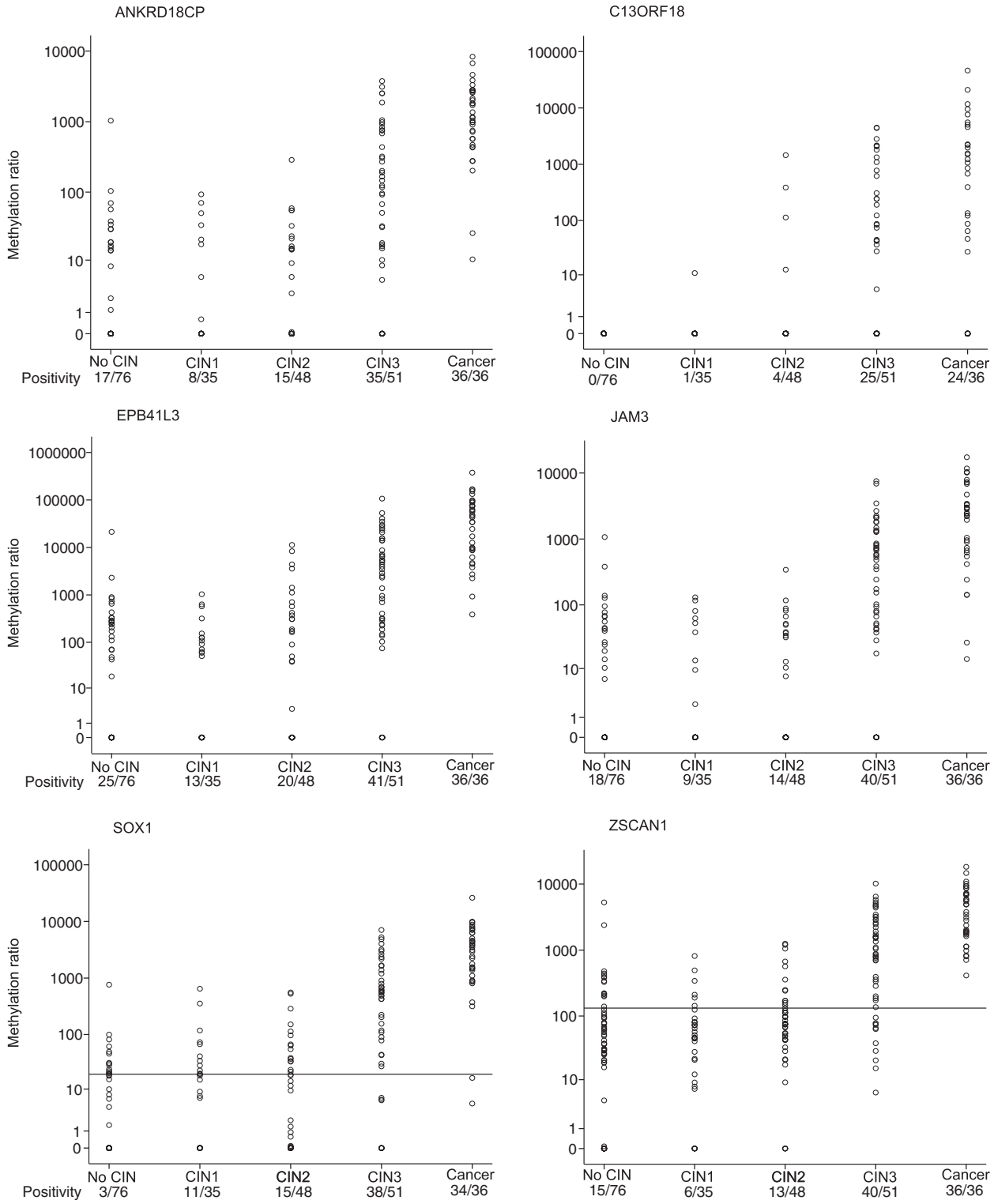

FIGURE 2 Methylation ratio of ANKRD18CP, C13ORF18, EPB41L3, JAM3, SOX1 and ZSCAN1 analyzed with QMSP in 246 scrapings from patient with no CIN lesion, CIN1, CIN2, CIN3 and cancer in the Chinese cohort. Relative levels of methylation significantly increases with severity of underlying histological lesion ($P \leq .001$). The horizontal solid lines represent threshold (19.1 for SOX1 or 132 for ZSCAN1)

cancer patients were methylation positive for ANKRD18CP, EPB41L3, JAM3 and ZSCAN1 (36/36). Methylation of SOX1 was tested positive in 34 out of 36 cervical cancer scrapings. In contrast, of the 76 samples of women with no CIN, most cases were methylation negative, and in all cases (0/76) for C13ORF18.

3.3 | The performance of methylation markers to detect CIN2+/CIN3+ lesions in scrapings with an abnormal cytology in Chinese women

In order to determine the frequency of DNA methylation in normal/CIN1 vs CIN2+, we selected scrapings of 205 women with an abnormal cytology (cytology ≥ ASCUS) (Table 1). All six individual methylation markers and three marker panels revealed a significant lower number of women with methylation in the normal/CIN1 vs women with CIN2+ lesions ($P < .0005$). The methylation frequency of each marker in both Chinese and Dutch cohorts is shown in Table S2. To evaluate the diagnostic value of the three Dutch methylation panels, the sensitivities and specificities to detect CIN2+ in the Chinese cohort were determined. In 205 patients with scrapings with an abnormal cytology, sensitivity to detect CIN2+ lesions was 79%, 76% and 72% for the C13ORF18/EBP41L3/JAM3, C13ORF18/ANKRD18CP/JAM3 and ZSCAN1/SOX1 methylation panel, and with a specificity of 57%, 65% and 68%, respectively (Table 2).

When we compared these results with the sensitivity and specificity to detect CIN2+ lesions of these same methylation panels in the Dutch cohort as reported previously,[15,18,19,23] the C13ORF18/EBP41L3/JAM3 and C13ORF18/ANKRD18CP/JAM3 panels revealed similar results ($P > .05$) (Table 2, Figure S2A). On the other hand, for the ZSCAN1/SOX1 panel, the sensitivity was higher in the Chinese cohort (72% vs 57%) accompanied with a lower specificity (68% vs 83%) ($P < .05$). This is mainly due to the higher sensitivity of ZSCAN1 and the lower specificity of SOX1 (Table 2, Figure S2A).

For the detection of CIN3+ lesions, the sensitivities for all the three methylation panels (C13ORF18/EBP41L3/JAM3, C13ORF18/ANKRD18CP/JAM3 and ZSCAN1/SOX1) were significantly higher (94%, 92% and 91%) without loss of specificity (53%, 61% and 66%, respectively) compared to the detection of CIN2+ lesions. In contrast to the detection of CIN2+, the sensitivities to detect CIN3+ lesions in patients with scrapings with an abnormal cytology were significantly higher in the Chinese than in the Dutch cohort with similar specificities (Table 2; Figure S2B).

3.4 | The performance of methylation markers to detect CIN2+/CIN3+ lesions in hrHPV-positive scrapings in Chinese women

In order to evaluate the diagnostic performance of these three methylation panels as a potential triage test in hrHPV-positive women, we selected 227 women with hrHPV-positive scrapings (Table 1). Again, methylation positivity of all markers and panels were positively associated with the severity of the underlying disease ($P < .0005$) (Table S3). The sensitivity to detect CIN2+ lesions of the three panels (C13ORF18/EBP41L3/JAM3, C13ORF18/ANKRD18CP/JAM3 and ZSCAN1/SOX1) varied between 71% and 79% with specificities between 63% and 67%. This revealed that the sensitivities and specificities to detect CIN2+ of all three marker panels were comparable between the Chinese and Dutch cohorts ($P > .05$) (Table 3 and Figure S3A).

For the detection of CIN3+ lesions in the Chinese cohort with hrHPV-positive women, the sensitivities for all the three methylation

TABLE 2 Sensitivity and specificity of methylation markers in cervical scrapings with abnormal cytology from Chinese and Dutch cohorts

	Chinese population (n = 205)				Dutch population (n = 215)			
	CIN2+ vs ≤CIN1		CIN3+ vs ≤CIN2		CIN2+ vs ≤CIN1		CIN3+ vs ≤CIN2	
	Sens (%)	Spec (%)	Sens (%)	Spec (%)	Sens (%)	Spec (%)	Sens (%)	Spec (%)
Methylation marker panels								
C13ORF18/EPB41L3/JAM3	79	57	94	53	73	72	83	64
C13ORF18/ANKRD18CP/JAM3	76	65	92	61	74	76	80	62
ZSCAN1/SOX1	72	68	91	66	57	83	70	78
Individual markers								
ANKRD18CP	64	77	83	74	65	86	69	71
C13ORF18	41	99	58	96	40	95	49	89
EPB41L3	73	64	89	62	69	79	81	61
JAM3	67	78	88	76	63	91	72	78
ZSCAN1[a]	68	82	89	78	48	89	59	85
SOX1[a]	66	73	85	72	55	88	68	82
HPV test								
hrHPV	95	18	95	13	79	42	78	33

Note: The sensitivity and specificity data were retrieved from a Dutch cohort of 215 women as reported previously.[15,19,23]

Abbreviations: CIN, cervical intraepithelial neoplasia; Sens, sensitivity; Spec, specificity.

[a]Using a threshold for positivity at a methylation ratio of 19.1 for SOX1 or 132 for ZSCAN1.

TABLE 3 Sensitivity and specificity of methylation markers and HPV16/18 genotyping in hrHPV-positive scrapings from Chinese and Dutch cohorts

	Chinese population (n = 227)				Dutch population (n = 152)			
	CIN2+ vs ≤CIN1		CIN3+ vs ≤CIN2		CIN2+ vs ≤CIN1		CIN3+ vs ≤CIN2	
	Sens (%)	Spec (%)	Sens (%)	Spec (%)	Sens (%)	Spec (%)	Sens (%)	Spec (%)
Methylation marker panels								
C13ORF18/EPB41L3/JAM3	79	63	93	58	76	72	86	60
C13ORF18/ANKRD18CP/JAM3	75	67	90	63	77	81	85	61
ZSCAN1/SOX1	71	67	89	65	62	83	76	74
Individual markers								
ANKRD18CP	64	79	82	75	65	86	74	71
C13ORF18	40	100	57	97	43	94	54	88
EPB41L3	73	67	89	64	72	78	85	68
JAM3	67	76	87	74	68	94	54	88
ZSCAN1[a]	67	82	88	78	53	92	67	82
SOX1[a]	65	78	83	74	59	86	74	76
HPV16/18 genotyping	77	71	82	59	78	53	83	42

Note: The sensitivity and specificity data were retrieved from a Dutch cohort of 152 women as reported previously.[15,19,23] The HPV16/18 genotyping data were retrieved from Boers et al.[19]

Abbreviations: CIN, cervical intraepithelial neoplasia; Sens, sensitivity; Spec, specificity.

[a]Using a threshold for positivity at a methylation ratio of 19.1 for SOX1 or 132 for ZSCAN1.

panels (C13ORF18/EBP41L3/JAM3, C13ORF18/ANKRD18CP/JAM3 and ZSCAN1/SOX1) were significantly higher (93%, 90% and 89%, respectively) without loss of specificity (58%, 63% and 65%, respectively) compared to the detection of CIN2+ lesions. The sensitivities and specificities to detect CIN3+ lesions in patients with hrHPV-positive scrapings between the Chinese and Dutch cohorts of two panels (C13ORF18/EBP41L3/JAM3 and C13ORF18/ANKRD18CP/JAM3) were comparable, while the sensitivity of ZSCAN1/SOX1 was higher in the Chinese cohort with a similar specificity compared to the Dutch cohort (Table 3, Figure S3B).

HPV16/18-specific genotyping has been reported as potential triage markers in hrHPV-positive scrapings.[24] In our Chinese cohort, 127 of 227 hrHPV-positive samples tested HPV16- or HPV18-positive. Of these women 21 had no disease, 7 had CIN1, 31 CIN2, 37 CIN3 and 31 cervical cancer (Table 1). The sensitivity and specificity of HPV16/18 genotyping in hrHPV-positive scrapings was 77% and 71% to detect CIN2+ lesions and 82% and 59% for the detection of CIN3+ lesions, respectively. The diagnostic performance of our methylation panels in the Chinese cohort was noninferior to HPV16/18 genotyping (all $P > .05$) (Table 3, Figure S3).

4 | DISCUSSION

In our study, we tested three previously identified CIN2+ specific methylation marker panels, primarily validated in a Dutch cohort of women with abnormal cytology results.[14-19] Highly similar diagnostic performance of methylation panels of C13ORF18/EBP41L3/JAM3 and C13ORF18/ANKRD18CP/JAM3 was shown in the Chinese population compared to the Dutch cohort. Combination of ZSCAN1/SOX1 showed higher sensitivity and lower specificity for CIN2+ to triage cytological abnormal scrapings from the Chinese population. The current results imply that analysis of our three methylation panels for identification of cervical lesions in Chinese population is promising.

Except for ZSCAN1/SOX1, the other two methylation panels (C13ORF18/EBP41L3/JAM3, C13ORF18/ANKRD18CP/JAM3) showed comparable diagnostic performance, despite some differences between the two cohorts. The Chinese cohort recruited women who went to the gynecologic clinic because of complaints (70%) or just for opportunistic screening (27%). As demonstrated in our previous study,[19] in the Netherlands (until 2017), population-based cervical cancer screening was assessed by cytological analysis. The Dutch cohort only consisted of women with an abnormal cytology result and/or hrHPV-positivity as triage test. According to the national guidelines for cervical cancer screening, only women with an abnormal cytology were referred to the gynecologist for colposcopy. Thus, hrHPV-positive women with normal cytology were missing in the Dutch cohort. Other potential differences might exist in quality of cytology/histology between Chinese and Dutch cytologists/histologists, which will influence the colposcopy referrals. However, the observed similarity in methylation status apparently was not influenced by these differences in composition between both cohorts. So, we assume that marker panel ZSCAN1/SOX1 behaves differently due to diverse hrHPV subtype infection in different populations.

HrHPV test is regularly used as a secondary test in women with an abnormal cytology result. However, low specificity of hrHPV test results in a large number of unnecessary colposcopy referrals. In our study, the performance of hrHPV test in abnormal cytology samples (sensitivity 95%, specificity 18%) for CIN2+ detection is similar to the result as analyzed from another Chinese cohort (Shandong province China: sensitivity 100%, specificity 15% and 22%).[25] Specificities (57%-68%) of our three marker panels were significantly higher than hrHPV testing (specificity of 18%) in the present study. Thus, we propose that methylation marker panels might be a potential triage strategy of women with cytological abnormal results in China to reduce the unnecessary referrals. Whether or not combination of these methylation marker panels with hrHPV testing will improve the diagnostic performance needs to be studied in future.

The application of hrHPV testing in China is increasing with huge numbers of hrHPV-positive women referred for colposcopy, thus triage testing in hrHPV-positive women is compulsory.[13] Cytology, p16/Ki67 dual-staining and HPV16/18 genotyping are the mostly reported triage possibilities in hrHPV-positive women. However, recent data showed that the cytologists were likely to deliver a biased cytological diagnosis with knowledge of hrHPV status, leading to ~50% unnecessary colposcopy referrals of women with a subsequent cytology triage test after primary hrHPV screening.[6] In addition, cytology has other main drawbacks such as requirement of high quality of cytological slides and high professional skills of involved technicians and finally its inability to be performed on self-samples.[26] The same limitations account for triage methods based on immunostaining such as p16/Ki67.[27-29] HPV16/18-specific genotyping has been already implemented as a triage test for hrHPV-positive individuals in the guideline of the American Society for Colposcopy and Cervical Pathology and is also recommended in China.[24] But, HPV16/18 genotyping is useless for cervical cancer patients with hrHPV infections other than HPV16/18 subtypes. In our study, the sensitivity and specificity of HPV16/18 genotyping was 77% and 71%, respectively, to detect CIN2+, which is concordant with the data published by Bu et al from the Guangdong Province, China (sensitivity 65%, specificity 67%)[30] and other studies analyzing other populations.[31,32] Our study shows that the diagnostic performance of three methylation panels was noninferior to the HPV16/18 genotyping to triage hrHPV-positive women. For triaging hrHPV-positive women in China, one might consider cotesting of HPV16/18 genotyping and methylation analysis, especially the potential application of methylation panels in hrHPV infections other than HPV16/18. However, the current results could not be directly translated into clinical applications due to the small size and hospital-based cohort in our present study. In a previous study from our group analyzing a Slovenian cohort of hrHPV-positive women, HPV16/18 genotyping combined with these methylation marker panels minimally improved sensitivity, but drastically decreased specificity.[23] This is in line with other studies.[30,33,34] However, large randomized studies are needed in future to confirm these results.

Methylation assays have been proven to be objective, reproducible and high-throughput, which can also be performed using the same DNA isolated for hrHPV testing either from clinician-collected or from self-sampled materials.[19,35,36] These advantages could help overcome the main barriers to cervical screening in developing countries.[37] Besides markers reported by our group, there are other methylation markers reported to be noninferior to current triage reflects as well.[38-41] Until now, there is no triage method that can identify all women with a high risk for high-grade CIN. One might also have to take into account the cost-effective effect for the application of methylation analysis in China. Improved sensitivity and specificity and large prospective randomized studies for potential markers and cost-effective analysis are needed before the introduction of methylation analysis into cervical cancer screening programs.

Validation of methylation markers in different populations is essential. The strength of our current study is the reproducibility of our previously reported methylation panels in an independent Chinese cohort. However, our study also has limits: we used a selected series of cervical samples from women who went to a gynecological outpatient clinic for a variety of reasons and therefore is not representative of a screening population. Our results cannot be directly translated to determine the clinical relevance and further studies in screening populations are warranted.

5 | CONCLUSIONS

Our methylation panels identified in a Dutch population are also applicable for triage testing in cervical cancer screening in China. The high reproducibility of established methylation panels enables its implementation in randomized controlled trials and further large prospective validation in population-based screening in future.

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CONFLICT OF INTERESTS

A.G.J.v.d.Z., E.S. and G.B.A.W. are inventors on patents related to the content of the manuscript. E.S. is a member of the scientific advisory board of Roche, Hologic Inc. and QCMD, and received travel reimbursements from Roche, Abbott, Hologic Inc. and QCMD. E.S. and G.B.A.W. are members of the scientific advisory board of CC Diagnostics and received a research grant from CC Diagnostics. The other authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Our study was approved by the ethics committee of Tianjin Central Hospital of Gynecology Obstetrics. Written informed consent was obtained from all of the women included in this study.

ORCID

G. Bea A Wisman https://orcid.org/0000-0002-4830-3401

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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