

# The clinical application value of FAM19A4/mir124-2 methylation test in hrHPV-positive women

Xiaohua He 1,2, Shaoheng Li 1, Xuanping Wang1, Yongping Mu 2\*

<sup>1</sup>Inner Mongolia Medical University, Hohhot 010000, Inner Mongolia Autonomous Region, China.

<sup>2</sup>Department of Clinical Laboratory Centre, the Affiliated Peoples Hospital of Inner Mongolia Medical University / Inner Mongolia Cancer Hospital, Hohhot 010020, Inner Mongolia Autonomous Region, China.

**Abstract:** Cervical cancer is one of the most common malignancies in women. The combination of human papillomavirus (HPV) genotyping with thinprep liquid-based cytology test (TCT) of cervical exfoliated cells and HPV vaccine helps greatly for the early diagnosis and prevention of cervical cancer. However, there is still a certain risk of missed diagnosis or over-diagnosis due to the limitations of its methodology. Therefore, women infected with high-risk HPV (hrHPV) need more reliable tests to identify whether the lesions will progress to cancer, which can guide early and accurate treatment and prevent over-treatment. Recently, studies have found that FAM19A4/mir124-2 methylation is closely related to cervical cancer, and its methylation degree increases with the severity of cervical lesions, which is expected to become a new biomarker of cervical cancer. Here, we summarized the clinical application value of FAM19A4/mir124-2 methylation test in hrHPV-positive women.

**KEYWORDS:** FAM19A4, mir124-2, DNA methylation, cervical cancer, cervical intraepithelial neoplasia Funding.

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## 1. Introduction

Epidemiological studies have shown that consistent infections with high-risk HPV is a necessary factor for cervical cancer [1]. However, the majority of hrHPV infections are transient and they can progress to invasive cervical cancer (ICC) only if genetic or epigenetic changes are induced by the accumulation of the host or viral genomes [2]. Therefore, it is essential to identify the lesions and reflect the risk of clinically relevant infection and cancer progression in order to avoid that gynecologists unnecessarily referral women with transient hrHPV infection. Studies [3] have shown that host genomic DNA methylation leads to the silencing of tumor suppressor genes, resulting in carcinogenesis in cervical cancer and precancerous lesions. DNA methylation, a major form of epigenetic inheritance regulation, has emerged as a biomarker that can distinguish the risk of high-grade cervical intraepithelial neoplasia (CINII/III+) progressing to cervical cancer [2,4,5]. DNA methylation is significantly higher in CINII/III+ compared to  $\leq$  CINI. As triage test, DNAmethylation has higher specificity than atypical squamous cells of undetermined significance or worse (ASCUS+) and higher sensitivity than HPV16/18 genotyping [6]. Hypermethylation of FAM19A4 and mir124-2 in host cell genomes has been extensively

studied. This paper reviews the clinical application value of FAM19A4/mir124-2 methylation test in hrHPV positive women, in order to provide ideas for its follow-up research.

## 2. method

### 2.1 Relationship between methylation of FAM19A4/mir124-2 and HPV and cervical cancer

FAM19A4 (family with sequence similarity 19 (chemokine (C-C motif)-like) member A4) is a member of the TAFAGene family encoding small molecule proteins, also known as TAF4, which is a secreted protein and is expressed at low levels in normal tissues [7]. FAM19A4, as a ligand of formyl peptide receptor 1 (FPR1), can promote macrophages' cytophagy and increase releasing reactive oxygen species. It is usually up-regulated in monocytes and macrophages stimulated by lipopolysaccharide, and involved in regulating a series of immune responses of the host, so as to protect the host and regulate the function of infection and stress responses [8, 9]. Genome-wide methylation studies of cervical cancer have found that the promoter hypermethylation of FAM19A4 is frequently detected in all cervical carcinoma [10]. Therefore, it is speculated that FAM19A4 gene may be involved in a series of immune responses after HPV infection, but its specific mechanism is still unclear, and further research and

confirmation are needed. In addition, FAM19A4 methylation has been proposed as an alternative biomarker for early detection of cervical cancer, especially for high-grade squamous intraepithelial lesions (HSIL) [11, 12], and as a new classification tool for self-samples of hrHPV positive women [13].

MicroRNAs (miRNAs) are short non-coding RNAs and can also be epigenetically modified through DNA methylation, which regulates CpG islands on miRNA promoters and changes their expression. Comprehensive analysis of epigenome and miRNA expression in cancer cells by microarray and sequencing technology revealed that miRNA is a common target of abnormal DNA methylation in cancer [14]. In the human genome, mir124-2 is associated with CpG islands, which are thought to be hypermethylated sites for cervical, colon, gastric, liver, and leukemia [15]. Studies have found that the positive rate of hypermethylation of mir124-2 is very low in CIN1 lesions and increases with the severity of CIN, which can be detected in all cervical cancers [16]. Although the current study found that the methylation level of FAM19A4/ mir124-2 was mainly affected by the severity of different cervical lesions, it has not been related to HPV genotyping [17-19]. However, since bias may be caused by the relatively small size of samples used in current studies, it is not clear whether HPV genotyping has a definite effect on the occurrence of methylation, and further research is needed.

### **2.2 FAM19A4/ mir124-2 methylation in screening for cervical cancer**

Currently, thinprep liquid-based cytology test (TCT) combined with HPV genotyping of cervical exfoliated cells is the main means of cervical cancer screening. Continuous infection of hrHPV is a prerequisite for cervical cancer or its precancerous lesions [20]. However, most hrHPV infections do not progress to disease, leading to a large number of unnecessary colposcopy referrals and over-diagnosis in cervical cancer screening based on the hrHPV test. The sensitivity and specificity of HPV16/18 genotyping in the identification of CINII+ lesions are low with only 58.9% and 58.2% respectively [11]. For patients with ASCUS, although the TCT test has a higher specificity, it still misses 30% of the diagnosis of CINII lesions [21]. Studies have shown that [22], in hrHPV induced cervical cancer, the level of DNA methylation increases in the pre-tumorigenic stage and reaches the highest in the cancer stage. Therefore, it is very meaningful to find specific molecular markers of hypermethylation in cervical cancer while non-methylation in normal cervix in order to supplement the deficiency of current early screening methods for cervical cancer. Recently, studies

have found that the methylation level of FAM19A4/ mir124-2 is related to the degree of CIN lesion, which can be effectively used to identify cervical (pre)cancer and is an effective screening method for cervical cancer in hrHPV-positive women [11, 13, 23, 24].

The promoter hypermethylation of FAM19A4/ mir124-2 is increased with the grade of cytological abnormalities in HPV-positive women [17, 19, 24]. In low-grade squamous intraepithelial lesions (LSIL) or CIN1, the positivity is 24%, while it increased to 66% in HSIL/CINIII and was seen in all cancers [24]. Through ROC analysis, the promoter methylation of FAM19A4 performed best in distinguishing high grade and non-high grade pathological change, i.e. the atypical squamous cells that cannot exclude a high-grade squamous intraepithelial lesion (ASC-H+) and no intraepithelial lesion or malignancy (NILM) or ASCUS/LSIL with an area under the ROC curve (AUC) of 0.732,

while ASCUS+ from NILM is 0.674 in HPV-positive women [19]. It distinguishes HSIL+ lesions from LSIL and below with an AUC of 0.81 (sensitivity 75.42%, specificity 81.44%) [17]. Interestingly, a combination of the positive FAM19A4 methylation with HPV16/18 genotypes substantially increased the rate of histologic HSIL+ detection from 25% to 77.27%. The rate further increased up to 90.91% when it was combined with HPV16/18/52/58 genotypes [19], demonstrating that FAM19A4 methylation test is a valuable marker of cervical (pre)cancer in hrHPV-positive women.

Luttmer et al. [11] compared the clinical performance of FAM19A4 methylation analysis to cytology and HPV16/18 genotyping combined with cytology, for CINIII+ detection in hrHPV-positive women, the sensitivities were 75.6%, 85.6% and 92.2%, respectively, with corresponding specificities of 71.1%, 49.8% and 29.4%, respectively. It is seen that the sensitivity of FAM19A4 methylation detection is between cytology and HPV16 / 18 genotyping combined with cytology, but the specificity is the highest. However, in women older than 30 years, the specificity was significantly higher than that of cytology (62.1% vs 47.6%) with comparable sensitivity (88.3% vs 85.5%). Similarly, Leeman and his colleagues [25] reported that FAM19A4/ mir124-2 methylation testing and its combination with HPV16 / 18 genotyping had increased the sensitivity (77.8% vs 93.1%) but decreased the specificity (69.3% vs 49.4%) for CINIII+ detection. It needs to be noted that, detection was more sensitive (89.3% vs 98.2%) with reduced specificity (61.1% vs 46.3%) in women aged  $\geq 30$  years. For women with LSIL and below, the sensitivity and specificity were 83.3% and 60.6%, respectively. In conclusion, FAM19A4/ mir124-

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2 methylation detection is more sensitive to CINIII+. Although its combination with HPV16/18 genotyping reduces the specificity, its sensitivity is greatly improved, which increases the detection rate of cervical cancer and precancerous lesions, and may realize early detection, diagnosis and treatment of cervical cancer.

### **2.3 FAM19A4/ mir124-2 methylation in follow-up of high-risk HPV-positive women**

Generally, cytology or hrHPV testing is mainly reviewed in 6, 12, and 24 months to improve the sensitivity of lesion detection, as the follow-up strategy in hrHPV positive women. Women may be referred directly or after triage for colposcopy and biopsy to identify HSIL/CINII-III or ICC attribute to an abnormal cytology or a positive hrHPV genotype [26]. Biopsy diagnosed HSIL/ CINII-

III is currently the indication for surgical removal of precancerous lesions [27]. However, most HSIL/CINII-III will not progress to ICC without treatment [28,29]. Therefore, a follow-up strategy that can provide accurate risk stratification and identify distinct subpopulations of women with HSIL /CINII-III may reduce harms, especially among young women of childbearing age.

The results of a 14 year follow-up of HPV positive women by De Strooper et al [23], using FAM19A4/ mir124-2 methylation testing and cytology showed a 100% power of FAM19A4/ mir124-2 methylation testing for cervical cancer stratified by baseline, implying a very low risk of cancer in woman who are HPV positive and methylation negative. Kaplan–Meier estimate that for women who are baseline methylation negative and baseline cytology negative, the risk of cervical cancer incidence was 1.7% and 2.4% respectively, i.e. the risk difference was 0.71%. This suggests that FAM19A4/ mir-124-2 methylation negativity has a similar ability to predict long-term cancer risk in HPV positive women as cytology negativity. It is also showed that FAM19A4/ mir-124-2 methylation testing merits can be considered as an objective follow-up or triage test for HPV based cervical screening programs.

### **2.4 FAM19A4 / mir124-2 methylation in monitoring progression in high-risk HPV positive women**

Because of lacking of predictive markers for cancer progression in CINII/III lesions, most hrHPV positive CINII/III lesions are treated clinically with similar surgical excision, that is, large loop excision of the transformation zone (LLETZ) or cold knife conization. However, this diagnostic-treatment trajectory is associated with considerable overtreatment because of the high rate of regression of CINII/III lesions. Spontaneous regression is expected in 44% – 50% of

CINII and 32% of CINIII [30 – 32], while only about 5% of untreated CINII and 12% -31% of untreated CINIII eventually progress to cervical cancer [29,30]. The majority of women treated in the cervix are of reproductive age, and it is of great clinical and social value to distinguish easily regressive CINII/III lesions from easily progressive CINII/III lesions.

Tainio et al [32] performed a summary analysis of CINII progression and regression rates from 3 to 60 months in over 3000 women across 36 studies. Overall, looking at the 24 months interval, there was a 50% regression rate (11 studies, 1470 women included) and an 18% progression rate (9 studies, 1445 women). In a subgroup analysis including 1069 women under the age of 30 years, the regression

rate was 60% and only 11% of patients had an exacerbation of the lesion. Loopik et al [33] showed that the long-term regression rate of CINII lesions even reached 71% in 211 women under 25 years of age in the included studies. FAM19A4/mir-124-2 methylation analysis can detect almost all cervical cancers, including rare histotypes and hrHPV negative carcinomas, which suggests that a negative FAM19A4/mir-124-2 methylation test result may exclude the existence of cervical cancer [18]. Considering its ability to predict long-term cancer risk in HPV positive women, patients with CINII/III lesions who are positive for FAM19A4/ mir-124-2 methylation are clinically at high risk of developing cancer and are recommended to be managed with aggressive interventions; instead, it is suggested that there might be self-healing in this patient, and follow-up observation is recommended. It can avoid overtreatment and reduce the psychological and economic burden on patients, leading to personalized diagnosis and treatment for different patients.

### **2.5 FAM19A4/ mir124-2 methylation assay methodology**

Methylation-specific polymerase chain reaction (MSP) [19]

The principle is that after bisulfite modification, the unmethylated cytosine in genomic DNA is converted to uracil, whereas the methylated cytosine unchanged. Accordingly, methylated primer pairs and unmethylated primer pairs could be designed to amplify modified DNA. PCR products could be separated on 1.5%-2% agarose electrophoresis gel, and the bands visualized by gel imaging system to determine the methylation state of the samples. If methylated primer pairs alone can amplify a fragment, it indicates that the assay site is hypermethylated. If only unmethylated primer pairs can amplify a fragment, it indicates the absence of methylation at the assay site. If both primer pairs can produce PCR positive product, the sample is indicated

to be partially methylated. This method is highly sensitive and economic, requires no special instruments, can be used for the detection of Formalin-fixed and paraffin-embedded (FFPE) samples, and is not limited by endonucleases, thus making it currently the most widely used method. Good primers are essential for experimental results, and false positives can result from incomplete bisulfite conversion.

Taqman probe-based quantitative PCR (qPCR) [13,17,24,25]

After bisulfite conversion of genomic DNA, qPCR was performed with a pair of FAM19A4/mir124-2 methylated primers and a pair of primers for the internal reference gene and the probes corresponding to them, usually with a housekeeping gene  $\beta$ -actin (ACTB) as the internal reference to determine the total amount of DNA. If the probes hybridize to DNA, it could release a fluorescent signal, which can be collected to obtain a cycle threshold (CT value) and an amplification curve of ACTB and FAM19A4/mir124-2 in order to analyze the methylation status of the sample. The  $\Delta$ CT value between the target gene and the reference gene can also be used to judge the methylation level of the sample. The smaller is the  $\Delta$ CT value, the higher is the methylation level of the sample. The greatest advantage of this method is its high throughput and sensitivity. Moreover, it eliminates the need for post PCR electrophoresis, hybridization, etc., reducing contamination and operational errors.

genomic sequencing

Commonly used methods are Sanger sequencing, pyrosequencing [34] and high-throughput sequencing. These methods are reliable and precise and can unambiguously determine the methylation status of every CpG site in a fragment of interest, but they require large amounts of clone sequencing, which are tedious and expensive.

### 3. Conclusion

FAM19A4/mir124-2 methylation test is expected to be a novel cervical cancer biomarker, as it has high sensitivity and specificity, and its methylation level increases with the severity of cervical lesions, which can be used for identifying effectively cervical cancer and precancerous lesions and can be used for predicting the risk of lesion progression. It is of great value to apply it alone or in combination with existing cervical cancer screening techniques in the early and follow up diagnosis and monitoring the progression of lesions. It is necessary to further develop relevant test products and protocols to provide objective triage guidance for women with cervical abnormalities detected in screening, so as to reduce harms, avoid overtreatment

and achieve more selectively individualized treatment.

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