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Original Article

Risk evaluation of cervical cancer progress by screening human papillomavirus DNA, E6/E7 mRNA and protein, and cell free ferrous protoporphyrin

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Abstract: Human papillomavirus (HPV) infection is a major cause of cervical cancer. We sought to evaluate the efficiency of HPV DNA, E6/E7 mRNA and protein, and cell free ferrous protoporphyrin (FH) tests for cervical cancer screening. Cervical specimens were collected from 186 Chinese women simultaneously undergoing biopsy examination and HPV DNA, E6/E7 mRNA and protein, and FH tests. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were determined for each test during the progress of cervical cancer. Diagnostic efficiencies were compared between the HPV and FH tests. Of the 186 participants, 83 women (44.6%) had negative (cervical intraepithelial neoplasia grade 0 [CIN0 or no CIN]) or low-grade squamous intraepithelial lesions (CIN1), and 103 women (55.4%) had high-grade squamous intraepithelial lesions (HSILs [CIN2/3]) or squamous cell carcinomas (SCCs). E6/E7 protein staining produced the lowest sensitivity (65.0%) and the highest specificity (86.7%) for identifying CIN2+ samples (HSILs and SCCs), with an area under the curve (AUC) of 0.759 (95% CI: 0.689-0.829). In contrast, the DNA test (AUC = 0.667, 95% CI: 0.587-0.748) produced the highest sensitivity (96.1%) and the lowest specificity (37.3%). HPV E6/E7 mRNA detection (sensitivity 91.3%, specificity 47.0%, PPV 68.1%, and NPV 81.3%; AUC = 0.691, 95% CI: 0.612-0.770) and FH tests (sensitivity 90.3%, specificity 45.8%, PPV 67.4%, and NPV 79.2%; AUC = 0.680, 95% CI: 0.601-0.760) were both better commercial diagnostic tools than the DNA-based assay for cervical cancer screening in the clinic. Thus, the degree of FH substances is an efficient and cost-effective predictor to estimate the progress of cervical cancer.

Keywords: Squamous intraepithelial lesion, cervical cancer, human papillomavirus, E6, E7, mRNA, ferrous protoporphyrin

Introduction

Cervical cancer is the fourth most common cancer in women. Indeed, 528,000 new cases were diagnosed worldwide in 2012 (http://www.wcrf.org/int/cancer-facts-figures/data-specific-cancers/cervical-cancer-statistics), and human papillomavirus (HPV) infection is its major cause [1, 2]. The up to 170 types of HPV [3] can be classified as high-risk (HR) and low-risk based on detection frequency [4]. In epidemiology studies, DNA from the common types 16, 18, 31, 33, 45, 52, and 58 of HR HPV are associated with most of the invasive cervical cancer. This is especially true for HPV type 16 (HPV-16), which predominates in squamous cell carcinoma (SCC), and HPV-18, which predominates in adeno- and adenosquamous-carcinoma (ADC) [5, 6].

Most HR HPV infections spontaneously regress in 6 to 24 months [7]. Only a small percentage of the infections persist over several years, and this is regarded as a risk factor [8]. Without clinical treatment of high-grade squamous intraepithelial lesions (HSILs), there is a risk for the

Table 1. Histological findings for the 186 women enrolled in the study

Histology result[a]	No. (%) of women	Age (mean ± standard deviation)
Normal (no CIN)	32 (17.2)	30-68 (49.8 ± 8.9)
LSILs (CIN1)	51 (27.4)	25-62 (40.6 ± 9.3)
HSILs (CIN2/3)	71 (38.2)	25-62 (42.0 ± 9.0)
SCCs	32 (17.2)	31-64 (43.9 ± 9.7)
Total	186	

[a]LSILs: Low-grade squamous intraepithelial lesions; HSILs: High-grade squamous intraepithelial lesions; SCCs: Squamous cell carcinomas; CIN: Cervical intraepithelial neoplasia.

development of SCCs and ADCs of the cervix [9, 10]. Thus, the high prevalence of HPV infection makes it difficult to predict lesions and carcinomas by DNA testing [11-14]. Cytological and histological examinations can detect and confirm cervical premalignant changes and cancers but have limited sensitivity and specificity for low-grade (LSILs) or borderline abnormalities in distinguishing if they will progress to invasive cancer or spontaneously regress [15, 16]. Indeed, these tests are still not reliable for prediction.

In etiology research, the development of cervical cancer is strongly correlated with the overexpression of two HPV oncogenes, E6 and E7 [17, 18]. In the viral genome, specific opening in the E2 open reading frame enables continuous expression of E6 and E7, which results in cell immortalization and transformation [19-21]. RNA assays for E6 and E7 have been suggested and tested for their higher prognostic value compared to DNA assays for disease progress prediction and cancer prevention [22-26].

Ferrous protoporphyrin (FH) is mainly present in cell mitochondria and is proposed to be contained in the protein hydrophobic core of cellular oxygen receptors to react with Fe^{2+} [27]. Compared to normal cells, tumor cells exhibit significant alterations in energy metabolism and mitochondrial respiration (*i.e.*, the Warburg effect) [28, 29]. Such cell reprogramming leads to FH precipitation from the complex center of the protein, and being in a cell-free state, this causes irreversible oxidative damage and malignant transformation of cells. FH substances interfere with the cell micro-environment and play an important role in the progression of tumorigenesis, such as tumor cell immortalization and invasion, genomic instability, and drug resistance. Therefore, FH detection has been suggested for cancer screening and clinical diagnosis. Here, we tested cell-free FH in cervical exudates to evaluate the stability of uterine epithelial cells and the risk of developing cervical cancer.

In this study, we screened HPV DNA, mRNA (E6/E7), protein (E6/E7), and free reduced iron protoporphyrin in female Chinese patients for the detection of HSILs and SCCs using biopsy diagnosis to compare the sensitivity, specificity, positive predictive values (PPVs), and negative predictive values (NPVs) of these tests. The diagnostic efficiency of the HPV and FH tests were also compared.

Materials and methods

Patients and sample collection

Cervical specimens were collected from 186 Chinese women (aged 22-68 years) during the period of February to November, 2014. All samples were obtained with written informed content. The study was approved by the Ethics Committee of Tianjin Medical University, and the experiments were conducted according to the principles approved.

All patients underwent colposcopy performed by specialized gynecologists. Histology diagnosis was conducted with specimens collected from colposcopy-directed biopsies by specialized pathologists. Cervical specimens for HPV DNA and mRNA tests were collected with a cervical brush transport kit (Digene, Gaithersburg, MD, US). Each sample was divided into two aliquots used for HR HPV DNA and mRNA detection, respectively. Cervical exudate was collected for FH assays. Biopsy examination, HPV DNA and mRNA tests, and FH assays were performed independently. The sensitivity of the diagnostic tests was defined as the proportion of histologically confirmed HSILs or SCCs (cervical intraepithelial neoplasia grade 2 or worse [CIN2+]) detected by the test. In the calculations of specificity and NPV, it was assumed that women with a negative cervical biopsy do not contain disease.

Histology and immunohistochemical staining

The cervical tissues were dehydrated with ethanol, embedded in paraffin, cut into 4- to 6-μm-thick slices, dewaxed with xylene, subjected to

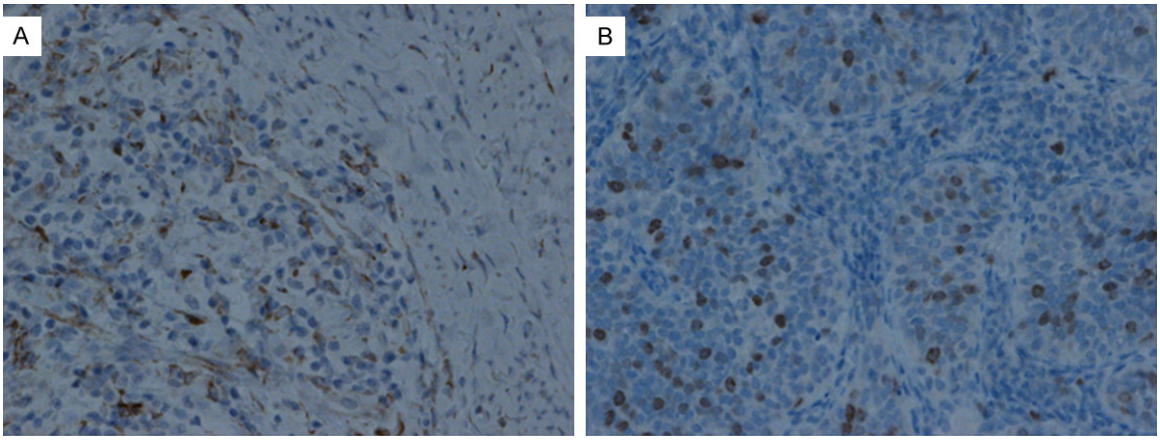

Figure 1. Immunohistochemical staining of human papillomavirus (HPV) E6 (A) and E7 (B) proteins (× 100).

Table 2. Outcome for the 186 women by the human papillomavirus (HPV) E6/E7 protein test

Histology result[a]	HPV E6/E7 protein (No. (%))[b]						
	E6		E7		E6 and/or E7		
	+	-	+	-	+	-	Total
CIN2+	49 (47.6)	54	52 (50.5)	51 (49.5)	67 (65.0)	36 (35.0)	103 (100)
CIN1-	6 (7.2)	77 (92.8)	9 (10.8)	74 (89.2)	10 (12.0)	73 (88.0)	83 (100)
Total	55 (29.6)	131 (70.4)	61 (32.8)	125 (67.2)	77 (41.4)	109 (58.6)	186 (100)

[a]CIN: Cervical intraepithelial neoplasia. [b]E6: Chi squared = 35.9, $P = 2.05 \times 10^{-9}$; E7: Chi squared = 32.8, $P = 1.04 \times 10^{-8}$; and E6 and/or E7: Chi squared = 50.6, $P = 1.11 \times 10^{-12}$.

Table 3. Outcome for the 186 women by the human papillomavirus (HPV) DNA test

Histology result[a]	HPV DNA (No. (%))[b]		
	+	-	Total
CIN2+	99 (96.1)	4 (3.9)	103 (100)
CIN1-	52 (62.7)	31 (37.3)	83 (100)
Total	151 (81.2)	35 (18.8)	186 (100)

[a]CIN: Cervical intraepithelial neoplasia. [b]Chi squared = 33.7, $P = 6.44 \times 10^{-9}$.

Table 4. Outcome for the 186 women by the human papillomavirus (HPV) E6/E7 mRNA test

Histology result[a]	HPV mRNA (No. (%))[b]		
	+	-	Total
CIN2+	94 (91.3)	9 (8.7)	103 (100)
CIN1-	44 (53.0)	39 (47.0)	83 (100)
Total	138 (74.2)	48 (25.8)	186 (100)

[a]CIN: Cervical intraepithelial neoplasia. [b]Chi squared = 35.1, $P = 3.10 \times 10^{-9}$.

gradient alcohol hydration, stained in hematoxylin and eosin (H&E), and then cleared in xylene. Paraffin-sectioned slides were baked for 40 min at 60°C, dewaxed using xylene, and immersed in a descending series of ethanol. The slides were then washed with phosphate-buffered saline (PBS). Antigen retrieval was achieved with 20 μg/ml proteinase K (Qiagen, Hilden, Germany) in PBS for 20 min at room temperature (RT). Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in PBS, and non-specific staining was blocked with goat sera. After washing, slides were incubated with lyophilized HPV-16 E6 peptides (60 nmol, Miltenyi-Biotec, Bergisch Gladbach, Germany) and E7 mouse monoclonal antibody (1:2 dilution; Invitrogen, Camarillo, CA, US), respectively, overnight at 4°C. The antibodies were removed by aspiration, and the slides were washed and incubated at RT for 30 min with horseradish peroxidase-labeled anti-rat secondary antibodies. Colorimetric development was performed with diaminobenzadine reagents according to the manufacturer's instructions (Dako, Carpinteria, CA, US).

HPV DNA detection

Detection of DNA from HR HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68

Table 5. Statistics of human papillomavirus (HPV) E6/E7 mRNA expression

Histology result[a] (n)	HPV mRNA[b]		
	Copy (mean ± standard deviation)	95% confidence interval	*P* value[c]
Normal (32)	552.6 ± 179.9	386.1-718.9	
LSILs (51)	7939.4 ± 11282.2	4177.8-11701.1	0.002
HSILs (71)	73692.6 ± 149851.8	36260.7-111124.4	0.001
SCCs (32)	116237.6 ± 149590.5	60379.6-172095.7	0.001

[a]LSILs: Low-grade squamous intraepithelial lesions; HSILs: High-grade squamous intraepithelial lesions; and SCCs: Squamous cell carcinomas. [b]Spearman's rho = 0.555, $P = 0.003$. [c]After Bonferroni correction, the significance level was $\alpha' = 0.017$.

Table 6. Outcome for the 186 women by the cell free ferrous protoporphyrin (FH) test

Histology result[a]	FH (No. (%))[b]		
	+	-	Total
CIN2+	93 (90.3)	10 (9.7)	103 (100)
CIN1-	45 (54.2)	38 (45.8)	83 (100)
Total	138 (74.2)	48 (25.8)	186 (100)

[a]CIN: Cervical intraepithelial neoplasia. [b]Chi squared = 31.2, $P = 2.28 \times 10^{-8}$.

was conducted using a *digene* Hybrid Capture® II High-Risk HPV DNA test (Digene) according to the manufacturer's instructions.

HPV E6/E7 mRNA assay

All samples were tested for the presence of the E6 and E7 transcripts of the HR HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 by use of a QuantiVirus® HPV E6/E7 RNA Assay kit (DiaCarta, Richmond, CA, US) according to the manufacturer's instructions.

FH assay

FH assays were conducted using an Epithelial FH Dyeing Kit (Dofmelo, Tsingdao, Shandong, China) according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were performed using SPSS statistical software v19.0. Results are presented as counts and percentages, with mean ± standard deviation (SD) and 95% confidence intervals (CIs) being estimated for continuous data. Results of HPV DNA, mRNA, and FH tests were compared with the histological findings by using the chi squared (χ^2) test. Histological results were grouped as normal-CIN1 (CIN1-) *vs.* CIN2-SCC (target condition, CIN2+). The expected values and 95% CIs for sensitivity, specificity, PPV, and NPV were calculated for the target condition (CIN2+). Quantitative data from HPV mRNA and FH tests were compared among the Normal, LSIL, HSIL, and SCC groups by using standard analysis of variance (ANOVA), and Spearman's correlations were estimated. The diagnostic performance of each test for effective cervical cancer screening was evaluated and compared using receiver operating characteristic (ROC) curve analysis. All statistical analyses were conducted at the significant level of $\alpha = 0.05$, and Bonferroni correction was applied for multiple comparison tests.

Results

Histological findings

Among the 186 patients, 32 (17.2%) biopsy samples revealed normal/benign findings (no CIN or CIN0), whereas 154 (82.8%) samples presented abnormalities, including 51 (27.4%) LSIL/CIN1, 71 (38.2%) HSIL/CIN2/3, and 32 (17.2%) cervical cancer (SCCs group) with FIGO stage Ia1 to IIIb period (**Table 1**).

HPV E6/E7 protein staining

All paraffin sections were tested for HPV-16 E6 protein and HPV-16 E7 protein (**Figure 1**). These experiments were performed by specialized pathologists from the Department of Pathology. HPV E6 protein was found in 47.8% (49/103) of the CIN2+ group and 7.2% (6/83) of CIN1-. Nine CIN1- samples displayed HPV-16 E7 protein (10.8%, 9/83), whereas it was found in 50.5% (52/103) of the CIN2+ samples. The estimates of sensitivity, specificity, PPV, and NPV of the HPV-16 E6 and/or E7 protein staining tests were 65.0% (67/103), 86.7% (72/83), 85.9% (67/78), and 66.7% (72/108), respectively. The outcome of E6/E7 proteins for CIN1- and CIN2+ is shown in **Table 2**.

Table 7. Statistics of cell free ferrous protoporphyrin (FH) scales

Histology result[a] (n)	FH[b]		
	Scale (0-3, mean ± standard deviation)	95% confidence interval	*P* value[c]
Normal (32)	0.11 ± 0.25	0.02-0.20	
LSILs (51)	0.62 ± 0.51	0.48-0.76	2.19×10^{-7}
HSILs (71)	1.89 ± 1.03	1.65-2.14	2.38×10^{-20}
SCCs (32)	2.47 ± 0.84	2.17-2.77	7.77×10^{-16}

[a]LSILs: Low-grade squamous intraepithelial lesions; HSILs: High-grade squamous intraepithelial lesions; and SCCs: Squamous cell carcinomas. [b]Spearman's rho = 0.725, $P = 2.62 \times 10^{-13}$. [c]After Bonferroni correction, the significance level was $\alpha' = 0.017$.

HPV DNA

HPV DNA test had a sensitivity of 96.1% (99/103), specificity of 37.3% (31/83), PPV of 65.6% (99/151), and NPV of 88.6% (31/35) for CIN2+ (HSILs and SCCs). The outcome of the HPV DNA test is shown in **Table 3**.

HPV E6/E7 mRNA

The HPV E6/E7 mRNA assay had a sensitivity of 91.3% (94/103), specificity of 47.0% (39/83), PPV of 68.1% (94/138), and NPV of 81.3% (39/48) for CIN2+ (HSILs and SCCs). The outcome for HPV E6/E7 mRNA assays is shown in **Table 4**.

The expression of the mRNAs was significantly increased in LSILs (7939.4 ± 11282.2, $P = 0.002$), HSILs (73692.6 ± 149851.8, $P = 0.001$), and SCCs (116237.6 ± 149590.5, $P = 0.001$) compared to the Normal samples (552.6 ± 179.9; see **Table 5**). The correlation coefficient between mRNA expression and cancer progress was of Spearman's rho (Normal, LSILs, HSILs, and SCCs) = 0.555 ($P = 0.003$).

FH dye test

The outcome of the FH tests is presented in **Table 6**. The estimates of sensitivity, specificity, PPV, and NPV were 90.3% (93/103), 45.8% (38/83), 67.4% (93/138), and 79.2% (38/48), respectively, for CIN2+ (HSILs and SCCs).

The scales of the FH test were significantly associated with the progress of cervical cancer ($P = 2.62 \times 10^{-13}$, Spearman's rho (Normal, LSILs, HSILs, and SCCs) = 0.725). The scales increased in LSILs (0.62 ± 0.51, $P = 2.19 \times 10^{-7}$), HSILs (1.89 ± 1.03, $P = 2.38 \times 10^{-20}$), and SCCs (2.47 ± 0.84, $P = 7.77 \times 10^{-16}$) compared to the Normal samples (0.11 ± 0.25; see **Table 7**).

All statistical results of the HPV and FH assays for cervical cancer screening are presented in **Table 8**. By ROC curve analysis (**Figure 2**), HPV E6 and/or E7 protein staining was the most accurate test for diagnosing women with HSILs or worse biopsy results among all the tests. The area under the curve (AUC) is 0.759 (95% CI: 0.689-0.829; shown in **Table 8**). For the clinical practice of cervical cancer screening, HPV E6/E7 mRNA detection (AUC = 0.691, 95% CI: 0.612-0.770) and FH tests (AUC = 0.680, 95% CI: 0.601-0.760) are both better performing diagnostic tools than the HPV DNA-based assay (AUC = 0.667, 95% CI: 0.587-0.748).

Discussion

In current study, we screened female Chinese patients with available HPV and cervical epithelial cell stability tests. In total, 151 (81.2%) HPV infections among the 186 subjects were identified with the DNA test, 91.4% (138/151) were detected with mRNA expression, and 51.7% (78/151) with HPV-16 E6 and/or E7 protein staining. The specificity and sensitivity of each test for the risk of high grade dysplasia or worse (HSIL+/CIN2+) were evaluated and compared.

Among the three HPV tests, protein staining produced the highest specificity (86.7%), and the DNA test produced the highest sensitivity (96.1%). Compared to the HPV DNA test, fewer false positives occurred in the HPV mRNA test, suggesting the better diagnostic relevance of the mRNA test for clinical applicability. Other than the HPV tests, the FH test displayed relatively high sensitivity (90.3%) and specificity (45.8%). All of these tests have significant ($P < 0.05$) correlations with the pathological progression of cervical cancer. Among them, FH scales of cervical exudates were the most tightly associated with the development of cervical changes; the correlation coefficients (Spearman's rho (Normal, LSILs, HSILs, and SCCs)) reached up to 0.725. The degree of FH sub-

Table 8. Statistics of the human papillomavirus (HPV) and cell-free ferrous protoporphyrin (FH) assays for cervical cancer screening (detection of high-grade squamous intraepithelial lesion or worse)

Statistics[a]	E6 and/or E7	95% CIs[b]	HPV DNA	95% CI	HPV mRNA	95% CI	FH	95% CI
Sensitivity (%)	65.0	56.3, 74.8	96.1	92.2, 99.0	91.3	85.4, 96.1	90.3	83.5, 95.1
Specificity (%)	86.7	79.5, 94.0	37.3	27.7, 48.2	47.0	36.1, 57.8	45.8	34.9, 56.6
PPV (%)	85.9	77.0, 93.6	65.6	58.3, 72.8	68.1	60.9, 75.4	67.4	59.4, 75.4
NPV (%)	66.7	57.4, 75.9	88.6	77.1, 97.1	81.3	70.8, 91.7	79.2	66.7, 89.6
AUC	0.759	0.689, 0.829	0.667	0.587, 0.748	0.691	0.612, 0.770	0.680	0.601, 0.760

[a]PPV: positive predictive value, NPV: negative predictive value, and AUC: area under the curve. [b]95% CIs: 95% confidence intervals.

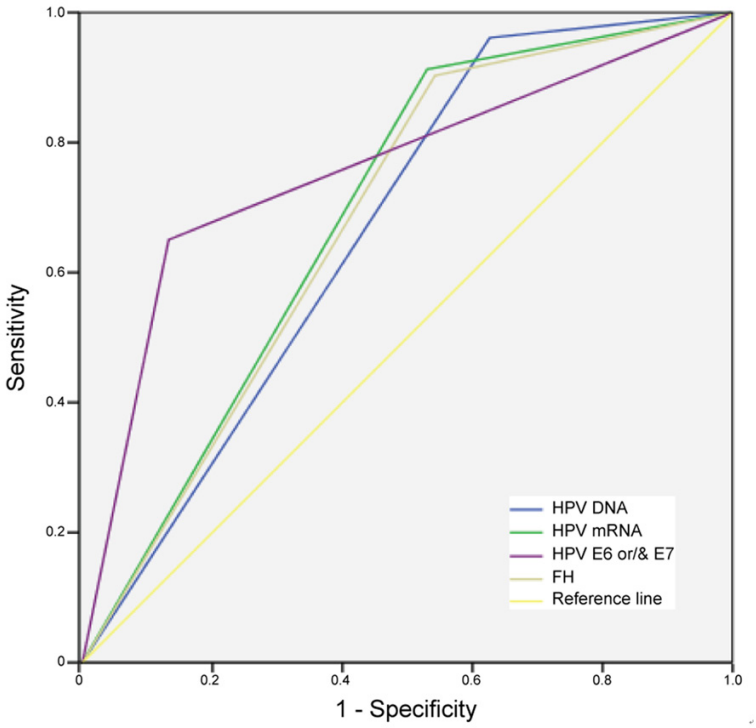

Figure 2. Receiver operating characteristic (ROC) curve for the human papillomavirus (HPV) and cell free ferrous protoporphyrin (FH) tests using the cut-off point of high-grade squamous intraepithelial lesion.

stances steadily increased in cervical exudates from Normal samples to SCCs, indicating that the FH assay may be even better than the HPV mRNA test for cervical cancer screening.

HPV DNA, HPV E6/E7 mRNA, and FH tests are all commercially available methods for use in the clinic. Our findings are in line with other reports that suggest that E6/E7 detection enables a better distinction between transient HPV infection and persistent gene expression that will progress to cervical lesions and cancer [30]. Moreover, we determined that the FH test performs better than HPV detection assays for clinical diagnosis, especially considering that the amount of FH substrate is highly correlated with the severity of cervical changes. However, due to the small sample size of our study, these conclusion need to be verified with a large consecutive cohort analysis in future research.

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Disclosure of conflict of interest

None.

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