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Original Article

Diagnostic Accuracy of p16 Expression in Differentiating Cervical Intraepithelial Neoplasia from Invasive Squamous Cell Carcinoma: A Descriptive Analysis at a Tertiary Care Hospital of Bangladesh

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Abstract

Background: Cervical cancer is one of the most common cancers that affect women worldwide. It is well established that high-risk human papillomavirus (HR-HPV) is the prime risk factor for the development of cervical cancer. Early and accurate diagnosis of cervical neoplasia is of utmost significance for prolongation of patient survival. A panel of immunomarkers has been developed and tested to overcome the limitations to histopathological diagnosis. Among them p16 is one of the commonly used immunomarkers now-a-days. In recent years, routine haematoxylin and eosin (H & E) stain coupled with p16 immunohistochemistry (IHC) have a major impact in the field of uterine cervical pathology and cancer screening.

Objectives: The present study was aimed to determine the p16 scores and its association with histological types, and grades of ISCC including diagnostic accuracy in differentiating ISCC from CIN lesions.

Materials and Methods: This descriptive type of cross-sectional study was conducted in the Department of Pathology, Rajshahi Medical College (RMC), Rajshahi, in collaboration with the Department of Pathology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, during a period of two years from January 2019 to December 2020 by using routine H & E staining and p16 IHC analysis to evaluate the expression of p16 in samples of cervical biopsies from 51 patients with histopathologically confirmed diagnosed cases of CIN and ISCC, considering the percentage of p16 positive cells and the reaction intensity.

Results: This study revealed that the aberrant expression of p16 increased from LSIL to ISCC, thus emphasizing its usefulness as an adjunct marker for predicting risk of developing cervical cancer in the test patients. Most of the cases of ISCC in this study expressed high level of p16, while only one patient with LSIL failed to express the marker. Notably, 90% of LSIL cases were low to moderate p16 positive, 100% of HSIL cases were moderate to high p16 positive, while most of the ISCC cases (81%) had high p16 expression. Among p16 positive cases, this is an attempt to verify direct association between lesion severity and reaction intensity. The frequency of positive cells and the reaction intensity were statistically significant ($P<0.001$) when compared among different histologic types. Most importantly, the present results clearly demonstrated that p16 IHC was capable of differentiating ISCC from CIN cases.

Conclusion: In addition to routine H & E staining and histopathological diagnosis of cervical lesions, p16 immunomarker could be used for risk assessment of histologically detected lesions and for distinguishing between ISCC and CIN specimens which, in turn, could help predict the progression of cervical lesions and thus monitor the screening of cervical cancer in the community.

Keywords: Cervical intraepithelial neoplasia (CIN), Invasive squamous cell carcinoma (ISCC), p16 immunomarker, Immunohistochemistry (IHC), Diagnostic accuracy of p16.

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Introduction

Cervical cancer is the second most common type of cancer in Bangladesh, with approximately 12,000 new cases detected every year, and over 6,000 deaths due to the severity of the disease.[1] According to an estimate, invasive squamous cell carcinomas (ISCC) constitute 80% of cervical cancers in the country.[1-2] ISCC is caused by persistent infections with high risk types of human papillomavirus (HR-HPV), particularly HPV 16 and 18, which encode two potent oncogenes, referred to as E6 and E7, which are required to induce and maintain neoplastic growth of cervical cancer cells.[3] For cervical lesions, however, the following grading system has been endorsed by WHO[4]: low-grade squamous intraepithelial lesion (LSIL) is used for cervical intraepithelial neoplasia CIN1 and high-

grade squamous intraepithelial lesion (HSIL) is used for CIN2 and CIN3. Earlier on, the progression rates of LSIL to HSIL and to ISCC have been documented.[5-6]

The use of p16 immunohistochemistry (IHC) is to increase diagnostic accuracy of dysplastic lesions.[7] The association between the expressions of p16 in cervical lesions and the role of p16 as prognostic markers for persistent HR-HPV infections has been evaluated by a number of findings,[6,8-9] thus implicating the efficacy of p16 immunomarker in the accurate interpretation of cervical biopsies that correlate with corresponding HPV infection status. P16 is mainly a tumour-suppressor protein and an antioncogene that acts on cyclin-dependent kinase (CDK), favouring cell-cycle arrest in the G1 phase.[10] P16 protein and its related gene are associated with HPV infection and its activity has been studied in different cancers including cervical carcinomas[11]. Genomic stress induces enhanced p16 protein expression. This triggers immediate cell cycle arrest. The enhanced expression of the HR-HPV E7 protein triggers a similar oncogenic stress stimulus. This results in increased expression of the p16. However, at the same time, E7 inactivates the cell cycle arresting activity of pRb gene. Thus the final part of the p16 mediated cell cycle arrest mechanism is inactivated and, as a result, the cells continue to proliferate, despite very high level of p16 protein.[12]

Various reports evaluated the potential of p16 IHC in the diagnosis of cervical cancer patients around the world. For example, the prevalence, diagnosis and management of cervical carcinoma in Bangladeshi women have been reported.[2,13-15] In India, samples of CIN and ISCC were diagnosed, analyzed and evaluated by the use of p16 immunomarker.[16-20] In Thailand[6] and Sudan[21], p16 immunochemistry was used to diagnose and classify cervical lesions. In Chinese and Korean women, p16 immunostaining has been proven to be useful for cervical cancer screening.[8-9] Further the efficacies of p16 immunohistochemical marker in the accurate interpretation of cervical biopsies and correlate the data with HPV infection status in China[22-23] and Japan[24] have been reported. Moreover, studies from European countries,[25] Nigeria[26] and Tanzania[27] used p16 immunochemical staining for differentiating cervical cancerous lesions from non-cancerous ones.

In general, we aimed to determine the validity of p16 immunomarker in diagnosing CIN and ISCC in 51 hospital patients at RMC. In addition, the specific objectives of the present study were: (i) to diagnose LSIL, HSIL and ISCC of cervix in biopsy specimens by histopathological examinations; (ii) to find out the intensity of p16 expression in LSIL, HSIL and ISCC cases; (iii) to find out association between p16 expression and histopathological diagnosis; and (iv) to estimate the diagnostic accuracy of p16 in differentiating ISCC from CIN biopsies.

Materials and Methods

This descriptive type of cross sectional study was carried out in the Department of Pathology, RMC, for a period of two years from January 2019 to December 2020. Sample size was determined using Cochran's formula[28-29] and accordingly, 51 cases were incorporated in the study by purposive sampling. Data were collected using a structured questionnaire containing the variables of interest. Women patients (aged 27-80 years; mean±SD 49.27±11.53 years) admitted in the Department of Gynecology and Obstetrics, Rajshahi Medical College Hospital (RMCH), diagnosed clinically and later on histopathologically as cases of CIN, LSIL, HSIL and ISCC were included in this study. Specimens were obtained from patients who had undergone cervical colposcopy directed biopsy and total abdominal hysterectomy with or without bilateral oophorectomy. Patient diagnosed as a case chronic cervicitis and/or other variants of cervical carcinoma and patients who received chemotherapy or radiotherapy before biopsy were excluded. Documents of the patient were obtained from the hospital records. Routine haematoxylin and eosin (H & E) stain was done in the Department of Pathology, RMC. The p16 immunostaining was performed at the Department of Pathology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka. The research protocol was submitted to the Institutional Review Board (IRB), Rajshahi Medical College (RMC) for approval. Subsequently, it was approved by the Ethical Review Committee (ERC) of the college. Ethical issues were dealt using a consent form duly signed by respondents.

Processing of the collected samples:

Paraffin blocks were sectioned in 4 μm thickness. After deparafinization with xylene and rehydration with decreasing graded alcohol, two sections were made for each case: one for H & E stain (Figs. 2, 4 and 6) and the other for immunohistochemical analysis with p16 immunomarker. For IHC, the sections were mounted on poly-L-lysin coated slides. All the microscopy was done and photomicrographs were taken by Olympus multi-headed microscope (Model u-MDO10R3, Olympus Corporation, Tokyo, Japan).

Immunohistochemical analysis:

For IHC, the sections were incubated with a primary monoclonal antibody p16 in appropriate dilutions directed against the p16 antigen. The reaction was considered positive when a chestnut brown colour was seen in the nucleus and/or cytoplasm. The result of p16 was scored by a semi quantitative scoring system. Two parameters were considered; reaction intensity and percentage of p16-positive cells. The nuclear and cytoplasmic scoring systems of the immunomarker were followed using techniques described earlier.[16-17] The final immunoreactive scores of the p16 expression was determined by adding the intensity and proportion of scores of the stained cells, with the minimum score of 0 and a maximum score of 8, where

scores of 0-2 were taken as low, 3-5 as moderate and 6-8 as high. Accordingly, three histologic types of the samples *viz*., LSIL, HSIL and ISCC were identified (Figs. 3, 5 and 7). In addition, three histologic grades of the ISCC namely, Grade-I (well differentiated), Grade-II (moderately differentiated) and Grade-III (poorly differentiated) were recognized and recorded.

Statistical analysis:

Collected data were processed and analyzed with the help of SPSS software for Windows (version 21.0). Descriptive statistics were presented as frequency with corresponding percentage for categorical data and as mean and standard deviation for quantitative data. The significance of p16 expression within the individual histologic types and within histologic grades of ISCC was analyzed using the Fisher's exact test, where P <0.05 was considered as statistically significant. Diagnostic accuracy parameters of the p16 immunomarker in differentiating ISCC from CIN lesions were computed by using the 'gold standard' formulae,[30] where 2×2 contingency table findings of the diagnostic modalities (a, b, c and d) were compared with those of the histopathological diagnosis made on biopsy materials taken from the cervical lesions, as follows: Sensitivity= a÷a+c×100; Specificity= d÷b+d×100; Positive predictive value= a÷a+b×100; Negative predictive value= d÷c + d×100; and Diagnostic efficiency = a+d÷a+b+c+d×100.

Results

The present study was primarily intended to find out the validity of p16 immunomarker in a total of 51 confirmed cases of CIN (n= 20; 39.21%, split into LSIL n=10; 19.61%, and HSIL n=10; 19.61%) and ISCC (n=31; 60.78%). Biopsy materials taken from lesions of the patients' cervix were diagnosed histologically. Scoring of p16 was then made and the findings of the study obtained from data analysis are documented below. It is a headline. Please make it bold and keep some space before the line

The immunohistochemical analysis of p16 has been split into the following four heads: (1) Distribution of p16 scores; (2) Association of p16 expression with histologic types; (3) Association of p16 expression with histologic grades of ISCC; and (4) Diagnostic accuracy of p16 in differentiating ISCC from CIN lesions, which are described in the following paragraphs.

(1) Distribution of p16 scores:

Table 1: Distribution of p16 scores

p16 expression	p16 scores	Frequencies (n)	Percentages (%)
Low	0	01	01.96
	1	00	0.00
	2	01	01.96
Moderate	3	04	07.84
	4	05	09.80
	5	11	21.57
High	6	08	15.69
	7	14	27.45
	8	07	13.73
	Total	51	100.00

Table 1 shows that 56.86% of the samples had high p16 scores between 6 to 8, while moderate scores between 3 to 5 had 39.21% cases and low scores between 0 and 2 represented only 3.92% cases.

(2) Association of p16 expression with histological types:

Table 2: Association of p16 expression with histological types

Histological types	0-2 (Low)	3-5 (Moderate)	6-8 (High)	Total	P value
LSIL	02 (20%)	08 (80%)	00 (00%)	10 (100%)	
HSIL	00 (00%)	06 (60%)	04 (40%)	10 (100%)	<0.001
ISCC	00 (00%)	06 (19%)	25 (81%)	31 (100%)	

Table 2 shows the association of p16 expression with histologic types. Among 10 cases of LSIL (Figs. 1-2), 20% cases had low expression, 80% had moderate expression and none showed high expression of p16. Of another 10 cases of HSIL (Figs. 3-4), 40% cases showed high expression and the rest 60% cases had moderate expression. Out of the remaining 31 cases of ISCC (Figs. 5-6), 25 (81%) cases had high expression and 6 (19%) had moderate expression of p16. The intensity and percentage of cells stained with p16 showed to increase from LSIL to ISCC. Data were analyzed using Fisher's exact test (calculated value= 24.834), where the value clearly demonstrated a highly significant association of p16 expression with the three histologic types (P <0.001).

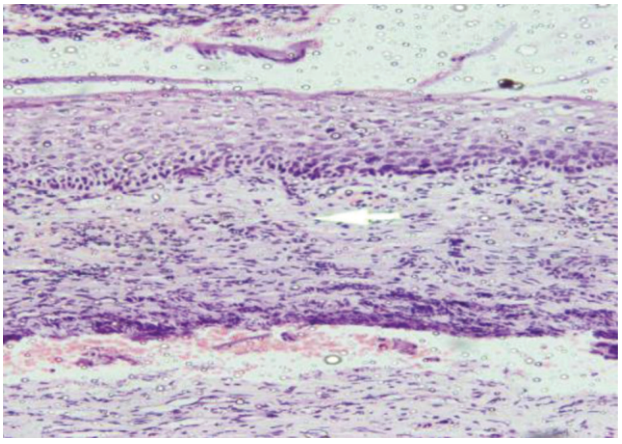

Figure 1: Photomicrograph of a low-grade squamous intraepithelial lesion (LSIL); H & E stain, ×100

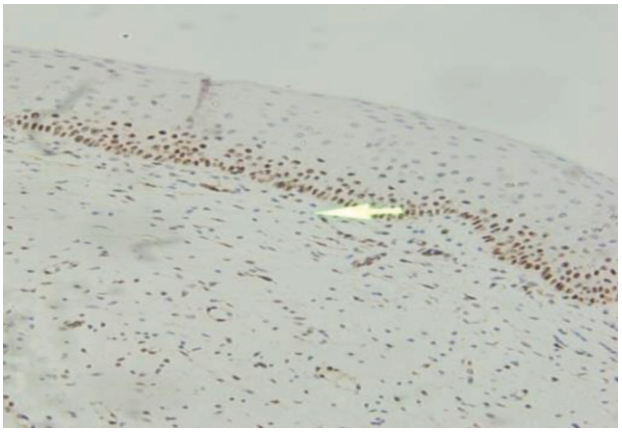

Figure 2: Photomicrograph of a low-grade squamous intraepithelial lesion (LSIL); p16 stain, ×100

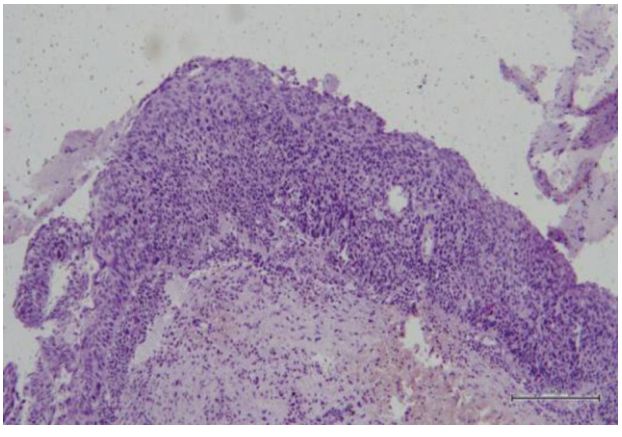

Figure 3: Photomicrograph of a high grade squamous intraepithelial lesion (HSIL); H & E stain, ×100

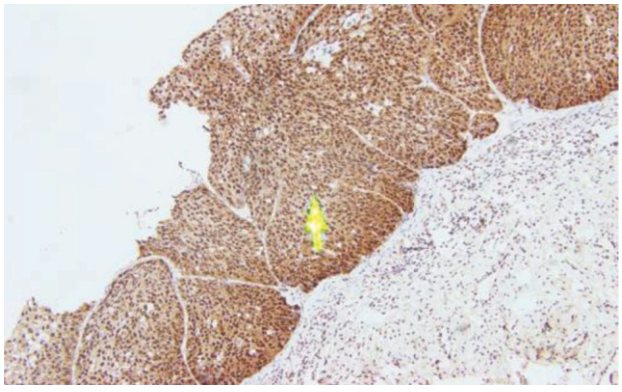

Figure 4: Photomicrograph of a high grade squamous intraepithelial lesion (HSIL); p16 stain, ×100

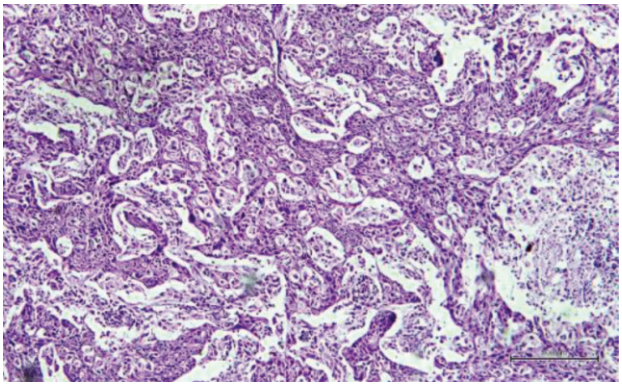

Figure 5: Photomicrograph of an invasive squamous cell carcinoma (ISCC); H & E stain, ×400

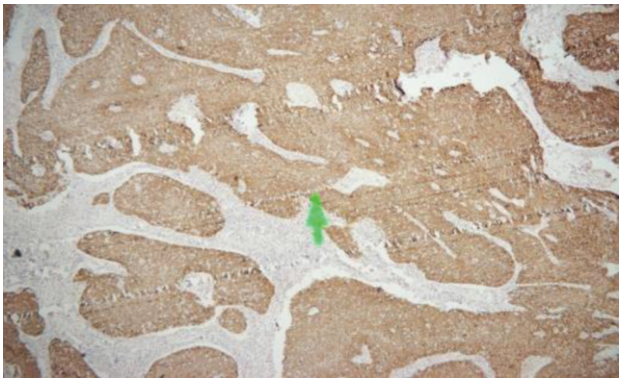

Figure 6: Photomicrograph of an invasive squamous cell carcinoma (ISCC); p16 stain, ×400

(3) Association of p16 expression with histological grades of ISCC:

Table 3: Expression of p16 in histological grades of ISCC

Histological grades	p16 expression		Total	P value
	3-5 (Moderate)	6-8 (High)		
Grade-I	03 (23%)	10 (77%)	13 (100%)	>0.05
Grade-II	02 (18%)	09 (82%)	11(100%)	
Grade-III	01 (14%)	06 (86%)	07 (100%)	

Association of p16 expression with histologic grades of ISCC has been presented in Table 3. Results revealed that out of 13 Grade-I cases, 3 (23%) showed moderate and the rest 10 (77%) cases showed high expression of p16. Of 11 Grade-II cases, however, 2 (18%) showed moderate and 9 (82%) showed high expression of p16 immunomarker. Out of 7 Grade-III cases, remarkably 6 (86%) showed high expression and only 1 (14%) showed moderate expression of p16. But none of the histologic grades showed low expression of p16 immunomarker. The experimental data were subjected to Fisher's exact test, where the calculated value (0.364) did not exceed the statistical significance level ($P > 0.05$).

(4) Diagnostic accuracy of p16 in differentiating ISCC from CIN lesions:

Table 4: Diagnostic accuracy of p16 in differentiating ISCC from CIN lesions

p16 expression	Histopathological diagnosis		Total
	ISCC	CIN	
High	25 (81%)=a	4 (20%)=b	29 (57%)=a+b
Low and moderate	6 (19%)=c	16 (80%)=d	22 (43%)=c+d
Total	31 (62%)= a+c	20 (39%)= b+d	51 (100%)= a+b+c+d

Data presented in Table 4 below demonstrated that 29 out of 51 cases (57%) were labelled as high for p16 expression, whereas 22 cases (43%) were designated as low and moderate for the immunomarker. Thus, the sensitivity of p16 in differentiating ISCC from CIN lesions was 80.65%, while the specificity was 80%. The positive and negative predictive values of the test were 86.20% and 72.72% respectively. Therefore, overall diagnostic efficiency of the test was found to be 80.39%.

Discussion

The present results revealed that 56.86% of the samples had high p16 scores between 6 and 8, while moderate scores between 3 to5 had 39.21% cases and low scores between 0 and 2 represented only 3.92% cases (Table 1). In a study in India, samples of control, CINI, CINII, CINIII and ISCC showed 0%, 50%, 70%, 90% and 100% p16 immunoreactivity, respectively[16] whereas the degree of histological dysplasia with high expression of p16 (75%) was noticed for ISCC but low expression (25%) for LSIL.[17] Again p16 expression was positive in 96% of invasive cancer, 66.6% in HSIL and 37.5% in LSIL.[18] In another study involving 75 cases,[20] p16 immunomarker expression was positive for 67 cases (89.3%), ambiguous for 5 (6.6%) and negative for 3 (4%) cases. These findings are in well agreement with the present results. The expression of p16 in 243 cervical tissues from CIN and cancer patients in Bangkok, Thailand was evaluated and classified into 53 non-dysplastic lesions, 106 CINI, 61 CIN2/3 and 23 ISCC categories, where p16 expression was demonstrated in 91.3% of ISCCs, 78.7% of CIN2/3 and 10.4% of CIN1 lesions[6]. Cervical biopsy reports of 1,154 cases from Beijing, China showed 331 negative cases for dysplasia, 462 positive for CINI, 176 for CIN2, 163 for CIN3 and 22 for ISCC, and there was significant increase in the expression of p16 ($P < 0.001$) from negative to ISCC cases, suggesting that p16 immunohistochemistry improves the diagnostic accuracy of cervical lesions.[8] In a report from the Republic of Korea[9], the efficacy of p16 immunohistochemical marker in the accurate interpretation of cervical biopsies was evaluated and the data were correlated with the HPV infection status. The study revealed that the positivity of p16 increased significantly with the severity of the cervical lesions in patients with HR-HPV infections ($P<0.001$), indicating that the immunomarker was efficient in advancing the diagnostic accuracy of cervical biopsies in such group of people. Apart from minor fluctuations in p16 expressivity values, these findings lend support to those of ours.

In a previous study, positive p16 reactivity was recorded in 80% of CIN3 cases, 83.9% of CIN2 cases, and 97.2% of CIN1 cases in S-W Nigeria,[26] which suggested that the use of p16 immunochemistry would be useful in the evaluation of cervical biopsies for benign mimics of HSIL that could aid proper pathological evaluation and help in patients triaging for follow up. However, the present p16 scores and expression percentages differ slightly from those mentioned above which might have sprang from the differences in the biopsy specimens and protocol for immunohistochemical analysis under study.

In this study, however, the association of p16 expression with histologic grades of ISCC was not statistically significant ($P > 0.05$), even though 9 of 31 cases showed high expression (82%) of the p16 immunomarker. This lends support to a similar report from India,[20] in which there was no statistically significant association ($P=0.877$) between histologic grades of ISCC and p16 expression.

In this study 29 out of 51 cases (56.86%) were labelled as high for p16 expression, whereas 22 cases (43.13%) were designated as low and moderate for the immunomarker. Thus, the sensitivity of p16 in differentiating ISCC from

CIN lesions was 80.65%, while the specificity was 80.00%. The positive and negative predictive values of the test were 86.20% and 72.72%, respectively. Therefore, overall diagnostic efficiency of the test was found to be 80.39%. The use of p16 immunomarker was statistically significant to differentiate between CIN1/LSIL and CIN2, CIN3/HSIL but not between CIN2 and CIN3 cases from Pune, India.[19] In a previous study, p16 positivity increased with histologic severity in 1079 Chinese women attending for cervical cancer screening in China,[22] in which the sensitivity and specificity of the immunomarker to detect CIN2+ in the entire population were 90.9% and 79.5%, respectively, thus emphasizing p16 as an efficient screening tool for detecting underlying cervical pre-cancer and cancer patients. In a recent study of 145 patients from Tanzania,[27] p16 IHC staining yielded 103 (71.0%) positive cases and there was a significant association between histopathological classes and p16 expression levels ($P < 0.001$), where p16 sensitivity and diagnostic accuracy values were 97.2% and 92.8%, respectively in differentiating cervical cancerous lesions from non-cancerous ones. These findings conform nicely to the results of the present study, implying that the immunopositivity of p16 immunomarker increases with the severity of cervical lesions and thus may play an important role in stratification of premalignant and malignant lesions of the cervix in the patients.

Conclusions

The present findings suggest that p16 immunomarker can be used as an adjunct to histopathology that could definitely improve reporting of grades of CIN as well as ISCC cases in cervical cancer screening in the country.

Limitations: Short duration of study and only a single hospital based information.

Conflict of interest: None.

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