

Original Article

Immunohistochemical analysis of proliferating cell nuclear antigen and minichromosome maintenance complex component 7 in benign and malignant salivary gland tumors

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ABSTRACT

Background: Proliferation markers have been used to determine the behavior and prognosis of benign and malignant tumors; this study was aimed to compare the immunohistochemical (IHC) expression of proliferating cell nuclear antigen (PCNA) and novel marker minichromosome maintenance complex component 7 (MCM7) in common salivary gland tumors including pleomorphic adenoma (PA), mucoepidermoid carcinoma (MEC), and adenoid cystic carcinoma (AdCC), to find a possible significant correlation between benign and malignant tumors.

Materials and Methods: In this cross-sectional study, a total of 90 cases, including 30 PAs, 30 MECs, and 30 AdCCs, were collected. The IHC expressions of PCNA and MCM7 were evaluated. Their expressions were compared with each other and between benign and malignant tumors. Statistical analysis was performed by Chi-square and Tukey's test. *P* value was considered 0.05.

Results: Out of 30 cases of PA, 28 cases (93.3%) were PCNA positive and 28 cases (93.3%) were MCM7 positive. In the AdCC cases, 29 cases (96.6%) were PCNA positive and 29 cases (96.6%) were MCM7 positive. In the MEC cases, all cases (100%) were PCNA positive and 23 cases (76.6%) were MCM7 positive. The labeling index (LI) of MCM7 and PCNA was evaluated, and this index was lower in MCM7 LI than PCNA in all tumors. The MCM7 and PCNA expression showed a significant difference in PA and MEC ($P < 0.001$).

Conclusion: PCNA expression was higher than MCM7 expression in salivary gland tumors. However, more studies are needed to evaluate the malignant activity of these tumors with group of markers such as MCM family members.

Key Words: Adenoid cystic carcinoma, minichromosome maintenance complex component 7, mucoepidermoid carcinoma, pleomorphic adenoma, proliferating cell nuclear antigen

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INTRODUCTION

The prevalence of salivary gland neoplasms is about 5% among the benign and malignant head-and-neck tumors.^[1] These tumors demonstrate variable

histopathological and also clinical characteristics, so their early diagnosis and treatment are crucial.^[2] Pleomorphic adenoma (PA) or benign

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mixed tumor is a benign neoplasm showing some degrees of morphological variations. It is the most common epithelial tumor (about 60%) in salivary glands.^[3] Its age of occurrence is approximately 30–50 years. It presents with a minor preference in women.^[4] Mucoepidermoid carcinoma (MEC) is one of the most common malignant salivary gland tumors and is the most common malignant salivary gland tumor in children. This tumor occurs more often in the 2–7 decades of life, is more common in the parotid gland, and is usually seen as an asymptomatic swelling. Adenoid cystic carcinoma (AdCC) is one of the most common (10%–25% of salivary gland tumors) and well-known salivary gland malignancies.^[5] It is more common in minor salivary glands and its age of occurrence is about 50–60 in the life cycle.^[4] At present, there are no detectible risk factors which lead to early diagnosis of these malignancies.^[6]

Proliferating cell nuclear antigen (PCNA) is a nuclear protein which helps delta DNA polymerase in DNA replication as a sliding clamp.^[7] This protein has a high concentration in late G1 phase and early S phase. It falls in the G2 and M phases of cell proliferation cycle.^[8]

Minichromosome maintenance complex component family (MCM2-MCM7) includes important binding proteins with a critical role in the initiation and progression of DNA replication. They also participate in controlling the cell cycle periodicity.^[9,10] They are highly expressed in the G1 and S phases. Then, the expression is decreased gradually and may not be even detectable in the G0 phase.^[11] It is implied that MCM expression is amplified in the proliferative cells, while they are deficient in the differentiated cells, suggesting that they may be useful as proliferative markers.^[12] The detected expression of MCM proteins in dysplastic and malignant cells indicates that these proteins can be used for detecting some carcinomas in clinical settings.^[13-15] Previous studies have recommended MCM7 as a diagnostic and prognostic marker in oral squamous cell carcinoma (SCC) and esophageal SCC.^[16-18] However, comparative MCM7 expression has not been evaluated between benign and malignant salivary gland tumors.

Dysregulations in cell cycle result in abnormal cell proliferation in tumorigenesis, and constant DNA replication plays an important role in the occurrence of neoplasms.^[19] Proteins with a role in cell cycle

are involved in various biological procedures. For instance, neoplasm formation or its progression can be used as proliferation markers to predict the biological behavior of tumors or to differentiate benign and malignant tumors.^[13] It has been reported that MCM2-7 plays a role in replicating genome only once in each cell cycle.^[20] The function of MCM proteins in the cell cycle regulation and DNA replication and its probable relation with pathological features, diagnosis, or prognosis of neoplasms may suggest it as a new marker of cell proliferation.^[21] PCNA has also been mentioned as a prognostic marker in salivary gland tumors in earlier studies,^[22-24] and the expression of MCM7 has only been evaluated in AdCC but not in other benign and malignant salivary gland tumors.

Detection of novel biomarkers in relation with the progression and invasion of tumors can assist in developing drugs which target molecular markers in these tumors. The aim of this study was to compare MCM7 and PCNA expression to evaluate a possible correlation in proliferation progress between benign and malignant salivary gland tumors and between the marker's expression and histologic grade and metastasis in MEC and AdCC.

MATERIALS AND METHODS

Specimen selection

The samples of this cross-sectional study were collected from 90 formalin-fixed, paraffin embedded tissue blocks of PA, MEC, and AdCC (thirty cases from each of them) obtained from the archives of the Pathology Department of Amir-Alam Hospital, Tehran University of Medical Sciences, Tehran, Iran. The study was approved by the Ethics Committee (ethical code: IR.SBMU.RIDS.REC.1394.66) of Dental School at Shahid Beheshti University.

The MEC samples were graded according to Auclair *et al.*^[25] and AdCC and classification of Cho *et al.*^[26] The numbers of all grades of MEC, AdCC, and metastatic cases are presented in Table 1.

Hematoxylin and eosin slides were reviewed in order to confirm the previous diagnosis. The clinicopathologic information of each case, including age, sex, tumor location, and histologic grades, was obtained from the patients' records and by reviewing the slides. Samples with incomplete data, insufficient paraffin-embedded tumor material, inappropriate fixation, and incisional biopsy were excluded.

Table 1: Characteristics of pleomorphic adenoma, mucoepidermoid carcinoma, and adenoid cystic carcinoma in cases

Variables	PA	MEC	AdCC	
Sex				
Male	19	16	6	
Female	16	14	24	
Age (mean)	38.16±16.35	40.63±20.90	43.73±15.43	
Site of tumor				
Palate	18	1	2	
Parotid	5	22	19	
Alveolar mucosa	3	0	6	
Sublingual	0	1	0	
Submandibular	1	1	1	
Tongue	0	2	1	
Hard palate	5			
Flour of the mouth	0	1	0	
Cheek	1	2	1	
Upper lip	2	0	1	
Histopathologic grade		High	Solid	5
		Moderate	Tubular	6
		Low	Cribriform	19
Size (cm)				
Range	1-4.5	1-7.5	0.7-7.5	
Mean	2.02	5.19	3.01	
Lymph node metastasis		3	2	

PA: Pleomorphic adenoma, MEC: Mucoepidermoid carcinoma, AdCC: Adenoid cystic carcinoma

Immunohistochemistry

For all specimens, μ 4 sections were cut and mounted on the silane-coated slides. The sections were deparaffinized with 100% xylene and rehydrated in graded ethanol series. They were then immersed in tris-buffered saline (TBS) of PH 6 and heated in a microwave oven at 750 watts for antigen retrieval. After cooling into room temperature, the sections were incubated with primary antibody MCM7 (Monoclonal mouse Anti-Human, Thermo scientific, Fremont, CA, USA) and PCNA (Monoclonal Mouse Anti-Human, Thermo Scientific, Fremont, CA, USA) at 1:2000 for an hour. After washing in TBS, the sections were treated with Dako Envision.

Diaminobenzidine chromogen was used to visualize the antibody expression, which was then counterstained with Meyer's hematoxylin. Oral SCC was used as positive control for both antibodies. For negative control, normal saline was used instead of the primary antibody.

Evaluation of immunohistochemistry

To evaluate the labeling index (LI), the percentage of positive nuclei was taken from 1000 tumor cells at \times 400 magnification and regarded as the LI. These stained cells were evaluated in five microscopic fields, which illustrated more intense staining. Furthermore,

the intensity of staining was evaluated as follows: 0 = negative, + = mild, ++ = moderate, and +++ = strong.^[27]

Statistical analysis

Statistical analysis was carried out on the tabulated data using SPSS-18 software (SPSS Inc., Chicago, IL, USA). MannWhitney, Chi-square, Bonferroni, and Tukey Honestly Significant Difference (HSD) tests were used for data analysis. The significance level of all tests was set as $P < 0.05$.

RESULTS

The general characteristics of 90 patients included in the present study are shown in Table 1.

PCNA was expressed in 28 (93.3%) PA samples, 29 (96.6%) AdCC samples and all MEC samples (100%). MCM7 was expressed in 28 (93.3%) PA cases, 29 (96.6%) AdCC cases, and 23 (76.6%) MEC cases.

The LI was lower in MCM7 than in PCNA for all three tumors, it means that the mean of stained cell nuclei in expressed samples of MCM was lower than PCNA samples. These differences were significant for the MEC and PA [$P < 0.001$; Table 2].

In PCNA, LI showed a significant difference between PA and AdCC ($P < 0.001$) and between MEC and AdCC [$P < 0.001$; Table 2].

The Tuckey-HSD test also indicated a significant difference in MCM7 expression between MEC and AdCC ($P = 0.001$).

In our study, of 30 MEC samples, 19 samples were of low grade, 7 samples were of intermediate grade, and 4 samples were of high grade [Table 1] PCNA LI showed no difference between histologic grades of MEC. It was the same for MCM7 LI [Table 3].

In this study, 19 AdCC samples showed cribriform pattern, 6 samples tubular and 5 samples were solid. There was no significant difference in PCNA LI between the histological grade of AdCC and it was the same for MCM7 LI [Table 4]

Data analysis showed no significant correlation of PCNA and MCM7 LI between each histological grades of AdCC [$P = 1.0$, $P = 0.2$ and $P = 0.6$; Table 3].

However, all the histological grades of MEC showed a lower expression in MCM7 than in PCNA [$P < 0.001$; Table 3].

Furthermore, there was not a significant difference between MCM7 and PCNA expression and lymph node metastasis in AdCC and MEC ($P > 0.05$).

Expression of MCM7 was different in components of MEC cases [Table 5].

The MCM7 and PCNA expressions in the cell nuclei of PA, MEC, and AdCC are demonstrated in Figures 1-6.

DISCUSSION

The routine gold standard of diagnosis in salivary gland tumors is histopathologic evaluation.^[1] Sometimes, using a proper biologic marker with immunohistochemistry method can be helpful for definitive diagnosis or predicting the aggressiveness and behavior of tumors. Some proliferation and biologic markers have been utilized as diagnostic and prognostic markers. Some of the previous studies have introduced MCM proteins as novel biologic markers for proliferating cells and tumors.^[28] Considering the evidence that MCM proteins have a critical role in DNA replication and cell cycle control,^[9,28] this study was designed to examine whether MCM7 proteins

Table 2: Minichromosome maintenance complex component 7 and proliferating cell nuclear antigen labelling index in pleomorphic adenoma, mucoepidermoid carcinoma, and adenoid cystic carcinoma

Salivary gland tumor	PA	MEC	AdCC
MCM7 LI	22.16±16.90%	12.80±23.06%	36.16±21.55%
PCNA LI	80.33±26.25%	88.36±17.96%	37.70±29.43%
<i>P</i>	<0.001	<0.001	0.417

PA: Pleomorphic adenoma, MEC: Mucoepidermoid carcinoma, AdCC: Adenoid cystic carcinoma, LI: Labeling index, PCNA: Proliferating cell nuclear antigen, MCM7: Minichromosome maintenance complex component 7

Table 3: Minichromosome maintenance complex component 7 and proliferating cell nuclear antigen labeling index in histopathologic grade of mucoepidermoid carcinoma

Grades MEC	Low grade	Intermediate grade	High grade
MCM7 LI	12.16%±22.35	14.71%±27.46	12.50%±25.00
PCNA LI	85.42%±22.03	94.71%±3.49	91.25%± 4.78
<i>P</i>	<0.001	<0.001	<0.001

MEC: Mucoepidermoid carcinoma, MCM7: Minichromosome maintenance complex component 7, LI: Labeling index, PCNA: Proliferating cell nuclear antigen

Table 4: Minichromosome maintenance complex component 7 and proliferating cell nuclear antigen labeling index in histopathologic grade of adenoid cystic carcinoma

AdCC grades	Tubular	Cribriform	Solid
MCM7 LI	43.33%±22.28	26.84%±21.48	26.00%±21.03
PCNA I	43.33%±31.25	40.79%±21.48	19.20%±10.52
<i>P</i>	1.000	0.220	0.631

AdCC: Adenoid cystic carcinoma, MCM7: Minichromosome maintenance complex component 7, LI: Labeling index, PCNA: Proliferating cell nuclear antigen

Table 5: Minichromosome maintenance complex component 7 and proliferating cell nuclear antigen labeling index in components of mucoepidermoid carcinoma

MEC components	IHC marker	
	MCM7	PCNA
Mucous cells (%)	0.07	87.87
Epidermoid cells (%)	12.77	88.77
<i>P</i>	<0.001	0.592

MEC: Mucoepidermoid carcinoma, MCM7: Minichromosome maintenance complex component 7, PCNA: Proliferating cell nuclear antigen, IHC: Immunohistochemical markers

could be more useful proliferative and diagnostic markers than PCNA in salivary gland tumors or not.

In this study, 93.3% of PA, 96.6% of AdCC, and 100% of MEC samples showed PCNA expression, which was in line with the study of Gordón-Núñez

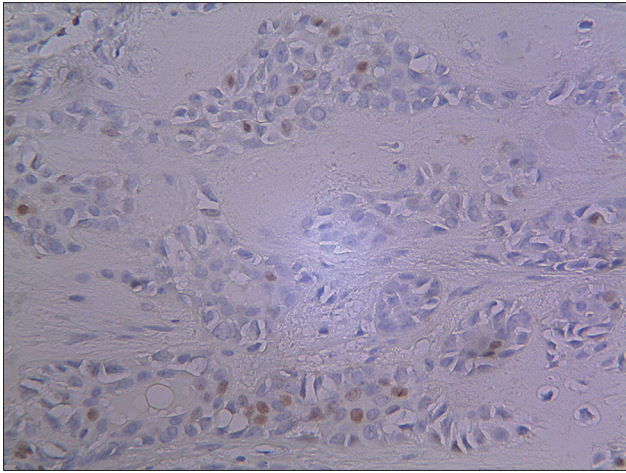


Figure 1: Minichromosome maintenance complex component 7 expression in cell nuclei of pleomorphic adenoma (×400).

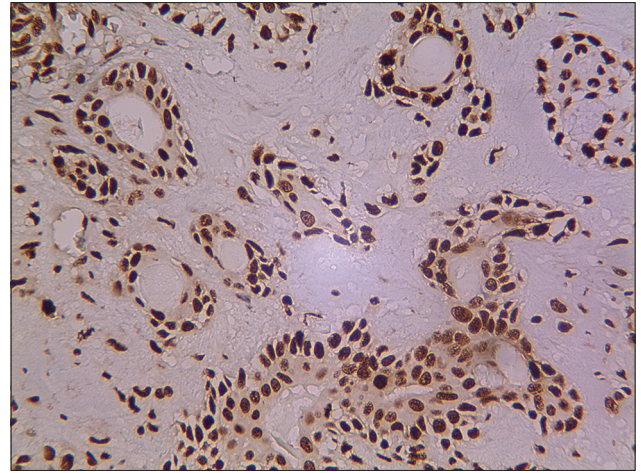


Figure 4: Proliferating cell nuclear antigen expression in cell nuclei of pleomorphic adenoma (×400).

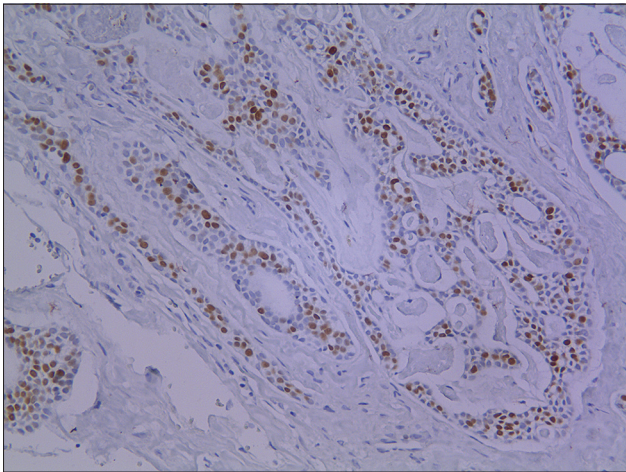


Figure 2: Minichromosome maintenance complex component 7 expression in cell nuclei of adenoid cystic carcinoma (×200).

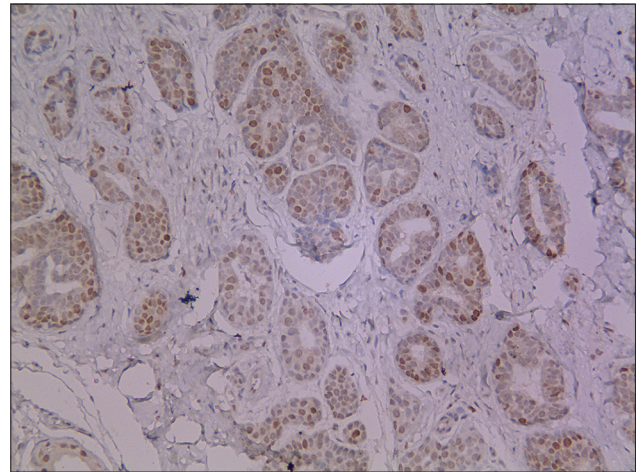


Figure 5: Proliferating cell nuclear antigen expression in cell nuclei of adenoid cystic carcinoma (×200).

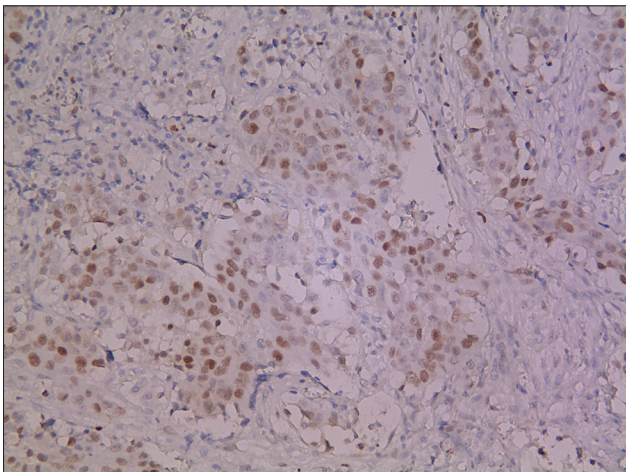


Figure 3: Minichromosome maintenance complex component 7 expression in cell nuclei of mucoepidermoid carcinoma (×200).

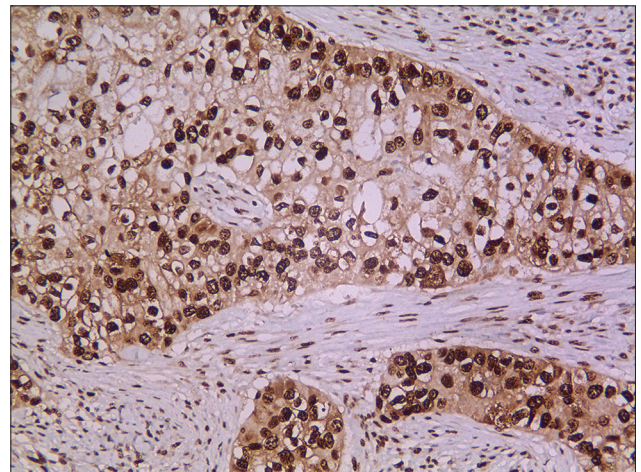


Figure 6: Proliferating cell nuclear antigen expression in cell nuclei of mucoepidermoid carcinoma (×200).

et al.^[29] and contrary to the studies of Russo *et al.*^[30], Perez *et al.*^[31] and Alves *et al.*,^[24] in which the PCNA

marker was expressed in all samples. The reason of contradiction could probably be due to the smaller

number of samples in the above studies (3 cases in Russo's study, 1 case in Perez's study, and 15 items in Alves's study) and different staining evaluation methods.

The LIs of PCNA in PA, MEC, and AdCC samples were 80.33%, 88.36%, and 37.75%, respectively. In our study, the LI of PA was less than that of MEC, but it was not statistically significant. Former studies have compared the percentage of stained tumor samples with each other not their LI (as it is referred to as the mean number of stained cells). Using this method, if few cells are stained in one sample, it is the same as the samples which are stained in almost all cells, so it is better to suggest a standard method for counting and comparing the stained cells with biomarkers to be more accurate and comparable with other studies. There was a significant difference between AdCC, MEC, and PA, which was consistent with the results of Russo *et al.*^[30] Russo *et al.* reported that PCNA expression was higher in poorly differentiated tumors, but AdCC and PA were not directly compared with each other. They compared all malignant salivary gland tumors with all benign tumors. Furthermore, the number of AdCC samples was much lower than that of PAs (3 AdCC/12 PA).

There were no statistically significant differences between different histopathologic variants in terms of PCNA expression. These results were consistent with the results of Cho *et al.*,^[26] which showed the higher expression of PCNA in the solid regions, but this difference was not statistically significant.

There was no statistically significant difference in the PCNA expression among the histopathologic variants of MEC, which was in contrast to the results of Alves *et al.*,^[24] Cardoso *et al.*,^[22] and Hicks and Flaitz.^[32] In all these studies, there a small sample size was recruited.

In this study, 93.3% of PA samples showed the MCM7 expression. About 76.6% of MEC samples and 96.6% of AdCC samples showed the expression of this marker. In this study, the LI of the MCM7 samples of PA, MEC, and AdCC were 22.16%, 12.80%, and 33.16%, respectively. Significant differences were observed in the expression of this marker between MEC and AdCC. In MEC samples, all epidermoid components showed a significant difference in MCM7 expression. In previous studies, the MCM7 marker expression has been higher in OSCC and SCC esophageal cancer than in dysplasia and normal

tissue.^[16,33,34] The reporting methods in these studies are different, and most of them have reported the number and percent of cases which showed expression not the LI. Until now, MCM7 has not been studied in the salivary gland tumors; it has only been investigated in AdCC.

Our results showed no significant difference in MCM7 expression with histopathologic grades between MEC and AdCC.

In general, in this study, the LI was significantly lower in MCM7 than in PCNA in salivary gland tumors. MCM proteins remain inactive in the G1 phase, until the S phase is activated to start replication. From the G1 phase to S phase, the MCM undergoes phosphorylation, and these changes help subsequent gathering of other replication members, while PCNA is observed in late G1 phase and in S phase. Its expression increases in these phases and then decreases in phase G2-M, but PCNA is still observed in these phases.^[35-37] PCNA is involved in both DNA replication and repair, which can lead to its presence in large quantities even in nonproliferating cells. Unlike PCNA, MCM proteins have a role only in the DNA replication process.^[38] The longer fluctuation and presence of PCNA during the cell cycle might be the cause of its higher expression than MCM7, which is more stable during the cell cycle.^[38]

In our study, no significant difference was seen in lymphatic metastasis between MCM7 and PCNA expression. Since the number of metastasis samples was low, it was not possible to make a proper conclusion.

It seems that it would be more precise if there were more cases to evaluate the difference between the grades of MEC and histologic types of AdCC. Further studies are suggested to include some cases of recurrent PA and compare the differences between the PA cases with and without recurrent PAs. With all controversies in former studies, it has been concluded that the prognostic significance of the whole MCM family is more accurate than that of one MCM protein individually.^[39]

CONCLUSION

Low expression of MCM7 in common salivary gland tumors and absence of relationship with malignancy in these tumors may suggest more studies on MCM family to find a more proper diagnostic marker in these

tumors. MEC cases showed a different expression of MCM7 in their components, and the expression was very low. Therefore, further studies are suggested to be conducted on this tumor. According to previous studies on PCNA, this marker seems to be more reliable in salivary gland tumors.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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