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The synergic effect of BRAF^{V600E} mutation and multifocality on central lymph node metastasis in unilateral papillary thyroid carcinoma

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Abstract. The purpose of this study is to evaluate the potential synergic effect of BRAF^{V600E} mutation and multifocality on central lymph nodes metastasis (CLNM) in the patients with unilateral papillary thyroid carcinoma (PTC). We enrolled 413 patients with unilateral PTCs who accepted prophylactic unilateral or bilateral central lymph node dissection (LND). Univariate and multivariate analyses were made to determine the association between related factors and CLNM. Then, all patients were divided into 4 groups based on their status of BRAF^{V600E} mutation and multifocality. Relative excess risk of interaction (RERI), attributable proportion (AP) of interaction and synergy index (SI) were applied to evaluate the interactive effect of these two factors on CLNM. Results showed that BRAF^{V600E} mutation and multifocality were independent risk factors for CLNM. A further study revealed that unilateral PTCs accompanying multifocality with BRAF^{V600E} mutation had the highest incidence of CLNM compared with other subgroups. Besides, RERI was 4.323 (95% CI = 1.276–7.369), AP was 0.523 (95% CI = 0.364–0.682) and SI was 2.469 (95% CI = 1.607 to 3.794), indicating a significant additive interaction of BRAF^{V600E} mutation and multifocality on CLNM. The present study has confirmed that BRAF^{V600E} mutation and multifocality are risk factors for CLNM in unilateral PTC. Additionally, unilateral PTC patients accompanying multifocality with BRAF^{V600E} mutation may have an increased risk of CLNM in clinically negative CLNM.

Key words: Papillary thyroid carcinoma, BRAF^{V600E} mutation, Multifocality, Central lymph node metastasis, Prophylactic lymph node dissection

THYROID CARCINOMA has become increasingly prevalent in recent years. In addition, papillary thyroid carcinoma (PTC), which is the most common histological type of thyroid cancer, has taken on a rapidly increasing incidence worldwide [1]. Fortunately, most PTC patients have relatively excellent clinical outcomes after surgery. However, cervical lymph node metastasis (LNM) is observed in 40~90% of patients in the first operation [2]. As is well known, LNM, which usually follows a regular pattern in PTC, is commonly found in Level VI. Then, it metastasizes to lateral compartment. Defined as the metastasis found in the lateral neck without central LNM, skip lesions are rare [3]. Central neck compartment (Level VI) is the most common occurrence site LNM, which firstly metastasizes to ipsilateral central compartment and then reaches contralateral counterparts in unilateral PTC. In fact, LNM has been proved to be an independent factor for regional recurrence [4-6]. Moreover, some studies have even reported that central lymph

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Standard Abbreviations: PTC, Papillary thyroid carcinoma; PTMC, Papillary thyroid microcarcinoma; LNM, Lymph node metastasis; CLNM, Central lymph node metastasis; LND, Lymph node dissection; CLND, Central lymph node dissection; RERI, Relative excess risk; AP, Attributable proportion; SI, Synergy index; HT, Hashimoto's thyroiditis

node metastasis (CLNM) is significantly associated with decreased survival [7-9]. It is acknowledged that therapeutic central lymph node dissection (CLND) may be beneficial for clinically suspicious CLNM. Nevertheless, it is controversial whether prophylactic CLND should be performed for clinically negative central compartment.

$BRAF^{V600E}$ mutation is widely accepted as a highly specific molecular marker for PTC [10]. Several studies have shown the positive relationship between $BRAF^{V600E}$ mutation and other aggressive factors, such as extrathyroidal extension, LNM, high TNM stages, and recurrence [11-13]. Thus, $BRAF^{V600E}$ mutation is considered as a powerful biomarker for predicting poor prognosis. Multifocality also frequently exhibits in PTC. Certain studies have indicated that multifocal carcinoma in PTC is positively associated with the incidence of CLNM [14, 15], while other studies have argued that there is no association between multifocality and CLNM [16]. In addition, multifocality has found to significantly increase the incidence of recurrence [17].

Some studies have investigated the relationship between $BRAF^{V600E}$ mutation and multifocality in PTC. Based on these study reports, the PTC that coexists with $BRAF^{V600E}$ mutation is more likely to have multifocality [18]. Nonetheless, most of those studies only explored the relationship between $BRAF^{V600E}$ mutation and multifocality, and the studies focused on the combined interaction of $BRAF^{V600E}$ mutation and multifocality on CLNM in PTC are still rare. Therefore, this study mainly aims to investigate the interactive effect of $BRAF^{V600E}$ mutation and multifocality on CLNM in unilateral PTCs.

Materials and Methods

Study population patients enrolling criteria

In our hospital, 413 patients were pathologically diagnosed to have PTC in unilateral thyroid. All these patients underwent ultrasound examination preoperatively, and none of them showed any sign of CLNM. Consequently, they all accepted total thyroidectomy or hemithyroidectomy accompanying prophylactic unilateral or bilateral CLND during the period between January, 2012 and February, 2015. Specifically, a total of 288 (69.7%) patients accepted total thyroidectomy. The rest 125 (30.3%) patients, who presented no evidence of nodule in their contralateral thyroid according to preoperative ultrasound and intraoperative exploration, underwent hemithyroidectomy. Meanwhile, 132 (32.0%) patients accepted bilateral CLND, and the others with unilateral CLND received routine intraoperative exploration on contralateral central compartment. The patients enrolled in this study had no history of thyroid or neck surgery, as well as other head or neck cancers. In this study, multifocality was defined as the presence of two or more tumour foci in unilateral thyroid. The results were identified by experienced pathologists through final pathological examination. We collected the pathological features, including Hashimoto's thyroiditis (HT), extrathyroidal extension, full tumor encapsulation, capsular invasion and lymphovascular invasion, which were identified by final pathological examination. As multifocal PTCs were found in the specimens, the dominant nodule was analysed. This study was approved by the Ehics Committee of Taizhou Cancer Hospital.

DNA isolation and $BRAF^{V600E}$ mutation analysis

Standard phenol–chloroform extraction and ethanol precipitation procedures were utilized to extract DNA from fresh thyroid samples. The forward and reverse primers of BRAF exon 15 were 5'-TCATAATGCTT GCTCTGATAGGA-3' and 5'-GGCCAAAAATTTAAT CAGTGGA-3' respectively. Polymerase chain reaction (PCR) was performed. We started with the primary denaturation step at 94°C for 3 min, which was followed by 35 cycles of denaturation at 94°C for 30 s. Then, we annealed the samples at 55°C for 30 s, and elongated them at 72°C for 1 min, which was followed by a final elongation step at 72°C for 5 min. To evaluate the quality of the PCR products, we utilized 2% agarose gel electrophoresis. The $BRAF^{V600E}$ mutation was confirmed through sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) on an ABI PRISM 3730XL DNA Analyzer (Applied Biosystems, Foster City, USA). In the present study, the $BRAF^{V600E}$ mutation status of multifocal PTCs was determined by the dominant tumor.

Statistical analysis

Categorical data were compared through χ^2 and Fisher exact tests, whilst continuous data were compared *via* independent two-sample *t* test. Logistic regression analysis was performed to estimate the odds ratios (OR) of certain parameters for CLNM. The results were presented as the OR with 95 % confidence interval (95% CI) and *p* value.

Logistic regression analysis and 95% CI were made to estimate the interaction between $BRAF^{V600E}$ mutation and multifocality in association with CLNM, and ad-

justed for tumor size, age, sex, HT, extrathyroidal extension, capsular invasion, full tumor encapsulation and lymphovascular invasion. Meanwhile, relative excess risk (RERI), attributable proportion (AP), and synergy index (S) were utilized to evaluate the interactive effect of $BRAF^{V600E}$ mutation and multifocality on the presence of CLNM in PTCs. RERI was applied to assess the excess risk attributed to the interaction relative to the risk without exposure. AP was used to measure the attributable proportion of the CLNM, which was caused by an interactive effect in patients exposed to those two factors. SI represents the excess risk resulted from the exposure to those two factors, when there is a biological interaction relative to the risk from the exposure to them without interactive effect. When there is no additive interaction, the 95% CI of RERI and AP include 0, or S includes 1. RERI > 0, AP > 0, or S > 1 is considered to signify the existence of biological interaction [19]. SPSS statistical software (version 18.0) was used for all the analyses. All tests were significant when $p < 0.05$.

Results

Comparison of clinicopathological factors between the unilateral PTCs with and without CLNM

The mean tumor size was 13.45 ± 10.2 mm, which ranged from 2 to 52 mm. Table 1 shows the clinicopathological factors of the 413 patients. There were 107 (25.9%) patients with HT, 111 (26.9%) patients with multifocality, 75 (18.2%) patients with extrathyroidal extension, 166 (40.2%) patients with CLNM and 209 (50.6%) patients with $BRAF^{V600E}$ mutation. Based on the comparison results of those clinicopathological factors between the patients with and without CLNM, the patients with a larger tumor size are more likely to develop CLNM ($p < 0.001$). Similarly, tumor size <10 mm is inversely associated with occurrence of CLNM ($p < 0.001$). HT and full tumor encapsulation seem to be negatively associated with CLNM ($p = 0.016$ and $p = 0.003$, respectivly). Multifocality, extrathyroidal extension and $BRAF^{V600E}$ mutation are positively associated with the presence of CLNM ($p < 0.001$, $p = 0.029$ and $p < 0.001$, respectively). It is also found that capsular inva-

Table 1 The relationship between clinicopathological factors and CLNM in Unilateral PTC

Characteristics	Total Number	CLNM Positive	CLNM Negative	*p* value
Total number	413	166 (40.2)	247 (59.8)	
Tumor size (mm)	13.45 ± 10.2	14.12 ± 9.5	12.31 ± 10.5	<0.001*
<10 mm	242 (58.6)	76 (45.8)	166 (67.1)	<0.001*
≥10 mm	171 (41.4)	90 (54.2)	81 (32.9)	
Age (years)	45.55 ± 11.81	44.21 ± 10.9	46.23 ± 12.1	0.514
<45 years	242 (58.6)	94 (56.6)	148 (59.9)	0.572
≥45 years	171 (41.4)	72 (43.4)	99 (40.1)	
Sex				0.387
Female	313 (75.8)	130 (78.3)	183 (74.1)	
Male	100 (24.2)	36 (21.7)	64 (25.9)	
HT	107 (25.9)	32 (19.3)	75 (30.4)	0.016*
Multifocality	111 (26.9)	61 (36.7)	50 (20.2)	<0.001*
Extrathyroidal extension	75 (18.2)	39 (23.5)	36 (14.6)	0.029*
$BRAF^{V600E}$ mutation	209 (50.6)	103 (69.9)	106 (51.0)	<0.001*
Capsular invasion	104 (25.2)	53 (31.9)	51 (20.6)	0.013*
Full tumor encapsulation	80 (19.4)	20 (12.0)	60 (24.3)	0.003*
Lymphovascular invasion	73 (17.7)	40 (24.1)	33 (13.3)	0.007*

Note: HT = Hashimoto' thyroiditis; CLNs = central lymph nodes; CLNM = Central lymph node metastasis; PTC = Papillary thyroid carcinoma. *represent the *p* value <0.05.

Table 2 Multivariate Analysis of Central Lymph Node Metastases in Unilateral PTCs

	OR	95% CI	*p* value
Age	1.412	0.823–2.633	0.312
Sex	0.761	0.552–1.725	0.352
Tumor size (mm)	1.426	1.163–1.685	0.031*
HT	0.831	0.532–1.324	0.298
Multifocality	2.681	1.261–4.287	0.003*
Extrathyroidal Extension	1.821	1.301–3.118	0.015*
$BRAF^{V600E}$ mutation	4.314	1.521–9.014	0.001*
Capsular invasion	1.623	1.421–2.981	0.018*
Full tumor encapsulation	0.412	0.264–0.672	0.008*
Lymphovascular invasion	1.782	1.275–3.689	0.012*

Note: HT = Hashimoto' thyroiditis; PTC = Papillary thyroid carcinoma. *represent the *p* value <0.05.

sion and lymphovascular invasion have a strong correlation with CLNM ($p = 0.013$ and $p = 0.007$, respectively). Besides, there is no other clinicopathological feature associated with CLNM.

To further evaluate the relationship between those clinicopathological factors and CLNM, a multivariate analysis was performed with the adjustment of age, tumor size, gender and other statistically significant factors presented in Table 1. The results given in Table 2 show that multifocality and $BRAF^{V600E}$ mutation are independent risk factors for CLNM (OR = 2.681, $p = 0.003$; OR = 4.314, $p = 0.001$). In addition to those two factors, tumour size ≥10 mm, extrathyroidal extension, capsular invasion and lymphovascular invasion are also risk factors for CLNM, while full tumor encapsulation is a protective factor.

Interactive effect of $BRAF^{V600E}$ mutation and multifocality on CLNM

To deeply investigate the interaction of $BRAF^{V600E}$ mutation and multifocality on CLNM, all the patients were divided into 4 groups according to the status of $BRAF^{V600E}$ mutation and multifocality, namely $BRAF^{V600E}$ mutation (+) multifocality (+) (Group 1), $BRAF^{V600E}$ mutation (–) multifocality (+) (Group 2), $BRAF^{V600E}$ mutation (+) multifocality (–) (Group 3) and $BRAF^{V600E}$ mutation (–) multifocality (–) (Group 4). The mean number of removed central lymph nodes and metastatic central lymph nodes was 7.0 ± 3.8 (3–19) and 2.3 ± 2.4 (0–17), respectively. There was no difference in the number of removed central lymph nodes and metastatic central lymph nodes between Group 1 and other three groups (Table 3). Finally, the results in Table 3 indicate that the incidence of CLNM in Group 1 was significantly higher than in other groups ($p = 0.030$, $p = 0.018$ and $p < 0.001$, respectively).

Table 4 shows that the probability of CLNM was the greatest in the patients with $BRAF^{V600E}$ mutation and multifocality (OR 8.265, 95% CI 4.731–14.437, $p = 0.001$) compared with those with other combinations after the adjustment for age and other confounders. The probability of CLNM was greater in the patients with only $BRAF^{V600E}$ mutation or multifocality (OR 2.721, 95% CI 1.697–4.362, $p = 0.001$; OR 2.221, 95% CI 1.206–4.090, $p = 0.010$) compared with those without $BRAF^{V600E}$ mutation and multifocality after the adjustment for confounders. Based on the abovementioned results, RERI was 4.323, with 95% CI ranging from 1.276 to 7.369, which indicates that there was a strong additive interaction of $BRAF^{V600E}$ mutation and multifocality on the presence of CLNM. Furthermore, there would be 4.323 relative excess risks contributed by the additive interaction. AP was 0.523 with 95% CI ranging

Table 3 The CLNM in 4 subgroups based on status of $BRAF^{V600E}$ mutation and Multifocality

Characteristics	Group 1	Group 2	Group 3	Group 4	p^a	p^b	p^c
Total number	75	36	134	168			
Number of removed CLNs	7.1 ± 4.3	7.2 ± 5.1	6.7 ± 4.4	6.9 ± 4.1	0.601	0.532	0.754
Number of metastatic CLNs[d]	2.4 ± 2.3	2.3 ± 2.2	2.6 ± 2.9	2.3 ± 2.8	0.665	0.561	0.318
CLNM (%)	51 (68.0)	16 (44.4)	67 (50.0)	32 (19.1)	0.030*	0.018*	<0.001*

Note: Group 1 is patients with $BRAF^{V600E}$ mutation (+) multifocality (+), Group 2 is those with $BRAF^{V600E}$ mutation (–) multifocality(+), Group 3 is those with $BRAF^{V600E}$ mutation (+) multifocality (–) and Group 4 is those with $BRAF^{V600E}$ mutation (–) multifocality (–). *p* value[a] represent Group 1 *vs.* Group 2; *p* value[b] represent Group 1 *vs.* Group 3; *p* value[c] represent Group 1 *vs.* Group 4; * represent the *p* value <0.05. (+) represent positive and (–) represent negative. Number of metastatic CLNs[d], only calculated patients with central lymph node metastasis. CLNM = Central lymph node metastasis. CLNs = central lymph nodes.

Table 4 Measures for estimation of synergic effect between $BRAF^{V600E}$ mutation and Multifocality for the risk of CLNM in Unilateral PTC

$BRAF^{V600E}$ Mutation	Multifocality	CLNM case	Total case	OR (95% CI)	*p* value
No	No	32 (19.1)	168	1.0	
Yes	No	67 (50.0)	134	2.721 (1.697–4.362)	0.001*
No	Yes	16 (44.4)	36	2.221 (1.206–4.090)	0.010*
Yes	Yes	51 (68.0)	75	8.265 (4.731–14.437)	0.001*
RERI	4.323 (1.276–7.369)				
AP	0.523 (0.364–0.682)				
SI	2.469 (1.607–3.794)				

Note: Adjusted for tumor size, age, sex, Hashimoto' thyroiditis, extrathyroidal extension, capsular invasion, full tumor encapsulation and lymphovascular invasion. PTC = Papillary thyroid carcinoma; CLNM = Central lymph node metastasis. * represent the *p* value <0.05.

from 0.364 to 0.682, suggesting that 52.3 % CLNM exposed to the two risk factors was caused by the additive interaction of $BRAF^{V600E}$ mutation and multifocality. SI was 2.469 (95% CI = 1.607 to 3.794).

Discussion

The incidence of $BRAF^{V600E}$ mutation usually ranges from 34.2% to 87.1%. Besides, it is widely accepted that $BRAF^{V600E}$ mutation is an important independent factor on CLNM in PTCs [10, 12, 20-26]. In this study, the incidence of $BRAF^{V600E}$ mutation was 50.6%, which was consistent with those in other studies. In terms of multifocality, it was considered as an independent factor for CLNM [15]. For this study, we compared clinicopathological factors in the patients with and without CLNM through univariate and multivariate analysis firstly. Subsequently, we identified that multifocality and $BRAF^{V600E}$ mutation were risk factors for CLNM in unilateral PTCs. In addition, tumor size and extrathyroidal extension were also important risk factors for CLNM, which was consistent with the findings of other researches [27-30].

Then, the results of the 4 subgroups were categorized by different statuses of $BRAF^{V600E}$ mutation and multifocality. It was found that the patients with $BRAF^{V600E}$ mutation and multifocality presented significantly the highest incidence of CLNM compared with other three groups. Specifically, the incidence of CLNM in Group 1 was 68.0%, which was more than triple that in Group 4 (19.1%). Obviously, it was significantly higher than those in the groups with only $BRAF^{V600E}$ mutation or multifocality (50.0% and 44.4%, respectively). The abovementioned results primarily prove the potentially combined effect of the two risk factors on CLNM. To deeply evaluate the additive effect of $BRAF^{V600E}$ mutation and multifocality, we utilized the value of RERI, AP and SI. According to the results, the PTC patients with both $BRAF^{V600E}$ mutation and multifocality were associated with a 4.323 times higher risk of CLNM compared with those who had no mutilfocality or $BRAF^{V600E}$ mutation in unilateral PTCs. In consequence, the increased risk of CLNM prompted by the presence of both multifocality and $BRAF^{V600E}$ mutation was significantly more than the interaction attributed to the existence of either only $BRAF^{V600E}$ mutation or multifocality. Thus, this significant additive interaction indicates that multifocality might contribute to an extra risk of CLNM for the unilateral PTC patients who also have $BRAF^{V600E}$ mutation.

Currently, there is no clear mechanism to explain the results of this study. Multifocal papillary cancers in thyroid can be divided into independently arising papillary cancers and those resulted from intrathyroidal spread [31]. In this study, the $BRAF^{V600E}$ mutation status of multifocal PTCs was only determined by the dominant tumour, rather than each focus in individuals. Although this approach may be controversial, according previous studies, most multifocal thyroid carcinoma share identical $BRAF^{V600E}$ status, ranging from 59.4–85.7% [31-35]. Additionally, the most common condition for the mixed $BRAF^{V600E}$ mutation tumour group is that the dominant tumour is BRAF-positive while other foci are partly or all BRAF-negative. Given this, the dominant tumour could also represent the $BRAF^{V600E}$ mutation status in the mixed $BRAF^{V600E}$ mutation tumour group to some extent [36]. In view of this, we thought that the $BRAF^{V600E}$ mutation status of the dominant tumour in the patients with multifocality can reasonably represent additional foci in them. $BRAF^{V600E}$ mutation was accepted as a strong promoter of aggressive behaviours for

PTC. On this basis, we hypothesized that $BRAF^{V600E}$ mutation might enrich the aggressive behaviours in each $BRAF^{V600E}$ mutation positive foci in individual patient. Due to the rich lymphatic channel system in thyroid, the potentially influenced area of each focus may enlarge and improve, which might finally cause the creased probability of CLNM. A further study on the potential mechanism will be needed in the future.

Currently, fine needle aspiration biopsy (FNAB) is the first choice for the preoperative diagnosis of multifocality with an acceptable accuracy, since it is even effective on the treatment of the tumour foci with a diameter of 5 mm or less [37]. Although whether $BRAF^{V600E}$ analysis could be routinely used in clinical practice is still controversial, it has been proved to be feasible preoperatively [38]. Moreover, a system meta-analysis has proved that preoperative $BRAF^{V600E}$ test can improve the diagnostic accuracy of FNAB [39]. For mutifocality PTCs, additional tumour foci are too small to be diagnosed preoperatively sometimes, so it is unpractical to perform $BRAF^{V600E}$ test for all tumour foci preoperatively. Given that the dominant tumour foci can represent other foci in individuals to some extent, the preoperative test of $BRAF^{V600E}$ mutation for the dominant foci seems to be a feasible choice. From another perspective, ipsilateral CLNM was considered as an important predictor for the presence of contralateral CLNM in unilateral PTC [40]. Additionally, the incidence of contralateral CLNM in unilateral PTC was only 5.3% (7/132) in this study, which is also very low in other studies [40]. Although some studies have suggested that there may be no difference in postoperative complications between unilateral and bilateral CLND for experienced surgeons, it is not necessary to routinely perform bilateral CLND for unilateral PTCs initially. For this reason, prophylactic unilateral CLND may be recommended as the first step for unilateral PTCs with high-risk factors of CLNM, such as multifocality and $BRAF^{V600E}$ mutation. Then, other appropriate methods, including intraoperative frozen biopsy and exploration, may be applied to further evaluate the contralateral central compartment. Taken together, the preoperative diagnoses of multifocality and the $BRAF^{V600E}$ mutation of the dominant tumour by FNAB may help to evaluate the central compartment in unilateral PTCs. Consequently, ipsilateral prophylactic central LND may be the first choice for the above mentioned suspicious unilateral CLNM PTCs.

In the present study, 58.6% of the enrolled patients had papillary thyroid microcarcinoma (PTMC), which is defined as the tumor with a diameter of less than 10 mm. The incidence of PTMC has dramatically increased recently, but it usually has an excellent prognosis. Commonly, if a primary suspicious thyroid malignancy is identified, surgery would be recommended. However, the most appropriate treatment of PTMC remains as a matter of debate. Corresponding strategies range from observation alone to surgical resection [41-43]. Therefore, the findings in the present study may help surgeon to make decision for unilateral PTMCs to some extent, and a research concentrating on the combined effect on unilateral PTMCs should be performed in future.

The present study also has some limitations. Firstly, a selection bias could occur, because this is a retrospective cross-section study. Secondly, some of the enrolled patients only underwent hemithyroidectomy. Nevertheless, preoperative ultrasound and intraoperative exploration showed a powerful evidence to ensure the absence of nodule in the contralateral lobe. Finally, our study only tested the dominant tumour in the patients with multifocality, which cannot provide absolutely accurate information about foci. However, the original aim of our study was to evaluate the potential effect of $BRAF^{V600E}$ mutation status in the dominant tumour and multifocality in central compartment. Therefore, the approach of $BRAF^{V600E}$ mutation test in the present study did not have a significant influence on the results. Undoubtedly, a multicentre study on a large number of patients with a long-term follow-up would be needed for a better understanding of the potential interaction of those two factors in unilateral PTCs.

In conclusion, $BRAF^{V600E}$ mutation and multifocality are risk factors for CLNM in unilateral PTCs. The risk of CLNM in unilateral PTC patients with both $BRAF^{V600E}$ mutation and multifocality seems to be higher than in other patients. Thus, ipsilateral prophylactic CLND may be appropriate for the unilateral clinically-negative PTCs with the above mentioned two factors in central compartment, as the first step.

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Disclosure

There is no conflicts of interest.

Reference

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