Original Article

Comparative immunohistochemical study of Bcl-X in ameloblastoma, keratocystic odontogenic tumor and adenomatoid odontogenic tumor

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Abstract

Objectives: Since its recognition as a physiologic process associated with tumor, among molecular mechanisms involved in tumor progression, defects in regulation of apoptosis have generated an accelerating volume of research that has sought to elucidate the role of programed cell death in pathogenesis and treatment of various tumors. Therefore, this study was performed to understand better the diverse biological profile of epithelial odontogenic tumors with the help of immunohistochemical expression of Bcl-X protein.

Materials and Methods: We studied Bcl-X protein expression in 45 cases of epithelial odontogenic tumors which included 15 cases each of ameloblastomas, keratocystic odontogenic tumor (KCOT) and adenomatoid odontogenic tumor (AOT) and correlated the expression with their growth pattern.

Results: Cytoplasmic staining of Bcl-X revealed overexpression in ameloblastoma when compared to KCOT and AOT. Percentage of positive cells showed a statistically significant difference, $P = 0.007$ between ameloblastoma and KCOT, whereas $P < 0.001$ between ameloblastoma and AOT. However, no significance was observed between KCOT and AOT ($P = 0.132$).

Conclusion: The present study supports the fact that epithelial odontogenic tumors show diverse growth profiles. An increased Bcl-X expression was seen in ameloblastoma compared to KCOT and least expression in case of AOT which could be indicative of more aggressive biological behavior and increased cell survival activity of ameloblastoma than KCOT and AOT. This signifies the diagnostic relevance of this biomarker and also could be a possible regulator of the proliferative compartment by contributing in tumor progression and cytodifferentiation of epithelial odontogenic tumors.

Keywords: Adenomatoid odontogenic tumor, ameloblastoma, Bcl-X, immunohistochemistry, keratocystic odontogenic tumor

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INTRODUCTION

Odontogenic tumors represent a spectrum of lesions ranging from benign and malignant neoplasms to dental hamartomas, all arising from odontogenic residues such as odontogenic epithelia and/or ectomesenchyme with variable amounts of dental hard tissues.[1] Odontogenic

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epithelium is responsible for tooth development under physiologic conditions and can give rise to tumors or cysts in the jaws in pathologic conditions. Of all the lesions of head and neck area that affect the maxillary and mandibular bones, the highly prevalent odontogenic tumors have been the focus of several studies that adopted different analytic approaches.[2] The interest in these lesions is high because of their similar radiographic and histopathologic features but different clinical behaviors.

Apoptosis, also known as programed cell death or physiologic cell death, plays a diverse role in embryogenesis, the development and maintenance of normal homeostasis as well as in oncogenesis within all multicellular organisms and is associated with the pathogenesis of various tumors. The growth rate of tissues is determined by proliferative activity and cell death. An imbalance among the antiapoptotic proteins such as Bcl-2 family members could induce dysregulation of apoptosis, which would contribute to oncogenesis and tumor development.[3-6]

Recent reports have documented the expression of Bcl-2 gene products in tooth germs and ameloblastomas by an immunohistochemical method, suggesting that these proteins have important roles in odontogenesis and tumor growth. Other Bcl-2 family proteins have not yet been examined extensively in odontogenic epithelium until recently.[7]

One such specific marker to identify proliferative activity and tumor aggressiveness by immunohistochemistry (IHC) is Bcl-X, a 20 kDa protein. Very little data exist on the expression of Bcl-X, a newly discovered member of the Bcl-2 antiapoptotic protein family in odontogenic tumors, and a very minimal amount of information is available in the literature to study and compare the Bcl-X expression between various odontogenic tumors and to correlate the expression to their diverse biological behavior and aggressive nature inherent in them.

This study, therefore, aimed to investigate whether the newly discovered Bcl-X protein is expressed by these epithelial odontogenic tumors, and if so whether or not there exists a significant difference in Bcl-X expression between ameloblastoma, keratocystic odontogenic tumor (KCOT) and adenomatoid odontogenic tumor (AOT) so as to reveal its possible role in progression and determination of the growth profile of these tumors and in an attempt to elucidate its influence on their biological behavior.

MATERIALS AND METHODS

The samples for this study involved the use of formalin-fixed paraffin-embedded tissues of histopathologically diagnosed 45 cases of epithelial odontogenic tumors retrieved from the Department of Oral Pathology and Microbiology, Yenepoya Dental College, Mangalore, India. These 45 cases which included ameloblastoma (15 cases), KCOT (15 cases) and AOT (15 cases) were confirmed and taken for IHC evaluation.

Immunohistochemistry

For IHC detection of Bcl-X, serial sections of 4-μm thickness were cut and mounted on poly-L-Lysine coated slides and were dried for 24 h at 37°C. Then, the sections were deparaffinized and rehydrated in xylene and descending grades of alcohol, respectively. Antigen heat retrieval was done by keeping the slides in a pressure cooker filled with boiling trisodium citrate buffer (pH 6.0) for 20 min. Peroxidase block is applied for 10 min. It is then washed with Tris buffer twice for 5 min each. Monoclonal anti-Bcl-X antibody (Ready-to-use vial, BioGenex) was used. The Super Sensitive™ Polymer-HRP IHC detection system (BioGenex Life Sciences Pvt. Ltd.) was used for the application of the biotinylated link antibody and peroxidase-labeled streptavidin, according to the manufacturer's instructions for the procedure. Visualization was performed using freshly prepared 3,3'-diaminobenzidine tetrahydrochloride chromogen for 10 min. The slides were then counterstained with Mayor's hematoxylin stain. For each batch of staining, a negative control where the primary antibody was replaced by Tris buffer saline, and a positive control of normal tonsil tissue was used.

Interpretation of staining

Cytoplasmic staining was considered positive for Bcl-X staining. The slides were viewed in a bright field microscope at a magnification of ×20 for analyzing intensity, localization and pattern of staining. A positive Bcl-X expression was designated for samples showing cytoplasmic staining. All the slides were methodically evaluated by two different observers to remove the inter- and intraobserver bias.

The photomicrographs for assessing the percentage of positive cells were taken at ×20 magnification using Olympus Camera sp-350 attached to microscope Olympus CX-41. One dense area of cells with maximum Bcl-X expression was randomly selected for analysis. In ameloblastoma slides, areas for positive cell counting were selected from peripheral ameloblasts such as cells and stellate reticulum-like cells; in KCOT slides, areas for positive cell counting were selected from basal cell layers, intermediate cell layers and superficial cell layers, whereas in AOT slides, areas for positive cell counting were selected from duct-like structures and polyhedral sheets of cells. The cells were counted manually using ImageJ software

(1.42q, NIH, USA). The percentage of Bcl-X expression was quantified by determining the number of positive cells expressing cytoplasmic Bcl-X staining among the total number of cells in a selected area. The qualitative, quantitative and semi-quantitative analysis of the stained sections was done by light microscopy and according to the immunoreactive score (IRS) given by Remmele and Stegner. In IRS scale, the intensity of staining (grades: 0–3) and percentage of positive cells (grades: 0–4) were taken into account. IRS was evaluated by obtaining the product of intensity grade and percentage of positivity grade for each case. The IRS represented a product of points given for the evaluated characters, and it ranged from 0 to 12. The IRS points were categorized into four groups based on expression, i.e., 0–1 – negative expression, 2–3 – positive weak expression, 4–8 – positive mild expression and 9–12 – positive strong expression [Table 1].

Statistical analysis

Data were entered and analyzed using SPSS software version 10.05 (SPSS Inc., Chcago, Illinois, USA). Chi-square test and ANOVA with *post hoc* least significant difference test were used to validate the comparison and correlation of Bcl-X expression between ameloblastomas, KCOTs and AOTs. Differences with a probability value of <0.05 were considered statistically significant.

RESULTS

Bcl-X expression was detected in all the three groups, and intensity of the staining varied from weak to strong in the studied sections. IHC results of the qualitative, quantitative and semi-quantitative analysis for Bcl-X expression in studied groups are summarized in Tables 2-4, and microscopic findings of them have been shown in Figures 1-4.

When the expression of Bcl-X was analyzed, 39 cases out of 45 showed positive staining (86.7%) and six cases (13.3%) showed the complete absence of staining. Among the different groups analyzed, ameloblastoma cases showed 93.3% of positivity. In KCOT, about 86.7% of positive staining and AOT showed 80% of positive staining results [Table 2].

The intensity grade was analyzed between the three groups; in ameloblastoma, out of 15 cases, four cases (26.7%) showed moderate intensity, whereas eight cases (53.3%) showed intense staining and only two cases (13.3%) cases showed mild staining. When the intensity grades of KCOT were analyzed, only one case (6.7%) had intense staining and six cases each (40%) showed moderate and mild staining, whereas in AOT, about nine cases (60%) of them showed mild staining and only three cases (30%) showed moderate staining [Table 3].

The percentage value of cells positive for Bcl-X staining was calculated out of total epithelial cells from each area. The final value of positive cells was considered for analysis. The mean values of percentage of positive cells from each group were statistically analyzed for comparison. The percentage of positivity value of ameloblastoma has a mean value of 63.33 and standard deviation (SD) - 22.077, KCOT has a mean value of 40.73 and SD - 21.855 and AOT has a mean value of 28.60 and SD - 21.033 [Table 4].

When the IRS was compared in between ameloblastoma and KCOT, most of the ameloblastoma cases, i.e., 40.0% of cases showed score 9 with positive strong expression of Bcl-X and maximum number of KCOT cases, i.e., 40% of them showed score 2 with positive weak expression of Bcl-X and 20% of cases showed score 4 and score 6 each

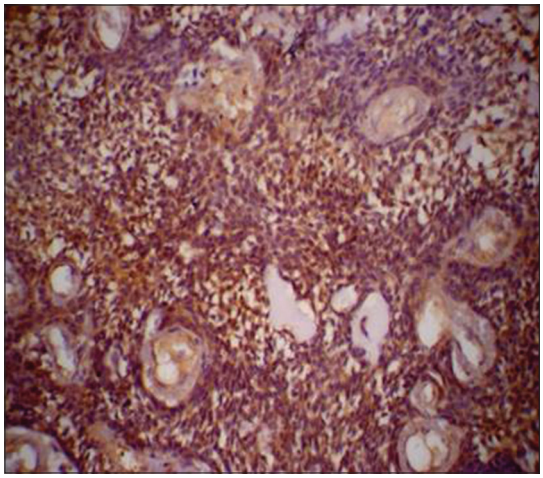

Figure 1: Photomicrograph showing Bcl-X expression in plexiform ameloblastoma tissue (×200)

Table 1: Comparison of immunoreactive score between ameloblastoma, keratocystic odontogenic tumor and adenomatoid odontogenic tumor

Group	IRS							Total	*P*		
	0	1	2	4	6	9	12		1 and 2	1 and 3	2 and 3
Ameloblastoma (1)	1	0	2	0	4	6	2	15	0.05 (S)	0.009 (S)	0.308 (NS)
KCOT (2)	2	0	6	3	3	1	0	15			
AOT (3)	3	0	9	0	3	0	0	15			
Total	6	0	17	3	10	7	2	45			

S: Significant, NS: Not significant, KCOT: Keratocystic odontogenic tumor, AOT: Adenomatoid odontogenic tumor, IRS: Immunoreactive score

Table 2: Total staining results in ameloblastoma, keratocystic odontogenic tumor and adenomatoid odontogenic tumor

Group	Staining		Total
	Negative staining	Positive staining	
Ameloblastoma	1	14	15
KCOT	2	13	15
AOT	3	12	15
Total	6	39	45

KCOT: Keratocystic odontogenic tumor, AOT: Adenomatoid odontogenic tumor

Table 3: Intensity grades in ameloblastoma, keratocystic odontogenic tumor and adenomatoid odontogenic tumor

Group	Intensity grades				Total	*P*
	No staining	Mild	Moderate	Intense		
Ameloblastoma	1	2	4	8	15	0.042 (S)
KCOT	2	6	6	1	15	
Ameloblastoma	1	2	4	8	15	0.004 (S)
AOT	3	9	3	0	15	
KCOT	2	6	6	1	15	0.423 (NS)
AOT	3	9	3	0	15	

S: Significant, NS: Not significant, KCOT: Keratocystic odontogenic tumor, AOT: Adenomatoid odontogenic tumor

Table 4: Comparison of percentage of positive cells in ameloblastoma, keratocystic odontogenic tumor and adenomatoid odontogenic tumor

Group	Mean	SD	*P*		
			1 and 2	1 and 3	2 and 3
Ameloblastoma (1)	63.33	22.077	0.007 (S)	<0.001 (S)	0.132 (NS)
KCOT (2)	40.73	21.855			
AOT (3)	28.60	21.033			
Total	44.22	25.684			

S: Significant, NS: Not significant, KCOT: Keratocystic odontogenic tumor, AOT: Adenomatoid odontogenic tumor, SD: Standard deviation

with positive mild expression and maximum number of AOT cases, i.e., 60% of them showed score 2 with positive weak expression of Bcl-X [Table 1].

DISCUSSION

Odontogenic tumors constitute a group of heterogeneous lesions that range from hamartomatous or nonneoplastic tissue proliferations to malignant neoplasms with metastatic capabilities. Odontogenic cysts are encountered relatively commonly in dental practice and odontogenic tumors, by contrast, are lesions of varying rarity within odontogenic tissues and constitute an important aspect of oral and maxillofacial pathology.[8,9]

Epithelial proliferations play a significant role in the behavior of odontogenic lesions. Proliferation activity is an important predictor of biologic behavior of pathologic condition and as a potential guide for therapy. A series of genetic and molecular alterations appear to promote the development and progression of tumors through multiple steps, and recent studies have identified various molecular alterations responsible for their development and

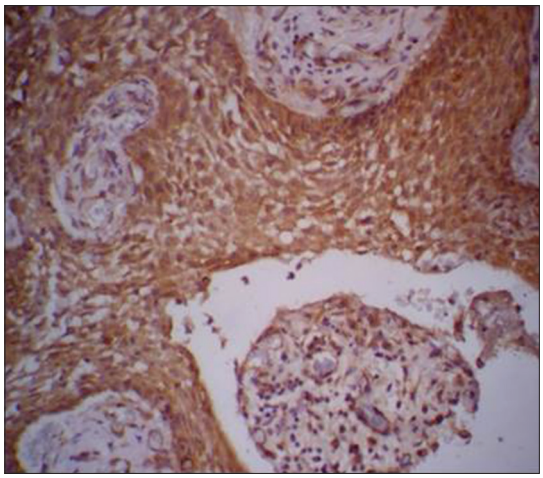

Figure 2: Photomicrograph showing Bcl-X expression in unicystic ameloblastoma tissue (×200)

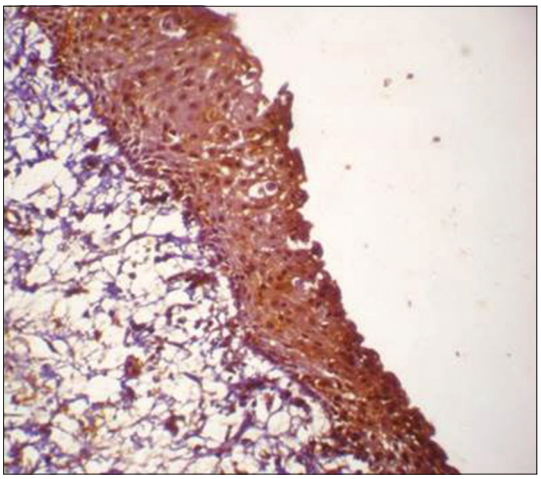

Figure 3: Photomicrograph showing Bcl-X expression in keratocystic odontogenic tumor tissue (×200)

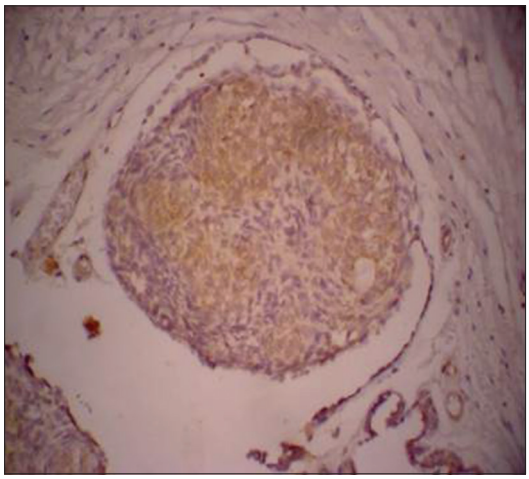

Figure 4: Photomicrograph showing Bcl-X expression in adenomatoid odontogenic tumor tissue (×200)

progression.[10,11] Determination of the factors responsible for this epithelial proliferation, using IHC, helps in investigating the differences between biological behavior of various tumors.[12]

Current studies of tumor biology suggest several basic mechanisms that may be used by neoplastic cells to provide a growth advantage over normal tissue. Neoplastic cells may show an increased rate of cell division and/or a decreased rate of programed cell death.[13] It is believed that tumor cells show a normal level of cell division and an increased expression of antiapoptotic proteins.

In the current study, an effort has been made to compare and correlate the growth potential of these different odontogenic tumors to assess the aggressiveness with the help of molecular studies that have offered interesting findings regarding their pathogenesis. There are a number of genetic and molecular changes that appear to promote the development and multistage progression of odontogenic tumors. The mechanisms by which these tumors grow and evolve include overexpression of antiapoptotic proteins such as Bcl-2 and Bcl-X.[14]

Ameloblastoma was selected for this study as it is considered as "enigmatic" with unknown etiology and though benign, deserves special attention because of particular biological behavior exhibiting greater infiltrative potential, high recurrence rate and capacity to metastasize or undergo malignant transformation when compared to its other epithelial counterpart AOT, which is now believed to be a result of metaplastic process rather than an epithelial-ectomesenchymal interaction.[15] In addition, a large size AOT supports the classification of the tumor as a benign neoplasm and not a hamartoma which has triggered a long-term debate whether it should be categorized as a hamartomatous malformation or a true benign tumor,[16] and KCOT has been compared in this study with ameloblastoma and AOT as it is now regarded as a benign neoplasm rather than a conventional cyst by Toller in 1967; based on its aggressive biological behavior, prone to recurrence and the genetics involved, it is reclassified as a tumor by Philipsen in 2005.[17] Therefore, a more detailed molecular study of these tumors can put some insight into the biological behavior and their aggressive nature.

It has been substantiated that apoptosis is a critical step in cell differentiation, cell turnover and in the maintenance of tissue homeostasis. Recent advances on cancer biology have shown that the process of tumorigenesis may involve not only increased cell proliferation but also decreased cell death or increased cell survival. Mutations of any of the genes encoding antiapoptotic proteins or any changes in the levels of their expression can lead to increased cell survival and contribute to growth advantage of the affected tissues compared to the neighboring ones.[18]

Extensive search in the literature revealed very few studies evaluating Bcl-2 and Bcl-X expression in odontogenic tumors and only one in odontogenic myxomas by Bast *et al*.[13] These investigators noted an increase in expression of Bcl-2 and Bcl-X and therefore suggested the production of these antiapoptotic proteins by the tumor cells to be a possible mechanism of disease progression providing a growth advantage.[13] A similar observation in our study points to the likely role of the same mechanism even in ameloblastoma, KCOT and AOT.

Dysregulated Bcl-X expression further induces DNA damage, affecting the cellular activity and allows the cell to remain in an antiapoptotic state and thus contributes to continuous growth. Therefore, Bcl-X dysregulation can be an important early event in the progression of odontogenic tumors, and the intensity of expression can be directly correlated with aggressiveness of the tumor.

In our study, the Bcl-X expression was seen more in ameloblastomas [Table 2] than KCOT and least in AOT cases. This observation of a decrease in Bcl-X-positive cells with a decrease in proliferative growth of tumors possibly reveals that Bcl-X is expressed more in epithelial cells that have an increased capacity for survival that could be more in ameloblastoma which was in compliance from studies by Chen *et al*.[19] and Lo Muzio *et al*.[20] that showed an increase in Bcl-2 expression in poorly differentiated oral squamous cell carcinoma (OSCC) than in well-differentiated OSCC reflecting a possibility that Bcl-2 was expressed more in keratinocytes that have an increased capacity for survival.

In the present study, Bcl-X immunoreactivity was expressed higher by the columnar cells (70.8%) in the periphery of tumor islands when compared to stellate reticulum cells (55.8%) [Figures 1 and 2], which is consistent with the findings of Florescu *et al*.[21] and Sindura *et al*.[22] with the Bcl-2 protein that was also seen by de Vicente *et al*.[23]

Similar studies in literature communicate that around 90% of ameloblastomas are positive for Bcl-2 in the peripheral layers of tumor islands found by Mitsuyasu *et al*.[24] and Sandra *et al*.,[25] which indicates that Bcl-2 and Bcl-X expression may be related to differentiation and proliferation of odontogenic epithelium, and their overexpression may be associated with the ameloblastomas

development maintaining stem cell population in peripheral layers of tumor islands.

However, our study findings were compatible with several other studies on ameloblastomas using various proliferative markers such as proliferating cell nuclear antigen by Kim and Yook[14] and Ki-67 by Sandra *et al*.[25] and Meer *et al*.,[26] which was higher in peripheral cells of ameloblastomas asserting that proliferative activity is higher in peripheral neoplastic cells compared to central neoplastic cells.

In cases of KCOT, expression of Bcl-X was found in the whole thickness of the epithelium in our study [Figure 3] which was in harmony with the study of Tekkesin *et al*.[27] with Bcl-2 protein, and the authors suggested that their results supported the notion of odontogenic keratocyst having a neoplastic nature and redefinition and reclassification as a tumor. This study clearly demonstrates that KCOT-like ameloblastoma demonstrates equivalent aggressive clinical and noticeable invasive behavior. Therefore, it is now considered as no longer a developmental cyst but as an odontogenic tumor.

In the present study, the Bcl-X expression in AOT cases showed mild-to-moderate positivity, and a varied expression was found in all these cases [Table 3]. Similar outcomes were seen by Tegginamani *et al*. with Bcl-2 protein.[16] It was reflected in his study that expression was present in most of the epithelial cells of AOT [Figure 4], and it behaves more aggressively in most cases that rules out AOT as a cyst. The whole concept of AOT behaving more aggressive regulating apoptosis and facilitating cell survival by expressing Bcl-X protein from this study could correlate to its biological behavior and could be considered as a benign neoplasm rather than a hamartoma or a cyst and progresses in a similar pathway indicative of a true neoplasm rather than a developmental anomaly.

Another distinctive and interesting finding of the present study is the localization of Bcl-X immunoreactive cells in these tumors. The detailed observation and analysis of the sample slides exhibited the presence of more immunoreactive cells in peripheral ameloblast-like cells when compared to stellate reticulum-like cells in ameloblastoma which was in agreement with many studies in the literature. In KCOT, it was more seen in basal cell layer compared to intermediate and superficial cell layer, and in AOT cases, duct-like cells displayed more immunoreactive cells compared to polyhedral epithelial cells. These observations could substantiate that all the cells which were positive for Bcl-X did not show uniform staining localization within a tissue and contribute to the fact that there are different levels of cellular differentiation and activity-inactivity within group of cells which do not directly correspond to localization, and the overall Bcl-X expression within a tissue sample rather depends on individual nature of the tumors.

After reviewing the literature, this appears to be the first study on comparison of Bcl-X expression in a group of epithelial odontogenic tumors. This study identifies the presence of Bcl-X protein in odontogenic epithelium with significant differences found between ameloblastoma, KCOT and other clinically indolent odontogenic tumor such as AOT.

As this oncoprotein Bcl-X regulates programed cell death by allowing the tumor cells to escape apoptosis, thereby promoting the cell survival and facilitating the growth advantage over the surrounding tissues and consequently resisting the therapeutic approach to radiation or chemotherapy, so we suggest a definite role of Bcl-X in the progression of these tumors.[4,5,13] The treatment modalities for the odontogenic lesions should target the neoplastic epithelium which could result in reduction of the extent of lesion and thus minimizing the significant functional, esthetic and psychological damage caused by these aggressive odontogenic lesions.

CONCLUSION

The results show variability and heterogeneous expression for Bcl-X protein in odontogenic tumors of epithelial origin. The Bcl-X expression had a significant difference between ameloblastoma, KCOT and AOT which could be suggestive of a difference in the growth profile, aggressiveness and increased cell survival ability of these odontogenic tumors. Further correlative studies using a panel of markers for other members of the Bcl-2 family are necessary to elucidate the specific molecular defects critical to the biology of these odontogenic tumors, which will have an impact on diagnosis and treatment.

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Conflicts of interest
There are no conflicts of interest.

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