Original Article

Evaluation of PD-L1 and PD-1 expression in aggressive eyelid sebaceous gland carcinoma and its clinical significance

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Purpose: Eyelid sebaceous gland carcinoma (SGC) is an aggressive but rare malignancy of ocular region. Over-expression of PD-L1 and PD-1 has been demonstrated in a variety of solid tumors including conjunctival melanoma. PD-L1 is an immunoinhibitory molecule that suppresses the effective T cells response against tumor antigen leading to the progression of tumors. Inhibitors of the interaction of PD-L1 and PD-1 are associated with good clinical response various carcinomas. The prognostic value of the PD-1/PD-L1 axis in SGC remains unexplored. The purpose of this study was to evaluate expressions of PD-1 and its ligand PD-L1 in SGC and correlate its expression with clinicopathological features and patients survival. **Methods:** The immunohistochemical expression of PD-L1 and PD-1 was evaluated in 30 SGC cases. **Results:** PD-L1 immunopositivity was detected in 41.9% of the SGC cases. PD-1 expression in tumor infiltrative lymphocytes (TILs) was observed in 53.3% samples. Tumor PD-L1 positivity, PD-1 expression in TILs and tumor size (>10 mm) was associated with reduced disease-free survival. On multivariate analysis only tumor size (>10 mm) and a combined positivity of PD-L1 in tumor cells and PD-1 in TILs with an odds ratio of 5.212 (95% confidence interval 1.449-18.737) continued to be significantly associated with SGC recurrence. **Conclusion:** PD-L1 is overexpressed in 50% of SGC cases. The combined tumor PD-L1 positivity and TILs showing PD-1 expression within the same SGC patient's samples predict high-risk SGC, suggesting that the up-regulation of PD-L1 in tumor cells and PD-1 positivity within the same SGC patient may aggravate tumor recurrence.

Key words: Eyelid, PD-1, PD-L1, sebaceous gland carcinoma

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Sebaceous gland carcinoma (SGC) of the eyelid arises from sebaceous glands of ocular adnexa.[1] Its significance among eyelid malignancies is due to its multifocal origin and pagetoid spread.[2,3] SGC is also considered to be one of the most aggressive eyelid tumor. It accounts for 1%-5.5% of all eyelid malignancies and is the most common eyelid malignancy with the reported rate of 31.2% in Indian population after basal and squamous cell carcinoma.[4] Incidence of SGC varies from 0.5% to 5% of all lid carcinomas in the USA and 28% in China.[1-5] The risk of metastasis and recurrence is approximately 10%-15% and the mortality rate is found to be 10%-40%. Treatment of SGC includes excision with clear margins. Radical surgical procedures like exenteration are reserved for the most advanced stages.[6-8]

Cancer escapes the immune responses by various mechanisms such as immune check point inhibition. One such check point of a particular interest is the interaction between programmed cell death ligand 1 (PD-L1) and its interaction with its receptor, programmed cell death receptor (PD-1).[9]

PD-L1 is a 40-kDa trans-membrane protein encoded by the CD74 gene on chromosome 9. It is expressed on natural killer cells, macrophages, myeloid dendritic cells, B cells, resting T-cells, epithelial cells and tumor cells.[10] PD-1 is a type of trans-membrane, inhibitory receptor for PD-L1 which belongs to the CD28/CTLA-4 subfamily of immunoglobulin superfamily which is expressed on T cells, B cells, monocytes, natural killer cells, dendritic cells and many tumor-infiltrating lymphocytes (TILs) and regulates autoimmunity and tolerance.[10]

In response to the immune attack, cancer cells overexpress PD-L1 which binds to a PD-1 receptor on T cells, inhibiting the activation of T-cells and induces the production of cytokine (such as IFN-γ and IL-2) thereby suppressing effective T-cell response against a tumor antigen.[11] Various studies have shown that blocking the interaction between PD-L1 and PD-1 pathway by many approved drugs such as nivolumab and pembrolizumab enhances the endogenous anti-tumor responses in non-small cell lung carcinoma and melanoma.[12,13] These drugs have shown therapeutic success in different malignancies including BCC and cutaneous melanoma.[14-16] Overexpression of PD-L1 has been reported in various types of tumor such as ovarian cancer, colorectal adenocarcinoma,

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non-small cell lung carcinoma, glioblastoma and has also been also been associated with poor prognosis in cutaneous melanoma and basal cell carcinoma.[17]

The immunohistochemical expression of PD-L1 in tumor cells has been considered as a predictive marker for tumor response to anti-PD-L1 or PD-1 immunotherapy in different malignancies.[18] However, little is known about the expression pattern of these immune regulatory molecules in eyelid SGC. In the present study, we aimed to investigate the expression PD-1/PDL-1 along will CD8 in eyelid SGC to determine the role of the PD-1/PD-L1 axis in eyelid SGC.

Methods

Patients and tissues

Thirty cases of eyelid SGC were selected for the study and carried out in accordance with Declaration of Helsinki principles. Informed consent was obtained from all of the patients participating in this study. The clinical features, radiological details and gross appearance of the selected patients were recorded. Tumor stage was determined according to the American Joint Committee on Cancer (AJCC, 7th edition) cancer staging criteria.[19] Haematoxylin and eosin-stained sections were analysed by light microscopy to confirm the diagnosis of SGC. The cases were classified as well or poorly differentiated on the basis of the extent of sebaceous differentiation and cytoplasmic vacuolations. The presence of pagetoid spread was also noted. All patients were followed up at 6-month intervals after surgical intervention for a mean period of 54.91 months (range, 12–89 months).

Immunohistochemistry

Unstained sections 4 μm thick were cut on Poly-lysine coated slides from formalin fixed paraffin-embedded blocks. These were deparaffinized in xylenes followed by rehydration through graded alcohols. Antigen retrieval was performed in citrate buffer (pH 6.0) for 20 minutes at 360W. After cooling, the slides were washed with TRIS-buffered saline, pH 7.5 and incubated with 0.3% hydrogen peroxide for 20 minutes, followed by incubation with the primary monoclonal antibodies against PD-L1 (clone E1L3N, cell signalling technology) and anti PD-1 (D4W2J, cell signalling technology) both at a dilution of 1:100 and were processed with Ultravision Quanto Detection (Thermo Scientific, Fremont, CA, USA) system. The immunohistochemical staining results for PD-L1 and PD-1 were evaluated on the basis of both percentage positivity and staining intensity. The percentage of immunostaining was based on the number of tumor cells showing positivity in 10 high-power fields. This was scored as 0 (<5%), 1+ (5%-25%), 2+ (26%-50%) and 3+ (51%-100%). The staining intensity was scored on a scale from 0 to 3+ in the tumor cells (0, negative/weak staining (if any); 1+, weak; 2+, medium; 3+ strong), and the scores obtained from the percentage positivity and staining intensity were added to create a single immunohistochemistry (IHC) score. The maximum score obtained in this system is 6 and the minimum is 0.[19] The tumors were regarded as immunopositive when an IHC score of 3, 4, 5 or 6 was obtained and as having a negative or reduced expression when the IHC score was 0-2 and for heterogeneous staining, the maximum intensity score was taken in arriving at the final score. PD-L1 and PD-1 staining were evaluated by one pathologist (S.S) and two observers simultaneously, and a consensus was reached for each IHC score. Human tonsillar tissue was used as positive controls for PD-1 [Fig. 1a], PD-L1 [Fig. 1g].

Statistical analysis

The Chi-square (χ^2) test was used to assess the association between immunohistochemical reactivity and clinicopathological characteristics. The Kaplan–Meier method was employed for survival analysis, and differences in survival were estimated with the log-rank test. A P value of <0.05 was considered to be statistically significant. The following clinicopathological factors were included in the survival analyses: sex, age, tumor size, tumor site, lymph node metastasis, clinical stage, histological differentiation and pagetoid spread. The univariate and multivariate analysis was performed with the Cox proportional hazard model to identify the factors that were useful in predicting disease-free survival rates. All of the statistical analyses were performed with MedCalc statistical software version 14.8.1 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014).

Results

Patient characteristics

The mean age of SGC study subjects was 57.2 years (range 25-88 years). Most of the tumors were localized to upper eyelid 22 (73.3%). A tumor size of >2 cm, which signifies poor prognosis, was seen in 50% cases. Light microscopy revealed 16 (53.3%) well differentiated SGC cases, 8 (26.6%) cases showed pagetoid spread. Lymph node metastasis was seen in 7 (23.3%) cases. Out of 30 cases, 9 (30%) patients were diagnosed with a recurrence and 1 died at a follow-up of 5 years (2011-2016).

Immunohistochemical expression of PD-1 in SGC

The immunohistochemical evaluation demonstrated high PD-1 expression in 16 out of 30 samples (53.3%). Positive staining of PD-1 was mainly located in tumor infiltrative cells [Fig. 1b]. Absence of PD-1 expression was observed in 46.6% cases [Fig. 1c].

Association between PD-1 protein expression and clinicopathological characteristic of SGC

Immunohistochemical evaluation of PD-1 expression on tumor infiltrative cells was not found to be statistically associated with the patient's gender and age, tumor size, histopathological differentiation, tumor stage, pagetoid spread or status of lymph node metastasis.

PD-1 immunoexpression and clinical outcome

Positive expression of PD-1 was observed in seven out of nine patients with recurrence (77%) and in one patient who died. Kaplan Meier survival analysis was carried out to determine the prognostic potential of PD-1 expression. There was no significant association between reduced disease-free survival in SGC cases with PD-1 overexpression (P = 0.006, log-rank analysis) [Table 1].

Immunohistochemical expression of PD-L1 in SGC

The data of IHC demonstrated high PD-L1 expression in 13 out of 30 (43.3%) cases studied. Positive staining of PD-L1 was mainly located in the cytoplasm and membrane of SGC

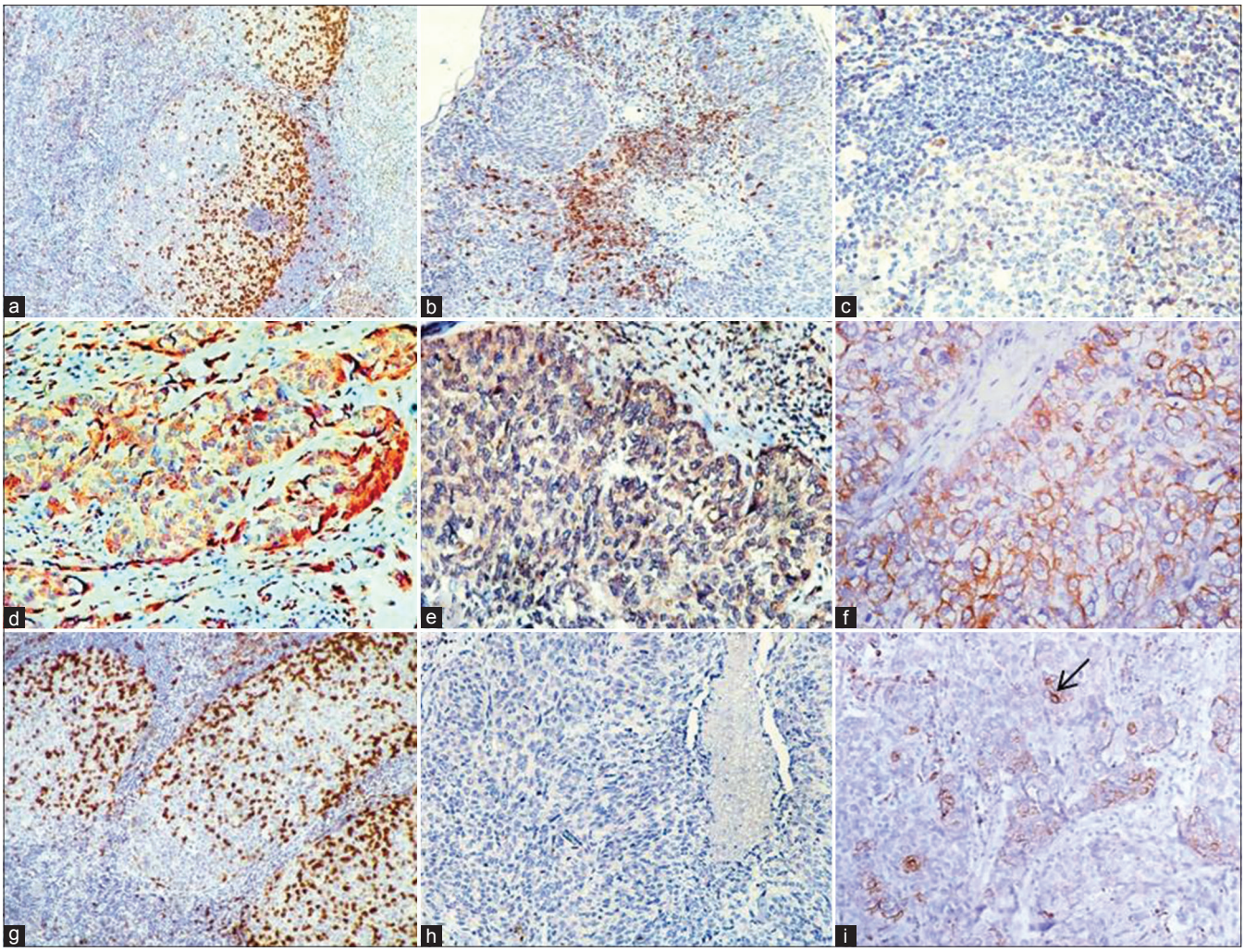

Figure 1: PD-1 immunostaining in (a) human tonsil and (b) PD-1 expression in tumor infiltrating lymphocytes surrounding the tumor nodule in a case of poorly differentiated sebaceous gland carcinoma (PDSGC) (c) Absence of PD-1 in a case of PDSGC (d) PD-L1 staining in tumor and stromal cells of well differentiated sebaceous gland carcinoma (WDSGC) (e and f) PD-L1 cytoplasmic and membranous immunostaning in PDSGC (g) Positive immunoreactivity of PD-L1 in human tonsil. (h) Absence of PD-L1 in a case of PDSGC (i) Heterogeneous staining of PD-L1 in a representative case of SGC

Table 1: Risk factor affecting disease-free survival in patients with SGC

	Univariate analysis			Multivariate analysis		
	OR	95% CI	*P*	OR	95%CI	*P*
Age ≥60 years	0.5924	0.1668-2.1031	0.4179			
Size ≥2 cm	5.2462	1.1069-24.864	0.0368*	5.2236	1.0999-24.8073	0.0385*
Histopathological differentiation	0.8900	0.2529-3.0386	0.85481			
Pagetoid spread	0.2895	0.0414-2.5851	0.2694			
Lymph node metastasis	1.3852	0.3573-5.3670	0.6372			
Upper eyelid involvement	0.7842	0.2023-3.0400	0.7251			
Surgical intervention	1.4882	0.3157-7.0659	0.6169			
PD-1	4.3076	0.9092-20.4088	0.0658			
PD-L1	6.4171	1.3586-30.3088	0.0189			
PD-L1 positive tumor and PD-1 positive TILs	5.2218	1.4716-18.5281	0.0109	5.2123	1.4499-18.7377	0.001*

*Significant; CI: Confidence interval; OR: Odds ratio

cells. [Fig. 1d-f]. Heterogeneous staining of PD-L1 was observed in few cases with tumor cells with strong PD-L1 staining intensity [Fig 1i], whereas other areas by tumor cells by lacking PD-L1 immunopositivity [Fig. 1h].

Association between PD-L1 protein expression and clinicopathological characteristic of SGC

PD-L1 expression was not found to be statistically associated with the patient's gender and age, tumor size, histopathological differentiation, tumor stage, pagetoid spread or status of lymph node metastasis.

PD-L1 immunoexpression and clinical outcome

PD-L1 membranous and cytoplasmic expression was observed in seven out of nine patients with recurrence (77%). Kaplan Meier survival analysis was carried out to determine the prognostic potential of PD-L1 expression. A significant association of reduced disease-free survival was seen in SGC cases with PD-L1 overexpression ($P = 0.0189$) [Table 1 and Fig. 2a].

Correlation between PD-1 immunopositivity in TILs and PD-L1 expression in tumor cells

Thirty cases were evaluated for both PD-L1 expression in tumor cells and PD-1 expression in TILs. PD-L1 expression in tumor cells was significantly associated with PD-1 expression in TILs ($P = 0.001$). The rate of co-expression of PD-L1 in tumor cells and PD-1 expression in TILs from the same specimen was 43% (13/30) [Table 2]. A significant association of reduced disease-free survival was seen in SGC cases showing co-expression of PD-L1 in tumor cells and PD-1 expression in TILs from the same specimen ($P = 0.0109$) [Fig. 2b].

Univariate and multivariate analysis to identify independent prognostic markers

PDL-1 expression ($P = 0.0189$), Size (>10 mm) of the tumor ($P = 0.0368$) and co-expression of PD-L1 in tumor cells and PD-1 expression in TILs from the same specimen ($P = 0.0109$) were factors found to be associated with reducing disease-free survival and promoting metastasis on the univariate analysis. When stepwise multivariate analysis was performed on these factors, only tumor size (>10 mm) with an odds ratio of 5.2226 (95% confidence interval l. 0999-24.8073) and SGC patients showing co-expression of PD-L1 and PD-1 in tumor cells and PD-1 expression with an odds ratio of 5.212 (95% confidence interval 1.449-18.737) continued to be significantly associated with an increased of SGC recurrence [Table 1].

Table 2: Comparison of PD-L1 and TILs with PD-1 immunostaining

IHC	PD-L1		
	Positive (n=13)	Negative (n=17)	P (χ^2 test)
PD-1 positive TILs (n=16)	13	03	0.001
PD-1 negative TILs (n=14)	0	14	

Discussion

Thirty cases of SGC were analyzed by immunohistochemically for the expression of PD-L1 and PD-1. Immunohistochemically, 43% SGC patients showed PD-L1 protein expression on tumor cells. Expression of PD-1 was localized to the tumor infiltrating lymphocytes in 53% cases. Further 42% of SGC cases were found to be positive for both PD-1 expressions in TILs and PD-L1 with in the same tumor section. Heterogeneous staining of PD-L1 was observed in few cases with some areas of the tumor with strong PD-L1 staining intensity, whereas other areas by tumor cells by lacking PD-L1 immunopositivity. Overexpression of PD-L1 has been reported in many different tumor types, such as Non-Small Cell Lung Carcinoma, Glioblastoma, Ovarian cancer, colorectal adenocarcinoma, melanoma including cutaneous squamous cell carcinoma.[20] The presence of PD-1 positive TILs and PD-L1 tumor cells indicate that the immune checkpoint may be activated in SGC cases.

Recent studies have suggested that for the PD-1 to exert its immune inhibitory effects their needs to be a PD-L1 expression by the tumor cells.[21] In our cases, we observed PD-1 positive TILs is predominantly maintained in tumors with high PD-L1 expressions and not in PD-L1 negative tumors. Such an association between the presence of PD-1 positive TILs and PD-L1 positive tumor cells reflects an immune-reactive milieu and have been observed in various tumors types and have been correlated with unfavorable prognosis.[22,23] Further various reports have suggested that objective response to anti PD-1/PD-L1 targeted therapy was only seen in PD-L1 positive tumors.[12,24]

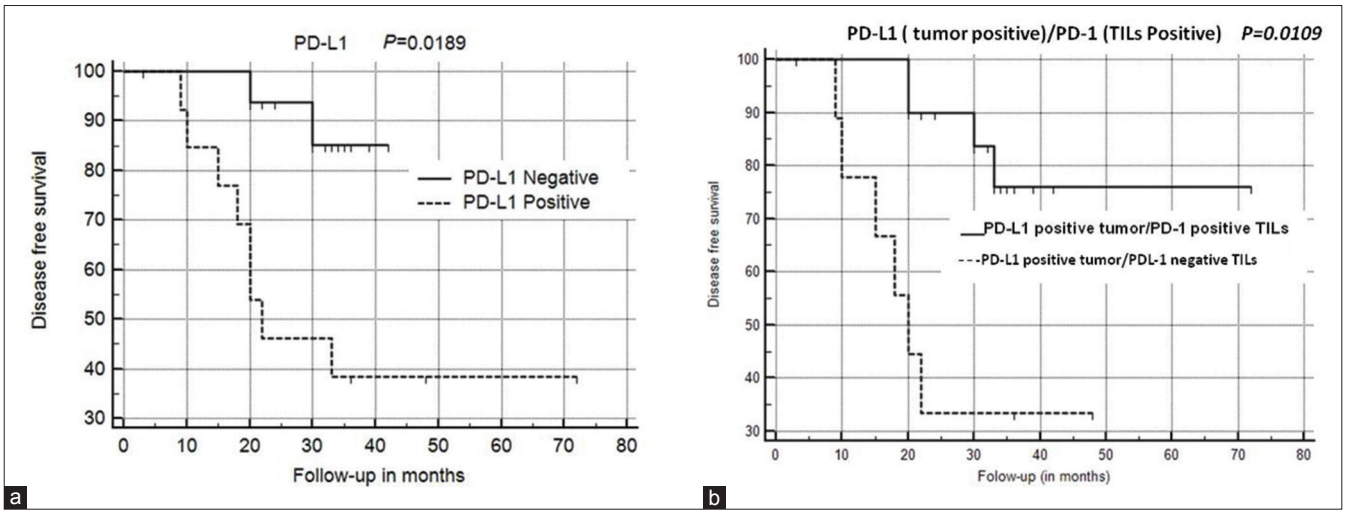

Figure 2: Kaplan-Meier analysis of the probability for disease-free survival shows reduced disease-free survival rates in SGC patients with PD-L1 expression in tumor (a) and in patients with both PD-L1 positive tumor along with PD-1 positive tumor infiltrative lymphocytes (b)

In line with former studies examining PD-L1 expression in various cancer including conjunctival melanoma[25] and basal cell carcinoma,[14] we found that high PD-L1 expression on tumor cells is common in SGC. The infiltration of PD-1 positive TILs and expression of PD-L1 in SGC both correlated reduced disease-free survival according to univariate analysis, however, on multivariate analysis only PD-L1 was found to be an independent prognostic indicator for SGC. Currently, the expression of PD-L1 on tumor cells is regarded as an immune-tolerance mechanism of the tumor, as it can attract PD-1 expressing immunoinhibitory TILs. Interestingly, the group with PD-L1 positive tumor cells/PD-1 positive TILs had a more unfavorable prognosis than the group with only PD-L1 positive or PD-1 positive expression. These results suggest that the presence of PD-L1 in tumor and PD-1 in TILs in the same patient is predictive of poor prognosis in SGC patients. So far, data on the importance of TILs in SGC are scarce and even fewer data exist on the expression pattern of PD-L1 and PD-1 in rare and aggressive eyelid tumors like SGC and their clinical significance in tumors occurring in the ocular region.

Conclusion

In summary, we provide a comprehensive view of the immunohistochemical expression of PD-L1 and PD-1 in SGC. However, a major limitation of our study is the small size of the cohort due to the rarity of SGC and heterogenous intensity of PD-L1 in SGC sections, indicating that an entire tumor block might be needed to determine PD-L1 expression in a tumor. The presence of PD-L1 immunopositvity in SGC specimens could assist patient selection for treatment with PD-1/PD-L1 checkpoint inhibitors. Hence, the significance of PD-L1 expression in SGC needs to be done in a larger sample size along with other molecular parameters for a better understanding of the role of PD-L1 mediated immune escape in SGC.

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Ethics

This study was conducted after approval had been obtained from the Institute Ethics committee, AIIMS, New Delhi, India (IEC-107/05.02.2016, RP-28/2016), and carried out in accordance with Declaration of Helsinki principles. Informed consent was obtained from all patients participating in this study.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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