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ORIGINAL ARTICLE

Expression profiles of miR-29c, miR-200b and miR-375 in tumour and tumour-adjacent tissues of head and neck cancers

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Abstract Altered expression of microRNAs (miRNAs) has been shown in many types of malignancies including the head and neck squamous cell carcinoma (HNSCC). Although there are many new and innovative approaches in the treatment of HNSCC, a clear marker of this disease is still missing. Three candidate miRNAs (miR-29c-3p, miR-200b-5p and miR-375-3p) were studied in connection with HNSCC using quantitative real-time PCR expression levels in 42 tissue samples of HNSCC patients and histologically normal tumour-adjacent tissue samples of these patients. Primary HNSCC carcinoma tissues can be distinguished from histologically normal-matched noncancerous tumour-adjacent tissues based on hsa-miR-375-3p expression (sensitivity 87.5 %, specificity 65 %). Additionally, a significant decrease of hsa-miR-200b-5p expression was revealed in tumour-adjacent tissue samples of patients with node positivity. Lower expression of hsa-miR-200b-5p and hsa-miR-29c-3p in HNSCC tumour tissue was associated with higher tumour grade. Consequently, survival analysis was performed. Lower expression of hsa-miR-29c-3p in tumour-adjacent tissue was associated with worse overall and disease-specific survivals. Lower expression of miR-29c-3p in tumourous tissue was associated with worse relapse-free survival. hsa-miR-375-3p seems to be a relatively promising diagnostic marker in HNSCC but is not suitable for prognosis of patients. Furthermore, this study highlighted the importance of histologically normal tumour-adjacent tissue in HNSCC progress (significant decrease of hsa-miR-200b-5p expression in tumour-adjacent tissue of patients with node positivity and low expression of hsa-miR-29c-3p in HNSCC tumour-adjacent tissue associated with worse prognosis).

Keywords Head and neck neoplasms · Carcinoma, squamous cell of head and neck · MicroRNAs · Biomarkers, tumour · Survival · Proportional hazards models

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer [1]. Despite new approaches in treatment of HNSCC, the overall 5-year survival rate for patients with HNSCC is only 50 % mostly because of the high rate of recurrences and advanced stage of disease by diagnosis [2]. Epidemiology of HNSCC has changed during the past 30 years; formerly, HNSCC was most commonly seen in older adults with a history of alcohol and tobacco use, and now it can be seen in younger adults in their 40s and 50s [3]. Thus, biomarkers with specific indications for diagnosis, prognosis and prediction of therapeutic response are desperately needed.

Many studies proved that the aberrations in the microRNA (miRNA) expression are tightly connected with pathogenesis of human cancers, including HNSCC [4, 5]. miRNAs are small RNA molecules (20–22 nucleotides) unable to encode proteins but managing significant catalytic, structural and post-transcriptional regulatory functions. They regulate target molecule by binding to target messenger RNA (mRNA) and inhibit protein translation or induce degradation of mRNA [6]. In this study, we focused on expression profiles of miR-29c-3p, miR-200b-5p and miR-375-3p. With detailed 5p arm and 3p arm and sequence presentation, we could achieve more reproducible results. The arm annotation is quite often lacking in other studies.

miR-29c belongs to the miR-29 family and is deregulated in many different types of cancer including nasopharyngeal carcinomas. miR-29c habitually has tumour-suppressive effect in those cancers [7–11]. The 3p arm of the miR-29 precursor is a prevailing product (miR-29c or miR-29c-3p), although the 5p arm (miR-29c* or miR-29c-5p) also objectively exists [12]. miR-200b-5p is a key regulator of epithelial-mesenchymal transition (EMT) involved in cancer metastasis and chemoresistance [13]. Furthermore, RNA-sequencing analysis revealed that enhanced expression of pri-miR-200b resulted in increased expression of both miR-200b-3p and miR-200b-5p. miR-200b-5p was not expressed in triple negative breast cancer cell lines with EMT features [14]. Hui et al. revealed that the downregulation of miR-375, presented in 91 % of HNSCC, would result in enhanced proliferation, deregulated growth and nonfunctional apoptosis [15]. Jung et al. also disclosed the miR-375-mediated suppression of multiple oncogenic pathways in HPV-associated carcinogenesis [16]. Downregulation of miR-375 may be a potential marker of metastasis occurrence and poor outcome in HNSCC [17].

In this study we focused on the evaluation of three miRNAs with supposed tumour-suppressive effect (miR-29c-3p, miR-200b-5p and miR-375-3p) as diagnostic and prognostic markers of HNSCC.

Materials and methods

Sample preparation

All procedures performed in this study were approved by the ethical committee of St. Anne's Faculty Hospital, Brno, Czech Republic, and were in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All surgical tissue samples were obtained from HNSCC patients after they signed the informed consent. Histologically verified primary HNSCC carcinoma tissues (T) and matched non-cancerous adjacent tissues (A) were collected. The tissue material harvested at surgery was placed into RNAlater solution for RNA stabilization and storage (Ambion, USA). The material was maintained cold, and RNA was isolated within 24 h.

Total RNA extraction, quantitative real-time PCR

We obtained total RNA from samples using TRIzol reagent (Invitrogen, UK). RNA concentrations and purity were determined by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). An optical density ratio at 260:280 nm was calculated to evaluate protein contamination of RNA. In addition to the ratio at 260:280 nm, measurements were taken also at 280 and 230 nm. Our 260:280 values were between 1.84 and 2.08. The A260/A230 ratio was greater than 1.5 in all samples. According to manufacturer's instructions, 10 ng of isolated RNA was transcribed using the TaqMan® miRNA reverse transcription kit (Applied Biosystems, USA), and 1.33 μl of the transcribed miRNA was used directly in the quantitive real-time PCR reaction. The primer and probe sets were selected from TaqMan miRNA expression assays hsa-miR-29c (assay ID: 000587), hsa-miR-200b (assay ID: 002274) and hsa-miR-375 (assay ID: 000564)) (Applied Biosystems, USA). The amplified DNA was analysed by the comparative Ct method using RNU44 (assay ID: 001094) as an endogenous control. The qRT-PCR was performed under the following amplification conditions: total volume 20 μl, initial denaturation 95 °C/10 min, then 45 cycles 95 °C/15 s, 60 °C/1 min with the 7500 real-time PCR system (Applied Biosystems, USA). Sequence of studied miRNAs is shown in Table 1.

Data normalization and statistical analysis

Log-transformed miRNA expression data were analysed using a paired test (tumour versus tumour-adjacent tissue) and using one factor ANOVA. Survival analysis was analysed using Cox proportional hazard regression with miRNA expression levels as covariates. Receiver-operator curves (ROC), cutoff, sensitivity and specificity were calculated using Cutoff Finder (http://molpath.charite.de/cutoff) according to [18]. Unless noted otherwise, p level < 0.05 was considered significant. Software Statistica 12 (StatSoft, Tulsa, OK, USA) was used for analysis.

Table 1 Sequence of studied miRNAs

miRNA	Sequence
hsa-miR-375-3p	UUUGUUCGUUCGGCUCGCGUGA
hsa-miR-29c-3p	UAGCACCAUUUGAAAUCGGUUA
hsa-miR-200b-5p	CAUCUUACUGGGCAGCAUUGGA

Results

Clinico-pathological characterization of HNSCC patients

In this study, in total, 42 biopsy samples of tumours from male patients with histologically verified spinocellular carcinoma and comprehensive patient history were used. Only patients fulfilling following criteria were included: descriptors of the tumour are present (histology, tumour staging, grading) and patients with no current or previous malignancy. Therapeutic strategy was not taken into account. Sampling was performed before the therapy begun (either chemo-, radiotherapy or surgery). Age of patients and HPV status was not taken into account. Tumour-adjacent tissue was verified histologically. Expression of the selected miRNAs in tumour tissue was compared with the control group consisting of matched tumour-adjacent histologically normal tissue (39 samples). Brief description of the cases is shown in Table 2. In the next step, the effect of clinico-pathological conditions of patients on the expression of the selected miRNAs was analysed.

miRNA expression pattern in tumour and tumour-adjacent tissues

The expression analysis of miRNA was performed to characterize the expression profile of selected miRNAs in the particular tissue type. A multivariate test revealed a significant effect of the tissue type on the miRNA expression pattern (F (6, 144) = 3.07, p = 0.007).

In accordance with the aim of this study, the expression of selected miRNAs in tumour tissue and histologically normal tumour-adjacent samples was assessed. miRNA expression for the tumour samples and the matched adjacent tissues (tumour = 39, adjacent = 39) were analysed using the paired t test analysis.

hsa-miR-375-3p and hsa-miR-29c-3p were both more expressed in tumour-adjacent tissues (11.59-fold higher expression, p = 0.0001 and 2.63-fold higher, p = 0.048 for miR-375 and -29c, respectively). No statistically significant change in expression of hsa-miR-200b-5p between adjacent and tumour tissues was found.

ROC (receiver-operator curves) analysis identified a sensitivity 87.5 % (95 % CI 94.5–73.9), specificity 65 % (95 % CI 77.9–49.5) and area under curve (AUC) = 0.74 for hsa-miR-375-3p and sensitivity 59.0 % (95 % CI 72.9–43.4), specificity 69.2 % (95 % CI 81.4–53.6) and AUC = 0.62 for hsa-miR-29c-3p (see Fig. 1).

Table 2 Characteristics of the samples of patients and controls. Note that tumour staging and grading refer to the first "tumour" group of patients only

Factor	Group	Number	Age (min–max)
Group	Tumour	42	63 (47–87)
	Tumour-adjacent	39	62 (47–87)
Gender			
	Male	42	63 (47–87)
Tumour grade			
	High	35	64 (47–87)
	Low	4	65 (56–79)
Tumour stage			
	T1–2	18	62 (47–83)
	T3–4	22	65 (47–87)
Node positivity			
	No	15	64 (51–83)
	Yes	20	64 (47–87)
Meta positivity			
	No	31	64 (47–87)
	Yes	4	61 (55–71)

miRNA expression and tumour staging

Consequently, the effect of tumour staging on the expression of the above-mentioned miRNAs was analysed. Tumour-adjacent tissues are involved in the development and progression of the tumour; therefore, the effect of tumour staging was not only related to the expression in HNSCC tumourous tissue but also to the expression in tumour-adjacent tissue samples. First, the effect of TNM T staging was analysed (T1–2 versus T3–4). Thirty-five tumour tissue samples (T1–2 = 15, T3–4 = 20) and 34 tumour-adjacent tissue samples (T1–2 = 15, T3–4 = 19) were involved in the analysis. No significant association between the selected miRNA expression and T stage was determined either in the tumour or in the tumour-adjacent tissue. Subsequently, the effect of node positivity was analysed. Thirty-five tumour tissue samples (N positive = 20, N negative = 15) and 33 tumour-adjacent tissue samples (N positive = 19, N negative = 14) were involved in the analysis. A significant decrease of hsa-miR-200b-5p expression was revealed in tumour-adjacent tissue samples of patients with node positivity (0.17-fold expression, 95 % CI 0.03–0.87; p = 0.035). In the next step, the effect of the presence of distant metastases was analysed. Thirty-five tumour tissue samples (M positive = 4, M negative = 31) and 33 tumour-adjacent tissue samples (M positive = 4, M negative = 29) were included in the analysis. No significant association between distant metastasis and the selected miRNA expression was determined either in the tumour or in the tumour-adjacent tissue.

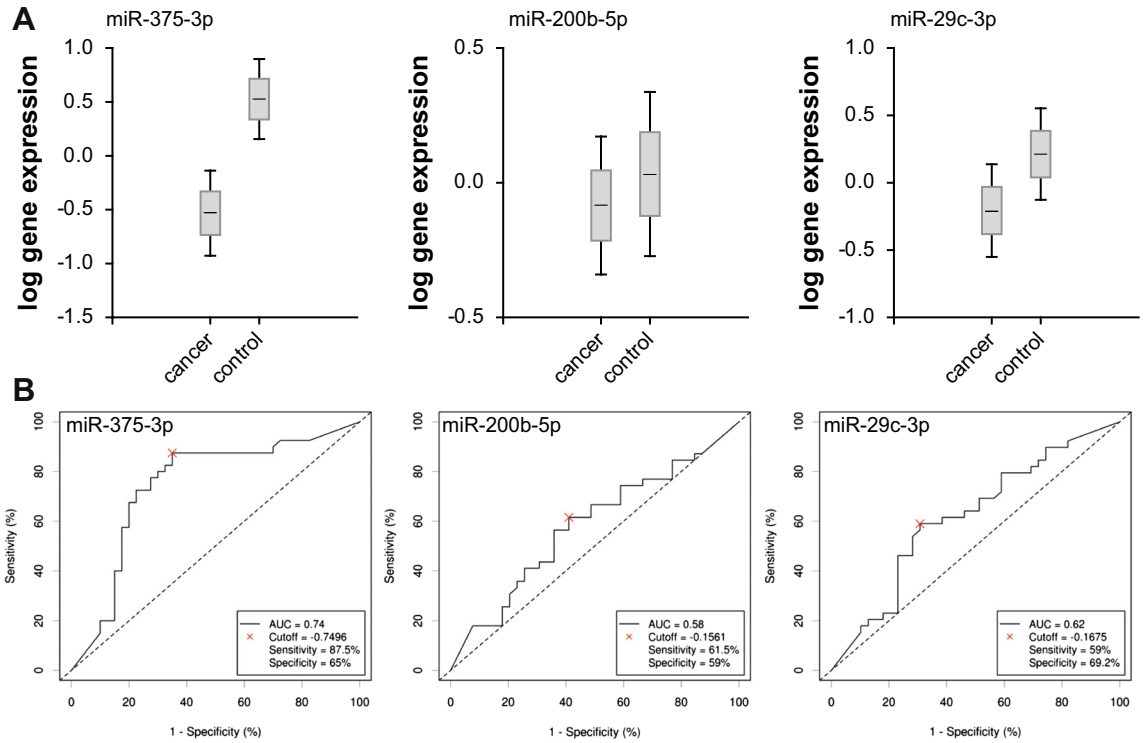

Fig. 1 Tissue gene expression of microRNAs. **a** Expression in tumour and tumour-adjacent (control) tissues. Displayed as logarithm of gene expression, mean ± 1and 2 SE. *Asterisks* indicate difference significant at $p < 0.05$ in paired test. **b** Area under curves, sensitivity and specificity for particular miRNAs

Gene expression and histological grading

Thirty-four tumour tissue samples (high grade = 30, low grade = 4) and 32 tumour-adjacent tissue samples (high grade = 29, low grade = 3) were involved in the analysis. Low expression of hsa-miR-200b-5p (0.03-fold change, 95 % CI 0.001–0.15; $p = 0.0001$) and hsa-miR-29c-3p (0.05-fold change, 95 % CI 0.001–0.63; $p = 0.023$) in tumour tissue was significantly associated with higher tumour grade (see Fig. 2).

Association between miRNA expression and disease-free and overall survivals

The prognostic value of miR-29c-3p, miR-200b-5p and miR-375-3p expressions on overall, disease-specific and recurrence-free survivals was studied by Cox proportional hazard regression. Similar to previous chapters, the hazard was calculated for the miRNA expression in tumour and tumour-adjacent tissues separately. Survival analysis showed a significant effect of

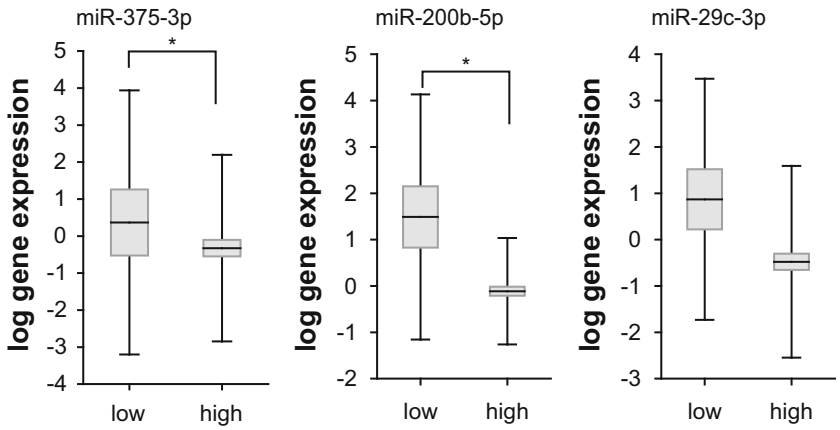

Fig. 2 Gene expression of miRNAs in tumour tissue in relation to tumour grade. Displayed as logarithmic gene expression, mean ± 1and 2 SE. High grade indicates grade > 2

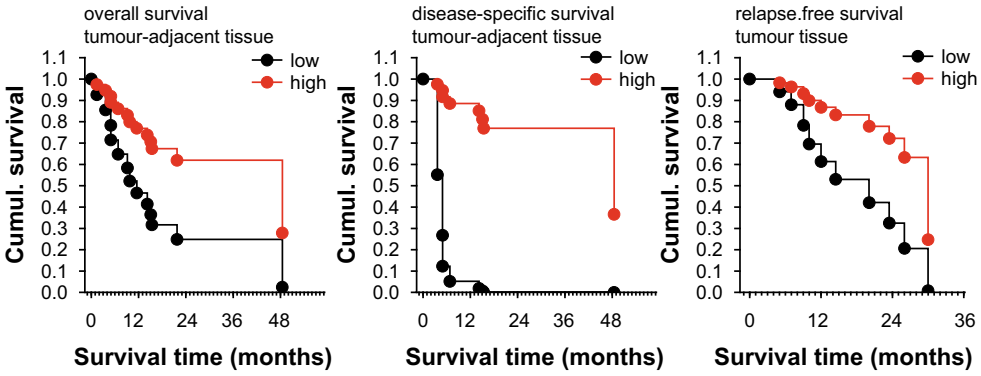

Fig. 3 Cox proportional hazard model for overall, disease-specific and recurrence-free survivals for miR-29c. miRNA expression as a continuous predictor. Note that overall and disease-specific survivals are displayed for tumour-adjacent tissue and relapse free for tumour tissue expressions. For detailed results see Table 3

miR-29c-3p on overall and disease-specific survivals in tumour-adjacent tissue (hazard ratio, HR = 0.27, 95 % CI = 0.01 to 0.85 and 0.07, 95 % CI = 0.01 to 0.59, respectively) (Fig. 3). In addition, there was a significant effect of miR-29c-3p on recurrence-free survival in tumour tissues (HR = 0.31, 95 % CI = 0.10 to 0.91). miR-200b and 375 were not associated with a hazard in overall, disease-specific and recurrence-free survivals. For details, see Table 3.

Discussion

MicroRNAs (miRNAs) are important regulators of gene expression. Downregulation of tumour suppressor miRNAs or overexpression of particular onco-miRNAs is involved in pathogenesis of human cancers and cause tumourigenesis in mouse models [19].

In this study, we focused on the expression profiles of presumed tumour suppressor miRNAs (miR-29c-3p, miR-200b-5p

Table 3 Cox proportional hazard model for overall survival, disease-specific survival and recurrence-free survival. Displayed for tumour tissue and tumour-adjacent tissue miRNA expressions separately

Outcome type	Tissue	miRNA	Hazard ratio (95 % CI)	*p* value
Overall survival				
	Tumour	miR-200b	1.00 (0.42 to 2.38)	0.993
		miR-375	1.32 (0.76 to 2.27)	0.321
		miR-29c	0.89 (0.47 to 1.70)	0.732
	Tumour-adjacent	miR-200b	0.84 (0.30 to 2.40)	0.755
		miR-375	2.17 (0.83 to 5.65)	0.113
		miR-29c	0.27 (0.01 to 0.85)	0.024
Disease-specific survival				
	Tumour	miR-200b	1.25 (0.51 to 3.08)	0.63
		miR-375	1.45 (0.74 to 2.81)	0.278
		miR-29c	0.80 (0.37 to 1.75)	0.573
	Tumour-adjacent	miR-200b	0.44 (0.04 to 4.23)	0.488
		miR-375	4.48 (0.90 to 22.33)	0.067
		miR-29c	0.07 (0.01 to 0.59)	0.014
Recurrence-free survival				
	Tumour	miR-200b	0.91 (0.14 to 5.23)	0.924
		miR-375	1.77 (0.67 to 468)	0.248
		miR-29c	0.31 (0.10 to 0.91)	0.034
	Tumour-adjacent	miR-200b	0.09 (0.00 to 7.77)	0.290
		miR-375	3.09 (0.10 to 96.39)	0.521
		miR-29c	1.14 (0.06 to 21.08)	0.928

CI confidence interval

and miR-375-3p) in HNSCC. Many of the miRNAs have gender-related expression levels, and oestrogen-dependent miRNA regulation is also well known [20, 21]. For these reasons, only male HNSCC patients were included into analysis. hsa-miR-375-3p and hsa-miR-29c-3p were both less expressed in tumour tissues. ROC (receiver-operator curves) analysis identified a sensitivity 87.5 %, specificity 65 % and AUC = 0.74 for hsa-miR-375-3p and sensitivity 59.0 %, specificity 69.2 % and AUC = 0.62 for hsa-miR-29c-3p. No statistically significant change in expression of hsa-miR-200b-5p between tumour-adjacent and tumour tissues was found. Downregulation of tumour suppressor miR-375 could lead to uncontrolled cancerous inhibitor of protein phosphatase 2A (CIP2A) expression and strengthened stability of MYC oncogene, which contributes to the promotion of tumourous phenotypes, such as increased proliferation, colony formation, migration and invasion [22]. Furthermore, it was shown that common anti-cancer drugs such as doxorubicin, 5-fluorouracil, trichostatin A or etoposide reactivated miR-375 and its primary transcript pri-miR-375 expression in tongue cancer cells [23]. Lower hsa-miR-29c-3p expression in tumour tissue is also in accordance with other studies [11, 24–26]. Missing significant difference in hsa-miR-200b-5p expression between tumour-adjacent tissues and HNSCC tumour tissues could be caused by the presence of activated cancer-associated fibroblasts (CAFs) in tumour-adjacent tissue. Tang et al. revealed that miR-200s are generally downregulated not only in breast cancer tissues but also in activated CAFs. Fibroblasts with downregulated miR-200s displayed accelerated migration and invasion [27]. In accordance, a decrease of hsa-miR-200b-5p expression in tumour-adjacent tissue samples was associated with node positivity in HNSCC patients. Low expression of hsa-miR-200b-5p and hsa-miR-29c-3p in tumour tissue was also significantly associated with higher tumour grade. Using the survival analysis, it revealed a significant effect of hsa-miR-29c-3p expression in tumour-adjacent tissue on the overall and disease-specific survivals and in tumour tissue on recurrence-free survival (higher expression of this miRNA was associated with better prognosis). Nevertheless, no significant effect of hsa-miR-375-3p or hsa-miR-200b-5p expressions was demonstrated using the survival analysis. It could be due to context-dependent effects of miRNAs. They are tumour suppressive in context of pro-tumourigenic pathways, but they also can confer a resistance to the chemotherapy [28]. For example, artificial overexpression of miR-375 in cervical cancer cells decreased paclitaxel sensitivity in vitro and also in vivo [29]. miR-141 and miR-200 expressions may lead to two counteracting effects: resistance to platinum compounds on the one hand but increased sensitivity to paclitaxel on the other [28, 30]. The negative correlation between hsa-miR-200b-5p and hsa-miR-200b-3p expressions and cisplatin sensitivity was revealed also in NCI60 platform by CellMiner (http://discover.nci.nih.gov/cellminer/).

Conclusions

In conclusion, hsa-miR-375-3p seems to be a relatively promising diagnostic marker inasmuch as primary HNSCC carcinoma tissues can be distinguished from histologically normal-matched noncancerous tumour-adjacent tissues based on these miRNA expressions. A close relationship was found between tumour miRNA expression profiles and circulating miRNAs [31, 32]. Consequently, hsa-miR-375-3p could be a promising target of further research of diagnostic markers in circulation.

Furthermore, this study highlighted the importance of histologically normal tumour-adjacent tissue in HNSCC progress, inasmuch as a significant decrease of hsa-miR-200b-5p expression was revealed in the tumour-adjacent tissue samples of patients with node positivity, and low expression of hsa-miR-29c-3p in HNSCC tumour-adjacent tissue was significantly associated with worse prognosis.

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Compliance with ethical standards

Conflicts of interest None

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