Biochimica et Biophysica Acta 1842 (2014) 686–690

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Piwi-like 1 and 4 gene transcript levels are associated with clinicopathological parameters in renal cell carcinomas

Omar Al-Janabi [a,1], Sven Wach [a,1], Elke Nolte [a], Katrin Weigelt [a], Tilman T. Rau [b], Christine Stöhr [b], Wolfgang Legal [a], Stefan Schick [c], Thomas Greither [d], Arndt Hartmann [b], Bernd Wullich [a], Helge Taubert [a,*]

[a] Department of Urology, Universitätsklinikum Erlangen, Friedrich-Alexander Universität Erlangen-Nürnberg, Erlangen, Germany
[b] Institute of Pathology, Universitätsklinikum Erlangen, Friedrich-Alexander Universität Erlangen-Nürnberg, Erlangen, Germany
[c] Tumour Centre at the Friedrich-Alexander University Erlangen-Nürnberg, Erlangen, Germany
[d] Center for Reproductive Medicine and Andrology, Martin Luther University Halle-Wittenberg, Halle, Germany

ARTICLE INFO

Article history:
Received 21 November 2013
Received in revised form 24 January 2014
Accepted 30 January 2014
Available online 7 February 2014

Keywords:
Piwi-like genes
Clear cell renal cell carcinoma
Quantitative RT-PCR
Age
Normal renal tissue
Tumorigenesis

ABSTRACT

Piwi-like gene family members (Piwil 1–4) are considered stem cell-associated genes/proteins. These are expressed predominantly in germline cells, but are re-expressed in different tumors. Piwil 1–4 gene expression has not previously been studied and correlated with clinicopathological parameters in renal cell carcinomas (RCC). The Piwil 1–4 transcript levels were analyzed by quantitative real-time PCR in 73 clear cell RCC (ccRCC) tissues and corresponding normal tissues. The transcript levels of Piwil 1, 2 and 4 were strongly and significantly correlated with each other, in both the tumor tissues and the normal tissues ($P < 0.001$; Spearman's rank test). Piwil 4 gene expression was significantly higher in the ccRCC tissues than that in the corresponding normal renal tissues ($P < 0.001$; Wilcoxon signed-rank test). When the ccRCC patient cohort was divided according to the median Piwil 1–4 expression into low- and high-expression groups and according to age into younger (≤64 years) and older patient groups (>64 years), the younger patients displayed significantly higher levels of Piwil 1 mRNA in comparison to the older patients ($P = 0.010$; Fisher's exact test). Interestingly, Piwil 1 expression was left–right polarized in the normal tissues but not in the tumor tissues ($P = 0.004$; Fisher's exact test). Altogether, associations were determined between the Piwi-like family member expression levels and clinicopathological parameters of ccRCC, suggesting a potential role for these genes/proteins in ccRCC diagnostics and tumorigenesis as well as in renal tissue embryology.

1. Introduction

The Piwi-like genes belong to the Argonaute gene family and comprise a family of four members in humans: Piwi-like 1 (Hiwi, Piwil 1), Piwi-like 2 (Hili, Piwil 2), Piwi-like 3 (Piwil 3) and Piwi-like 4 (Hiwi 2, Piwil 4) [1]. Piwi-like genes/proteins are expressed predominantly in the germline. Piwi-like proteins can catalyze an amplification loop (ping-pong cycle) of small RNAs, the so-called piRNAs. Both piRNAs and piwi-like proteins function as a piwi ribonucleoprotein complex in transposon repression through target degradation and epigenetic silencing (reviewed in [2,3]). Only Piwil 4 mRNA expression has been described in several human somatic tissues [4]. In contrast, the expression of all four Piwil genes has been reported in prostate and pancreatic tissues in rhesus macaques [5]. Importantly, Piwil gene re-expression is common to many tumor entities [6]. Piwi-like protein overexpression has been linked to cell physiological parameters in tumors, e.g., Piwil 1 protein to cell proliferation in gastric cancer [7] and Piwil 2 to anti-apoptotic signaling in different cancers [8]. In addition, the detection of Piwi-like proteins has been associated with clinical parameters and/or poor prognosis in several cancers such as breast, cervical, ovarian, endometrial, colon, colorectal, esophageal, gastric or liver cancer and gliomas, seminomas and sarcomas (reviewed in [6]). However, Piwi-like gene mRNA expression has been studied to a much lesser extent in tumor tissues. Piwil 1 mRNA was detected in seminomas, gastric, liver and pancreatic cancers, gliomas and sarcomas [7,9–13]. Piwil 2 gene expression was detected in breast, cervical, prostate, gastrointestinal, ovarian and endometrial cancer, as well as soft tissue sarcomas [8,14,15] but it was not detected in bladder carcinomas or bladder carcinoma cell lines [16]. Piwil 3 transcript expression has only been validated in soft tissue sarcomas [14]. Nevertheless, the Oncomine database (www.oncomine.org/) contains entries that suggest Piwil 3

Abbreviations: Piwil, Piwi-like; ccRCC, Clear cell renal cell carcinoma

* Corresponding author at: Department of Urology, Universitätsklinikum Erlangen, Friedrich-Alexander Universität Erlangen-Nürnberg, D-91054 Erlangen, Germany. Tel.: +49 9131 8523373; fax: +49 9131 8523374.
E-mail addresses: Omar.Al-Janabi@uk-erlangen.de (O. Al-Janabi), Sven.Wach@uk-erlangen.de (S. Wach), Elke.Nolte@uk-erlangen.de (E. Nolte), Katrin.Weigelt@uk-erlangen.de (K. Weigelt), Tilman.Rau@uk-erlangen.de (T.T. Rau), Christine.Stoehr@uk-erlangen.de (C. Stöhr), Wolfgang.Legal@uk-erlangen.de (W. Legal), stefan.schick@tuz.imed.uni-erlangen.de (S. Schick), Thomas.greither@uk-halle.de (T. Greither), Arndt.Hartmann@uk-erlangen.de (A. Hartmann), Bernd.Wullich@uk-erlangen.de (B. Wullich), Helge.Taubert@uk-erlangen.de (H. Taubert).
[1] Both authors contributed equally.

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http://dx.doi.org/10.1016/j.bbadis.2014.01.014

expression in breast, colon, ovary and brain cancers. Piwil 4 mRNA expression was previously reported in cervical cancer and soft tissue sarcomas [14,17], and the Oncomine database also reports Piwil 4 expression in breast, liver and brain cancers. To date, a comparative analysis of the impact of Piwi-like gene mRNA expression on tumorigenesis and tumor progression in RCC has not yet been performed, although Piwil 2 mRNA was reported in one tested RCC sample [8]. Therefore, we aimed to investigate the expression levels of all four Piwil genes in ccRCC tissues and to analyze the correlations between the expression levels of these genes with each other and with clinicopathological parameters.

2. Methods

2.1. Patients and clinical samples

Piwil 1, Piwil 2, Piwil 3 and Piwil 4 mRNA expression levels were analyzed in ccRCC and corresponding normal renal tissue samples from 73 non-selected ccRCC patients. Of these 73 ccRCC patients, all were studied for Piwil 1, Piwil 2 and Piwil 4, and 72 patients were studied for Piwil 3 mRNA expression in tumor and corresponding normal tissues. All patients in this study underwent radical nephrectomy at the Clinic of Urology of the University Hospital Erlangen, Germany. This study was performed in compliance with the Helsinki Declaration. The use of tumor tissues for research was approved by the Internal Review Boards of the Medical Faculty of the University Clinic of Erlangen. All patients gave written informed consent.

The median age of the patients at surgery was 65 years (range, 39–88 years). The median follow-up time after primary tumor resection was 24 months (range, 0–56 months). During the follow-up period, 18 of the 73 patients (25%) died. The tumors were staged and graded according to the TNM Classification of Malignant Tumors [18]. A detailed description of the ccRCC patients' clinicopathological data is given in Table 1.

2.2. Determination of Piwil mRNA tissue expression levels by quantitative PCR

All tissue samples were provided by the tissue bio-repository (stored at −80 °C) at the Comprehensive Cancer Center (CCC), Erlangen-Nürnberg, University Hospital Erlangen. Hematoxylin and eosin-stained tissue sections were reviewed by an experienced uropathologist (TTR). Only tumor tissue samples that were composed of more than 70% cancer cells were included in the study. Normal control tissues that comprised the renal cortex, including parts of the normal tubular system, were derived from the opposite pole of the nephrectomy specimen. RNA extraction was performed with Trizol reagent (Ambion/Life Technologies, Darmstadt, Germany) according to the manufacturer's instructions. All RNA preparations were treated with RNase-free DNaseI (Roche, Mannheim, Germany). RNA isolation quality, cDNA preparation and qRT-PCR quality were checked according to the MIQE guidelines as suggested by Bustin et al. [19] and are described in Supplementary data 1. RNA yields and purity were determined with a microliter spectrophotometer (NanoDrop 1000; Thermo Scientific, Wilmington, DE). cDNA synthesis was performed with the Dynamo cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, 1 μg of total RNA was reverse-transcribed in a total volume of 20 μl and was used as template cDNA in subsequent PCR reactions. Quantitative PCR reactions were performed in a total volume of 10 μl, using gene-specific primers and hybridization probes for Piwil 1 (Hs01041737_m1), Piwil 2 (Hs00216263_m1), Piwil 3 (Hs00908825_m1) and Piwil 4 (Hs00381509_m1) that were purchased from Applied Biosystems (Applied Biosystems, Foster City, CA, USA). To allow relative quantification, each sample was analyzed in parallel for the expression of specific Piwil transcripts and the endogenous reference gene HPRT1 (Hs99999909_m1, Applied Biosystems). The quantitative PCR reactions were performed in 96-well plate format with the StepOne plus real-time PCR system (Applied Biosystems; Supplementary data 1). The relative Piwil transcript expression levels were calculated according to the ΔΔCt method [20] in which one sample served as reference.

2.3. Statistical analysis

Correlations between the continuous biological markers variables were calculated with Spearman's rank correlation test (r_s). Differences between the Piwil member mRNA expression levels in malignant vs. normal tissues from ccRCC patients were estimated with the Wilcoxon signed-rank test and between different age groups with the Mann–Whitney *U*-test. Relationships between the biological marker expression levels and the clinicopathological parameters were assessed with Fisher's exact test. Specifically, the Piwil member median expression levels were used to separate samples into low- and high-expression groups (Table 2). Receiver operating curve (ROC) analyses were performed to determine the ability of mRNA expression values to distinguish between tumor and normal tissue samples. All calculations were performed with the SPSS 21 statistical package (SPSS-Science, Chicago, IL, USA). *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Piwil member mRNA expression levels in malignant and normal tissues from renal cell carcinoma patients

The median-normalized relative Piwil 1, Piwil 2, Piwil 3 and Piwil 4 mRNA expression levels in tumor tissues were 2.70 (0.00–259.12), 2.15 (0.01–39.55), 0.92 (0.00–637.39) and 16.93 (0.00–142.95),

Table 1
Relationships between clinicopathological parameters and Piwil gene family member mRNA expression levels in tumor tissues from renal cell carcinoma patients.

Clinicopathological parameters	No. cases	Piwil 1 low/high	No. cases	Piwil 2 low/high	No. cases	Piwil 3 low/high	No. cases	Piwil 4 low/high
Age	73	***P* = 0.010**	73	*P* = 0.816	72	*P* = 0.638	73	*P* = 0.352
≤64 years	37	12/25	35	17/18	35	16/19	35	15/20
>64 years	36	23/13	38	20/18	37	20/17	38	21/17
Tumor grade	72	*P* = 0.256	72	*P* = 0.648	71	*P* = 1.000	72	*P* = 0.898
1 + 2	51	24/27	51	26/25	50	25/25	51	25/26
3 + 4	21	13/8	21	11/10	21	10/11	21	11/10
Tumor stage	73	*P* = 0.854	73	*P* = 1.000	72	*P* = 0.593	73	*P* = 0.566
pT1 + 2	48	25/23	48	24/24	47	22/25	48	22/26
pT3 + 4	25	12/13	25	13/12	25	14/11	25	14/11
Localization	72	*P* = 0.355	72	*P* = 1.000	71	*P* = 0.813	72	*P* = 0.344
Left	33	19/14	33	17/16	32	17/15	33	19/14
Right	39	18/21	39	20/19	39	19/20	39	17/22
Patient status	73	*P* = 1.000	73	*P* = 0.595	72	*P* = 0.786	73	*P* = 1.000
Alive	55	28/27	55	29/26	54	26/28	55	27/28
Dead	18	9/9	18	8/10	18	10/8	18	9/9

Statistical analysis was performed with Fisher's exact test. Significant values are marked in bold.

Table 2
Relative Piwil family member mRNA expression levels in malignant and corresponding normal tissues from ccRCC patients.

	No. patients	Median[a]	Range
Piwil 1		$P = 0.089$	
Malignant tissue	73	2.70	0.00–259.12
Normal tissue	70	4.17	0.00–215.47
Piwil 2		$P = 0.914$	
Malignant tissue	73	2.15	0.01–39.55
Normal tissue	70	1.75	0.00–23.89
Piwil 3		$P = 0.923$	
Malignant tissue	72	0.92	0.00–637.39
Normal tissue	70	0.67	0.00–525.84
Piwil 4		**$P < 0.001$**	
Malignant tissue	73	16.93	0.00–142.95
Normal tissue	70	5.87	0.00–70.75

[a] *P*-values were estimated with the Wilcoxon signed-rank test. Significant value is marked in bold.

respectively (Table 2). The median-normalized transcript levels of Piwil 2–4 were lower in normal tissues than that in tumor tissues. However, the levels of Piwil 1 were somewhat higher in normal tissues. We detected the following normalized mRNA expression values for Piwil 1–4 in normal tissues median (range): 4.17 (0.00–215.47), 1.75 (0.00–23.89), 0.67 (0.00–525.84) and 5.87 (0.00–70.75) (Table 2).

3.2. Relationships between Piwil member mRNA expression levels in malignant and normal tissues from clear cell renal cell carcinoma patients

The relative Piwil gene family member mRNA expression levels in the malignant tissue were strongly and significantly positively correlated with each other (bivariate linear correlation analysis; Spearman's Rho test). The relative Piwil 1 mRNA expression levels correlated significantly with those of Piwil 2 ($r_s = 0.438$; $P < 0.001$) and Piwil 4 ($r_s = 0.375$; $P = 0.001$). Additionally, a significant positive correlation was observed between the relative Piwil 2 mRNA expression levels and those of Piwil 4 ($r_s = 0.785$; $P < 0.001$) and weakly with Piwil 3 ($r_s = 0.240$; $P = 0.042$; Supplementary Table 1). When we compared the Piwil 1–4 mRNA expression levels in normal tissues, the associations between Piwil 1, 2 and 4 were comparable to those in the tumor tissues. The relative Piwil 1 mRNA expression levels correlated significantly with those of Piwil 2 ($r_s = 0.485$; $P < 0.001$) and Piwil 4 ($r_s = 0.464$; $P < 0.001$). Additionally, the Piwil 2 transcript levels correlated significantly with those of Piwil 4 ($r_s = 0.527$; $P < 0.001$; Supplementary Table 1).

A comparison of the Piwil 4 mRNA expression levels in malignant and normal tissues revealed that the transcript levels were significantly higher in the malignant tissue samples ($P < 0.001$; Wilcoxon signed-rank test; Table 2). An ROC analysis, using the median Piwil 4 mRNA expression (11.08) as the cutoff value, yielded a sensitivity of 67.1% and a specificity of 67.1% for discriminating between malignant and corresponding normal tissues ($P < 0.001$). The area under the curve was 0.746 (95% CI = 0.665–0.827; Supplementary Fig. 1). However, we found no significant differences in the Piwil 1, Piwil 2 and Piwil 3 mRNA expression levels between the malignant and corresponding normal ccRCC tissues (data not shown).

3.3. Associations between Piwil 1 mRNA expression levels in tumor tissues and renal cell carcinoma patients clinicopathological data

For the statistical analyses, all Piwil member expression levels were stratified according to their median values to separate ccRCC patients into groups with low and high tumor expression of Piwil 1–4 mRNA (Tables 1 and 2).

We found no associations between clinicopathological parameters such as the tumor stage, tumor grade or survival (overall and disease-specific survival) and Piwil 1–4 gene expression (data not shown). However, we detected a significant association between the age at diagnosis and the Piwil 1 transcript levels. Patients were stratified into two groups according to the median age at diagnosis to form groups of ≤64 years and >64 years. The younger ccRCC patient group (≤64 years) displayed significantly higher expression levels of Piwil 1 mRNA compared to the older group (>64 years; $P = 0.010$; Fisher's exact test; Fig. 1). However, there were no significant differences between the two age groups with respect to Piwil 2, Piwil 3 and Piwil 4 mRNA expression (data not shown). Similarly, patients with high Piwil 1 mRNA expression levels were younger at diagnosis than those with low Piwil 1 transcript levels (60.0 vs. 68.0 years, respectively). In summary, the ccRCC patients with high Piwil 1 expression levels experienced tumor onset 8 years earlier than did the group with low tumor expression of Piwil 1.

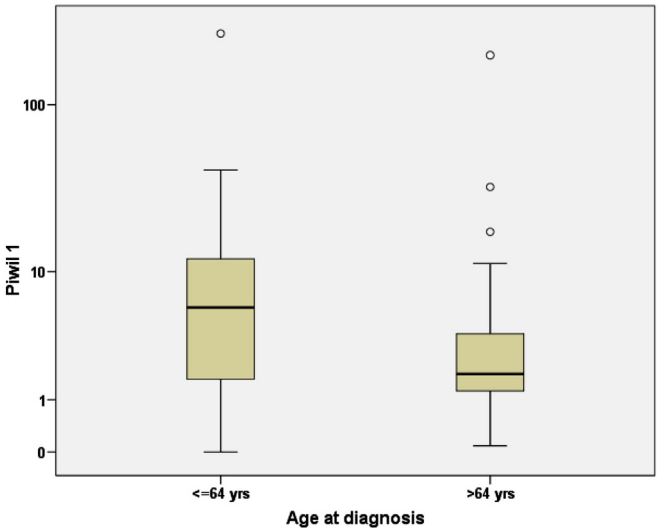

Fig. 1. Box plot: Piwil 1 gene expression and age at diagnosis. Piwil 1 expression levels were significantly different between the two age groups (≤64 years vs. >64 years) when the patients were separated according to the median age ($P = 0.022$; Mann–Whitney *U*-test). When the ccRCC patients were further separated according to median Piwil 1 mRNA expression in patients with low or high expression, significant differences were observed in both groups when they were compared according to age ($P = 0.010$; Fisher's exact test).

3.4. Associations between Piwil 1 mRNA expression levels in normal renal tissue and left–right polarity

While incorporating additional information about the normal tissue locations, we detected an association between the Piwil 1 expression levels in normal renal tissues and the tissue location, such that the Piwil 1 transcript levels were significantly higher in normal tissues derived from the right-sided kidneys than from left-sided kidneys ($P = 0.001$; Mann–Whitney *U*-test). After dividing the normal tissues according to the median Piwil 1 expression to form low- and high-expression groups, we detected also a difference between the left- or right-sided origins of the normal tissues ($P = 0.004$; Fisher's exact test). However, similar associations with left–right polarity were not detected for Piwil 2–4 in the normal tissues or for any of the Piwil gene members in the tumor tissues.

4. Discussion

In this study, the Piwil 1–4 expression levels were analyzed in ccRCC tissues and corresponding normal tissues and were correlated with each other as well as with histomorphological and clinicopathological parameters. We found strong, significant positive correlations between the mRNA levels of Piwil 1, 2 and 4, but not Piwil 3, in tumor tissues. Additionally, strong significant positive associations between the transcript levels of Piwil 1, 2 and 4 were detected in normal renal tissues.

Comparable results have also been found for soft tissue sarcoma (STS) patients, in whom significant correlations were detected between Piwil 2 and Piwil 4 expression levels in tumor tissues and in tumor-adjacent non-malignant tissues ([14] and unpublished results). Currently, it is not known whether the Piwil gene family members can interact with each other or if they are regulated in a concerted manner. The significant correlations between the Piwil 1, 2 and 4 mRNA levels in the normal renal tissues suggest a general rather than a tumor-specific interaction or regulation.

The expression levels of all Piwil family members, with the exception of Piwil 1, were higher in the tumor tissues than that in the corresponding normal renal tissues. However, only the expression levels of Piwil 4 differed significantly between the corresponding malignant and normal renal cell tissue. A former study of STS patients also revealed higher Piwil 2–4 mRNA expression levels in tumor tissues, compared to normal tissues, but these differences were not significant [14]. Differences in expression between tumor and non-malignant tissues and therefore a diagnostic impact of Piwil mRNA expression have been reported in two other tumor entities. Piwil 1 (Hiwi) mRNA levels were significantly higher in hepatocellular carcinomas than that in adjacent normal hepatic tissues [21]. Furthermore, Piwil 2 transcript levels were significantly increased in papillary thyroid carcinomas compared to matched adjacent non-cancerous epithelium [22].

We did not detect significant associations between the Piwil 1–4 expression levels and the ccRCC patients' clinicopathological parameters (including survival), except for the age at diagnosis. Significantly higher Piwil 1 mRNA expression levels were detected in the younger ccRCC patients after the patients were separated according to the median age at diagnosis ($\leq$64 years vs. >64 years). Our results show that ccRCC patients with higher Piwil 1 transcript levels had a significantly earlier age of tumor onset (8 years) than did patients with lower tumor transcript levels, suggesting a role for Piwil 1 in ccRCC tumorigenesis. However, no correlations were found between the Piwil 1 protein expression and the ages of cervical or hepatocellular carcinoma patients [21,23]. The following different roles have been suggested for Piwi-like proteins during tumorigenesis: transcriptional silencing of tumor-suppressing genes through epigenetic mechanisms, post-transcriptional regulation of oncogenes and tumor suppressor genes, regulation of genomic stability and promotion of cell proliferation in cancer cells, leading to aneuploidy during mitosis (reviewed in [6]). Practical evidence that Piwil 1 (Hiwi) can function in a pro-tumorigenic manner was recently presented. The overexpression of Hiwi in sarcoma precursors inhibited their differentiation in vitro and generated sarcomas in vivo. The authors reported that Hiwi-associated DNA hyper-methylation with subsequent genetic and epigenetic changes favored a tumorigenic state [24].

An interesting result was the significantly different Piwil 1 mRNA expression levels that were measured in normal renal tissues that originated on either the left- or the right-handed side. Interestingly, this association was not detected in tumor tissues, regardless from which body site the tumors originated. Because our finding was related to normal tissue alone, it indicates a role for Piwil 1 in the regulation or implementation of left–right polarity in the kidney and possibly in the whole human body. We are only able to offer a hypothesis to explain this effect. The bone morphogenic protein (Bmp) signaling pathway is crucial for inducing and maintaining dorsal–ventral patterning equilibrium (reviewed in [25]). Zili (the homolog of Piwil 2 in zebrafish) antagonizes Bmp signaling to regulate dorsal–ventral patterning during early embryogenesis in zebrafish [25]. However, Ziwi expression (the homolog of Piwil 1 in zebrafish) is only specific to the gonads in adult zebrafish [26]. Furthermore, Bmp signaling pathway regulates left–right axis formation in chick embryos [27]. Therefore, it is tempting to speculate that Piwi-like genes/proteins might have an impact on body polarity by interfering with bone morphogenetic proteins. Piwi-like 2 might affect the dorsal–ventral axis, whereas Piwi-like 1 might instead play a role in the induction or maintenance of left–right polarity. However, further studies are necessary to test this hypothesis.

In summary, Piwil transcript levels can associate with each other, as shown for Piwil 1, 2 and 4, and with the age of tumor onset (Piwil 1), or they can differ between tumor and normal tissues (Piwil 4) and between the left and right kidney (Piwil 1) in normal tissues. Therefore, Piwil family member gene expression has a potential role in ccRCC diagnostics and tumorigenesis as well as the embryology of renal and possibly other tissues.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.01.014.

Acknowledgements

We would like to thank Rudolf und Irmgard Kleinknecht-Stiftung and Dr. Robert Pfleger Stiftung for supporting HT, the "ELAN-Fonds des Universitätsklinikums Erlangen" for supporting SW and EN, the "American Journal Experts, LLC" for providing language-editing service of our manuscript and V. Popp for technical assistance.

Competing interests

All authors disclose that they have no actual or potential conflicts of interest, including any financial, personal or other relationships with other people or organizations that could inappropriately influence (bias) this work.

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