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Original Article

Decreased HCRP1 expression is associated with poor prognosis in breast cancer patients

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Abstract: Background: Downregulation of hepatocellular carcinoma related protein 1 (HCRP1) has been reported to be associated with a poor prognosis in a variety of malignant tumors. The purpose of this study was to assess HCRP1 expression in breast cancer and to examine its possible correlation with commonly used prognostic factors, particularly epidermal growth factor receptor (EGFR). Methods: Immunohistochemical analysis was performed on tumors from 194 patients with primary breast cancer. HCRP1 expression was analyzed along with major clinicopathological variables. Results: HCRP1 protein expression was shown to be correlated with age ($P = 0.001$), histological grade ($P = 0.005$), tumor progression ($P = 0.013$), and death ($P = 0.001$), but not with tumor size, lymph-node metastasis, or Ki67 status. Kaplan-Meier survival curves showed that lower HCRP1 expression was significantly correlated with increased short-term survival ($P < 0.001$), and both univariate and multivariate analyses revealed that HCRP1, tumor size, lymph-node metastasis, and human epidermal growth factor receptor-2 (HER-2) were independent prognostic factors (all $P < 0.05$). In addition, low HCRP1 expression was much more frequent in triple negative breast cancer (TNBC; 63.89%) than in luminal (16.95%) and HER-2 overexpression phenotypes (7.5%; $P < 0.001$), and significant correlations between HCRP1 and survival time were observed for the TNBC group ($P < 0.004$). Furthermore, an inverse relationship between HCRP1 and EGFR expression was found both for the complete set of all cases ($P < 0.001$), and for each phenotype analyzed individually ($P < 0.05$). Conclusion: Our results suggest that HCRP1 may play an important role in EGFR regulation and that its decreased expression is an independent predictor of breast cancer, especially in TNBC patients.

Keywords: Breast cancer, HCRP1, EGFR, triple negative breast cancer, prognostic marker

Introduction

Breast cancer is the most common malignant cancer and the second leading cause of cancer death in women worldwide. Triple negative breast cancer (TNBC) represents a phenotype lacking expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2); it is associated with the shortest relapse-free and overall survival durations [1, 2]. Owing to the lack of clinical targets, no standard treatment method is available for TNBC. Chemotherapy is still the best option, but drug resistance drastically reduces its therapeutic effectiveness [3-5]. Therefore, there is an urgent need to identify new therapeutic targets in order to improve the outlook for these patients.

Numerous studies have shown that epidermal growth factor receptor (EGFR) is highly expressed in TNBC, and its expression is positively associated with a poor clinical outcome and aggressive tumor properties [6, 7]. We have previously shown that EGFR can enable breast cancer cells to become insensitive to different types of P-glycoprotein related drugs [8], making anti-EGFR therapy a promising treatment for patients with chemorefractory TNBC. In fact, anti-EGFR therapies have been reported to improve outcomes in other cancers, including colorectal cancer, non-small-cell lung can-

Table 1. Correlation of HCRP1 with clinicopathological factors (n = 194)

Factors	Total	HCRP1 expression		r	*P-value*
		low	high		
All cases	194	46	148		-
Age at surgery, yr					
≤ 35	17	6	11	0.242	0.001
36-50	106	33	73		
≥ 51	71	7	64		
Tumor size					
< 2 cm	71	12	59	-0.126	0.079
2-5 cm	101	27	74		
> 5 cm	22	7	15		
Axillary lymph-node metastasis metastasis					
0	85	17	68	-0.089	0.219
1-3	43	10	33		
4-9	42	12	30		
> 9	24	7	17		
Histological grade					
I	6	0	6	-0.200	0.005
II	150	31	119		
III	38	15	23		
Progression					
Absent	145	28	117	-0.178	0.013
Present	49	18	31		
Ki67 status					
1+	118	22	96	-0.134	0.062
2+	51	17	34		
3+	23	7	16		
4+	2	0	2		
Death					
No	168	33	135	-0.243	0.001
Yes	26	13	13		

cer, and squamous-cell carcinoma of the head and neck [9-12]. Several studies have failed to find any definite association between EGFR gene amplification and EGFR overexpression in breast cancer, indicating that reduced degradation of EGFR could be the underlying mechanism [13-15].

Hepatocellular carcinoma related protein 1 (HCRP1), a member of the membrane-trafficking complex, endosomal sorting complexes required for transport (ESCRT)-I, has been reported to play an important role in mediating the internalization and degradation of ubiquitinated membrane receptors such as EGFR [16]. HCRP1 is downregulated in hepatocellular carcinoma and several ovarian cancer cell lines, and loss of HCRP1 expression can result in cetuximab (monoclonal antibody against EGFR) resistance in ovarian cancer cell lines [17, 18]. One study showed that oral and oropharyngeal squamous cell carcinoma patients with low HCRP1 expression had significantly shorter overall and recurrence-free survival times [19]; however, the status and function of HCRP1 in breast cancer remains unclear, especially in cases with overexpression of EGFR.

In the present work, we investigated the expression of HCRP1 in 194 patients to examine its relationship with EGFR expression and to determine its viability as a prognostic marker for breast cancer.

Materials and methods

Clinical samples

Our study included 194 patients with primary breast cancer who were diagnosed at the Department of Pathology, Shandong Provincial Hospital affiliated to Shandong University, between 2006 and 2009. The age of patients at the time of diagnosis ranged from 28 to 85, with the median being 49.6 years. Follow-up data were available for all cases. The average observation time for overall survival was 65 months for patients still alive at the time of analysis, and it ranged from 20 to 84 months. During the follow-up period, 24 patients (12.4%) died, and 48 (24.7%) experienced disease progression in the form of either metastasis or local recurrence. None of the patients received chemotherapy or radiotherapy prior to surgery. The tumor node metastasis (TNM) system was used classify the stage of the cancer [20, 21]. After surgery, patients were

followed up with a blood examination that included the tumor marker, CA153. Imaging modalities were also used and included a chest x-ray every 3-6 months. The histology classification was evaluated according to the WHO Classification of Tumors of the Breast, Fourth Edition [22], and the grading was assessed according to the Nottingham grading system [23, 24]. Patients who died from unrelated causes were excluded from this study. Patient characteristics are shown in **Table 1**. Three major phenotypes of breast cancer were classified based on the immunostaining results described below.

Antibodies

Rabbit polyclonal anti-HCRP1 antibodies (1:100) were obtained from Abcam (Cambridge, MA, USA) and rabbit monoclonal antibodies anti-EGFR (1:50) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibodies for ER (1:100), PR (1:100), HER-2 (1:100), and Ki67 (1:50) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Immunohistochemistry

Sections of 4 mm in thickness were cut from formalin-fixed, paraffin-embedded blocks and then deparaffinized in xylene and rehydrated using a series of graded washes with ethanol. After inhibition of endogenous peroxidase and antigen retrieval (microwave irradiation in 0.01 M citrate buffer at pH 6.0), the sections were incubated with each primary antibody at 4°C overnight and this was followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako Cytomation, Denmark). Slides were then developed for 5 minutes with the chromogen, 3, 3'-diaminobenzidine (DAB), and counterstained with hematoxylin to distinguish the nucleus from the cytoplasm. Normal liver tissues were used for the positive and negative controls, after being stained with and without primary antibodies, respectively.

Evaluation of stained samples

The immunostainings were examined independently by three clinical pathologists (Qin, YJ, Wang, QX and Zhang, QH). For HCRP1, a staining value (0-9) was calculated as the intensity of positive cytoplasmic staining (negative = 0; weak = 1; moderate = 2; strong = 3) multiplied by the percentage of immunostained tumor cells (< 10% = 1; 11%-50% = 2; > 51% = 3). The intensity of the staining was assessed in 100 cells. The final staining scores were classified according to the following scale: score 0 ⇒ value 0, score 1+ ⇒ values 1-3; score 2+ ⇒ values 4-6; score 3+ ⇒ values 7-9. All cases were divided into two groups of low (score 0 or 1+) and high (2+ or 3+) expression [18].

The expression of ER and PR was scored as "negative" (nuclear staining in < 1% of tumor cells) or "positive" (nuclear staining in ≥ 1% of tumor cells) [25]. Scoring of EGFR was the same as for ER and PR, except that membrane rather than nuclear staining was evaluated and the cutoff for positivity was staining in ≥ 10% of tumor cells. The Ki67 status was evaluated based on the percentage of positive nuclear stained tumor cells: score 1+ for < 25%, score 2+ for 26-50%, score3+ for 51-75%, and score4+ for > 75% [26].

The expression of HER-2 was scored as "negative" (no reactivity or membrane staining in < 10% of tumor cells), "1+" (faint/barely perceptible staining in > 10% of tumor cells), "2+" (weak to moderate membrane staining in > 10% of tumor cells), and "3+" (uniform intense membrane staining of > 10% of invasive tumor cells). Samples giving a result of 2+ were retested using fluorescence in situ hybridization (FISH). Samples were determined to be positive for HER-2 if the immunohistochemistry score was 3+ or the FISH amplified ratio of HER-2 to CEP17 was > 2.2 [27].

Statistical analysis

A chi-square test was applied to evaluate associations between the immunoreactivity of HCRP1 and other markers, and to evaluate the clinicopathological characteristics of patients. Kaplan-Meier survival analysis was used to estimate the prognostic relevance of HCRP1 and the survival difference between groups was assessed by the log-rank test. Univariate and multivariate Cox regression analysis were performed to evaluate differences in all possible risk factors for death. SPSS 15.0 software was used for all statistical analyses. For all tests, $P < 0.05$ was considered to indicate statistical significance.

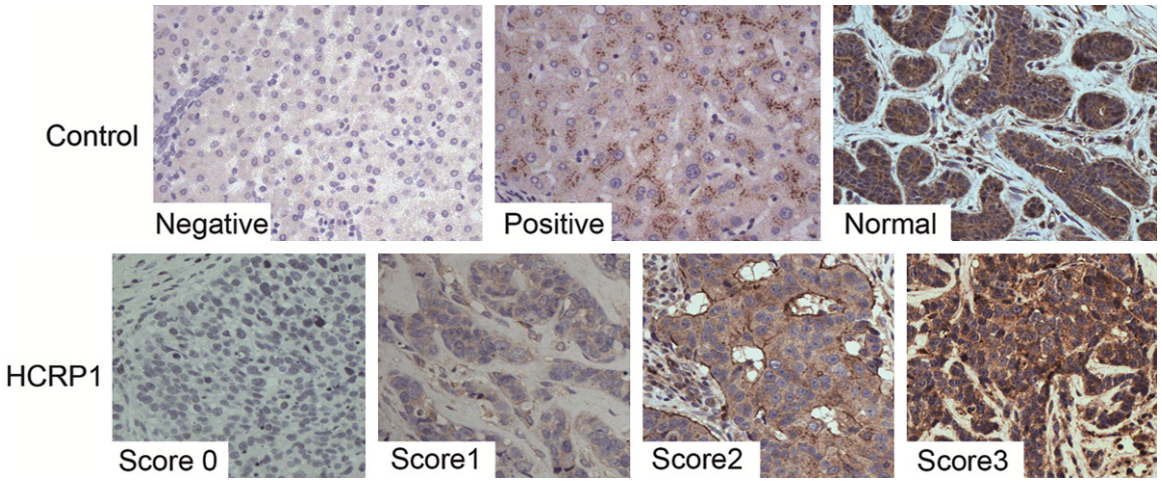

Figure 1. HCRP1 immunostaining. Representative images of tissue sections stained for HCRP1 and scored 0, 1, 2, and 3 (lower row). Representative images of positive, negative, and normal controls (upper row). Sections of formalin-fixed, paraffin-embedded human liver samples were used as positive controls. Negative controls were incubated with immunoglobulin fractions in place of polyclonal primary antibodies. In normal control breast tissue, positive staining was detected in cytoplasm with moderate to strong staining (194/194). Magnification was 400× in all cases.

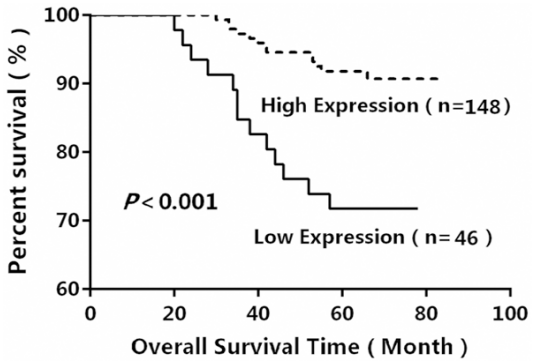

Figure 2. Kaplan-Meier plot of overall survival of 194 patients. Kaplan-Meier plot of overall survival of 194 patients with breast cancers stratified by HCRP1 expression level. A log-rank test showed significant differences between low and high expression groups ($P < 0.001$).

Results

HCRP1 expression in breast cancer

HCRP1 expression was analyzed in the tissue of 194 primary breast cancer patients and its immunoreactivity was readily detected in the cytoplasm. In normal breast tissue, HCRP1 expression was always moderate to strong. Representative images of HCRP1 staining are shown in **Figure 1**. In invasive fields, HCRP1 expression was detected at low levels in 23.71% of cases and at high levels in 76.29% of cases. No significant differences were found to correlate with tumor size, lymph-node metastasis, or Ki67 status. However, we did observe significant correlations of HCRP1 with age ($P = 0.001$), histological grade ($P = 0.005$), tumor progression ($P = 0.013$), and death ($P = 0.001$) (**Table 1**).

HCRP1 and survival

A Kaplan-Meier curve of the overall survival of 194 patients, stratified by HCRP1 expression level, was plotted, and a log-rank test showed significant differences between low and high expression groups ($\chi^2 = 12.620$; $P < 0.001$; **Figure 2**). The five-year survival rate was 71.74% in the low expression group and 91.83% in the high expression group. The hazard ratio was 3.659 (95% CI: 2.148-13.970) and 0.273 (95% CI: 0.072-0.466) in the low and high expression groups, respectively.

A set of well-known clinicopathological factors with prognostic significance including age, histological grade, tumor size, lymph-node metastasis, progression, ER, PR, HER-2, and Ki67 status, were used to assess the strength of HCRP1 as a as either an independent prognostic marker or one that is predictive in concert with others. In univariate survival analyses, HCRP1, Ki67, histological grade, tumor size, lymph-node metastasis, progression, ER, and

Table 2. Univariate and multivariate survival analysis of influencing factors (n = 194)

Variable	Univariate	Multivariate		
	P-value	*P-value*	HR	95% CI
HCRP1	0.001	0.021	0.272	0.119-0.620
Age	0.264			
Ki67	0.002	0.235		
Histological grade	0.022	0.759		
Tumor size	< 0.001	0.046	1.999	1.011-3.951
Lymph-node metastasis	< 0.001	0.007	1.917	1.199-3.066
ER	0.001	0.697		
PR	0.068			
HER-2	0.002	0.003	2.409	1.347-4.308
Tumor Progression	0.001	0.163	3.503	1.087-11.287

HER-2 were significantly associated with overall survival, whereas the patients' age at diagnosis and PR expression did not show a statistically significant correlation.

Multivariate survival analysis using the Cox proportional hazards model was performed for all factors found significant by the univariate analyses. The results showed that low expression of HCRP1, larger tumor size, presence of lymph-node metastasis, and HER-2 overexpression were associated with poor overall survival, suggesting that downregulation of HCRP1 could be used as an independent prognostic marker for breast cancer patients (**Table 2**).

HCRP1 expression in different disease phenotypes

In order to assess the HCRP1 expression status in different phenotypes of breast cancer, a panel of immunohistochemical biomarkers were used to classify 194 breast cancer cases into three major phenotypes [27]: luminal (ER positive), HER-2 overexpression (ER negative, PR negative, and HER-2 positive), and TNBC (ER negative, PR negative, and HER-2 negative). Amongst the 194 cases, 60.82% were luminal, 18.56% were TNBC, and 20.62% were HER-2 overexpressing. Representative immunostaining of ER, PR, and HER-2 in human breast carcinoma samples are shown in **Figure 3**. A Chi-square test was used to evaluate the differences in HCRP1 expression between the phenotypes (**Table 3**); low expression was much more prevalent for TNBC (63.89%) than for the luminal (16.95%; $P < 0.001$) and HER-2 overexpression groups (7.5%; $P < 0.001$). There was no statistically significant correlation between HCRP1 expression and death or survival time in the luminal and HER-2 overexpression cases (**Table 4**); however, significant correlations with death and survival time were observed for HCRP1 in the TNBC group ($P = 0.004$ for death; $P = 0.003$ for survival).

Correlations between HCRP1 and EGFR expression

In order to investigate the relationship between HCRP1 and EGFR expression in breast cancer, we measured the expression of EGFR using immunohistochemical staining. Out of all cases 29.90% were positive for EGFR and 24.58%, 63.89%, and 15.00% were found to have luminal, TNBC, and HER-2 overexpression phenotypes, respectively. Notably, 41 of 58 EGFR-positive cases (70.69%) showed downregulation of HCRP1, whereas HCRP1 was detected at high levels in 131 of 136 EGFR negative breast cancer patients (96.32%). A strong inverse relationship between HCRP1 and EGFR was found when all cases were analyzed together ($P < 0.001$), and also when the three phenotypes were analyzed individually (luminal, $P < 0.001$; TNBC, $P = 0.016$; HER-2, $P < 0.001$) (**Table 5**).

Discussion

In this study, immunohistochemical staining was used to examine HCRP1 expression in 194 breast cancer patients. We found that 148 exhibited high HCRP1 levels and 46 showed low HCRP1 expression. Meanwhile, HCRP1 was always expressed moderately to strongly in the cytoplasm of normal breast ductal gland epithelial cells, making it possible to be used as an internal control in clinical work.

To evaluate the clinicopathological significance of HCRP1 expression, we investigated if there were correlations between HCRP1 levels and parameters of known pathological significance. Low HCRP1 immunostaining scores correlated significantly with patients under 50 years old ($P = 0.001$), higher histological grade ($P = 0.005$), as well as higher incidence of tumor progression ($P = 0.013$) and death ($P = 0.001$), but not with other parameters including tumor size, lymph-node metastasis, and Ki67 status. In agreement with previous research [18, 19], we

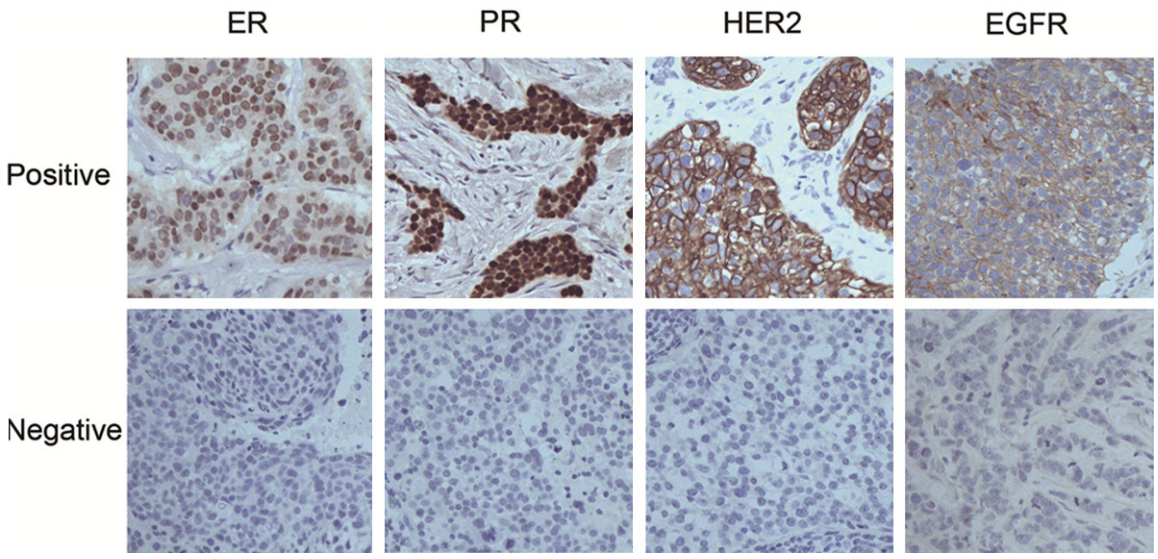

Figure 3. Representative immunostaining of commonly used diagnostic biomarkers in human breast carcinoma samples. ER and PR immunoreactivity were detected in the nucleus and HER-2 and EGFR on the cell membrane. Magnification was 400× in all cases.

Table 3. Expression of HCRP1 for different breast cancer phenotypes (n = 194)

Classification	Total	HCRP1 expression		χ^2	*P-value*
		low	high		
Luminal	118	20	98	40.920	< 0.001
TNBC	36	23	13		
HER-2 overexpression	40	3	37		

Table 4. Correlation of HCRP1 with death and survival time (n = 194)

HCRP1	Death		Survival time	
	r	*P*-value	r	*P*-value
Luminal	0.018	0.848	-0.043	0.642
TNBC	-0.466	0.004	0.479	0.003
HER-2 overexpression	-0.301	0.059	0.148	0.362

found that patients with low expression of HCRP1 had significantly decreased five-year overall survival rates compared to high-expression cases ($P < 0.001$), and both univariate and multivariate survival analyses strongly supported ($P = 0.001$ and $P = 0.021$ respectively) the hypothesis that reduced expression of HCRP1 in breast cancer is a prognostic factor for reduced survival.

TNBC accounts for approximately 15-25% of all breast cancer cases and it is associated with poor prognosis and insensitivity to currently available treatments such as hormone therapy or drugs targeting HER-2 [28, 29]. In the present study, 23 of 36 TNBC cases (63.89%) showed low HCRP1 expression, whereas only 20 out of 118 luminal cases (16.95%) and 3 out of 40 HER-2-overexpression cases (7.5%) exhibited low levels. These results suggest that HCRP1 may be a useful supplementary marker for differentiation of breast cancer phenotypes. Additionally, significant correlations of HCRP1 with death and survival were observed in the TNBC group, indicating that TNBC patients with low HCRP1 expression may have a higher risk of death and a shorter survival times than those of with other disease phenotypes.

Wittinger *et al.* reported low HCRP1 levels in 25 of 39 (64.10%) cases of ovarian cancer highly expressing HER-2, and that downregulation of HCRP1 mRNA/protein eventually led to increased levels of activated EGFR and HER-2 in the SK-OV-3 and MDAH-2774 ovarian cell lines [18]. In contrast to their results, we found that almost all breast cancer tissue positive for HER-2 (37 of 40) showed moderate to strong HCRP1 expression. As HER-2 is unique amongst EGF-family receptors in not having to undergo ligand binding [30], whether HCRP1 lose function in HER-2 regulation in breast cancers still need further investigation.

Table 5. Correlations between HCRP1 and EGFR expression (n = 194)

	HCRP1	EGFR		r	*P-value*
		negative	positive		
All	low	5	41	-0.721	< 0.001
	high	131	17		
Luminal	low	0	20	-0.791	< 0.001
	high	89	9		
TNBC	low	5	18	-0.398	0.016
	high	8	5		
HER-2 overexpression	low	0	3	-0.678	< 0.001
	high	34	3		

In agreement with a number of other studies [6, 7], we have found that EGFR expression was markedly increased in TNBC patients. Moreover, high expression of EGFR was significantly associated with low HCRP1 levels. This result was found for all three phenotypes examined, and it is consistent with the previously demonstrated role of HCRP1 as a key regulator of EGFR degradation [16-18]. It indicates the potential of HCRP1 as a new therapeutic target for TNBC, but further studies are required to clarify the significance of HCRP1 protein expression and/or gene amplification in EGFR-positive breast cancer.

This study is the first to examine the expression of HCRP1 in primary breast cancer and it was found that low HCRP1 levels were associated with shorter five-year overall survival rates, showing that HCRP1 may represent a useful independent prognostic marker for breast cancer. Our results showed that low HCRP1 was much more prevalent in TNBC than in other phenotypes, but the data also suggest that reduced HCRP1 expression is a more general event occurring in most EGFR positive breast cancers. As the size of our sample was relatively small, further studies are necessary to elucidate the mechanisms of HCRP1-EGFR regulation. In addition, methods for modulating HCRP1 levels deserve consideration as an alternative strategy for anti-EGFR treatment of breast cancers, especially for those that are multidrug resistant.

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Disclosure of conflict of interest

None.

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