Estrogen receptor β promoter methylation: a potential indicator of malignant changes in breast cancer

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Abstract

Introduction: Estrogen receptor β (ERβ) always lacks expression in estrogen-dependent tumors, which may result from gene inactivation by methylation. In this study, we aimed to determine whether aberrant methylation of the ERβ promoter is associated with decreased ERβ gene expression in breast cancer.

Material and methods: ERβ methylation status was determined for 132 pairs of breast cancer and adjacent normal tissues via the MethyLight method. Additionally, mRNA relative expression was quantified by real-time polymerase chain reaction (RT-PCR) to determine whether aberrant methylation had a negative correlation with expression. The correlation of ERβ promoter methylation and clinical parameters is also discussed.

Results: Methylation was observed in 96 (72.7%) breast cancer samples, and the median percentage of fully methylated reference (PMR) among methylated tissues was 0.83. Meanwhile, 94 (71.2%) adjacent normal tissues were methylated and the median PMR was 0.48. Compared to adjacent normal tissues, the methylation level of breast cancer was significantly higher ($p < 0.001$) and mRNA expression was much lower ($p < 0.001$). There was a significant correlation between ERβ methylation and mRNA expression in adjacent normal breast tissues ($p = 0.004$). In addition, the methylation rate of cancer tissues whose maximum diameter < 3 cm was significantly higher than those > 3 cm ($p = 0.025$).

Conclusions: ERβ promoter methylation level varies between cancerous and adjacent normal breast tissues. There was significant downregulation of ERβ methylation expression in pre-cancerous stages of breast cancer. Therefore, demethylation drugs may offer a potential strategy for preventing the development of pre-cancerous cells.

Key words: breast cancer, estrogen receptor β, methylation, percentage of fully methylated reference.

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Introduction

Estrogen receptor β (ERβ) has been recognized as a member of the nuclear receptor superfamily, which has also included the traditional estrogen receptor α since 1996 [1]. These estrogen receptors have common structural features but play different biological roles that are mediated via their DNA-binding domains, which interact with specific DNA elements, such as the estrogen-response element, to activate downstream target genes [2]. ERβ has, unlike estrogen receptor alpha, different functions and a wider distribution among a variety of tissues, including breast, colon, esophagus, stomach, brain, lung, prostate, testis, pancreas and blood vessels [3]. In breast tissues, ERβ is expressed in luminal epithelium, myoepithelium, fibroblasts and lymphocytes in stromal cells [4]. The ERβ expression level is down-regulated in malignant breast tissue [5]. Although the final conclusion is still inconsistent about the relationship of ERβ expression level and prognosis as well as treatment response, ERβ has been accepted as a tumor suppressor gradually [6]. Recent research strongly supported that loss of ERβ could be one of the key elements leading to breast epithelial cell malignancy. It suggested that ERβ has a significant role in inhibition of invasion, stimulation of apoptosis, and prevention of oncogenic transformation in rapid differentiating prostatic epithelial cells [7].

DNA epigenetic alternation including gene-specific hypermethylation and hypomethylation has a significant influence on neoplastic transformation [5, 7]. Gene silencing due to DNA hypermethylation of CpG islands in the promoter regions is a common mechanism of gene regulation in breast carcinogenesis [8, 9]. Aberrant DNA methylation is regarded as one of the most common molecular abnormalities in breast cancer, and hypermethylation of CpG islands results in the loss of expression of some crucial genes [9]. Studies in vitro show that ERβ methylation probably participates in the epigenetic regulation of ERβ expression [10–15], and methylation of the ERβ promoter is an early event in malignant transformation of breast tissue [15]. Different methylation models in cancer cell lines and breast cancer tissues have been classified for two ERβ promoters, exon 0K and 0N, which could generate different ERβ isoforms with diverse splicing sites [10]. However, few studies of ERβ methylation have been conducted on paired cancerous and normal tissues from a single patient.

In this study, we aimed to investigate the relationship and consistency between promoter methylation status and ERβ expression levels, along with clinical parameters, of paired cancerous and normal tissues in breast cancer patients.

Material and methods

Patients and samples

The study protocol was approved by the Institutional Review Board and all participants signed a written consent form. Tissues of 132 breast cancer objects were collected from September 2010 to October 2011 at the Anyang Cancer Hospital in the Henan province of China. All participants were Chinese Han women, mean age 50.91 ±9.88 years, and baseline characteristics are shown in Table I. None of them had received preoperative hormonal therapy. Cancerous and adjacent normal tissues were collected from each patient during surgery. Normal breast tissues were obtained at least 1 cm distant to the edge of cancerous breast tissue. Subsamples of tissues were removed prior to DNA extraction for conventional fixed, embedded and H + E staining. Histopathological categories including benign and malignant characterizations were verified by two experienced pathologists. Each tissue was divided into two parts, and one was placed in RNA Stabilization Reagent (Qiagen, Valencia, CA), while the other was stored at –80°C until extraction.

DNA extraction and bisulfite modification

Genomic DNA was extracted from tissues by the standard method of proteinase K digestion and phenol-chloroform extraction [16]. In brief, fresh tissues were diced and DNA was extracted after the tissue pieces were digested overnight. The purity and concentration of extracted DNA were determined from its optical density by use of a Nano-Drop spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). Sodium bisulfite conversion of approximately 800 ng of extracted genomic DNA was performed for the EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA, USA) and diluted to a final concentration of 25 ng/µl.

PCR and Sanger sequencing for promoter 0K

After sodium bisulfite conversion, genomic DNA methylation status of promoter 0K was verified according to PCR-based sequencing on 20 pairs of cancerous and adjacent normal tissues. Hot Start Taq DNA polymerase mixture (Qiagen, Valencia, CA) was used to amplify the promoter 0K fragment (295 bp) on bisulfite converted DNA. Primers for promoter 0K are listed in Table II. PCR products were purified using a PCR clean-up gel extraction column (Macherey-Nagel GmbH & Co, Düren, Germany). The sequences were determined using a capillary sequencer (ABI Prism 3100).

Table I. Correlation between clinical parameters and the rate and PMR median values of methylated tissues

Clinical parameters		Number (%)	Adjacent normal tissues				Cancerous tissues			
			Methylated number (%)	P	PMR median	P	Methylated number (%)	P	PMR median	P
Age	≤ 50	66 (50.0)	46 (69.7)	0.507	0.456	0.555	44 (66.7)	0.118	0.804	0.544
	> 50	66 (50.0)	48 (72.7)		0.491		52 (78.8)		0.688	
Menstrual years	< 35	40 (30.3)	28 (70.0)	0.821	0.463	0.884	31 (77.5)	0.675	0.448	0.590
	≥ 35	22 (16.7)	16 (72.7)		0.465		16 (72.7)		0.600	
	Missing	70 (53.0)	50 (71.4)		0.506		49 (70.0)		0.872	
Menopause	No	66 (50.0)	47 (71.2)	0.933	0.534	0.830	46 (69.7)	0.378	0.895	0.938
	Yes	64 (48.5)	46 (71.9)		0.463		49 (76.6)		0.448	
	Missing	2 (1.5)	1 (50.0)		0.040		1 (50.0)		0.078	
Pregnancies	≤ 2	55 (41.7)	36 (65.5)	0.161	0.642	0.626	39 (70.9)	0.700	0.263	0.319
	> 2	73 (55.3)	56 (76.7)		0.428		54 (74.0)		1.100	
	Missing	4 (3.0)	2 (50.0)		0.179		3 (75.0)		0.207	
Birth	≤ 2	84 (63.6)	57 (67.9)	0.143	0.620	0.592	61 (72.6)	0.931	0.368	0.121
	> 2	45 (34.1)	36 (80.0)		0.363		33 (73.3)		1.574	
	Missing	3 (2.3)	1 (33.3)		0.040		2 (66.7)		0.203	
Family history of cancer	No	92 (69.7)	62 (67.4)	0.120	0.402	0.130	65 (70.7)	0.224	0.824	0.442
	Yes	37 (28.0)	30 (81.1)		0.955		30 (81.1)		0.929	
	Missing	3 (2.2)	2 (66.7)		0.083		1 (33.3)		0.078	
Location	Left	60 (45.5)	46 (76.7)	0.230	0.272	0.010	45 (75.0)	0.647	0.335	0.223
	Right	70 (53.0)	47 (67.1)		0.833		50 (71.4)		0.833	
	Missing	2 (1.5)	1 (50.0)		0.054		1 (50.0)		10.097	
Tumor diameters	≤ 3 cm	73 (55.3)	52 (71.2)	0.916	0.599	0.085	59 (80.8)	0.025	0.833	0.493
	> 3 cm	54 (40.9)	38 (70.4)		0.253		34 (63.0)		0.452	
	Missing	5 (3.8)	4 (80.0)		0.423		3 (60.0)		1.174	

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Table I. Cont.

Clinical parameters		Number (%)	Adjacent normal tissues				Cancerous tissues			
			Methylated number (%)	P	PMR median	P	Methylated number (%)	P	PMR median	P
Pathological types	Invasive	120 (90.9)	86 (71.7)	0.436	0.482	0.537	89 (74.2)	0.101	0.540	0.596
	Non-invasive	10 (7.6)	6 (60.0)		0.468		5 (50.0)		1.025	
	Missing	2 (1.5)	2 (100.0)		3.223		2 (100.0)		7.950	
Lymph node metastasis	No	47 (35.6)	33 (70.2)	0.816	0.416	0.754	33 (70.2)	0.479	0.448	0.627
	Yes	79 (59.8)	57 (72.2)		0.534		60 (75.9)		0.895	
	Missing	6 (4.5)	4 (66.7)		0.095		3 (50.0)		5.373	
ERα	Negative	46 (34.8)	33 (71.7)	0.862	0.440	0.455	34 (73.9)	0.927	0.971	0.406
	Positive	82 (62.1)	60 (73.2)		0.517		60 (73.2)		0.704	
	Missing	4 (3.0)	1 (25.0)		0.416		2 (50.0)		0.412	
PR	Negative	57 (43.2)	43 (75.4)	0.527	0.387	0.811	44 (77.2)	0.389	0.341	0.988
	Positive	71 (53.8)	50 (70.4)		0.599		50 (70.4)		1.023	
	Missing	4 (3.0)	1 (25.0)		0.416		2 (50.0)		0.412	
C-erbB-2	Negative	81 (61.4)	62 (76.5)	0.146	0.599	0.276	61 (75.3)	0.437	1.025	0.606
	Positive	45 (34.1)	29 (64.4)		0.308		31 (68.9)		0.448	
	Missing	6 (4.5)	3 (50.0)		0.416		4 (66.7)		0.427	
P53	Negative	61 (46.2)	46 (75.4)	0.472	0.621	0.477	49 (80.3)	0.082	0.448	0.206
	Positive	66 (50.0)	46 (69.7)		0.411		44 (66.7)		0.971	
	Missing	5 (3.8)	2 (40.0)		3.425		3 (60.0)		0.776	
Ki67	Negative	47 (35.6)	33 (70.2)	0.797	0.495	0.701	35 (74.5)	0.681	0.575	0.435
	Positive	76 (57.6)	55 (72.4)		0.440		54 (71.1)		0.852	
	Missing	9 (6.8)	6 (66.7)		0.475		7 (77.8)		0.776	
Total		132 (100)	94 (71.2)		0.482		96 (72.7)		0.804	

PMR – percentage of fully methylated reference, ERα – estrogen receptor α, PR – progesterone receptor, C-erbB-2 – human epidermal growth factor receptor-2, P53 – P53 protein, Ki67 – Ki67 proliferation antigen.

Methylation assay for promoter 0N

Methylation analysis of promoter 0N was performed by the MethyLight method [17]. Three sets of primers and probes designed specifically for bisulfite-converted DNA were used, and an ACTB set was used to normalize for input DNA. The sequences of primers and probes are shown in Table II. Specificity of the reactions for methylated DNA were confirmed separately by a control human genomic DNA which had been treated with DNA methyltransferase SssI (New England Biolabs, Beverly, MA). The percentage of fully methylated molecules at a specific locus was calculated by dividing the promoter 0N: ACTB ratio of a sample by the promoter 0N: ACTB ratio of SssI-treated control DNA and multiplying by 100. Percentages of fully methylated reference (PMR) values were used to quantity the methylation level of each sample. PMR > 0 means that the measurement and gene are methylation positive.

RNA extraction and real-time RT-PCR

Total RNA was isolated from tissues that were stored in RNA stabilization reagent (Qiagen, Valencia, CA) using TRIzol reagent (Invitrogen, Carlsbad, CA) after a quick liquid nitrogen grind. The quality and quantity of RNA were determined from its optical density in an ethidium bromide-stained 1% agarose gel, and 2 µg of RNA was subsequently used to generate cDNA with the M-MLV reverse transcription kit (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was performed with a LightCycler 480 System (Roche Diagnostics GmbH, Mannheim, Germany). The LightCycler 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) assay was used for total ERβ amplification and GAPDH was used as a reference gene. The 20 µl reaction system contained 10 µl of SybGreen mix, 10 pmol of each primer, and 50–100 ng of cDNA template. The primers used are listed in Table II. Relative expression levels of total ERβ were calculated by $N = 2^{-\Delta Ct(GAPDH - ER\beta)}$ [18], where N is the relative quantity of mRNA expression.

Statistical analysis

Non-parametric statistics were used because the distribution of PMR and *N* value were not normal. The Spearman correlation coefficient and paired rank sum test were adopted for analysis of methylation and expression levels in cancerous and adjacent normal tissues. Pearson χ^2 and Wilcoxon rank sum tests were used to analyze the relationship between methylation levels and clinical parameters. All statistical analyses were conducted using SPSS 17.0 software, and *p* < 0.05 was considered as statistically significant.

Table II. Primer and probe sequences for detection of promoter methylation and mRNA expression

Detection step	Gene	Forward primer sequence	Reverse primer sequence	Probe oligo sequence
Promoter 0K PCR	Promoter 0K	GTTGGGGTTATTTCGGGGTTGTT	CCTCCAACAAAACAAACACATTCA	
Promoter 0N Q-PCR	ACTB	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTT	6FAM-ACCACCACCCAACACACAATAACAAACACA-TAMRA
	Promoter 0N	TTTGAAATTTGTAGGGCGAAGAGTAG	ACCCGTCGCAACTCGAATAA	6FAM-CCGACCAACGCTCGCCG-TAMRA
cDNA Q-PCR	GAPDH	TCCCTGAGCTGAACG GGA AG	GGAGGAGTGGGTGTCGCTGT	
	ERβ	TGGGCACCTTTCTCCTTTAGTGG	GCTTCACACCAGGGACTCTTTTGAG	

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Results

Histopathology

All cancerous tissue samples were correctly classified and benign samples included normal and proliferative breast cells. All procedures were confirmed by two experienced pathologists independently.

Promoter 0K methylation

Twenty participants were selected by a random number table (accounting for 15% of all patients), and their paired cancerous and adjacent normal tissues were used for promoter 0K methylation assay. No methylation was observed from the sequencing chart.

Promoter 0N methylation and mRNA expression

The DNA MethyLight Q-PCR assay was conducted on 132 pairs of breast cancer and adjacent normal tissues. Methylation positive breast cancer tissues (n = 96) had a median PMR value of 0.83, while adjacent normal breast tissues (n = 94) which were methylation positive had a median PMR value of 0.48 (p < 0.001) (Figure 1 A). The median mRNA relative expression level value (N*1000) of cancerous tissues was 0.18 compared with 7.30 of adjacent normal tissues (p < 0.001) (Figure 1 B). However, several cases presented an opposite change of PMR or mRNA levels (e.g. cancer tissue with a high mRNA level or low methylation).

One hundred and eleven pairs of breast tissues were included for the analysis due to the lack of 21 samples for mRNA expression level. From the results, there was no significant correlation between promoter 0N methylation PMR and mRNA expression levels in the breast cancer group (p = 0.899), but there was a significant correlation in normal breast tissue (p = 0.004).
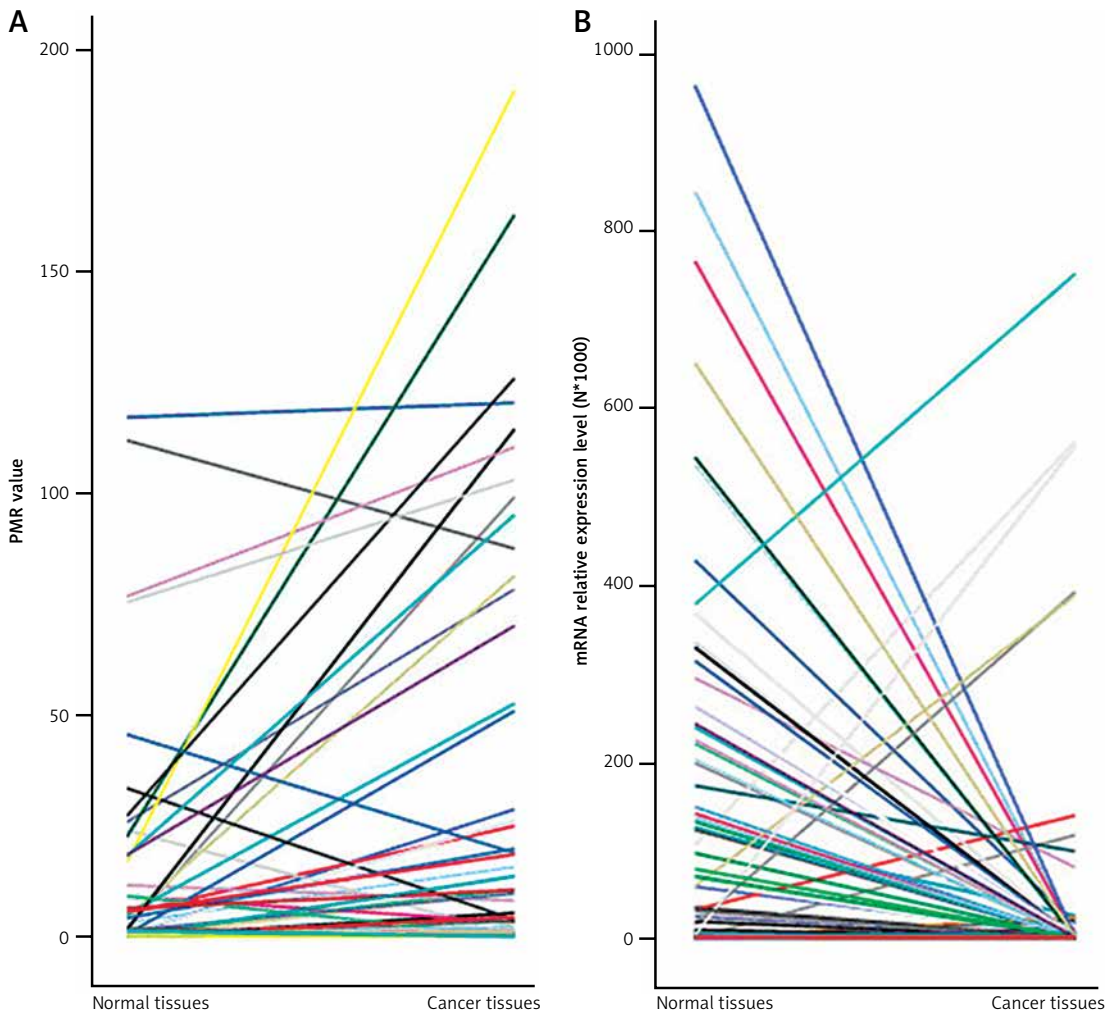
Figure 1. Estrogen receptor beta methylation PMR values and mRNA relative expression levels. Each line stands for a participant. The start point (adjacent normal breast tissues) and end point (breast cancerous tissues) mean PMR value (**A**) or mRNA relative expression levels (N*1000) (**B**)

PMR – percentages of fully methylated reference.

Methylation and clinical parameters

The rate and PMR median values of methylated tissues were calculated for associations with clinical parameters. The rate of ERβ methylation in cancer tissues with maximum diameters < 3 cm was significantly higher than those > 3 cm ($p = 0.025$). However, the PMR median values in both groups showed no statistical significance ($p = 0.493$). Also, the PMR of left breast cancer was relatively higher than the other side ($p < 0.01$), although the rate of methylation did show significance ($p > 0.01$).

Discussion

The ERβ promoter region has been cloned with an increased CG content [19], and further investigation showed 2 exons (0N and 0K) existing in the ERβ promoter region [11]. The methylation pattern for exon 0N differs in cancerous and normal breast tissues, in contrast to exon 0K [10, 15]. A previous study verified that promoter 0K did not methylate either in benign or malignant breast cells [10]. As a scattered CG dinucleotide distribution in the promoter 0K, a PCR-based sequencing method was designed to depict the GC methylation status in the promoter 0K region. After scanning, no methylation occurred in any of the 20 paired tissues in our study, and this was consistent with the former results [10]. Promoter 0K and 0N could transcribe different mRNA isoforms which diverge in their 5'-untranslated regions, and the isoform change in carcinogenesis might be caused by a different methylation pattern [20]. We quantified promoter 0N methylation to evaluate the DNA methylation status of CpG islands with the MethyLight method, and used established probes and primers in the promoter 0N region [21, 22]. Widschwendter *et al.* demonstrated that 79% of breast cancer tissues were methylated with a median PMR value of 0.1 [22]. In our study, similar results with an ERβ methylation rate for breast cancer tissue of 72.3% and a median PMR value 0.17 were proved, which confirmed the reliability for the further analyses.

Recent studies indicated that ERβ mRNA expression levels were down-regulated by promoter methylation, and re-expression occurred with the addition of DNA methyltransferase inhibitors [10, 12]. Transcriptional silencing of ERβ is necessary for cancer progression in breast cancer and other hormone-sensitive cancers [13]. Promoter methylation in cancerous and pre-cancerous tissues results in transcriptional silencing of ERβ, whereas no methylation occurs in normal breast tissue [14, 15]. Therefore, ERβ is increasingly believed to act as a tumor suppressor gene [12]. Rody *et al.* speculated that methylation of the ERβ promoter is an uncommon focal event, based on the absence of methylation in benign breast tissue from breast cancer patients [15]. However, we found that there was no difference in methylation rate between cancerous and adjacent normal breast tissues. This inconsistency could have resulted from different methods, patient sources, or differences in sample size. We can deduce that the surrounding tissue of breast cancer differs from normal breast tissues without ERβ promoter methylation [23]. Therefore, adjacent normal breast tissues may be in a pre-cancer stage and ERβ methylation may be an early indicator of pre-malignant changes.

Compared with adjacent normal tissue, a significant decline of ERβ expression in breast cancer tissue was proved in our study (Figure 1 B), which was also reported previously [24, 25]. According to the correlation analysis, there was a relationship between methylation level and mRNA expression in adjacent normal breast tissue. The possible explanations might be as follows: first, compared with normal breast tissues, cancerous tissues included more complicated cell types, not only cancer cells in different differentiation stages but also normal duct cells, glandular cells and stromal cells. The ERβ expression level varies significantly in different cells. Second, the extracted tissues were not micro-dissected, which may result in an uncontrolled proportion of malignant cells. As a result, the kind of relationship in cancerous tissues might be not obvious. Finally, DNA methylation occurs in early carcinogenesis [26, 27]; therefore, the push power from methylation might decline during the tumor progression due to multiple uncontrollable factors. In our study, the rate of promoter 0N methylation in cancer tissues with maximum diameter < 3 cm was significantly higher than those > 3 cm. It may result from the smaller tumors always being in the early stage of tumor development. Unlike DNA genetics, epigenetic changes are reversible and ERβ re-expression is evident in the presence of DNA methyltransferase inhibitors [10, 12, 28]. Therefore, demethylation drugs may be a potential means for altering the development of pre-cancerous cells.

In conclusion, a relationship between methylation level and mRNA expression in adjacent normal breast tissue was observed in the study. Also, the level of ERβ promoter methylation varied between breast cancerous and adjacent normal tissues. The reversion of ERβ methylation in pre-cancerous stages may suggest a significant role in the prevention of breast cancer.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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