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Original Article

Impact of NFKB1 and NFKBIA gene polymorphism and additional gene-gene interaction on liver cancer risk in Chinese population

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Abstract: Aims: To investigate the role of genetic polymorphisms of NFKB1 and NFKBIA genes and additional gene-gene interactions on susceptibility of liver cancer. Methods: Logistic regression was performed to investigate association between SNPs in NFKB1 and NFKBIA genes and liver cancer risk and generalized multifactor dimensionality reduction (GMDR) was used to analyze the gene-gene interaction. Results: Liver cancer risk was significantly higher in carriers of T allele in NFKBIA-rs696 than those with CC genotype (CT+TT versus CC), adjusted OR (95% CI)=1.49 (1.15-1.87), and was higher in carriers of del allele in NFKB1-rs28362491 than those with ins/ins genotype (del/ins+del/del versus ins/ins), adjusted OR (95% CI)=1.62 (1.26-2.03), and higher in carriers of G allele in rs230496-NFKB1 than those with AA genotype (AG+GG versus AA), adjusted OR (95% CI)=1.61 (1.31-2.02). Liver cancer risks were the highest in participants with rs696-CT or TT and rs28362491-ins/del or del/del genotype, compared to subjects with rs696-CC of and rs28362491-ins/ins genotype, OR (95% CI)=4.62 (2.94-6.58). Haplotype containing the rs696-T and rs3138053-A alleles was associated with increased liver cancer risk, OR (95% CI)=1.56 (1.19-2.08), *P*=0.0015, and haplotype containing the rs28362491-del and rs230496-G alleles was also associated with increased liver cancer risk, OR (95% CI)=2.36 (1.65-3.85), *P*<0.001. Conclusions: rs696 and rs28362491 polymorphism and their additional interaction were associated with increased liver cancer risk. Haplotype containing the rs696-T and rs3138053-A alleles, rs28362491-del and rs230496-G alleles were associated with increased liver cancer risk.

Keywords: NFKBIA, NFKB1, liver cancer, interaction, haplotype

Introduction

Liver cancer is the fifth most common cancer and the third most common cause of cancer-related death worldwide. It threatens the health and safety of worldwide populations [1]. According to World Health Organization report in 2012, annually more than 700000 patients died from liver cancer, and this number is rising year by year [2]. Incidences of liver cancer were different in different countries or populations, and the incidences of liver cancer were the highest in circum-Pacific of Asia and South of Sahara [3]. Several risk factors for liver cancer have been reported previously, including chronic infections of hepatitis B virus (HBV) and hepatitis C virus (HCV), which were the most important risk factors for liver cancer [4, 5]. Although the liver cancer is a rapidly fatal disease, early diagnosis is important for better prognosis, treatment and recovery [6].

Chronic inflammation, which plays an important role in hepato-carcinogenesis, has been wildly accepted as a main risk factor. However, the molecular and cellular mechanisms linking inflammation and liver cancer remain unclear. Recent studies have suggested that nuclear factor-κB (NF-κB) may play an important role in bridging the actions of growth factors and chronic inflammation to liver cancer [7, 8]. One of these susceptibility loci [9, 10] is found on chromosome 4q24 and harbors, and was a member of the NF-κB family-NFKB1, which was originally identified as a nuclear factor bound to the enhancer of the immunoglobulin κ-light

chain gene [11] specific to B cells [12]. NFKB1 is a major transcription regulator for the immune system, including differentiation, cell adhesion, proliferation and apoptosis [13], and binds to REL, RELA or RELB to form the NF-κB complex. This complex could be inhibited by IκB proteins (e.g. NFKBIA), which located on chromosome 14q13, and could inactivate NF-κB by cytoplasmic trapping. Previously, some genetic variations of NF-κB have been reported to be associated with several types of cancer risk, such as breast, prostate, stomach, mouth and so on. However, little is known about the association between genetic polymorphisms of NF-κB genes and susceptibility of liver cancer. To our knowledge, till now, only one study focused on impact of NFKB1 and NFKBIA gene polymorphism on liver cancer in Chinese population was conducted previously. In addition, no study focused on the impact of interaction among several single nucleotide polymorphisms (SNPs) of NFKB1 and NFKBIA gene on susceptibility of liver cancer were conducted. So the aim of this study was to investigate the role of genetic polymorphisms of NFKB1 and NFKBIA gene and additional gene-gene interactions on susceptibility of liver cancer based on Chinese Han population.

Materials and methods

Subjects

This was a case-control study. Participants were consecutively recruited between January 2011 and September 2014 from Qingdao No. 6 People's Hospital. A total of 826 subjects (356 males, 470 females), including 286 liver cancer patients and 540 control subjects were selected, the mean age for all participants was 70.6±15.4 years. Liver cancer cases were diagnosed by combination of pathological examination, ultrasound and clinical manifestations. Identification of the stage of tumor is based on International Classification of Disease, Ninth Revision (ICD-9), codes of 155.0 (primary malignant neoplasms), 155.1 (malignant neoplasms of the intrahepatic bile ducts) or 155.2 (unspecified malignant neoplasms of the liver). Controls were matched by sex, age and ethnic background. Normal controls with family history of liver cancer were excluded. All controls were free of any cancer at the time of cancer diagnosis for the corresponding case. Informed consent was obtained from all participants.

Body measurements

Data on demographic information, lifestyle risk factors and family history of all kinds of cancer for all participants were obtained using a standard questionnaire administered by trained staffs. Cigarette smokers were those who self-reported smoking cigarettes at least once a day for 1 year or more. Alcohol consumption was expressed as the sum of milliliters of alcohol per week from wine, beer, and spirits. Body weight, height and waist circumference (WC) were measured. BMI (body mass index) was calculated as weight in kilograms divided by the square of the height in meters. WC was measured two times at 1 cm above the umbilicus at minimal respiration by trained observers; the mean of the two WC measurements was utilized in the analysis. Blood samples were collected in the morning after at least 8 hours of fasting. All plasma and serum samples were frozen at -80°C until laboratory testing. All analysis was performed by the same lab.

Genomic DNA extraction and genotyping

We selected SNPs according to the following criterions: 1) within the NFKBIA and NFKB1 gene, which have been reported previously; 2) and minor allele frequency (MAF) greater than 2%. Three SNPs of NFKBIA gene and three SNPs of NFKB1 gene were selected for genotyping in the study: rs696, rs3138053, rs2273650, rs28362491, rs230530 and rs230496. ABI Prism7000 software and allelic discrimination procedure was used for genotyping of forementioned six SNPs. Genomic DNA from participants was extracted from EDTA-treated whole blood, using the DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A 25 μl reaction mixture including 1.25 ul SNP Genotyping Assays (20×), 12.5 μl Genotyping Master Mix (2×), 20 ng DNA, and the conditions were as follows: initial denaturation for 10 min and 95°C, denaturation for 15 s and 92°C, annealing and extension for 90 s and 60°C, 50 cycles. All SNPs were detected by Taqman fluorescence probe. Probe sequences of all SNPs were shown in **Table 1**.

Quality control

Several quality control methods were used in the process of information collection, as well as

Table 1. Description and Probe sequence for 6 SNPs used for Taqman fluorescence probe analysis

SNP ID	Chromosome	Functional Consequence	Nucleotide substitution	Probe sequence
NFKBIA (IκBα is an inhibitor of NFKB1)				
rs696 2758 G.>A	14:35401887	Utr variant 3 prime	C>T	5'-CCTACCACAATAAGACGTTTTGGGC [C/T] AGGCAGTGTGCAGTGTGGATATAAG-3'
rs3138053	14:35405648	Upstream variant 2 KB	A>G	5'-ACGATCCTTTTTCTGCGGGAGCACA [A/G] TGTAGGTCAGATAGCATAAACGAAT-3'
rs2273650	14:35401592	Utr variant 3 prime	C>T	5'-AACAATACATTATGTACACCATTTA [C/T] AGGAGGGTAACACAAACCTTGACAG-3'
NFKB1				
rs28362491 294 ins/del	4:102500998	Intron variant, upstream variant 2 KB	-	5'-CTCCGTGCTGCCTGCGTTCCCCGACC [-/ATTG] ATTGGGCCCGGCAGGCGCTTCCTGG-3'
rs230530	4:102532823	Intron variant	A>G	5'-TTTTTAGCACCAAACATCTTAATTT [A/G] CATTCAAATAAATGAGAACCACCAT-3'
rs230496	4:102567334	Intron variant	A>G	5'-TGTCTGGATTTGCTTGAGACAGCCC [A/G] GTTTGCCCCTGACCTAATTGTTTAT-3'

Table 2. General characteristics of 826 study participants in case and control group

Variables	Case group (n=286)	Control group (n=540)	*p*-values
Age (year)	70.8±16.7	71.9±16.1	0.357
Males, N (%)	130 (45.4)	226 (41.8)	0.357
Smoke, N (%)	81 (28.3)	145(26.8)	0.652
Alcohol consumption, N (%)	93 (32.5)	160 (29.6)	0.392
WC (cm)	84.7±18.4	86.2±18.8	0.272
BMI (kg/m^2)	24.6±9.2	25.1±9.0	0.451

Note: Means ± standard deviation for age; WC, waist circumference; BMI, body mass index.

the assays of genomic DNA extracting and genotyping (SNPs), including: 1) questionnaire investigation were administered by staffs, who were trained for more than 6 times; 2) before the formal investigation, we conducted a pre-survey for 100 participants; 3) data input was administered by double-person and double-equipment, to decrease mistake by consistency check; 4) when we finished genotyping for all SNP and participants, we extracted about 1/10 of the subjects for consistency check, and the consistency rate was 100%.

Statistical analysis

The mean and standard deviation (SD) were calculated for normally distributed continuous variables, and percentages were calculated for categorical variables. The categorical data were compared using χ^2 test. Further, continuous variables were compared using Student's t test. Hardy-Weinberg equilibrium (HWE) and haplotype analysis were performed by using SNPstats (available online at http://bioinfo.iconcologia.net/SNPstats). Logistic regression was performed to investigate association between SNP and liver cancer. Generalized multifactor dimensionality reduction (GMDR) was used to analyze the interaction among six SNPs, cross-validation consistency, the testing balanced accuracy, and the sign test, were calculated to assess each selected interaction.

Results

A total of 826 subjects (356 males, 470 females), including 286 liver cancer patients and 540 normal subjects were selected. The mean age of all participants was 70.6±15.4 years. Participants characteristics stratified by cases and controls are shown in **Table 2**. The distributions of gender, smoking and drinking were not significantly different between cases and controls. The means of age, WC and BMI were also not significantly different between cases and controls.

All genotypes were distributed according to Hardy-Weinberg equilibrium in controls (all *p* values were more than 0.05). The frequency of rs696-T allele in NFKBIA gene was significantly higher in liver cancer cases than that in controls (28.7% *vs* 20.4%), and the frequencies of rs28362491-del allele and rs230496-G allele of NFKB1 gene were also significantly higher in

Table 3. Genotype and allele frequencies of 6 SNPs between case and control group

SNP	Genotypes and Alleles	Frequencies N (%)		OR (95% CI)*	Bonferroni-corrected *p*-values	H-W test for controls
		Control group (n=540)	Case group (n=286)			
NFKBIA (IκBα is an inhibitor of NFKB1)						
rs696	CC	340 (63.0)	148 (51.7)	1.00	0.002	0.523
	CT	180 (33.3)	112 (39.2)	1.27 (1.03-1.64)		
	TT	20 (3.7)	26 (9.1)	2.02 (1.34-3.26)		
	CT+TT	200 (37.0)	138 (48.3)	1.49 (1.15-1.87)	0.001	
	C	860 (79.6)	408 (71.3)			
	T	220 (20.4)	164 (28.7)			
rs3138053						
	AA	336 (62.2)	162 (56.6)	1.00	0.501	0.432
	AG	176 (32.6)	102 (35.7)	1.04 (0.87-1.48)		
	GG	28 (5.2)	22 (7.7)	1.23 (0.81-1.62)		
	AA+GG	204 (37.8)	124 (43.4)	1.10 (0.86-1.54)	0.494	
	A	848 (78.5)	426 (74.5)			
	G	232 (21.5)	146 (25.5)			
rs2273650						
	CC	330 (61.1)	167 (58.6)	1.00	0.602	0.226
	CT	178 (33.0)	99 (34.7)	1.02 (0.83-1.45)		
	TT	32 (5.9)	19 (6.7)	1.18 (0.73-1.65)		
	CT+TT	210 (38.9)	118 (41.4)	1.06 (0.80-1.49)	0.509	
	C	838 (77.6)	433 (76.0)			
	T	242 (22.4)	137 (24.0)			
NFKB1						
rs28362491						
	ins/ins	349 (64.6)	146 (51.0)	1.00	<0.001	0.480
	ins/del	165 (30.6)	108 (37.8)	1.41 (1.17-1.80)		
	del/del	26 (4.8)	32 (11.2)	2.28 (1.65-2.97)		
	ins/del+del/del	191 (35.4)	140 (49.0)	1.62 (1.26-2.03)	<0.001	
	ins	863 (79.9)	400 (69.9)			
	del	217 (20.1)	172 (30.1)			
rs230530						
	AA	329 (60.9)	164 (57.3)	1.00	0.472	0.379
	AG	182 (33.7)	102 (35.7)	1.05 (0.78-1.41)		
	GG	29 (5.4)	20 (7.0)	1.10 (0.73-1.68)		
	AA+GG	211 (39.1)	122 (42.6)	1.06 (0.76-1.46)	0.5092	
	A	840 (77.8)	430 (75.2)			
	G	240 (22.2)	142 (24.8)			
rs230496						
	AA	353 (65.4)	147 (51.4)	1.00	<0.001	0.562
	AG	164 (30.4)	112 (39.2)	1.45 (1.24-1.96)		
	GG	23 (4.2)	27 (9.4)	2.04 (1.50-2.67)		
	AA+GG	187 (34.6)	139 (48.6)	1.61 (1.31-2.02)	<0.001	
	A	870 (80.6)	406 (71.0)			
	G	210 (19.4)	268 (29.0)			

*Adjusted for gender, age, smoke, alcohol, BMI and WC.

Table 4. Best gene-gene interaction models, as identified by GMDR

Locus no.	Best combination	Cross-validation consistency	Testing accuracy	*p-values**
2	rs696, rs28362491	10/10	0.6217	0.0100
3	rs696, rs28362491, rs230496	9/10	0.5399	0.0547
4	rs696, rs28362491, rs230496, rs230530	8/10	0.5399	0.1719
5	rs696, rs28362491, rs230496, rs230530, rs3138053	9/10	0.5590	0.0547
6	rs696, rs28362491, rs230496, rs230530, rs3138053, rs2273650	8/10	0.4958	0.3770

*Adjusted for gender, age, smoke, alcohol, BMI and WC.

Table 5. Interaction analysis for rs696 and rs28362491 by using logistic regression

rs696	rs28362491	OR (95% CI)	*p-values**
CC	ins/ins	1.00	-
CT or TT	ins/ins	1.78 (1.29-2.67)	<0.001
CC	ins/del or del/del	2.23 (1.48-2.89)	<0.001
CT or TT	ins/del or del/del	4.62 (2.94-6.58)	<0.001

*Adjusted for gender, age, smoke, alcohol, BMI and WC.

liver cancer cases than that in controls (30.1% *vs* 20.1%, 29.0% *vs* 19.4%). Logistic regression analysis showed that liver cancer risk was significantly higher in carriers of T allele of NFKBIA-rs696 than those with CC genotype (CT+TT versus CC), adjusted OR (95% CI)=1.49 (1.15-1.87), Bonferroni-corrected *p*-value was 0.001. In addition, we also found liver cancer risk was also significantly higher in carriers of del allele of rs28362491-NFKB1 than those with ins/ins genotype (del/ins+del/del versus ins/ins), adjusted OR (95% CI)=1.62 (1.26-2.03) (Bonferroni-corrected *p*-value less than 0.001), was also higher in carriers of G allele of the rs230496-NFKB1 than those with AA genotype (GA+GG versus AA), adjusted OR (95% CI)=1.61 (1.31-2.02), Bonferroni-corrected *p*-value less than 0.001. However, we did not find any significant association between the others SNP and liver cancer after covariates adjustment (**Table 3**).

We employed the GMDR model to investigate the impact of the interaction among 6 SNPs on liver cancer risk after adjustment for covariates. **Table 4** summarizes the results obtained from GMDR analysis for gene-gene interaction, we found that there was a significant two-locus model (*P*=0.0100) involving rs696 and rs28362491, indicating a potential gene-gene interaction between rs696 and rs28362491. Overall, the two-locus models had a cross-validation consistency of 10 of 10, and had the testing accuracy of 62.17%. **Table 5** summarizes the results obtained from interaction analysis between two SNP by using logistic regression. We found that subjects with CT or TT of rs696 and ins/del or del/del of rs28362491 genotype have the highest liver cancer risk, compared to subjects with CC of rs696 and ins/ins of rs28362491 genotype, OR (95% CI)=4.62 (2.94-6.58), after covariates adjustment.

Pairwise LD analysis between SNPs was measured, and D' value between rs696 and rs3138053 was 0.514, D' value between rs28362491 and rs230496 was 0.626. The most common haplotype in NFKBIA was rs696-C and rs3138053-A haplotype, the haplotype frequencies were 0.5696 and 0.4502 in case group and control group, and the most common haplotype in NFKB1 was rs28362491-ins and rs230496-A haplotype, the haplotype frequencies were 0.5105 and 0.4428 in case group and control group. Haplotype containing the rs696-T and rs3138053-A alleles was associated with increased liver cancer risk, OR (95% CI)=1.56 (1.19-2.08), *P*=0.0015, and haplotype containing the rs28362491-del and rs230496-G alleles was also associated with increased liver cancer risk, OR (95% CI)=2.36 (1.65-3.85), *P*<0.001, after adjustment for gender, age, smoke, alcohol, BMI and WC (**Tables 6** and **7**).

Discussion

In the current study, we investigated the impact of NFKBIA and NFKB1 gene polymorphism on liver cancer risk. We found that NFKBIA and NFKB1 gene polymorphisms were associated with increased liver risk. Liver cancer risks were significantly higher in carriers of T allele of NFKBIA-rs696 than those with CC genotype, and also significantly higher in carriers of del allele of the rs28362491-NFKB1 than those

Table 6. Haplotype analysis on association between NFKBIA and liver cancer

Haplotypes	rs696	rs3138053	Frequencies		OR (95% CI)	*p*-values*
			Control group	Case group		
H1	C	A	0.5696	0.4502	1.00	--
H2	T	A	0.2219	0.3017	1.56 (1.19-2.08)	0.0015
H3	C	G	0.1782	0.1913	1.32 (0.95-1.78)	0.080
H4	T	G	0.0303	0.0567	1.49 (0.93-1.42)	0.105

*Adjusted for gender, age, smoke, alcohol, BMI and WC.

Table 7. Haplotype analysis on association between NFKB1 and liver cancer

Haplotypes	rs28362491	rs230496	Frequencies		OR (95% CI)	*p*-values*
			Control group	Case group		
H1	ins	A	0.5105	0.4428	1.00	--
H2	del	A	0.2688	0.2809	1.28 (0.95-1.77)	0.286
H3	ins	G	0.1858	0.2095	1.39 (0.79-1.86)	0.528
H4	del	G	0.0349	0.0669	2.36 (1.65-3.85)	<0.001

*Adjusted for gender, age, smoke, alcohol, BMI and WC.

with ins/ins genotype and higher in carriers of G allele of the rs230496-NFKB1 than those with AA genotype. However, we did not find any significant association between the others SNP and liver risk before and after covariates adjustment. NFKB1 gene, which located at chromosome 4q23-q24 and composed of 24 exons [14], is a non-DNA binding protein. Several genetic polymorphisms in NFKB1 have been reported, and in these polymorphisms, rs28362491 was the mostly studied SNP, several associations were reported between rs28362491 and many types of cancer, including breast, prostate, stomach, mouth and so on [15-17]. But little is known about the relationship between NFKB1 and NFKBIA gene and liver cancer. To our knowledge, the current study was the second study focused on the association between NFKB1, NFKBIA gene and liver cancer risk in Chinese population. Previously, just one study conducted by Gao et al [18] involved in this association, they indicated that rs28362491 del and rs230496-G were associated with higher risk of liver cancer, liver cancer risk was higher in carriers of the NFKB1 GA and AA (rs230525-rs230530) haplotypes, but they did not found any association between NFKBIA variants and risk of live cancer. Cheng et al [19] indicated that the NFKB1 rs28362491 polymorphism was associated with increased hepatocellular carcinoma (HCC) risk. Marcos et al [20] suggested that the deletion allele of the rs28362491 polymorphism was associated with higher risk of developing alcoholic liver cirrhosis (ALC) through an increase in inflammation. Recently, a meta-analysis also indicated the NFKB1-rs28362491 polymorphism was associated with the higher incidence of cancer in Caucasian and Asian populations [21]. The rs28362491-Ins promoter polymorphism increasing the risk of cancer may result from its positive regulation of NF-κB expression. In a study on HBV-induced hepatocarcinogenesis, the prevalence of NFKB1-rs28362491 polymorphism was higher in HCC patients than that in healthy controls [22]. The results of the current study was consistent with the results of the study by Gao et al [18], which was also conducted in Chinese Han population, however, they did not find any SNP of NFKBIA gene associated with liver cancer. In the current study, we found that NFKBIA gene polymorphism was associated with increased liver risk, which was significantly higher in carriers of T allele of NFKBIA-rs696 than those with CC genotype.

Liver cancer was influenced by many genetic factors and both NFKBIA and NFKB1 genes were associated with increased liver cancer risk, so it was necessary to investigate the impact of gene-gene interaction among several SNPs of NFKBIA and NFKB1 gene on liver cancer risk. We employed the GMDR analysis to investigate the impact of the interaction among 6 SNPs on liver cancer risk, and we found that there was a significant gene-gene interaction between rs696 and rs28362491, subjects with CT or TT of rs696 and ins/del or del/del of rs28362491 genotype have the highest liver cancer risk, compared to subjects with CC of

rs696 and ins/ins of rs28362491 genotype. To our knowledge, this is the first study on the impact of gene-gene interaction between NFKBIA and NFKB1 gene on liver cancer susceptibility. Loci that are located nearby on the same chromosome may be in linkage disequilibrium (LD). This means that alleles at these loci are not inherited in an independent manner but certain allele combinations occur more often than expected by random segregation. The implication of LD in association studies is that knowledge of variation at a certain position also gives knowledge of variation at linked loci. The haplotype analysis represents a much more powerful approach than analysis for SNP and provides important information on recombination (physical exchange of DNA during meiosis), vital for locating disease-causing mutations by linkage. Because the single SNP regression demonstrated that NFKBIA and NFKB1 gene significantly affect the development of liver cancer risk, haplotypes were inferred to capture possible allelic associations. In current study, we found that the most common haplotype in NFKBIA was rs696-C and rs3138053-A haplotype, and the most common haplotype in NFKB1 was rs28362491-ins and rs230496-A haplotype, haplotype containing the rs696-T and rs3138053-A alleles, and haplotype containing the rs28362491-del and rs230496-G alleles were statistically associated with increased liver cancer risk. IκBα was the inhibitor of NFKB1, and could be encoded by NFKBIA gene [23]. The dysfunction or down regulation of IκBα will lead to over activation of NF-κB. Till now, epidemiological studies on NFKBIA were relatively rare. A 2758G/A polymorphism (rs696) in 30-untranslated region might regulate the expression of IκBα and thus affect the activation of NF-κB. Sun and colleagues [24] suggested that higher frequency of AG genotype was associated with colorectal cancer in Chinese old patients.

Several limitations of this study should be considered. Firstly, limited number of SNP in NFKBIA and NFKB1 gene was chosen in this study. More SNPs should be included in the further studies. Secondly, environmental factors should be included in the gene-environment analysis, including lifestyle, diet and activity factors. Thirdly, the etiology of liver cancer might be different for men and women, so the gender difference on the association of genetic polymorphisms with liver cancer should be investigated in the future studies.

In conclusion, the results of current study indicated that rs696 in NFKBIA and rs28362491 and rs230496 in NFKB1 gene polymorphism were associated with increased liver risk. There was a significant gene-gene interaction between rs696 and rs28362491, subjects with CT or TT of rs696 and ins/del or del/del of rs28362491 genotype have the highest liver cancer risk, compared to subjects with CC of rs696 and ins/ins of rs28362491 genotype. Haplotype containing the rs696-T and rs3138053-A alleles, and haplotype containing the rs28362491-del and rs230496-G alleles were associated with increased liver cancer risk.

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Disclosure of conflict of interest

None.

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