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ORIGINAL ARTICLE

Combination of Interleukin 1 Polymorphism and *Helicobacter pylori* Infection: an Increased Risk of Gastric Cancer in Pakistani Population

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Abstract *Helicobacter pylori* is one of the major risk factors involved in the development ofgastritis and gastric cancer (GC). *H. pylori* infection leads to increased production of pro-inflammatory cytokines by the host. Carriage of specific polymorphisms in cytokine genes may be associated with host susceptibility to the development of GC. We investigated the role of host genetic factors including polymorphisms of IL-1B and IL-1RN in correlation with gastritis and GC in *H. pylori* infected Pakistani population. A total of 230 gastritis cases and 100 GC cases were genotyped for IL 1B-511 and IL-1RN penta-allelic variable number of tandem repeats (VNTRs). A combination of *IL-1B-511**T and *IL-1RN**2 alleles (OR 19.064; 95% CI 2.319–156.7; p = 0.001) in *H. pylori* infected individuals had markedly increased risk of GC development. In Pakistani population, an increased risk of GC development is associated with the carriage of *IL-1B-511**T and *IL-1RN**2 alleles. Synergistic effect of *H. pylori* infection and *IL-1B-511**T/*IL-1RN**2 genotypes was also observed in association with significantly higher risk of developing GC. Further prospective and large scale studies are needed to establish the clinical impact of these findings.

Keywords *Helicobacter pylori* · Gastritis · Gastric cancer · Cytokines · Polymorphism

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Introduction

Helicobacter pylori infection affects more than half of the world's population and shows a strong association with peptic ulcer disease (PUD) and the development of gastric cancer (GC). However, only a small proportion of the infected population develops PUD or GC. It is reported that only 3% and 10–20% of *H. pylori* infected people are at higher risk of developing GC and PUD, respectively [1–3], if *H. pylori* eradication is not successful.

There are many factors involved in the progression of *H. Pylori*-associated gastritis into precancerous and cancerous outcomes; therefore, vast majority of *H. pylori* infected population does not develop GC. A variety of host genetic and environmental factors are implicated which in combination with *H. pylori* infection enhance the risk of developing GC [4–9]. There are several *H. pylori*-associated virulence factors including motility, urea hydrolysis, adhesion, cytotoxin associated gene A (*cagA*) and vacuolating cytotoxin A (*vacA*) etc., which are involved in colonization in extreme acidic environment of stomach.

Human genetic polymorphism has also been found to be associated with the severity of many diseases. Several genes including interleukins *(IL)-1B, IL-1RN, IL-8, IL-10* and *TNF* α, which are responsible for proinflammatory response have

been studied and reported to have single nucleotide polymorphisms (SNPs) in association with the severity of inflammation and GC. SNPs in these genes may down-regulate the cytotoxin response and upregulate the ILs production, particularly IL-1B, which suppresses the acid produced by gastric epithelial cells in in-vitro and in-vivo studies [10–12]. Up-regulated production of IL-1B may result in neutralization of the acidic environment of stomach and facilitate the colonization of *H. pylori* which spreads from antrum towards corpus. Three genes *IL-1A, IL-1B* and *IL-1RN* are found in *IL-1* gene cluster and respectively code for IL-α, IL-B and IL-1 receptor antagonist. Binding of IL-1α and IL-1B is inhibited by *IL-1RN* when it binds with IL-1 receptors, and injurious proinflammatory effects of IL-1B are also neutralized by *IL-1RN*. Therefore, polymorphisms of IL-1B and *IL-1RN* genes influence inflammatory response, causing high IL-1B/*IL-1RN* ratio, which results in gastric hypochlorhydria, which may then facilitate the development of precancerous lesions and finally GC [5, 13–16]

Promoter regions of *IL-1B* and *IL-1RN* genes are mainly known to have SNPs which alter the expression of genes and affect inflammatory response, which can vary from population to population because of occurrence of different allele frequencies in specific populations. *IL-1B* gene has been reported to carry three different SNPs: a T-C base transition at IL1B-31 and C-T base transition at *IL-1B-511* and *IL-1B-3954* [5, 17, 18]. Almost 86 base paired variable number of tandem repeats (VNTRs) are present in the *IL-1RN* gene [19]. In allele specific studies, *IL-1RN2, IL-1B-511 T, IL-1B-31 C* and *IL-1B-3954 T* genotypes were reported to show strong association with increased production of proinflammatory cytokines, hypoacidity and increased risk of developing GC, particularly intestinal type [5, 20–24]. Similarly, *TNF α-308 A* allele is over-expressed with polymorphism in *H. pylori* positive patients resulting in the amplification of proinflammatory response and the inhibition of gastric acid production, promoting further colonization of *H. pylori*. It has also been reported that individuals carrying *TNF α-308 A* allele are at higher risk of developing GC [10, 22, 25, 26].

Materials and Methods

Patients and Sample Collection

This prospective study involved a total of 330 subjects, divided into two groups: one group comprised of 230 subjects with dyspepsia, who underwent eosophago-gastroduodenal endoscopy for upper gastrointestinal symptoms in the endoscopy unit of Dow University of Health Sciences (DUHS), Civil Hospital, Karachi. Two biopsies each from the gastric antrum and the corpus of every individual were collected in 10% formalin for histological analysis and DNA extraction. The second group comprised of 100 subjects with GC, who reported to Ziauddin University Hospital, Karachi. Seventy two small endoscopic and 28 resected gastric tissues were collected from both cancerous and histopathologically normal lesions from these patients. This research was conducted with the permission granted by the ethical review board of the University of Karachi, Pakistan and written consents were also obtained from all the participants of the study.

Histopathology

Sections (3–4 um thick) from formalin fixed paraffin embedded (FFPE) tissues stained with Haematoxylin and Eosin staining for evaluating histological features of gastric inflammation according to the updated Sydney system [27]. Details of the histopathological procedures and definitions followed in this study are similar to those already published by our group [28].

Table 1 PCR primers and probes used in this study

Target gene	Primer sequence (5′-3′)	Probe sequence (5′-3′)	Product size (bp)	PCR condition	Reference
B-globin	ACACAACTGTGTTCACTAGC CAACTTCATCCACGTTCACC	-	110	94 °C, 30 s; 51 °C, 30 72 °C, 30 s (40 cycles)	[28]
ureA	GCCAATGGTAAATTAGTT CTCCTTAATTGTTTTTAC	-	411	94 °C, 1 min; 45 °C, 1 min; 72 °C, 1 min (40 cycles)	[28]
IL-1B-511 C/T	TCCTCAGAGGCTCCTGCAAT TGTGGGTCTCTACCTTGGGTG	FAM-TGTTCTCTGCCTCGGG AGCTCTCTG-BHQ1 JOE-CTGTTCTCTGCCTCAGGA GCTCTCTGTC-BHQ1	-	95 °C, 15 s; 60 °C, 30 s; 72 °C, 30 s (40 cycles)	[22]
IL-1RN	CCCCTCAGCAACACTCC GGTCAGAAGGGCAGAGA	-	-	94 °C, 30 s; 58 °C, 30 s; 72 °C, 30 s (40 cycles)	[29]

Table 2 The main demographic characteristics of the study population

	Gastritis (Controls)	Gastric Cancer	*p* value
Number of patients	230	100	
Sex			<0.001
Males, n (%)	117 (50.8)	70 (70)	
Mean age ± SD, yrs	37.58 ± 13.64	55.7 ± 14.78	
Females, n (%)	113 (49.1)	30 (30)	
Mean age ± SD, yrs	36.5 ± 13.73	57.9 ± 10.00	
H. pylori positive by PCR, n (%)	113 (49.1)	55 (55)	0.195
Males, n (%)	63 (55.7)	37 (67.2)	0.508
Females, n (%)	50 (44.2)	18 (32.7)	0.092

DNA Extraction and Polymerase Chain Reaction (PCR) for *H. pylori*

DNA was extracted from gastric biopsies and 3 to 5 μm sections of tissues. FFPE tissue sections were deparaffinized with xylene, washed with 100% ethanol to remove xylene traces and air dried. Samples were homogenized and added to a mixture of 20ul 20% SDS, 80 μl protein kinase buffer (0.375 M NaCl, 0.12 M EDTA, pH 8.0), 40 μl of Proteinase K (10 mg/ml), and sterile water. The mixture was incubated at 55 °C for 24 h for digestion by proteinase K. The next day, 100 μl of 6 M NaCl was added, the mixture was centrifuged at 13,000 rpm and the supernatant was transferred to another sterile tube where the DNA was precipitated by adding 1 ml of 100% ethanol and the suspension was centrifuged at 14,000 rpm. The DNA pellet was washed with 70% ethanol, air dried, resuspended in 50 μl of 1× TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) and stored at −20 °C until the polymerase chain reaction procedure (PCR) was employed. DNA samples were used for detection of *H. pylori* infection by assessing for the presence of the *ureaseA* gene using specific primer sequences (Table 1). A PCR mixture containing 2–3 μl DNA sample, 0.5 μl each of forward and reverse primers, 12.5 μl of 2× master mix (kapaTaq ready mix, Kapa Biosystem, Boston, USA) and nuclease free water was subjected to PCR amplification under the conditions described in Table 1. PCR for human β- globin gene was performed as a control for the quality of sample and DNA extraction [28].

Table 3 The main histopathological features on biopsy specimens from the study population

Pathological features	Numbers (percentages)	*H. pylori* positive, n (percentages)
Severity of chronic gastritis	*n* = 230	
Mild	117 (50.8)	29 (24.7)
Moderate	63 (27.3)	38 (60.3)
Severe	50 (21.7)	46 (92)
Neutrophilic activity	*n* = 86	
Mild	31 (36)	11 (35.4)
Moderate	33 (38.3)	19 (57.5)
Severe	22 (25.5)	20 (90.9)
Intestinal metaplasia	*n* = 14	
Mild	4 (28.4)	0 (0)
Moderate	3 (21.4)	2 (66.6)
Severe	7 (50)	6 (85.7)
Gastric Cancer	*n* = 100	
Intestinal type	61 (61)	46 (75.4)
Diffuse type	39 (39)	9 (23)

Qualitative Real Time PCR for the Detection of Specific Polymorphisms in *IL-1B-511*, *IL-1RN* Genes

Polymorphic regions in *IL-1B-511 were amplified through* real time PCR (Stratagene-MxPro). Final reaction mixture of 25 μl consisted of 12.5 Master Mix, 0.5 μl of each probe, 2 μl of each forward and reverse primers, 3–5 μl of DNA sample and required volume of nuclease free water.

VNTRs of *IL-1RN* were identified by primer-specific amplification. Base pair (bp) size of amplified DNA products detected on 2% agarose gel were correlated with number of 89 bp repeated units; i.e. allele 1 = 4 repeats (410 bp), allele 2 = 2 repeats (240 bp), allele 3 = 5 repeats (500 bp), allele 4 = 3 repeats (325 bp), and allele 5 = 6 repeats (595 bp). Primer, probe sequences and PCR conditions are shown in Table 1.

Statistical Analysis

All data were cross tabulated and the mean values of variables were compared. Hardy-Weinberg Equilibrium (HWE) and odds ratios (ORs) with confidence interval (CI) of 95% were computed to indicate which carrier of proinflamatory cytokine alleles is more likely to develop GC in association with *H. pylori* infection. *P* value from two sided Fisher's Exact test was calculated in this case. A *p* value of <0.05 was considered statistically significant. IBM SPSS 20 for Windows 7 was used for statistical analysis.

Table 4 Comparison of cytokine polymorphisms among *H. pylori* positive and negative cases of gastritis and gastric cancer

Disease	Genotype	*H. pylori* + VE *N* = 113	*H. pylori* –VE *N* = 117	Odd Ratio (95% CI)	Relative Risk	*p* value (Two sided Fisher's Exact test)
Gastritis	***IL-1B-511***					
	T/T	4	2	2.11 (0.379–11.754)	1.370	0.44
	C/C	5	11	0.446 (0.150–1.328)	0.619	0.195
	C/T	104	104	1.44 (0.592–3.562)	1.222	0.504
	IL-1RN					
	A1	33	41	0.765 (0.439–1.333)	0.870	0.397
	A2	28	31	0.914 (0.505–1.653)	0.955	0.880
	A1/2	52	45	1.364 (0.807–2.306)	1.169	0.286
Gastric cancer	***IL-1B-511***					
	T/T	11	8	1.156(0.421–3.175)	1.066	0.804
	C/C	-				
	C/T	44	37	0.865 (0.315–2.375)	0.938	0.804
	IL-1RN					
	A1	3	4	0.591 (0.125–2.791)	0.766	0.698
	A2	47	36	1.469 (0.516–4.183)	1.203	0.594
	A1/2	5	5	0.8 (0.216–2.957)	0.9	0.750

N Number of cases

CI Confidence Interval

Results

Demographic and Pathological Features

The main demographic and clinical features of the study population are given in Table 2. Males were slightly predominant (117 vs. 113 females) and their mean age was 37.58 ± 13.64 years in the chronic gastritis group. In GC group, males were markedly predominant (70 vs. 30 females) and the mean age was 55.7 ± 14.78 years. There was no statistically significant difference in *H. pylori* positivity among chronic gastritis and GC groups and among males and females as shown in Table 2.

The semiquantitative grading of various histopathological features of chronic gastritis and types of GC along with the frequencies of *H. pylori* infection are given in Table 3. Notably, no case of chronic atrophic gastritis (CAG) or gastric epithelial dysplasia (GED) was noted in this series. Intestinal metaplasia (IM) was also rare in this study.

Association of *H. pylori* with Gastritis and Gastric Cancer

PCR confirmed the presence of *ureA* gene in 113 (49.1%) cases out of 230 gastric biopsies from patients with chronic gastritis, while in GC tissues, 55 (55%)

Table 5 Frequencies of *IL-1B-511* and *IL-1RN* genotypes in gastritis and gastric cancer cases

Genotype	Gastric cancer *N* = 100	Gastritis *N* = 230	Odd Ratio (95% CI)	Relative risk	*p* value (Two sided Fisher's Exact test)
IL-1B-511					
T/T	19	6	8.757 (3.379–22.697)	2.862	<0.001
C/C	0	16	-	-	0.004
C/T	81	208	0.451 (0.232–0.877)	0.605	0.028
IL-1RN					
A1	7	74	0.159 (0.070–0.359)	0.231	<0.001
A2	83	59	14.151 (7.76–25.78)	6.464	<0.001
A1/2	10	97	0.152 (0.075–0.308)	0.232	<0.001

N Number of cases

CI Confidence Interval

Table 6 Specific combination of *IL-1B-511* and *IL-1RN* in gastritis and gastric cancer cases

Genotype	Gastric cancer $N = 100$	Gastritis $N = 230$	Odd Ratio (95% CI)	Relative Risk	p value (Two sided Fisher's Exact test)
IL-1B-511/*IL-1RN*					
TT/A1	7	3	5.695 (1.442–22.550)	2.409	0.01
TT/A2	12	3	10.318 (2.844–37.441)	2.864	<0.001
Others	81	224	0.114 (0.044–0.296)	0.349	<0.001

N Number of cases

CI Confidence Interval

cases were positive for *ureA* gene. The difference in the prevalence of *H. pylori* among the two groups was not significantly different ($p = 0.195$), as shown in Table 2.

Specific Cytokine Polymorphism and *H. pylori* Infection Susceptibility

The effect of specific cytokine polymorphisms on susceptibility to *H. pylori* infection was determined by comparing the frequencies of polymorphisms in *H. pylori* positive and negative gastritis and GC cases. No significant association was observed between *H. pylori* infection susceptibility and specific cytokine polymorphisms, as shown in Table 4.

IL-1B-511 and *IL-1RN* Alleles Association with Gastric Cancer

A comparison of *IL-1B-511* and *IL-1RN* genotype frequencies in gastritis and GC cases showed that those subjects carrying *IL-1B-511* T/T alleles and *IL-1RN* A2 alleles have 8.757 and 14.151-fold increased risk of developing GC, respectively, as shown in Table 5. GC and control cases deviated from HWE due to excess of heterozygosity ($p < 0.01$). No significant difference between gastritis and GC cases was observed.

Combined Effect of *IL-1B-511* T/T and *IL-1RN* A1 and A2 in Progression of Gastric Cancer

Combined effect of *IL-1B-511* T/T and *IL-1RN* A1 and A2 was determined and it was observed that those individuals carrying both *IL-1B-511* T/T and *IL-1RN* A1 alleles were at 5.695 times increased risk of developing GC. The risk was increased 10.318-fold if the individuals carried *IL-1B-511* and *IL-1RN* A2 alleles (Table 6).

Combined Effect of *H. pylori* Infection and *IL-1B-511* T/T and *IL-1RN* A2 Alleles in Gastric Cancer Risk

An analysis of the association between *H. pylori* infection and GC and gastritis cases revealed that there was slightly increased risk of developing GC in *H. pylori* positive cases (OR = 1.2) as compared with *H. pylori* negative cases (OR = 0.7), but the difference was not statistically significant as shown in Table 7. However, significant association was observed if *H. pylori* infected individuals carried *IL-1B-511* T/T and *IL-1RN* A2 alleles, increasing the risk of developing GC 6.183 and 17.835-fold, as shown in Table 8.

Combined Effect of *IL-1B-511* T/T and *IL-1RN* A2 Alleles in *H. pylori* Positive Gastritis and Gastric Cancer Cases

Significant increase in the risk of GC development was observed in *H. pylori* infected individuals carrying a combination of *IL-1B-511* T/T and *IL-1RN* A2 alleles with the OR of 19.064, as shown in Table 9.

Discussion

A study of host genetic factors involved in the development and progression of GC in any population can solve the riddle that why only small portion of population develops gastric complications and GC after being infected by *H. pylori* [5]. Host factors, in addition to environmental and bacterial factors, are involved in initiating immune and inflammatory response

Table 7 *H. pylori* infection and risk of gastric cancer

H. pylori	Gastric Cancer $N = 100$ (%)	Gastritis $N = 230$ (%)	Odd Ratio (95% CI)	p value (Two sided Fisher's Exact test)
Negative	45 (45%)	117 (50.8%)	0.7 (0.493–1.266)	0.340
Positive	55 (55%)	113 (49%)	1.2 (0.79–2.027)	0.340

N Number of cases

CI Confidence Interval

Table 8 Frequency of *IL-1B-511* and *IL-1RN* genotypes in *H. pylori* positive gastritis and gastric cancer cases

Genotype	Gastric Cancer *H. pylori* positive *n* = 55	Gastritis *H. pylori* positive *n* = 113	Odd Ratio (95% CI)	Relative Risk	*p* value (Two sided Fisher's Exact test)
IL-1B-511					
T/T	11	4	6.813 (2.059–22.544)	2.55	0.01
C/C	0	5	-	-	0.174
C/T	44	104	0.346 (0.134–0.894)	0.541	0.04
IL-1RN					
A1	3	33	0.140 (0.041–0.480)	0.212	<0.001
A2	47	28	17.835 (7.526–42.262)	7.285	<0.001
A1/2	5	52	0.117 (0.044–0.316)	0.195	<0.001

N Number of cases

CI Confidence Interval

against *H. pylori* infection. In addition, there are also certain endogenous factors which suppress acid production and facilitate *H. pylori* existence in the stomach [29]. Interleukin-1B is the proinflammatory cytokine which is acid suppressor and is up-regulated in *H. pylori* infection [30, 31]. Its injurious inflammatory effects are counterbalanced by *IL-1RN*. Polymorphisms in these cytokine genes disturb the regulatory mechanisms resulting in the production of unfavorable levels of IL-1B/IL-RN [32, 33]. One of the three biallelic polymorphisms in IL-1B is C-T base transition at position −511 [34], which is also investigated in this study along with the specific allele (VNTR) in *IL-1RN* gene [12]. Figueiredo et al. reported that *IL-1B511**T and *IL-1RN**2 are highly associated with increased risk of GC in Caucasian population [35]. However, in Japanese population, *IL-1B-511* polymorphism was not found to have any association with GC. In Taiwan, independent role of *IL-1RN**2 carriage in addition to *H. pylori* infection was suggested to increase the risk of developing GC [21]. El-Omar et al. reported that IL-1B-31*T/*IL-1RN**2 increase the risk of GC, They further revealed that carriage of *IL-1B-511**T and *TNFα*-308A was also associated with increased risk of non-cardia GC which is greater in homozygotes [22]. Machado et al. found that IL-1 polymorphisms are more commonly associated with intestinal type GC as compared to diffuse type [24].

Our study is the first from Pakistan with information regarding the prevailing frequencies of cytokine polymorphisms in relation with *H. pylori* infection in gastritis and GC patients. Our data shows that IL-1B C/T (90.4%) allele is higher in gastritis patients along with high frequencies of *IL-1RN*1/2 (42.1%) in the same group. No association was found between specific alleles and the propensity of acquiring *H. pylori* infection. In case of GC patients, the frequency of *IL-1RN**2 (83%) was observed high with similar findings regarding the frequency of IL-1B C/T (81%) with no significant association with *H. pylori* infection. Regardless of *H. pylori* infection, frequency distribution in gastritis and GC patients revealed a significant association of *IL-1B-511**T (OR 8.757; 95% CI 3.379–22.697; $p < 0.01$) and *IL-1RN**2 (OR 14.151; 95% CI 7.7625.78; $p < 0.01$) with high risk of GC, which is consistent with findings of El-Omar et al. [22]. We also evaluated the synergistic effect of *IL-1B-511* and *IL-1RN* genotypes. Statistical analysis in this regard showed that carriage of TT genotype of *IL-1B-511* and A2 genotype of *IL-1RN* (OR 10.318; 95% CI 2.844–2.864; $p < 0.001$) had increased risk of GC than carriage of TT genotype of *IL-1B-511* and A1 genotype of *IL-1RN* (OR 5.695; 95% CI 1.442–22.55; $p = 0.01$) or other genotypic combinations (OR 0.114; 95% CI 0.044–0.296; $p < 0.001$). Isolated

Table 9 Specific combinations of *IL-1B-511* T/T and *IL-1RN* alleles in *H. pylori* positive gastritis and gastric cancer cases

Genotype	Gastric Cancer *H. pylori* positive *N* = 55	Gastritis *H. pylori* positive *N* = 113	Odd Ratio (95% CI)	Relative Risk	*p* value (Two sided Fisher's Exact test)
IL-1B-511/IL-1RN					
TT/A1	3	3	2.115 (0.413–10.839)	1.558	0.394
TT/A2	8	1	19.064 (2.319–156.7)	30.7	0.001
Others	44	109	0.147 (0.044–0.486)	0.392	0.001

N Number of cases

CI Confidence Interval

H. pylori infection in the absence of high risk cytokine genotype carriage carried only a slightly increased risk of GC (OR 1.2; 95% CI 0.79–2.027; $p = 0.340$) which was not statistically significant. On the other hand, in *H. pylori* infected individuals, carriage of *IL-1B-511**T (OR 6.813; 95% CI 2.059–22.544; $p = 0.01$) genotype was found to have statistically significant association with increased risk of GC, which is in concordance with another study reporting OR with 95% CI of 3.2 (1.27–8.05) [36]. *IL-1RN**2 allele (OR 17.835; 95% CI 7.526–42.262; $p < 0.01$) seems to have more profound role in increasing GC risk. OR in our study was found much higher than the findings of Chen et al. who reported OR in case of IL-1RN*2 with 95% CI of 2.2 (1.3–3.8) [21]. In addition, a combination of *IL-1B-511**T and *IL-1RN**2 (OR 19.064; 95% CI 2.319–156.7; $p = 0.001$) in *H. pylori* infected individuals had markedly higher risk of GC, which was also reported by Ruzzo et al., who found OR with 95% CI of 6.49 (2.01–20.4) [36].

There are certain limitations in this study which must be kept in mind when interpreting results. This is a cross-sectional study with no information on treatment and follow-up of patients. Details on the grades and stages of GC were also not analyzed. There is a relatively low prevalence of *H. pylori* infection in GC patients. The prevalence of *H. pylori* infection in GC patients varies widely. One possible reason may the relatively higher prevalence of diffuse type of GC in this study (39%), the type which is typically not associated with *H. pylori* infection. Despite the above limitations, this is one of the largest studies on this subject from this region of the world and will help understand the molecular epidemiology of GC.

Based on the findings of our study, it can be concluded that *H. pylori* infection in individuals with the carriage of *IL-1B-511**T/*IL-1RN**2 genotypes carries a high risk of developing GC. Further prospective and large scale studies are needed to establish the clinical impact of these findings.

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Compliance with Ethical Standards

Conflict of Interest No conflict of interest exists.

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