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Molecular Analysis of Interleukin-10 Gene for -1082 G/A Polymorphism in Breast Cancer Patients

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ABSTRACT

One in every nine Pakistani women has become breast cancer patients. Genetic polymorphisms of the Interleukin-10 (*IL-10*) gene were studied at position -1082 (G/A) in breast cancer patients in the Pakistani population. Breast cancer patients (n= 200) were recruited from different local tertiary care hospitals along with healthy subjects (n=100) for molecular analysis of the human *IL-10* gene mutation at the -1082 (G/A) position. Extraction of genomic DNA was done from patients and healthy subjects. An allele-specific polymerase chain reaction determined polymorphism at a position (-1082 G/A). PCR products were analyzed by agarose gel electrophoresis to detect polymorphism. Further analysis was done by sequencing the PCR products from some randomly selected samples. Urban locality, drinking of non-filtered water, poor hygiene, and poor socioeconomic status are the risk factors for the onset of breast cancer in the Pakistani population. Molecular analysis of the human *IL-10* gene revealed that the prevalence of homozygous normal GG allele was 19%, heterozygous GA was 29% and homozygous mutant AA was 52% in breast cancer patients. However, in healthy individuals, GG allele was found in 70%, GA in 20%, and AA in 10% population. These findings proposed that the *IL-10* gene (AA polymorphism) at the -1082 position is most prevalent in local breast cancer patients in Pakistan.

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Authors' Contribution
MA, NM and IRM conceptualized the study. MA and GM performed data curation. MA, NM and IRM performed the formal analysis and did the investigation. NM, GM and IRM administered and suervised the project. NM and IRM performed validation of results and data visualization. MA wrote the original draft. NM, GM and IRM reviewed and edited the manuscript.

Key words
Interleukine-10, Breast cancer, Polymorphism, Homozygous, Heterozygous

INTRODUCTION

Breast cancer is defined as the uncontrolled proliferation of epithelial tissue of breast cells. It is the most prevalent cancer among females all over the world. Breast cancer may affect males as well (Azamjah *et al*., 2019). Studies showed the various risk factors associated with breast cancer are familial (77.6%), smoking, poor diet (47.1%), food additives (45.4%) and hormonal disturbance (46.3%) (Thomson *et al*., 2014). In developed countries, breast cancer is the top most common cancer and one hundred times more common in females than males (Bray *et al*., 2018). In Pakistan, one in nine women of Pakistan has become breast cancer patients during their lifetime (Menhas and Umer, 2015).

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Single nucleotide polymorphisms (SNPs) are important changes in the human genome and affect more than 1% of the entire population (Wang *et al*., 1998). Any alteration in the genes can be detected and SNPs are used as genetic markers (Taylor *et al*., 2001; Srinivasan *et al*., 2016). SNPs work as genetic marker because it is related with the functions of those genes that are associated with the onset of various complex diseases as, Alzheimer, blood pressure, diabetes, various forms of cancers, migraine and schizophrenia (Kaur *et al*., 2019). Interleukins (IL) also called cytokines are a very diverse group of proteins that control or cascade the functions of innate and adaptive types of immunity. Numerous kinds of white blood cells produce a variety of interleukins but a specific lymphocyte produces a very specific form of IL in the body and this specific IL can also cascade so many diverse types of cells (Zhang and An, 2007; Arango and Descoteaux, 2014).

Interleukin-10 (*IL-10*) gene produces a protein which is a cytokine that is produced initially by monocytes and later by lymphocytes. This cytokine shows the multiple spectra of immunoregulation and inflammation (Saxena *et al*., 2015). The Th1 cytokines such as IL-1B, IL-2, IL-12, TNF-A, and IFN-y are very important in the process of cell-mediated immunity. They play an important role against viruses and intracellular pathogens in the host

defense system. MHC2 or major histocompatibility molecules class 2 are the antigens that are present on the surface of various antigen-presenting cells (APC) such as macrophages, various B cells, and endothelial cells. They play an important role in the immune response (Carey *et al*., 2012).

The expression of Th1 cytokines, MHCII antigens is down-regulated by IL-10, and it acts as a co-stimulatory molecule on macrophages. The survival of B cell, proliferation, and antibody production is also enhanced by IL-10 (Couper *et al*., 2008). IL-10 cytokine can block NF-kappa B production, the regulation of the JAK-STAT signaling pathway also takes place with the help of IL-10 (Carey *et al*., 2012).

Several studies showed that single nucleotide polymorphism at position (-1082 G/A) at the promoter site of the *IL-10* gene influence the expression of the RNA and hence important anticancerous molecular pathways in the cells (Crawley *et al*., 1999; Hoffmann *et al*., 2001; Kingo *et al*., 2005). It may lead to the pathogenesis of breast cancer hence IL-10 polymorphism is considered a very important biomarker for the breast cancer (Howell *et al*., 2007). So, we have selected IL-10 as a biomarker for our current research by checking the frequency of polymorphism of the *IL-10* gene (-1082 G/A) in the promoter region in breast cancer patients of Pakistan. There is a need to further expand the research on larger population of breast cancer patients.

MATERIALS AND METHODS

All procedures complied through the statement of Helsinki and the study protocol was accepted in the board of advanced studies and research, university of Sargodha, Sargodha, Pakistan. Permission from the ethical committee, the University of Sargodha was obtained for the research work. The subjects were divided into patients (n=200) and healthy controls (n=100). primers required to target the *IL-10* gene (-1082 G/A) were searched from the literature (Settin *et al*., 2009) and were further evaluated using different bioinformatics tools i.e., Primer3 software (http://frodo.wi.mit.edu/primer3/) and then synthesized (Macrogen, Korea). Individuals included in the current research were based on inclusion-exclusion criteria. The inclusion criteria involved freshly diagnosed breast cancer patients in which chemotherapy has not been started, while those in which chemotherapy has been started were excluded from the study.

Collection of blood samples

Blood samples were collected from 200 breast cancer patients from different local tertiary care hospitals and 100 healthy individuals as control. Clinical history and laboratory findings data were collected of cancer patients. After informed consent, an appropriate amount of venous blood (1-3 ml) was collected using 5ml disposable syringes into EDTA vacutainer tubes and stored at -20˚C until further use.

Genomic DNA isolation

Genomic DNA was extracted using the standard protocol of the DNA extraction kit (Vivantis, Cat No. GF-BD-100). A spectrophotometer determined the DNA concentration at 260 nm and then DNA was stored at -20˚C until further use.

Genotyping

Allele-specific PCR is a technique that is used to detect specific point mutations. To do allele-specific PCR for the detection of Interleukin-10 (*IL-10*) gene polymorphism at -1082 (G/A) position**,** one forward primer and two allele-specific reverse primers were used. The sequences of primers used for IL-10 SNP analysis were: Forward primer 5′-AGCAACACTCCTCGTCGCAAC-3′ and two allele-specific primers 5′-CCTATCCCTACTTCCCCC-3′ (G) and 5′-CCTATCCCTACTTCCCCT-3′ (A) (Settin *et al*., 2009). Each PCR reaction total volume was 25 µL and involved, deionized autoclaved H_2O (15 µL), PCR Buffer (2 µL), dNTPs (stock 2mM) 2 µL, $MgCl_2$ (stock 25mM), 2.5 µL, Genomic DNA 2 µL (3 µg), forward primer (stock 10pmol/µL) 0.5 µL, each of reverse primer (stock 10pmol/µL) 0.5 µL and Taq polymerase (stock 5U/uL) 0.5 µ/L.

All ingredients were thoroughly mixed and placed in the thermal cycler for PCR reaction. PCR temperature cycling conditions involved initial denaturation at 95°C for 04 min, then 30 cycles and each cycle involved denaturation at 95°C for 40 sec, annealing at 61°C for 60 sec, extension at 72°C for 60 sec, and the final extension at 72°C for 10 min before termination of PCR reaction at 4°C. The amplified PCR products were 179 bp size for the *IL-10* gene and were analyzed on 2% agarose gel. Amplified PCR product (179 bp) of the *IL-10* gene representing alleles G and allele A. Sequencing analysis was also performed for five randomly selected breast cancer patient samples.

Sequence analysis

Five random samples of breast cancer were sequenced using ABI sequencer by using primers IL-10 (F) 5′-ATCCAAGACAACACTACTAA-3′ and IL-10(R) 5′-TAAATATCCTCAAAGTTCC-3′ (Turner *et al*., 1997). The amplified product size by using sequencing primers was 588 bp. Sequencing results were further analyzed for SNP identification after peaks corrections.

RESULTS

Distribution of breast cancer patients

Table I summarizes the frequency distribution of breast cancer patients (n=200) with cancer stage and age groups. Chi-squared test (χ^2) was used to find the differences in breast cancer patients with cancer stage and age groups. The p-value of .05 significant level was used to find out the significance. The value of chi-square (χ^2) for breast cancer stages is 600 with p values of .000*** which represent significant differences among breast cancer stages. The value of chi-square (χ^2) for different age groups of breast cancer patients is 200 with a p-value of .000*** that indicates significant difference among different age groups related to breast cancer disease.

Results show a higher prevalence of breast cancer in urban areas. Unfiltered drinking water, poor hygienic diet, and low socioeconomic status are the risk factors associated with breast cancer.

In the breast cancer patients, no association was observed regarding the residential area. ALT level was normal as the normal level of bilirubin, so these are not the risk factors for breast cancer. AST and ALP levels were high in the patients so these are the risk factors for the onset of breast cancer. While bilirubin, ALT, serum creatinine, and blood urea nitrogen levels remain normal. The age between 36 to 50 years of onset of menopause is the most significant risk factor for the growth of breast cancer as shown in Table II.

Table I. Distribution of breast cancer patients with cancer stage and age-wise (n=200).

Category	Frequency	Percentage (%)	chi-squared test (χ2)	P value
Distribution of breast cancer patients' cancer stage wise				
Stage 1	28	14	600	.000***
Stage 2	74	37		
Stage 3	68	34		
stage 4	30	15		
Distribution of breast cancer patients age-wise				
Group 1 (20-40 Y)	84	42	200	.000***
Group 2 (41-60 Y)	84	42		
Group 3 (61-80 Y)	32	16		

Polymorphism of IL-10 *gene with PCR*

In the *IL-10* gene mutation -1082 (G), reverse primer (G) is attached to a template and amplifies the target. In contrast, in the case of -1082 (A) mutation, reverse primer (A) is attached to the point of mutation, resulting in a product size of 179 bp for both mutation specific reverse primers.

Table II. Environmental, blood, and clinical parameters of breast cancer patients. (n=200).

Factors	Category	Cases	(%)
Environmental factors			
Residential area	Factory	68	34
	No factory	132	66
Locality	Urban	120	60
	Rural	80	40
Water	Filtered	60	30
	Unfiltered	140	70
Dietary Factors	Hygienic	60	30
	Unhygienic	140	70
Socio economic status	Poor	104	52
	Not Poor	96	48
Blood parameters			
ALT	High	58	29
	Normal	90	45
	Low	52	26
Bilirubin	High	2	1
	Normal	193	96.5
	Low	5	2.5
AST	High	68	34
	Normal	56	28
	Low	76	38
ALP	High	94	47
	Normal	48	24
	Low	58	29
Serum creatinine	High	8	4
	Normal	117	58.5
	Low	75	37.5
Clinical parameters			
Age	20-35 Y's	46	23
	36-50 Y's	92	46
	>50 Y's	62	31
Menstrual cycle/M	one/month	42	21
	> one/M	46	23
	Menopause	112	56
Diagnostic time of breast cancer	0-0.5 Y's	46	23
	0.6-01 Y's	126	63
	> 01 Y's	28	14
Family history	Positive	38	19
	Negative	162	81
Tumor type	T1	26	13
	T2	74	37
	T3	72	36
	T4	28	14

Out of a total of 200 patients with breast cancer, homozygous allele GG was found in 38(19%) patients, while found homozygous mutant allele AA in 104 (52%) patients while 58 (29%) were found to have heterozygous both G and A alleles as shown in Table III.

Table III. Frequency distribution of genotype and alleles in a study group.

	Controls (n=100) (%)	Females with breast cancer (n=200) (%)	chi-squared test (χ2)	P value
Frequencies distribution of genotype				
AA	10 (10%)	104 (52%)	81.191	.000***
AG	20 (20%)	58 (29%)		
GG	70 (70%)	38 (19%)		
Frequencies distribution of alleles				
G	160 (80%)	134 (33.5%)	113.5129	.000***
A	40 (20%)	266 (66.5%)		

Table III summarize the frequency distribution of genotype AA, GA, and GG and the frequency distribution of Allele A and allele G. Chi-squared test (χ^2) was used to find the differences in genotype AA, GA, and GG and the Allele A and allele G. p-value of .001 significant level were used to find out the significance.

Out of 100 cases of a control group, genotype AA was found in the 10 (10%) individuals, genotype AG was found to be in the 20 (20%) individuals and genotype GG was found to be in the 70 (70%) individuals. In the females with breast Cancer (n=200), genotype AA was found in 104 (52%) patients, genotype AG was found in 58 (29%), and genotype GG was found in 38 (19%) patients. The results indicate the chi-square value (χ^2) of 81.191 with a p-value of .000*** which indicates significant differences among genotypes of healthy and breast cancer patients. The prevalence of genotype AA (52%) was highest among breast cancer patients while genotype AA was only (10%) in the control group. The prevalence of genotype AG in the breast cancer patients was found to be (29%) while in the control group genotype AA was 20%. The occurrence of genotype GG was 19% in the breast cancer patients while in the control group genotype GG was 70% (Fig. 1).

The allele A prevalence in breast cancer patients is 266 (66.5%) and allele G is 134 (33.5%). While in the control group the A allele is 40 (20%) and allele G is 160 (80%). The value of chi-square (χ^2) is 113.5129 with a p-value of .000*** which indicates significant differences among allele categories. The occurrence of the A allele is high in the breast cancer patients while is low in the control and the G allele is high in the control while is low in the breast cancer patients (Fig. 2).

Sequencing of breast cancer patients was also done by random sampling and results are shown in (Fig. 4).

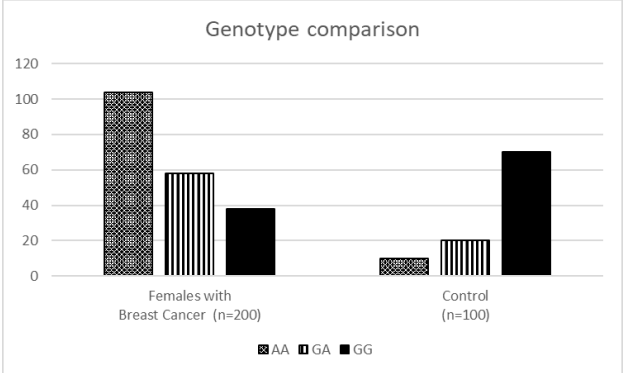

Fig. 1. Comparison of genotype AA, GA, and GG in a study group.

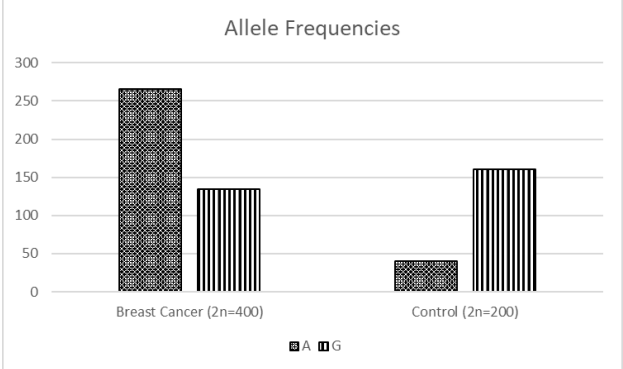

Fig. 2. Comparison of Allele A and G in a study group

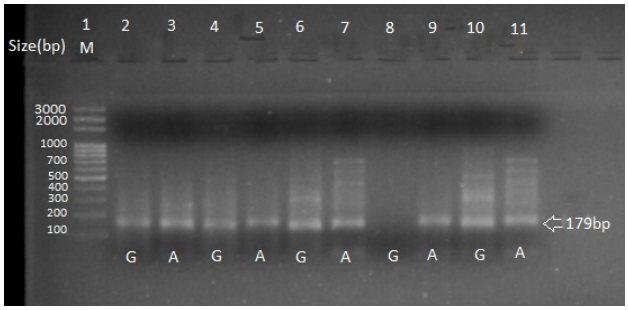

Fig. 3. Agarose gel electrophoresis (2%) showing *IL10* gene polymorphism. Lane 1 represents 100 bp ladder, Lanes 2 and 3 contain heterozygous samples 1, Lanes 4 and 5 contain heterozygous sample 2, Lanes 6 and 7 contain heterozygous sample 3, Lanes 8 and 9 contain homozygous (AA) sample 4 and, Lanes 10 and 11 contain heterozygous sample 5.

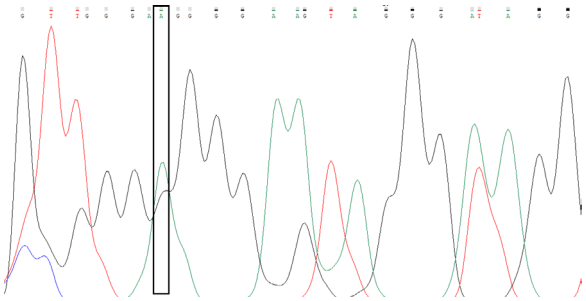

Fig. 4. The presence of single nucleotide polymorphism (A nucleotide) in breast cancer patient sample at -1082 position of *IL10* gene.

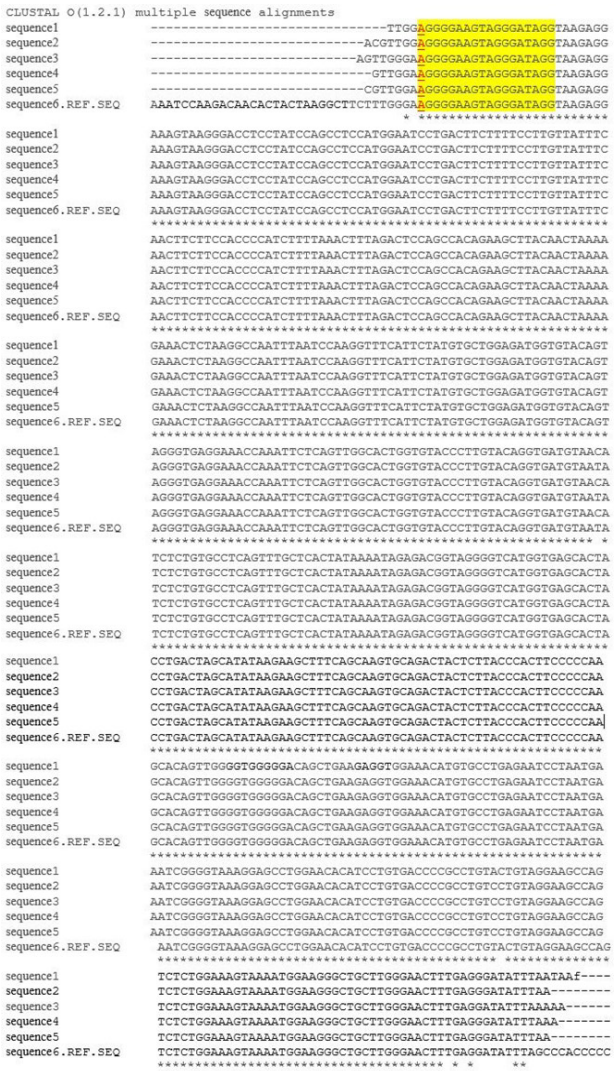

Fig. 5. ClustalW analysis results are showing. Molecular analysis of *IL10* gene (-1082 G/A) polymorphism is highlighted with red color representing the A allele. Allele-specific primers are yellow highlighted.

Amplicon sequencing and analysis

To verify the SNP validation, sequencing was performed. After the sequencing, results were obtained by the Sanger sequencing method. It was further analyzed for SNP identification for *IL-10* gene (-1082 G/A) polymorphism. Wild type possesses GG genotype, mutant possesses AA genotype, and heterozygous has both GA genotype. Sequencing results are displayed in (Fig. 4). All breast cancer samples were possessed (A) SNP at -1082 locus. Figure 5 shows the Clustal W results.

DISCUSSION

The present research was conducted on patients with breast cancer to analyze the prevalence of (-1082 G/A) polymorphism in the *IL-10* gene. The analysis of (-1082 G/A) polymorphism in the promoter region of *IL-10* was carried out by using allele-specific PCR through optimized conditions. we have selected 200 patients with breast cancer, and 100 healthy individuals for this research according to inclusion criteria. The clinical history of these patients was also preserved.

In women, breast cancer has the highest prevalence with about twenty-five percent of the total cancer cases reported. In 2018 more than six million deaths and two million new breast cancer cases were reported. In developed countries, breast cancer is the top most common cancer and one hundred times more common in women than men (Bray *et al*., 2018). It has been reported that in the USA the expected incidence of this cancer is 30% and the death rate is 14% in females (Rebecca *et al*., 2018).

One in nine women of Pakistan has become breast cancer patients during their lifetime (Menhas and Umer, 2015). Pakistan is the top country in Asia with the maximum breast cancer rate. The total number of breast cancer cases in adult females aged from 21 to 89 years registered was 3,889. Advanced stage of breast cancer was also found in young females which delay prognosis (Badar *et al*., 2005). Every year, a significant number of breast cancer cases in rural areas is seen due to inheritance transmitted from mothers to their daughter's urban females, and rural Pakistani women, facing cervical, ovarian, and uterine cancers (Farhana *et al*., 2005). New cases of breast cancer were 25928 (14.5%) with 13725 (11.7%) deaths in Pakistan (Sung *et al*., 2020).

IL-10 was considered to be a critical cytokine, playing an immunoregulatory and modulating role in activating and suppressing immune responses and is produced by activated T-cells, B cells, monocytes, and thymocytes (Lyer and Cheng, 2012). Several types of immune cells are proliferated and differentiated by IL-10 activity, also involved in the tumor-promoting and tumor-inhibiting

properties result in the playing role in the tumor development and metastasis process in the body (Gonzales *et al*., 2018). The function of this cytokine (IL-10) is altered when any type of alteration or polymorphism occurs in the promotor region, changing the downstream transcriptional activity signaling and cellular events result in the development of a human disorder. Previous studies show the polymorphism at IL-10 (-1082 G/A) was associated with osteosarcoma (Cui *et al*., 2015), lymphoma (Yu *et al*., 2014), gastric cancer (Kuo, 2014; Li *et al*., 2014; Ni *et al*., 2012), oral cancer (Tsai *et al*., 2014), nasopharyngeal carcinoma (Tsai *et al*., 2013). Another study shows the role of IL-10 (-1082 G/A) in the development of chronic lymphoid leukemia in the late stage among Russians (Ovsepyan *et al*., 2015).

The current research investigated the role of the genotype of the *IL-10* gene in the development of breast cancer. The data shows that the AA genotype is associated with breast cancer progression (Table III). This is the first study in Pakistan that examine the association of *IL-10* genotype with breast cancer. To ensure the reliability and accuracy of the present research, a large sample size is required among childhood cancer studies. From the molecular viewpoint, changes in the genotype of the *IL-10* gene may alter the normal functions of IL-10 protein, and its downstream signaling pathway and cellular activity result will be the progression of breast cancer among females.

A study found on the Chinese population shows that *IL-10* gene (-1082 G/A) polymorphism was significantly linked by the progression of breast cancer (Kong *et al*., 2010), and it is similar to our present study that AA genotype was found to be in 52% of the breast cancer patients. This argument is further supported by another study of the Italian population that *IL-10* -1082 (AA) genotype was associated with the increased risk of breast cancer (Giordani *et al*., 2003). Another study conducted in the northern chinses population shows that the AA genotype of *IL-10* (-1082 G/A) rs 1800896 was significantly connected with the higher risk of breast cancer as related to the GG genotype (Tian *et al*., 2017) as the same outcome of the present research work in the Pakistani population. Smith *et al*. (2004) also showed that the AA genotype of *IL-10* polymorphism at (-1082 G/A) was the critical factor in the development and progression of breast cancer in the UK population as revealed by the present study.

CONCLUSION

Molecular analysis of *IL-10* (-1082 G/A) polymorphism showed a risk factor for breast cancer patients in the Pakistani population. Nonfiltered water, poor hygiene, age (26-50 Y's), menopause, have significant factors for breast cancer. In a future perspective, this study will also helpful in the gene therapy and drug design if more elaborate molecular analysis and mechanism is found out as well.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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