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Study Of Genetic Polymorphism In Oxidative Stress Related Genes And Their Association With Gastrointestinal Cancer: A Case Control Study From Rural Population Of South Western Maharashtra.

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Abstract: Oxidative stress is known to be one of the major factors involved in the development and progression of cancer. Superoxide dismutase and Catalase plays an important role in the primary defence against oxidative stress. The present study was therefore undertaken to investigate the association between polymorphism of superoxide dismutase (SOD1, SOD2, SOD3) and Catalase (CAT1 & CAT2) and the gastro intestinal cancer risk in the rural population of the south western Maharashtra. The present case-control study included 200 confirmed gastrointestinal (GI) cancer patients and 400 age and gender matched healthy controls. The polymorphism in the Superoxide Dismutase and Catalase genes was studied out by PCR-RFLP method. When we studied the genotypic frequency of SOD1, SOD2, & SOD3, SOD2 (rs1141718) showed the negative association with the GI cancer risk [OR: 0.3097, CI: 0.1727-0.5553, p <0.0001]. There was no significant association found between SOD1 & SOD3 of superoxide dismutase and CAT1 & CAT2 of Catalase gene polymorphism and susceptibility of GI cancer. The present study shows no significant association of SOD1 (G allele of rs 2070424), SOD3 (A allele of rs2536512), CAT1 (T allele of rs7943316) and CAT2 (rs1001179) with the development of gastrointestinal cancer in the rural population of south western Maharashtra from India. However, SOD2 (rs1141718) shows the negative association with the development of GI cancer.

Keywords: Genetic polymorphism, Superoxide dismutase, Catalase, PCR-RFLP, Gastrointestinal Cancer

1. INTRODUCTION:

Gastrointestinal cancer refers to the malignant condition of gastrointestinal tract and its accessory organs. In comparison with cancer caused in other system of body, GI cancer is responsible for more deaths[1-2]. GI cancer is the third most common cancer among the men and fifth common cancer among the females in India occupying approximate 5% of all types of cancer[3]. It is the one of the leading cause of death in southern region of India than northern region[4]. Etiology of gastric cancer includes diet, lifestyle, chewing, smoking, drinking habits and *Helicobacter Pylori* infection.[5] In addition to these host genetic factors may contribute as a most critical agent in gastric carcinogenesis.[6] Tobacco and alcohol consumption in various forms also contribute as major risk factor of GI cancer. Consumption of tobacco in any form (smoking or chewing) generates free radicals in the form of reactive oxygen species (ROS). ROS are highly reactive molecules, at higher concentration they may damage cellular structure including DNA and alter their function, which is a major risk factor for development of cancer.[7-8] To protect the cellular damage caused by these ROS, aerobic organism have anti oxidant systems, which include enzymatic and non enzymatic antioxidants. Among the two major enzymatic antioxidant systems, superoxide dismutase (SOD) converts superoxide into hydrogen peroxide, while the other Catalase (CAT) reduces this H_2O_2 to water [9-10] thus protecting the damage caused by ROS. A single nucleotide polymorphism (SNP) in Superoxide dismutase or Catalase genes may affect the stability and activity of the antioxidant activity of the enzyme, leading to altered activity if the functional protein for ROS detoxification. Different types of cancer are associated with transitions in several SNPs of SOD isoforms including the transitions of A to G at 251position of codon 251 in Exon 10 of SOD1, Transition of T to C in Ile58Thr polymorphism at codon 58 in Ex3 of SOD2, transition mutation of G to A at position 172 in Ala to Threonine substitution in SOD3 gene.[11-15] Similarly, the point mutation of A to T in codon 326 of Ex7 in the promoter region of CAT gene and C262T polymorphism in 262 region of promoter of CAT gene are associated with multiple cancer risk. [16-22] As there are very few studies from India that report association of polymorphism in SOD and CAT with the risk of gastrointestinal cancer, the present study was undertaken to investigate the association between polymorphism of SOD1, SOD2, SOD3 and CAT1 & CAT2 genes and the GI cancer risk in the rural population of the South Western Maharashtra of India.

2. MATERIALS AND METHODS

Study subjects

In a present case-control study 200 histologically confirmed GI cancer patients and 400 healthy age and sex matched controls were drafted from Department of Oncology of Krishna Hospital & Medical Research Centre during the year 2018-2019. All untreated GI cases in the study ranged in age from 20-80(Mean±SD 59±13.33) years were enrolled. The demographic information including age, sex, economic status, place of residence, dietary habits, family history, tobacco and alcohol consumption status and other confounding risk factors were collected through personal interviews in the form of structured questionnaire. Informed consent was obtained from each subject.

Genomic DNA isolation from whole blood

Genomic DNA extraction was carried out from whole blood samples of cases and controls collected in sterile EDTA containing vacutainer. The extraction was carried out by method where the whole blood was processed with red blood cell lysis buffer (10mM Tris -HCl pH-7.6, 320mM sucrose, 5mM $MgCl_2$, 1% triton X-100, pH 7.6), thereafter the sample was treated with the nucleic lysis buffer (10mM Tris- HCl, 11.4mM sodium citrate, 1mM EDTA,

1% SDS, pH-8.0). The sample was further treated with the 20mg/ml of Proteinase K and subsequently RNase A (20mg/ml) followed by DNA precipitation with the ethanol. The purified DNA was checked on 1% agarose gel for its quality and quantity. The DNA was used for further genotyping by polymerase chain reaction (PCR) and Restriction fragment Length Polymorphism (RFLP).

Genotyping assays.
Single nucleotide polymorphisms in SOD1(Cu, Zn-SOD),SOD2(Mn-SOD),SOD3(EC-SOD), and CAT genes were studied by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Each PCR reaction was carried out separately in 20 microliter reaction mixture containing 100 nanogram (ng) of purified genomic DNA template, 1X PCR buffer,0.2 mM each dNTP, 1U Taq DNA polymerase (Genei,Merck Biosciences) and 10 picomole (Forward & Reverse) primes. The primers were selected to amplify the regions of DNA that contain polymorphic sites of interest as shown in the table 1. After performing PCR, the PCR products were analyzed by agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer. The agarose gels were stained with ethidium bromide and visualized under UV Transilluminator and photographed in gel documentation system (Bio-Rad Laboratories). After confirmation of DNA amplification, each PCR product was digested with an appropriate restriction enzyme as shown in table-1. 10µl of the PCR products were digested at 37°C overnight with specific restriction enzymes in 20 µl reaction mixtures containing buffer supplied with each restriction enzyme. After overnight incubation, digestion products were then separated on a 2-3% low EEO agarose (Sigma) gel at 100 V for 30 min stained with ethidium bromide and photographed in Gel Documentation System.

Statistical analysis
The polymorphism in SOD and CAT genes and there association with the risk of developing GI cancer was studied by logistic regression model using SPSS software (Version 11.0). The association was estimated by calculating the odds ratio (OR) with 95% confidence intervals (CI) after adjustment of variables to determine the cancer risk associated with genotypes. *p* value <0.05 was considered statistically significant.

3. RESULTS:

Characteristics of the study subjects:
Demographic characteristics of the study subjects are shown in the table 2. During the study period, 200 GI cancer patients met the eligibility criteria for this study and 400 healthy disease free controls were selected to match these cases. The age (Mean±SD) in years was 59±13.33 for cases and 57.46±11.64 for controls. There were no statistically significant differences between gender (p=0.133) and age (p=0.307) of the cases and control group. While the data analysis showed significant relationships between chewing habit [p<0.001], drinking habit [p<0.001] and family history [p<0.001] with the risk of GI cancer.

Association of superoxide dismutase and catalase genotype variants with GI cancer:
The frequency distribution of SOD1 (Cu, Zn-SOD), SOD2 (Mn-SOD), SOD3 (EC-SOD) genes and catalase genes were determined in GI cases and healthy control group as shown in Table 3.
SOD1 (Cu, ZnSOD) cd251 Ex-10 rs2070424:
The genotype frequency for SOD1 (Cu, Zn-SOD) homozygous AA is 67.5% in cases and 62.8% in controls whereas in homozygous variants is 0.5% in cases and 2.8% in controls. The frequency of heterozygous AG is 32.2% in cases and 34.5% in controls.

When we compared the genotypic frequency of heterozygous A/G with homozygous A/A (OR: 0.86, CI: 0.59-1.23, p: 0.42) and the genotypic frequency of homozygous variant G/G with the homozygous A/A (OR: 0.16, CI: 0.215-1.32, p: 0.05) we did not find the association with development of the GI cancer.

SOD2 (MnSOD) C399T cd399 Ex-3 rs1141718:

The genotype frequency of SOD 2 for homozygous T/T is 74.5% in cases and 49.8% in controls whereas as the allele frequency for homozygous variant is 11.0% in cases and 17.2% in controls. Similarly the frequency of heterozygous TC is 14.5% in cases & 33.0% in controls, which is significantly low in cases as compared to controls.

The comparative study of genotypic frequency of homozygous variant C/C (OR: 0.3097, CI: 0.1727-0.5553, p <0.0001) allele and heterozygous T/C allele C/C(OR: 0.2934, 95% CI: 0.1862-0.4623, p <0.0001) with the homozygous T/T, showed the negative association of the codon 399 of exon-3 of SOD2 (rs1141718) for the development of the GI cancer. Similarly the combination of the C/T+T/T (OR: 0.0024, CI: 0.00015-0.03952, p: <0.0001) revealed the decreased risk of GI cancer.

SOD3 (EC-SOD) Ex3 rs2536512:

When we studied the genotype frequency of SOD3 homozygous GG is 14.5% in cases and 13.5% in controls, where as homozygous AA is 26.2% in cases and 34.2% in controls. Similarly the allele frequency of heterozygous GA is 59.4% in cases and 52.2% in controls, did not show the significant difference.

The genotype frequency of homozygous variant AA and heterozygous A/G with reference to homozygous GG (OR:0.6932, CI: 0.3983-1.206, p:0.1937 and OR:1.069, CI:0.6458-1.770, p: 0.7949 respectively) showed no significant risk of developing the GI cancer.

CAT1 cd 326 Ex7 rs7943316:

The genotype frequencies of AA, AT and TT of A326T genotype in codon 326 of exon7 of CAT1, were observed to be 12.5%, 72.5% & 15.0% respectively among the cases and 27.5%, 52.5% & 20% respectively in the controls which did not show any significant difference. When compared to the homozygous AA, the genotypic frequency of homozygous variant TT (OR: 1.650, CI: 0.9019-3.019, p= 0.1024) showed no association with the GI cancer risk.

CAT2 C262T promoter rs1001179:

For genotypes of 262 promoter of CAT2 gene polymorphism the frequencies of CC, CT & TT were 45.0%, 22% & 33% respectively in cases and 26.5%, 44.5% & 29.0% respectively among the controls.

The comparative analysis of heterozygous CT (OR: 0.2911, CI: 0.1887-0.4491; p<0.0001), homozygous TT (OR:0.6701, CI:0.443-1.012, p:0.0568) and combined CC/TT+TT/TT (OR:0.4407, CI:0.3086-0.6293, p<0.0001) genotypes with homozygous CC, did not revel any significant association with GI cancer risk.

4. DISCUSSION:

The host genetic factors are found to play the crucial role in increasing the risk for many cancers including GI cancer.[23] Recent studies on antioxidant enzymes state that diseases like diabetes [24-25] , age related diseases [26-27] and cancers[28-29] are connected with defects in antioxidant pathways. Among the antioxidant enzymes SOD & CAT plays a vital role in oxidant defence mechanism and can promote or suppress tumour formation in human gastric mucosa.[30] There is limited literature available on polymorphism in oxidative stress related genes and their role in cancer development. Therefore the present study was undertaken to determine the association of polymorphism in three isoforms of SOD and catalase genes with the risk of GI cancer in rural population of south western Maharashtra. In this case control study we observed that amongst three SNPs of superoxide dismutase genes, SOD1rs2070424

and SOD3rs2536512 are not the risk factor for developing a GI cancer. However, few studies from Iran have reported positive association between polymorphism in SOD1 rs2070424 gene and colorectal cancer [20], while some studies reveal the negative association of SOD1 rs2070424 and Gastric cancer.[31] In agreement to our results when considered for polymorphism in SOD3 rs2536512 and its association with gastric cancer in Iranian population did not show any susceptibility.[32] Few Indian studies have proved association of SOD1 rs2070424 & SOD3 rs2536512 with cervical cancer risk [33] but till date no Indian studies have reported the association of SOD gene family with the GI cancer risk.

In our study Manganese superoxide dismutase (SOD2 rs1141718), gene polymorphism showed decreased risk of GI cancer development. Our studies seem to be in agreement with previous studies that suggest vital role of SOD2 in protecting the cells from ROS-induced oxidative damage and is known to be tumour suppressor.[34-36] In a Polish case-control study the results have showed no association of SOD2 with Gastric cancer [37], similarly the study from Turkey reveals no association of SOD2 with prostate cancer.[16] In contrast to other reports on association of SOD2 with GI cancer in Chinese population[38], breast cancer[11,29,39-40], bladder cancer[41] our results showed polymorphism in SOD2 is associated with GI cancer. This implies that the distribution of polymorphism in SOD2 varies among different races and regions. When we assessed two SNPS of catalase genes, CAT1rs7943316 and CAT2rs1001179 showed no significant association with risk of GI cancer. Our results seem to be in agreement with the findings from Saadat *et al.* noted no association of CAT2 with gastric cancer[31] and colorectal cancer[20] the from Iranian population. Similarly findings from a German case-control study support our data proving no association of CAT gene with Colorectal Cancer.[42] However, Meta analysis by Wang in 2016 reported no positive relationship between CAT2 polymorphism risk of breast cancer, head and neck cancer, haematological malignancies, digestive system cancer and brain cancer, however there was a significant association between the CAT C262T gene and prostate cancer.[43]

5. CONCLUSION:

This study indicates that polymorphism in SOD1 (G allele of rs 2070424), SOD3 (A allele of rs2536512), CAT1 (T allele of rs7943316) and CAT2 (T allele of rs1001179) do not contribute to the advancement of gastrointestinal cancer in the rural population of Maharashtra. However, our results conferred the negative association of SOD2 (rs1141718) with the development of GI cancer. This analysis of negative correlation of SOD2 C399T and GI cancer can be confirmed using larger sample size with the including more detailed patient clinical information and detailed demographic status.

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Conflicts of interest: None declared

*Ethical Clearance***:** The study protocol was approved by Institutional Ethics Committee of KIMSDU, protocol number 164/2017-2018.

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Table 1: Details of PCR and RFLP procedure

Gene	FP/RP	PCR condition	PCR product	Restriction enzyme	Restriction product
SOD1 Cu, Zn-SOD cd251 Ex-10 rs2070424	5'-agt act gtc aac cac tag ca-3' 5'-cca gtg tgc ggc caa tga	95^0C- 5 min, 30 cycles of 95^0C- 30 sec, 58^0C- 30 sec, 72^0C- 30 sec, 72^0C- 5 min	570 bp	1 U MspI 37°C incubation 16 hours	WT: 570bp HT:570bp, 369bp,201bp VT: 369bp,201bp

	tg-3’				
SOD2 C399T cd399 Ex-3 rs1141718	5’-agc tgg tcc cat tat cta ata g-3’ 5’-tca gtg cag gct gaa gag at-3’	95^0C- 5 min, 35 cycles of 95^0C- 30 sec, 52^0C- 30 sec, 72^0C- 1 min, 72^0C- 5 min	139 bp	1 U EcoRV 37°C incubation 16 hours	WT: 117bp, 22bp HT: 139bp, 117bp, 22bp VT: 139bp
SOD3 EC-SOD Ex3 rs2536512	5’-gac atg tac gcc aag gtc ac -3’ 5’-aac tgg tgc acg tgg atg-3’	95^0C- 5 min, 35 cycles of 95^0C- 30 sec, 65^0C- 30 sec, 72^0C- 1 min, 72^0C- 5 min	245 bp	1 U BssHII 37°C incubation 16 hours	WT: 183bp, 62bp HT:245bp, 183bp,62bp VT:245bp
CAT1 cd 326 Ex7 rs7943316	5’-aat cag aag gca gtc ctc cc -3’ 5’-tcg ggg agc aca gag tgt ac -3’	95^0C- 5 min, 35 cycles of 95^0C- 30 sec, 55^0C- 30 sec, 72^0C- 30 sec , 72^0C- 5 min	250 bp	1 U HinFI 37°C incubation 16 hours	WT: 177bp, 73bp HT: 250bp,177bp,73bp VT: 250bp
CAT2 C262T promoter rs1001179	5’-aga gcc tcg ccc cgc cgg acc g-3’ 5’-taa gag ctg aga aag cat agc t-3’	95^0C- 5 min, 30 cycles of 95^0C- 20 sec, 66^0C- 30 sec, 72^0C- 30 Sec, 72^0C- 5 min	185 bp	1 U SmaI 37°C incubation 16 hours	WT: 155bp, 30bp HT:185bp VT:185bp, 155bp,30bp

Table 2. Distribution of selected demographic characteristics of gastrointestinal cancer cases and healthy controls from rural areas of south-western Maharashtra in India.

Variable		**Cases**	**Controls**	**Chi Square Value**	**p-value based on X^2**
Age (Mean±SD) yrs		**59±13.33**	**57.46±11.64**		
Age	<= 50 yrs	51(25.5%)	102(25.5%)	3.60	0.307
	51- 60 yrs	50(25%)	118(29.5%)		
	61- 70 yrs	67(33.5%)	136(34.0%)		
	>= 71 yrs	32(16.0%)	44(11.0%)		
Gender	Male	113(56.5%)	200(50.0%)	2.25	0.13
	Female	87(43.5%)	200(50.0%)		
Economic Status	Rich	2(1.0%)	114(28.5%)	131.29	<0.001
	Middle class	39(19.5%)	158(39.5%)		
	Poor	159(79.5%)	128(32.0%)		

Diet	Vegetarian	36(18%)	116(29.0%)	8.52	0.003
	Mixed	164(82%)	284(71.0%)		
Chewing Habit	Yes	134(67%)	119(29.8%)	75.86	<0.001
	No	66(33%)	281(70.2%)		
Drinking Habit	Yes	38(19%)	25(6.2%)	23.06	<0.001
	No	162(81%)	375(93.8%)		
Family History	Yes	44(22%)	0(0.0%)	94.96	<0.001
	No	156(78%)	400(100.0%)		
Education	illiterate	77(38.5%)	70(17.5%)	59.41	<0.001
	SSC	85(42.5%)	141(35.2%)		
	HSC	26(13%)	80(20.0%)		
	graduate	13(6.4%)	109(27.2%)		

Table 3. Genotype frequencies of SOD and CAT gene variants and their association with gastrointestinal cancer patients and healthy controls.

Gene	**Genotype**	**Cases (n=200)**	**Controls (n=400)**	**Chi square**	**p Value**	**Odds Ratio (95% CI)**	**p value**	**Adjusted odds ratio (95% CI)**	**p value**
SOD1 cd251 Ex-10 rs2070424	AA/AA	135(67.5%)	251(62.8%)	4.09	0.129	1 (Reference)		1 (Reference)	
	AA/GG	64(32.0%)	138(34.5%)			0.86(0.59-1.23)	0.4232	0.93 (0.63-1.37)	0.733
	GG/GG	1(0.5%)	11(2.8%)			0.16(0.21-1.32)	0.0553	0.29(0.35-2.42)	0.253
	AA/GG +GG/GG	65(11%)	149(25%)	1.311	0.2522	0.81(0.56-1.16	0.2522	0.85(0.58-1.24)	0.414
SOD2 C399T cd399 Ex-3 rs1141718	TT/TT	149(74.5%)	199(49.8%)	34.522	<0.001	1 (Reference)		1 (Reference)	
	CC/TT	29(14.5%)	132(33.0%)			0.29(0.18-0.46)	<0.0001*	0.40(0.24-0.66)	<0.0001
	CC/CC	22(11.0%)	69(17.2%)			**0. 30(0.17-0.55)**	**<0.0001***	0.62 (0.34-1.10)	0.107
	TT/CC +TT/TT	51(9%)	201(33%)	33.528	<0.0001	0.33(0.23-0.49)	<0.0001*	0.46(0.30-0.69)	<0.0001
SOD3 Ex3 rs2536512	GG/GG	29(14.5%)	54(13.5%)	4.815	0.09	1 (Reference)		1 (Reference)	
	GG/AA	120(59.4%)	209(52.2%)			1.06(0.64-1.77)	0.7949	0.90(0.52-1.56)	0.73
	AA/AA	51(25.5%)	137(34.2%)			0.69(0.39-1.20)	0.1937	0.57(0.31-1.03)	0.065
	GG/AA +AA/AA	171(29%)	346(57%)	0.1119	0.738	0.09(0.5654-1.498)	0.738	0.79(0.47-1.32)	0.379
CAT1 cd 326 Ex7 rs7943316	AA/AA	25(12.5%)	110(27.5%)	24.166	<0.001	1 (Reference)		1 (Reference)	
	AA/TT	145(72.5%)	210(52.5%)			3.03(1.87-4.92)	<0.0001	2.31(1.38-3.87)	0.001
	TT/TT	30(15.0%)	80(20.0%)			1.65(0.90-3.01)	0.1024	1.06(0.55-2.02)	0.854
	AA/TT +TT/TT	175(29%)	290(48%)	17.204	<0.0001	2.65(1.65-4.26)	<0.0001	2.001(1.21-3.29)	0.006
CAT2	CC/CC	90(45.0%	106(26.5	32.91	<0.001	1 (Reference)		1 (Reference)	

C262T promoter rs1001179		)	%)	6					
	CC/TT	44(22%)	178(44.5 %)			0.29(0.18-0.44)	<0.0001	0.40(0.24-0.64)	<0.0001
	TT/TT	66(33.0%)	116(29.0 %)			0.67(0.44-1.01)	0.0568	0.75(0.48-1.16)	0.202
	CC/TT +TT/TT	110(18%)	294(49%)	20.747	<0.0001	0.44(0.30-0.62)	<0.0001	0.57(0.39-0.83)	0.004

*indicates significant p<0.05, p value determined based on x^2