Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 18977 - 18985
Received 25 April 2021; Accepted 08 May 2021.

Role of Toll like Receptor 2 Gene Polymorphism and Expression and Interleukin 6 Gene Expression in Diagnosis of Colorectal Cancer

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Abstract
Background: The immune system lays a major role in colorectal cancer (CRC) development. Toll-like receptor 2 (TLR2), is predominantly expressed in tissues involved in immune function. We targeted to compare TLR2 receptor polymorphism and levels of TLR2 receptor expression between cancerous tissues and normal one and detect the relation between TLR2 gene polymorphism and its gene expression in cancerous tissue.**Patients and Methods:** This study is a case-Control Study that was conducted in the duration from May 2018 to March 2020 in Medical Biochemistry and General surgery Departments, Faculty of Medicine, Zagazig University. The study included 50 participants. They were classified into 2 groups; control group (25 apparently healthy control and cases group (25 colorectal cancer patients). The tissue samples were obtained as normal mucosa/cancer specimens by colonoscopy. TLR2 genes expression on tissue samples was performed by Real time PCR, using specific primers.**Results:** there was statistically significant difference (P< 0.05*) between case and control groups regarding TLR2 polymorphism with(DD&DI) had higher frequency among colorectal patients (64%) and II type more frequency in control group (80%). TLR2 expression (p<0.05*) had higher levels were detected in colorectal cancer patients 1.5 (1.05-3.5) versus 0.91 (0.71-1.32) in control group. Higher levels IL6 (p<0.001*) were detected in colorectal cancer patients 2.41 (1.31-4.27) versus 0.81 (0.38-1.51) in control group. There was statistically significant difference association between TLR2 polymorphism and TLR2 expression among colorectal patients (p<0.05) with higher level of TLR2 expression was reported in DD genotype.**Conclusion:** TLR2 polymorphism has influence on the incidence of colorectal cancer in Egypt and increase the TLR2 expression in tumor tissue. Also increased TLR2 expression in CRC patient with positive correlation is associated with downstream IL6 expression so it may be used as a marker for colorectal cancer progression.
Keywords: gene polymorphism, TLR2, colorectal cancer

Introduction

Colorectal cancer (CRC) is the second most common cancer in women after breast cancer and the third most common in men, with 1.8 million new cases in 2018 **(1)**. Although CRC is potentially a curable cancer, it remains the fourth most common overall cause of cancer-related deaths **(2**). In Egypt, incidences are within the same worldwide range and CRC represents 3.47% of male cancers and 3% of female cancers with significantly higher rates in males than in females **(3**). The continuous search for CRC risk factors revealed an interaction between genetic factors and a variety of environmental exposures. The immune system lays a major role in CRC development, and the association of innate immunity with CRC **(4**). Toll-like receptors (TLRs) play a vital role in immune responses, being involved in the regulation of inflammatory reactions and activation of the adaptive immune response to eliminate infectious pathogens and cancer debris **(5**). One of the most studied of these innate immunity receptors is Toll-like receptor 2 (TLR2), which is considered as a member of the family of pattern recognition receptors. It is predominantly expressed in tissues involved in immune

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function and plays substantial roles in mediating innate immunity and inflammatory response (**6**). Upon binding with the bacterial lipopolysaccharide (LPS), it induces the production of effector molecules as part of the immune response including pro-inflammatory cytokines and Chemokines (**7).**Interleukin-6 (IL-6) is a cytokine with multifaceted effects playing a remarkable role in the initiation of the immune response. The increased level of this cytokine in the elderly seems to be associated with the chronic inflammatory setting of the microenvironment in aged individuals. IL-6 also represents one of the main signals in communication between cancer cells and their non-malignant neighbors within the tumor niche (**8).**

Subjects and Methods

The study was done in Medical Biochemistry and General surgery Departments, Faculty of Medicine, Zagazig University. This study is a case-Control Study that was conducted in the duration from May 2018 to March 2020. The study included 50 participants. They were classified into 2 groups; control group (25 apparently healthy control and cases group (25 colorectal cancer patients).The tissue samples were obtained as normal mucosa/cancer specimens by colonoscopy.

TLR2 genes expression on tissue samples was performed by Real time PCR, using specific primers.

Blood samples were analyzed prior to endoscopy for

IL6 expression: Real-time quantitative reverse transcription PCR (qRT-PCR) was performed in order to quantities the mRNA levels. PCR was performed in SYBR Green PCR Master Mix (PE Applied Biosystems) containing each of forward and reverse primers, and 30 ngcDNA.The TLR2-196 to -174del polymorphism was investigated using allele-specific polymerase chain reaction (PCR).

Detection of TLR2-196 to -174del andTLR4+896A/G polymorphism gene

All the reagents were highly purified analytical PCR-materials. All the tubes, tips pipettes used for DNA extraction were DNAse, RNAse free tubes to avoid contamination purchased from Gentra (Minneapolis, USA).

DNA Extraction

DNA was isolated using the Genomic blood DNA extraction Mini Kit purchased from INTRONBIO.

DNA Quantitation

For determination of DNA concentration and evaluation of DNA purity, 20 µl of each extracted DNA sample was added to 1 ml of deionized water in quartz cuvette and absorbance was measured at 260 and 280 nm wavelengths using Milton royspectronic 3000 array. DNA has am maximum absorbance at 260 nm as the resonance structures of Pyrimidine and purines bases are responsible for the absorbance. An absorbance of 1.0 at 260 nm gives DNA concentration 50 µl /ml. proteins absorb maximally at 280 nm due to the presence of tyrosine, phenylalanine and tryptophan and absorption at this wavelength is used for detection of protein in DNA samples. This is done by determination of the a260/ a280 ratio. This ratio for pure double –stranded DNA was customarily taken to the between 1.8 and 1.9 **(9).**Detection of TLR2-196 to -174del gene polymorphism was done using allele-specific polymerase chain reaction (PCR)

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PCR for *TLR2* -196 to -174 *del* was as follows: Initial denaturation step at 95 °C for 5 min, Amplification was carried out by 35 cycles at 95 °C for 30 s, at 60 °C for 40 s, and at 72 °C for 40 s and followed by a final elongation cycle at 72 °C for 7 min.
Detection of TLR2-196 to -174del gene polymorphism in the Digested samples was done by Gel Electrophoresis

Statistical analysis
All data were collected, tabulated and statistically analyzed using SPSS 20.0 for windows (SPSS Inc., Chicago, IL, USA). Quantitative data were expressed as the mean ± SD & median (range), and qualitative data were expressed as absolute frequencies (number) & relative frequencies (percentage). Independent samples Student's t-test was used to compare between two groups of normally distributed variables while Mann Whitney U test was used for non-normally distributed variables. Kruskal Wallis test was used to compare between more than two groups of non- normally distributed variables. Percent of categorical variables were compared using Chi-square test or Fisher's exact test when appropriate. Spearman's rank correlation coefficient was calculated to assess relationship between various study variables, (+) sign indicate direct correlation & (-) sign indicate inverse correlation, also values near to 1 indicate strong correlation & values near 0 indicate weak correlation. All tests were two sided. P-value< 0.05 was considered statistically significant (S), p-value ≥ 0.05 was considered statistically insignificant (NS).

Results
The study included 50 participants; control group (25 apparently healthy control and cases group (25 colorectal cancer patients). Both groups were matched as there was no statistically significant difference (P≥ 0.05) between them regarding age and sex, ensuring homogeneity of both groups.

Table (1): Demographic characteristics of the studied groups (n=50)

Variables	Case group(n=25)	Control group(n=25)	Test	P value
Age (years) Mean± SD (minimum-maximum)	63.52±14.44 (35-88)	60.6±13.56 (36-88)	[a]0.737	0.465
Sex: No (%)				
● Male	18 (72%)	13 (52%)	[b]2.12	0.145
● Female	7 (28%)	12 (48%)		

[a] Independent t-Test [b] Chi square test (X^2)

Table (2): TLR2 polymorphism among the studied groups (n=50)

Variables	Case group (n=25)		Control group (n=25)		Test	P value	P between types
	No	%	No	%			
TLR2 polymorphism:					[b]	0.006	Ref
● II (n=29)	9	36.0	20	80.0	10.1	*	P1= 0.008*
● DI	11	44.0	4	16.0			P2=0.017*
	5	20.0	1	4.0			

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• (n=15) • DD (n=6)							

[b] Chi square test (X^2) p1 between II and DI p2 between II and DD

Table (2) shows that there was statistically significant difference (P< 0.05*) between case and control groups regarding TLR2 polymorphism with (DD &DI)higher frequency among colorectal patients with about 64% and II type more frequency in control group with 80%.

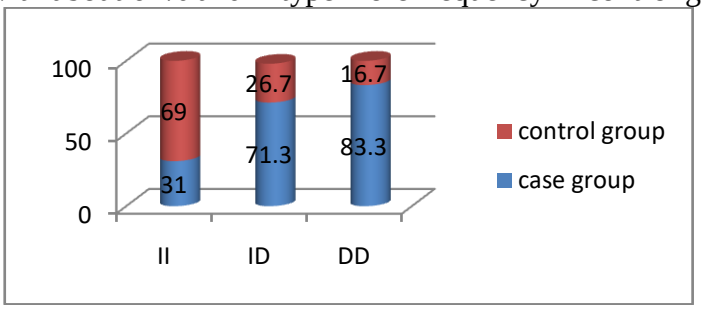

Figure (1): Distribution of TLR2-196 to -174del gene polymorphism among the studied groups

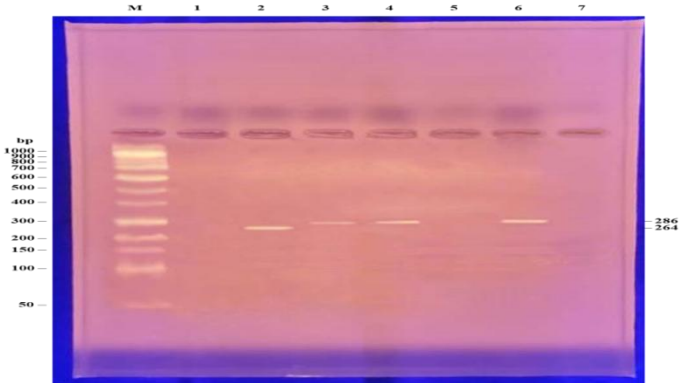

Figure (2): Agarose gel electrophoresis picture with ethidium bromide showing the PCR product in which there is analysis of TLR2-196 to -174del gene polymorphism in the control group Lane M: DNA size marker (50-bp) ladder, Lane (2,3) shows heterozygous type ID with one band at 264 bp and one band at 286, Lane (4,5,6,7) shows wild type II with one band at 286.

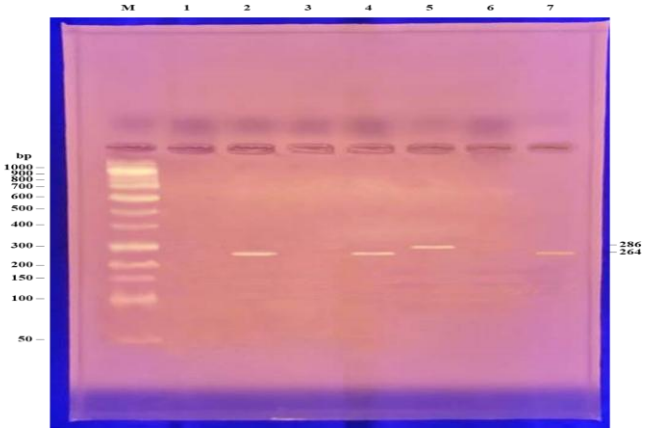

Figure (3): Agarose gel electrophoresis picture with ethidium bromide showing the PCR product in which there is analysis of TLR2-196 to -174del gene polymorphism in the case

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group Lane M: DNA size marker(50-bp) ladder, Lane (2,3) represents homozygous type DD as single band at 264, Lane (4,5) represents heterozygous type with one band at 264 and the other at 286, Lane (6,7) show polymorphic type with one band at 264.

Table (3): Comparing TLR2 expression between the studied groups (n=50)

Variables	Case group (n=25)	Control group (n=25)	Test	P value
TLR2 Mean± SD Median (IQR)	 2.3±1.75 1.5(1.05-3.5)	 1.03±0.51 0.91(0.71-1.32)	[c]-2.73	0.006*

[c]Mannwhitney test

As shown in table 3 and fig (5), there was statistically significant difference between the studied groups regarding TLR2 expression ($p<0.05$*) with higher levels were detected in colorectal cancer patients 1.5 (1.05-3.5) versus 0.91 (0.71-1.32) in control group.

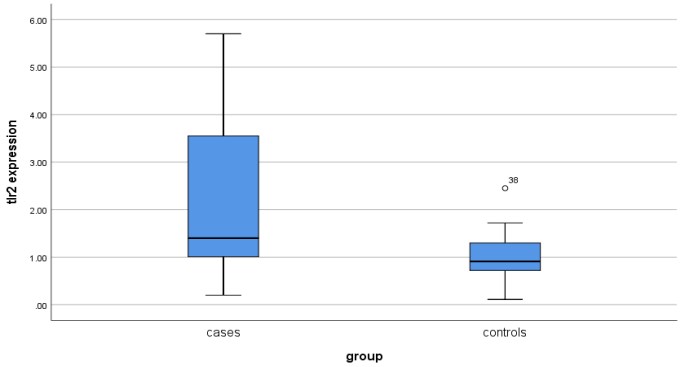

Figure (4): Box blot illustrating TLR2 expression in the studied groups

Table (4): Comparing Il6 between the studied groups (n=50)

Variables	Case group (n=25)	Control group (n=25)	Test	P value
IL6 Mean± SD Median (IQR)	 2.89±1.76 2.41(1.31-4.27)	 0.8±0.5 0.81(0.38-1.51)	[c]-5.36	<0.001*

[c]Mannwhitney test

As shown in table 4 and fig (6), there was highly statistically significant difference between the studied groups regarding IL6 ($p<0.001$*) with higher levels were detected in colorectal cancer patients 2.41 (1.31-4.27) versus 0.81 (0.38-1.51) in control group.

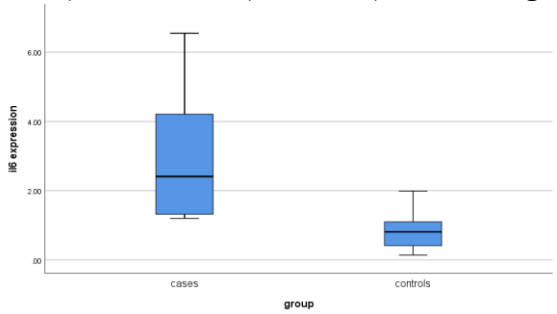

Figure (5): Box blot illustrating IL6 levels in the studied groups

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Table (5): TLR2 polymorphism effect on TLR2 expression among colorectal patients (n=25)

Variables	TLR2 expression		Test	P value
	Median	IQR		
TLR2 polymorphism: DD (n=5) ID (n=11) II (n=9)	2.6 1.6 1.2	2.5-4.5 1.2-3.5 0.5-1.4	[d] 7.57	0.023 *

[d]Kruskal Wallis ANOVA p<0.05 (statistically significant)

This table shows that there was statistically significant difference association between TLR2 polymorphism and TLR2 expression among colorectal patients (p<0.05) with higher level of TLR2 expression was reported in DD genotype.

Table (6): Correlation between TLR2 and IL6 in colorectal cancer patients

Variables	Il6 level	
	R	P
TLR2 expression	0.552	0.004*

Table 6 and figures 7 show that there was statistically significant positive correlation between TLR2, expression and IL6 with (r was 0.552).

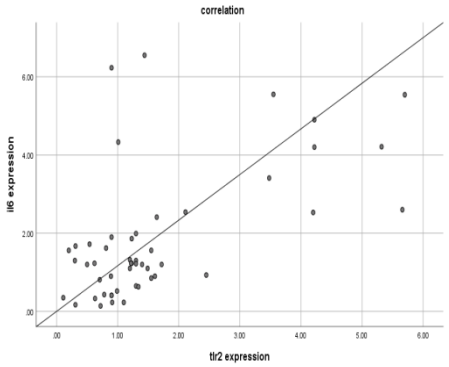

Figure (6): correlation between IL6 levels and TLR2 expression in colorectal cancer patients

Discussion

Colorectal cancer survival in developed countries has increased given improved screening and treatment, although 17% of stage II and 36% of stage III tumors still recur within 5 years. Identifying patients that need thorough follow-up and adjuvant therapy remains important, whereas other patients could be spared unnecessary treatment and possibly follow-up (10). Toll-like receptors (TLR) represent a large family of pattern recognition receptors expressed on immune cells and epithelial cells and play a crucial role in the innate immune response since they detect pathogens and induce pro-inflammatory immune responses (11). Several studies showed that TLRs play a role in cancer pathogenesis. TLRs express on tumor cells, while dying tumor cells release endogenous TLR ligands, thus activating TLR signaling and promoting tumorigenesis (12). Interleukin-6 (IL6) is proposed to play a key role in chronic inflammation and carcinogenesis. (13) From this point of view our study was carried out to investigate the influence of the toll-like receptor TLR2 polymorphisms on their gene expression in colorectal cancer samples.Regarding age and sex our study reveals that there was no statistically significant difference (P≥ 0.05) between case and control groups, ensuring homogeneity of both groups. This result goes hand with hand with Proença et al. that found that gender was not significantly different between the groups but in disagree with our result

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they found that statistically significant differences were found between the group aged younger than 60 years and the group greater than or equal to 60 years(14). However Oslen et al., showed that the median age of cancer colon was 68 years and there was a slight predominance of male patients (15). In agree with our study Semlali et al., showed that the male to female ratio was not significantly different between cases and controls also the mean ages did not differ considerably (16). Metwally et al., demonstrate that In Egypt, incidences are within the same worldwide range and CRC represents 3.47% of male cancers and 3% of female cancers with significantly higher rates in males than in females (3). Regarding TLR2 polymorphism our study shows that there was statistically significant difference (P< 0.05*) between case and control groups with higher frequency of DD genotype among colorectal patients with 83.3% and more II genotype among control group. This finding goes hand with hand with Proença et al., which found that TLR2-196 to -174del was associated with increased CRC risk (14). Also Juliana et al., illustrate that For TLR2 -196 to -174 del, the genotype (ins/del and del/del) and allele (del) frequencies were increased statistically (P < 0.01) in gastric cancer group (33.4% and 19% respectively) than in control group (16.0% and 8.5% respectively) (17). However, and in opposite side Semlali et al., showed that All genotype and allele frequencies for the three SNPs of TLR2were similar between normal and colon cancer patients (16). Li et al., (2014) demonstrate that both TLR2 and TLR4 in cancer patients are implicated in carcinogenesis and antitumor treatment (18). Regarding TLR2 expression our study revealed that there was statistically significant difference between the studied groups regarding TLR2 expression (p<0.05*) with higher levels were detected in colorectal cancer patients 1.5(1.05-3.5) versus 0.91(0.71-1.32) in control group.In agreement with our result Proença et al. observed significantly increased TLR2relative gene expression in tumor tissue (RQ = 2.36) compared to adjacent normal tissue (14). In another study Nihon-Yanagi et al. found a higher TLR2 expression in cancer samples at each stage compared with that in the normal mucosa. Furthermore, TLR2 was more intensively expressed in stage II and III tumors (19). Meng et al. also found that high levels of TLR2 expression in tumor tissues of colorectal cancer (CRC) patients have been reported (20). Our study revealed that there was statistically significant difference relation between TLR2 polymorphism and TLR2 expression among colorectal patients (p<0.05) with higher level of TLR2 expression was reported in DD genotype.This result go hand with hand with Proença et al. who found that Individuals with at least one polymorphic allele TLR2-196 to -174del had more than two times higher TLR2 expression in tumor tissue (RQ = 2.19; P = 0.03) compared to those with the wild genotype (14). Regarding IL6 our result revealed that, there was highly statistically significant difference between the studied groups (p<0.001*) with higher levels were detected in colorectal cancer patients 2.41 (1.31-4.27) versus 0.81 (0.38-1.51) in control group.Chang et al., reported that high levels of IL-6 and IL-8 secretion in CRC patients also correlated with higher risks of recurrence (21). Knupfer and Preiss found that IL6 promote mitosis, proliferation, metastasis, migration, and angiogenesis and make a microenvironment which is good for the metastasis (22). Also Olsen et al., found that IL6mRNA was significantly more highly expressed in tumor compared to normal adjacent tissues (paired data) (p<0.001), mean difference in log10 ratio: 0.52 (15). Our study found positive correlation between TLR2 expression and IL6 with (r was 0.552) respectively.Our finding go hand with hand with Chang et al., which found higher levels of expression of TLR1, TLR2, TLR4 and TLR8 mRNA were related to the up regulation of inflammatory cytokines IL-6 and IL-8gene expression in the tissue and the up regulation of IL-6 in the blood (21).

Conclusion

We concluded from our study that TLR2 polymorphism has influence on the incidence of colorectal cancer in Egypt and increase the TLR2 expression in tumor tissue. We found also

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increase TLR2 expression in CRC patient with positive correlation with their downstream IL6 expression so it may be used as a marker for colorectal cancer progression.

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