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Research Article

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Involvement of *TLR4* Polymorphisms on Colorectal Cancer Treatment

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Abstract

The important role of polymorphisms on immunity genes in the susceptibility to various diseases has been widely described. Both polymorphisms *D299D* and *T399I* of *TLR4* are shown associated with inflammatory bowel diseases as well as colorectal cancer. Previously, we have shown that *TLR4* polymorphisms are significantly associated with disease presentation of colorectal cancer such as late stage, differentiation as well as lymph, node and metastasis. Our study aimed to investigate an association between *TLR4 D299G* and *T399I* polymorphisms in Tunisian patients with colorectal cancer treatment. We found that *T399I* and *D299G* polymorphism of *TLR4* were significantly associated with adjuvant chemotherapy and radical surgery. We also showed that mutant alleles of *T399I* and *D299G* combined genotypes and haplotypes may affect the effectiveness of therapy. Finally, we showed no significant longer survival and *TLR4* polymorphisms. In conclusion, we suggest that polymorphisms in *TLR4* gene may be predictive of treatment type.

Keywords

Colorectal cancer; Treatment; Chemotherapy; Surgery; Toll like receptor 4; Polymorphisms

Abbreviations

TLR4: Toll like Receptor 4; LPS: Lipopolysaccharide; CRC: Colorectal Cancer; CD: Crohn's Disease; UC: ulcerative Colitis; MyD88: Myeloid Differentiation Factor 88; 5FU: 5-Fluorouracil; NF-κB: Nuclear Factor κB; TRIF: TIR Domain-containing Adaptor Protein inducing Interferon-β; PI3K: Phosphatidylinositide 3-Kinases; MAPK: Mitogen-activated Protein Kinases

Introduction

Transmembrane receptors *TLR4* (Toll-like receptors) plays an important role in the innate immune response that allows the host defense against infectious diseases of bacterial origin. Two missense mutations *Asp299Gly* (*D299G*) and *Thr399IIe* (*T399I*) were identified in the *TLR4* gene, which result in an amino acid substitution in the third exon of the gene. These two mutations disrupt the extracellular region of the receptor at the site of binding with the ligand. Indeed, it has been shown that these two variants are associated with a reduced sensitivity to lipopolysccharides (LPS) and an attenuated immune response causing a decrease in the secretion of proinflammatory cytokines [1].

In fact, both *D299G* and *T399I* polymorphisms are shown associated with inflammatory bowel disease (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC) [2] and increase the risk of some cancers such as breast cancer, gastric cancer and colorectal cancer [3-5]. However, in our previous study, we have shown no significant association between *D299G* and *T399I* polymorphisms and the incidence of colorectal cancer in the Tunisian population [6].

In addition to the role of *TLR4* polymorphisms in colorectal cancer, these mutations are also involved in the presentation of this disease. In fact, *D299G* and *T399I* are significantly associated with clinical features such as differentiation and architecture of the tumor and with advanced stage of the colorectal cancer. Moreover, it has been shown that these polymorphisms present a risk factor to lymph node and metastasis in patients with colorectal cancer [6-8].

Colorectal cancer is considered as the major cause of mortality and morbidity in the world. In Tunisia, colorectal cancer is the first digestive cancer with nearly 40% of cases occurring after 60 years with a male predominance. It occupies the fourth rank in males after lung, bladder and prostate cancers, and the second in women after breast cancer. Surgery is the main treatment for colorectal cancer. The procedure involves the resection of the affected segment of colon with healthy margins colon. Moreover, in case of delay in diagnosis, colorectal cancer is locally advanced and total resection becomes impossible and chemotherapy is necessary [9]. The modalities of surgery and adjuvant therapies are based on tumor extension and the possible existence of a revealing complication. Furthermore, it has been shown that overexpression of *TLR4* in the tumor microenvironment can serve as a biomarker of disease progression as well as the target of therapy. In fact, induction of *TLR4* signaling by LPS could improve the therapeutic results in patients with colorectal cancer [10].

Therefore, the purpose of this study was to investigate the possible interaction of *D299G* and *T399I* polymorphisms of *TLR4* with treatment of colorectal cancer in Tunisian population.

Material and Methods

Subjects

A group of patients/control is collected from the Salah Azaiez hospital and Charles Nicolle hospital of Tunis (Tunisia). Patients concerns 100 unrelated sporadic CRC cases (45 women, 55 men, with age range 58 ± 14) with no family cancer histories: they were classified on the bases of their histopathological profiles. Consent for the genetic study was obtained from volunteers.

DNA extraction and genotyping of polymorphisms

Genomic DNA was extracted from peripheral blood leukocytes using conventional proteinase K digestion and phenol/chloroform extraction method. A NanoDrop (ND-1000) is used to quantify DNA. *TLR4* variants *D299G* and *T399I* in patients and controls subjects were genotyped using specific primers for each polymorphism. PCR products were then analyzed using a primer extension method (SNaPshot) [6] .

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Statistical analysis

The data were analyzed using SPSS software (version 11.5.). Significance of the association was determined by Pearson's chi-squared test χ^2 , Fisher's exact test and Anova test. A value of p<0,05 was considered significant.

Results

In our study we evaluate the association between *TLR4 D299G* and *T399I* polymorphisms and treatments of CRC patients which were stratified according to surgery, neoadjuvant chemotherapy, adjuvant chemotherapy and preoperative radiotherapy.

We found that the majority of patients were submitted to radical surgery. All patients with *TLR4 T399I* polymorphism were operated radically, similarly to those with *D299G* polymorphism except one patient who submitted to palliative surgery (Table 1). In fact, we showed a significant association between *D299G* polymorphism of *TLR4* and surgery (p= 0,037; RR 6,49 (0,8-53,52)) unlike *T399I* polymorphism which shows no significant association with this type of treatment (p>0,05). In addition, we observed that all patients with *T399I* polymorphism and eleven from thirteen patients with *D299G* polymorphism had chemotherapy. Therefore, we showed a significant association between *D299G* and *T399I* polymorphisms and adjuvant chemotherapy treatment (p= 0,04; RR 4,26 (0,8-20,4) and p=0,042; RR1,11 (1,02-1,2)) respectively (Tables 1 and 2).

Indeed, we showed that *TLR4* polymorphisms could be a risk factor for surgery and neoadjuvant chemotherapy resulting in inefficiency of treatment.

However, we did not find a significant association between *D299G* and *T399I* polymorphisms of *TLR4* gene and neoadjuvant chemotherapy and preoperative radiotherapy (p>0,05) (Tables 1 and 2).

We next examined the additive effect these two variants of *TLR4* gene on CRC risk. The combined frequency of genotypes harbouring *D299G* and *T399I* polymorphisms was significantly associated with adjuvant chemotherapy (p= 0,035) (Table 3). Then, we analyzed the distributions of common *TLR4* haplotypes and their effects on CRC treatments. Similarly, CG and TG haplotypes showed a significant association with CRC treatments, especially with radical surgery (p= 0,038) and adjuvant chemotherapy (p= 0,029) (Table 4). Finally, we examined a possible association between *TLR4* polymorphisms and overall survival (OS). However, we showed no significant association between *TLR4 D299G* and *T399I* polymorphisms and survival of patients with or without treatment (Data not show).

Discussion

Colorectal cancer is one of the major cancer types for which new immune-based cancer treatments are currently in development. The most common treatment for colorectal cancer is surgery. Radiation therapy, which may be prescribed in rectal cancer, is more often associated with concomitant chemotherapy preoperatively.

Table 1: Association between *TLR4 T399I* polymorphism and CRC treatments.

	T399I TLR4		
Genotypes	**CC**	**CT**	**p value**
Surgery			
Palliative and non-palliative surgery n=32	32	0	0.092
radical surgery n=68	62	6	
Neoadjuvant chemotherapy			
No n=75	71	4	0.46
Yes n=25	23	2	
Adjuvant chemotherapy			
No n=40	40	0	**0.042** RR 1.11 (1.02-1.2)
Yes n=60	54	6	
Preoperative radiotherapy (dose in Gy)			
No n=75	72	3	0.16
Yes n=25	22	3	

Table 2: Association between *TLR4 D299G* polymorphism and CRC treatments.

	D299G TLR4		
	AA	**AG**	**p value**
Surgery			
Palliative and non-palliative surgery n=32	32	1	**0.037** RR 6.49 (0.8-53.52)
radical surgery n=68	56	12	
Neoadjuvant chemotherapy			
No n=75	65	10	0.58
Yes n=25	22	3	
Adjuvant chemotherapy			
No n=40	38	2	**0.04** RR 4.26 (0.8-20.4)
Yes n=60	49	11	
Preoperative radiotherapy (dose in Gy)			
No n=75	66	9	0.41
Yes n=25	21	4	

Table 3: Association between combined genotype of *D299G* and *T399I* polymorphisms and CRC treatments.

Combined genotypes (*D299G/T399I*)	**Surgery**		**p-value**
	palliative and non-palliative surgery	**radical surgery**	
CCAA	31	57	0,056
CCAG	1	5	
CTGG	0	6	
Combined genotype (*D299G/T399I*)	**Neoadjuvant chemotherapy**		**p-value**
	NO	**YES**	
CCAA	66	22	0,7
CCAG	5	1	
CTGG	4	2	
Combined genotype (*D299G/T399I*)	**Adjuvant chemotherapy**		**p-value**
	NO	**YES**	
CCAA	38	50	**0,035**
CCAG	2	4	
CTGG	0	6	
Combined genotype (*D299G/T399I*)	**Preoperative radiotherapy (dose in Gy)**		**p-value**
	NO	**YES**	
CCAA	67	21	0,31
CCAG	5	1	
CTGG	3	3	

Table 4: Association between *TLR4 D299G* and *T399I* haplotypes and CRC treatments.

Haplotypes	**Surgery**		**p-value**
	palliative and non-palliative surgery	**radical surgery**	
CA	31	56	**0,038**
CG	1	6	
TG	0	6	
Haplotypes	**neoadjuvant chemotherapy**		**p-value**
	NO	**YES**	
CA	65	22	0,7
CG	6	1	
TG	4	2	
Haplotypes	**Adjuvant chemotherapy**		**p-value**
	NO	**YES**	
CA	38	49	**0,029**
CG	2	5	
TG	0	6	
Haplotypes	**Preoperative radiotherapy (dose in Gy)**		**p-value**
	NO	**YES**	
CA	66	21	0,3
CG	6	1	
TG	3	3	

Chemotherapy can be prescribed as a preventive measure to prevent metastasis when the tumor grows in depth. Indeed, chemotherapy drugs are used in the treatment of several cancers because of their ability to block the uncontrolled growth of cancer cells. A recent study shows that the involvement of the immune system plays a crucial role in the effectiveness of these drugs [11].

The immune system is a biological system whose main role is to protect organism against external aggressions. These aggressions may be in the range of microbes such as viruses, bacteria, fungi, parasites or order of cancer cells. To adapt and improve treatment by immunotherapy in human cancers, it is necessary to understand the role of immunity genes polymorphisms such as Toll-like receptors *TLR4*. In fact, the *TLR4* gene is located on chromosome 9 in the region (*9q33.1*). The existence of a mutation in this gene leads to changes in the responses against pathogens. There are two major polymorphisms: *Asp299Gly* (*D299G*; *rs4986790*) and *Thr399IIe* (*T399I*; *rs4986791*) that affect the extracellular domain of *TLR4* protein thus leading to a decreased ability to detect bacterial components [12]. *TLR4* signaling is strongly involved in inflammatory processes [13-17]. Previous studies have revealed that inflammation induced TLR are involved in carcinogenesis [3,18,19]. Additionally, we lately showed that *TLR4* polymorphisms (*D299G* and *T399I*) were associated with a severe form of colorectal cancer, particularly with advanced stage, lymph nodes and metastasis [6-8,20].

Here, we demonstrated the association between *TLR4* polymorphisms and colorectal cancer treatments. We showed that

TLR4 D299G and *T399I* variants are associated with a poor prognosis for treatment according to surgery and adjuvant chemotherapy (p= 0,04; RR 4,26 (0,8-20,4) and p= 0,042) respectively. Moreover, by studying haplotypes of *TLR4* variants, we found that haplotypes with mutated alleles are also associated with radical surgery and chemotherapy ((p= 0,038 and p= 0,029) respectively). However, the combined genotypes of these two variants of *TLR4* are significantly associated only with the adjuvant chemotherapy (p= 0,035). This poor response to treatment in patients with the mutated alleles of *D299G* and *T399I* could be explained by the disruption of the *TLR4* signaling pathway. Indeed, Davoodi et al. [21] showed that 5-Fluorouracil (5-FU) increased *TLR4* expression to induced apoptosis in colorectal cancer cells in the presence and absence of LPS. They also found that wild type *TLR4* expressing cells are more sensitive to 5-FU treatment compared to cells expressing *TLR4* variants (*D299G* and *T399I*). On the other hand, Apetoh et al. [22,23] showed that *TLR4* polymorphism predicts early relapse after chemotherapy in breast cancer patients. Bergmann et al. [24] demonstrated that head and neck cancer patients with *TLR4* wild-type genotype showed significantly longer disease-free survival.

Microbial products have been utilized as adjuvants to stimulate TLR signaling and activating immune responses to enhance tumor immunotherapy. Okamoto et al. [25] also showed an antitumor activity of *TLR4*/IFN-γ signaling using streptococcal agent OK-432. However, *TLR4*, the receptor for lipopolysaccharide, activate both MyD88-dependent and TRIF dependent or MyD88-independent pathways. MyD88 is an adaptor protein for *TLR4* signaling implicated on NF-kB, MAPK and PI3K pathways activation driving tumor survival and paclitaxel chemoresistance in epithelial ovarian carcinoma cells [26-29]. Rajput et al. [30] showed that the inhibition of *TLR4* may enhance the response to chemotherapy against breast cancer based on Paclitaxel, a known *TLR4* ligand. In fact, they showed that the paclitaxel kill not only tumor cells but also improves their survival by activating *TLR4*/MyD88-dependent pathway. On the other hand, Huang et al. [31] demonstrated that atractylenolide-I, *TLR4*-antagonizing agent, sensitizes epithelial ovarian carcinoma cells to paclitaxel by blocking *TLR4*/MyD88-dependent pathway.

Finally, activation of *TLR4* signaling is needed for host protection against pathogens. TLR ligands can be employed as immunological adjuvants in tumors effective immunotherapy. Nevertheless, TLR activation may be considered as a two-edged sword. In fact, *TLR4* plays an ambivalent role with both antitumor and pro-tumor effect.

We observed no significant association between *TLR4 D299G* and *T399I* polymorphisms and survival of patients with or without treatment. Bergmann et al. [32] showed a significant association between *TLR4 D299G* polymorphisms and recurrence of disease as well as overall survival in patients with head and neck squamous cell carcinomas. They also showed a significant association between longer DFS and patients with *D299G* wild-type genotype and under adjuvant systemic therapy. However, no evidence for significant survival differences between *TLR4* genotypes in patients without adjuvant systemic therapy.

In conclusion, we showed a significant association between TL4 polymorphisms (*D299G* and *T399I*) and colorectal cancer treatment, particularly with surgery and chemotherapy. We suggest that mutant alleles of *TLR4* polymorphisms might be predictive of the choice of nature therapy. Further investigations and an advanced exploration of the relationship between *TLR4* and tumor micro environment are now needed to clarify the mechanisms of tumor progression and metastasis and to develop more effective therapeutic approaches and new therapeutic targets in cancer therapy.

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