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Haplotyping of PD-1 Polymorphisms in Egyptian Patients with Colorectal Cancer: A Case-Control Study

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- PD-1
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Abstract

Background: As being the third-most common cancer type in males and the second-most common cancer type in females worldwide, the incidence of colorectal carcinoma is increasing and associated with poor prognosis. Programmed cell death protein 1 (PD-1) gene encodes an inhibitory cell surface receptor that is thought to be implicated in influencing the cancer cells' evasion of the host immune system. The current work was conducted to evaluate the association of PD-1 single nucleotide polymorphisms, PD-1.3G/A (rs11568821) and PD-1.5 C/T (rs2227981) haplotypes with both the susceptibility and the onset of colorectal cancer in the Egyptian population. **Methods and Results:** The PD-1.3 G/A and, PD-1.5 C/T polymorphisms were investigated in 100 colorectal cancer patients and 100 healthy controls by allelic discrimination technique through TaqMan real-time polymerase chain reaction. The lowest overall risk of colorectal cancer was associated with the haplotype G/T while the A/T haplotype showed the highest risk among the four being insignificant. However, the A/T haplotype was significantly associated with an increased risk of the early onset disease by 4.4 times as compared to the reference haplotype G/C. Moreover, the PD1.5C/T SNP was in a significant linkage disequilibrium with PD1.3G/A SNP (D = 0.0255, D'= 0.151, r= 0.1298 and *p*= 0.0094). **Conclusion:** Among the Egyptian population, the G/T haplotype was considered protective acquiring the least risk for colorectal cancer, while the mutant A/T haplotype was not only associated with a higher risk of the disease but also with a significantly higher risk to exhibit early onset colorectal cancer.

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INTRODUCTION

In 2020, more than 1.9 million new colorectal cancer (CRC) cases and 935,000 deaths were estimated to represent about one in 10 cancer cases and deaths. *[1]* Furthermore, CRC is the third-most common cancer type in males and the second-most common cancer type in females worldwide. *[2]* Major risk factors for CRC include changes in lifestyle factors and diet, e.g., shifts toward an increased intake of animal-source foods and a more sedentary lifestyle, leading to decreased physical activity and increased prevalence of excess body weight due to lifestyle factors related to economic growth and industrialization. *[3]*

Meanwhile, CRC a relatively higher incidence in economically developed countries, is associated with a higher life expectancy, education, and human developmental index (HDI) due to better diagnosis of CRC. [4]

Patient survival is extremely dependent on the tumor stage at the time of diagnosis. At an early stage, just 40% of CRC cases are diagnosed and nearly 50% of presently diagnosed patients will develop into metastatic cancer. [5]

Metastatic CRC remains the fourth most common cause of death from the disease. [6] Despite the recent progress in diagnosis and treatment, including the introduction of targeted therapies, the prognosis of these advanced CRC cases remains poor. [7] Improvement in molecular biology has helped clarify some of the genetic mechanisms related to colorectal carcinogenesis. [5]

Maintaining the totality of genomic DNA is necessary for correct cell function. Genome disorders can predispose to the development of several malignancies, including CRC. The CRC can be initiated by DNA damage induced by chemical agents, smoking, alcohol consumption, and abnormal fat metabolism. [8,9]

Aggressive tumors can survive and proliferate in difficult environmental conditions. [10] The tumor releases several factors which support the formation of angiogenesis and disrupts nearby tissue architecture, this encourages invasion and metastatic spread. The role of the immune system is known to destroy cancer cells by helping innate effectors or some specific defensive cells or to allow for the presentation of tumor antigens to T cells. [11]

Immune organization of aggressive cancerous growth is often raised by tumor expression of ligands that hamper effector immune responses throughout the stimulation of immune checkpoints. One of such checkpoints is programmed death-1 (PD-1) that is considered to have a central role in the tumor microenvironment where the tumor-specific T cells continue to keep their antitumor functions. PD-1, expressed by activated T cells, is an inhibitory cell surface receptor involved in the regulation of T cell activity during immunity and tolerance.[12]

The programmed death-1 (*pdcd1*) gene is located on human chromosome 2 on a segment (2q33~q37) encoding a 50–55 KD trans-membranous glycoprotein. [13] Several important polymorphic loci have been identified, including 2 in the exon 5 region (7785C/T, 7625T/C,) and 1 in the intron region (7146G/A). [14]

Programmed death-1 has been suggested to be implicated in influencing the cancer cells' evasion of the host immune system after contact with its two ligands. Moreover, polymorphisms or modifications in this gene can influence its task. For instance, single nucleotide (SNP) is one of the most important factors affecting cancer

susceptibility. In this regard, the latest studies have focused on the information that PD-1 polymorphisms are linked with susceptibility to many types of cancer, for instance, gastric cancer [15], breast cancer [16], esophageal cancer [17], hepatocellular carcinoma.[18] and cervical cancer.[19]

The ethnic differences among populations in *pdcd1* gene polymorphisms remain an important research point to understand the molecular basis and pathogenesis of cancer. However, there is a limited number of studies that reported an association between *pdcd1* gene polymorphisms and CRC. [20 - 22] In addition, the available data lacks any possible associations of the gene haplotypes with the risk of CRC in the studied ethnicities. So, the current work was conducted to evaluate the association of PD-1 single nucleotide polymorphisms, PD-1.3G/A (rs11568821) and PD-1.5 C/T (rs2227981) haplotypes with both the susceptibility and the onset of colorectal cancer in the Egyptian population.

1. Subjects and Methods:

2.1. Calculation of sample size:

The sample size was calculated by OSSE (http://osse.bii.a-star.edu.sg/calculation1.php). The calculation was based on a previous study [20] that investigated the role of PD-1.3 A/G gene polymorphisms in the susceptibility and progression of CRC, with 'A' allele frequency of 61% vs. 41% in case vs. control. A sample size of 100 CRC cases and 100 control participants achieves an 81.2% power with 5% significance level. We aimed also to study the linkage disequilibrium between this SNP and PD-1.5 C/T which was found to be associated with CRC.[21] Sample size of 200 (100 cases, 100 control subjects) achieves 90% power to detect a hypothesized disease haplotype frequency of 0.2 assuming a relative risk (RR) of 2 at a disease prevalence of 0.01 and 0.05 α-level as calculated by Power for genetic association software (PGA1, version 2).

2.2. Subjects:

This case-control study was conducted with a cohort of two hundred subjects, 100 CRC patients recruited from Mansoura University Hospitals in addition to 100 age and sex-matched healthy volunteers who were enrolled as a control group. The diagnosis of CRC was confirmed via histopathological examination. The study protocol was approved by the Institutional Research Board of Faculty of Medicine, Mansoura University (irb: R.20.08.976). This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and informed written consent was obtained from all patients and controls. All biochemical investigations were performed at Medical Biochemistry Department, Mansoura Faculty of Medicine, Mansoura, Egypt.

Any subject with one or more of the following conditions was excluded from the study: autoimmune disorders, hereditary nonpolyposis colorectal cancer (HNPCC), or a previous history of any chronic diseases. CRC patients who had undergone prior chemotherapy or radiotherapy were also excluded.

The patients enrolled in the current study were CRC patients whose diagnosis and cancer status was confirmed by histopathological examination and whose complete medical records were available. All participants in this study were subjected to full history taking and general examination.

The experimental work was carried out in the Medical Biochemistry and Molecular Biology Department, Mansoura Faculty of Medicine in the period between August 2020 and March 2021.

2.3. Blood sampling and Genomic DNA extraction:

Two milliliters of venous blood samples were withdrawn by venipuncture from all subjects included in this study and delivered to 0.2 ml K_2EDTA- containing tubes and then stored at -80°C until being utilized for DNA extraction.

Genomic DNA was extracted from all blood samples using GeneJet whole blood genomic DNA purification kit; ***ThermoScientific, USA, Cat. No K0781.*** Extracted DNA samples were stored at −20 °C for further use.

2.4. *Genotyping of PD1 Single Nucleotide Polymorphisms:*

In this study, PD1.3G/A (rs11568821) and PD1.5C/T (rs2227981) SNPs of the PD1 gene were investigated. Genotyping of both gene variants was performed by allelic discrimination through TaqMan real-time polymerase chain reaction (7500 Real-Time PCR System, Applied Biosystems, USA) using predesigned TaqMan SNP Genotyping Assays. Each SNP assay was done using two specific forward and reverse primers and two TaqMan® probes differing in sequence only at the SNP site. The oligonucleotide probe was designed to hybridize with the target polymorphism. One probe was complementary to the wild-type allele and the other to the variant allele. The two probes were labeled with a fluorescent dye (either VIC or FAM). The PD1.3G/A (rs11568821) probes sequence: ACATGGGCCGGGCACCCCCGGAGAC[C/T] GCAGGTGGGCT GGGGCCCCAGATCA; the C probe was labeled with VIC for detection of the wild G allele while the T probe was labeled with FAM for detection of the variant A allele. The PD1.5C/T (rs2227981) probe sequence: GTGGCTGGGCACTCCGAGGGCCGTC[A/G] GCTGAGCCCCTGCGGGCG GGGGATG; the A probe was labeled with VIC for detection of the variant T allele while the G probe was labeled with FAM for detection of the wild C allele.

2.5. Statistical analysis of the data:

Analysis of the data was done using Statistical Package for Social Science (SPSS) version 21 and SNPStats software. The SNPs were tested for Hardy-Weinberg equilibrium then their genotypic and allelic disease association analysis was performed. Association between categorical variables was tested using Chi-square test for comparison of qualitative data. The data were presented in the form of crude odds ratio (COR), p values and 95% confidence interval (CI). Multiple SNPs analysis, including haplotypes and linkage disequilibrium, was done. Multiple inheritance models (co-dominant, dominant, recessive, over-dominant and log-additive) were tested to select the strongest result that indicates the best inheritance model. For all above mentioned statistical tests done, p values of ≤ 0.05 were considered significant.

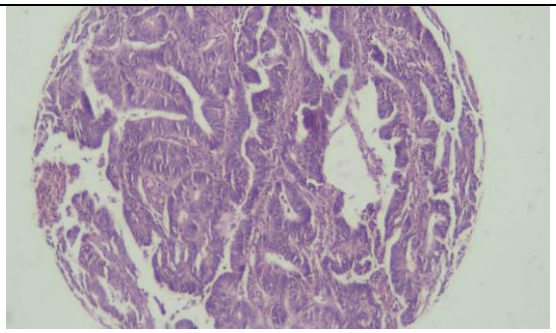
3. Results:

This study included 100 patients with CRC 57 males and 43 females with ages ranging from 30 to 73 years (mean ± SD, 55.9 ± 10.4 years) and 100 sex- and age-matched controls (54 males and 46 females) with an age range from 34 to 73 years (mean ± SD, 54.04±10.13 years). Of the 100 patients, 27 (27%) cases were presented with early onset CRC at ages < 50 years while CRC was presented in 73 (73%) cases at ages ≥ 50 years (late-onset CRC).

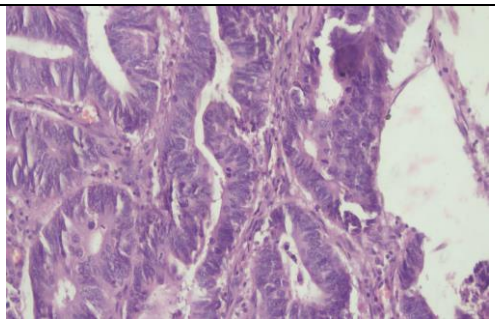
On histopathological diagnosis, most of the cases were diagnosed as adenocarcinoma (80 cases, 80%) while 16 cases (16%) were diagnosed as mucoid adenocarcinoma. In addition, 4 cases (4%) were diagnosed as pure signet ring cell

carcinoma. The adenocarcinoma showed malignant acinar structures with desmoplastic stroma and the malignant cells represented variable degrees of atypia and pleomorphism. The tumor grades were either grade 1 (28 cases, 28%) or grade 2 (72 cases, 72%) **(Figure 1; a and b).** Mucoid adenocarcinoma cases showed pools of mucin with floating malignant cells of which some had signet ring appearance. Mucin pools represented more than 50% of tumor mass **(Figures 1; c and d).** Pure signet ring cell carcinoma was formed predominantly of signet ring cells in more than 50% of tumor mass **(Figure 1; e and f).**

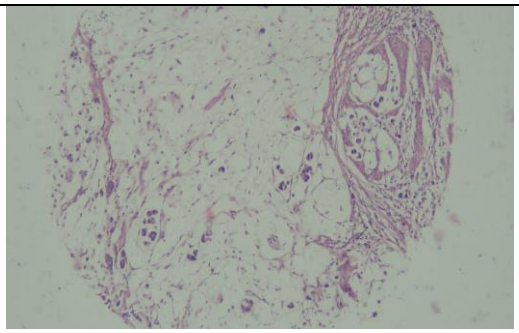
The TNM staging of CRC patients revealed 18 (18%) patients presented with stage I, 29 (29%) patients with stage IIa while stage IIb was detected only in 5 (5%) patients. As regards stages IIIa, IIIb and IIIc, they were detected in 6 (6%), 27 (27%) and, 15 (15%) patients respectively. According to the tumor location, cecal and/or ascending masses were found in 38 (38%) cases as well as 7 (7%) and 36 (36%) cases with transverse and descending colon masses respectively. The recto-sigmoid masses were detected in 5 (5%) cases while rectal masses were found in 14 (14%) cases.



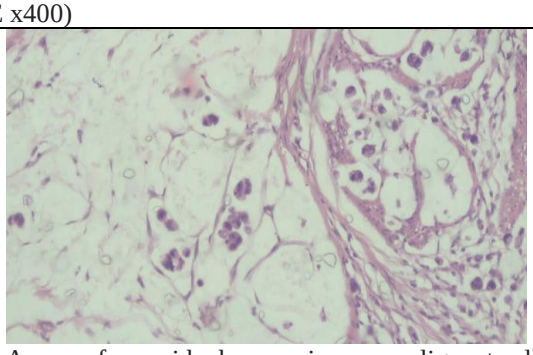
(a) A case of adenocarcinoma showing malignant acinar structures with desmoplastic stroma (Hx &E x100)



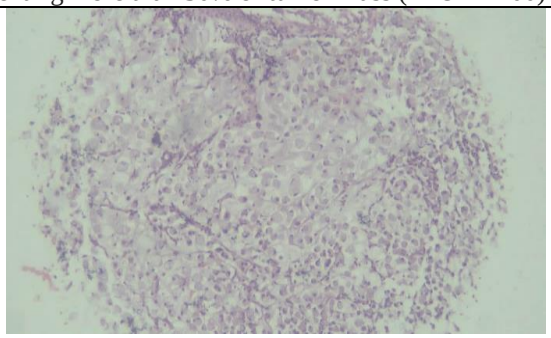
(b) A case of adenocarcinoma showing malignant cells with variable degrees of atypia and pleomorphism (Hx &E x400)



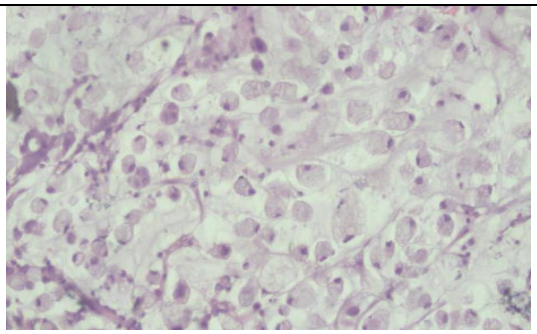
(c) A case of mucoid adenocarcinoma showing pools of mucin representing more than 50% of tumor mass (Hx&E x100)



(d) A case of mucoid adenocarcinoma, malignant cells with some have signet ring appearance. (Hx&E x200)



(e) A case of pure signet ring cell carcinoma is formed predominantly of signet ring cells in more than 50% of tumor mass (Hx&E X100)



(f) a case of pure signet ring cell carcinoma is formed predominantly of signet ring cells in more than 50% of tumor mass (Hx&E X400)

Figure 1: Histopathological diagnosis of colorectal carcinoma cases.

The PD1.3 G/A genotypes and allele distribution Exact test for Hardy–Weinberg equilibrium (HWE) shows that the control group was compatible with the HWE (P = 0.17). The genotype and allele distribution of the PD1.5 C/T polymorphism of the control group deviated from the HWE ($p < 0.0001$) as the mutant homozygous genotype TT was absent among all studied individuals.

The frequency of the heterozygous genotype AG and the mutant homozygous genotype AA as well as the A allele of the PD1.3G/A SNP was significantly higher in the CRC patients than in the controls. Those with AA genotype had a 8.5 times higher odds to exhibit CRC and the mutant A allele was considered as a risk allele with 1.7 times higher odds to exhibit CRC. However, the heterozygous genotype CT and the T allele distribution of the PD1.5C/T polymorphism was lower in the CRC patients than in the healthy controls. The CT genotype and the T allele were considered as protective variants having low odds (0.36 and 0.5 respectively) to exhibit CRC **(Table 1).**

The five genetic models; co-dominant, dominant, recessive, over-dominant, and log-additive were applied to study the association between different models of inheritance for PD1.3G/A and the risk for CRC. The codominant and the recessive models represented the best inheritance models for a statistically significant association between PD1.3G/A and CRC while the dominant model (the wild homozygous genotype GG) was associated with the lowest CRC risk **(Table 2).** The mutant homozygous genotype TT of PD1.5C/T was absent among the studied population, hence its inheritance models could not be studied.

On comparisons between early and late onset CRC, statistically significantly higher proportions of TNM earlier stages (stage I and II) were noted in both types. Regarding the association between the studied SNPs and the onset of the disease, there was a significant association between the CT genotype of PD1.5 C/T with the early onset CRC patients **(Table 3).** Binary logistic regression was run to ascertain the effects of PD1.5 CT versus the CC genotype on the likelihood that the participants would exhibit early onset CRC. This showed a statistically significant association (COR [95% CI] = 2.5 [1.01 - 6.17], p= 0.048, OR [95% CI] was 2.6 [1.04 – 6.57], p = 0.041).

A significant global haplotype difference between CRC patients and controls was observed in PD1.3G/A and PD1.5C/T SNPs. The haplotype G/T was associated significantly with the lowest overall risk of CRC. Despite the A/C and A/T haplotype were insignificantly associated with the CRC risk, the A/T haplotype showed the highest risk among the four haplotypes **(Table 4).** However, haplotyping study in relation to the onset of CRC revealed that the A/T haplotype was significantly associated with an increased risk of the early onset disease by 4.4 times as compared to the reference haplotype G/C **(Table 5).** Moreover, the PD1.5C/T SNP was in a significant linkage disequilibrium with PD1.3G/A SNP (D = 0.0255, D'= 0.151, r= 0.1298 and p= 0.0094).

Table (1): PD1.3G/A and PD1.5C/T genotypes and alleles frequencies in CRC patients (n=100) versus the controls (n=100):

Genotype/Allele		Control group No (%)	CRC group No (%)	*p*	χ^2	*p*	COR (CI 95%)
PD1.3G/A	GG	54(54%)	53(53%)	0.887	0.020	**<0.001***	1.00 (Reference)
	AG	43(43%)	22(22%)	**0.002***	**10.051**		0.52 (0.28 – 0.99)
	AA	3(3%)	25(25%)	**<0.001***	**20.100**		8.5 (2.4 – 29.8)
	G	151 (76%)	128 (64%)	**0.012***	6.268	**0.012***	Reference
	A	49 (24%)	72 (36%)	**0.012***	6.268		1.7 (1.1 – 2.7)
PD1.5C/T	CC	39 (39%)	64 (64%)	**<0.001***	**12.511**	**<0.001***	Reference
	CT	61 (61%)	36 (36%)	**<0.001***	**12.511**		0.36 (0.2 – 0.64)
	TT	0(0%)	0(0%)	-	-		-
	C	139 (70%)	164 (82%)	**0.004***	8.506	**0.004***	Reference
	T	61 (30%)	36 (18%)	**0.004***	8.506		0.5 (0.3 – 0.8)

Data are absolute frequency (N). P value: Chi-Square test. COR = Crude odds ratio (Binary logistic regression). CI = Confidence interval.

Table (2): Inheritance model analysis of PD1.3G/A in association with the risk for colorectal cancer:

Inheritance model	Genotype	Control	CRC	*OR (95% CI)*	*p*	AIC
Co-dominant	GG	54(54%)	53(53%)	1.00	**<0.0001***	256.1
	AG	43(43%)	22(22%)	0.46(0.24-0.89)		
	AA	3(3%)	25(25%)	9.02(2.55-31.98)		
Dominant	GG	54(54%)	53(53%)	1.00	0.99	283.5
	AG+AA	46(46%)	47(47%)	1.00(0.57-1.76)		
Recessive	GG+AG	97(97%)	75(75%)	1.00	**<0.0001***	259.6
	AA	3(3%)	25(25%)	11.71(3.37-40.64)		
Over-dominant	GG+AA	57(57%)	78(78%)	1.00	**0.0005***	271.3
	AG	43(43%)	22(22%)	0.33(0.18-0.63)		
Log-additive	---			1.56(1.05-2.33)	**0.025***	278.5

p: probability, OR: odd's ratio, CI: confidence interval, (*): indicates significance, AIC: Akaike information criterion.

Table (3): Patient's criteria and genotyping of PD1.3G/A and PD1.5C/T in early and late onset CRC:

Characteristic	Early onset CRC	Late onset CRC	*p* value
N	27	73	
Sex			0.781
Male	16 (59.3%)	41 (56.2%)	
Female	11 (40.7%)	32 (43.8%)	
TNM stage			**0.025***
I & II	19 (70.4%)	33 (45.2%)	
III	8 (29.6%)	40 (54.8%)	
Tumor site			0.389
Cecal / Ascending	10 (37%)	28 (38.4%)	
Transverse	1 (3.7%)	6 (8.2%)	
Descending	8 (29.6%)	28 (38.4%)	
Rectosigmoid	3 (11.1%)	2 (2.7%)	
Rectal	5 (18.5%)	9 (12.3%)	
PD 1.3			0.463
GG	12 (44.4%)	41 (56.2%)	
AG	6 (22.2%)	16 (21.9%)	
AA	9 (33.3%)	16 (21.9%)	
PD 1.5			**0.045***
CC	13 (48.1%)	51 (69.9%)	
CT	14 (51.9%)	22 (30.1%)	

Data are N (%), Test of significance is Chi-Square test (Fisher's exact test for tumor site).

Table (4): The haplotype frequencies of PD-1 polymorphism and the CRC risk:

PD1.3 G/A	PD1.5 C/T	Control group	CRC group	Frequency	OR (95% CI)	*p*	Global haplotype association *p*
G	**C**	0.53	0.57	0.55	1.00	--	**<0.0001 ***
A	**C**	0.17	0.25	0.20	0.84 (0.41-1.72)	0.63	
G	**T**	0.23	0.07	0.14	0.20 (0.09 - 0.45)	**0.0002** *	
A	**T**	0.08	0.11	0.09	0.96 (0.29-3.19)	0.95	

OR: odd's ratio (adjusted by sex), CI: confidence interval, p: probability and (*): indicates significance.

Table (5): The haplotype frequencies and early onset CRC risk:

Haplotype		Frequency			Association	
PD1.3 G/A	**PD1.5 C/T**	**Total**	**EOCRC**	**LOCRC**	**OR (95% CI)**	***p* value**
G	**C**	0.5632	0.4888	0.5989	Reference	-
A	**C**	0.2518	0.2520	0.2504	0.74 (0.3-1.8)	0.51
G	**T**	0.0718	0.0668	0.0723	1.1 (0.25-5.03)	0.89
A	**T**	0.1082	0.1925	0.0784	4.4 (1.4-14.2)	**0.014***

EOCRC= Early onset CRC, LOCRC= Late onset CRC, OR= odds ratio (adjusted by sex), CI=confidence interval, p: probability and (*): indicates significance.

4. Discussion:

Worldwide, an estimated 19.3 million new cancer cases and almost 10.0 million cancer deaths occurred in 2020. Although colorectal cancer (CRC) ranked the third most common among the newly diagnosed cancer cases, CRC had the second-highest overall mortality rate worldwide. [1] Although great progress has been made in recent years, CRC survival remains unsatisfactory due to high metastasis and recurrence. Understanding the underlying molecular mechanisms of CRC tumorigenesis and metastasis has become increasingly important. [23]

Under normal conditions, the immune checkpoints (ICs), including programmed cell death protein 1 (PD-1), inhibit the immune response and prevent from its overactivation. This mechanism can be used by cancer cells to avoid recognition and destruction, a discovery made by prof. Honjo, the Nobel Prize winner in 2018. [24] PD-1 plays a vital role as an immunosuppressive receptor and a negative regulator of the immune responses. It promots self-tolerance through modulating the activity of T-cells, activating apoptosis of antigen-specific T cells, and inhibiting apoptosis of regulatory T cells.[25] It is belonging to immunoglobulin superfamily [18] and is expressed on CD4+ T cells, CD8+ T cells, NKT cells, B cells, and monocytes. [26] The inherited genetic variants in the regulatory regions of ICs genes may affect both expression and structure of ICs on immune cells and can be considered as risk factors of cancer development. [24]

Most of the individual studies and meta-analysis regarding PD-1 SNPs in relation to cancer were performed on Asian populations and the reported significant differences in distribution of their genotypes could be true only for individuals of Asian origin. [24] In this context, the current study aimed to focus a possible association between the PD-1.3G/A (rs11568821) and PD-1.5 C/T (rs2227981) haplotypes with the susceptibility and the onset of colorectal cancer in Egyptian population.

On studying the genotype frequency of PD1.3G/A SNP, the frequencies of the heterozygous

genotype AG, and the mutant homozygous genotype AA as well as the A allele were significantly higher in the CRC patients than the controls while the frequencies of the heterozygous genotype CT and the T allele of the PD1.5C/T SNP were lower in the CRC patients with the lack of the homozygous TT genotype (Table 1). This lack of the mutant TT genotype made it inconsistent with the HWE with the inability to detect the best model of inheritance of that SNP. A finding that was reported before on the same population by Abo El-khair et al. [27] Several deviated SNPs have been detected to be out of the equation, however, they should not be excluded from the research, but additional analysis is needed on larger cohort. [28]

The antitumor CD8+ T cells exhibit preferential expression of PD-1 protein leading to their exhaustion and functional impairment, which in turns lead to attenuated tumor-specific immunity with disseminating tumor progression. [29, 30] An intron four location of the PD1.3G/A represents an enhancer-like structure where four imperfect tandem repeats are placed. This region contains binding sites for the transcription factors involved in hematopoietic differentiation and inflammation. [31] The presence of PD-1.3 A allele disrupted the binding site in the first repeat leading to aberrant PD-1 expression and deregulated lymphocyte activity. [32] However, the PD1.5C/T polymorphism, located in exon 5, is a synonymous variation (Ala268Ala) that does not modify the amino acid structure of the protein. [33]

The present study is one of the few studies investigating a possible association between both SNPs and CRC. Hashemi et al. [34] meta-analysis included 9 studies of PD-1.3G/A (rs11568821) and 16 studies of PD-1.5 C/T (rs2227981) polymorphisms in association with different types of cancers. Despite revealing that the wild variant of both polymorphisms was associated with an overall protection against cancer, they only reported two studies that were conducted on the Iranian population in association with CRC. [20, 21]

Yousefi et al. [20] investigated the PD-1.3G/A SNP in CRC patients and reported a higher frequency of AA genotypes while Mojtahedi et al. [21] studied the PD1.5C/T SNP and detected nonsignificant association with CRC.

On the other hand, Lamami et al. [22] suggested that PD-1.5 C/T polymorphism may be associated with the risk and progression of CRC in Turkish population.

Conflicting results were reported in other types of cancers, Savabakr et al. [15] and Haghshenas et al. [16] detected a significant association between the T allele of the PD1.5C/T SNP with gastric cancer, and thyroid cancer in Iranian population respectively. In Chinese population, the PD1.5C/T SNP was reported by Li et al. [19] to increase cervical cancer risk while Hua et al. [35] reported lower association risk with breast cancer. Other studies showed nonsignificant association between both polymorphisms and cancer susceptibility including the study of Haghshenas and coworkers [16] who studied the PD-1.3G/A SNP and Pirdelkhosh et al. [36], Fathi et al. [37] and Karami. et al [38] who reported nonsignificant differences in genotypic or allelic analysis at both positions with cancer.

Moreover, Ma et al. [39] and Gomez et al. [40] demonstrated no consistent differences of the PD1.5 genotype frequencies in Chinese cancer patients and in Brazilian cancer patients respectively.

The inheritance model analysis revealed a statistically significant association between PD1.3G/A and CRC under the best-fitting co-dominant and recessive models with the mutant homozygous AA genotype showing a higher cancer risk than other models of inheritance (Table 2). This was consistent with the finding of Hashemi et al. [34] who indicated that the heterozygous (AG) and dominant (GG) genetic models of this variant significantly decreased the overall cancer risk. Their pooled analysis involving PD1.5C/T polymorphism revealed that this variant significantly decreased the overall cancer risk in the recessive genetic models (TT). While, this meta-analysis included Asian, Caucasian, and Southern American ethnicity, our ethnic population, the Egyptian, was not included when studying both SNPs.

According to our data, earlier stages were detected more frequently either in early onset or late onset CRC patients which could be attributed to the advanced technologies of early diagnosis and the awareness of the population about the worldwide cancer prevalence (Table 3). The early-onset CRC is associated with aggressive tumor characteristics and distal location, but these patients tend to have better survival than older onset patients. [41]

On studying the association of both SNPs and the onset of CRC, only individuals with CT genotype of PD 1.5 C/T have 2.6 times higher odds to exhibit early-onset rather than late-onset CRC as compared to carriers of CC genotype (Table 3). Taking into consideration this inconsistent result, more research on this issue is needed on a larger cohort of early and late-onset CRC patients to confirm or deny this finding.

The studied polymorphisms showed specific haplotyping results related to the Egyptian ethnicity considering the completely different genotype distribution among populations. A significant global haplotype association with CRC risk in the overall frequencies was detected and the G/T haplotype was significantly associated with the lowest CRC risk having odds of 0.20 to exhibit the disease. Despite being statistically insignificant, the A/T haplotype was associated with a higher risk of CRC (Table 4) and was significantly associated with a 4.4 folds higher risk to exhibit early onset CRC (Table 5). This was not expected as the T allele is thought to be a protective allele that was associated with a lower CRC risk but the detection of a significant linkage disequilibrium between the studied SNPs could explain this haplotype effect. The linkage disequilibrium between polymorphisms may alter the pdcd1 gene expression. [32] The analysis of SNPs will allow to evaluate the phenotypic effect of haplotypes on immune cells which may allow to evaluate if a specific haplotype is associated with higher, lower, or neutral risk of cancer development. [24]

To the best of our knowledge, multiple SNPs analysis with haplotyping of the PD1.3 or PD1.5 in CRC was studied for the first time in the current study regarding both the disease and the Egyptian ethnicity. However, in non-small cell lung cancer and squamous cell carcinomas of head and neck studies done by Pirdelkhosh et al. [36] and Fathi et al. [37] respectively, nonsignificant associations were found in the frequencies of haplotypes between Iranian patients and controls. Differences in ethnic background, and type of cancer may contribute to between-study heterogeneities.

In recent years, immunotherapy has been revolutionized by a new approach that works by blocking the immune checkpoints (IC) receptors

including PD-1 to elicit an anti-cancer response, a promising finding that has opened new perspectives for cancer treatment. Inherited genetic markers such as SNPs may also be useful in stratification patients into groups which will benefit from immunotherapy. [24] Accordingly, the results of our study could help the era of colorectal cancer immunotherapy considering the PD-1 as a potential target in the patients with risky haplotypes.

5. Conclusions:

Despite years of research on possible association of *pdcd1* SNPs and the risk of cancer development, few studies reported this association to the risk of CRC, and we think that no studies investigated these associations in Egyptian ethnicity. In this respective, this study was the first to announce a potential hidden effect of PD-1.3G/A (rs11568821) and PD-1.5 C/T (rs2227981) haplotypes on the risk of CRC in Egyptian. As a conclusion from this study, the G/T haplotype was considered protective as it acquired the least risk for CRC, while the mutant A/T haplotype was not only associated with a higher risk of CRC but also with a significantly higher risk to exhibit early onset disease, a point that could be studied on larger cohorts and compared with other populations. Based on these findings of the potential interactions between the studied SNPs and CRC, we postulate that it may be possible to select CRC patients in need of more intensive monitoring according to the detected haplotype.

6. Limitations and future perspectives:

This study has some limitations, which should be taken into consideration while interpreting its findings. The limited cohort size (n=200) available for this study emphasizes the importance of confirmative studies in larger cohorts of patients. The lack of the homozygous TT genotype of the PD-1.5 C/T (rs2227981) could be a unique finding among the Egyptian ethnicity but still in need for further investigation among this population.

Based on these limitations, we also propose future directions of the research broadening and complementing presented topic through analysing the SNPs which will cover the whole *pdcd1* gene. Further insight into the associated mechanisms may enable deeper understanding the impact of these variations on CRC tumorigenesis and progression. Very important, future studies should include the functional relevance of the inherited variations in the context of pdcd1 gene expression that should be well-studied and understood.

Identifying appropriate biomarkers of CRC risk may help in establishing personalized cancer immunotherapy through patient stratification and can provide them with promising survival benefits. Our results suggest that different pdcd1 SNPs may be biomarkers for identifying patients likely to benefit from the emerging anti PD-1 immunotherapy strategies. Therefore, it could be an encouraging key area for further research involved in more exploration of PD-1 inhibitors as a therapy for candidate CRC patients who carry selected PD-1 haplotypes distribution. Expectedly, the future approaches could be based on the patient, that will lead to more effective cancer immunotherapy.

7. References:

1. **Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F**. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021 May; 71(3): 209-49. doi: 10.3322/caac.21660.

2. **World Health Organization**: International Agency For Research on Cancer: Cancer Today. Data visualization tools for exploring the global cancer burden in 2020. Available from: https://gco.iarc.fr/today/home.
3. **Siegel RL, Miller KD, Goding Sauer A, et al**. Colorectal cancer statistics. CA Cancer J Clin. 2020; 70: 145-64.
4. **Song M and Chan AT**: Environmental factors, gut microbiota, and colorectal cancer prevention. Clin Gastroenterol Hepatol 17: 275-89, 2019.
5. **Gonzalez-Pons M, Cruz-Correa M**. Colorectal cancer biomarkers: where are we now? Biomed Res Int 2015; 149014.
6. **Singh PP, Sharma PK, Krishnan G, Lockhart AC**. Immune checkpoints and immunotherapy for colorectal cancer. Gastroenterol Rep (Oxf) 2015; 3: 289-97.
7. **Kocian P, Šedivcov M, Drgč J, et al**. Tumor-infiltrating lymphocytes and dendritic cells in human colorectal cancer: their relationship to KRAS mutational status and disease recurrence. Hum Immunol 2011; 72: 1022-8.
8. **Charames GS, Bapat B**. Genomic instability and cancer. Curr Mol Med 2003; 3: 589-96.
9. **Mik M, Dziki L, Malinowska K, Trzcinski R, Majsterek I, Dziki A.** Polymorphism of MSH2 Gly322Asp and MLH1 -93G>A in non-familial colon cancer – a case-controlled study. Arch Med Sci 2017; 13: 1295-302.
10. **Coussens LM, Zitvogel L, Palucka AK**. Neutralizing tumor- promoting chronic inflammation: a magic bullet? Science 2013; 339: 286-91.
11. **Tesniere A, Apetoh L, Ghiringhelli F, et al.** Immunogenic cancer cell death: a key-lock paradigm. Curr Opin Immunol 2008; 20: 504-11.
12. **Nishimura H, Nose M, HiaiH, Minato N, Honjo T**. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 1999; 11: 141-51.
13. **Okazaki T, Honjo T.** PD-1 and PD-1 ligands: from discovery to clinical application. Int Immunol 2007; 19: 813-24.
14. **Liu X, Hu LH, Li YR, et al**. Programmed cell death 1 gene polymorphism is associated with ankylosing spondylitis in Chinese Han population. Rheumatol Int. 2011; 31: 209-13.
15. **Savabkar S, Azimzadeh P, Chaleshi V, et al.** Programmed death-1 gene polymorphism (PD-1.5 C/T) is associated with gastric cancer. Gastroenterol Hepatol Bed Bench 2013; 6: 178-82.
16. **Haghshenas MR, Naeimi S, Talei A, et al**. Program death 1 (PD1) haplotyping in patients with breast carcinoma. Mol Biol Rep 2011; 38: 4205-10.
17. **Qiu, H, Zheng L, Tang W, Yin P, Cheng F, Wang L**. Programmed death-1 (PD-1) polymorphisms in Chinese patients with esophageal cancer. Clin Biochem 2014; 47: 612-7.
18. **Li, Z.; Li, N.; Zhu, Q.; Zhang, G.; Han, Q.; Zhang, P.; Xun, M.; Wang, Y.; Zeng, X.; Yang, C.; et al.** Genetic variations of PD1 and TIM3 are deferentially and interactively associated with the development of cirrhosis and HCC in patients with chronic

HBV infection. Infect. Genet. Evol. 2013, 14, 240–6.

19. **Li XF, Jiang XQ, Zhang JW, Jia YJ.** Association of the programmed cell death-1 PD1.5 C>T polymorphism with cervical cancer risk in a Chinese population. Genet Mol Res 2016; 15. DOI: 10.4238/gmr.15016357.
20. **Yousefi AR, Karimi MH, Shamsdin SA, Mehrabani D, Hosseini SV, Erfani N, et al.** PD-1Gene Polymorphisms in Iranian Patients with Colorectal Cancer. Lab Med .2013; 44(3):241–4.
21. **Mojtahedi Z, Mohmedi M, Rahimifar S, Erfani N, Hosseini SV, Ghaderi A**. Programmed death-1 gene polymorphism (PD-1.5 C/T) is associated with colon cancer. Gene. 2012 Oct 25; 508(2): 229-32. doi: 10.1016/j.gene.2012.07.059.
22. **Lamami, Y., Mesediyeva, R., Arikan, S., Ercan, Ş., Kıyan, H., Tatar, C., Nayci, A., Farooqi, A., Yaylim, İ., and Kiran, B**. Preliminary report: one of the PD-1 gene variants may be a valuable marker for colorectal cancer. Archives of Medical Science - Civilization Diseases. 2018; 3(1), pp.34-40. https://doi.org/10.5114/amscd.2018.75533
23. **Ji Y, Lv J, Sun D, Huang Y.** Therapeutic strategies targeting Wnt/β-catenin signaling for colorectal cancer (Review). Int J Mol Med. 2022 Jan;49(1):1. doi: 10.3892/ijmm.2021.5056.
24. **Wagner M, Jasek M, Karabon L**. Immune Checkpoint Molecules-Inherited Variations as Markers for Cancer Risk. Front Immunol. 2021 Jan 14;11:606721. doi: 10.3389/fimmu.2020.606721.
25. Han Y, Liu D, Li L. PD-1/PD-L1 pathway: current researches in cancer. Am J Cancer Res. 2020 Mar 1;10(3):727-42.
26. **Chamoto, K.; Al-Habsi, M.;** Honjo, T. Role of PD-1 in Immunity and Diseases. Curr. Top. Microbiol. Immunol. 2017; 410, 75–97.
27. **Abo El-Khair SM, Sameer W, Awadallah N, Shaalan D**. Programmed cell death 1 gene polymorphism as a possible risk for systemic lupus erythematosus in Egyptian females. Lupus. 2019 Oct;28(12):1427-1434. doi: 10.1177/0961203319878493.
28. **Turner S, Armstrong LL, Bradford Y, et al.** Quality control procedures for genome-wide association studies. Curr Protoc Hum Genet. 2011; 68: 1–18.
29. **Thommen, D.S.; Schumacher, T.N.** T Cell Dysfunction in Cancer. Cancer Cell. 2018; 33, 547–562.
30. **He, Q.F.; Xu, Y.; Li, J.; Huang, Z.M.; Li, X.H.;Wang, X.** CD8+ T-cell exhaustion in cancer: Mechanisms and new area for cancer immunotherapy. Brief. Funct. Genom. 2019; 18, 99–106.
31. **Prokunina L, Gunnarsson I, Sturfelt G, et al.** The systemic lupus erythematosus-associated PDCD1 polymorphism PD1.3A in lupus nephritis. Arthritis Rheum 2004; 50: 327–38.
32. **Salmaninejad A, Khoramshahi V, Azani A, Soltaninejad E, Aslani S, Zamani MR, et al.** PD-1 and cancer: molecular mechanisms and polymorphisms. Immunogenetics.2018; 70(2):73–86. doi: 10.1007/s00251-017-1015-5
33. **Lin SC, Yen JH, Tsai JJ, et al**. Association of a programmed death 1 gene polymorphism with the development of rheumatoid arthritis,

but not systemic lupus erythematosus. Arthritis Rheum 2004; 50: 770–5.

34. **Hashemi, M., Karami, S., Sarabandi, S., Moazeni-Roodi, A., Małecki, A., Ghavami, S., & Wiechec, E.** Association between PD-1 and PD-L1 Polymorphisms and the Risk of Cancer: A Meta-Analysis of Case-Control Studies. Cancers .2019; 11(8), 1150. https://doi.org/10.3390/cancers11081150
35. **Hua, Z.; Li, D.; Xiang, G.; Xu, F.; Jie, G.; Fu, Z.; Jie, Z.; Da, P.; Li, D**. PD-1 polymorphisms are associated with sporadic breast cancer in Chinese Han population of Northeast China. Breast Cancer Res. Treat. 2011; 129: 195–201.
36. **Pirdelkhosh, Z.; Kazemi, T.; Haghshenas, M.R.; Ghayumi, M.A.; Erfani, N**. Investigation of Programmed Cell Death-1 (PD-1) Gene Variations at Positions PD1.3 and PD1.5 in Iranian Patients with Non-small Cell Lung Cancer. Middle East J. Cancer 2018; 9: 13–17.
37. **Fathi F, Faghih Z, Khademi B, Kayedi T, Erfani N, Gahderi A**. PD-1 Haplotype Combinations and Susceptibility of Patients to Squamous Cell Carcinomas of Head and Neck. Immunol Invest. 2019 Jan;48(1):1-10. doi: 10.1080/08820139.2018.1538235.
38. **Karami S, Sattarifard H, Kiumarsi M, Sarabandi S, Taheri M, Hashemi M, Bahari G, Ghavami S.** Evaluating the Possible Association between PD-1 (Rs11568821, Rs2227981, Rs2227982) and PD-L1 (Rs4143815, Rs2890658) Polymorphisms and Susceptibility to Breast Cancer in a Sample of Southeast Iranian Women. Asian Pac J Cancer Prev. 2020 Oct 1;21(10):3115-23. doi: 10.31557/APJCP.2020.21.10.3115.
39. **Ma Y, Liu X, Zhu J, et al**. Polymorphisms of co-inhibitory molecules (CTLA-4/PD-1/PD-L1) and the risk of non-small cell lung cancer in a Chinese population. Int J Clin Exp Med, 2015; 8, 16585-91.
40. **Gomez GVB, Rinck-Junior JA, Oliveira C, et al**. PDCD1 gene polymorphisms as regulators of T-lymphocyte activity in cutaneous melanoma risk and prognosis. Pigment Cell Melanoma Res. 2018;31:308–17. https://doi. org/10.1111/pcmr.12665
41. **Burnett-Hartman AN, Powers JD, Chubak J, Corley DA, Ghai NR, McMullen CK, Pawloski PA, Sterrett AT, Feigelson HS**. Treatment patterns and survival differ between early-onset and late-onset colorectal cancer patients: the patient outcomes to advance learning network. Cancer Causes Control. 2019 Jul;30(7):747-55. doi: 10.1007/s10552-019-01181-3.