Association of Gastric Cancer with Multidrug Resistance 1 Gene C3435T Polymorphism in Egyptian Population

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Abstract: Background: Gastric cancer is a disease with high death rate making it the second most common cause of death worldwide. Host genetic factors play a key role in gastric carcinogenesis. The multidrug resistance 1(MDR1) gene mediates the expression of p-glycoprotein, which has a role in active transport of various substrates including xenobiotics and so has a protective function in many tissues and organs like gastrointestinal epithelial cells. C3435T polymorphism of MDR1 gene influences p-glycoprotein expression and activity in gastrointestinal tract. **Objectives:** The objective of this study was to investigate the association of MDR1 gene C3435T polymorphism with gastric cancer incidence and its clinicopathological features in the Egyptian population. **Subjects and Methods:** In our study 24 gastric cancer patients were diagnosed and compared with 20 healthy volunteers as a control group. Genomic DNA was extracted by Gene JET Genomic DNA purification kit. The *MDR1 C3435T* gene polymorphism was studied by a polymerase chain reaction- restriction fragment length polymorphism method (PCR-RFLP). **Results:** Among the clinicopathological features of patients group we found that MDR1 mutant genes CT&TT showed higher frequencies within stages T3&T4 and also in patients with distant metastasis, although these differences did not reach statistical significance. Positive distant metastasis showed statistically significant higher frequency of T allele versus negative metastasis among patients group with odds ratio (OR)=5.176, 95% confidence interval(CI)=1.000 - 27.064 (p=0.038). **Conclusions:** Our data add to the growing literature of the relationship between genetic variation in MDR1 gene and the susceptibility to gastric cancer and suggests the association between MDR1 gene polymorphism and clinicopathological features of gastric cancer where patients with the mutant genotypes were more in the advanced cases.

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1. Introduction:

Stomach cancer (also known as gastric cancer) is a disease in which the cells forming the inner lining of the stomach become abnormal and start to divide uncontrollably forming a tumor mass[(1)]. It is a disease with a high death rate (800,000 per year) making it the second most common cause of cancer death worldwide after lung cancer [(2)]. *Helicobacter pylori* (*H. pylori*) is a bacterium that infects the human stomach and is classified as a carcinogen, although only a small number of infected persons actually develop gastric cancer. This suggests that genetic factors may also play an important role in the gastric carcinoma [(3)]. Diabetes mellitus has been associated with an increased risk of gastric cancer by 77% in patients from East Asia; however no relationship has been identified for Western patients [(4)].

Adenosine triphosphate Binding Cassette (ABC) transporters are transmembrane proteins that utilize the energy of adenosine triphosphate (ATP) hydrolysis. They carry out certain biological processes such as translocation of various substrates across membranes**.** P-glycoprotein (Pgp) was one of the first members of the ABC superfamily to be studied.Over expression of Pgp was linked to multidrug resistance gene in mammalian cell lines and human cancers [(5)]. Pgp is expressed at low levels in most tissues except at the apical surface of epithelial cells lining the colon, small intestine, pancreatic ductules, bile ductules, kidney proximal tubules, and the adrenal gland [(6)]. Epithelial cells with excretory roles generally express Pgp. It is also located in the endothelial cells of the blood brain barrier**,** the blood testis barrier and the blood-mammary tissue barrier [(7 & 8)]. Pgp is expressed at high levels at the luminal surface of secretory epithelial cells in the pregnant endometrium, as well as the placenta, where it may provide protection for the fetus [(9)].The tissue localization of Pgp suggests that the protein plays a physiological role in the protection of susceptible organs from toxic xenobiotics through the secretion of metabolites and xenobiotics into bile, urine and the lumen of the gastrointestinal tract by active pump outside the cell. Cells adapt to the presence of toxic xenobiotics in their environment by up regulation of drug efflux pumps, such as Pgp, which provides them with a long-term survival

advantage [10]. Pgp has the ability to interact with hundreds of structurally diverse substrates which are generally nonpolar, weakly amphipathic compounds, and include natural products, anticancer drugs, steroids, fluorescent dyes, linear and cyclic peptides and ionophores. Potential physiological substrates for Pgp include peptides, steroid hormones, lipids, and small cytokines, such as interleukin-2, intereukin-4, and interferon-ϒ [11 & 12].

The human Multidrug resistance 1 (MDR1) gene, a stable MDR phenotype induced, is activated after short-term exposure of cells to a variety of environmental insults. The role of p-glycoprotien in protection from toxicants explains why its polymorphism play a role in increasing risk of various diseases including the tumors [13]. The MDR1 gene is found on chromosome 7q21.1. Several polymorphisms of this gene have been characterized and a single base polymorphism in exon 26 (C3435T) is suggested to influence P-gp expression and activity [14].

The aim of the present study was to demonstrate whether C3435T polymorphism of MDR1gene influences the risk of development and progression of gastric cancer in Egyptian population.

2. Subjects and Methods:

Subjects

Twenty-four patients including 14 males and 10 females with gastric cancer were enrolled in our study, after taking the consent and all of them had available gastric specimens for histopthological diagnosis and tumor staging of gastric cancer. The control group comprised 20 healthy age and sex matching volunteers who were willing to participate in this study. They were 11 males and 9 females.

Methods

Clinicopathological features:

Histopthological diagnosis and tumor staging of gastric cancer were accomplished according to guidelines and criteria established by Fenoglio-Preiser *et al.,* 2000 and the American Joint Committee on Cancer (AJCC) 2010 [15 &16]. *H. pylori* infection status was determined by histology; the slides were stained with haematoxylin and eosin (Fig. 1) and Giemsa (Fig. 2); the latter was used to confirm presence or absence of *H.pylori* [17]. Data such as sex, age, diet, diabetes, previous stomach surgery, smoking behavior and presence or absence of distant metastases were recorded by a registry form at the time of enrolment. Subjects with any other malignancy were excluded. A written informed consent was obtained from all participants.

DNA extraction:

Genomic DNA was extracted from peripheral blood leukocytes, using Gene JET Genomic DNA purification kit, United States (Cat. #K0721, #KO722, Lot 00099443, Fermentas Life Sciences) according to the manufacturer's instructions [18].

MDR1 (C3435T) genotype analysis:

The polymorphism of the *MDR1 (C3435T)* gene was conducted by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The C3435T variant of the MDR1 gene was identified with primers: 5′-ACTCTTGTTTTCAGCTGCTTG-3′ as the forward primer and 5′-AGAGACTTACATTAGGCAGTGACT-3′ as the reverse primer [10].

For PCR reactions, 200 ng of genomic DNA was amplified in 50 µL of reaction mixture containing 250 µM each of dNTPs (dATP, dCTP, dGTP, and dTTP), 250 ng of each primer, 1.5 mM $MgCl_2$, and 1 U *Taq* polymerase. PCR amplification consisted of an initial 5-min denaturation at 94 °C, followed by 35 cycles of denaturation at 94°C for 90 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The terminal extension was performed at 72°C for 10 min. All reactions were done using the thermal cycler Applied Biosystems (Perkin- Elmer 9600, USA). The PCR product was then digested with *fast digest Mbo*I restriction enzyme (Cat. #FD0814, Lot 00094071, Fermentas Life Sciences) for 5-15 minutes in 37°C. The products were then resolved on 2% agarose gel electrophoresis containing ethidium bromide,then visualized using UV transilluminator. DNA molecular weight marker (QIAGEN GelPilot 50 bp Ladder (100) {cat no. 239025}) was used to assess the size of PCR-RFLP products[10]. After digestion by *Mbo*I restriction enzyme, appearance of 2 bands at 130 and 76 bp represented the C allele while undigested 206-bp fragment indicated the presence of the T allele as shown and illustrated in figure 3.

Statistical methods:

Data were analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc.,Chicago, IL). Numerical data were expressed as mean, standard deviation, and range. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using student t-test. Odds ratio (OR) with its 95% confidence interval (CI) was used for risk estimation. A p-value<0.05 was considered significant [19.

3. Results

Twenty-four patients including 14 males (58.3%) and 10 females (41.7%) with gastric cancer were enrolled in our study. The control group comprised 20 healthy subjects including 11(55%) males and 9 (45%) females. The age range of gastric cancer patients was from 30 to 70 years with a mean age of 55.2 ± 11.7 while the control group was 26 to

60 years with a mean age of 51 ± 11.4 were involved in our study (Table 1).

Among the risk factors of gastric cancers, both diabetes and *H.pylori* which showed a frequency of 9 cases (37.5%) while smoking and dietary showed frequencies of 11 cases (45.8%) and 13 cases (54.2%), respectively among patients group. As regards the clinicopathologic features, 15 cases (62.5%) were stage T3 & T4 and 14 cases (58.3%) had distant metastases.

The frequency of the wild genotype CC was 52.2% versus 47.8% for mutant genotypes CT&TT among patients group while the mutant heterozygous CT and homozygous TT genotypes were found to have higher frequencies versus CC genotype 70.0% versus 30.0% among control group yet this difference didn't reach the statistical significance (OR 2.545, 95% CI 0.723- 8.961, p=0.142) (Table 2) (Fig. 4). Higher C allele frequency in patients group was observed when compared with healthy controls, although this difference didn't reach statistical significance (OR 2.077, 95% CI 0.849 - 5.080, p=0.107) (Table 3) (Fig. 5).

No significant statistical association between the different studied risk factors and MDR1 genotypes was observed among gastric cancer patients (p>0.05) (Table 4).

Among the clinicopathologic features of patients group we found that MDR1 mutant genes CT&TT showed higher frequencies within stages T3&T4,and in distance metastasis, although these differences were not statistically significant (OR 4.500, 95%CI 0.670-30.230, p=0.193) and (OR 2.545, 95%CI 0.723- 8.961, p=0.133), respectively (Table 5). Positive distant metastasis showed statistically significant higher frequency of T allele versus negative metastasis (39.9% versus 11.1%) among patients group (OR 5.176, 95%CI 1.000 - 27.064, p=0.038) (Table 6)(Fig. 6).

Table (1): Statistical comparison between patients group and control group as regards age and sex

Variable	Patients group	Control group	*p*-value
Sex male : female (%/%)	14:10(58.2%:41.7%)	11:9(51%:49%)	0.741
Age mean ± SD(y)	55.2±11.7	51.0±11.4	0.237

Table (2): Genotype frequency of multidrug resistance protein 1 (MRD1) gene polymorphism (C3435T) in patients group and control group

Genotype	Genotype frequency(%) Patients group	control group	OR(95%CI)	*P*-value
CC	13(52.2%)	6(30.0%)	2.545 (0.723-8.961)	
CT&TT	11(47.8%)	14(70.0%)		0.142

Table (3): Allele frequency of multidrug resistance protein 1 (MRD1) gene polymorphism (C3435T) in patients group and control group

Allele	Patients group frequency(%)	Control group frequency(%)	OR(95%CI)	*p*-value
C	35 (71.7%)	22 (55%)	2.077(0.849-5.080)	0.107
T	13 (28.3%)	18 (45%)		

Table (4): Association between genotype frequency of multidrug resistance protein 1 (MRD1) gene polymorphism (C3435T) and risk factors in patients group

Variable	Genotype frequency(%) Wild (CC)	Mutant(CT&TT)	OR(95%CI)	*p*-value
Smoking				
Positive	6(54.5%)	5(45.5%)	0.972(0.194-4.872)	0.973
Negative	7(53.8%)	6(46.2%)		
Diabetes				
Positive	4(44.4%)	5(55.6%)	1.875(0.352-9.981)	0.675
Negative	9(60.0%)	6(40.0%)		
Diet				
Positive	6(46.2%)	7(53.8%)	2.042(0.395-10.553)	0.392
Negative	7(63.6%)	4(36.4%)		
H.pylori				
Positive	3(33.3%)	6(66.7%)	4.000(0.693-23.089)	0.206
Negative	10(66.7%)	5(33.3%)		

Table (5): Association between genotype frequency of Multidrug resistance protein 1 (MRD1) gene polymorphism (C3435T) and clinicopathologic features of gastric cancer in patients group

Variable	Genotype frequency(%) Wild (CC)	Mutant (CT&TT)	OR(95%CI)	*p*-value
Surgery				
Positive	6(40%)	9(60%)	0.222(0.033-1.493)	0.193
Negative	6(66.7%)	3(33.3%)		
Staging				
T1,T2	6(66.7%)	3(33.3%)	4.500(0.670-30.230)	0.193
T3,T4	6(40.0%)	9(60.0%)		
Distant metastasis				
Positive	5(35.7%)	9(64.3%)		
Negative	7(70.0%)	3(30.0%)	0.268(0.046-1.548)	0.133

Table (6): Association between allele frequency of Multidrug resistance protein 1 (MRD1) gene polymorphism (C3435T) and clinicopathologic features of gastric cancer in patients group

Variable	Allele frequency(%) C	T	OR(95%CI)	*p*-value
Surgery				
Positive	63.3%	36.7%	0.247(.047-1.294)	0.083
Negative	87.5%	12.5%		
Staging				
T1,T2	87.5%	12.5%	4.053(0.773-21.256)	0.083
T3,T4	63.3%	36.7%		
Distant metastasis				
Positive	60.7%	39.3%		
Negative	88.9%	11.1%	5.176(1.000-27.064)	0.038

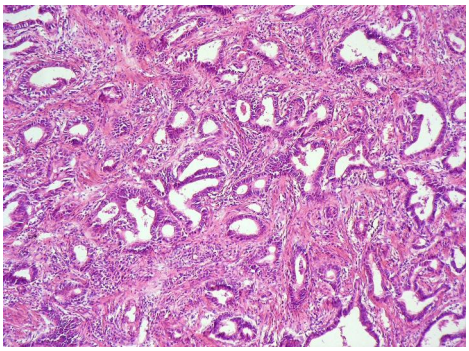
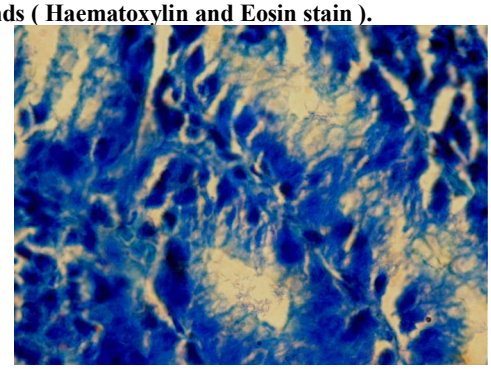


Fig (1): Well differentiated gastric adenocarcinoma (intestinal type); the infiltrating branching and tortious neoplastic malignant glands show increased nuclear/cytoplasmic ratios, and hyperchromatism. There is a desmoplasti stromal reaction to the infiltrating glands (Haematoxylin and Eosin stain).



Fig(2): A gastric carcinoma case accompanied by infection with *Helicobacter pylori*, the arrow points to rod-shaped organisms that are present along the luminal surfaces of the epithelium and in the luminal mucus stained by *Giemsa stain (oil lens)*, where they are stained bluish-purple.

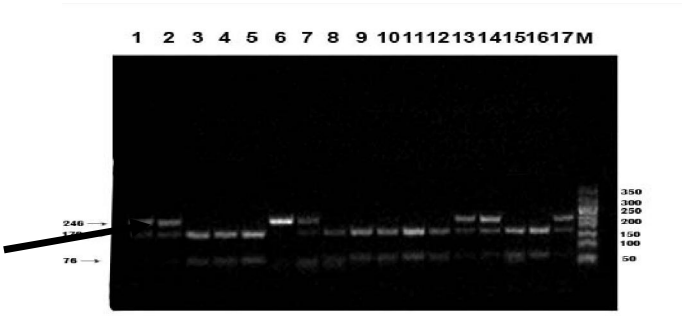


Fig (3): PCR-RFLP of the *MDR1(C3435T)* gene polymorphism using MboI restriction enzyme
M: DNA molecular weight marker: 50-350 bp
Lane 1, 2, 7, 13, 14, 17: Heterozygous mutant (C/T): 3 bands 246, 170 & 76bp
Lane 6: Homozygous mutant (T/T): one band at 246bp
Lane 3, 4, 5, 8, 9, 10, 11, 12, 15, 16: Homozygous wild type (C/C): 2 bands at 170and 76 bp

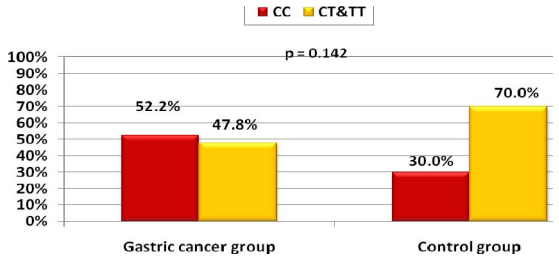


Figure (4): MRD1 gene polymorphism (C3435T) in the gastric cancer and control groups

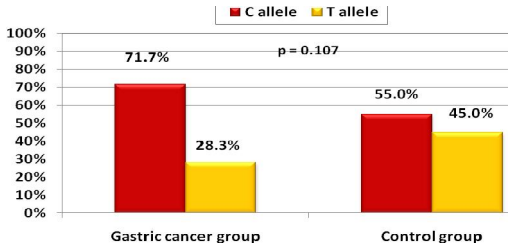


Figure (5): Allele frequency of MRD1 gene polymorphism (C3435T) in the gastric cancer and control groups

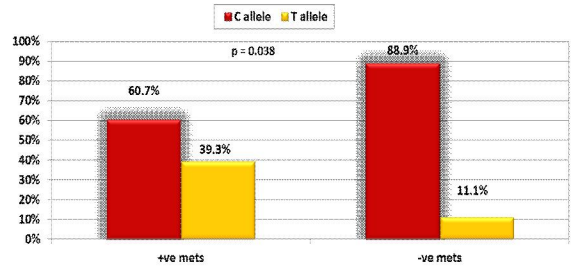


Figure (6): Metastasis and allele frequency of MRD1 gene polymorphism (C3435T) in the gastric cancer group

4. Discussion

Gastric cancer is a significant health problem world-wide. Although the incidence and mortality rates of this malignancy have been decreasing over the last few decades, it still remains second only to lung cancer as the leading cause of cancer death worldwide (20). The highest incidence of stomach cancer is in Asia and, Latin America and Caribbean; and the lowest incidence in Africa and Northern America (21). Infection with *H pylori* bacteria seems to be a major cause of stomach cancer. Long-term infection of the stomach with this germ may lead to inflammation, chronic atrophic gastritis and pre-cancerous changes of the inner lining of the stomach. Patients with stomach cancer have a higher rate of *H pylori* infection than people without this cancer. Even so, most people who carry this germ in their stomachs never develop cancer (22).

P-glycoprotein is an important ATP-dependent membrane transporter, which is involved in the absorption, distribution, and elimination of numerous substrates and acts as an energy-dependent efflux pump that exports its substrates out of the cell [23]. The multidrug resistant 1 gene encodes P-gp, 170-KDa member of adenosine triphosphate-binding cassette, super-family of membrane transporters. The most important physiological role of P-gp is the protection of organism against toxic xenobiotic [10]. Differences of P-gp expression and activity may reflect genetic polymorphism. In our study we aimed to evaluate the association of MDR1 gene, C3435T polymorphism with gastric carcinoma and assess the relationship between its genotypes and clinicopathological features of gastric cancer. Twenty four gastric cancer patients were enrolled in our study. Twenty healthy age and sex matched volunteers were selected as control group.

Our results in this study showed that the frequency of the wild genotype CC was 52.2% versus 47.8% for CT&TT genotypes among patients group while the mutant heterozygous CT and homozygous TT genotypes were found to have higher frequencies versus CC genotype 70.0% versus 30.0% among control group yet this difference didn't reach statistical significance (p=0.142). Higher C allele frequency in patients group was observed when compared with healthy controls, although this difference didn't reach statistical significance (p=0.107).

Some researchers [10] reported that, according to their studies on MDR1 gene polymorphism worldwide, allele and genotype frequencies of C3435T polymorphism depends strongly on the ethnicity of the investigated population. Within the European white population, C allele frequency ranges from 37% in Greeks to 62% in polish population and in Asian the allele frequency varies from 34% in southwest Asians to 61% in Japanese.

In our study no significant association between different studied risk factors including *H pylori*, diabetes, diet or smoking and MDR1 genotypes was observed among our patients with gastric cancer (p>0.05). In agreement with our results some researchers [10] found that no significant association between *H pylori* and MDR1 genotypes was observed among patients with gastric cancer (p=0.948). Some studies [24] found that *H pylori* eradication treatment can reduce the risk of gastric cancer, however other studies [25] found that gastric atrophy was linked to an increased risk of gastric cancer incidence and demonstrated that it is possible that there are two types of gastric cardia adenocarcinoma, one linked to gastric atrophy and *H pylori* infection, and one which resembles esophageal adenocarcinoma, on which gastric atrophy has no effect or even a protective effect. Marimuthu *et al.,* [26] found that there was no increase of stomach cancer incidence or mortality in diabetics in studies conducted in Europe or the US. However Chen *et al.,* [27] found in a cohort study that there was a 37% gastric cancer risk reduction for people diagnosed with diabetes for less than four years, but a 76% risk increase for those having diabetes for over four years previously.

Among the clinicopathologic features of our patients group we found that MDR1 mutant genes CT&TT showed higher frequencies within stages

T3&T4,and in patients with distance metastasis although these differences were not statistically significant(p=0.193 and 0.133,respectively). Positive distant metastasis showed statistically significant higher frequency of mutant T allele versus negative metastasis (39.9% versus 11.1%) among patients group (p=0.038).

In agreement with our results some researchers [20], found that the frequency of T3435T was significantly lower in gastric cancer patients than control (OR=0.43, 95% CI=0.23-0.79,P=0.007), yet they contradicted our study as regards clinicopathological features where MDR1 T3435T genotype was associated with reduced risk of more advanced cancer stages (OR=0.31, 95%CI=0.13-0.73,p=0.007)and lymph node metastasis (OR=0.28, 95%CI=0.13-0.65,p=0.008)of gastric cancer. In this context, they reported that their results were unexpected because p-glycoprotein in gastric mucosa has a protective effect and plays a significant role for dysplastic cells to survive by preventing intracellular accumulation of potentially toxic substances as metabolites including various carcinogens, while T3435T polymorphism was related to low expression of p-glycoprotein which would increase the risk of gastric cancer and its clinicopathological severity. This explanation supports our results concerning MDR1 gene polymorphism and clinicopathological severity in gastric cancer patients.

In the study conducted by Sabahi *et al.,* [10], the association between MDR1 gene polymorphism C3435T and gastric cancer was investigated, and the polymorphic homozygous T/T genotype showed an association with the incidence of gastric cancer (OR=1.73, 95% CI=1.123-2.680; p=0.015). The homozygous T allele was associated with more than 2-fold lower MDR1 expression levels compared with homozygous C/C samples. They reported that according to the protective role of P-gp against toxic substrates, the association of C3435Tand p-gp expression with gastric cancer may seem to be a high possibility. The C/C genotype may have a protective factor against gastric cancer. The challenge in the field is to define the cancer relevant gene polymorphism in a given tumor type and to design selective inhibitors, which target cancer cells but leave out normal cells.

5. Conclusion and Recommendations:

From what proceeded it was expected that the prevalence of C/C genotype in gastric cancer patients (52.2%) would be significantly lower than that in control (30%) but this paradox in our study may be due to small sample size of gastric cancer patients. The results of the present study suggests that of MDR1 gene C3435T polymorphism may be related to gastric cancer severity where patients with mutant genotypes CT and TT and carriers of T allele were more at risk for developing advanced gastric cancer clinicopathological features.

Data of this study suggested the relationship between genetic variation in MDR1 gene and the susceptibility to gastric cancer and also the association between MDR1 gene polymorphism and clinicopathological features of gastric cancer where patients with the mutant genotypes were found in more advanced cases. Larger and more detailed studies are required to confirm our results. It is necessary to confirm whether MDR1 C3435T polymorphism has any prognostic value that could help for the individualization of clinical outcome of cancer chemotherapy relating it to the progression free survival and the overall survival in gastric cancer patients.

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