Journal Homepage: - www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI: 10.21474/IJAR01/4131
DOI URL: http://dx.doi.org/10.21474/IJAR01/4131

RESEARCH ARTICLE

POLYMORPHISMS OF ESTROGEN BIOSYNTHESIS AND METABOLIZING GENES IN EGYPTIAN WOMEN WITH BREAST CANCER.

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Manuscript Info

Manuscript History

Received: 04 March 2017
Final Accepted: 02 April 2017
Published: May 2017

Key words:-
CYP17; CYP1A1; CYP1B1; COMT; Cancer breast; Egyptian women.

Abstract

Polymorphisms of genes responsible for biosynthesis and metabolism of estrogen including CYP and COMT groups might play a role in breast cancer. This study aims to investigate the association of CYP17 MspA1I, CYP1A1 MspI, CYP1B1 G>C, and COMT G>A polymorphisms with breast cancer in Egyptian women. Participants were in the form of 152 Egyptian women with cancer breast in addition to 100 healthy controls. They were subjected to DNA analysis using PCR-RFLP technique to characterize genetic polymorphisms of CYP17 MspA1I, CYP1A1 MspI, CYP1B1 G>C, and COMT G>A. Interestingly all these polymorphisms showed a positive association with cancer breast but in a variable degrees. Highest association was found with CYP1B1 C allele (p = 0.000, OR=10.26, 95% CI = 5.98 – 17.8) followed by COMT A allele (p = 0.000, OR=6.66, 95% CI= 4.09 – 10.9) then Cyp1A1 MspI C allele (p = 0.000, OR=4.46, 95% CI=2.68 -7.47) and lastly the CYP17 MspA1 C allele (p = 0.058, OR=1.46, 95% CI =1.0 – 2.1). Regarding clinical presentation, COMT A allele carriage was significantly higher among cases with positive lymph nodes (p=0.02) and in pre-menopausal cases (p= 0.020) while CYP 17 MspA1I C allele carriage was significantly higher among cases with negative breast feeding (P= 0.043). We can come to a conclusion that rare alleles of estrogen biosynthesis and metabolizing genes particularly CYP1B1 G>C, and COMT G>A followed by CYP1A1 MspI, and CYP17 MspA1I are associated with breast cancer among Egyptian women.

Introduction:-

Breast tumors were described by the Egyptians then subsequently by Greeks and Romans since ancient times (Hosny, Elkaffas , 2002). Moreover, the most known cancer among women was breast cancer (BC), as per the Egyptian National Cancer Institute (NCI) series (Elatar, 2002). Known established BC risk factors include inherited mutations such as the genes of BRCA1 and BRCA2, estrogens lifelong exposure (late menopause and early

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menarche), alcohol, smoking, postmenopausal obesity in addition fatty diet (Fredslund et al.; 2012). Estrogen metabolism is affected by four possible pathways that might contribute to cancer evolution. These include the 16 alpha hydroxylation pathway, the pathway of 2-hydroxylation, the pathway of 4-hydroxylation through redox cycling, besides 4-hydroxyestradiolquinone- adenine/guanine adduct depurination pathway **(**yue, et al; 2013**)**. Single nucleotide polymorphisms (SNPs) in genes of estrogen biosynthesis and metabolism might have an effect on levels of circulating estrogen and affect the susceptibility of breast cancer (Bozina, et al; 2009). The human CYP17 gene found on 10q24.3 chromosome (Fan, et al; 1992), encodes for cytochrome p450c17A that mediates activities vital for the endogenous steroid hormones production, such as estrogen and androgen (Miller, 1998)**.** The most studied single nucleotide polymorphism (SNP) in CYP17 gene was a T to C (A1 to A2) substitution at 34 bp (base pair) upstream of the translation initiation site of CYP17 (rs 743572) (Cary et al; 1994). This substitution might create an additional SP1-type (CCACC box) binding site in promoter region, enhancing transcription, and expression of CYP17, hence an increased estrogen level (Carey, et al; 1994**,** Feigelson et al; 1998).

The Cytochrome P450 1A1 (CYP1A1) gene, located at 15q22-q24, (Kawajiri et al; 1986). It is used in estrone and estradiol conversion to 2-hydroxyestradiol (Cavalieri, et al; 2001). It also activates metabolism of polycyclic aromatic hydrocarbons to aryl epoxides by its aryl hydrocarbon hydroxylase activity (Law, 1990). A well-known single nucleotide polymorphism affecting this gene is the 6235T>C also known as CYP1A1*2A polymorphism in the 3'untranslated region, could be identified by Msp I restriction enzyme (Reding, et al; 2012). This polymorphism was more studied in association with risk of breast cancer (dos Santos, et al; 2011, da Fonte de Amorim, et al; 2002). The CYP1B1 gene is located at 2p21-p22 chromosome. It encodes for the main cytochrome p450 enzyme which catalyzing the formation of estrogen metabolite called 4-hydroxy estrogen, that has carcinogenic effect in animal models due to its high hormonal activity (Kristensen, et al; 2000). Moreover, 4-hydroxyestradiol can undergo redox cycling (Liehr, et al; 1986) that causes the formation of free radicals as well as reactive semiquinone/ quinone intermediates which are potentially noxious to the DNA (Cavalieri, et al; 1997). Val 432 Leu (G > C) polymorphism of the cytochrome p450 1B1 (CYP1B1) gene was 2.4- to 3.4-fold higher catalytically active than the wild type one (Mitrunen, Hirvonen , 2003).

Catechol-O-Methyltransferase enzyme (COMT) activates the O-methylation of 2- and 4-OHE2 (Yager, Liehr, 1996). COMT activity is found highly in kidney and liver, and it is also present at high levels in RBCs, brain, the mammary gland and uterine endometrium. The COMT gene, found on 22q11.1–q11.2 chromosome, has a G>A single-nucleotide polymorphism in codon 158/108 of the membrane bound/cytosolic form (Butterworth , Dragunow, 1996). It causes an amino acid change, Val>Met , which determining high- and low-activity alleles of the enzyme (Lachman, et al; 1996). It was proved that the COMT M*et* allele, the low-activity and heat-labile enzyme, was four to five fold less effective in catechol substrates methylation in vitro (Scanlon, et al;1979) . The decreased COMT activity might cause accumulation of 4-OHE2 which may confer high breast cancer risk, however, the results remained controversial (He, et al; 2012).

This study is aiming to test for the association between polymorphisms of CYP17 MspA1I, CYP1A1 MspI, CYP1B1 G>C, and COMT G>A genes and breast cancer in Egyptian women.

Materials and Methods:-

This work is a case controlled study involving 152 Egyptian women with breast cancer admitted in the Oncology Center, College of Medicine, Mansoura University, Egypt. Breast cancer cases were compared to 100 unrelated healthy women which are age and locality matched. All these participants were exposed to thorough history analysis of items like age, socioeconomic level, education, work, nutrition, smoking habits, consanguinity, family history, parity, abortion, age at menarche, menopause, breast feeding, use of oral contraceptives, stage and grade of cancer, number of lymph nodes, estrogen receptors, progesterone receptors and site of metastases.

For all participants, DNA extraction was done from their peripheral bloods samples. Characterization of CYP17 MspA1I, CYP1A1 MspI, CYP1B1 G>C & COMT G>A gene polymorphisms was done by suing PCR_RFLP (polymerase chain reaction, restriction fragment length polymorphism) technique as being described in table 1, fig 1-4.

Statistical Analysis:-

SPSS program version 17 was used for analyzing the data statistically. Comparing genotype and allele frequencies among cases and controls was done using the Fisher's Exact and Chi square tests with the odds ratio (OR) and 95%

confidence intervals (95% CI). Testing for genetic equilibrium was done using Hardy-Weinberg Equilibrium (HWE). p value <0.05 was considered significant.

Results:-

Interestingly all studied polymorphisms showed a positive association with cancer breast in Egyptian women but in variable degrees. Highest association was found with CYP1B1 C allele (53.3% vs. 10.0%, p = 0.000, OR=10.26, 95% CI = 5.98 – 17.8) followed by COMT A allele (51.0% vs. 13.0%, p = 0.000, OR=6.66, 95% CI= 4.09 – 10.9) followed by Cyp1A1 MspI C allele (37.8 % vs. 22.0 %, p = 0.000, OR=4.46 , 95% CI=2.68 -7.47) and lastly the CYP17 MspA1I C allele (41.8 % vs. 33.0 %, p = 0.058, OR=1.46, 95% CI =1.0 – 2.1) (tables 2-4). All these genes were positively associated with breast cancer in the dominant model, i.e. significant higher frequency of COMT GA+AA (91.4% vs. 26.0%; p=0.000, OR=30.43, 95%CI= 14.0-76.4); CYP1B1 GC+CC (73.1% vs. 20.0%; p=0.000, OR=10.8, 95% CI= 5.66-20.87); higher frequency of CYP1A1 MspI TC+CC (56.6% vs. 24.0%; p= 0.000, OR=4.13, 95% CI= 2.3-7.5) and CYP17 MspA1I TC+CC (72.4% vs. 58.0%; P=0.026, OR=1.9, 95% CI =1.07-3.34).

Comparing cases regarding the frequency of genotypes of CYP17 MspA1I, CYP1A1 MspI, Cyp1B1 G>C, and COMT G>A gene polymorphisms in relation to all studied demographic, clinical parameters and laboratory parameters (table 6), no significant association was found except for that of CYP 17 MspA1I C allele carriage (CT+CC) that was significantly higher among cases with negative breast feeding (P= 0.043, OR= 6.474, 95% CI=0.85-135.69) and COMT A allele carriage (GA+AA) that was significantly higher among cases with positive lymphadenopathy p=0.02, OR= 0.12, 95% CI= 0.01-0.930 and also in premenopausal cases compared to postmenopausal cases (P= 0.020, OR=0.190, 95% CI=0.028-0.959).

Discussion:-

This study, to our knowledge is the first report of testing the association of polymorphic genetic variants involved in biosynthesis and metabolism of estrogen with breast cancer among Egyptian women. The results showed a significant increase of the frequency of certain genotypes among patients compared to controls.

The highest degree of association was found with C allele carriage of CYP1B1 polymorphism. The same was reported among American CC homozygous women (Sigurdson, et al; 2009); Nigerian premenopausal women **(**Okobia, et al; 2009**);** Han Chinese women (Jiao, et al; 2010**);** Greenlandic Inut premenopausal women (Ghisari, et al; 2014). On the other hand, negative association was observed in other studies in American women **(**Reding, et al; 2009**);** German women **(**MARIE-GENICA, 2009); Polish women **(**Ociepa-Zawal, et al; 2009**);** Brazilian pre or postmenopausal women **(**dos Santos, et al; 2011**);** Caucasian Slovenian postmenopausal women **(**Cerne, et al; 2011**);** Nigerian postmenopausal women **(**Okobia, et al; 2009**);** Thai women (Sangrajrang, et al; 2009); Han Chinese women (Sun , et al; 2015). On the contrary, a reverse association with the G; and not the C allele was observed among American women of African ethnicity (Van Emburgh, et al; 2008). A reduced risk was also observed among African American women with the CYP1B1 CC genotype in comparison with those who had at least one G allele (Kato, et al; 2009).

The second gene to show positive association with breast cancer was COMT low production allele A carriage. Also COMT AG+AA genotypes were common in cases with positive lymphadenopathy and in premenopausal women. The same positive association was observed among American premenopausal women specially the heaviest ones (Thompson, et al;1998**);** Turkish premenopausal women with sporadic breast cancer (Sazci, et al; 2004**);** Chinese premenopausal women **(**Wan, et al; 2014**);** Brazilian women (dos Santos, et al; 2011**);** Chinese women (Wang, et al; 2010). However, in a meta-analysis study; it was observed that no significant association was reported in all genetic models in overall, European, Asian populations **(**Ding, et al; 2010); while Caucasian population had a significantly decreased risk of breast cancer (He, et al; 2012). Similarly, negative association was reported among Caucasian American women (Sigurdson, et al; 2009, Reding, et al; 2009**);** Portuguese women **(**Silva, et al; 2006**);** French women (Delor, et al; 2010**);** Slovenian women (Cerne, et al; 2011**);** Greenlandic Inut women (Ghisari, et al; 2014**);** German Postmenopausal women (MARIE-GENICA, 2009); Thai women (Sangrajrang, et al; 2009); Indian women (Syamala, et al; 2010**,** Naushad, et al; 2011; Syrian women (Lajin, et al; 2011). On the contrary, American women with COMT (AA) showed decreased breast cancer risk among postmenopausal females especially leanest ones (Thompson, et al; 1998**,** Wu, et al; 2003).

The third association was with the CYP1A1 MspI C allele carriage. Other studies have shown similar results and found positive association in American women of African ethnicity (Taioli et al; 1999**);** Polish premenopausal women aged below 50 years **(**Ociepa-Zawal, et al; 2009**);** Brazilian premenopausal women **(**dos Santos, et al; 2011**);** Kazakh women **(**Balmukhanov, et al; 2013**);** On the contrary to this finding, negative association was reported in African American women **(**Kato, et al; 2009**);** German women **(**MARIE-GENICA, 2009**);** Russian women **(**Balmukhanov, et al; 2013**)**; Brazilian women with sporadic breast cancer **(**Oliveira, et al; 2015**);** Thai women (Sangrajrang, et al; 2009).On the other hand, decreased risk with the rare allele was observed among Brazilian women (da Fonte de Amorim, et bal; 2002).

The least association was found with CYP 17 MspA1I C allele positive genotypes (CC+CT) were associated with increased breast cancer risk among Egyptian breast cancer women particularly within cases with negative breast feeding. Other studies have shown similar results reporting positive association with the CYP **17** MspA1I C allele carriage with the development of breast cancer. Examples include the findings reported in American post-menopausal women **(**Chen, et al; 2008**);** Brazilian postmenopausal women **(**dos Santos, et al; 2011**);** Iranian women **(**Hosseini, et al; 2009**);** Indian women younger than 40 years **(**Chakraborty, et al; 2007**);** Chinese postmenopausal females (Zhang, et al; 2009**).**

On the contrary to this findings, negative association was reported in some other studies in American women **(**Kato, et al; 2009**);** German women (MARIE-GENICA, 2009); Turkish women (Karakus, et al; 2015); Portuguese women (Silva, et al; 2006); Indian women (Syamala, et al; 2010); Indian women older than 40 years (Chakraborty, et al; 2007**);** and Thai women **(**Sangrajrang, et al; 2009**).** Reduced risk was reported in Greenlandic Inut women (Ghisari, et al; 2014).

These wide variations in genetic associations might be due to genomic diversity in subjects of different ethnicities; nonetheless it might also arise from biased selection criteria and low power studies. Although this study might also suffer some limitations related to a relatively small sample size and lack of expression analysis, we could conclude that cancer breast in Egyptian women is associated with rare alleles of estrogen biosynthesis and metabolizing genes including CYP1B1 G>C, and COMT G>A followed by CYP1A1 MspI, and CYP17 MspA1I.

Table 1:- PCR primer and conditions used for identification of studied gene polymorphisms.

Polymorphism	***Primers***	***Conditions of PCR***	***PCR Product***	***Restriction enzymes***	***Fragments after digestion***
Cyp17 MspA1I	F: (5'-CAT TCG CAC TCT GGA GTC-3') R: (5'-AGG CTC TTG GGG TAC TTG-3')	Denaturation at 94^0C for 5 min, 30 cycles at 94^0C for 1 min, 58^0C for for 1 min and 72^0C for 1 min and one final cycle of extension at 72^0C for 5 min.	459 bp	MspA1I	- 459 bp for TT (A1A1) wild type. - 335 and 124 bps for CC (A2A2) homozygous gnotype. - 459, 335 and 124 bps for TC (A1A2) heterozygous genotype.
CYP1A1 MspI	F :(5'-TAG GAG TCT TGT CTC ATG CCT -3') R: (5'-CAG TGA AGA GGT GTA GCC GCT -3')	Initial denaturation at 95^0C for 10 minutes, followed by 35 cycles at 95^0C for 30 sec, 55^0C for 30 sec and 72^0C for 1 minute and a final extension of 72^0C for 7 minutes.	340 bp	MspI	- 340 bp for TT wild type - 200 and 140 bp fragments for CC homozygous genotype - 340, 200, 140 bp for TC heterozygous genotype.
CYP1B1 G>C	F: (5'- TCA CTT GCT TTT CTC TCT CC-3') R: (5'- AAT TTC	by initial denaturation at 95^0C for 10 minutes, followed by 35 cycles at 95^0C for 1 minute, 58^0C	650 bp	Eco57I	650 bp for GG wild type 340 and 310 for CC homozygous genotype 650, 340

	AGC TTG CCT CCT G-3')	for 1 minute and 72^{0}C for 1 minute and a final extension of 72^{0}C for 7 minutes.			and 310 bp for GC heterozygous.
COMT G>A	F: (5'-TAC TGT GGC TAC TCA GCT GTG C-3') R: (5'-CTG AAC GTG GTG TGA ACA CC-3').	94°C for 3 minutes and then cycled 35 times at 94°C for 30 seconds, 60°C for 20 seconds, and 72°C for 30 seconds, followed by 72°C for 5 minutes.	237 bp	NlaIII	114 bp for G allele96, 18 bp for A allele

Table 2:- Genotype frequencies of CYP17 MspA1I gene polymorphisms in a sample of Egyptian women with breast cancer compared to controls

***CYP17 T>C* Genotypes**	***BC cases n=152(%)***	***Controls n=100(%)***
TT	42(27.6)	42(42)
TC	93(61.2)	50(50)
CC	17(11.2)	8(8)
T Allele	177(58.2)	134(67)
C Allele	127(41.8)	66(33)
Statistics	*P*	*OR (95% CI)*
CC vs. TC vs. TT	*0.058*	
TC+CC vs. TT (Dominant)	0.026*	1.9 (1.07 – 3.34)
TC vs. TT+CC (Overdominant)	0.1	1.58 (0.9 – 2.7)
CC vs. TT+TC (Recessive)	0.65	1.34 (0.5 – 3.56)
C Allele vs. T Allele	0.058$^{\#}$	1.46 (1.0 – 2.1)
HWE	χ^2=10.09, p<0.05*	χ^2=1.7, p>0.05

*significant p<0.05; # near significant; HWE: Hardy-Weinberg Equilibrium

Table 3:- Genotype frequencies of CYP1A1 MspI gene polymorphisms in a sample of Egyptian women with breast cancer compared to controls

CYP1A1 T>C Genotypes	**BC Cases n= 152(%)**	**Controls n=100(%)**
TT	66(43.4)	76(76)
TC	57(37.5)	24(24)
CC	29(19.1)	0(0)
T Allele	189(62.2%)	176(88%)
C Allele	115(37.8%)	24(22%)
Statistics	*P*	*OR (95% CI)*
CC vs.TC vs. TT	<0.00001**	
TC+CC vs. TT (Dominant)	0.000**	4.13 (2.3 – 7.5)
TC vs. CC+TT (Overdominant)	0.035*	1.9 (1.04 – 3.5)
CC vs. TT+TC (Recessive)	0.000**	NA
C Allele vs. T Allele	0.000**	4.46 (2.68 - 7.47)
HWE	χ2=6.25, p<0.05	χ2=1.86, p>0.05

*significant p<0.05; **significant p<0.001, HWE: Hardy-Weinberg Equilibrium

Table 4:- Genotype frequencies of CYP1B1 G>C gene polymorphisms in a sample of Egyptian women with breast cancer compared to controls

CYP1B1 G>C Genotypes	**BC Cases n= 152(%)**	**Controls n=100(%)**
GG	41(26.9%)	80(80%)
GC	60(39.5%)	20(20%)
CC	51(33.6)	0(0%)
G Allele	142(46.7)	180(90%)
C Allele	162(53.3)	20(10%)
Statistics	p	OR (95% CI)
GG vs. GC vs. CC	<0.00001**	
GC+CC vs. GG (Dominant)	0.000**	10.8(5.66- 20.87)
GC vs. GG+CC (Overdominant)	0.002**	2.61 (1.4 – 4.9)
CC vs. GG+GC (Recessive)	0.000**	NA
C Allele vs. G Allele	0.000**	10.26(5.98 – 17.8)
HWE	$\chi 2$=6.5, p<0.05*	$\chi 2$=1.23, p>0.05

*significant p<0.05; **significant p<0.001, HWE: Hardy-Weinberg Equilibrium

Table 5:- Genotype frequencies of COMT G>A gene polymorphisms in a sample of Egyptian women with breast cancer compared to controls

COMT G>A	**BC Cases n= 152(%)**	**Controls n=100 (%)**
Genotypes		
GG	13(8.6)	74(74)
GA	123(80.9)	26(26)
AA	16(10.5)	0(0)
Alleles		
G	149(49)	173(87)
A	155(51)	27(13)
Statistics	p	OR (95% CI)
GG vs. GA vs. AA	<0.00001**	
GA+AA vs. GG (Dominant)	0.000**	30.43 (14.0 – 76.4)
GA vs. GG+AA (Overdominant)	0.000**	12.07 (6.3 – 23.06)
AA vs. GG+GA (Recessive)	0.002*	NA
A Allele vs. G Allele	0.000**	6.66 (4.09 – 10.9)
HWE	$\chi 2$=58.25, p<0.0001**	$\chi 2$=2.23, p>0.05

*significant p<0.05; **significant p<0.001, HWE: Hardy-Weinberg Equilibrium

Table 6:- Distribution of dominant model of each gene polymorphism among cases according to clinical and demographic parameters.

Parameter	**CYP17 gene CT+CC vs. TT** P, OR(95%CI)	**CYP1A1 gene CT+CC vs. TT** P, OR(95%CI)	**CYP1B1 gene GC+CC vs. GG** P, OR(95%CI)	**COMT gene GA+AA vs. GG** P, OR(95%CI)
Family history (+ve vs. -ve)	0.917, 1.056 (0.335-3.208)	0.139, 0.473(0.153-1.410)	0.479, 1.426(0.474-4.198)	0.064, 3.19(0.73-13.199)
Consanguinity (+ve vs. -ve)	0.895, 0.941(0.346-2.5)	0.213, 0.59(0.23-1.46)	0.182, 1.78(0.69-4.48)	0.680, 0.721(0.104-3.765)
Abortion (+ve vs. -ve)	0.285, 0.642(0.262-1.548)	0.281, 1.465(0.69-3.12)	0.526, 1.28(0.56-2.94)	0.064, 0.174(0.01-1.36)
Breast feeding (+ve vs. -ve)	0.043*, 6.474 (0.85-135.69)	0.104, 0.420(0.127-1.351)	0.851, 1.121(0.308-4.427)	0.123, 0.344(0.073-1.809)
Parity (+ve vs. -ve)	0.209, 1.74(0.679-4.597)	0.328, 0.697(0.317-1.531)	0.359, 0.692(0294-1.640)	0.703, 0.786(0.204-3.257)

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Age at menarche (young vs. old)	0.977, 0.923(0.417-2.035)	1.0, 1.013(0.499-2.055)	0.798, 0.846(0.378-1.885)	0.417, 1.893(0.534-6.792)
Smoking (+ve vs. -ve)	0.168, 0.606(0.278-1.316)	0.270, 0.695(0.345-1.398)	0.122, 0.567(0.259-1.240)	0.428, 0.623(0.177-2.36)
Menopause (+ve vs. -ve)	0,224, 0.638(0.289-1.4)	0.237, 1.476(0.736-2.965)	0.073, 0.509(0.226-1.139)	0.020*, 0.190(0.028-0.959)
Estrogen receptor (+ve vs. -ve)	0.961, 1.018(0.47-2.24)	0.979, 0.991(0.49-2.01)	0.522, 1.273(0.572-2.848)	0.111, 0.397(0.106-1.432)
Progesterone receptor (+ve vs. -ve)	0.876, 0.944(0.430-2.077)	0.986, 1.006(0.495-2.045)	0.414, 1.365(0.608-3.088)	0.089, 0.374(0.10-1.349)
Stage Early vs. late	0.041*, 0.289(0.086-0.961)	0.098, 3.405(0.84-15.977)	0.738,1.535(0.373-7.288)	1.0, 1.344(0.159-29.699)
Grade Early vs. late	1.0,1.547(0.155-37.468)	0.763,0.5(0.056-3.824)	0.877,0.542(0.070-4.843)	0.081,0.121(0.014-1.177)
Metastasis (+ve vs. -ve)	0.557, 0.757(0.267-2.076)	0.673, 0.840(0.344-2.031)	0.966, 0.981(0.360-2.612)	0.062, NA
Lymph nodes (+ve vs. -ve)	0.449, 0.750(0.332-1.681)	0.541, 1.23(0.603-2.504)	0.081, 0.499(0.21-1.167)	0.02*, 0.12(0.01-0.930)

*significant p<0.05; **significant p<0.001, HWE: Hardy-Weinberg Equilibrium

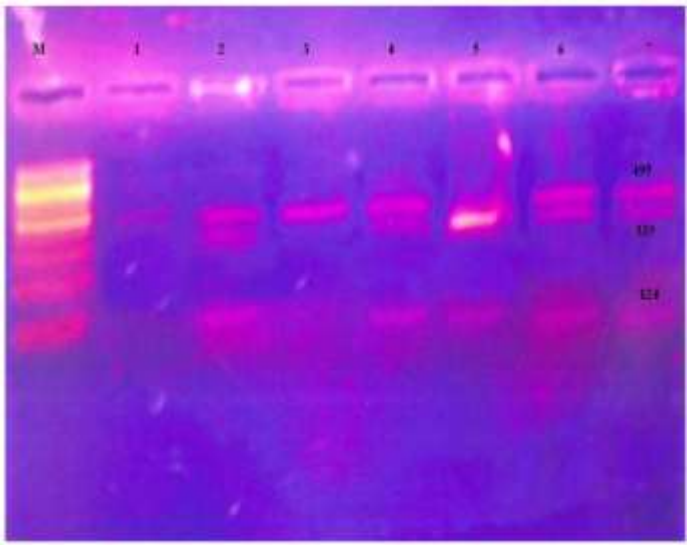

Fig1:- CYP17 MspA1I PCR product digestion using MspA1I. Lane M : 100 bp DNA ladder marker. Lane 1,3: wild TT genotype, lane 2,4,6,7 TC heterozygous genotype and lane 5: homozygous CC genotype

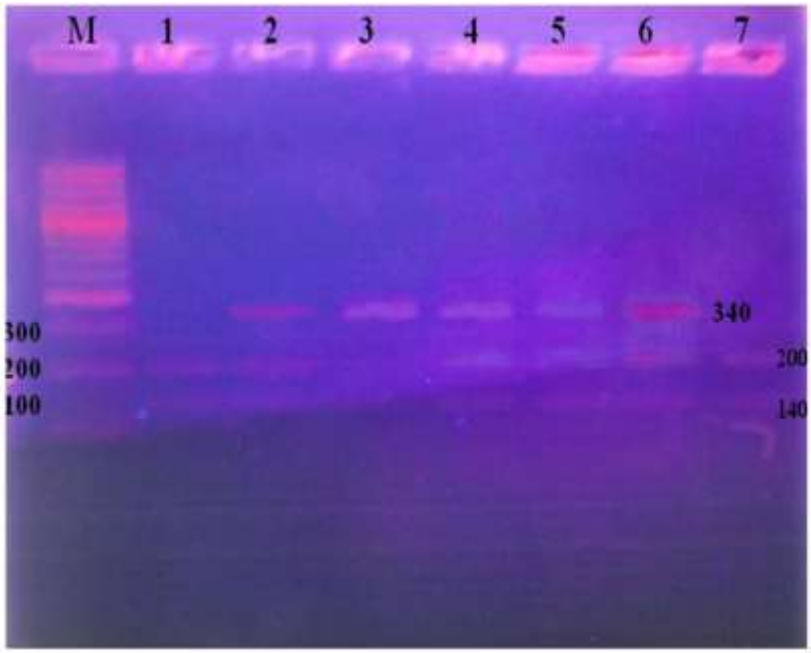

Fig 2:- CYP1A1 T>C polymorphism digestion using MspI enzyme. Lane M 100 bp DNA ladder marker. Lane 3 : wild TT genotype. Lane 2,4 - 6 : heterozygous TC genotype. Lane 1,7: homozygous mutant CC genotype.

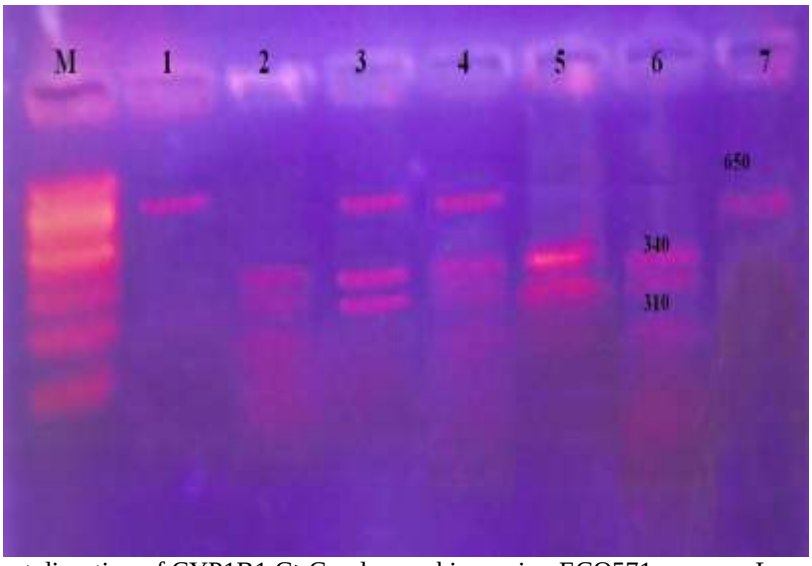

Fig 3:- PCR product digestion of CYP1B1 G>C polymorphism using ECO571 enzyme. Lane M : 100 bp DNA ladder marker. Lane 1, 7: wild type GG, lane 2,5,6 :homozygous mutant CC genotype and Lane 3,4: heterozygous GC genotype.

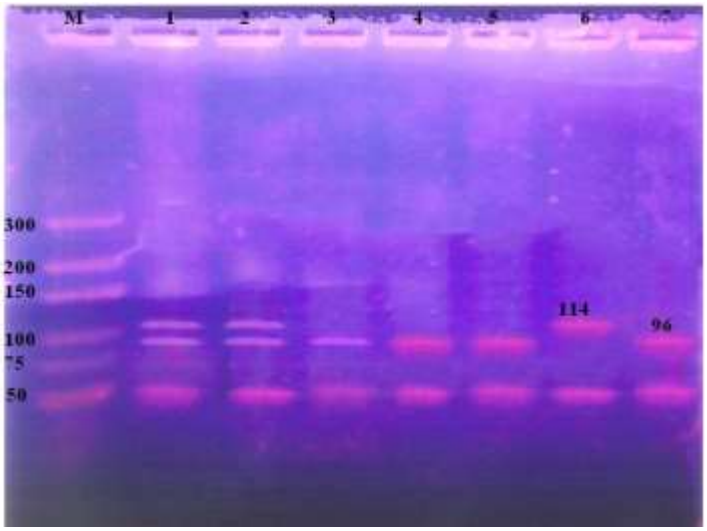

Fig 4:- PCR product digestion of COMT G>A polymorphism using NlaIII enzyme. Every digested PCR Product has 27, 42 and 54 bp fragments. The presence of G allele gives fragment at 114 bp, while the presence of A allele gives 96 and 18 bp fragments. Lane M: DNA ladder marker, lane 1,2: heterozygous GA genotype, lane 6: wild type GG and lane 3-5,7 homozygous mutant AA genotype. The other fragments run out of the gel.

Acknowledgement:-

The authors are very grateful to of all cases and healthy control subjects who participated in this work, and to the team of General Surgery & Oncology Department, Faculty of Medicine , Mansoura University for their great help and support throughout this study.

Conflict of Interest: The authors state that this study is completely free from all issues related to interest conflict**.**

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