Association of matrix metalloproteinases 3 and 9 single nucleotide polymorphisms with breast cancer risk: A case-control study

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Abstract. Two single nucleotide polymorphisms (SNPs) of matrix metalloproteinase (MMPs) 3 and 9 are functionally implicated in the progression of various types of cancer, including breast cancer (BC). However, the roles of these SNPs remain controversial. In addition, they also vary between one population and another. Therefore, the present study aimed to investigate the possible association between MMP3-1171 5A/6A and MMP9-1562 CT SNPs and the risk of BC among Egyptians, and to elucidate the alteration of MMP3 and MMP9 gene expression in patients with BC. The present case-control study enrolled 162 patients with BC and 146 control subjects. Restriction fragment length polymorphism-PCR was performed for analysis of the selected SNPs, gene expression of MMP3 and MMP9 was also assessed in 50 patients and 50 control subjects by reverse transcription-quantitative PCR. The frequencies of 5A/6A genotype and 5A allele of MMP3 were significantly higher in patients with BC compared with in healthy subjects. On the other hand, the distributions of MMP9 genotypes and alleles were not significantly different among patients and healthy subjects. Compared with healthy subjects, the expression levels of the two genes were found to be upregulated in patients with BC. Therefore, the present study indicated that MMP3-1171 5A/6A SNP, not MMP9-1562 C>T SNP may be a risk factor for developing BC among Egyptian females.

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Abbreviations: BC, breast cancer; SNPs, single nucleotide polymorphisms; MMP, matrix metalloproteinase; RT-qPCR, reverse transcription-quantitative PCR; HWE, Hardy Weinberg Equation; ER, estrogen receptor; PR, progesterone receptor; Her-2, human epidermal growth factor receptor 2

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Key words: single nucleotide polymorphisms, matrix metalloproteinase, breast cancer, risk factor, Egyptian

Introduction

Worldwide, breast cancer (BC) is the most frequently diagnosed cancer and it is the primary cause of cancer-deaths among females (1). In Egypt, BC is the prevalent female cancer with a percentage of about 38.8% of all cancers types in women, with increasing mortality rates (up to 1.3% in the years from 2000 to 2011 (2,3). The risk for developing BC has been reported to be associated with many susceptible single nucleotide polymorphisms (SNPs) (4,5). Therefore, deep insights into the mechanisms underlying the involvement of SNPs in cancer susceptibility may lead to better understanding of the molecular pathogenesis of the disease. Moreover, SNPs can be implicated as powerful biomarkers in the prediction and therapy of various cancer diseases (6).

Two integral members of matrix metalloproteinase (MMPs', family MMP3, and MMP9) have a crucial role in the development and metastasis of cancer. MMP3 is a 54 kDa enzyme synthesized in the connective tissue, leading, subsequently; to the activation of other MMPs such as gelatins B and the release of some cell surface molecules including E cadherin. Furthermore, it is implicated in the degradation of many extra cellular components such as collagen III and IV, which may render it as an effective tumor promoter (7). MMP9 is the most complex enzyme of MMPs family with 92 kDa (8), where, under physiological conditions, it binds to gelatin, collagen and laminin of matrix leading to their degradation. However, in pathologic conditions, this function mediates tumor invasion (9).

The role of MMP genes functional polymorphisms in cancer susceptibility has been previously investigated. However, better understanding of the association between MMPs SNPs and BC pre-disposition and prognosis still needs further studies (10). Actually, some of promoter-located SNPs

may lead to allele-specific effects on the expression of MMPs such as-1562 C>T substitution in MMP9 promoter (11), and adenosine-insertion/deletion-at position-1171 in the MMP3 promoter sequence (12). Consequently, these two SNPs attracted researchers to study their possible roles in the progression of BC. However, to the best of our knowledge, the published results, regarding the association between both SNPs and BC risk is still inconclusive and controversial as it had been reported that they vary greatly from one population to another, along with the lack of previous studies investigating such association in the Egyptian population, which urged us to investigate the possible association between both MMP3-1171 5A/6A and MMP9-1562 C/T SNPs with BC susceptibility among a sample of Egyptian females.

Materials and methods

This case-control study enrolled 162 BC patients and 146age-matched cancer-free women. Patients were recruited from Cancer Management and Research Department, Medical Research Institute and Ayadi Al-Mostakbal Hospital (Alexandria-Egypt). In addition, research methodology and sampling in this work were approved by the Research Ethic Committee, Medical Research Institute/Alexandria University. Prior to sample collection, an informed consent was obtained from each participant enrolled in the study. Clinicopathological data of patients including age, pathological type, grade, lymph node status, ER, PR and Her-2 status were also collected. A blood sample from each participant was withdrawn on EDTA-coated tube and stored at -80˚C till time of investigations.

DNA extraction. Extraction of genomic DNA was performed using Invitrogen Pure Link Genomic DNA mini kit (Thermo Fischer Scientific, Inc.) following manufacturer instructions. Assessment of DNA purity and concentration were performed by measuring sample absorbance at 260/280 nm with Nano Drop® ND-1000 UV-Visible Spectrophotometer (Thermo Fischer Scientific, Inc.). The extracted DNA was stored at -20˚C till genotyping.

Genotyping of MMP3-1171 5A/6A SNP. Restriction fragment length polymerase chain reaction was performed for genotyping of MMP3. DNA was amplified by PCR using BIO-RAD T100 Thermal-cycler (Thermo Fischer Scientific, Inc.). The reaction was carried-out in a final volume of 25 μl consisting of 12.5 μl of 2x Dream Taq green master mix with dual dye (Thermo Fischer Scientific, Inc.), 100 ng DNA template and 0.5 μmol of forward/reverse primer (13) (Table I). The thermal profile was as follows: 3 min initial denaturation at 94˚C, followed by 35 cycles of denaturation for 30 sec at 95˚C, annealing at 56.1˚C for 30 sec, and extension at 72˚C 30 sec, then a final extension at 72˚C for 10 min. For digestion; 10 μl of PCR products were incubated with 5 U of the enzyme PsyI (Promega Corporation) for 2 h at 37˚C. After digestion, the different genotypes of MMP3 were visualized on 3% agarose gel, the homozygous 6A/6A genotype produced a large fragment at 129 bp whereas the homozygous 5A/5A genotype had shorter fragments at 97 and 32 bp. Heterozygote 5A/6A genotype generated three bands at 129, 97 and 32 bp (Fig. 1A).

Genotyping of MMP9-1562 C/T. For the analysis of MMP9-1562C/T polymorphism, PCR was carried out in 25 μl reaction volume as described previously using the suitable primers pair (14). The PCR cycling conditions were as follows: 3 min of initial denaturation at 95˚C, then 35 cycles of denaturation for 30 sec at 95˚C, then annealing for 30 sec at 58˚C and extension for 30 sec at 72˚C, followed by 10 min at 72˚C as final extension. For digestion, 10 μl the PCR product were incubated with 5 U of the restriction enzyme Sph1 (Promega Corporation) at 37˚C for 2 h. Upon digestion, The CC genotype produces single large fragment at 460 bp, whereas TT genotypes produces shorter fragment at 202 bp, and the heterozygote genotype CT generates a combination of three fragments (460 bp, 202 bp and 258 bp bands).

MMP3 and MMP9 gene expression. MMP3 and MMP9 gene expression was investigated in 50 patients and 50 cancer-free control subjects. Total RNA was extracted from the whole blood using Invitrogen PureLink RNA mini kit, following the manufacturer's instructions. Purity of the extracted RNA was assessed via NanoDrop® ND-1000 UV-Visible Spectrophotometer (both from Thermo Fischer Scientific). Reverse transcription-quantitative PCR was performed using Top real TM one step RT-qPCR kit (SYBR-Green with low ROX.RT qPCR). PCR was performed using 10 μl reaction mix, 2 μl of RNA, 1 μl of each appropriate primer (15) (Table I), 1 μl 2x enzyme mix and 5 μl of water. The thermal profile was as follows: Hold 30 min at 50˚C, initial denaturation 10 min 95˚C, followed by 45 cycles of a denaturation step at 95˚C for 5 sec and an annealing step at 50˚C for 30 sec. qPCR reactions were carried out in duplicates for each sample, then the relative expression of MMP3 and MMP9 was normalized to GAPDH as a house keeping gene and fold-change was calculated with the $2^{-\Delta\Delta Cq}$ method (16).

Statistical analysis. Statistical analyses were carried out using SPSS 22.0 software. Hardy Weinberg equation (HWE) was performed to compare the frequencies of observed to expected genotype in the studied groups, Chi Square (χ^2) test used to compare genotypes distribution between the two study groups. The association between different genotypes and cancer risk was assessed by calculating odd ratio (OR) at corresponding 95% confidence interval (95% CI). Kruskal Wallis test was used to analyze the variation of gene expression among different genotypes of MMP3 and MMP9. The associations between both of different genotypes of MMP3, MMP9 and gene expression with the clinicopathological characteristics were analyzed using Chi Square (χ^2) test. Kaplan-Meier analysis was used to study the associations between the different genotypes of MMP3 and MMP9 and disease-free and overall survival rates. $P<0.05$, was statistically considered significant.

Results

Clinicopathological characteristics of patients. The clinicopathological parameters of all patients are represented in Table II. Concerning the histological grade, about three quarters of patients were of grade II, whereas one quarter

Table I. RFLP-PCR and reverse transcription-quantitative PCR primers for MMP3, MMP9 and GAPDH.

Genes	RFLP-PCR primers (5'-3') (13,14)[a]	Reverse transcription-quantitative PCR primers (5'-3') (15)[a]
MMP3	F: CTTCCTGGAATTCACATCACTGCCACCACT	F: GTCTCTTTCACTCAGCCAAC
	R: GGTTCTCCATTCCTTTGATGGGGGGAAAGA	R: ATCAGGATTTCTCCCCTCAG
MMP9	F: GCCTGGCACATAGTAGGCCC	F: CCTTCCTTATCGCCGACAAG
	R: CTTCCTAGCCAGCCGGCATC	R: TGAACAGCAGCATCTTCCCC
GAPDH	-	F: GACCTGCCGTCTAGAAAAAC
	-	R: TTGAAGTCAGAGGAGACCAC

[a]References for the primers used in RFLP-PCR and quantitative PCR. MMP, matrix metalloproteinase. RFLP-PCR, restriction fragment length polymorphism-PCR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; F, forward; R, reverse.

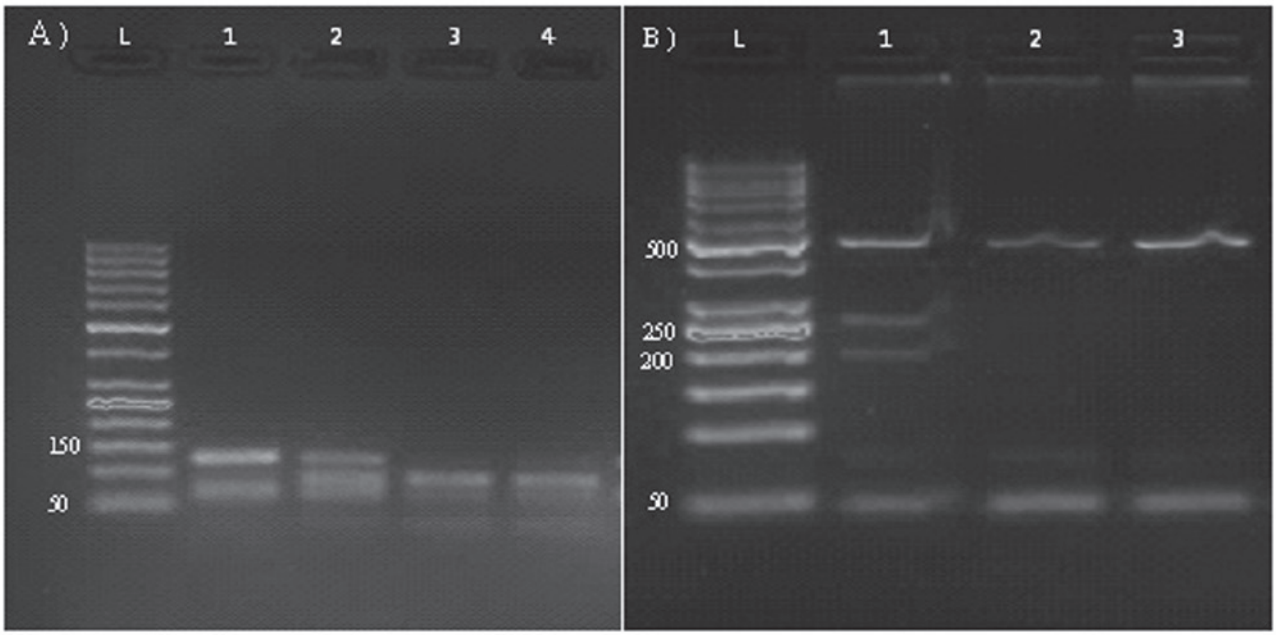

Figure 1. Agarose gel electrophoresis showing digestion of PCR products of MMP3 and MMP9 genes. (A) Agarose gel (3%) stained with ethidium bromide revealing digestion of PCR product using the *Psy*1 restriction enzyme. Lane L is a 50 bp DNA ladder, lane 1 is the homozygous 6A/6A genotype, lanes 3 and 4 are homozygous 5A/5A genotype, lane 2 is the heterozygous 5A/6A genotype. (B) Agarose gel (2%) stained with ethidium bromide revealing digestion of PCR product using *Sph*I restriction enzyme. Lane L is the 50 bp DNA ladder, lane 1 is the heterozygous CT genotype, and lanes 2 and 3 are the homozygous CC genotype.

of them were of grade III. With respect to pathology type, invasive ductal carcinoma was found in more than 95% of patients. More than half of patients had positive axillary lymph nodes. With regarding to receptors' status, positive ER and PR were found in more than half of patients, whereas almost two third of patients were negative to HER-2 expression, and no triple negative BC patients were found among enrolled patients.

Distribution of genotypes and allelic frequencies of MMP3-1171 5A/6A SNP. The genotypes distributions in the healthy females did not significantly differ from those predicted by HWE (P=0.164), in BC patients the distribution deviated from that expected by HWE (P=0.005). The distributions of MMP3 genotypes and allele frequencies are presented in Table III. Our results indicated a significant difference in distribution of 5A/6A, 6A/6A, and 5A/5A genotypes between BC patients and the healthy females groups (P=0.006, 0.003, and 0.011, respectively). Furthermore, results also indicated that both 5A/6A and 5A/5A genotypes are associated with higher BC risk (OR=2.545, 95% CI=1.379-4.695, P=0.0028, OR=5.760, 95% CI=1.267-26.187, P=0.0234, respectively) as compared with 6A/6A genotype. The odds ratio for 5A allele indicated that it is associated with 3.021-fold increased BC risk as compared with the 6A allele.

Distribution of genotype and allelic frequencies of MMP9-1562 C> T SNP. Fig. 1B shows only two genotypes of-1562 C>TMMP9 polymorphism, where no homozygote TT

Table II. Clinicopathological parameters of patients with BC.

Clinicopathological parameters and characteristics	Patients, n (%)
Histological grade	
II	121 (74.7)
III	41 (25.3)
Involved axillary lymph nodes	
Negative	67 (41.4)
Positive	95 (58.6)
Pathological type	
Invasive ductal carcinoma	157 (96.9)
Invasive lobular carcinoma	5 (3.1)
ER status	
Negative	54 (33.3)
Positive	108 (66.7)
PR status	
Negative	65 (40.1)
Positive	97 (59.9)
HER-2 status	
Negative	117 (72.2)
Positive	45 (27.8)

BC, breast cancer; ER, estrogen receptor; PR, progesterone receptor.

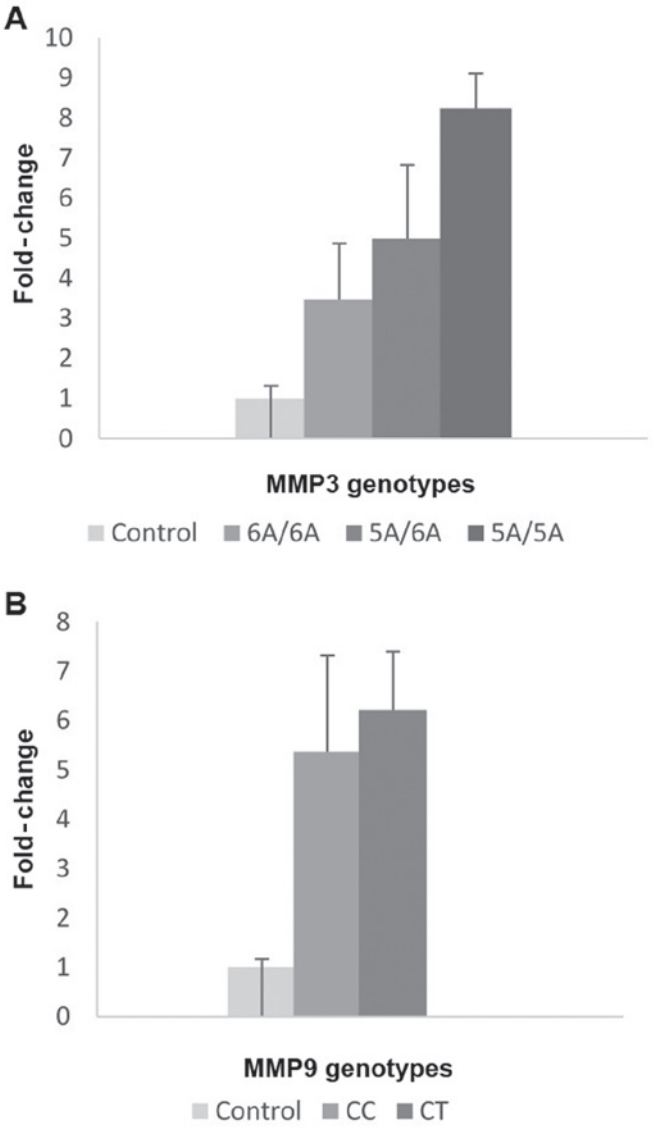

Figure 2. Fold-change in gene expression among different genotypes of (A) MMP3 and (B) MMP9 in 50 patients with BC compared with healthy subjects. Results are expressed as the mean ± SEM. Kruskal Wallis test was used to analyze the variation of gene expression among different genotypes of MMP3 and MMP9. BC, breast cancer; MMP, matrix metalloproteinase.

genotype was found among the enrolled subjects in the study. The distribution of MMP9 genotypes did not deviate from those expected by HWE in both patients and control groups (P=0.287, P=0.484 respectively). The results represented in Table III indicated that the distribution of CC and CT genotypes did not significantly differ when comparing cancer patients and healthy women groups (P=0.249), the distribution of C and T alleles did not show any significant difference between cancer and control group (P=0.266). Furthermore, the results of this study also indicate that MMP9 polymorphism is not associated with BC risk, however, the T allele may be a weak risk factor for progression of the disease (OR=1.442, 95% CI=0.754-2.759).

Gene expression of MMP3 and MMP9. The gene expression of MMP3 showed an insignificant up regulation in patients as compared to the healthy subjects, however, the expression in patients with 5A/5A genotype was about two times higher than6A/6A genotype patients (Fig. 2A). The expression of MMP9 in patients was significantly up regulated in patients (P=0.021). Moreover, the expression in CT genotype patients was found to be higher as compared to patients suffering from CC genotype (Fig. 2B).

Association of MMP3 and MMP9 gene polymorphisms and gene expression with clinicopathological features. Genotypes distribution of MMP3 and MMP9 polymorphisms with respect to patient's clinical characteristics are represented in Table IV. With regard to MMP3, 6A/6A genotype was associated with grade II. But, regarding lymph nodes (LNs) status, about half of patients with negative LNs were of 6A/6A genotype, and it was noted that 6A/6A genotype was associated with the expression of ER, PR, and more than 50% of patients with positive Her-2 had 6A/6A genotype. With respect to MMP9, CC genotype was associated with the less aggressive pathological type grade II. Furthermore, the majority of patients with negative LNs had CC genotype, concerning the receptors status CC genotype associated with the presence of ER and PR.

Table V illustrates the correlation between both of MMP3 and MMP9 gene expression and patient's clinical characteristics. Regarding MMP3, the expression was significantly associated with positive PR and Her-2 (P=0.032, P=0.034 respectively). However, no significant link was found with the other characteristics. On the other hand, MMP9 was significantly associated with Her-2 receptor, but no other significant association between its expression and the other clinicoplathological parameters was noted.

Table III. Distribution of MMP3 and MMP9 genotypes and alleles frequency in patients with BC and healthy females.

Genes	Genotype	Patients, n (%) (n=162)	Controls, n (%) (n=146)	χ^2	P-value	OR	95% CI
MMP3	6A/6A	110 (67.901)	126 (86.301)	14.516	0.003[a]	Reference (1)	
	5A/6A	40 (24.691)	18 (12.321)	7.678	0.006[a]	2.545[a]	1.379-4.695
	5A/5A	12 (7.407)	2 (0.69)	6.452	0.011[a]	5.760	1.267-26.187
Alleles	5A	64 (19.753)	22 (7.534)	19.089	<0.001[a]	3.021[a]	1.808-5.048
	6A	260 (80.246)	270 (92.465)			0.331	0.198-0.553
MMP9	CC	137 (84.567)	130 (89.042)	1.332	0.249	Reference (1)	
	CT	25 (15.432)	16 (10.958)			1.483	0.7573-2.9028
	TT	0 (0)	0 (0)				
Alleles	C	299 (92.284)	276 (94.521)	1.237	0.266	0.693	0.363-1.326
	T	25 (7.716)	16 (5.479)			1.442	0.754-2.759

[a]Statistically significant (P<0.05). A χ^2 test was used to compare the genotype distribution among subjects studied. BC, breast cancer; MMP, matrix metalloproteinase; OR, odds ratio.

Table IV. Associations of MMP3 and MMP9 genotypes with clinicopathological parameters of patients with BC.

	MMP3 genotypes			MMP9 genotypes	
Characteristics	5A/6A	6A/6A	5A/5A	CC	CT
Grade					
II	28	84	9	108	13
III	16	22	3	29	12
χ^2	3.905	3.364	0.001	8.05	
P-value	0.049	0.061	0.980	0.005[a]	
Axillary lymph node status					
Negative	28	35	4	59	8
Positive	14	73	8	78	17
χ^2	14.975	10.702	0.344	1.067	
P-value	<0.001[a]	0.001[a]	0.557	0.302	
ER status					
Negative	17	33	4	42	12
Positive	27	73	8	95	13
χ^2	0.764	0.669	≥0.999	2.682	
P-value	0.382	0.414	≤0.001[a]	0.091	
PR status					
Negative	23	37	5	51	14
Positive	21	69	7	86	11
χ^2	3.711	3.475	0.013	3.102	
P-value	0.052	0.063	0.910	0.078	
Her-2 status					
Negative	31	76	10	96	21
Positive	18	25	2	40	5
χ^2	2.809	1.224	0.798	1.218	
P-value	0.089	0.271	0.370	0.288	

[a]Statistically significant (P<0.05). χ^2 test was used to study the association between genotypes and clinicopathological parameters. BC, breast cancer; ER, estrogen receptor; MMP, matrix metalloproteinase; PR, progesterone receptor.

Survival analysis. The median of disease-free and overall survival rates showed no significant difference between the different genotypes of MMP3-1171 5A/6A and MMP9-1562 C> T SNPs (Fig. 3).

Table V. Associations between MMP3 and MMP9 gene expression and clinicopathological parameters of patients with BC.

Genes	Positive LNs	ER status	PR status	HER-2 status	Vascular invasion
MMP3					
χ^2	0.19	2.82	4.60	4.47	0.91
P-value	0.66	0.09	0.03[a]	0.03[a]	0.34
MMP9					
χ^2	0.81	0.40	0.01	5.43	0.42
P-value	0.37	0.53	0.91	0.02[a]	0.52

[a]Significant association. BC, breast cancer; ER, estrogen receptor; LN, lymph node; MMP, matrix metalloproteinase; PR, progesterone receptor.

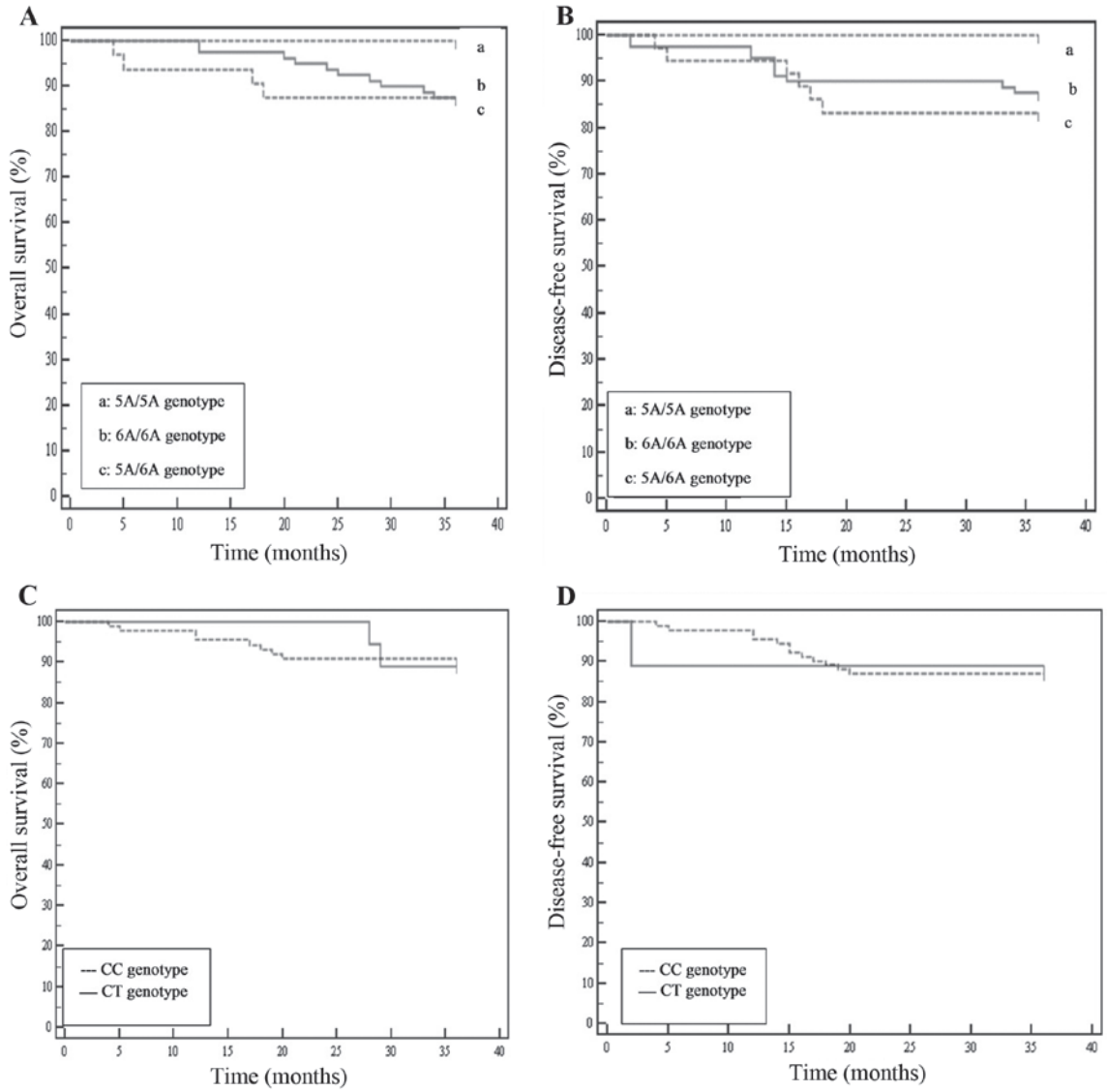

Figure 3. Survival analysis for MMP3 and MMP9 genotypes. (A) Overall and (B) disease-free survival for MMP3 genotypes. (C) Overall and (D) disease-free survival for MMP9 genotypes. Kaplan-Meier analysis was used to study the associations between the MMP3 and MMP9 genotypes and disease-free and overall survival rates. MMP, matrix metalloproteinase.

Discussion

Compared to western females, BC in Egyptian females may show some different characteristics, where the disease may have different clinical, pathological and molecular profiling (17). A suitable explanation of such inter-individuals variations of tumor characteristics may be obtained through analysis of different SNPs, which may also facilitate the

prediction the patient's prognosis (8). Furthermore, and along with investigating the variations in gene expression, SNPs analysis may be promising predictive and prognostic markers, which may lead to better management of the disease (18).

Most of the reported SNPs in MMPs genes are functionally implicated in the progression of BC (19-21) particularly; those found in the promoter region and alter gene expression, which may result in imbalance in the MMP system, which, in turn, leads to excessive degradation of ECM and deregulation of ECM dynamics in cancer development (22).

The present study indicates that 5A/6A and 5A/5A genotypes of MMP3 are associated with an increased risk for developing BC. These results are in line with previous findings of AbdRaboh in 2016, who reported that 5A allele is associated with increased risk of BC in a sample of BC patients in UAE (23). In addition, Padala in 2017, reported that 5A/6A genotype increased the risk of BC development among Indian population (22).

With regard to MMP9, the present results are consistent with that of Toroghi and his co-workers in 2017, who reported that MMP-9 polymorphism [-1562 C>T] is not associated with the BC incidence in the Iranian population (9). Many studies investigated the links connecting the MMP-9 promoter polymorphism [-1562 C>T] with cancers. However, the published results are inadequate and inconclusive to confirm the relation of this SNP with the increased risk of cancer (24,25). Lei *et al* (20) found a moderate increase in BC risk in TT homozygote genotype, whereas Przybylowska *et al* 2006 negated the association between T allele and cancer development (26). However, MMP9 polymorphism was found to act as genetic modifiers for the prognosis of BC in the Chinese population (27).

The present study reveals an insignificant upregulation of MMP3 expression but a significant upregulation of MMP9 expression in the BC patients. Such upregulation of both genes may be attributed to the fact saying that MMPs are greatly implicated in the cancer development and progression (28). Aberrant MMP expression is related to elevated risk of different types of cancers (29,30). In addition, gene expression of MMP3 and MMP9 is reported to be up-regulated and to be implicated as prognostic marker in different types of cancers such as gastric, melanoma, lung, colorectal, and BC (24,31,32).

The 5A allele is associated with elevated transcriptional activity (33). However, insertion of an adenosine at the-1171 position in promoter region reduces its transcriptional activity to about half, as such insertion at the polymorphic site increases the binding affinity of the promoter for the repressor ZBP-89, which is one of the transcription factor that regulate the activity of the MMP3 gene expression (34). Moreover, the levels of both MMP3 mRNA and protein were reported to be significantly elevated in the skin tissue of individuals with 5A/5A genotype than 6A/6A individuals, and intermediate in 5A/6A heterozygotes individuals (35).

On the other side, several cis elements in the promoter region of MMP9 have been reported to regulate its expression at the transcription level. These include 2 AP-1 sites (2533, and 279, bound by transcription factors c-Fos and c-Jun), a PEA3 motif (2540, recognized by transcription factor Ets), and a consensus sequence (2600) for binding of nuclear factor-kB. C-1562T polymorphic site is also an important regulatory element through acting as a binding site for transcription repressor proteins (36). Where, The C to T substitution at position-1562 in MMP-9 promoter region increases its transcriptional activity as T-allele lacks the ability of binding to the repressor protein (26). The T allele promotes BC progression through its enhancing effects on the MMP9 expression and activity, which, in turn, increases ECM degradation and invasion (14,24).

Additionally, a direct association between increased expression of MMP9 protein and bad prognosis among BC patients (37) was reported. Furthermore, differential MMP9 expression modulates the degree of cellular differentiation and results in the increased aggressiveness of BC (38). This may be attributed to the fact saying that the-1562 C/T and -1171 6A/5A SNPs in the MMP9 and MMP3 can modify the binding affinity of some transcription factors, leading, in turn, to alteration in their gene expression (39,40). Moreover, the up regulation of MMP3 and MMP9 expression resulting from high transcriptional activity of either 5A allele or T allele may promote the development and growth of various malignancies (31,41).

The results of survival analysis are in accordance with the finding of Padala *et al* (22), who reported decreased survival rate associated with risk genotypes of MMP3 and MMP9 SNPs, while statistically, it is insignificant. In addition Song *et al* (42) reported a positive association between the expression of MMP9, the increased risk of cancer recurrence and the reduced the survival rates of BC patients.

To the best of our knowledge, this is the first study to investigate the association of *MMP-3* promoter-1171 5A/6A, *MMP-9* promoter-1562C>T SNPs and the risk of BC among Egyptians. It was found that 1171-5A/6A MMP3 SNP is associated with an increased risk of BC. However, polymorphism in MMP9 is not associated with the risk. In addition, the absence of homozygous TT genotype among the study population may indicate the importance of studying SNPs among different populations as the genetic composition was reported to be greatly varied from one population to another.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

FAERI designed the study and wrote the protocol with the assistance of MH and NAAEM. NAEI collected the samples and participated in the laboratory investigations. NAAEM and ME interpreted the clinical data of patients. FAERI and SEE contributed to laboratory investigations and statistical analysis. MAE provided the laboratory equipment and consultation as well as interpretation of results. FAERI wrote the manuscript

and NAAEM and MH proofread it. All authors provided advice and approved the final manuscript.

Ethics approval and consent to participate

The research methodology in the present work was approved by Research Ethical Committee of the Medical Research Institute, Alexandria University (10RG #:10RG0008812; Alexandria, Egypt) and written informed consent was obtained from each participant enrolled in the study prior to sample collection. Experimental procedures and sampling followed the international and national regulations in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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