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RESEARCH ARTICLE

ASSOCIATION STUDY BETWEEN ANGIOTENSIN II TYPE 2 RECEPTOR (T1247G and A5235G) POLYMORPHISMS AND BREAST CANCER AMONG EGYPTIAN FEMALES

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Abstract

Background: Breast cancer is the most common type of cancer among women. Many types of cancer are associated with polymorphisms of the renin-angiotensin system (RAS). Angiotensin II (Ang II) is a vasoconstrictor, a mitogen and an angiogenic factor. Ang II exerts its effect through two receptors (AGTR 1-2). T1247G and A5235G are two SNPs within the 5′-region of the AGTR2 gene**.**

Objectives: Our aim was to investigate the association between (T1247G and A5235G) SNPs with breast cancer among Egyptian females.

Patients and Methods: One hundred Egyptian women were included in the present study; 50 cases and 50 controls. Data about age, menstrual and family history were obtained. TNM category and axially LN status were also assessed in breast cancer patients. All subjects were genotyped for AGTR2 (T1247G and A5235G) SNPs using the Custom TaqMan SNP Genotyping assays.

Results: For A5235G, women carrying genotypes AA/AG were more likely to develop breast cancer than GG carriers (OR = 4.125, 95% CI: 1.473-11.555). A significant association was observed between (A5235G) genotypes and both TNM category and axillary LN. Also, a significant association between (T1247G) genotypes and TNM category with the mutant GG genotype being predictor of poor outcome. Multivariate logistic regression analysis proved that (A5235G) SNP genotypes significantly contribute to the prediction of breast cancer risk.

Conclusions: Our study suggests that RAS is implicated in the development and in the invasion of breast cancer.

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Introduction:-

The octapeptide Angiotensin II (Ang II) is the major biologically active component of the renin-angiotensin system (RAS).(Rodrigues-Ferreira *et al.*, 2012a)

The RAS and Ang II have a great role in regulation of blood pressure, tissue remodeling and inflammatory pathologies.(Lemarie and Schiffrin, 2010; Rodrigues-Ferreira *et al.*, 2012b) Ang II can be released in blood stream or produced locally by stromal and endothelial cells, thus excerting both paracrine and autocrine effects. (Rodrigues-Ferreira *et al.*, 2012b)

Ang II exerts its biological functions though binding to three main G-protein-coupled receptors known as angiotensin II receptor type 1 (AGTR1), type 2 (AGTR2) and angiotensin II receptor type 4 (AGTR4). The AGTR2 shows only 34% of their nucleic acid sequence with the AGTR1. (Guimond and Gallo-Payet, 2012)

The AGTR1 is abundantly expressed in adults in heart, adipose tissue, kidney and adrenal gland. (Lemarie and Schiffrin, 2010) In contrast, the AGTR2 are highly expressed in fetal tissues. However, their expression decreases under normal conditions in adults being restricted to adrenal grand, kidney, uterine myometrium and brain. (Carey *et al.*, 2000)

Ang II can regulate cell turnover by promoting cell growth and proliferation as well as apoptosis and programmed cell death. It can be explained by the opposite action of Ang II on AT1 and AT2 receptors. Signaling of Ang II via AGTR1 can induce vasoconstriction, cell growth, and inflammation. However, binding of Ang II to type 2 receptor counteracts actions mediated by AGTR1, promoting vasodilatation, apoptosis and antigrowth effects. The growth inhibiting effects of AGTR2 are due to activation of phosphatases, resulting in the inactivation of extracellular-signal- regulated kinases.(Carey *et al.*, 2000; Lemarie and Schiffrin, 2010)

In human, the gene encoding AGTR1 is located on chromosome 3q21-25, and have 5 exons. (Molina Wolgien Mdel *et al.*, 2014) The AGTR2 gene is mapped to human chromosome Xq22-q23. It has 3 exons and exon 3 has the entire reading frame. The cDNA for the AGTR2 encodes for a protein of 363 amino acids with a molecular weight of 41 KDa. (Li *et al.*, 2012)

T1247G (NCBI Ref. SNP ID: rs 5950584) is a non-coding single nucleotide polymorphism in which there is a T/G transversion at position 1247 within the AGTR2 gene. (Wolgien *et al.*, 2013; Molina Wolgien Mdel *et al.*, 2014)

Another gene polymorphism of AGTR2 is an A/G transition at position 5235 (A5235G) within the intronic region of the AGTR2 gene. (NCBI Ref. SNP ID: rs 1403543). (Wolgien *et al.*, 2013; Molina Wolgien Mdel *et al.*, 2014)

Most components of the RAS are expressed locally in a wide variety of tumors including breast tumors.(Tahmasebi *et al.*, 2006; Rhodes *et al.*, 2009; George *et al.*, 2010) Angiotensin II is considered among the growth promoting and tissue remodeling factors that may be subverted in cancer. In addition, both AGTR1 and AGTR2 are present in tumors and may be up-regulated in some.(Vinson *et al.*, 2012) To date, most studies have mainly focused on the role of AGTR1 receptor in cancer, while the role of AGTR2 has been largely neglected. (Rodrigues-Ferreira *et al.*, 2012a)

The significant association of AGTR2 signaling with different types of cancer has been proved in several studies.(Tahmasebi *et al.*, 1999; Bose *et al.*, 2009; Dolley-Hitze *et al.*, 2010; Neo *et al.*, 2010; Gallagher *et al.*, 2011) Recent studies showed that the effects of AT2 receptors in cancer remain controversial whether AGTR2 antagonizes, or mimic, the effects of AGTR1 subtype on cancer cell proliferation and invasion.(Arrieta *et al.*, 2008; Clere *et al.*, 2010; Doi *et al.*, 2010; Pickel *et al.*, 2010)

Still little is known about the effects of AT2 receptors in breast cancer. AT2 receptors expression has been shown to be significantly increased in breast hyperplasia and cancer. (Rodrigues-Ferreira *et al.*, 2012a) In addition, the use of angiotensin receptor blockers effectively reduce tumor growth, angiogenesis and metastasis.(Singh *et al.*, 2015) AGTR2 may thus represent a new therapeutic target that improve breast cancer diagnosis and treatment planning. (Rodrigues-Ferreira *et al.*, 2012a; Singh *et al.*, 2015)

In Egypt, breast cancer is estimated to be the most common cancer among females accounting for 37.7% of their total with 12,621 new cases in 2008. (Zeeneldin *et al.*, 2013)It has been suggested that breast cancer is associated with gene polymorphism of some RAS components.(de Noronha *et al.*, 2012) However, until now, no data are available regarding the association between AGTR2 (T1247G and A5235G) polymorphisms and breast cancer in Egyptian females.

Aim of the study:-

Studying the association between angiotensin II type 2 receptor (T1247G and A5235G) polymorphisms and breast cancer among Egyptian females.

Subjects & Methods:-

- **Subjects**

One hundred eligible Egyptian women were included in the present study; 50 breast cancer cases and 50 healthy matched controls. They were recruited from the outpatient clinic of the MRI hospital. This study has been approved by the Ethics Committee of Alexandria University and a written informed consent was obtained from all participants. Those patients taking ACEIs and ARBs as well as those with other malignancies or lung, liver and renal diseases were excluded from the study.

Detailed information about demographic factors, menstrual and family history were obtained. Medical and drug history with special stress on the use of hormonal replacement therapy, OCPs, and antihypertensive drugs was taken. TNM category and axially LN were also assessed in breast cancer patients. All cases had their tumor surgically and histologically confirmed.

Routine laboratory investigations

The following investigations were done using RxL Max Dimension autoanalyzer: fasting serum glucose, serum urea and creatinine, serum activity of alkaline phosphatase as well as serum transaminases. (Burtis *et al.*, 2012) Serum CA 15.3 was conducted on ADVIA Centaur® XP, using two step sequential chemiluminescent immunometric assay.(Slev *et al.*, 2006)

Molecular studies:-

- **DNA extraction**

Genomic DNA was extracted from peripheral blood leukocytes using Pure Link™ Genomic DNA Mini Kit . The concentration and purify of extracted DNA was assessed using a Nanodrop 2000 spectrophotometer and then stored at -20ºC till genotyping.

- **Genotyping**

Allelic discrimination of the two studied polymorphisms of AGTR2 was done using TaqMan® SNP genotyping assays specific to each SNP (Shen *et al.*, 2009) (Applied Biosystems, Palo Alto, CA, USA) (T1247G rs5950584; A5235G rs1403543).

Rotorgene Q Real time PCR system (Applied Biosystems, Palo Alto, CA, USA) was employed to obtain specific sequences amplification according to the following steps: 10µl of TaqMan® universal PCR Master Mix (2X) was added followed by 0.5µl of specific assay mix (40X) (T1247G and A5235G) containing primers and probes. Then 1-20ng of extracted DNA and sterile water were added to complete a total volume of 20µl. The thermal profile was a such: An initial step at 95ºC for 10 minutes (hold), followed by 40 cycles of denaturing to 95ºC for 15 seconds and annealing/ extension at 60ºC for 1 minute.

Statistical analysis of the data (Kotz *et al.*, 2006):-
Data were fed to the computer and analyzed using IBMSPSS software package version 20.0. (Kirkpatrick and Feeney, 2013) Qualitative data were described using number and percent. Quantitative data were described using range, mean, standard deviation and median. Chi-square test or Fisher's Exact and Monte Carlo correction was used to compare between different groups. For normally quantitative variables, to compare between two studied groups, Student t-test and for abnormally quantitative variables, Mann Whitney test was applied. Odd ratio was used to calculate the ratio of the odds and 95% Confidence Interval of an event occurring in one risk group to the odds of it occurring in the non-risk group. Univariate and Multivariate logistic regression was assessed. Significance of the obtained results was judged at the 5% level.

Results:-

The mean age of cases and controls was 52.62±9.53 and 50.18±7.92 years, respectively. There was no statistical significant difference between both groups regarding age (p=0.167). In addition, there were no significant differences between both groups for other variables and routine laboratory investigations. (Table 1, 2) A statistically significant difference in both age of menarche and age of menopause between breast cancer patients and controls was found (p=0.001, 0.003, respectively). (Table 1)

AGTR2 (A5235G) polymorphism:-

The breast cancer patients had a higher frequency of the wild genotype (AA) than controls (40% vs 20%). They also had a higher percentage of heterozygous genotype AG than controls (48% vs 44%) and lower frequency of mutant genotype GG than controls (12% vs 36%). A statistical significant difference in genotype frequencies was observed between the two groups (p=0.003) with AA/GG carriers being more likely to develop breast cancer than GG carriers (OR = 4.125, 95% CI: 1.473-11.555). (Table 3)

The allele frequency was also analyzed for AGTR2 (A5235G) SNP. We found a statistical significant difference in allele frequencies between cases and controls (p= 0.002). The patients had higher frequency of the A allele than controls (64% vs 42%) and the A allele was associated with increased breast cancer risk than G allele (OR = 2.455, 95% CI: 1.389 – 4.339).

A statistical significant association was observed between (A5235G) SNP genotypes and both TNM category and axillary LN (p=0.027, 0.003, respectively). It was concluded that GG genotype is a predictor of a better prognosis and the presence of A allele may be linked to a more advanced stage of cancer.

No significant deviation from Hand-Weinberg equilibrium was observed in either case and control groups and the allele frequency was balanced among both groups. (p=0.768 and 0.493) (Table 5)

Multivariate logistic regression analysis was done for breast cancer patients to measure the strength of association between genotypes frequencies, age of menarche, age of menopause and breast cancer. It was demonstrated that age of menarche and AGTR2 (A5235G) SNP genotypes followed by the age of menopause significantly contribute to the prediction of breast cancer risk (p = 0.002, 0.004, 0.016, respectively). (Table 6)

AGTR2 (T1247G) polymorphism:-

The (T1247G) SNP genotypic distributions were as follows: 74% for the TT, 22% for the heterozygous TG and 4% for the GG, respectively, in breast cancer patients and 70% for the TT, 28% for the homozygous TG, and 2% for the GG, respectively in controls.

There was no statistical significant difference in genotype frequencies among patients and controls (p = 0.298). Likewise, no significant difference (p = 0.845, OR =0.926 with 95% CI: 0.431-1.993) in the AGTR2 (T1247G) allele frequencies was found among both groups, as illustrated in (Table 3).

The (T1247G) SNP association with breast cancer progression was studied. We observed a significant association between genotypes and TNM category (p = 0.024) with the mutant GG genotype being predictor of poor outcome for TNM category only. It was concluded that presence of G allele is strongly associated with breast cancer progression. (Table 4)

Finally, an HWE test was performed, and it confirmed that the genotype frequencies didn't deviate significantly from equilibrium in either case and control groups (p = 0.331 and 0.768). (Table 5)

Discussion:-

Breast cancer is the most common cancer among women affecting up to one third of them during their life span. (Mahdi *et al.*, 2013) It is included about 10% of all cancers and 23% of women cancers in developed countries. (Coley, 2008) Among American women, breast cancer is the second killer cancer after lung cancer. (Jemal *et al.*, 2010) Several genetic, environmental and life style factors could contribute to occurrence of breast cancer. (Shen *et al.*, 2014) About 5-10% of breast cancers are likely to be hereditary and caused by abnormal gene. (Mahdi *et al.*, 2013) It was proved that increased expression of some genes due to polymorphisms increases the risk to breast cancer development. (Mahdi *et al.*, 2013)

Recently, numerous studies proved that RAS is not only a linear proteolytic cascade, but instead, a complex humoral system that control different intracellular pathways. (Ribeiro-Oliveira *et al.*, 2008)

Accumulating evidence suggests that the RAS is involved in the development and progression of several kinds of cancer.(Uemura and Kubota, 2012) Many types of cancer have been found to be associated with polymorphism of (RAS) genes. (de Noronha *et al.*, 2012)

T1247G and A5235G are two non-coding SNPs, located within the intronic region of AGTR2 gene. (Molina Wolgien Mdel *et al.*, 2014)

These two SNPs could regulate the binding affinity of the transcription machinery to the promoter region of AGTR2. This in turn affects mRNA transcript processing by alternative splicing mechanisms. Therefore, these genetic alternations may interfere with Angiotensin II signaling through AGTR2. (Molina Wolgien Mdel *et al.*, 2014)

In the present study, we aimed to find the association between AGTR2 (T1247G, A5235G) polymorphisms and breast cancer in a sample of 100 Egyptian females; 50 breast cancer cases and 50 controls.

In our study, there was no statistical significant difference between patients and controls regarding age, family history, other variables as well as routine investigations.

However, a significant difference in age of menarche and menopause between patients and controls was found (p=0.001 and 0.003, respectively). As for A5235G SNP, comparing the genotype frequencies among the studied groups revealed a statistically significant difference (p = 0.003) between both groups, where the breast cancer patients have a higher frequency of AA and AG genotype and a lower frequency of the homozygous mutant gene (GG) than controls (12% vs 36%). It was concluded that AA/AG carriers are more likely to develop breast cancer than GG carriers. (Table 3)

Furthermore, there was a statistically significant difference in allele frequencies of A5235G SNP between control and breast cancer cases, where the patients had significantly higher frequency of the wild A allele than control (p = 0.002). It was proved that the A allele presence was associated with increased risk of breast cancer development (OR=2.455, 95% CI :1.389-4.339). The genotypes and allele frequencies of A5235G were balanced among both groups and no deviation from HWE was observed. In this study, the association between AGTR2 (A5235G) SNP with clinical variables such as TNM category and axially LN presence was assessed. A statistically significant association between genotypes and both clinical variables was proved (p=0.027, 0.003, respectively) with GG genotype being a strong predictor of better prognosis. (Table 4)

Finally, multivariate logistic regression analysis was done considering age of menarche, age of menopause and A5235G genotypes as the independent variables. Meanwhile, the breast cancer risk was the dependent variable. It was proved that age of menarche as well as genotypes followed by age of menopause significantly contribute to prediction of breast cancer risk. (p= 0.002, OR: 0.582, 95% CI: 0.424-0.797), (p= 0.004, OR: 13.412, 95% CI: 2.286-78.670), (p= 0.016, OR: 1.363, 95% CI: 1.059-1.755), respectively. (Table 6)

So far, only limited studies have evaluated the association between AGTR2 (A5235G) SNP and breast cancer, yet they also yielded inconsistent results.

In agreement with the present study; Molina Wolgien et al., (Molina Wolgien Mdel *et al.*, 2014) studied the association of the (A5235G) SNP with breast cancer among Brazilian women. It was proved that A5235G was associated with breast cancer risk with AA/ AG carriers more likely to be diagnosed with breast cancer more than polymorphic GG carriers. The G allele was considered to be protective. It was suggested that this SNP may alter susceptibility to the disease. It was also concluded that A5235G was associated only with axillary LN status. A statistically significant association was observed between allelic distributions and axillary LN presence (p=0.0003), in which the genotype GG might be considered a predictor of a better prognosis, and allele A may be linked to a more aggressive type of cancer.(Molina Wolgien Mdel *et al.*, 2014) In contrast to our study, they found that allele frequency deviate significantly from equilibrium in both case and control groups in HWE (p=0.0007 and 0.04).

On the other hand, de Noronha et al., (de Noronha *et al.*, 2012) evaluated the association between AGTR2 gene (A5235G) SNP and breast cancer among Brazillian women using custom TaqMan® SNP genotyping assays. They found that A5235G polymorphisms was not associated with breast cancer risk at all.

Another study was conducted by Wolgien et al.,(Wolgien *et al.*, 2013) to investigate any possible association between allelic distributions of (A5235G) SNP with ACE1 and 2 plasma levels among women with breast cancer. In concordance with our study, it was observed that allelic distribution of this SNP was associated with breast cancer risk. A significant correlation of (A5235G) SNP genotype distribution with plasma levels of ACE1 was proved (p<0.0001). ACEs plasma levels were also correlated with clinical variables and high level of ACE2 was associated with better prognosis. (Wolgien *et al.*, 2013)

In our case-control study, comparing the genotype frequencies of (T1247G) SNP among the studied groups revealed no statistically significant difference (Table 3; P= 0.298) between the breast cancer patients (TT=74%, TG= 22%, GG=4%) and control group (TT=77%, TG=28%, GG=2%). Furthermore, there was no statistically significant difference in allele frequencies of AGTR2 (T1247G) polymorphism between both groups (Table 3; p=0.845).

The genotype distributions as well as allele frequencies of (T1247G) polymorphism were both found to be in concordance with Hand-Weinberg equilibrium (p= 0.941, 0.086). In our study, we found a statistically significant association between (T1247G) genotypes and TNM category only (p=0.024) with the mutant GG genotype being predictor of poor outcome for TNM category only.

It was concluded that although AGTR2 (T1247G) was not associated with breast cancer risk, there was a strong association with TNM category, and the presence of G allele was associated with advanced stages of breast cancer.

Several studies tried to detect the impact of AGTR2 (T1247G) polymorphism on breast cancer risk and its progression; however they revealed conflicting results.

Molina Wolgien and colleagues (Molina Wolgien Mdel *et al.*, 2014) didn't find any significant association between AGT2R (T1247G) SNP and breast cancer risk (p=0.45). However, a significant association between allelic distribution and both TNM category and axially LN status was observed (p<0.0001). They suggested a strong association of this SNP with tumor progression with the GG genotype being predictor of poor outcome for both variables. The G allele seemed to be more strongly associated with axillary lymph node metastasis than with TNM category. The HWE test confirmed that both genotype and allele frequencies were balanced among patients and controls in their study.

On the other hand, Noronha et al., (de Noronha *et al.*, 2012) and his colleagues suggested a significant association between (T1247G) polymorphism with breast cancer risk in the Brazilian populations. They considered T1247G as a possible target for assessing breast cancer risk. (de Noronha *et al.*, 2012)

These discrepancies in results might be attributed to different sample sizes, genetic heterogeneity among different populations. In addition, the false positive results, lack of adjustment for other breast cancer confounding factors in some studies as well as use of different methods and techniques for genotyping also contribute to variable results.

From the previously mentioned studies, only limited data are available regarding the association between T1247G and A5235G polymorphisms and breast cancer. Further large scale studies are recommended among other ethnics to get strong evidence about the association of both SNPs and breast cancer risk and progression.

In conclusion, the present study demonstrated that AA and AG genotypes of AGTR2 (A5235G) SNP together with age of menarche and menopause can be considered as significant predictors for breast cancer risk among Egyptian females. Results also revealed that the two non-coding SNP of AGTR2 were associated with a least one clinical variable that reflects breast cancer progression.

Therefore, AGTR2 gene (T1247G and A5235G) SNPs can be used as potential tool for improving breast cancer diagnosis and treatment planning.

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