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THE ASSOCIATION BETWEEN THE ANGIOTENSIN-CONVERTING ENZYME GENE INSERTION/DELETION POLYMORPHISM AND THYROID CANCER RISK

Clinical Research

Mohamed Youssry Master Student, Biochemistry Department, Faculty of Science, Port-Said University.

Faten Zahran* Professor, Biochemistry Department, Faculty of Science, Zagazig University. *Corresponding Author

Mohamed Elgharib Professor of Organic Chemistry, Faculty of Science, Port-Said University.

Enas Oraby Fellow of Biochemistry,Toxicology Department, Emergency Hospital, Mansoura University.

Sahar Hamed Professor of immunology and molecular biology, Urology &Nephrology Center, Mansoura University.

ABSTRACT

Angiotensin I-converting enzyme (ACE) gene plays an important role in the pathogenesis of cancers. The association between ACE insertion/deletion (I/D) polymorphism and the risk of various cancers has been studied. The aim of this study was to identify possible correlation between thyroid cancer and the *ACE* I/D gene polymorphism, to evaluate whether the *ACE* I/D polymorphism could be a marker for early diagnosis and prognosis. As well as we analyzed the oxidative status of TC both directly, by measuring malondialdehyde (MDA), and indirectly, by evaluating the total antioxidant capacity (TAC), these in addition to thyroid hormones level of FT4 and TSH. ACE I/D was amplified with specific primer sets recognizing genomic DNA from thyroid cancer (n=52), thyroiditis (n=56) and control (n=66) volunteers. A significant increase (P< 0.0001) in thyroid hormones as well as MDA was observed in both thyroiditis and TC group when they compared to healthy group. While TAC show a significant decrease (P< 0.0001) in both thyroiditis and TC group when they compared to healthy group. Results showed no significant association between the ACE DD genotype and the risk of thyroid cancer when compared to control group (DD vs not DD: OR = 1.3, 95% CI = 0.24- 7.07, P= 0.54). While, thyroiditis group exist a significant risk for ACE DD genotype when compared to healthy group (OR 6.000, 95% CI = 1.413- 25.481; P=0.012). We conclude that the ACE gene I/D polymorphism are not related with the TC. Thus it cannot be regarded as an early diagnostic parameter or as a risk estimate for TC predisposition. MDA may have risk estimate for TC in addition to regular hormonal measure. The risk estimate for ACE gene I/D polymorphism was documented to the thyroiditis group.

KEYWORDS

ACE insertion/deletion gene polymorphism- Thyroid cancer - Thyroiditis – Oxidative stress – Thyroid hormones.

Introduction

Angiotensin-converting enzyme (ACE, gene ID: 1636) is a zinc-dependent carboxypeptidase, and its expression is observed in several malignancies. The association of ACE gene I/D polymorphism with cancer susceptibility has been widely evaluated; however the results are not often reproducible **(Li et al., 2015).** The ACE gene is located on chromosome 17q23.3, composed of 26 exons and 25 introns and expands over 20 kb. The ACE gene has been described with an insertion and deletion polymorphism (I/D) of a 287 bp Alu repeat within intron 16 **(Rigat et al., 1992).** By the polymorphism three different genotypes may exist, they are homozygous II and DD genotypes, and heterozygous ID genotype. Several studies have been conducted to identify the relationship between the ACE I/D polymorphism in cancer **(Zhang, et al., 2014),** including lung **(Wang, et al., 2015),** breast **(Li et al., 2015),** digestive system **(Wang, et al., 2015),** renal cells **(Decker, et al., 2015)** and hepatocellular **(Yuan et al., 2013).**

Oxidative stress, caused by an imbalance in reactive oxygen species (ROS) produced during normal cell metabolism and/or efficiency of scavenger antioxidant defense, is implicated in the pathogenesis of many chronic diseases **(Izmedu et al., 2015).** In hyper metabolic state the tissue oxidative injury was associated with hyperthyroidism. Available data indicate that hyperthyroid tissues exhibit an increased ROS production. When the hyperthyroid tissues increase their metabolic capacity, mitochondrial ROS generation is increased as a side effect of the enhanced electron carrier's level. Investigations of antioxidant defense system have returned controversial results **(Venditti P and Di Meo, 2006).** Tissue susceptibility to oxidative challenge was increased with any thyroid hormone-linked biochemical changes. This may explain the injury and dysfunction of the tissue suffer under stressful conditions. Mitochondria, as a primary target for oxidative stress, might account for hyperthyroidism linked tissue dysfunction **(Mancini et al., 2016).**

The aim of this study is to identify a possible correlation between TC formation risk and ACE I/D gene polymorphism and evaluate whether it could be suggested as an early diagnostic marker. These are in addition to the effect of OS and thyroid hormones in TC production.

Materials and Methods

To conduct this study we used 3 groups, they were healthy (n=66), thyroiditis (thyroid Inflammation) (n=56) and TC patients (n=52) underwent surgical thyroidectomy. This study was approved by the Mansoura University's Ethical Committee and informed, written consent was obtained from all patients and volunteer participants.

ACE I/D Polymorphism Genotyping

Peripheral blood (2 ml) was collected into EDTA tubes in Departments of surgery, Mansoura University. Genomic DNA was extracted from peripheral whole blood using Wizard® Genomic DNA Purification Kit-A 1125 (Promega, NY, USA). Prepared DNA samples were quantified (µg) and qualified (260/280 and 260/230) using of Nanodrop. Then, DNA samples were stored at +4°C until genomic studies were conducted.

The sequences of the forward primer was (5'-CTGGAGACCACTCCCATCCTTTCT-3') and reverse primer was (5'-GATGTGGCCATCACATTCGTCAGAT-3'), (prepared by eurofins, genomics, Germany). PCR was performed in a final volume of 50 µl that contained 25 µl master mix, ≈500 ng of genomic DNA, 12.5 pmol of each primer and 5% dimethylsulphoxide (DMSO). Amplification was performed using a Gene Amp PCR system (Thermo Scientific ARKTIK thermal cycler). Samples were denatured for 7 minute at 94°C and then cycled 30 times through the following steps: 45 seconds at 94°C, 1 minute at 58°C, and 1 minute at 72°C as previously described **(Rigat et al., 1992).** Two bands were obtained from PCR products; they were 490-bp which represents the insertion and 190-bp which represent the deletion. Both bands were visualized on a 1.5 % agarose- gel containing GelStar™ Nucleic Acid Gel Stain (LONZA, Rockland, ME, USA, Cat No: 50535) **(Figure 1).** A second PCR amplification was performed for each DD type with a primer pair that recognizes an insertion-specific sequence forward primer was (5'-TGGGACCACAGCGCCCGCCACTAC-3'); reverse primer was (5'-TCGCCAGCCCTCCCATGCCCATAA-3'), with identical PCR conditions except for an annealing temperature of 61°C and the

absence of 5% DMSO **(Figure 2).**

Assessment of thyroid function

Thyroid stimulating hormone (TSH) and free thyroxine (FT4) were determined in plasma samples by ELISA (IMMUNOSPEC, CA, USA) following manufacturer's instructions. Normal thyroid function (euthyroidism) was defined as normal TSH levels (0.4–4.5 mU/L) and FT4 (19–25.60 pmol/L).

Evaluation of oxidative stress

Total antioxidant capacity (TAC) and lipid peroxidation products, malondialdehyde (MDA) were determined both in plasma samples as well as tissue homogenate of thyroid using corresponding diagnostic kits (Biodiagnostic, Egypt) following manufacturer's instructions. TAC and the pink chromogen produced by the reaction of thiobarbituric acid with MDA were measured spectrophotometrically (SpectraMax M5, Molecular Devices) at 520 nm and 532 nm, respectively.

Tissue oxidation was assessed by measuring both TTAC and TMDA in thyroid tissue homogenate for the diseased groups only. The thyroid tissue homogenized in 10% (w/v) PBS (pH 7.4) using a minihard held homogenizer. The homogenates were centrifuged at 14000 rpm, 4°C, for 15 min, and the supernatants obtained were collected and stored at -80 ºC for further analysis.

Statistical analysis

Frequency and statistical analysis were performed with SPSS 16.0. Data were obtained as mean ± standard error (SE). A $p<0.05$ was defined as statistically significant. Expected and observed frequencies of genotypes and alleles were compared with Chi-Square analysis and Fisher's exact tests. Nominal values were analyzed with Student-T test. The sample size was calculated with the assumption that the prevalence of TC is 20-30% of the population. To provide 80% power with a 0.05 alpha error, a minimum of 50 participants was found to be adequate, thus our study population (n=52) was readily in the correct power.

Results

A total of 52 patients with TC, 56 with thyroiditis and 66 controls were recruited for this study. The mean age of patients and healthy controls were summarized in **Table 1.** No significant difference was found between patients and controls females while both diseased groups' males were bit older than in healthy group.

Products amplified with the first and second primer sets were analyzed using 1.5 % agarose gel electrophoresis **(Figure 1 and 2).** The genotype and allele frequencies of *ACE* gene polymorphism among study patients and control groups are shown in **Table 2.** The observed genotype frequencies of *ACE* gene polymorphism showed no significant association between the ACE I/D polymorphism and the risk of thyroid cancer when compared to control (DD vs not DD: OR = 1.3, 95% CI = 0.24- 7.07, P= 0.54; ID vs not ID: OR = 0.32, 95% CI = 0.45- 5.44, P= 34). While a significant risk for thyroiditis when compared with either control or thyroid cancer groups (OR 6.000, 95% CI = 1.413- 25.481; P=0.012 and OR =4.400, 95% CI = 1.020- 18.988; P=0.040) respectively. Patients with D allele showed a significant risk for thyroiditis when compared to control (OR =1.855, 95% CI = 1.112-3.092; P=0.012). On the other hand, the results showed no significant risk association between the *ACE* D allele in thyroid cancer and control or thyroiditis groups.

Baseline thyroid functions and oxidative stress parameters in the study groups

Table 3 represents the means and standard error of the means in different parameters measured in different studied groups. Compared with the control subjects, the levels of TSH, FT4 and MDA were significantly increased in patients groups ($p<0.0001$), whereas TAC levels were significantly decreased ($p<0.0001$). The effect of different ACE Genotype on thyroid hormonal levels, TSH level and FT4 level in different studied groups were illustrated in **Figure 3.** The effect of different ACE Genotype on total antioxidant and oxidative stress in different studied groups were illustrated in **Figure 4. Table 4** represents the means and standard error of the means for thyroid tissue homogenate of TTAC and TMDA. TTAC measure shows no difference between both diseased groups, while, TMDA show a significant increased in TC group when compared to thyroiditis group.

Discussion

Here in we have compared the effects of the *ACE* I/ D polymorphism in thyroiditis, TC patients and healthy controls. Angiogenesis was found to be essential for tumor progression and proliferation **(Tal and Segars, 2014),** as well as ACE activity has been related with tumor growth **(Fishchuk and Gorovenko, 2013).** Such as that *ACE* inhibitors and angiotensin receptor blockers have been shown to suppress tumor growth **(Mc Menamin et al., 2012).**

The ACE gene variations have been implicated in several chronic diseases, including coronary heart diseases **(Bahramali et al., 2016),** hypertension **(Goessler et al., 2015),** renal diabetes **(Shaikh et al., 2014)** and in Idiopathic Nephrotic Syndrome **(El-Gayar et al., 2016).** Significant differences were observed in the DD genotype and D allele of the ACE gene and these several chronic diseases. The DD homozygous genotype was found to be related to serum and tissue ACE increased levels. The evidence indicates that the D allele is associated with elevated plasma and serum ACE levels **(Rigat et al., 1992).**

In relation to cancer, *ACE* gene I/D polymorphism has been widely investigated in Caucaisians and Asian populations. Meta-analysis reports conducted with the Chinese population reveals a lack of association between *ACE1* I/D polymorphism and breast **(Li et al., 2015),** lung **(Wang et al., 2015),** digestive **(Wang et al., 2015),** cancers and uterine leiomyoma **(Gültekin et al., 2015).** A recent meta-analysis from Zhang et al. **(2014),** demonstrated that *ACE1* I/D polymorphism was related to Caucasians but not to Asians origin when studying different cancer types, including breast, lung, colorectal, gastric and prostate cancers **(Zhang et al., 2014).** In contrast, Gan et al. **(2014)** reported that the D allele was significantly associated with increased lymph node metastasis and advanced clinical stage for gastric cancer in the Chinese population **(Gan et al., 2014).** Furthermore, the D allele was related with increased risk of metastasis in colorectal cancer patients in Chinese subjects **(Zheng et al., 2017).** Thus, discrepancies exist for different types of cancers. **Fishchuk and Gorovenko (2013),** document in Ukrainian women there was an associated of increased risk for D allele and the formation of breast cancer**.** The carcinogenic effect of D allele might be due to its amplifier affects on *ACE* gene expression. This may lead to increasing angiotensin II production, which in turn drives to vasoconstriction, high blood pressure, cell proliferation and neovascularization **(Yapijakis et al., 2013).**

Based on the evidence described above, here in we investigated the role of *ACE1* I/D polymorphism in thyroiditis, TC patients. Contrary to must studies resulting with an unfavorable D allele; our study suggests that the *ACE* polymorphism has no effect on the formation of thyroid tumor, and cannot be regarded as an early diagnostic parameter for TC predisposition. This could be due to the benign nature of the TC. However, we found no study concerning this correlation, so it is seems that this study is the first to document the correlation between the *ACE* polymorphism and thyroid cancer.

To the best of our knowledge, this is the first study to evaluate MDA and TAC in association with thyroiditis and TC. In this study, the most important findings are that patients with thyroiditis and TC have elevated MDA levels and low TAC levels which when compared to the healthy ones. These two parameters are playing a role on OS. No reports are available in the literature to comment on the simultaneous measurement of these parameters in TC. Thyroid hormones are associated with the oxidant and antioxidant status of the human organism. The findings obtained from in vitro and in vivo studies show that thyroid hormones have a strong impact on OS **(Erem et al., 2015).** Thyroiditis or TC is associated with an increase of parameters of OS in either tissue/ plasma compared with control subjects including lipid peroxides. In conclusion, we found some important differences in the OS parameters and lipid profile between the patients and healthy controls. Increased MDA levels in both patients groups represent increased lipid peroxidation which might play an important role in the pathogenesis of the TC patients. Also, MDA can be used as a reliable marker of OS and oxidative damage in the studied groups.

As OS plays a role in the pathogenesis of many chronic diseases including thyroid diseases. Previous studies indicate that patients with either hyperthyroidism or hypothyroidism have increased risk for the presence of oxidative stress **(Erem et al., 2015; Izmedu et al., 2015).** In this study, oxidative stress was found to be increased in both patient

groups compared with controls which indicate increased oxidative stress. This increase in MDA was associated with thyroid hormones. This in contrast to the findings of **Izmedu et al., (2015)** where they found the elevated MDA was related to elevated plasma lipids. Thus, lipid oxidation in the tested groups may have been directly caused by the increase of thyroid function. Interestingly, the total antioxidant capacity (TAC) were increased in healthy controls and decreased dramatically in the different studied patients groups, a finding contrary to Torun et al., **(2009)** but similar to Lakshmi et al., **(2013).** It is also suggested that the depletion of antioxidant defense may be more severe in TC group (TSH≥ 35 mU/L) than in control (32). This could be due to high concentrations of thyroid hormones in TC group.

In conclusion, the ACE gene I/D polymorphism are not related with the TC. Both DD and ID genotypes were having no difference comparing control. Thus it cannot be regarded as an early diagnostic parameter or as a risk estimate for TC predisposition. MDA may have risk estimate for TC in addition to regular hormonal measure where all show an elevated level in TC group. TAC level was regress in the diseased groups indicating the lack in the antioxidant defense in these groups. On the other hand, the risk estimate for ACE gene DD genotype and D allele were documented to the thyroiditis group.

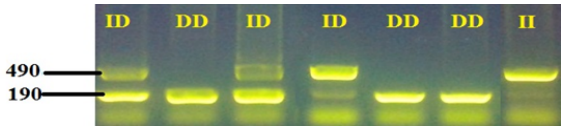

Figure 1: Agarose gel electrophoresis of the first PCR product determining the ACE genotypes. The figure shows different patients genotypes which representative by 1.5 % agarose gel stained with GelStar™ Nucleic Acid Gel Stain and photographed under ultraviolet trans-illumination after PCR amplification with specific primers. The upper band of 490 bp is the I allele and the lower band of 190 bp is the D allele (arrows). The II genotype is shown as a single upper band, the DD genotype as a single lower band, and the DI type as a double band.

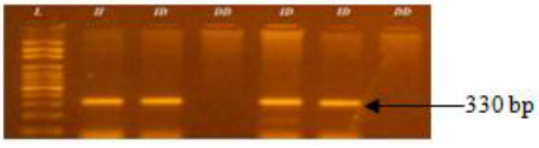

Figure 2: Agarose gel electrophoresis of the second PCR product. It shows the results of different samples from the 1st PCR identified as DD genotype using an insertion-specific primer to differentiate if it is real DD or mis-genotype from ID. The sample in lane 1 is the Ladder, in lane 2 is a standard for II and that in lane 3 is a standard for DI were previously identified by 1st PCR, followed by four samples (lanes 4 through 7) identified as DD with the insertion-spanning primer. A DI sample previously misclassified as DD is in lane 5, 6.

Table 1: Numbers and mean age of both genders in different studied groups

Group Genotype	Thyroiditis	Carcinoma	Thyroiditis	Carcinoma
Total				
Mean	1.6	1.64	1.8	2.7
SE	0.05	0.048	0.096	0.24
P		0.35		0.0001

Table 2: The Distribution of the ACE Genotypes and Allelic Frequency in the Different Studied Groups

ACE Genotype	Group's # (%)			
	Control	Thyroiditis	Carcinoma	Risk Estimate.
II	12 (18.2)	6 (10.7)	4 (7.7)	
Not II	54 (81.8)	50 (89.3)	48 (92.3)	
Risk Estimate				
OR		.540	.375	1.440[a]
95% CI		(0.122- 2.393)	(0.069- 2.037)	(0.221- 9.388)[a]
P		0.327	0.219	0.536[a]
ID	48 (72.7)	32 (57.1)	42 (80.8)	
Not ID	18 (27.3)	24 (42.9)	10 (19.2)	
Risk Estimate				
OR		0.500	0.317	1.575 [a]
95% CI		(0. 171- 1.459)	(0.456- 5.444)	(0.093- 1.085) [a]
P		0. 157	0. 343	0. 057 [a]
DD	6 (9.1)	18 (37.5)	6 (12)	
Not DD	60 (90.9)	30 (62.5)	46 (88)	
Risk Estimate				
OR		6.000	1.304	4.400 [a]
95% CI		(1.413- 25.481)	(0. 241- 7.069)	(1.020- 18.988) [a]
P		0.012	0.543	0.040 [a]
D	60 (45.5)	68 (60.7)	54 (51.9)	
I	72 (54.5)	44 (39.3)	50 (48.1)	
Risk Estimate				
OR		1.855	1.304	1.431 [a]
95% CI		(1.112- 3.092)	(0. 664- 1.860)	(0.834- 2.456) [a]
P		0.012	0.39	0.127 [a]

OR: Odds Ratio; CI= Confidence Intervals; P= significance of diseased groups against healthy group; a: Risk Estimate between Thyroiditis and Carcinoma.

Table 3: Means and standard error of the means in different parameters measured in different studied groups.

Group Measured	Healthy	Thyroiditis	Carcinoma
TSH (µU/mL)			
Mean	2.6	7.39	35.37
SE	0.08	0.59	4.21
P		0.0001	0.0001
P1			0.001
FT4 (ng/dL)			
Mean	0.71	2.5	1.96
SE	0.05	0.23	0.12
P		0.0001	0.0001
P1			0.04
TAC (U/mL)			
Mean	4.38	1.84	1.38
SE	0.19	0.04	0.03
P		0.0001	0.0001
P1			0.0001
MDA(nmol/mL)			
Mean	0.46	2.08	2.6
SE	0.04	0.19	0.13
P		0.0001	0.0001
P1			0.03

N= Number of cases in each group; SE= standard error of the means; P= significance of diseased groups against healthy group; P1= significance between diseased groups.

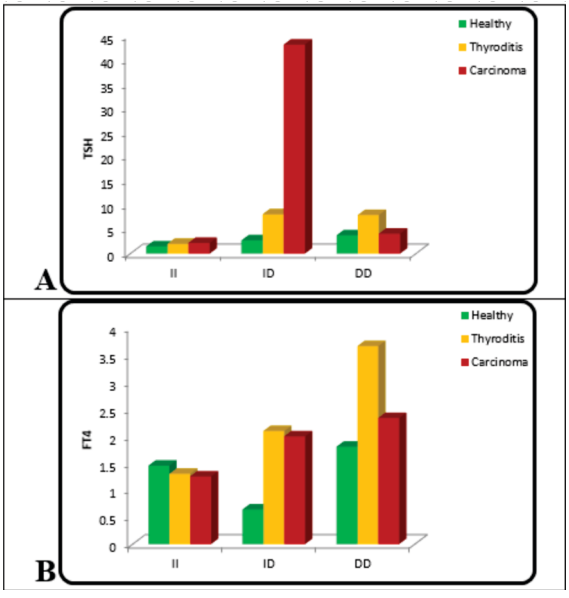

Figure 3: Effect of different ACE Genotype on thyroid hormonal levels, TSH level (A) and FT4 level (B) in different studied groups.

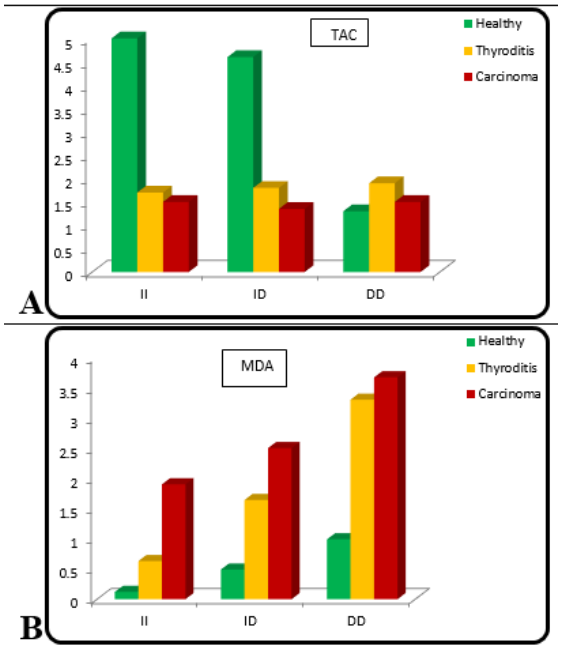

Figure 4: Effect of different ACE Genotype on total antioxidant, TAC level (A) and oxidative stress, MDA level (B) in different studied groups.

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