CD44 GENE POLYMORPHISM IN EGYPTIAN PATIENTS WITH VITILIGO

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Abstract

Vitiligo is an autoimmune polygenic disorder, characterized by loss of pigmentation due to melanocyte destruction. Multiple genes and environmental triggers are thought to play a role in inducing vitiligo. Etiological hypothesis suggested for etiopathogenesis of vitiligo include gender, immunological, neuro-hormonal and other environmental factors. Genes possibly play a role in all aspects of pathogenesis of vitiligo. Most genes associated with vitiligo are involved in immune regulation and immune targeting of melanocytes. These genes and environmental factors differ across different population. Vitiligo is the most common depigmenting disorder, with a prevalence of approximately (0.5-2%) in the world population.

CD44 gene has been linked with the development of several autoimmune disorders. CD44 is encoded by single gene, located on chromosome 11p13. CD44 is a cell surface glycoprotein with various functions, and is involved in a number of biological processes including lymphocyte migration, extravasations, homing, activation, and apoptosis, especially those involving a role in T-cell development. The purpose of the present work was to investigate the association between an inherited genetic polymorphism at CD44 gene and vitiligo in Egyptian patients. The present study included 50 vitiligo patients and 49 apparently healthy, age and sex matched individuals. This sample of individuals was selected randomly from population in Qalyubia Governate. Results of the present study showed a statistically insignificant difference between vitiligo patients and control groups regarding the frequency of CD44 genotypes and alleles.

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Introduction:-
Vitiligo is an acquired non-contagious disease characterized by progressive patchy, multifocal depigmentation of skin, with or without depigmentation of overlying hair and mucous membranes results from loss of melanocytes from the involved areas. Although non-contagious, the phenotype of this disorder has high impact on the psychological behavior of the affected individuals than their physical ability more often lead to social isolation (Jahan et al., 2013).

Etiological hypotheses suggested for etiopathogenesis of vitiligo include genetic, immunological, neuro-hormonal, cytotoxic, oxidative stress and melanocytorrhagy. Genes play a role in all aspects of pathogenesis of vitiligo. Most genes associated with susceptibility to vitiligo are involved in immune regulation and immune targeting of melanocytes. The autoimmune and genetic hypothesis was initially supported by clustering of vitiligo cases in certain families (Multiplex vitiligo families) and increased frequency of autoimmune diseases in patients of vitiligo as in their first degree relatives (with or without vitiligo) more so in multiplex vitiligo families (Spritz, 2012).

CD44 is encoded by single gene, located on chromosome 11p13 (Tijink et al., 2006). CD44 is a cell surface glycoprotein with various functions and involved in a number of biological processes including lymphocyte migration, extravasations, homing, activation and apoptosis, especially those involving a role in T-cell development (Baaten et al., 2010). CD44 is a receptor for hyaluronic acid (HA) and can also interact with other ligands, such as osteopontin, collagens and matrix metalloproteinases (MMPs) (Oxley and Sackstein, 1999). CD44 are reported as cell surface markers for some breast cancer and prostate cancer. In breast cancer research CD44+/CD24 expression is commonly used as a marker for breast cancer stem cells and is used to sort breast cancer cells into a population enriched in cells with stem like characteristics (Li et al., 2007) and has been seen as an indicator of increased survival time epithelial ovarian cancer patients (Sillanpää et al., 2003).

Vitiligo is a CD8+ T-cell mediated autoimmune disease and promotes the longevity of memory T-cell responses to melanoma. Studies have found that HA specific CD8+ T-cells are adaptively transferred into mice expressing HA as a self-antigen in the pancreas and HA specific T-cells proliferate in draining lymph nodes and upregulated CD44 (Hernandez et al., 2001).

The significant association of (rs10768122) SNP at the 3′-UTR CD44 gene was found with generalized vitiligo (GV) in Caucasians (Jin et al., 2012). The role of CD44 gene in patients with vitiligo is not well fully investigated.

Aim of the Work:
The aim of the present study was to investigate the role of CD44 (rs10768122) gene polymorphism in the susceptibility to vitiligo among group of Egyptian patients.

Subjects and Methods:-
Types of the study:
This study was a prospective case-control study.

Study population:
This study was conducted on a total number of 99 subjects that were classified into two groups:
1. Vitiligo group: Included 50 vitiligo patients (Group A).
2. Control group: Included 49 apparently healthy individuals with matched age and sex (Group B).
Patients were selected from the outpatient dermatology clinic of Benha University Hospitals.

Ethical Considerations:
An informed consent was obtained from all participants. The study was approved by the local ethics committee on research involving human subjects of Benha Faculty of Medicine.

Inclusion criteria:
Vitiligo male and female patients with different degrees of severity and different clinical types (Vitiligo vulgaris, acrofacial and vitiligouniversalis). Diagnosis of vitiligo was clinically and by using wood's light examination. The disease extent was assessed by body surface area (BSA).
Exclusion criteria:
Any subject was excluded from the study if he/she was:
1. Patients with other systemic diseases such as hypertension and diabetes mellitus
2. Patients with other autoimmune diseases such as systemic lupus erythematosus (SLE), type 1 DM, autoimmune thyroid disease, rheumatoid arthritis (RA) and Addison's disease (AD).
3. Patients suffering from cancers such as (breast cancer, prostate cancer or epithelial ovarian cancer).

Methods:
All patients were subjected to the following:
Detailed history taking from patients:
1. Personal history: name, age, sex, occupation, residence and special habits of medical importance.
2. History of present illness: onset, course and duration of vitiligo.
3. Family history of vitiligo.
4. History of medications intake.

Clinical examination:
1. General examination to exclude other autoimmune or systemic diseases.
2. Local examination (dermatological examination):
3. Description of vitiligo lesions including site and clinical type.
4. Any other associated skin disorders.
5. Disease extent according to BSA.

Laboratory investigations:
All studied subjects were tested for the genotypes of CD44 (rs10768122) gene polymorphism.

Sampling:
Three ml venous blood was collected from each subject by clean venipuncture using disposable plastic syringe and placed on ethylene diamine tetra-acetic salt (EDTA) (1.2 mg/mL) as an anticoagulant and stored at -20°C for subsequent molecular biology study.

DNA extraction:
DNA extraction from whole blood by Quick-gDNA<sup>TM</sup>MiniPrep; (<b>Zymo Research, Cat.# D3024</b>) according to manufacturer protocol.

Components of the kit (50 preps):

| Component                        | Quantity |
|----------------------------------|----------|
| Genomic Lysis Buffer             | 50mL     |
| DNA Pre-Wash Buffer              | 15mL     |
| g-DNA Wash Buffer                | 50 mL    |
| DNA Elution Buffer               | 10 mL    |
| Zymo-Spin<sup>TM</sup> Columns   | 50       |
| Collection Tubes                 | 50       |

Reagents preparation:
Genomic Lysis Buffer was prepared by adding beta-mercaptoethanol to a final dilution of 0.5% (v/v) by adding 250 µL per 50 ml buffer.

Whole blood genomic DNA purification protocol:
1. 400µL of Genomic Lysis Buffer was added to 100 µL of whole blood (4:1) and mixed by vortex for 5 seconds. Samples were let stand for 10 minutes at room temperature.
2. The prepared mixture was transferred to a Zymo-Spin<sup>TM</sup> column in a collection tube and centrifuged for 1 min at 10,000 x g. The collection tube containing the flow-through solution was discarded and then the column was placed into a new collection tube.
3. 200 µL of DNA Pre-Wash Buffer was added to the spin column and centrifuged for 1 min at 10,000 x g. The flow-through was discarded and placed the column back into the collection tube.
4. 500 µL of the g-DNA Wash Buffer was added to the spin column and centrifuged for 1 min at 10,000 x g. The collection tube containing the flow-through solution was discarded and the column was transferred to a sterile
1.5 mL microcentrifuge tube.

5. 100 µL of DNA Elution Buffer was added to the spin column. Then was incubated for 5 mins at room temperature and was centrifuged for 30 sec at maximum speed.

6. The purification column was discarded. The purified DNA was stored at -20°C for subsequent steps.

**Genotyping of CD44 (rs10768122) gene polymorphism:**

Genotyping of CD44 (rs10768122) gene polymorphism was performed using TaqMan® SNP Genotyping Assay. (Thermo Fisher, Cat.# 435179, Assay ID: C_16021387_20), according to the manufacture protocol. Principle of TaqMan® SNP Genotyping Taq DNA-polymerase with its 5’ nuclease activity can cleave the minor groove binding (MGB) oligonucleotide fluorogenic labeled probes. Each probe contains a fluorescent reporter dye and a quencher dye when it hybridizes to its complementary amplified target the enzyme cleaves it liberating the reporter dye, causing an increase in fluorescence intensity. While in the intact probe, the proximity of the quencher reduces the fluorescence signal observed from the reporter dye due to Förster resonance energy transfer (FRET) (Livak, 2003).

**Assay contents:**

Taq Man® SNP Genotyping Assay (40X) (Cat.# 4351379):
The assay contains 2 specific primers targeting the region flanking the polymorphic sequence of interest, 2 MBG-fluorescent probes to distinguish between the two alleles (MGB stabilizes the double-stranded structure formed between the target and the probe). Each probe is labeled with a different fluorophore; VIC® dye, linked to the 5’ end of the Allele 1 probe and FAM™ dye, linked to the 5’ end of the Allele 2 probe.

Taq Man® Genotyping Master Mix (Thermo Fisher, Cat.# 4371355): contains AmpliTaq Gold® DNA polymerase-Ultra Pure (UP), deoxyribonucleotide triphosphates (dNTPs), ROX™ passive reference in buffer.

**Performing PCR:**

Enzymatic amplification of the extracted DNA was performed by PCR on PikoReal 24, Thermo Scientific thermal cycler with the following conditions:

| AmpliTaq Gold® DNA polymerase activation | PCR cycles (40 cycles) |
|----------------------------------------|------------------------|
| Hold                                   | Denaturation            |
| 95°C for 10 min                         | 95°C for 15 sec         |
|                                        | 60°C for 1 min          |

**Interpretation of results:-**

Each sample genotype was called according to the fluorescence liberated from the corresponding dye which indicates the alleles present in the sample as following:

| A substantial increase in | Indicates               |
|---------------------------|-------------------------|
| VIC-dye fluorescence only  | Homozygosity for Allele 1|
| FAM-dye fluorescence only  | Homozygosity for Allele 2|
| Both VIC- and FAM-dye fluorescence | Allele 1-Alele 2 heterozygosity |

The genotyped data was exported to Excel sheets and organized for statistical analysis.

**Statistical Analysis:**
The statistical analysis of data was done using Excel program (MicrosoftOffice 2013) and IBM SPSS (statistical package for social science) program (SPSS, Inc., Chicago, IL, USA) version 20.

Qualitative data were represented as number and percentage. Chi-square test was used to compare groups. Quantitative data were represented by mean, ± SD, median and range. Comparisons between groups were done using t-test or Mann-Whitney (for non-parametric). Deviations from Hardy–Weinberg equilibrium expectations were determined using the chi-squared test. Odds ratio and 95% confidence interval were calculated.

Linear regression analysis was done for prediction of risk factors. N.B: P is significant if < 0.05 at confidence interval 95%.
Results:
The present study included 50 patients with vitiligo, in addition to 50 control subjects.

| Patients N=50 | Control N=49 | P Value |
|---------------|--------------|---------|
| Age(years)    |              |         |
| Median        | 31.5         | 26      | 0.565 |
| Range         | 6-66         | 15-59   | 0.565 |
| Age groups    |              |         |
| ≤20 years; N(%)| 12           | 8       | 0.0542 |
| >20 years; N(%)| 38           | 41      | 0.0542 |
| Gender        |              |         |
| Male; N(%)    | 15           | 21      | 0.140 |
| Female; N(%)  | 35           | 28      | 0.140 |

There was no statistically significant difference between patients and control groups regarding demographic data as shown in table 1.

1. Regarding the course of the disease, it was found that 92% of patients had progressive course and only 8% of them had stationary course.
2. The median of the disease duration was from 1-43 years.
3. On clinical examination, the most common affected site was the lower limbs 86%.
4. The most common clinical type in was vitiligo vulgaris that was observed in 68%. Family history was observed in only 6% of patients as shown in table 2.

| Patient (N=50) | Control |
|----------------|---------|
| Assessment of heterozygosity and Hardy-Weinberg equilibrium for studied SNP(rs10768122). |         |
This sample of individuals was selected randomly from population in Qaliobeya Governorate. Applying Hardy Weinberg equation, revealed that (rs10768122) genotypes in both cases and control subjects were independent (i.e., they are in HW equilibrium (HWE)) table 3. There is no evidence to reject the assumption of HWE in the sample.

Table 4: Comparison of CD44 (rs10768122) genotypes regarding demographic data in vitiligo group.

| Age(years); Median(range) | N=13 | N=25 | N=12 | P value |
|---------------------------|------|------|------|---------|
| ≤ 20 years; N (%)         | 12.5 | 36.2 | 18.8 | 0.301   |
| > 20 years; N (%)         | 92.3 | 19   | 76   | 0.155   |

Gender

| Gender | N=13 | N=25 | N=12 | P value |
|--------|------|------|------|---------|
| Male   | 3    | 23.1 | 9    | 0.541   |
| Female | 10   | 76.9 | 16   |         |

No significant differences were found between CD44 (rs10768122) genotypes regarding demographic data in studied vitiligo patients as shown in table 7 (P= 0.301, 0.155, 0.541).

Table 5: Association analysis of CD44 (rs10768122) (genotypes, alleles) between patients and control groups.

| Patients (n=50) | Control (n=49) | P value | OR   | 95%CI |
|-----------------|----------------|---------|------|------|
| N               | %              | N       | %    |      |
| GG              | 12             | 24      | 9    | 18.4 | 0.284 |
| GA              | 255            | 50      | 22   | 44.9 | 0.332 |
| AA              | 13             | 26      | 18   | 36.7 | 0.247 |
| G               | 49             | 49      | 42   | 40.8 |       |
| A               | 51             | 51      | 58   | 59.2 |       |

There was no significant association between CD44 (rs10768122) genotypes and alleles among studied vitiligo patients and control group (P=0.284, 0.332, 0.247).

Table 6: Comparison of CD44 (rs10768122) genotypes regarding clinical data in vitiligo group.

| Course of the disease | 4) AA | 5) AG | 6) GG | P Value |
|-----------------------|-------|-------|-------|---------|
| 7) Positive family history; N(%) | 1     | 7.7   | 2     | 8      | 0      | 0      | 1      |
| 8) Age of onset (years); Median Range | 21   | 6-47  | 25   | 3-64   | 19.5   | 0-52   | 0.325  |
| 9) ≤20 years; N(%) | 6     | 46.2  | 6     | 24     | 6      | 50     | 0.239  |
| 10) >20years; N(%) | 7     | 53.8  | 19    | 76     | 6      | 50     |        |
| 11) Duration (years); Median (range) | 6    | 2-42  | 3     | 1-30   | 3      | 1-27   | 0.175  |
| 12) Stationary; N(%) | 0     | 0     | 2     | 8      | 2      | 16.7   | 0.254  |
| 13) Progressive; N(%) | 13   | 100   | 23    | 92     | 10     | 83.3   |        |

Clinical types

| 14) Face; N(%) | 2     | 15.4  | 3     | 12     | 1      | 8.3    | 1      |
| 15) Trunk; N(%) | 11    | 84.6  | 13    | 52     | 8      | 66.7   | 0.127  |
| 16) Upper limb; N(%) | 8    | 61.5  | 16    | 64     | 5      | 41.7   | 0.417  |
| 17) Lower limb; N(%) | 12   | 92.3  | 20    | 80     | 11     | 91.7   | 0.653  |

| OR | 95%CI |
|----|------|
| 1.846 | 0.602-5.662 |
| 1.573 | 0.630-3.928 |
| 1.393 | 0.794-2.444 |

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There was no statistically significant difference of CD44 (rs10768122) genotypes regarding different clinical data in studied patients as shown in table 6.

Table 7:- Regression analysis for the prediction of vitiligo activity.

|                      | β     | P Value | 95% CI         |
|----------------------|-------|---------|----------------|
| Age (years)          | -0.003| 0.939   | -0.070-0.065   |
| Gender (males versus females) | 0.488 | 0.700   | -2.043-3.018   |
| Age of onset (years) | -0.035| 0.297   | -0.103-0.032   |
| Duration (years)     | 0.127 | 0.061   | -0.006-0.260   |
| Gene (AG+GG versus AA) | 0.870 | 0.270   | -0.700-2.440   |

Linear regression analysis was conducted for prediction of extent of vitiligo. Age, gender, age of onset, duration and CD44 (rs10768122) genotypes were applied as covariates for prediction of vitiligo activity. However, none of these covariates was considered as risk factor for higher BSA involvement in studied vitiligo patients.

Discussion:-
Vitiligo is an acquired skin disorder caused by the disappearance of pigment cells from the epidermis that gives rise to well define white patches which are often symmetrically distributed. Vitiligo affects approximately (1%) of the world’s population. Both adults and children are affected with no predilection for sex or ethnicity. The average age at onset lies around the second to the third decade of life. Vitiligo susceptibility is a complex genetic trait that may include genes involved in melanin biosynthesis, response to oxidative stress and regulation of autoimmunity. The importance of genetic factors for vitiligo susceptibility is evident by reports of its significant familial association (Alikhan et al., 2011).

Most popular theory of the vitiligo development is a multifactorial hypothesis according to which genetic condition predispose vitiligo macules to occur as a result of specific environmental factors (Spritz et al., 2011). Studying the polymorphisms of certain genes incorporated in the immune system detected a significant role in vitiligo susceptibility (Onay et al., 2007). It has been proposed that genes at different loci may contribute to the pathogenesis of vitiligo(Shajil et al., 2006).

CD44 is encoded by single gene, located on chromosome 11p13 (Tijink et al., 2006). CD44 is involved in cell proliferation, cell differentiation, cell migration, angiogenesis, lymphocyte homing to peripheral lymphoid tissues, presentation of cytokines, chemokines and docking of proteases at the cell membrane as well as in signaling for cell survival (Rangaswami et al., 2006). It was demonstrated that cross linking of CD44 with antibodies led to monocyte release of IL-1 and TNF, and subsequently it has been demonstrated that HA binding leads to secretion of cytokines,chemokines, growth factors and matrix modifying enzymes (Horton et al., 1999). HA, the principal ligand of CD44. Furthermore, HA has been shown to affect toll like receptor signaling Jiang et al., 2011).

CD44 dependent primary adhesion mainly of T-cells correlated with systemic disease. Therefore, circulating T-cells bearing activated CD44 might provide a marker for autoimmune or chronic inflammatory disease activity (Estess et al., 1999).

In this study we provided a new investigation of CD44 (rs10768122) gene polymorphism and vitiligo susceptibility among Egyptian patients.
The present study was conducted on a total number of 99 Egyptian subjects divided into two groups. Vitiligo group included 50 patients (15 males and 35 females). Control group, included 49 ages and sex-matched apparently healthy Egyptian subjects (21 males and 28 females).

Results of current study showed that there was no statistically significant difference between patients and control groups regarding different age groups. Results of the present study disagreed with results of the study conducted by Handa and Dogra, (2003); who showed that adults and children of both sexes are equally affected.

Results of current study showed that there was no statistically significant difference between patients and control groups regarding gender. These results were in agreement with the results of a study conducted by Nunes and Esser, (2011); who found that female predominance (65.9%) was noticed, the greater number of reports among females was explained by the social and psychological consequences in females affected by vitiligo. In contrast, study conducted by Sehgal and Srivastava, (2007); who reported male predominance among vitiligo patients. Also, results of the present study disagreed with results of the study conducted by Liu et al. (2005); who have found that there are no differences between gender.

No statistically significant difference was noted between patients and control groups regarding the mean age in years. This was in agreement with results of a study conducted by Nunes and Esser, (2011); who found that a mean age was 31.3 years of the vitiligo patients. Results of the present study disagreed with results of the study conducted by Dogra and Kumar, (2005); who found that mean age of vitiligo patients was 55 years.

Family history was reported in present study as 6%, this result agreement with Karelson et al. (2012); who mentioned that most cases of vitiligo are sporadic, 10% of the patients have positive family history. However, disagreed with results of a study conducted by Misri et al.(2009); who found that 57% of the vitiligo patients have positive family history.

Results of the current work no statistically significant correlation was found neither between CD44 (rs10768122) genotypes frequency nor age of onset, duration, course, extent or clinical types of vitiligo.

Results of the present study showed that there was no statistically significant difference between patients and control groups regarding the genotype frequencies of CD44 gene polymorphism. In our patients group, the normal genotype was (CD44-AA) which is the dominant genotype and was detected in (26%), while the heterozygous mutant genotype (CD44-AG heterotype) was detected in (50%), and the homozygous mutant genotype (CD44-GG homotype) was detected in only (24%).

Results of the present study were in disagree with the results of the study conducted by Jin et al., (2012); who showed that CD44 gene (rs10768122) was associated with GV in Caucasians ancestry. There was statistically significant difference between the vitiligo patients and controls (P= 6.13×10−8, OR= 1.24). Ethnicity probably plays the key role for this difference from our study result.

**Conclusion:**
From the results of the present study, we can conclude that CD44 (rs10768122) variant is not associated with vitiligo susceptibility among the studied group of Egyptian patients.

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