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RESEARCH ARTICLE

ASSOCIATION BETWEEN VITAMIN D RECEPTOR GENE POLYMORPHISMS AND OSTEOPOROSIS IN POST MENOPAUSAL WOMEN ATTENDING ZAGAZIG UNIVERSITY HOSPITALS

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Abstract

Aim:-To assess the association between the vitamin D receptor gene polymorphisms and osteoporosis in Egyptian postmenopausal women, to determine the genotypes most frequently associated with decreased bone mineral density and vertebral fracture. In addition, to assess the modulatory role of high daily calcium intake on the genetic predisposition of osteoporosis.

Patients and methods:- A total number of 92 Egyptian postmenopausal women with and without osteoporosis were diagnosed by bone mineral density measurement were subjected to identification of VDR genes (FOKI and BSMI) polymorphism by PCR technique followed by RFLP analysis.

Results:- The frequencies of BB, Bb and bb genotypes (BSMI polymorphism) in osteoporotic female were 58.7%, 32.6% and 8.7%, respectively. While, in controls their frequency was 8.7%, 17.4 % and 73.9%, respectively. The BB genotype was higher in patients than in controls (P = 0.000) while the bb genotype was significantly higher in controls than in patients. Regarding the FOKI polymorphism the frequencies of FF, Ff and ff genotypes among patients were 21.7%, 45.7% and 32.6%, respectively while their frequency in controls were 13%, 17.4% and 69.6%, respectively. We find out that the main predictors of osteoporosis are genotypes BB, Bb, ff in and number of pregnancy

Conclusions:- Postmenopausal females carrying B+ve or f+ve genotype were more risky to develop osteoporosis.

Introduction:-

Osteoporosis is a highly prevalent skeletal disorder characterized by compromised bone strength predisposing individuals to an increased risk of fractures. Fractures related to osteoporosis may be associated with chronic pain and leads to decreased quality of life, as well as significant morbidity and mortality (Lesliea and Suzanne., 2014).

Bone mineral density (BMD) decreases with advancing age among both women and men. Women in particular, suffer accelerated bone loss after menopause. While changes in sex hormone levels are considered very important in the development of osteoporosis, other factors, including genetic ones ,nutrition, body weight, muscle function and calcium absorption ,are thought to play a significant role as well (Mansour et al .,2010).

The action of vitamin D is mediated through binding to its nuclear receptor (VDR). The VDR gene, located on chromosome 12, is made up of 5.6 kb. In response to hormone binding, the VDR regulates the transcriptional activity of 1, 25 (OH)2 D3-responsive genes by complexing with a vitamin D response element located in the promoter region of target genes(Chow et al .,2014).

Vitamin D is a steroid hormone that maintains calcium homeostasis and modulates cellular proliferation and differentiation in a number of normal and malignant cells (Mostafa and Hegazy., 2014). Several polymorphisms have been identified in the VDR gene, and their functional significance and potential effects on disease susceptibility have been investigated (Uitterlinden et al., 2013).
The aim of this study was to evaluate pattern of VDR polymorphism in postmenopausal Egyptian females for possible genetic role.

Subjects and methods:-

This study was carried out on 92 postmenopausal female attending the outpatient clinic of both Rheumatology & Rehabilitation Department and Gynecology& Obstetrics Department, at Zagazig university hospitals

All participated subject considered postmenopausal if they had no menstruation for at least 6 months without any other obvious physiological or pathological cause. They were diagnosed according to the World Health Organization (WHO) definitions and criteria for osteoporosis.

The WHO definitions:-

The World Health Organization in 1994 has defined the following categories based on bone density in white women osteoporosis **(WHO organization study group., 1994)**:

- Normal bone: T-score greater than -1.
- Osteopenia: T-score between -1 and -2.5.
- Osteoporosis: T-score less than -2.5.
- Established (severe) osteoporosis includes the presence of a non-traumatic fracture.

Exclusion criteria included-

- Endocrinological disorders (such as hyperthyroidism, hypo- and hyperparathyroidism, cushing`s syndrome, hypogonadism and diabetes mellitus type I).
- Severe liver or gastroenteral diseases.
- Sever cardiac disease.
- Other skeletal diseases (Paget's disease, osteogenesis imperfecta and Rheumatoid Arthritis),
- Current pharmacological treatment with corticosteroids, cytotoxic chemo therapy anabolic androgenic steroids, estrogens or estrogen-related molecules, anticonvulsants before enrollment
- Consent: A written consent was taken from all subjects

All subjects were subjected to the following**:-**

Full history taking:-

With special stress on Marital State, Current Smoking, alcohol consumption, menstrual History, Pregnancy number, Contraception and number of years since menopause. Risk Factor, Drugs, History Of parental hip Fracture, history Of Fracture After age Of 30, Inflammatory Arthritis , Associated Medical Condition (i.e hypertension. DM). Environmental factors: Sedentary life &Low sun exposure, immobility.

Dietary assessment of calcium intake:-

Using modified FFQ from the Calcium Calculator TM, called the Calcium Assessment Tool (CAT). Calcium intake was estimated in terms of absolute amounts (g/day and mg/day). The data of ca Food frequency questionnaire were interpreted via the ca assessment tool on web site http://bcdairyfoundation.ca/interactive/calcium-calculator/ (Hung et al., 2011)

Anthropometrical assessment:-

Height, Weight, And Calculation of body mass index: weight (kg)/ height (m^2).

Radiological Assessment:-

Vertebral fracture assessment by Semiquantitative approach:-

A visual semiquantitative grading of vertebral fractures was done by two independent observers, one who was considered experienced and the other inexperienced but trained to investigate interobserver variance. Vertebrae T4 - L4 were graded on visual inspection and without direct vertebral measurement.

Grade 0 (normal): no reduction. **Grade 0.5:** was given to designate a borderline deformed vertebra**. Grade 1 (mildly deformed):** minimal fracture, 20%-25% height decrease. **Grade 2(moderately deformed)**: moderate fracture, 25%-40% height decrease. **Grade 3 (and severely deformed)**: severe fracture, greater than 40% height decrease.

For fracture versus non fracture comparison, a vertebral body was considered to be fractured if graded 1or higher; it was considered normal if graded 0 or 0.5.In addition, **a spinal fracture index (SFI)** was calculated for each patient by summing the individual vertebral deformity scores and dividing by the number of vertebrae evaluated (Genant et al., 1993).

BMD:-

For all the patient was measured using dual energy X-ray absorptiometry (DEXA) NORLAND Eclips (two site scanner) by (NORLAND Corporation) at Radiology Department, Zagazig University Hospitals .

The evaluated areas included the lumbar spine (L2–L4) and hip (femoral neck and total hip).

Laboratory examination:-

Thyroid and Parathyroid hormone, Serum Ca and phosphorus level, and serum 25-OHD3

VDR gene polymorphism:-

Five ml of venous blood sample was obtained from each participant, all blood samples were stored immediately at – 20 º c till the time of DNA extraction.

DNA extraction:-

Using Gene JET whole blood genomic DNA purification mini kit (Thermo SCIENTIFIC)

The FOKI polymorphism in exon 2 and BSMI polymorphism in intron 8 were determined following a protocol developed by other investigators (Mansour et al., 2010).

Polymerase Chain Reaction (PCR):-

DNA amplification:-

Using Dream Taq green PCR Master Mix (Thermo SCIENTIFIC)

The following temperature scheme was performed for the amplification using thermal cycler (Gene Amp, PCR system 9700): After denaturing the DNA for1-2 min at 94 ºC, the reaction mixture was subjected to 35 cycles of denaturation for 30 s at 94ºC, 30 s annealing at 61ºC and 1 min extension at 72 ºC. The detection of presence of amplification band (265bp for FOKI) & (850bp FOR BSMI) was done by agarose gel electrophoresis.

The Fast Digest FOK I enzyme was supplied by **Thermo Scientific** and is stored at -20 c enzyme recognizes and digests the following site:

5'...G G A T G(N)9 ...3'
3'...C C T A C(N)13 ...5' ↑

The Fast Digest Mva1269 I enzyme was supplied by **Thermo Scientific** and is stored at -20 c enzyme recognizes and digests the following site:

5'...G A A T G C N↓...3'
3'...C T T A C↑G N...5'

Detection of the amplified bands using 1.5% agarose gel containing ethidium bromide by performing
E/P on the gel electrophoresis apparatus electrodes were attached to by the power supply (Consort E 844). and visualized by UV transillumination.

Interpretation:-

FOKI:-

Samples with a single 265-bp band were identified as F/F homozygous genotype for FOKI gene, samples with 3 bands of 265, 196, 69-bp were typed as F/f heterozygous, and those with 2 bands of 196 and 69 were typed as f/f homozygous.

BSMI:-

Samples with a single 850-bp band were identified as B/B homozygous genotype for BSMI gene, samples with 3 bands of 850, 650, 200-bp were typed as B/b heterozygous, and those with 2 bands of 650 and 200 were typed as b/b homozygous

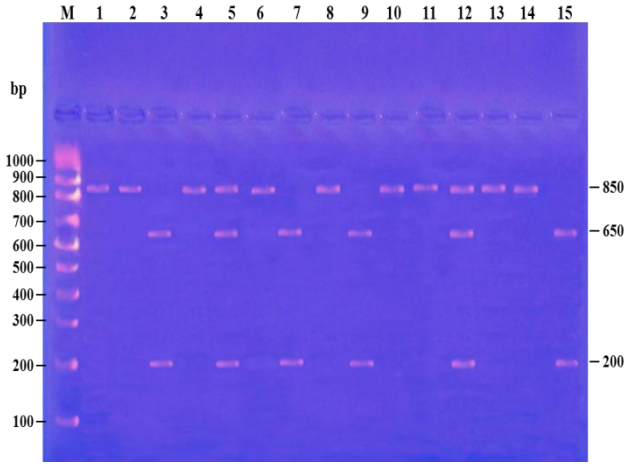


Fig (1): Gel electrophoresis (2%) of PCR–RFLP Technique of amplified BSMI genotype with heterozygous pattern Bb in lanes 5 and 12 while, lane 3 ,7,9 and 15 shows homozygous bb mutation and the remaining lanes the 3rd possibility of VDR polymorphism BB homozygous for absence of mutation.

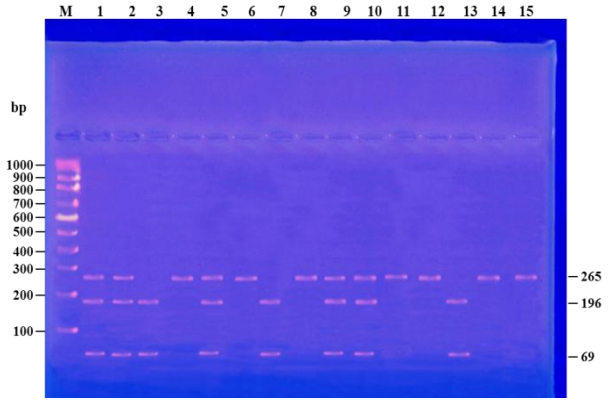


Fig (2): Gel electrophoresis (2 %) of PCR–RFLP technique of amplified FOKI genotype revealed homozygous pattern ff in lanes 3, 7and 13 heterogenous pattern Ff in lanes 1,2,5,9 and 10 while the remaining lanes showed the wild type of FF.

Statistical methods:-

Data were collected, revised, verified and then edited on P.C. Statistical Package for social science (SPSS) program version 19 was used for analysis of data. Data was summarized as mean, SD. Non parametric test (Mann–Whitney U and kruskal-wallis) were used for analysis of two quantitative data and Chi square for qualitative data. One way ANOVA was done for analysis of more than two variables followed by post hoc test for detection of significance. Logistic regression was done to detect predictors of osteoporosis. P-value is considered significant if <0.05*.

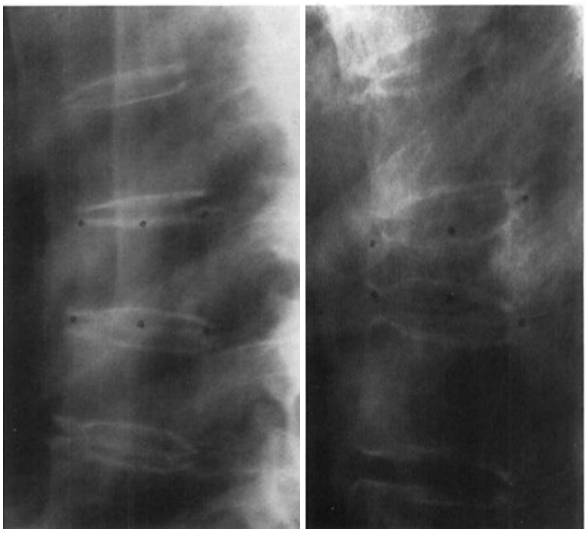
Result:-

Background characteristics of our participants (age, body mass index, marital status**,** education level and working status) showed no significant difference between the two tested groups (data not illustrated). Also we studied different risk factors for developing osteoporosis such as number of pregnancies and daily calcium intake which yielded highly significant and significant results respectively, while the duration of menopause (years), parental hip fracture did not give significant difference between osteoporotic and control group(data not illustrated). Distribution of the different genotypes and alleles is shown in (table 1). We found that BSMI polymorphism differed significantly between patients and controls. The frequencies of BB, Bb and bb genotypes (BSMI polymorphism) in osteoporotic female were 58.7%, 32.6% and 8.7%, respectively. While, in controls their frequencies were 8.7%, 17.4 % and 73.9%, respectively. The BB genotype was higher in patients than in controls (P = 0.000) while the bb genotype was significantly higher in controls than in patients. Regarding the FOKI polymorphism the frequencies of FF, Ff and ff genotypes among patients were 32.6%, 45.7% and 21.7%, respectively, While, in controls their frequencies were 69.6%, 17.4% and 13% respectively. High statistical difference was found in the B and b and F and f allele frequency between groups (P = 0.000) (table 2).

When the osteoporotic females were subdivided according to their genotypes, we found significant decrease in BMD femoral and lumbar in B+ve genotype and a significant decrease in T score femoral and highly significant decrease in BMD femoral in f+ve genotype (tables 3).

Studying the combination of genotypes as shown in Table 4, we found highly significant statistical difference in the frequency of combined genotypes FFBB, FFbb **(**p value =0.001 and 0.000 respectively) This table indicates that the most risky combination of genotypes is FFBB then Ff Bb. Whereas the most protective was FF+bb.

When we assessed the spinal fracture index (SFI) in our tested groups, there were highly significant difference between the cases and control (table 5). In addition, there was a highly significant association between SFI and BB genotype of the BSMI polymorphism and ff genotype of the FOKI polymorphism (table 6). In table 7 the main predictors of osteoporosis were the number of pregnancy, genetic factors specially BB, Bb and ff genotypes but daily ca intake and genotypes FF, Ff were not found to be predictors for osteoporosis occurrence**.** When we assessed dietary calcium intake; Using modified FFQ there was significant difference between the two groups regarding their daily calcium intake in food items, but when comparing this finding with BSMI and FOKI polymorphisms there were no significant relation to any of the different genotypes of both polymorphisms (data not illustrated).
Further studying of the effect of the most risky combined genotype from our results (ff BB) on BMD (T – score hip) we found that BMD was significantly lower among patients with ff BB genotype compared with other genotypes($p<0.01$)(table 8).



Fig(3): Semi-quantitative visual grading of vertebral deformities. Mild, grade 1**,** wedge fracture of thoracic vertebra (right). Severe, grade 3, c wedge fracture of thoracic vertebra(left).

Discussion:-

Osteoporosis is a common disease characterized by low bone mass and defects in the microarchitecture of bone tissue, which impairs bone strength and leads to an increased risk of fragility fractures (Ji and Yu., 2015). Osteoporosis is an important health problem. It is a multifactorial disease caused by hormonal and genetic factors. One of the suggested candidate gene involved in the pathogenesis is polymorphism of vitamin D receptor (VDR). The gene encoding the VDR is located on chromosome 12q and has several allelic variants including BSMI, FOKI (Mansour et al .,2010).

In our study, on studying the BSMI polymorphism we found that BB genotype was higher significantly in patients than in controls, while the bb genotype was significantly higher in controls than in patient, the frequency of B allele in our study was significantly higher in osteoporotic patients than controls (69% vs 16%). The b allele was higher in controls than patients (76% vs 23%). In concordance with our results Hosseinpanah et al., 2014, they found that the B+ve are the commonest genotype in both pre and postmenopausal osteoporotic females with a total percent of (57%). These findings are also supported by others (Pouresmaeili et al., 2013) who reported that B+ genotypes were the commonest among their osteoporotic females (47.6%). Also in our study of FOKI gene polymorphism there was significant difference in the frequency of f+ve genotype between patients and control. In addition, the frequency of f allele in our study was significantly higher in osteoporotic patients than controls. Reporting similar results Kurt et al., 2012 reported that ‘‘FF’’ genotype frequency was higher in their control group. The frequency of f allele in their result was higher in osteoporotic patients than controls. In our results, we found that the f allele of VDR FOKI is significantly associated with decreased T- score of BMD in hip area and lumbar spine when compared to F allele. The supporting evidence for our result is the recent meta-analysis done by Wang e al., 2013 they reached the conclusion that f allele of VDR FOKI compared to F allele was significantly associated with decreased BMD.

Our patients were further subdivided into three groups according to the genotypes for each polymorphism, we found significant relation between BSM I with both hip and lumber BMD. The BMD was significantly higher in bb genotype in comparison to the B+ve genotype Hosseinpanah et al., 2014 also reported that the bb variant had higher BMD at both hip and lumbar regions than the BB variant. Similarly, the BMD was higher in FF genotype. Wang e al., 2013 reported that postmenopausal Asian women with the FOKI ff genotype had a significant BMD loss compared to women with FF, Ff genotypes. On studying combined genotypes of BSMI and FOKI of VDR polymorphism. It was found that the combined genotype FF BB was more frequent among patients(28.3%) while it was only 4.3 % among controls, and the difference was statistically significant (P =0.001).a consistent result were given by Abd El Sadek et al., 2012 who also showed that the combined genotype FF BB was highly frequent among osteoporotic patients (20%) while it was not present among controls, and the difference was statistically significant (P =0.04).

The index for vertebral fracture in our tested groups, differ significantly between the cases and control. The highly significant association was between SFI and both BSMI and FOKI polymorphism of vitamin D receptor was confirmed recently by a systematic review by Mohammadi et al., 2014 but they did not signify a particular genotype to be more related to the risk of osteoporotic fracture.

In conclusion, VDR gene (BSMI and FOKI) polymorphism plays an important role in osteoporosis in Egyptian postmenopausal females. The BB genotype was higher in patients than controls and the bb genotype is a protective genotype. The FF genotype was predominant among post menopausal females and ff genotype was associated with osteoporosis. Currently, however, the mechanisms by which VDR alleles regulate BMD remain poorly understood.

Table 1: Distribution of different genotypes of BSMI and FOKI polymorphism in both groups.

	Osteoporosis patients (N=46)	Controls (N=46)	OR (95% CI)	p
bb	4(8.7%)	34(73.9%)	1.0	
Bb	15(32.6%)	8(17.4%)	15.9(3.6-78.8)	0.000
BB	27(58.7)	4(8.7%)	40.5 (7.5-256.5)	0.000
	Osteoporosis patients (N=46)	Controls (N=46)	OR (95% CI)	p
ff	15(32.6%)	32(69.6%)		
Ff	21(45.7%)	8(17.4%)	5.6(1.82-17.8)	0.000
FF	10(21.7%)	6(13%)	3.56(1.0-13.7)	0.02

* P-value is significant if <0.05.

** Highly significant if <0.01.

Table 2: Alleles frequency of FOK I and BSMI in tested groups.

	Group I	Group II	OR (95% CI)	p
f	41	20		
F	51	72	2.89(1.45-5.81)	0.000
b	23	76		
B	69	16	14.6(6.6-31.3)	0.000

Table 3: Comparison between the studied parameters in different BSMI genotypes:

	BB Median (range)	Bb Median (range)	bb Median (range)	K	P	Sig.
T- score hip	-3.20 (-4.97) – (-2.81)	-3.00 (-3.95) - (-2.60)	-2.36 (-3.30) - (-0.93)	8.690	0.013	S
T-score lumber	-2.09 (-3.33) – (- 0.5)	-1.59 (-2.41)- (-0.34)	-1.49 (- 2.71)- (-0.89)	5.431	0.066	S
	FF Median (range)	Ff Median (range)	ff Median (range)	K	P	Sig.
T-score hip	-3.19 (-3.71)- (-2.81)	-3.04 (-4.07)- (-2.24)	-3.36 (-4.97)–(-2.20)	6.416	0.040	S
T-score lumber	-1.81 (-3.25)– (-0.51)	- 1.38 (-3.33)– (-0. 34)	-2.64 (-3.30) -(-0.94)	8.369	0.015	S

Table 4: Combined genotypes of BSMI and FOKI polymorphism of patients and controls.

Variables	osteoporosis N (%)	normal N (%)	P-value
FF+BB	13(28.3)	2(4.3)	0.001
FF+Bb	2(4.3)	4(8.7)	0.67
FF+bb	0	26(56.5)	0.000
Ff+BB	7(15.2)	0	0.01
Ff+Bb	12(26.1)	3(6.5)	0.01
Ff+bb	2(4.3)	5(10.9)	0.43
ff+BB	7(15.2)	2(4.3)	0.15
ff+Bb	1(2.2)	1(2.2)	1.0
ff+bb	2(4.3)	3(6.5)	1.0

Table 5: spinal fracture index in the studied groups.

	osteoporosis Median(range)	normal Median(range)	Z	P	Sig.
Spinal fracture index	0.1530 (0.00 - 0.6150)	0.1530 (0.00 – 0.5380)	2.03	0.04	S

Table 6: The relation of genotype to spinal fracture index.

	BB Median (range)	Bb Median (range)	bb Median (range)	K	P	Sig.
spinal fracture index	0.453 (0.00-0.615)	0.230 (0.00 – 0.615))	0.230 (0.230 – 0.384)	8.004	0.018	S
	FF Median (range)	Ff Median (range)	ff Median (range)	K	P	Sig.
spinal fracture index	0.376 (0.00 – 0.584)	0.325 (0.00 – 0.615)	0.507 (0.430– 0.584)**)**	7.988	0.018	S

Table7: Logistic Regression for the association of BSM I with daily calcium intake (mg/ day), and number of pregnancy.

	B	S.E.	sig	exponent of B 95% CI odds ratio (lower- upper)
daily calcium intake(mg/ day)	.884	.783	.259	.522-11.216
number of pregnancy	2.601	.904	.004	2.291-79.274
bb			.000	
Bb	2.491	.935	.008	12.072 (1.932-75.416)
BB	4.785	1.120	.000	119.677 (13.332-1074.3)
ff			.023	
Ff	-1.460	.985	.138	0.232 (0.034-1.600)
FF	1.229	1.176	.296	3.419 (0.341-34.294)

Table (8): Relationship between BMD and combined genotype among osteoporotic patients.

	ff BB (N.=7) Median (range)	Mixed genotype (N.= 39) Median (range)	Mann-Whitney Test	P	Sig.
T-score hip	-3.51 (-3.27) - (-4.97)	-3.1 (-2.20) - (- 4.07)	2.983	0.003	HS

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