Relationship between Matrilin-3 (MATN-3) Gene Single Nucleotide Six Polymorphism, Transforming Growth Factor Beta2 and Radiographic Grading in Primary Osteoarthritis

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Abstract: Objective: Assess serum level of Transforming growth factor beta 2 (TGF-β2) and Matrilin-3 (MATN3) SNP6 polymorphism in osteoarthritic patients Background:.Osteoarthritis (OA) is a musculoskeletal disease characterized by pain and joint stiffness. TGF-β 2 is involved in chondrogenesis and osteogenesis, It has found thatMATN3 gene and protein expression was correlated with the extent of tissue damage in OA. Findings suggest that regulation of MATN3 expression is essential for maintenance of the cartilage extracellular matrix microenvironment Subjects and Methods:72 cases of primary OA (56 with knee OA and 16 with generalised OA were compared with that of 18 healthy controls. Radiographs were scored with the Kellgren-Lawrence scale. Serum TGF-β2 was measured by using (ELISA), levels of marker were correlated to radiographic grading of disease and MATN3 SNP6 polymorphism was determined by (PCR-RFLP).Results: MATN3 SNP6 polymorphism and serum level of TGF-β2were higher in OA compared with controls. Genotype, NN and N allele frequency were higher in patients with OA compared with controls. NN genotype and N allele frequency were higher in knee osteoarthritis than generalised OA.Significant positive correlation between level of TGFβ2 and radiographic grading in group with knee OA, but no correlation between serum level of TGFβ2 and radiographic grading in generalised OA.Conclusion:MATN3 SNP6 polymorphism and TGF-β2 implicated in the pathogenesis of osteoarthritis. Association of N/N genotype with primary osteoarthritis emphasizes on the need for prospective study include larger sample size to confirm the results of the present study.

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Key words: Matrilin-3,Transforming growth factor beta 2, and primary osteoarthritis.

Introduction:

Osteoarthritis (OA) is a multifactorial, inflammatory and degenerative disorder of the joint [(1-3)]. Osteoarthritis (OA) is a late-onset musculoskeletal disease characterized by gradual thinning and loss of articular cartilage of the synovial joints with a concurrent alteration in the physiology of several other joint tissues, including the subchondral bone and the synovium[(4)].

Furthermore, OA is probably not a single disorder, but rather a group of overlapping distinct diseases. These diseases are the consequences of mechanical or biological events that destabilize the normal coupling of synthesis and degradation of extracellular matrix in articular cartilage and subchondral bone[(5)]

Osteoarthritis is one of the major public health problems. Clinically, the predominant symptoms of osteoarthritis are joint pain, limitation of movement, tenderness, crepitus, occasional effusion, and variable degrees of local inflammation, but without systemic effects[(6)].

Many predisposing factors may contribute to osteoarthritis progression. such as advanced age, obesity, bone density, hormone level, mechanical factors, past history of trauma, and genetic susceptibilities[(7)]. Many studies have also revealed a role of the inflammatory process in the pathogenesis of OA[(8,9)].

The matrilin (MATNs) are a family of oligomeric extracellular matrix (ECM) proteins consisting at least of four related proteins[(10).]

Matrilin-3 (MATN3) is the least complex member of the matrilin family, consisting of only one von Willbrand factor A (vWFA) domain, four epidermal growth factor (EGF) like domains, and a C-terminal coiled-coil domain A[(11).] It has been found that enhanced matrilin-3 gene and protein expression was correlated with the extent of tissue damage in osteoarthritis patients. These findings suggest that tight regulation of matrilin-3 expression is essential for maintenance of the cartilage ECM microenvironment[(12).]

Plain radiographs are the standard method for assessing OA presence, severity, and progression. These can be scored either for individual radiographic features such as osteophytes, joint space narrowing, subchondral sclerosis, and subchondral cysts, or by

using a global scale that accounts for these features, such as the Kellgren-Lawrence (K-L) grade[9]. However, plain radiographs have several drawbacks in OA assessment, including insensitivity to change for short term follow up and lack of tight association with patient outcomes such as pain and disability. Biomarkers represent a potentially more sensitive way to evaluate and follow OA patients for the purposes of clinical research and therapeutic trials. Serum and urine biomarkers provide a rapid and relatively non-invasive method of repeated assessment over time, and may be more responsive to therapeutic interventions than the longer-term changes seen on radiographs[10,11].

Transforming growth factor-β stimulates proteoglycans and collagen type II and affects the expression of matrix metalloproteinases in human fibroblasts[12]. Moreover, TGF-β is strongly related with osteophytes formation, the hallmark of osteoarthritis[9].

Aim of the work:

The goal of this study is to assess serum level of Transforming growth factor beta 2 (TGF-β2) and Matrilin-3 (MATN3) SNP6 polymorphism in osteoarthritic patients and control group

Subjects and Methods:

Seventy two primary OA patients (56 patients with knee OA and 16 patients with generalised OA (affection of other joints beside knee)) with a mean age of 50.43 ± 9.40 and eighteen apparently healthy normal subjects with a mean age of 45.72 ± 13.36 were included in the study. Patients were recruited from outpatient clinic of Rheumatology and Rehabilitation department, Menoufia University in the period between September 2013 and November 2014. Informed consents were taken from both the patients and control group subjects before the beginning of the study.

All the studied groups were subjected to the following:

- Relevant clinical data were collected.
- Radiological examination of the affected joint. Knee radiographs were evaluated according to the Kellegren-Lawrence radiographic grading scale. In this scale 0 corresponds to osteoarthritis with no osteophytes, joint space narrowing, subchondral sclerosis. 1 to minute osteophytes of doubtful clinical significance.2 to definite osteophytes with unimpaired joint space 3 to definite osteophytes with moderate joint space narrowing, and 4 to definite osteophytes with severe joint space narrowing and subchondral sclerosis[8].

- **Serum level of TGF-B2 by sandwich ELISA technique.**

The TGF-β2 ELISA Kit is a solid phase enzyme-linked immunosorbent assay based on the sandwich principle. Prior to testing the standards and patient samples are diluted in assay buffer, acidified with HCl and then neutralized with NaOH. Afterwards, the neutralized standards and samples are added to the antibody coated (polyclonal) microtiter wells. After the first incubation the unbound sample material is removed by washing. Then a biotinilated mouse anti TGF-β2 antibody and the Streptavidin peroxidase Enzyme complex are incubated in succession. An immuno enzyme sandwich complex is formed. After incubation the unbound conjugate is washed off. Having added the substrate solution, the intensity of colour developed is proportional to the concentration of TGF-β2 in the patient sample[9].

- **Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method was used to determine Genotyping of MATN3 SNP6.**

I- DNA extraction.

Total DNA was extracted from EDTA treated blood sample using Thermo Scientific Gene JET Genomic DNA Purification Kit (Thermo Fisher Scientific, MA, USA).

II- Polymeras chain reaction.

The 501 base pair (bp) fragment encompassing the SNP 6 polymorphic site was amplified using the amplification master mix which consisted of:1µL of each of primers (Biosearch Technologies 2199 South Mcwell Blvd, Petaluma, USA).

Forward:5'GGACAGGATCCCACAAAAAG3'
Reverse5'-GAAAGAGGGGCTACAACAGG-3'25 µl of Dream Taq Green PCR master mix (Promega corporation 2800 Woods Hollow Road Madison,Wl53711-5399 USA)18 µl of nuclease-free Water Then 5µl of extracted DNA was added to the corresponding reaction tube.`PCR cycling conditions:The PCR amplification was performed on pre-programmed thermal cycler (Perkin Elmer Gene Amp PCR System 2400 Thermal Cycler) under the following conditions: an initial denaturation step at 95°C for 10 min, followed by 35 cycles, each cycle consisted of denaturation at 95°C for 1 min, annealing at 61°C for 1 min and extension at 72°C for 1 min. Final extension at 72°C for 10 min was carried out.Confirmation of successful PCR amplification was done by 2% agarose gel electrophoresis. Five µl of normal range (100 bp) ladder(Thermo scientific, Gene Ruler 100 bp DNA ladder) and 5 µl of PCR product were applied. Then electrophoresis was done for the voltage applied was 100 volts for 10 minutes then 80 volts for about 25 min.

III- PCR/RFLP

After PCR, the products are digested with a specific restriction enzyme BSEYI (Bio Lab new England NEB-R0635), so the following reaction

components were mixed gently at room temperature:

The digestion mixture contained :PCR product (10 µl), Restriction enzyme buffer (5 µl),Restriction enzyme BSEYI (2 µl),Nuclease-free water (33 µl),Then mixed gently and incubated for 1 hour at 37oC.

IV- Genotype determination:

The reaction mixture was loaded directly and electrophoresed on 2.5% agarose gel containing ethidium bromide and visualized under UV illumination. Restriction enzyme digestion of the fragment and electrophoresis allow the products of the different genotypes to be visualized.Wild-type genotype (CC) which coded as bb produced double band at 149 and 352 bp, heterozygotes (CN) which coded as Bb produced three bands at 501, 149 and 352 bp, and homozygote polymorphic genotype (NN) which coded as BB produced only one band at 501 bp.

Statistical analysis

Results were collected, tabulated and statistically analyzed by statistical package SPSS version 20.

Results:

This study included 90 subjects; 10 males and 80 females, their ages ranged between 26-70 years. The studied subjects were divided into two groups, group I(osteoarthritis group) included 72 patients with primary OA subdivided into Group Ia (knee OA) included 56 patients and Group Ib (generalized OA) included 16 patients and group II (control group) included 18 apparently healthy subjects.

Table (1) showed no statistical significant differences were found between the studied groups regarding age and gender (p= 0.17, 0.41 respectively). Group I was statistically higher than group II regarding weight and BMI (P= 0.002, 0.002 respectively); Also, group I was statistically higher than group II regarding TGFβ2 (P= 0.001). Regarding genotypic distribution of MATN-3 gene in the studied groups. In patients group which included 70 subjects, three genotypes were found, 49 patients had the genotype NN (68.1), 14 patients CN (19.4%) and 9 patients CC (12.5%) and in control group which included 18 subjects, the genotypes were as follow: 5 subjects had the genotype NN (27.8%), 9 subjects CN (50%)and 4 subjects CC (22.2%), there was a highly statistical significant difference between both groups (*p*=0.006). And regarding allele frequencies The N allele was more represented in patients (77.8%) than in controls (52.8%). However, C allele showed higher frequency in controls (47.2%) compared with patients (22.2%).Hence, statistically, there was highly significant difference in the distribution of both alleles (N and C) between patients and controls (p=0.003).

Table (1): Demographic criteria, clinical and laboratory parameters of the studied groups

Variable	**Group I (diseased) (n=72)**	**Group II (controls) (n=18)**	Test	P value
Age (year) X ± SD	50.43±9.41	45.72±13.37	**t-test** 1.41	0.17
Gender (n-%) Male Female	 7 (9.7%) 65 (90.3%)	 3 (16.7%) 15 (83.3%)	**FE** 0.70	0.41
Weight (kg) X ± SD	80.66 ± 12.18	71.16 ± 7.43	**t-test** 3.15	0.002
BMI(kg/m2) X ± SD	30.56 ± 3.15	26.73 ± 3.15	**t-test** 3.27	0.002
TGFβ2 (Pg/ml) X ± SD	758.69 ± 769.86	194.64 ± 127.97	**U** 3.39	0.001
MATN-3 genotypes (n-%) NN CN CC	 49 (68.1) 14 (19.4%) 9 (12.5%)	 5 (27.8%) 9 (50.0%) 4 (22.2%)	**X^2** 10.10	0.006
MATN-3 allele(n-%) N C	n=144 112 (77.8%) 32 (22.2%)	n=36 19 (52.8%) 17 (47.2%)	**X^2** 9.09	0.003

n: number X ± SD: mean ± standard deviation FE: Fisher's Exact test
P value: probability of error T test: student's t test U:Mann WhitneyX2 : qui square

In table (2), depicts non significant (P=0.07) increased level of TGFβ2 in group Ib rather than group Ia, meanwhile there was significantly (P=0.002) increased level of TGFβ2 in group Ia rather than group II and significantly(P=0.002) increased level of TGFβ2 in group Ib rather than group II. About the genotype distribution of MATN3 SNP6 was: NN genotype was statistically higher in group Ia (80.4%) comparing with group Ib (25.0%) meanwhile, CN genotype was statistically higher in group Ib (50.0%) than in group Ia (10.7%). No statistical difference was found in CC genotype between group Ia (8.9%) and group Ib (25.0%). Regarding allele frequencies, N allele was more represented in group Ia (85.7%) than in group Ib (50.0%); while C allele was higher in group Ib (50.0%) than in group Ia (14.3%), about the distribution of both alleles (N and C) there were statistical significant differences between group Ia and group Ib (P<0.001) and between group Ia and group II, meanwhile there was no statistical difference between group Ib and group II. There was a statistical significant difference between groups Ia, Ib and II regarding Radiographic grading.

Table(2): Statistical comparison between different studied groups regarding TGF β2, genotype distributions, allele frequencies and radiographic grading

Parameter	Group Ia (Knee) (n = 56)	Group Ib (Generalized) (n=16)	Group II Control (n= 18)	Test	P value
TGFβ2 (Pg/ml)	699.43± 831.81	955.00 ± 484.48	194.64±127.98	U 1.81 3.14 3.14	 0.07[1] 0.002[2] 0.002[3]
MATN-3 genotypes				X^2	
NN	45 (80.4%)	4 (25.0%)	5 (27.8%)	18.05	<0.001[1]
CN	6 (10.7%)	8 (50.0%)	9 (50.0%)	17.92	<0.001[2]
CC	5 (8.9%)	4 (25.0%)	4 (22.2%)	0.05	0.97[3]
MATN-3 allele	N =112	N = 32	N=36	X^2	
N	96 (85.7%)	16 (50.0%)	19 (52.8%)	18.37	<0.001[1]
C	16 (14.3%)	16 (50.0%)	17 (47.2%)	17.06	<0.001[2]
				0.05	0.82[3]
Radiographic grading				X^2	
Grade 1	40 (71.4)	5 (31.3%)		8.78	0.03[1]
Grade 2	7 (12.5%)	4 (25.0%)			
Grade 3	4 (7.1%)	3 (18.8%)			
Grade 4	5 (8.9%)	4 (25.0%)			

U = Mann Whitney
1 = comparing Knee osteoarthritis and generalized osteoarthritis
2 = comparing Knee osteoarthritis and control
3 = comparing generalized osteoarthritis and control

Table (3) shows the genotype distribution exhibited a significant difference between OA patients and control groups. The genotype of NN increased the risk of OA (odds ratio [OR] = 4.36, 95% confidence interval [CI] = (0.98 – 19.42); P = 0.04. Also the study found that N Allele was significantly different among OA patients compared with control individuals (OR= 3.13 (1.46 – 6.72); P = 0.003).

About genotype distribution among knee OA and generalised OA there was significant difference between both groups. NN genotype was more risky in knee osteoarthritis than generalised OA (odds ratio [OR] = 9.0, 95% confidence interval [CI] = (1.7 – 47.6); P = 0.02. On comparing allele distribution among Knee OA and generalised OA groups, N allele was significantly more presented in knee OA compared to generalised OA patients. [OR] = 6.0, 95% confidence interval [CI] = (2.51 – 14.35); P<0.001

Table (4) shows that there was significant positive correlation between TGFβ2 and age, weight, BMI, radiographic grading in group I, radiographic grading in group Ia and radiographic grading in group Ib but no significant positive correlation between TGFβ2 and ESR. r: correlation coefficient.

In table (5), there was no statistical significant association between the genotype distribution of MATN3 SNP6 and both of TGFβ2 level and radiographic grading.

Regarding the sensitivity and specificity of serum TGF β2 to differentiate between grade 1, 2 as mild OA and grade 3, 4 as sever OA, at cut-off value 990.0 pg/ml, the sensitivity, specificity and accuracy were 81.3% and 88.7% and 86.6%, respectively.

Receiver Operator Characteristic curve has shown a sensitivity of 75.4% and specificity of 50.0% and accuracy 78.0% when a cutoff point was 195.0 pg/ ml of serum TGF-β2 is used to differentiate between diseased and controls.

Table (3): Odds ratio of genotypes and alleles of the studied polymorphism

Parameter	**Group I (diseased) (n=72)**	**Group II (controls) (n=18)**	**Test**	**P value**	**Odds ratio**	**95% CI**
MATN-3 genotypes NN CN CC	 49 (68.1) 14 (19.4%) 9 (12.5%)	 5 (27.8%) 9 (50.0%) 4 (22.2%)	X^2 4.17 0.25	 0.04 0.73	 4.36 0.69 Ref (1.0)	 (0.98 - 19.42) (0.16 - 2.93)
MATN-3 allele N C	N=144 112 (77.8%) 32 (22.2%)	N=36 19 (52.8%) 17 (47.2%)	X^2 9.09	 0.003	 3.13	 (1.46 - 6.72)
	Knee (n = 56	**Generalized (n=16)**	**Test**	**P value**	**Odds ratio**	**95% CI**
MATN-3 genotypes NN CN CC	 45(80.4%) 6 (10.7%) 5 (8.9%)	 4 (25.0%) 8 (50.0%) 4 (25.0%)	X^2 **8.42** **0.35**	 0.02 0.68	 9.0 0.55	 (1.7 – 47.6) (0.25- 0.82)
MATN-3 allele N C	N =112 96 (85.7%) 16 (14.3%)	N = 32 16 (50.0%) 16 (50.0%)	X^2 **18.37**	 <0.001	 6.0	 (2.51 – 14.35)

Table (4): Spearman correlation between serum TGFβ2 and age, weight, BMI, ESR and radiographic grading

Parameter	**TGFβ2**	
	r	P value
Age(years)	0.26	0.02
Weight (kg)	0.24	0.04
BMI **(kg/m2)**	0.33	0.006
ESR	0.13	0.23
Radiographic grading	0.73	<0.001
Radiographic grading in knee group	0.63	0.000
Radiographic grading in generalized group	0.45	0.07

Table(5):Statistical relation between genotype distributions of (MATN-3) gene, TGFβ2 and radiographic grading

	Genotypes			Test	P value
	NN N = 49	**CN N = 14**	CC N = 9		
TGFβ2(Pg/ml) X ± SD	694.78±797.63	878.57±862.32	898.89±424.35	K 3.85	0.15
Radiographic grading Grade 1 Grade 2 Grade 3 Grade 4	 34 (69.4%) 6 (12.2%) 4 (8.2%) 5 (10.2%)	 7 (50.0%) 3 (21.4%) 1 (7.1%) 3 (21.4%)	 4 (44.4%) 2(22.2%) 2 (22.2%) 1 (11.1%)	X^2 4.26	0.64

K = Kruskal Wallis test

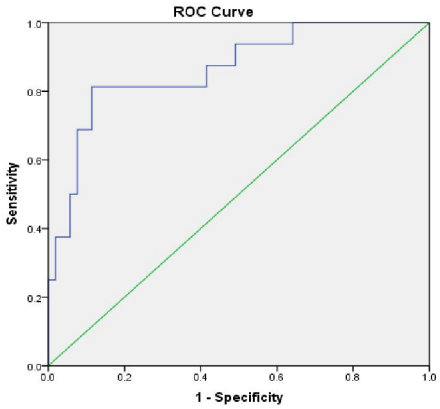
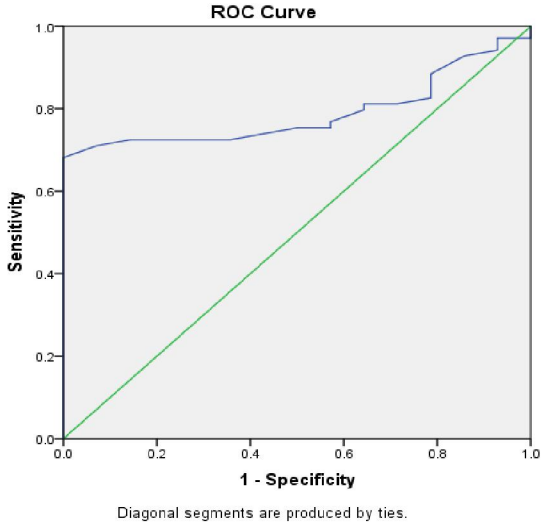


Fig (1): ROC curve between grades 1and 2 on one hand and grades 3 and 4 in the other hand regarding serum TGF β2



Fig(2): ROC curve between group I (diseased) and II (control) regarding serum TGF β2:

Discussion:

This work aimed to study the relationship between both TGF-β2 and matrilin-3 (MATN3) SNP 6polymorphism on one side and primary osteoarthritis on other side and their correlation with radiographic grading of disease. In this study the studied groups were well matched as regard ages and gender showing no statistically significant difference. The mean age of patient group was (50.43 ± 9.40), while in control group was (45.72 ± 13.36).

Similar studies showed high mean age of osteoarthritic patients as in Stilianos and coworkers study[17]. Also in another study by Nelson and colleagues[13], the mean age of the studied group was high.

Age is one of the strongest risk factors for OA of all joints [8].The increase in the incidence and prevalence of OA with age probably is a consequence of cumulative exposure to various risk factors and biologic changes that occur with aging Lawrence et al.,([19].

In this study the mean weight was statistically higher in patients (80.66 ± 12.18) when compared with controls (71.16 ± 7.43). In addition, the mean BMI was statistically higher in patients (30.56 ± 3.15) than in controls (26.73 ± 3.15), which is in agreement with Gu et al., study[20], who reported that the mean BMI of patients and control groups were (20.1±8.2),(19.9±8.30) respectively. In Muthuri et al., study[21], Obesity predisposed to knee OA with an overall odds ratio (OR) of 3.91 (95 % confidence interval (CI) 3.32 - 4.56). Primary OA is more prevalent in obese people. Increased loading on the joint probably is the main mechanism by which obesity causes knee or hip OA. Overloading the knee and hip joints could lead to cartilage breakdown and failure of ligamentous and other structural support [8].

In this study women constituted 90.3% of patients and 83.3% of controls. This was supported by Stilianos et al.([17] study. These findings reflect normal distribution of disease between males and females as OA prevalence is greater in women than men especially after menopause, increase of OA prevalence is associated with age of menopause, an association between low serum estrogen levels and radiographic knee OA was reported in postmenopausal women[22].

In the current study a correlation of TGF-β2 with radiographic grading was implied and found that serum TGF-β2 concentrations, besides being higher in osteoarthritis, were increased in serum of osteoarthritics proportionally to radiographic grading in knee osteoarthritis group. Similar results were reported by Stilianos et al.(17) who reported higher level of TGF- β 2 in patients compared to controls. In contrast to our results Otterness et al.,[23] found that serum TGF-β1 did not discriminate between subjects with and without hip or knee OA defined using American College of Rheumatology radiographic criteria.

Scharstuhi et al.,[24]found that inhibition of endogenous TGF-β leads to enhanced cartilage proteoglycan loss. This can be the result of either up-regulation of cartilage-degrading proteases or down-regulation of the natural inhibitors of these enzymes. This indicates a protective role for endogenous TGF-β on cartilage. Moreover, with the aid of the soluble form of the TGF-β-receptor II as a TGF-β antagonist, they identified endogenous TGF-β as the main contributor to osteophyte development during experimental OA.

On the contrary of this study, Lin et al.,[25] found that there is no significant difference in gene

expression of TGF β 2 in human chondrocytes between normal and OA patients. The current study showed that there was significant positive correlation between serum level of TGFβ2 and Kellegren-Lawrence radiographic grading in knee osteoarthritis group. In agreement with the obtained results, Stilianos et al.,[17] study showed positive correlation between serum level of TGF-β 2 and kellegren-Lawrence radiographic grading scale.

The obtained results showed there was statistically significant difference in distribution of the genotypes and allele frequencies between patients and healthy control.The NN genotype was higher in patients compared with controls (P =0.04).

Regarding odds ratio of genotypes, NN genotype is more risky for osteoarthritis 4.36 times than CC with (CI:0.98 – 19.42).The obtained results partially agreed with a study done by Gu et al.,[20]who found that NN carrier tends to be associated with the increased osteoarthritis.In a study done by Stefa´nsson et al.([27]concluded that the SNP6 was also significant for patients with OA.

In the current study N allele is more risky for osteoarthritis 3.13 times than C allele with(CI: (1.46 – 6.72).Against these results, Gu et al.([20] study, there was no significant difference between controls and the OA (P =0.131, OR= 0.845, 95% CI:0.678–1.052).

Regarding SNP6,Allele frequency was significantly different among knee OA patients compared with generalised OA.N allele was significantly more presented [OR] = 6.0, 95% confidence interval (CI :2.51 – 14.35); P <0.001.

The NN genotype was higher in knee OA group compared with generalised OA group (P=0.02). In agreement to these results, Gu et al.[20] study, NN genotype was higher in knee osteoarthritis (P= 0.021, OR = 2.402, 95% CI :1.141–5.060). Also in Minafra et al.[26] study, found the distribution of SNP6 genotypes had some relationship with knee OA.This suggests that this polymorphism can influence the disease progression.

Conclusion:

It could be concluded that MATN3 SNP6 polymorphism and TGF-β2 could be implicated in the pathogenesis of osteoarthritis.The observed association of N/N genotype with primary osteoarthritis emphasizes on the need for further prospective study that include larger sample size to confirm the results of the present study

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