

Osteoprotegerin gene polymorphisms and otosclerosis: an additional genetic association study, multilocus interaction and meta-analysis

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Abstract

Background Otosclerosis (OTSC) is among the most common causes of a late-onset hearing loss and alteration in the osteoprotegerin (OPG) expression was suggested in the implication of OTSC pathogenesis. A case-control association study of single nucleotide polymorphisms (SNP) in the OPG gene was performed in a Tunisian-North African population composed of 183 unrelated OTSC patients and 177 healthy subjects.

Results Rs3102734 and rs2073618 were significantly associated with OTSC which were predominantly detected in females after multiple corrections. The haplotypes A-A-C-G ($p = 0.0001$) and A-A-C-C ($p = 0.0004$) were significantly associated with OTSC suggesting a reduced risk in females. Multilocus association revealed that: rs2073618 in OPG, rs39335, rs39350 and rs39374 in RELN, rs494252 in chromosome 11 and rs1800472 in TGF β 1 showed significant OTSC-associated alleles in the Tunisian samples. In addition, meta-analysis for rs2073618 SNP in Tunisian, Indian and Italian populations revealed evidence of association of the rs2073618 polymorphism with OTSC (Odds ratios of 0.826 (95% CI [0.691–0.987], $p = 0.0035$)).

Conclusions Our findings suggest that rs3102734 and rs2073618 variants are associated with OTSC in an additional North African ethnic Tunisian population. Meta-analysis for rs2073618 based on three ethnic population revealed evidence of association with OTSC.

Background

Hearing loss (HL) in humans significantly reduces the quality of life and often leads to social isolation. One of the associated causes of acquired hearing impairment is otosclerosis (OTSC). OTSC is characterized by late-onset progressive sensorineural, conductive or mixed HL. The onset of this disease appears principally in the third decade while the hot spot age is in the sixth decade [1].

OTSC is an inflammatory bone remodeling disorder in the otic capsule of the middle ear, which in normal cases undergoes very little after development and ossification of the tissue. The exact mechanism that controls bone metabolism in the otic capsule and turnover within the auditory structures remains largely unknown. Bone is a dynamic tissue controlled by various biochemical, biomechanical and hormonal stimuli. Bone homeostasis is coordinated at the cellular level by a balance between resorption mediated by the osteoclasts and formation mediated by the osteoblasts. An imbalance between both processes occurs under certain pathological conditions that affect the skeleton, which leads to the development of bone diseases.

Animal models and genetically altered mice studies during the past twenty years have greatly deepen our knowledge on the factors that regulate the activity and formation of osteoclasts. In particular, the discovery of the receptor activator of nuclear- κ B ligand (RANKL)/RANK/osteoprotegerin (OPG) signaling axis provided a clue on the role played by the osteoblasts in these processes [2]. OPG, also known as osteoclastogenesis-inhibiting factor, is a cytokine receptor and a member of the tumor necrosis factor

(TNF) receptor superfamily encoded by the TNF receptor superfamily member 11B (*TNFRSF11B*) gene. The OPG protein is implicated in different signal transduction interfering biological responses, including apoptosis, cytotoxicity, differentiation and proliferation. It interacts with two common TNF family ligands, TRAIL [3] and RANKL [4]. The soluble receptor OPG acts as an endogenous decoy receptor towards RANKL, it binds RANKL and thus preventing interaction with and stimulation of RANK [2, 5]. Therefore, OPG inhibits osteoclast differentiation and maturation and induces the apoptosis of activated osteoclasts as demonstrated *in vitro* [6] and *in vivo* [7].

A deficiency in the OPG/RANKL composition induces a range of skeletal diseases such as osteoporosis and bone metastases [8]. Thus, the concentration of RANKL and OPG in bone is a major determinant of bone mass [9]. In addition, TNF-related apoptosis-inducing ligand (TRAIL) could put off the inhibitory activity of OPG. OPG prevents interaction between TRAIL and the Death Receptors and blocking TRAIL-induced apoptosis of several cell lines *in vitro* [3].

Although OPG plays an important role in controlling bone turnover, it is considered as a relevant candidate for genetic variations in the mechanisms of OTSC. For instance, OPG knockout mice, have displayed abnormal bone remodeling in the otic capsule similar to human temporal bones with OTSC [10], and overexpression of OPG in transgenic mice caused osteopetrosis [2]. In addition, OPG is expressed at high levels within the inner ear as detected in mice and is secreted to the perilymph and the surrounding bone which may serve to inhibit active bone remodeling within the otic capsule [11]. Thus, OPG can therefore be considered as a potent inhibitor of abnormal bone remodeling.

Previous Italian study [12] showed no associations between the rs2073618 (N3K) polymorphism in the *OPG* and OTSC. However, a recent Indian study revealed OPG polymorphisms in OTSC with sex-specific association of rs2073618 in males and rs3102734 in females [13]. Therefore, the contribution of OPG to OTSC remains controversial. Within this study, we aimed to address these shortcomings by performing a replication association study of OPG variants in OTSC by comparing a group of otosclerotic and control Tunisian-North African population. In addition, we performed a comprehensive meta-analysis of available case and control samples from previous studies under different genetic models to further evaluate *OPG* rs2073618 SNP with OTSC.

Results

OPG SNPs association with OTSC

We evaluated the association of previously documented OPG SNPs (rs2228568, rs7844539, rs3102734, rs2073618) with OTSC in a Tunisian population. HWE analysis revealed no deviations of the genotype frequencies in the control group for each SNP ($p > 0.001$).

Statistical power calculations estimated at 10% significance level that the population study had good power to detect SNP effects with rs3102734 and rs2073618 at 56.8% and 65.8%, respectively while rs2228568 and rs7844539 had limited power to detect an effect at 10.2% (Table 1).

Case-control association analyses revealed no statistically significant genetic differences between patients with OTSC and controls for the polymorphisms rs2228568 ($p = 0.483$) and rs7844539 ($p = 0.483$) within exon 4 of the OPG gene (Table 1). Statistically significant associations in rs3102734 ($p = 0.013$) and rs2073618 ($p = 0.007$) were detected in exon 1 of OPG gene from patients with OTSC compared to healthy controls.

Both SNPs in exon 1 showed significant allelic association with OTSC with ($p = 0.007$, OR = 0.45 [0.24–0.84]), and ($p = 0.0029$, OR = 1.57 [1.15–2.15]) for rs3102734 and rs2073618, respectively. The minor allele frequencies (MAFs) in the patients with OTSC accounted $p = 0.041$ for rs3102734 and $p = 0.71$ for rs2073618, while the respective MAFs in the healthy controls was $p = 0.09$ and $p = 0.619$, respectively. In addition, interactions of genotype and gender in rs3102734 and rs2073618 revealed a significant gender effect only in females (Table 2).

OPG haplotypes association with OTSC and linkage disequilibrium analysis

In order to evaluate the potential effects of SNPs allelic combinations on the risk of OTSC, haplotype analysis was first performed on the four described OPG SNPs (rs2228568, rs7844539, rs3102734, rs2073618) with OTSC in the Tunisian population.

Five significant haplotypes resulted within OPG gene on which two haplotypes verified sex-specific association of rs2073618 with OTSC. The genotype analysis for c.9C>G showed haplotypes A-A-C-G with “G” allele ($p = 0.0001$) and A-A-C-C with “C” allele ($p = 0.0004$) in females suggesting a reduced risk in females but not in males (Table 3). Analysis of the haplotype A-A-C-G showed that the disease-associated “G” allele in the Tunisian samples is in correlation with haplotype analysis as previously reported in the Indian population but with different sex-specific association.

Multilocus association with OTSC

To estimate the multilocus association effect of the significant associated SNPs rs2073618 (C>G) and SNPs in 3 different regions previously reported to be associated with OTSC in Tunisian population rs39335 (A>G), rs39350 (C>T) and rs39374 (A>G) in *RELN*, rs494252 in chromosome 11 (G>T) [14] and rs1800472 in *TGF β 1* (C>T) [15], allele combinations were examined with risk of OTSC. Statistical analysis was evaluated for the six SNPs (Table 4). Thus, four significant combinations were associated with OTSC (Table 5). Among them, three indicated significant differences between OTSC cases and healthy controls groups with sex-specific association including G-C-A-C-A-G ($p = 0.0349$) and C-C-A-T-G-G ($p = 0.0094$) with males, C-C-A-C-A-G ($p = 0.0008$) with females. Furthermore, analysis of this multilocus association showed significant OTSC-associated alleles in the Tunisian samples including both alleles “G” and C for rs2073618, “A” and “G” for rs39374, “C” and “T” for rs39350 with only reference allele for rs1800472 “C”, rs39335 “A” and, rs494252 “G”.

Furthermore, pairwise linkage disequilibrium was calculated according to D' and r^2 statistics for all possible two-way comparisons between all evaluated SNPs rs2073618 (C>G) in *OPG*, rs39335 (A>G), rs39350 (C>T) and rs39374 (A>G) in *RELN*, rs494252 in chromosome 11 (G>T) and rs1800472 in *TGF β 1* (C>T).

The degree of LD between evaluated SNPs resulted in strong linkage disequilibrium ($D' > 0.8$) for different two-way combinations. The SNP rs1800472 was found to be in complete LD with three SNPs rs2073618, rs39350 and rs39374, also the SNP rs494252 was in complete LD with rs39350 and rs39374 (Table 6).

Meta-Analysis of rs2073618 SNP

A meta-analysis for rs2073618 (c.9C>G) SNP was performed based on a Tunisian (the present study), Indian and Italian studies. Combining studied populations showed a total of 528 cases and 511 controls. Statistical significance was evaluated through Z and p-value. Association results were in the same direction as previously found in Indian population. A random effects model was used with significant heterogeneity. The forest plot shows correlation of rs2073618 SNP with OTSC, yielded a significant summary OR of 0.826 (95% CI [0.691–0.987] $p = 0.0035$) (Figure 1). The combined results suggested in significant association of rs2073618 (c.9C>G) with OTSC in various genetic models under dominant model $p = 0.006$, OR = 0.701 95% CI [0.545–0.901], recessive model $p = 0.076$, OR = 0.737 95% CI [0.527–1.032], heterozygous model $p = 0.022$, OR = 0.729 95% CI (0.557–0.955), homozygous $p = 0.02$ OR = 0.647 95% CI [0.449–0.933].

Sensitivity analysis was estimated via sequential calculation after excluding each study to evaluate the effects of individual one to the overall meta-analysis correlation. Egger's regression intercept was -4.09 and not significant p-value (2-tailed) about 0.63 suggesting that the variant is a non-sensitive one. However, publication bias was assessed by visual inspection of the Begg's funnel plots for asymmetry suggesting no publication bias between studies (Figure 2).

Discussion

Metabolic bone diseases and injuries are major causes of human skeletal malformations resulting in abnormal mineralized tissue microarchitecture. These are serious health concerns with a severe socio-economic impact [16–18]. Amongst others, OTSC affects several millions worldwide with late-onset of HL and represents therefore a major problem which requires more attention. In recent years, a lot of effort has been made to identify the disease-causing genes of OTSC, resulting in the determination of ten loci. Mapping these monogenic loci has not resulted in the recognition of any causative gene to date. Genome-wide association studies have identified some genetic risk factors.

OPG is a glycoprotein which inhibits osteoclast formation, maturation, osteolysis and induces the apoptosis of activated cells. The main function of OPG is to regulate the normal bone turnover with balanced bone resorption and formation. OPG is secreted by osteoblasts and osteogenic stromal cells

and protects the skeleton from excessive bone resorption by binding to RANKL and preventing it from interacting with RANK, the osteoclastic cell surface receptor [19]. Alteration in the OPG gene expression has been suggested to be involved in OTSC. For instance, research in animals genetically unable to produce OPG revealed HL and histopathology of the temporal bone consistent with that observed in OTSC [10]. Karosi et al. [20] reported a reduced or missing OPG gene expression in the tissue obtained from otosclerotic patients, however, the exact mechanism by which the control of the altered OPG expression in OTSC patients is not fully understood.

Within this study, we aimed to evaluate the association of OPG gene single-nucleotide polymorphisms with OTSC. For that purpose, we performed a replication association study by screening for OPG variants in a group of a North African Tunisian subpopulation affected with OTSC and comparing to a control group of healthy patients. The association results of SNP in exon 4 (rs2228568 and rs7844539) of *OPG* gene in Tunisian OTSC patients confirm the previous observations within the Indian population that provided no significant association with OTSC. This observation can be attributed to the limited statistical power in our population to detect an effect of the SNP of exon 4.

Priyadarshi et al. [13] reported a sex-specific association between the OPG gene polymorphisms c.9C>G (rs2073618) and c.30+15C>T (rs3102734) with OTSC in an Indian population. Our result reveals that rs2073618 SNP within the Tunisian population is in line with the previously reported polymorphism in Indian population. In addition, the haplotype interaction analysis of OPG SNP that was associated with OTSC in the Tunisian population showed disease-associated alleles in the Tunisian samples confirmed previous results. The rs2073618 was the only SNP genotyped in Indian and Italian populations. A meta-analysis based on the genotype and allele frequency distribution was performed within this study and further provided evidence of correlation of the rs2073618 SNP and OTSC suggesting that OPG may have an important role in the pathogenesis of OTSC.

Population-based association studies were carried out and identified a number of genes associated with OTSC in a number of populations including British, Belgian, Tunisian, and Indian. Among these, members of the transforming growth factor (TGF) superfamily, including TGF β 1 and bone morphogenetic proteins (BMP2, BMP4). Further evidence for the role of the TGF superfamily in the progression of OTSC has been evidenced by the several studies [15, 21–23] of protein expression, showing the presence of the TGF superfamily in active otosclerotic foci. In addition, genetic association analysis confirmed genetic variants in the *RELN* gene with OTSC in a number of populations [14, 24–26] including British, Italian, Belgian-Dutch and Tunisian populations. Within this study, we analyzed haplotype combinations with the risk of OTSC and we found that four regions interacted with the disease.

A previous study assessed the relationship between OPG variants and bone mineral density (BMD) or osteoporotic fractures in postmenopausal Chinese population and showed among nine SNP genotyped a significant association of rs2073618 with both BMD and osteoporotic fractures [27]. In addition, the same SNP in the OPG gene was associated with a decreased BMD in a case-control study performed in Mexican-Mestizo women with rheumatoid arthritis [28]. Taken together, alteration in the OPG gene is

related to abnormal bone metabolism and a number of skeletal pathologies including not only OTSC but also age-related bone diseases such as osteoporosis and rheumatoid arthritis.

To the best of our knowledge, this is the first study to demonstrate an association of SNP in the OPG with OTSC in a North African population and further supports previous reports suggesting linking of OPG gene polymorphism with OTSC in an Indian population. Further investigations include the functional verification of the SNP in OTSC by cloning and insertion of the SNP to detect whether the OPG promoter SNP (rs2073618) affects DNA-protein complex formation and promoter activity. In addition, with regard to the importance and synergy of the RANK/RANKL/OPG in bone turnover, the SNP of *RANK/RANKL* should be verified in OTSC patients.

Conclusions

In conclusion, we evaluated the association of OPG SNPs and OTSC and we found that rs2073618 SNP is linked to the onset of OTSC. This further supports the hypothesis that OPG plays an important role in bone turnover, metabolism within the otic capsule and alteration that may lead to the development of bone malformations including OTSC.

Methods

Patients were recruited by the Otolaryngology Department of the University Hospital of Sfax (Tunisia). The study population consists of 183 unrelated OTSC patients and 177 unrelated control subjects. The diagnosis of OTSC was based on clinical and audiological investigation and confirmed during surgery as previously described [14–15].

SNPs genotyping assays

Peripheral blood samples (5mL) were collected from all study subjects in EDTA tubes. Genomic DNA was extracted using standard phenol-chloroform protocol. Four SNPs (rs2228568, rs7844539, rs3102734, rs2073618) in OPG gene were selected for a replication study in a Tunisian population based on association data from previous studies [12–13].

Analysis of SNPs was performed using different allelic discrimination genotyping assays. All polymorphisms of the OPG gene were amplified from genomic DNA using selected primers (Table 7). For both SNPs in exon 4 rs7844539 (c.817+8A>C) and rs2228568 (c.768A>G) Sanger sequencing was used to detect variations. For rs3102734 (c.30+15C>T), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was performed using *HaeIII* restriction enzyme. While rs2073618 (c.9C>G) was genotyped using allele-specific polymerase chain reaction.

Association analysis in OPG

For the four studied SNP (rs2228568, rs7844539, rs3102734, rs2073618) in *OPG* gene, allele and genotype frequencies were calculated by direct counting method. The Hardy-Weinberg equilibrium (HWE) for genotype frequency in control groups was examined using *HWE calculator* <http://www.oege.org/software/hwe-mr-calc.shtml> as previously described [29]. Statistical power calculation was determined for case-control groups with significance level desired at 10% using a web browser program <http://osse.bii.a-star.edu.sg/>. Statistical analysis of association characteristics of the study cohort was performed using SPSS version 17.0 for Windows, Chicago, IL). A p-value (p) of 0.05 was fixed. Multivariate logistic regression analysis was done to evaluate associations between genotypes in OTSC and control samples and to investigate covariate interactions with gender. Statistical evaluation of level odds ratios (OR) and 95% confidence interval (95% CI) for the allelic model were calculated using *2BY2* program by Fisher's exact test [30] to evaluate the risk of OPG polymorphisms and OTSC ($p < 0.05$).

Association analysis in OPG, RELN, chromosome 11 and TGF β 1

Analysis of specific associated polymorphisms with OTSC, previously reported in the same Tunisian population, in *OPG*, *RELN*, chromosome 11 and *TGF β 1* were evaluated using *SNPalysse V8 Pro* (Dynacom, Chiba, Japan). Allelic combination analysis among OTSC case and control groups were evaluated by the maximum-likelihood method using the expectation-maximization (EM) algorithm. All statistical analyses were two-tailed with statistical significance level fixed at $p < 0.05$. Permutation p-values were calculated by comparing combination frequencies among case and control groups based on 10000 replications. Only significant combinations that are males, females and both sex-based analysis with frequency within $[0, 10^{-4}]$ in case or control groups were considered.

Pairwise linkage disequilibrium (LD) analysis of $|D'|$ and r^2 coefficients was assessed for six variants (rs2073618, rs1800472, rs39335, rs39350, rs39374, rs494252) using *SNPalysse V8 Pro* (Dynacom, Chiba, Japan). This analysis was estimated according to Hardy-Weinberg equilibrium model.

Meta-analysis of OPG SNP (rs2073618)

A meta-analysis was performed for rs2073618 (c.9C>G) SNP to investigate its genetic effect of OPG gene on previous studied OTSC populations. It was performed following the fixed-effect model or the random-effects [31] using *comprehensive Meta-Analysis Software V2* (Biostat Inc, Englewood, USA). The evaluation of heterogeneity of inter-study variations was done with Cochran's Q-test, as a simple Chi-square test [32]. The null hypothesis is that all studies are evaluating the same effect while the rejection of the null hypothesis ($p < 0.05$) would reveal heterogeneity between studies. In addition, an indicator of heterogeneity (I^2) was evaluated to measure the level of inconsistency across studies [33–36].

Abbreviations

HL: Hearing loss

OTSC: Otosclerosis

RANKL: Receptor activator of nuclear- κ B ligand

RANK: Receptor activator of nuclear- κ B

OPG: Osteoprotegerin

TNF: Tumor necrosis factor

TRAIL: Tumor necrosis factor related apoptosis inducing ligand

SNP: Single nucleotide polymorphism

HWE: Hardy–Weinberg principle

OR: Odds ratio

MAF: Minor allele frequency

TGF β 1: Transforming growth factor beta 1

BMP: Bone morphogenetic proteins

BMD: Bone mineral density

Declarations

Ethics approval and consent to participate

This study was approved by the ethical committee of the University Hospital of Sfax, and written informed consent was obtained from all participants of this study.

Consent for publication

Not applicable

Availability of data and materials

The data that support the findings of this study are not publicly available. Data are however available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AB, AT: Conceived the study, performed the experiments, analyzed the data and wrote the manuscript draft. FJ: Assisted and performed additional experiments. AC, IA, AG, IC: ascertained patients, diagnosis and sample collection. KH, NS: contributed in the data analysis. AS, MAM: Collected the samples. PVR: Assisted in supervising the data analysis. SM: Supervised the experimental part and acquired the funding of the project.

All the authors approved the final version of the manuscript.

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Tables

Table 1: Genotype and allele frequencies of the analyzed polymorphisms of *OPG* gene for cases with Otosclerosis and healthy controls.

SNP ID	Genotype Frequency (%)		X ² -Test p<0.05	OR (95% CI)	Allele Frequency (%)		p-value		
	Cases	Controls			Cases	Controls			
rs2228568 (c.768A>G)	A/A	78.6	76	0.483			0.48		
	A/G	18.1	22.4		A	87.6		87.2	
	G/G	3.3	1.6		G	12.4		12.8	
rs7844539 (c.817+8A>C)	A/A	78.6	76	0.483			0.48		
	A/C	18.1	22.4		A	87.6		87.2	
	C/C	3.3	1.6		C	12.4		12.8	
rs3102734 (c.30+15C>T)	C/C	92.9	84.2	0.013	Reference		0.007		
	C/T	6	14.1		0.379 (0.04-3.613)	C		95.9	91.2
	T/T	1.1	1.7		0.287 (0.116-0.708)	T		4.1	8.8
rs2073618 (c.9C>G)	C/C	14.8	19.9	0.007	Reference		0.0029		
	C/G	30.1	40.4		0.494 (0.235-1.037)	C		29.8	40.1
	G/G	55.1	39.8		0.482 (0.265-0.876)	G		70.2	59.9

SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval. Statistical significance could not be obtained with the otosclerosis (OTSC, cases) and control samples in the polymorphisms rs2228568 (p = 0.483) and rs7844539 (p = 0.483), while significant values were detected in the rs3102734 and rs2073618 (p < 0.05). Both SNP revealed allelic associations with OTSC.

Table 2: Interactions between genotype and gender of the analyzed polymorphisms of the *OPG* gene

SNP ID	Interaction gender	p-value (Females)	p-value (Males)
rs2228568 (c.768A>G)	0.642	0.442	0.467
rs7844539 (c.817+8A>C)	0.642	0.442	0.467
rs3102734 (c.30+15C>T)	0.059	0.046	0.096
rs2073618 (c.9C>G)	0.602	0.002	0.416

SNP, single-nucleotide polymorphism; Significant gender effect of the polymorphisms rs3102734 and rs2073618 was obtained in females only and are indicated in bold.

Table 3: *OPG* haplotype structure and frequencies

Haplotype	Haplotype frequency								
	Overall			Females			Males		
	Cases	Controls	p-value	Cases	Controls	p-value	Cases	Controls	p-value
A-A-C-G	0.6219	0.4859	0.0135	0.6383	0.3706	0.0001	0.5765	0.6842	0.2658
A-A-C-C	0.2228	0.335	0.0209	0.2132	0.4254	0.0004	0.2489	0.1842	0.4373
A-A-T-G	0.0439	0.0642	0.4015	0.0484	0.0836	0.2638	0.0298	0.0526	0.5453
C-G-C-C	0.0576	0.032	0.2958	0.0549	0.0376	0.5611	0.0669	0.0263	0.3632
C-G-T-G	0.0069	0.0119	0.6137	0.0062	0.0183	0.3465	0.0097	0	0.5439

Four SNPs on the following order (rs2228568, rs7844539, rs3102734, rs2073618) were used to analyze the haplotypes. Significant haplotype analysis was obtained in females for A-A-C-G and A-A-C-C (values indicated in bold).

Table 4: Statistics evaluations for six selected SNPs

	Number of genotypes	Number of alleles	Exact p-value	FDR q-value
rs2073618	3	2	2.6037 E-2	9.55 E-2
rs1800472	2	2	1	6.29 E-1
rs39335	3	2	7.4535 E-2	1.06 E-1
rs39350	3	2	3.1866 E-1	3.61 E-1
rs39374	3	2	4.2345 E-1	4.31 E-1
rs494252	2	2	1	5.94 E-1

FDR, false discovery rate. Six SNPs on the following order (rs2073618, rs1800472, rs39335, rs39350, rs39374, rs494252) were used to analyze association with OTSC

Table 5: Combinations and frequencies of significant associated SNPs in *OPG*, *RELN*, *Chromosome 11* and *TGFβ1* with OTSC.

Haplotype	Haplotype frequency								
	Over all			Females			Males		
	Cases	Controls	p-value	Cases	Controls	p-value	Cases	Controls	p-value
G-C-A-C-A-G	0.2703	0.2322	0.4113	0.2715	0.2367	0.5416	0.1387	0.3055	0.0349
G-C-G-C-A-G	0.1923	0.1662	0.5235	0.1888	0.1735	0.7609	0.2541	0.1662	0.2594
C-C-A-C-A-G	0.0669	0.1869	0.0004	0.0476	0.1732	0.0008	0.2363	0.1019	0.0609
C-C-A-T-G-G	0.1215	0.0533	0.0281	0.1077	0.0779	0.4392	0.0179	0.1569	0.0094

Haplotype combinations analysis revealed significant differences between OTSC and controls with sex-specific associations in C-C-A-C-A-G for females and G-C-A-C-A-G and C-C-A-T-G-G for males.

Table 6: Linkage disequilibrium between significant associated SNPs in *OPG*, *RELN*, *Chromosome 11* and *TGFβ1* with OTSC.

	rs2073618	rs1800472	rs39335	rs39350	rs39374	rs494252
rs2073618						
rs1800472	1					
rs39335	-0.4537	0.3508				
rs39350	0.2804	-1	-0.8309			
rs39374	0.1915	-1	-0.9229	0.8643		
rs494252	-0.0263	0.2859	-0.7712	-1	-1	

Results in bold indicate $D' > 0.8$: SNPs in strong linkage disequilibrium.

Table 7: Primers sequences used for SNPs genotyping in OPG gene

SNP	Sequence 5'- 3'	PCR-Product (bp)
OPG Ex1 rs3102734	Forward : TGCCGGGACGCTATATATAAC	226
	Reverse : TTCTCCCCGCCGGTCCGCT	
OPG Ex1 rs2073618	Forward 1: CGGGGACCACAATGAACAAC	273
	Forward 2: CGGGAACCACAATGAACAAG	
	Reverse: CTCTCTCTTGCTGTCTTCCAT	
OPG Ex4 rs2228568 rs7844539	Forward : TTACAAGGAAACTGGGGAGC	437
	Reverse : TGTGTGAGGAGGCATTCTTC	

Figures

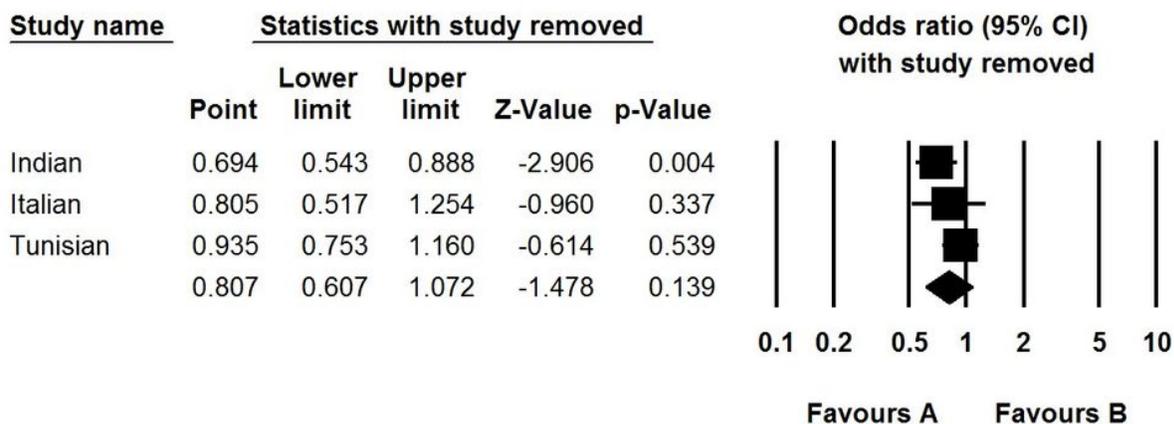


Figure 1

Forest plot of Indian, Italian and Tunisian (current study) studies for rs2073618 in the OPG gene. The association results were in line with the previously reported Indian study and a correlation between rs2073618 SNP with OTSC was obtained.

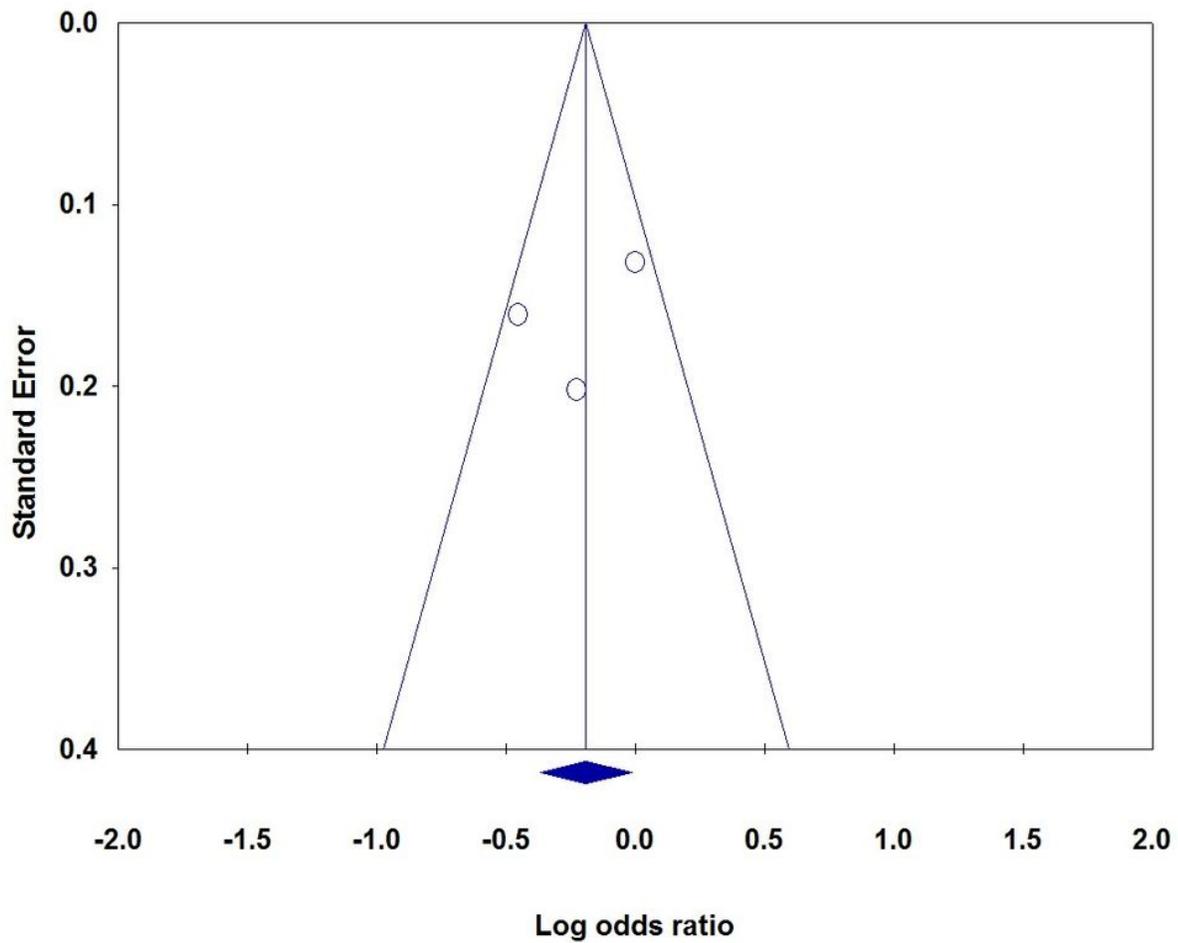


Figure 2

Begg's funnel plot of standard error by Log odds ratio of rs2073618 SNP in the OPG gene. No publication bias was obtained between the different studies (Indian, Italian and Tunisian).