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Cytotoxic T lymphocyte-associated antigen 4 rs231775 polymorphism and osteosarcoma

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Systematic reviews on osteosarcoma have concluded that *CTLA4* rs231775 AA genotype influences risk for the disease in the Chinese population. Remarkably, rs231775 shows different frequencies in different human populations. Therefore, it would be interesting to know whether this SNP is related to the risk of osteosarcoma in other populations. The present study aimed to evaluate the association between rs231775 and the susceptibility of osteosarcoma in the Spanish population. We performed an updated meta-analysis including a total of 538 cases and 623 controls.

The genotypic association analyses showed that the *CTLA4* rs231755 was associated with osteosarcoma susceptibility in the Spanish population. When meta-analysis was performed, the results displayed that *CTLA4* rs231775 AA genotype was associated with the risk of developing osteosarcoma in all analyzed populations (OR=2.07; 95% CI: 1.48-2.89).

The rs231775 AA genotype could be considered as a susceptibility marker in osteosarcoma.

Key words: CTLA4, rs231775, +49A/G and osteogenic sarcoma

Osteosarcoma is the most common primary malignant tumor of bone, mainly occurring in the second decade of life. The precise etiology of the disease remains partially unknown [1], but genetic factors seem to be involved in the development of the disease [2]. To date, several candidate gene association studies have been performed in osteosarcoma, analyzing more than 5000 SNPs. From all studies, rs231775, in the cytotoxic T-lymphocyte antigen-4 (*CTLA4*) gene, is one of the few SNPs found consistently associated with osteosarcoma risk in at least two different studies. Both publications reported that the AA genotype was associated with increased risk of osteosarcoma in the Chinese population [3, 4]. Interestingly, the rs231775 A allele has been shown to occur at different frequencies in different human populations: in the Chinese population the frequency is 0.37 while in Caucasians it is 0.63 (http://www.ncbi.nlm.nih.gov). Therefore, if the A allele is of risk for osteosarcoma Chinese patients, it would be interesting analyzing it in the Caucasian population, in which the frequency of the A allele is nearly twice as high.

The presented study aimed to evaluate whether rs231775 is associated with the risk of osteosarcoma in Spanish population. Moreover, we performed an updated meta-analysis to include and consider all the results published so far, following the PRISMA guidelines [5].

Materials and methods

Association study. A total of 99 Spanish patients diagnosed of osteosarcoma at the University Clinic of Navarra and 125 controls coming from the C.001171 collection of the Carlos III Health Institute were included in the analysis. Informed consent was obtained from all patients, or from their parents, before sample collection, following the Spanish Organic Law 15/1999. Genomic DNA was extracted from peripheral blood using standard procedures. *CTLA4* rs231775 genotyping was performed by PCR (FW: AAGGCTCAGCTGAACCTGGC and RV: CTGCTGAAACAAATGAAACCC) followed by restriction with *BstEII*. Duplicates were included in each assay. PCR products and digestion fragments were visualized after

electrophoresis on 3% agarose gels. The study was approved by the local ethics committees (105/2009) and was carried out according to the Declaration of Helsinki.

Systematic review and meta-analysis– Search strategy. We performed an exhaustive bibliographic search to identify studies that analyzed the association between the *CTLA4* rs231775 polymorphism and osteosarcoma susceptibility. We used the keywords and subject terms ("bone tumor" OR osteosarcoma) AND (polymorphism* OR SNP*), (rs231775 OR +49G>A) and (*CTLA4* or "Cytotoxic T lymphocyte antigen protein 4") AND ("bone tumor" OR osteosarcoma) for Pubmed (www.ncbi.nlm.nih.gov/pubmed) searches for papers published until June 2016. All references cited in these studies were then reviewed to possibly identify additional publications.

Inclusion and exclusion criteria. Original studies that investigated the association between the rs231775 polymorphism and osteosarcoma risk with enough data to calculate crude OR values were included. Reviews, meta-analyses and studies analyzing other regions or variants were excluded.

Data extraction. For each article, we gathered year of publication, first author, country of origin, ethnicity of population, sample size and genotype and/or allele frequencies. All data were independently extracted by two investigators and reached conformity on all items through consultation.

Quality assessment. The quality of included studies was assessed independently by two investigators by scoring according to a "methodological quality assessment scale" (Supplementary Table 1), which was modified from previous meta-analyses [6,7]. In the scale, five items, including the representativeness of cases, source of controls, sample size, quality control of genotyping methods and Hardy-Weinberg equilibrium (HWE) were carefully checked. Quality scores ranged from 0 to 10 and a higher score indicated better quality of the study. Scores > 5 were considered acceptable.

Statistical analysis. The data were statistically processed by R v2.15 software (http://www.R-project.org). Genotype frequencies in cases and controls were compared using a $\chi 2$ test. The deviation from HWE was also calculated by a $\chi 2$ test (in the control population). The effect sizes of the associations were estimated by the ORs from univariate logistic regression. We examined four genetic models to analyze the association between rs231755 and osteosarcoma risk in the Spanish population: (1) the codominant model compares major allele homozygotes *vs* heterozygote and *vs* minor allele homozygotes, (2) the dominant model compares major allele homozygotes *vs* heterozygotes + minor allele homozygotes, (3) the recessive model compares major allele homozygotes + heterozygotes *vs* minor allele homozygotes, (4) the log-additive model compares major allele homozygotes *vs* heterozygotes *vs* minor allele homozygotes. The major allele in the Spanish population was different from that showed by the Chinese population (A *vs* G). Therefore, to compare the effect of rs231755 A allele on osteosarcoma risk among populations, we took GG and GG+AG as reference genotypes (OR=1) and performed the following comparisons: GG *vs* AA + AG and GG+AG *vs* AA. For the meta-analysis, we used the genetic models described above plus allele model (which compares major *vs* minor allele). In all cases the significance level was set at 5%. The overall pooled OR and corresponding 95%CI were estimated using Mantel-Haenszel´s method with random effects model. The heterogeneity was quantified using the I^2 statistic (0-25% no heterogeneity, 25-50% moderate heterogeneity, 50-75% large heterogeneity and 75-100% extreme heterogeneity). Begg's funnel plot and Egger's test [8] were performed. These tests analyze the intervention effect estimates from individual studies against some measures of each study's size or precision. This means that effect estimates from small studies will therefore scatter more widely at the bottom of the graph, with the spread narrowing among larger studies. In absence of bias, the plot should resemble a symmetrical funnel. If there is bias, the plot will have an asymmetrical appearance.

Results

Genotype association study. The genotyping success rate was 85.26% (66 patients and 125 controls). Genotype frequencies in controls were consistent with those expected from the HWE (p>0.05). The genotypic association analyses showed that the *CTLA4* rs231755 was associated with osteosarcoma susceptibility in the Spanish population (p<0.05, under codominant and dominant models) (Table 1). The most significant result was obtained under the codominant model (AA *vs* AG) with an OR value of 0.39 (95% CI: 0.21-0.74). Supporting this result, the AG+GG genotype conferred protection to osteosarcoma susceptibility (OR=0.45; 95% CI: 0.24-0.83) under the dominant model (AA *vs* AG+GG).

Table 1. Association between genotype frequencies of *CTLA4* rs231775 and risk of osteosarcoma in the Spanish population.

Genotype	N (%) controls	N (%) cases	OR (95% CI) cod	P cod	OR (CI 95%) rec	P rec	OR (95% CI) dom	P dom	OR (IC 95%) log	P log
AA	37 (29.6)	32 (48.5)	1							
AG	83 (66.4)	28 (42.4)	0.39 (0.21-0.74)	0.005	AA/AG	0.16	AA	0.01	0.65 (0.38-1.11)	0.11
GG	5 (4.0)	6 (9.1)	1.39 (0.39-4.98)		GG 2.40 (0.70-8.18)		AG/GG 0.45 (0.24-0.83)			
Total	125 (100)	66 (100)								

Genotype distributions, odds ratio (OR; 95% confidence interval) and corresponding P values for logistic analyses of four different models are shown. Logistic regressions were applied in codominant (cod) (AA vs AG, AA vs GG), recessive (rec) (AA+AG vs GG), dominant (dom) (AA vs AG+GG) and log-additive (log) (AA vs AG vs GG) models.

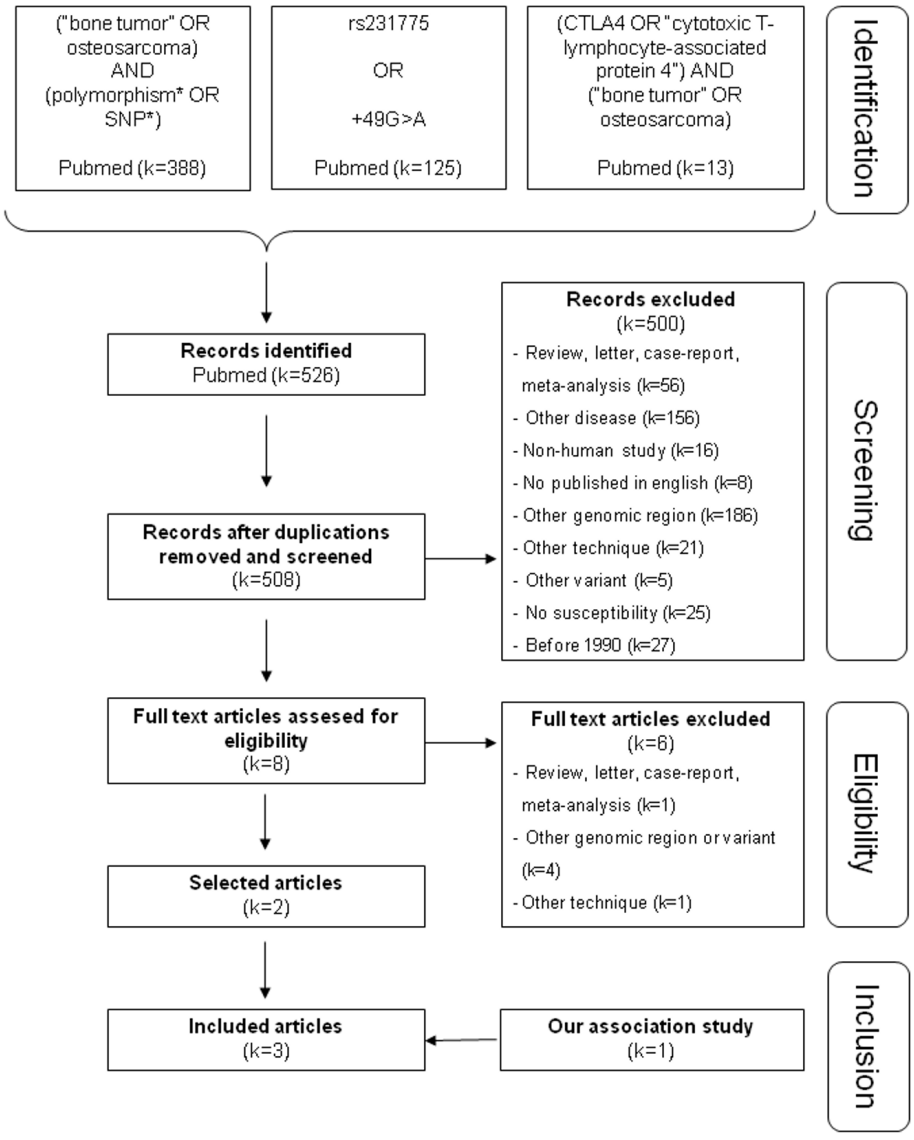

Figure 1. Flow-chart of study selection.

Meta-analysis. The original search provided 526 records. After eliminating duplications, 508 records remained. Of these, 500 were discarded after reviewing the abstracts because they did not meet the required criteria for inclusion. The full texts of the remaining 8 studies were examined in detail. Of these, we identified a total of 2 studies that investigated the association between *CTLA4* rs231775 and the risk of osteosarcoma. Both studies analyzed Chinese populations. We added the Spanish dataset to the meta-analysis (Figure 1). The characteristics of the three studies are presented in Table

Table 2. Characteristics of eligible studies in meta-analysis.

First author	Year	Country	Ethnicity	Genotyping method	HWE	Quality score	Reference
Bilbao-Aldaiturriaga	2016	Spain	Caucasian	PCR-RFLP	Y	Y	-
Wang	2011	China	Chinese	PCR-RFLP	Y	Y	(3)
Liu	2011	China	Chinese	PCR-RFLP	Y	Y	(4)

Y, yes; N, no

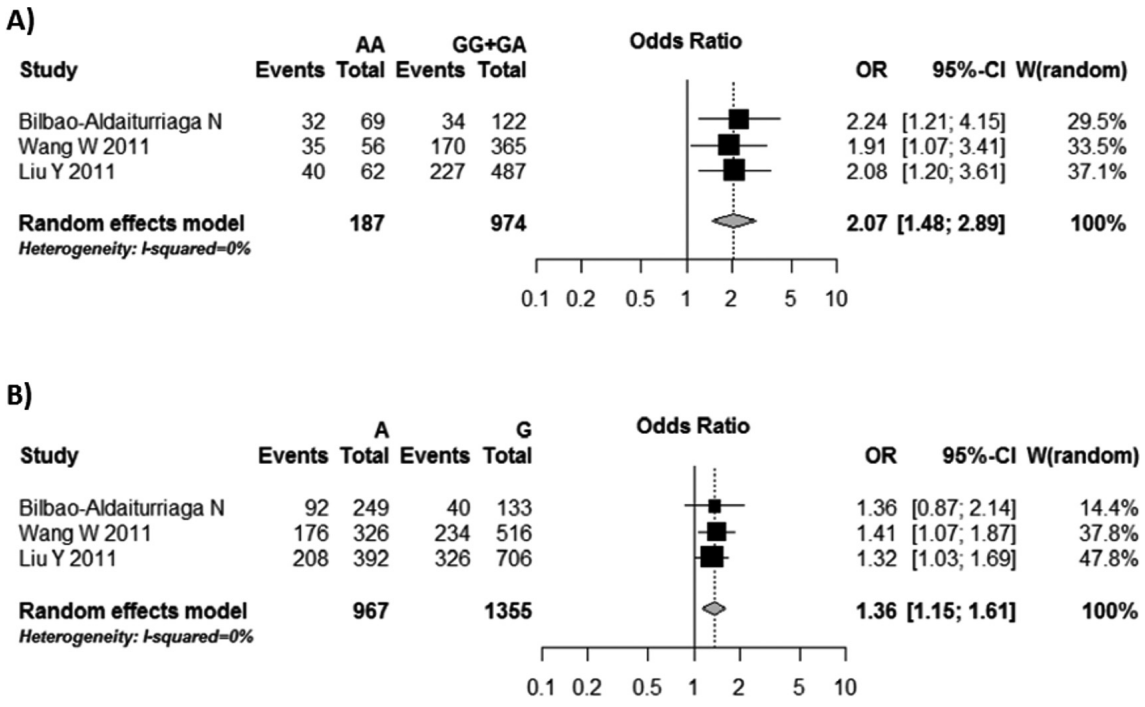

Figure 2. Forest plot for meta-analysis of the association between *CTLA4* rs231775 polymorphism and osteosarcoma risk comparing a) GG+AG *vs* AA and b) G *vs* A.

2. The distribution of genotypes in the controls of each study was in agreement with HWE (p>0.05). The three populations displayed significant association with osteosarcoma risk when the genotypes GG+AG *vs* AA when compared. In all populations, the AA genotype increased the risk of osteosarcoma (OR>1) (Table 3).

The meta-analysis finally included a total of three populations with 538 osteosarcoma patients and 623 controls. The results showed that AA genotype as well as A allele, increased the risk of osteosarcoma (OR=2.07; 95% CI:1.48-2.89 and OR= 1.36; 95% CI:1.15-1.51, respectively). The forest plots of the two significant tests (GG+AG *vs* AA and G *vs* A) are shown in Figure 2. The heterogeneity among studies was 0% under the allelic and genotypic tests (Figure 2).

Publication bias. The shapes of funnel plot did not reveal obvious evidence of asymmetry indicating that biases from publication may not have influence on the results (Figure 3).

Discussion

This study confirms that the *CTLA4* rs231775 AA genotype is associated with the risk of developing osteosarcoma. To date two previous studies had tested the hypothesis that the *CTLA4* rs231775 polymorphism was relevant to osteosarcoma, but these studies were performed in Chinese populations and there were no data in Caucasian population yet. Here, we present the results of the association study between rs231775 and osteosarcoma in Spanish population. Remarkably,

Table 3. Association between genotype frequencies of *CTLA4* rs231775 and risk of osteosarcoma in the studies included in the meta-analysis.

Population	Freq A allele controls	Genotype	N(%) controls	N(%) cases	$OR_{GG\ vs\ AA+AG}$ (IC 95%)	P	$OR_{GG+AG\ vs\ AA}$ (IC 95%)	P	Ref
		AA	37 (29.6)	32 (48.5)					
Spain	0.63	AG	83 (66.4)	28 (42.4)	GG	0.19	GG+AG	0.01	-
		GG	5 (4.0)	6 (9.1)	AA+AG 0.42 (0.12-1.42)		AA 2.24 (1.21-4.15)		
		AA	22 (7.8)	40 (15.0)					
China	0.33	AG	140 (49.6)	128 (47.9)	GG	0.05	GG+AG	0.03	(4)
		GG	120 (42.6)	99 (37.1)	AA+AG 1.49 (0.99-2.22)		AA 1.91 (1.07-3.41)		
		AA	21 (9.7)	35 (17.1)					
China	0.35	AG	108 (50)	106 (51.7)	GG	0.19	GG+AG	0.01	(3)
		GG	87 (40.3)	64 (31.2)	AA+AG 1.26 (0.89-1.77)		AA 2.08 (1.20-3.61)		

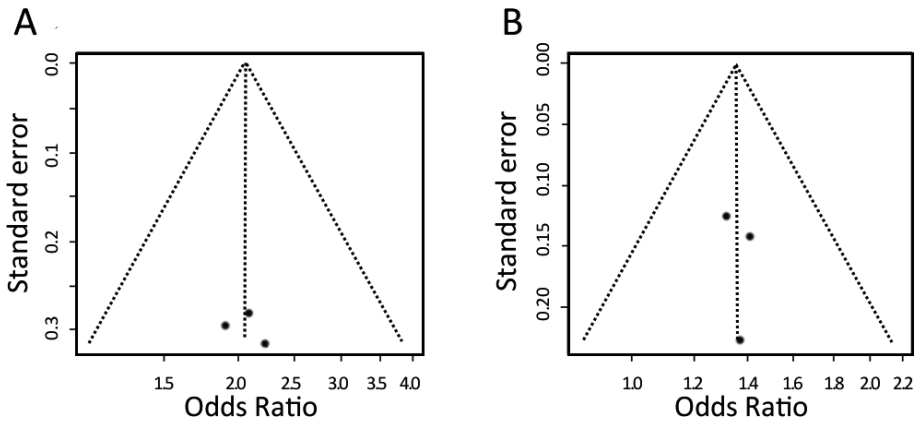

Figure 3. Funnel plots of the Egger's test of *CTLA4* rs231775 polymorphism for publication bias A) GG+AG *vs* AA comparison and B) G *vs* A comparison.

rs231775 was also significantly associated with osteosarcoma in the Spanish population.

Of note is that rs231775 A allele occurs at different frequencies at different populations. While in the two previous Chinese populations analyzed the frequency of A allele was 0.33 and 0.35, respectively, Spanish population showed a frequency of 0.63 (similar to other Caucasian populations). Anyway, when we performed a meta-analysis including all the populations studies to date (two Chinese and our Spanish population) we observed that individuals with A allele and AA genotype had an increased risk of developing osteosarcoma (OR=1.36 and 2.07, respectively) compared to those G allele carriers. *CTLA4* plays important roles in downregulating T-cell activation, thereby attenuating antitumor responses and increasing cancer susceptibility [9]. Interestingly, the polymorphism rs231775 is located at position +49 in exon 1 of *CTLA4* and causes an amino acid exchange (threonine to alanine) in the peptide leader sequence, which theoretically might alter the *CTLA4* function. Indeed, a functional study on multiple sclerosis displayed that A allele increased the CTLA4 production [10], downregulating T cell activation. Therefore, it is logical to think that AA genotype may be involved in the risk of osteosarcoma. In line with these results, *CTLA4* rs231775 AA genotype has been already correlated with the pathogenesis of other bone tumors as Ewing's sarcomas [11, 12]. Moreover, this polymorphism has been associated with other types of cancer, such as colorectal [13], lung [14], cervical [15], gastric [16] or breast cancer [17].

Our meta-analysis has several strengths. First, we used a comprehensive search strategy with well defined inclusion and exclusion criteria. Second, two reviewers performed the study selection and data extraction independently and discrepancies were resolved by consensus. Third, we assessed the quality of the included studies by predefined criteria and the score of included studies here was high. Moreover the heterogeneity among studies was 0% and there was not publication bias. Finally, all genotype data extracted from the studies are reported in the meta-analysis. Nevertheless, there are still some limitations. For instance, although we included the Spanish population to the meta-analysis, the number of cases is still relatively small; which is inherent to the low incidence of this tumor.

In conclusion, the current meta-analysis suggests that *CTLA4* rs231775 is associated with the risk of developing osteosarcoma and could therefore consider as susceptibility marker in osteosarcoma. Future studies are needed to confirm these results.

Supplementary information is available in the online version of the paper.

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Supplemental material

Supplemental Table 1. PRISM check list showing the guidelines followed in the present meta-analysis

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	3
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	4
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	4,5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	4,5
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	4
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	5,6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	5,6
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	5,6
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	6
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	-

RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Table 2
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Figure 3
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Table 3, Figure 2
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	7
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	8
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	-
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	9
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	10
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	10
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	11

Supplemental Table 2. Scale for methodological quality assessment

Criteria	Score
1.Representativeness of cases	
OS diagnosed according to acknowledged criteria	2
Mentioned the diagnosed criteria but not specifically described	1
Not described	0
2.Source of controls	
Population or community based	3
Hospital-based OS-free controls	2
Healthy volunteers without total description	1
OS-free controls with related diseases	0.5
Not described	0
3.Sample size	
>100	2
25-100	1
<25	0
4.Quality control of genotyping methods	
Repetition of partial/total tested samples with a different method	2
Repetition of partial/total tested samples with the same method	1
Not described	0
5.Hardy-Weinberg equilibrium (HWE)	
Hardy-Weinberg equilibrium in control subjects	1
Hardy-Weinberg disequilibrium in control subjects	0