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Sharon A. Savage[1,*], Lisa Mirabello[1], Zhaoming Wang[2], Julie M. Gastier-Foster[3,4], Richard Gorlick[5], Chand Khanna[6], Adrienne M. Flanagan[7,8], Roberto Tirabosco[8], Irene L. Andrulis[9], Jay S. Wunder[9], Nalan Gokgoz[9], Ana Patiño-Garcia[10], Luis Sierrasesúmaga[10], Fernando Lecanda[10], Nilgün Kurucu[11], Inci Ergurhan Ilhan[11], Neriman Sari[11], Massimo Serra[12], Claudia Hattinger[12], Piero Picci[12], Logan Spector[13], Donald A. Barkauskas[14], Neyssa Marina[15,16], Silvia Regina Caminada de Toledo[17], Antonio S. Petrilli[17], Maria Fernanda Amary[9], Dina Halai[9], David M. Thomas[18], Chester Douglass[19], Paul S. Meltzer[6], Kevin Jacobs[2], Charles C. Chung[2], Sonja I. Berndt[1], Mark P. Purdue[1], Neil E. Caporaso[1], Margaret Tucker[1], Nathaniel Rothman[1], Maria Teresa Landi[1], Debra T. Silverman[1], Peter Kraft[19], David J. Hunter[19], Nuria Malats[20], Manolis Kogevinas[21,22,23,24], Sholom Wacholder[1], Rebecca Troisi[1], Lee Helman[6], Joseph F. Fraumeni Jr.[1], Meredith Yeager[2], Robert N. Hoover[1], and Stephen J. Chanock[1]

[1]Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

[2]Cancer Genomics Research Laboratory, National Cancer Institute, Division of Cancer Epidemiology and Genetics, SAIC-Frederick, Inc., NCI-Frederick, Frederick, MD, USA

[3]Nationwide Children's Hospital, Columbus, OH, USA

[4]The Ohio State University, Department of Pathology and Pediatrics, Columbus, OH, USA

[5]Albert Einstein College of Medicine, The Children's Hospital at Montefiore, Bronx, New York, USA

[6]Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

[7]UCL Cancer Institute, Huntley Street, London, UK

[8]Royal National Orthopaedic Hospital NHS Trust, Stanmore, Middlesex, UK

[9]University of Toronto, Litwin Centre for Cancer Genetics, Samuel Lunenfeld Research Institute, Mt Sinai Hospital, Ontario, Canada

[10]Department Of Pediatrics, University Clinic of Navarra, Universidad de Navarra, Pamplona, Spain

*Corresponding Author: Sharon A. Savage, M.D., F.A.A.P., Senior Investigator, Clinical Genetics Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, 6120 Executive Blvd. EPS 7018, Rockville, MD 20892, Tel: 301-496-5785, savagesh@mail.nih.gov.

[11]A.Y. Ankara Oncology Training and Research Hospital, Department of Pediatric Oncology, Ankara, Turkey

[12]Laboratory of Experimental Oncology, Orthopaedic Rizzoli Institute, Bologna, Italy;

[13]University of Minnesota, Minneapolis, MN, USA

[14]Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

[15]Stanford University, Palo Alto, CA, USA

[16]Lucile Packard Children's Hospital, Palo Alto, CA, USA

[17]Pediatric Oncology Institute, GRAACC/UNIFESP, São Paulo SP Brazil

[18]Sir Peter MacCallum Department of Oncology, University of Melbourne, East Melbourne, Victoria, Australia

[19]Harvard School of Public Health, Boston, MA, USA

[20]Centro Nacional de Investigaciones Oncologicas, Madrid, Spain

[21]Centre for Research in Environmental Epidemiology (CREAL), Barcelona, Spain

[22]IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain

[23]CIBER Epidemiologia y Salud Pública (CIBERESP), Barcelona, Spain

[24]National School of Public Health, Athens, Greece

Abstract

Osteosarcoma is the most common primary bone malignancy of adolescents and young adults. In order to better understand the genetic etiology of osteosarcoma, we performed a multi-stage genome-wide association study (GWAS) consisting of 941 cases and 3,291 cancer-free adult controls of European ancestry. Two loci achieved genome-wide significance: rs1906953 at 6p21.3, in the glutamate receptor metabotropic 4 [GRM4] gene ($P = 8.1 \times 10^{-9}$), and rs7591996 and rs10208273 in a gene desert on 2p25.2 ($P = 1.0 \times 10^{-8}$ and 2.9×10^{-7}). These two susceptibility loci warrant further exploration to uncover the biological mechanisms underlying susceptibility to osteosarcoma.

Main Text

Osteosarcoma is the most common primary malignant bone tumor in children and young adults, affecting approximately four persons per million per year in the U.S.[1]. The peak incidence correlates with the pubertal growth spurt, occurring earlier in females than in males. It is more common at sites of rapid bone growth. Tall stature and high birth weight are proven risk factors[2], and osteosarcoma is a syndrome-associated malignancy in the Li-Fraumeni syndrome, Rothmund-Thomson syndrome, and hereditary retinoblastoma cancer susceptibility syndromes. Several small case-control studies have reported preliminary associations of common genetic variants with osteosarcoma risk in biologically-plausible pathways [3-11], e.g., growth and DNA repair, but statistical power has been limited by small sample sizes[12].

We developed an international, multi-institutional collaborative study of osteosarcoma to conduct a GWAS (Supplementary Table 1). Germline genomic DNA was extracted from either blood or buccal cells drawn from osteosarcoma case series using standard methods. Participating subjects provided informed consent under the auspices of local Institutional Review Boards. Control subjects were selected from previously-scanned cancer-free adults

over the age of 55 years drawn from large case control and cohort studies as per an approach successfully used for Ewing sarcoma[13] and pediatric acute lymphoblastic leukemia (ALL)[14].

Genotyping of all cases was conducted using the Illumina OmniExpress SNP microarray over time resulting in two parts, known as Stage 1A and 1B, due to the staggered receipt of samples in this multi-institutional study design (Supplementary Figure 1, Online Methods). Stage 1A genotyped 910 available osteosarcoma cases from five studies. Based on quality control filtering (including locus or sample missing rates, sample heterozygosity, Hardy-Weinberg equilibrium, and sex verification) and assessment of underlying population substructure (STRUCTURE and principal components analyses), 596 cases of European ancestry were advanced to the primary GWAS analysis (Supplementary Figures 2 and 3); 97 African American and 99 Hispanic cases were identified and excluded from the present analysis. A later download of samples, known as Stage 1B consisting of 218 osteosarcoma cases, was also scanned with the OmniExpress SNP microarray (Supplemental Table 1). The same quality filtering and population substructure analyses were applied, resulting in an additional 98 cases of European ancestry for the final analysis. The choice of SNPs for follow-up was generated based on the first set (Stage 1A) but for the final analysis, we present the results of the full set of 694 cases (namely, the combined Stages 1A and 1B).

Overall, 698,968 SNPs passed quality control metrics for the full set of 694 cases for final analysis. The number of SNPs overlapping with pooled controls was 510,856, 510,856, 310,384 and 304,092 for the USA, Spain, Italy and UK components, respectively, and were used in the association analyses (see Online Methods).

We combined already scanned controls drawn from two large cohort studies, Prostate, Lung, Colon and Ovarian Cancer Prevention Trial [PLCO][15] and the American Cancer Society Cancer Prevention Study-II [CPSII][16] scanned on Illumina Omni 2.5M array and three European studies (i.e., Spanish Bladder Cancer Study[17] on the HumanHap 1M SNP microarray, Environment and Genetics Lung Cancer Etiology Study [EAGLE][18]/on the HumanHap 550 SNP microarray and the Wellcome Trust Case Control Consortium [WTCCC][19] with the HumanHap 550 SNP microarray). We selected 2,703 controls based on comparable quality control metrics for comparison with the scanned cases. A systematic assessment of the underlying population substructure was conducted as described above to remove highly admixed individuals (non-European admixture of >20%) from a model to investigate cases of European background (Supplementary Figures 2 and 3).

Association analyses were performed using a 1-degree of freedom trend test adjusted by sex and eigenvectors. Optimized TaqMan assays were designed based on SNP associations observed in the first set of cases in the scan, known as Stage 1A (Supplementary Table 2). This set of 30 SNPs was genotyped in a follow-up set of 247 osteosarcoma cases (Supplemental Table 1) and 588 controls drawn from PLCO and NHS. A fixed effect meta-analysis was applied to the combined results of the two scanned sets comprising Stage 1 (596 cases in Stage 1A and 98 cases in Stage 1B yielding a total of 694 total, unique cases) with the replication stage of 30 SNPs with $P < 1.0 \times 10^{-4}$.

In a fixed effect meta-analysis of all cases and controls of European ancestry, 941 cases and 3,291 cancer-free adult controls, we observed two regions that achieved genome-wide significance on chromosomes 6p21.3 and 2p25.2 (Table 1 and Supplementary Table 2).

The locus on 6p21.3, marked by rs1906953 is associated with susceptibility to osteosarcoma ($P=8.0\times10^{-9}$, odds ratio (OR) 1.57, 95% confidence interval (CI) 1.35-1.83) (Table 1). The SNP, rs1906953, is located in intron 7 (IVS 7-32066 C>T) of the glutamate receptor metabotropic 4 (*GRM4*) gene (Figure 1A). Our most significant SNP resides within the

gene, *GRM4*, which is within a distinct haplotype block from the *HLA* Class II region, which is more than 1Mb telomeric on 6p21.3. It is also notable that between the *HLA* Class II region and our signal for OS is a distinct region containing *BAK1*, which in published GWAS studies has been conclusively associated with platelet counts[20-22], chronic lymphocytic leukemia[23], and testicular germ cell tumors[24].

The reference allele (C) of *GRM4* is highly conserved and its allele frequency is higher in more ancient populations; the global minor allele frequency (MAF) is 0.29 in the 1000 Genomes Project (Phase 1 genotype data from 1094 individuals[25]) data, with a greater frequency in individuals of Asian or African ancestry (MAF 0.46 and 0.59, respectively). While there are four surrogates in individuals of European background (r^2>0.6 and within ±500kb) in the 1000 Genomes data, the index SNP, rs1906953, maps to a DNaseI hypersensitivity region in the Encyclopedia of DNA Elements (ENCODE)[26] data set, raising the distinct possibility that the variant is within a region that is accessible to open chromatin (Supplementary Table 3) and could manifest active regulatory elements. One intronic surrogate SNP (rs73410895) is predicted to alter known regulatory motifs (Supplementary Table 3).

GRM4 is a plausible candidate gene that has been implicated in intracellular signaling and inhibition of the cyclic AMP (cAMP) signaling cascade. In mice, a cAMP-dependent protein kinase (Prkar1α) is an osteosarcoma tumor suppressor gene involved in tumorigenesis[27,28], suggesting the cAMP pathway is important in osteosarcoma. Although, glutamate signaling is best characterized in the central nervous system (CNS), where it is known to be involved in the excitability of gonadotropin releasing hormone neurons, it also occurs in bone[29]. The GRM4 receptor is expressed in bone osteoblast (bone-building) and osteoclast (bone-resorbing) cells, suggesting that glutamate signaling is involved in cell differentiation and regulation of bone formation and resorption[30]. *GRM4* is expressed in human osteosarcoma cells[31] and associated with poor prognosis in colorectal cancer[32], pediatric CNS tumors[33], rhabdomyosarcoma, and multiple myeloma[34], as well as cancer cell proliferation *in vitro*[35].

A second signal was observed in an intergenic region on chromosome 2p25.2 (Figure 1B); rs7591996 (OR 1.39, 95% CI 1.23-1.54, *P*=1.0 × 10⁻⁸) achieved genome-wide significance. A second SNP, rs10208273, was moderately correlated with the first signal (r^2=0.32 in HapMap 3 release 2) and approached genome-wide significance (OR 1.35, 95% CI 1.21-1.52, *P*=2.9 × 10⁻⁷) (Figure 1B and Table 1). The risk allele frequency for rs7591996 in our cases of European ancestry was 0.50 and 0.45 for the controls. rs7591996 has a global MAF of 0.38 in the 1000 Genomes data; the risk allele is the minor allele in CEU individuals and the major allele in individuals of Asian or African ancestry (MAF 0.35 and 0.14, respectively). Both rs7591996 and rs10208273 occur in a region of relatively low linkage disequilibrium (Figure 1B). The 1000 Genomes data for European populations documents at least 27 surrogate SNPs (r^2>0.6 and within ±500kb). Based on ENCODE[26] data, the two index SNPs do not localize to a region of active regulatory elements or transcription factor binding sites. However, several of the surrogate SNPs alter known regulatory motifs and transcription factor binding sites (Supplementary Table 3) and may affect regulation. These findings suggest that further sequencing and fine-mapping will be required to determine which variants will be optimal for functional studies needed to explain the direct association.

Finally, we report a third locus that is promising but does not yet achieve genome-wide significance, rs17206779, (OR 0.75, 95% CI 0.68 to 0.84, *P*=5.1×10⁻⁷) located in the ADAM metallopeptidase with thrombospondin type 1 motif, 6 (*ADAMTS6*) gene (g. 64447777C>T) on chromosome 5q12.3. It is notable that variation in genes encoding members of the ADAMTS protein family have been associated with height[36], a known risk

factor for osteosarcoma[2]. Further data are required to confirm this locus, and then fine-map the region prior to conducting functional studies.

To further explore the two significant regions reported in this manuscript, we imputed SNPs based on the 1000 Genomes data (March 2012 release) together with the DCEG Imputation Reference Set version 1[37] using the IMPUTE2 program[38] across a 1 Mb region centered on each index SNP (see Online Methods). A subsequent association analysis did not identify new signals that are substantially stronger than the genotyped SNPs for both 2p25.2 and 5q12.3 regions (Supplementary Figure 4). Although there appear to be stronger signals in the imputed data for the *HLA* Class II region (Supplementary Figure 4a), there is a strong recombination hotspot separating it from the region marked by the index SNP rs1906953. In this regard, it will be critical to pursue more detailed mapping and genotyping of the HLA class II region to determine if there is an independent signal in addition to that which resides in the *GRM4* gene.

An assessment of loci previously reported in candidate gene association studies[3-12] did not reveal that the reported loci approached genome-wide significance (Supplementary Table 4). It is notable that the SNPs in the 8q24.3 locus as well as the multi-cancer 8q24.21 region appear to be promising candidates[10]; the strongest signal of a set of highly correlated SNPs on 8q24.21, rs11777807, demonstrated a $P = 4.6 \times 10^{-5}$, and rs369051 on 8q24.3 had a $P = 1.16 \times 10^{-4}$. Further sample sizes are needed to determine if these are stable or perhaps due to chance.

We have conclusively identified two susceptibility regions, on chromosome 2p25.2 and 6p21.3, the latter harboring a plausible candidate gene, *GRM4*, in a multistage GWAS of osteosarcoma. It is noteworthy that the loci we have identified demonstrate estimated odds ratios greater than 1.5, values greater than those observed for most adult cancer GWAS. Our findings are consistent with the high risk estimates reported for Ewing's sarcoma[13] and ALL[14], other rare pediatric tumors. Further investigation of these associated loci is warranted to uncover the biological mechanisms underlying susceptibility to osteosarcoma.

Online Methods

Genome-wide SNP Genotyping

Genome-wide SNP genotyping of osteosarcoma cases was conducted using the Illumina OmniExpress Beadchip at the NCI Cancer Genomics Research Laboratory (CGR) in the Division of Cancer Epidemiology and Genetics (DCEG), National Cancer Institute, Bethesda, USA. Genotype analysis occurred in two stages because of the sequential receipt of samples. Stage 1A consisted of 910 unique cases from the Children's Oncology Group (COG), Toronto Study, PamplonaCUN, Rizzoli (Italy), Ankara (Turkey) and University College London (UCL). Adult, cancer-free controls were drawn from previously scanned studies in the US (add references), namely, the Prostate, Lung, Colon and Ovary Prevention Trial (PLCO) and the Cancer Prevention Study-II (CPS-II) of the American Cancer Society, both scanned on the Omni 2.5 M SNP microarray; European controls were drawn from the EAGLE (Italy) scanned on the HumanHap550, the Welcome Trust Case-Control Cohort-UK (WTCCC-UK) scanned on the Human Hap550 and the NCI-Spanish Bladder Cancer Study, scanned on the Human Hap 1 M SNP microarray chip. Stage 1B consisted of unique 218 cases scanned on the Omni Express Beadchip drawn from UCL, Rizzoli, PamplonaCUN studies plus two additional studies from the PeterMac (Australia) and Brazil. Cancer-free adult controls were drawn from the PLCO and the Nurses Health Study (NHS), scanned on the Illumina 2.5 M Omni and the OmniExpress, respectively.

Each participating study obtained informed consent from study participants and approval from its Institutional Review Board (IRB) for this study and obtained IRB certification permitting data sharing in accordance with the NIH Policy for Sharing of Data Obtained in NIH Supported or Conducted Genome-Wide Association Studies (GWAS). The CGEMS data portal provides access to individual level data from the NCI scan ONLY to investigators from certified scientific institutions after approval of their submitted Data Access Request.

Quality Control Assessment

Systematic quality control was conducted which included QC steps specific for the performance of different arrays at distinct times. For SNP assays, exclusions included those with less than 90% of completion rate, and SNPs with extreme deviation from fitness for Hardy-Weinberg proportion ($P < 1 \times 10^{-7}$). There were 29 duplicated cases in Stages 1A and 1B; concordance rates were 99.96%.

Analysis of Stage 1A—In the QC analysis of stage 1A, samples were excluded based on: 1) completion rates lower than 94% (n=28 samples); 2) abnormal heterozygosity values of less than 20% or greater than 31% (n=8); 3) expected duplicates (n=23 pairs); 4) abnormal X chromosome heterozygosity (n=1); 5) phenotype exclusions (due to ineligibility or incomplete information) (n=57). Genotypes for all subject pairs were also computed for close relationships (1-2nd degree) using GLU qc.ibds module with an IBD0 threshold of 0.70; no first-degree relatives were identified in the cases scanned. Utilizing a set of 12,000 un-linked SNPs (pair-wise $r^2 < 0.004$) common to the GWAS chips used herein[33], 264 subjects with less than 80% European ancestry were excluded based on STRUCTURE analysis[34] and PCA[35]; majority of these represent 97 cases of African American ancestry and 99 of Hispanic ancestry. The final participant count for the association analysis for Stage 1A was 596 cases and 2,703 controls of European ancestry. 698, 968 SNPs were available after the QC filtering. The number of SNPs overlapping with pooled controls are follows, 510,856, 510,856, 310,384 and 304,092 for the USA, Spain, Italy and UK components, respectively, were used in the downstream association analyses. PCA analyses per components did not reveal significant differences between cases and controls using the same 12,000 SNPs described above.

Analysis of Stage 1B—Similar quality control metrics were applied to the second set of additional scanned cases, known as Stage 1B. For the current analysis, an additional 98 cases were added to the association analysis of individuals of European ancestry.

Quality control metrics for previously scanned cancer-free adult controls—The pooled PLCO controls were scanned on the Illumina Human Omni2.5 array, we excluded samples with missing rate > 6%; excluded SNPs with missing rate > 10%; excluded samples with mean heterozygosity > 21% or < 16%; excluded one from each of cryptic related pair; excluded non-CEU admixed individuals based on the STRUCTURE analysis. The pooled SPBC controls were scanned on Illumina 1M SNP array and the genotype QC were previously described[17]; the pooled EAGLE controls were scanned on Illumina 550K SNP array and the genotype QC were previously published[18]; the pooled WTCCC controls were previously described[39].

Replication and TaqMan genotyping

A follow-up analysis of the 30 SNPs selected for replication (based on Stage 1A) was conducted using the TaqMan genotyping assays (ABI, Foster City, CA) that were optimized in the CGR pipeline. A total of 247 cases and 550 controls were analyzed; the studies included 99 histologically confirmed osteosarcoma cases and 65 hospital-based cancer-free

pediatric controls from the National Osteosarcoma Etiology Study Group plus 148 cases drawn from the studies used in the scan in which there was insufficient DNA for scanning. Cancer-free controls were also selected from the PLCO and NHS studies (previously analyzed on the Omni2.5M and OmniExpress, respectively).

Statistical analysis

Associations between the SNPs and the risk of osteosarcoma were estimated using unconditional logistic regression by the odds ratio (OR) and 95% confidence interval (CI) using the multivariate unconditional logistic regression assuming a trend genetic model (the effect of the variant by log-additive model with 1 degree of freedom). PCA analysis revealed four significant ($p<0.05$) eigenvectors when included in the NULL model. The main effect model was adjusted by sex, country, four eigenvectors, based on significance with $p<0.05$ observed in the NULL model. For the replication studies, the analysis was adjusted for sex only.

For stage 1 (A + B), in addition to the joint analysis detailed as above, a fixed effect meta-analysis was performed to combine results for each country (component) to facilitate filtering out artifacts by checking for the heterogeneity across countries. Similar meta-analysis was also performed to combine scan (discovery) association results with taqman (replication) association results for those 30 SNPs.

The estimated inflation factors, λ and adjusted λ_{1000} of the test statistic for the combined Stage 1A and 1B were 1.036 and 1.033.

Recombination hotspots were identified in the vicinity of osteosarcoma associated loci on 6p21.31 and 2p25.2 using SequenceLDhot[40], a program that uses approximate marginal likelihood method[41] and calculates likelihood ratio statistics at a set of possible hotspots. We tested 5 unique sets of 100 control samples drawn from controls. PHASE v2.1 program was used to calculate background recombination rates[42,43] and LD heatmap was visualized in r^2 using snp.plotter program[44].

Imputation

In order to further interrogate the loci associated with osteosarcoma, we imputed additional SNPs within 1 Mb on either side of the implicated SNPs using IMPUTE2 software and the reference data from both the 1000 Genomes project[13] and the DCEG Imputation Reference Set version 1[37]. SNPTEST was used to analyze the posterior SNP dosages from IMPUTE2, adjusting for sex, country, and four eigenvectors, as described above[45].

Data Analysis

Data analysis and management was performed with GLU (Genotyping Library and Utilities version 1.0), PLINK, Eigenstrat, IMPUTE2, and SNPTEST.

URLs

CGEMS portal: http://cgems.cancer.gov/

CGF: http://cgf.nci.nih.gov/

GLU: http://code.google.com/p/glu-genetics/

EIGENSTRAT: http://genepath.med.harvard.edu/~reich/EIGENSTRAT.htm

STRUCTURE: http://pritch.bsd.uchicago.edu/structure.html

PLINK: http://pngu.mgh.harvard.edu/~purcell/plink/

SequenceLDhot: http://www.maths.lancs.ac.uk/~fearnhea/Hotspot/

snp.plotter: http://cbdb.nimh.nih.gov/~kristin/snp.plotter.html

PHASE v2.1: http://www.stat.washington.edu/stephens/phase/download.html

IMPUTE2: http://mathgen.stats.ox.ac.uk/impute/impute_v2.html

SNPTEST: https://mathgen.stats.ox.ac.uk/genetics_software/snptest/snptest.html

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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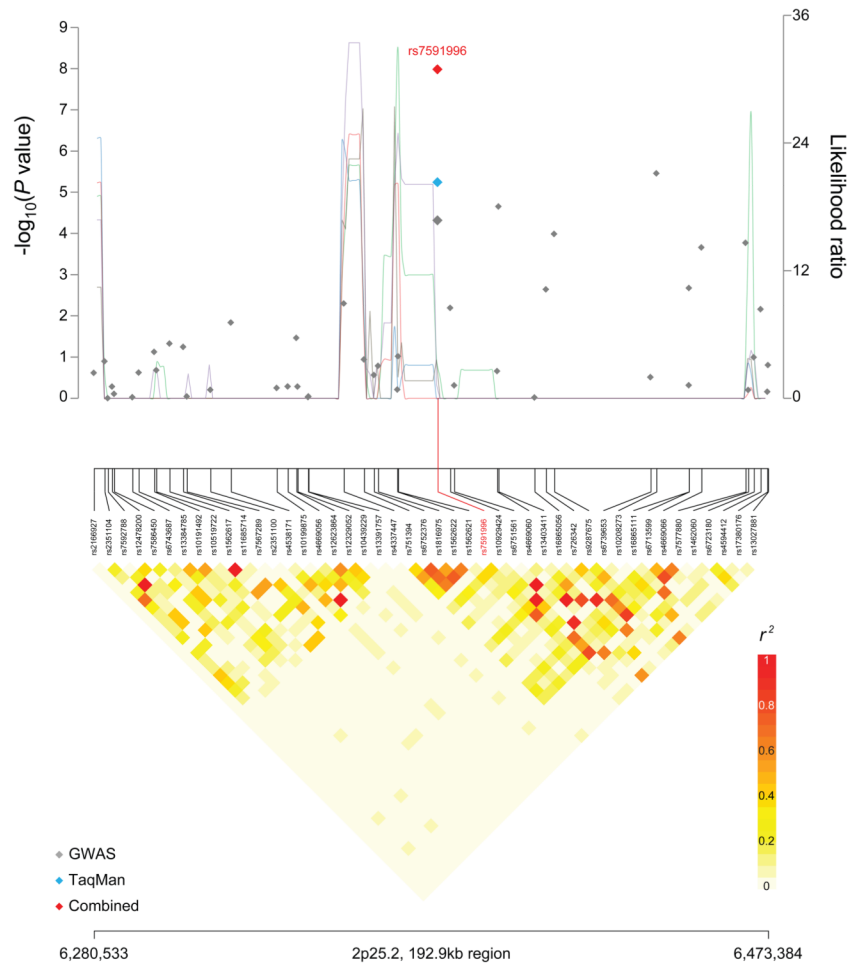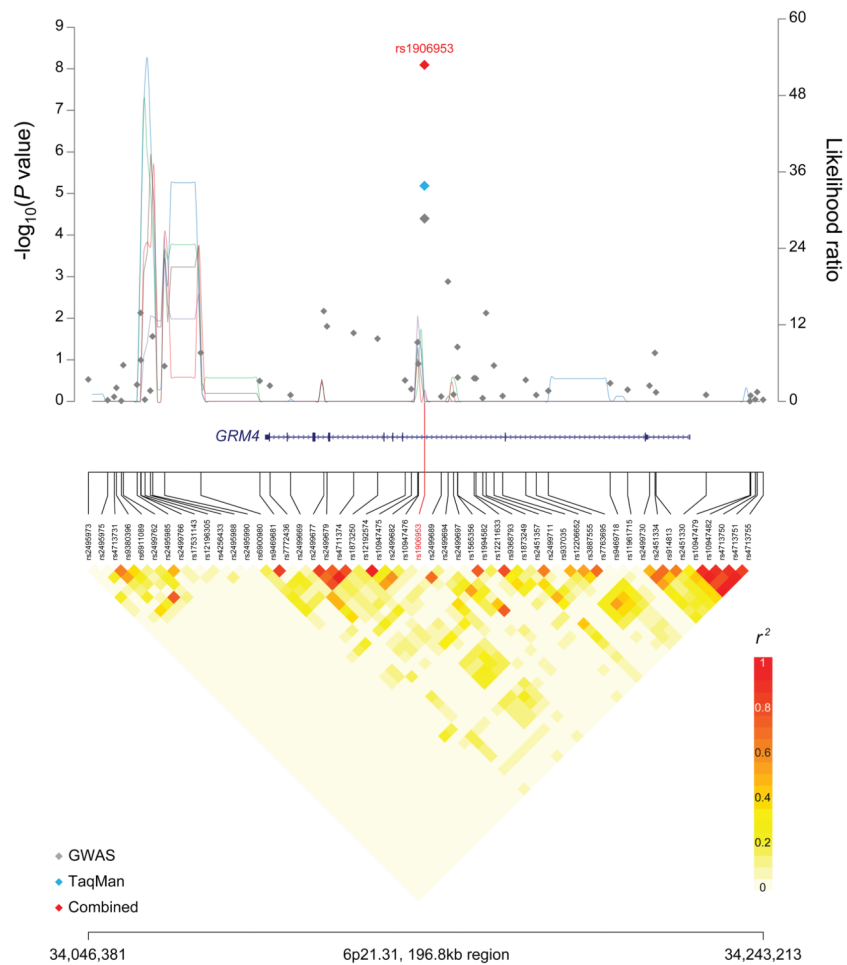
Figure 1. Regional plots of loci associated with osteosarcoma
Regional plots of association results, recombination hotspots and linkage disequilibrium for the (a) 6p21.31:34,046,381–34,243,213 and (b) 2p25.2:6,280,533–6,473,384 osteosarcoma susceptibility loci. Association results from a trend test in –log10*P* values (y axis, left; gray diamonds, combined GWAS result; sky blue diamonds, TaqMan result; red diamonds, combined result) of the SNPs are shown according to their chromosomal positions (x axis). Linkage disequilibrium structure based on controls (n=2,703) was visualized by snp.plotter software. The line graph shows likelihood ratio statistics (y axis, right) for recombination hotspot by SequenceLDhot software and 5 different colors represent 5 tests of 100 controls without resampling. Physical locations are based on NCBI human genome build 36. Gene annotation was based on the NCBI RefSeq genes from the UCSC Genome Browser.

Table 1

Summary of combined association results for 941 cases and 3,291 controls

dbSNP locus	Position[a]	Allele[b]	Stage	Cases EAF	Controls EAF	P	OR (95% CI)
rs1906953 6p21.31	34144424	C/T	Discovery	0.19	0.15	4.0×10^{-05}	1.45 (1.21-1.73)
			Replication	0.21	0.12	6.5×10^{-06}	2.00 (1.47-2.72)
			Combined			8.0×10^{-09}	1.57 (1.35-1.83)
rs7591996 2p25.2	6378872	A/C	Discovery	0.50	0.45	4.8×10^{-05}	1.30 (1.15-1.47)
			Replication	0.55	0.43	5.7×10^{-06}	1.69 (1.35-2.13)
			Combined			1.0×10^{-08}	1.39 (1.23-1.54)
rs10208273 2p25.2	6441445	G/A	Discovery	0.36	0.30	3.5×10^{-06}	1.37 (1.2-1.56)
			Replication	0.34	0.29	2.7×10^{-02}	1.31 (1.03-1.67)
			Combined			2.9×10^{-07}	1.35 (1.21-1.52)
rs17206779 5q12.3	64483533	T/C	Discovery	0.53	0.47	2.2×10^{-05}	1.32 (1.16-1.49)
			Replication	0.54	0.46	6.7×10^{-03}	1.37 (1.09-1.72)
			Combined			5.1×10^{-07}	1.33 (1.19-1.47)

The discovery stage consists of the combined scans of Stage 1A (596 cases) and Stage 1B (98 cases) for a total of 694 scanned osteosarcoma cases and 2,703 cancer-free adult controls, all of European ancestry. The independent replication set consisted of 247 osteosarcoma cases genotyped by TaqMan and 588 controls previously scanned on Illumina Omni2.5 or OmniExpress. The combined results report the data from the meta analysis of Stage 1 and the TaqMan replication (941 cases and 3,291 controls).

Abbreviations: EAF, effect (coded) allele frequency; OR, odds ratio; CI, confidence interval.

[a]Build 36 position;

[b]Reference/Effect alleles.