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Frequency of Pathogenic Germline Variants in Cancer-Susceptibility Genes in Patients With Osteosarcoma

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IMPORTANCE Osteosarcoma, the most common malignant bone tumor in children and adolescents, occurs in a high number of cancer predisposition syndromes that are defined by highly penetrant germline mutations. The germline genetic susceptibility to osteosarcoma outside of familial cancer syndromes remains unclear.

OBJECTIVE To investigate the germline genetic architecture of 1244 patients with osteosarcoma.

DESIGN, SETTING, AND PARTICIPANTS Whole-exome sequencing (n = 1104) or targeted sequencing (n = 140) of the DNA of 1244 patients with osteosarcoma from 10 participating international centers or studies was conducted from April 21, 2014, to September 1, 2017. The results were compared with the DNA of 1062 individuals without cancer assembled internally from 4 participating studies who underwent comparable whole-exome sequencing and 27 173 individuals of non-Finnish European ancestry who were identified through the Exome Aggregation Consortium (ExAC) database. In the analysis, 238 high-interest cancer-susceptibility genes were assessed followed by testing of the mutational burden across 736 additional candidate genes. Principal component analyses were used to identify 732 European patients with osteosarcoma and 994 European individuals without cancer, with outliers removed for patient-control group comparisons. Patients were subsequently compared with individuals in the ExAC group. All data were analyzed from June 1, 2017, to July 1, 2019.

MAIN OUTCOMES AND MEASURES The frequency of rare pathogenic or likely pathogenic genetic variants.

RESULTS Among 1244 patients with osteosarcoma (mean [SD] age at diagnosis, 16 [8.9] years [range, 2-80 years]; 684 patients [55.0%] were male), an analysis restricted to individuals with European ancestry indicated a significantly higher pathogenic or likely pathogenic variant burden in 238 high-interest cancer-susceptibility genes among patients with osteosarcoma compared with the control group (732 vs 994, respectively; $P = 1.3 \times 10^{-18}$). A pathogenic or likely pathogenic cancer-susceptibility gene variant was identified in 281 of 1004 patients with osteosarcoma (28.0%), of which nearly three-quarters had a variant that mapped to an autosomal-dominant gene or a known osteosarcoma-associated cancer predisposition syndrome gene. The frequency of a pathogenic or likely pathogenic cancer-susceptibility gene variant was 128 of 1062 individuals (12.1%) in the control group and 2527 of 27 173 individuals (9.3%) in the ExAC group. A higher than expected frequency of pathogenic or likely pathogenic variants was observed in genes not previously linked to osteosarcoma (eg, *CDKN2A*, *MEN1, VHL*, *POT1, APC*, *MSH2*, and *ATRX*) and in the Li-Fraumeni syndrome-associated gene, *TP53*.

CONCLUSIONS AND RELEVANCE In this study, approximately one-fourth of patients with osteosarcoma unselected for family history had a highly penetrant germline mutation requiring additional follow-up analysis and possible genetic counseling with cascade testing.

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Supplemental content

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The peak incidence of osteosarcoma (OMIM 259500) occurs during the pubertal growth spurt.[1-3] Osteosarcoma risk factors include tall height,[4,5] high birth-weight,[4,5] previous radiotherapy,[6] and at least 8 established cancer predisposition syndromes,[7,8] including autosomal-dominant disorders (Li-Fraumeni syndrome [OMIM 151623],[9,10] hereditary retinoblastoma [OMIM 180200],[11,12] and Diamond-Blackfan anemia [OMIM 105650][13,14]) and autosomal-recessive disorders (primarily DNA helicase disorders,[15-18] such as Rothmund-Thomson syndrome [OMIM 268400], RAPADILINO syndrome [OMIM 266280], Werner syndrome [OMIM 277700], and Bloom syndrome [OMIM 210900]). Candidate gene and genome-wide association studies suggest that common single-nucleotide polymorphisms are also associated with osteosarcoma,[19-21] affirming a complex underlying architecture for its genetic etiology but one that appears to be weighted disproportionately toward rare variants.

An earlier study reported that 4% of patients with osteosarcoma younger than 30 years with an unknown family history of cancer carried a pathogenic germline variant of *TP53* (OMIM 191170) that was known to be or highly likely to be associated with Li-Fraumeni syndrome; in addition, 6% of those patients carried rare likely pathogenic *TP53* variants.[22] A survey of 72 candidate genes across 1162 sarcomas, including 124 osteosarcomas, observed that 217 individuals (18.7%) had a pathogenic or likely pathogenic (pathogenic/likely pathogenic) germline variant in autosomal-recessive or autosomal-dominant genes; 7% of variants were in autosomal-dominant genes.[23] Previous studies estimated that approximately 8% to 10% of all children with cancer carry a pathogenic germline variant in a known cancer-susceptibility gene.[24,25] The frequency of pathogenic/likely pathogenic variants in children with osteosarcoma was reported to be between 3 of 42 patients (7.1%)[25] and 7 of 39 patients (17.9%).[24]

Key Points

Question What is the frequency of pathogenic or likely pathogenic germline genetic variants in known cancer-susceptibility genes in a large population of patients with osteosarcoma who were unselected for family history?

Findings In this next-generation exome sequencing study of 1244 patients with osteosarcoma, 28.0% of patients in the discovery set carried a rare pathogenic or likely pathogenic germline variant in a cancer-susceptibility gene compared with 12.1% of individuals without cancer who were comparably sequenced and 9.3% of individuals of non-Finnish European ancestry identified through the Exome Aggregation Consortium database.

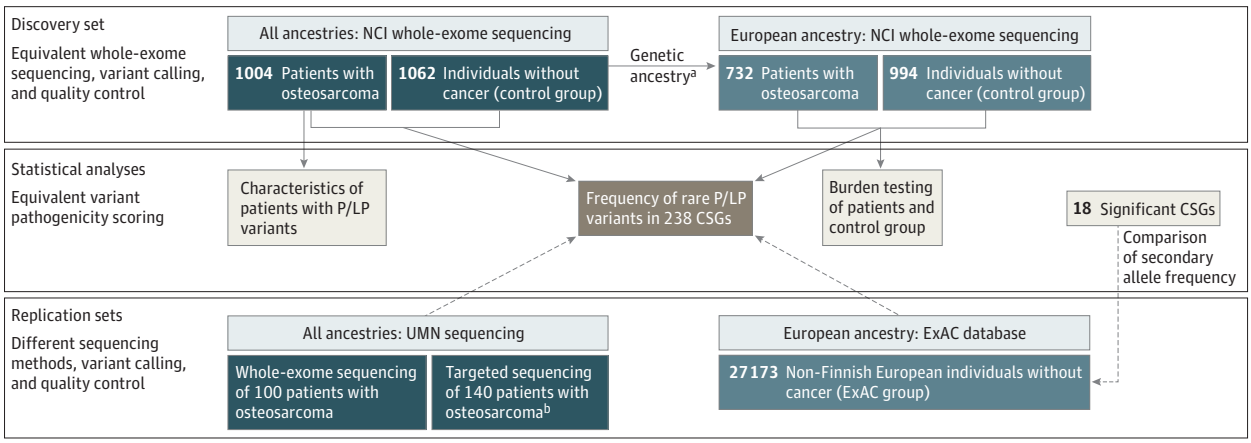
Meaning A higher than expected frequency of patients with osteosarcoma carrying a pathogenic or likely pathogenic germline variant suggests germline genetic testing may be warranted for individuals with osteosarcoma.

We used a 2-phase approach to evaluate rare germline variants in 1244 patients with osteosarcoma, beginning with assessment of 238 cancer-susceptibility genes followed by burden testing for an additional 736 candidate genes. We compared the frequency of pathogenic/likely pathogenic variants in patients with those of 1062 individuals without cancer (the control group), and for significant findings, with 27 173 individuals of non-Finnish European ancestry who were identified through the Exome Aggregation Consortium (ExAC) database[26] (the ExAC group; **Figure 1**).

Methods

The NCI Retrospective Study of Genetic Risk Factors for Osteosarcoma was approved by the institutional review board

Figure 1. Overview of Study Samples and Design



CSG indicates cancer-susceptibility gene; ExAC, Exome Aggregation Consortium database; NCI, National Cancer Institute; P/LP, pathogenic/likely pathogenic; and UMN, University of Minnesota.

[a] Genetic ancestry was determined using the available microarray from single-nucleotide polymorphism genome-wide association studies or whole-exome sequencing data from structure and principal component analyses. Individuals with more than 80% European ancestry were considered European.

[b] Sequencing of 238 cancer-susceptibility genes.

of the National Institutes of Health. All of the participants in the Genetic Epidemiology of Osteosarcoma study provided written informed consent, and the study was approved by the institutional review board of the University of Minnesota. The study was also approved by the respective local institutional review boards, and all participants provided written informed consent.

Study Populations

A total of 1244 patients with osteosarcoma were assembled from 10 participating centers and studies, including the National Cancer Institute retrospective Children's Oncology Group study of genetic risk factors for osteosarcoma[19] (United States); the Genetic Epidemiology of Osteosarcoma study of the Children's Oncology Group[21] (United States); the Clinica Universidad de Navarra (Pamplona, Spain); the Instituto de Oncologia Pediatrica, Grupo de Apoio ao Adolescente e a Crianca com Cancer/Universidade Federal de Sao Paulo[27] (Sao Paulo, Brazil); the Childhood Cancer Survivor Study[28] (United States); the National Cancer Institute Bone Disease and Injury Study of Osteosarcoma[29] (United States); the Unidad Nacional de Oncologia Pediatrica[30] (Guatemala City, Guatemala); the Royal National Orthopaedic Hospital NHS Trust and University College London Cancer Institute (Middlesex, United Kingdom); the Istituto Ortopedico Rizzoli (Bologna, Italy); and the Ankara Oncology Training and Research Hospital (Ankara, Turkey; eMethods and eTable 1 in the Supplement). Of those, 782 patients were previously reported in a genome-wide association study,[19,27] which included 48 patients from the Instituto de Oncologia Pediatrica, Grupo de Apoio ao Adolescente e a Crianca com Cancer /Universidade Federal de Sao Paulo. A total of 462 additional patients were included, drawn from the Childhood Cancer Survivor Study, the NCI Bone Disease and Injury Study of Osteosarcoma, the Hospital Infantil Manuel De Jesus Rivera (Managua, Nicaragua), and from the Unidad Nacional de Oncologia Pediatrica. Each center provided data on patient and clinical variables, which were harmonized across studies.

A total of 1004 patients who underwent whole-exome sequencing at the National Cancer Institute were included as a primary discovery set, and 240 additional (nonoverlapping) patients with osteosarcoma[21] comprised a replication set of patients who underwent whole-exome sequencing (n = 100) or targeted sequencing (n = 140) at the University of Minnesota (Figure 1; eMethods in the Supplement). Patients from the replication sets were drawn from the Genetic Epidemiology of Osteosarcoma study of the Children's Oncology Group (United States). Neither family history nor tumor sequence data were available for the patients in this study.

The 1062 individuals without osteosarcoma who were assigned to the control group were assembled internally from 4 participating studies. This group included 994 adults of European ancestry (mean [SD] age at enrollment, 64.6 [7.2] years) who were drawn from 3 large studies: the Prostate, Lung, Colon and Ovarian Cancer Prevention clinical trial (United States),[31] the American Cancer Society Cancer Prevention Study II (United States),[32] and the Environment and Genes in Lung Cancer Etiology study (Italy).[33] In addition, 68 individuals were enrolled from the Instituto de Oncologia Pediatrica, Grupo de Apoio ao Adolescente e a Crianca com Cancer/Universidade Federal de Sao Paulo study and were drawn from the same population as the 48 patients with osteosarcoma from Sao Paulo, Brazil (eMethods and eTable 1 in the Supplement).

The population substructure was determined for the patient group and the control group using the available single-nucleotide polymorphism microarray data or whole-exome sequencing data based on structure and principal component analyses, as previously described.[19,34] Individuals with more than 80% European ancestry were considered European (Figure 1; eTable 1 in the Supplement).

The population frequency of pathogenic/likely pathogenic germline variants was estimated for 238 cancer-susceptibility genes using publicly available noncancer whole-exome sequencing data from the ExAC database.[26] Variant data for each gene were analyzed for secondary comparisons with individuals in the ExAC group using similar pathogenicity scoring and in silico analysis.

Sequencing

Whole-exome sequencing was performed on a discovery set of 1004 patients and 1062 individuals in the control group using germline DNA extracted from either leukocytes or buccal samples between April 21, 2014, and July 1, 2017, at the National Cancer Institute (eMethods in the Supplement; Figure 1).[34-36] All analyses evaluated variants with minor allele frequencies of less than 0.01 that passed quality-control filters.[34,35,37] For the patient replication sets, we used buccal sample DNA to conduct whole-exome sequencing on 100 patients with osteosarcoma and targeted sequencing of 238 cancer-susceptibility genes on an additional 140 patients at the University of Minnesota from August 1, 2017, to September 1, 2018 (eMethods in the Supplement).

Genes and Variants

We assembled a set of 238 cancer-susceptibility genes, including 114 cancer-predisposing genes,[38] 14 genes associated with Diamond-Blackfan anemia,[34,39-41] and 110 cancer-associated genes previously described[25,42,43] or reported to have germline associations in the Catalogue of Somatic Mutations in Cancer[44] (eTable 2 in the Supplement). These genes were grouped by mode of inheritance: 141 genes were autosomal-dominant, 45 were autosomal-recessive, 25 were autosomal-dominant and autosomal-recessive, 11 were X-linked, 1 was Y-linked, and 15 had de novo or unknown inheritance patterns (eTable 2 in the Supplement). An additional 736 candidate genes were evaluated, including 140 genes associated with osteosarcoma that were identified through the Human Genome Epidemiology (HuGE) phenopedia[45] and manual curation of published reports and 596 genes somatically altered in pediatric bone cancers or recurrent in any pediatric cancer that were identified through the Catalogue of Somatic Mutations in Cancer[44] and annotation of published osteosarcoma somatic data[46-49] (eTable 3 in the Supplement).

A stepwise pipeline was constructed to evaluate each rare variant that passed quality-control filters in the genes of interest. Variants were classified as pathogenic, likely patho-

genic, of uncertain significance, likely benign, or benign based on previous reports[24,25] and recommendations from the American College of Medical Genetics and Genomics and the Association for Molecular Pathology[50] (eMethods and eTable 4 in the Supplement). An in silico prediction algorithm was also used to further filter the variant of uncertain significance category as damaging or not damaging (eMethods in the Supplement). All pathogenic/likely pathogenic variants are summarized in eTable 5 in the Supplement.

Statistical Analyses

We analyzed the 1004 patients with osteosarcoma in the discovery set, which included 732 patients of European ancestry, with the 1062 individuals in the control group, which included 994 patients of European ancestry (Figure 1; eMethods in the Supplement). The replication set consisted of 240 patients with osteosarcoma who had germline whole-exome sequencing or targeted sequencing data available, and we performed secondary patient comparisons with individuals in the ExAC group.[26]

Rare-variant burden tests were conducted on the 732 European patients in the discovery set and the 994 European individuals in the control group using burden and optimal sequence kernel association tests.[51] The comparisons between the patient group and the ExAC group were restricted to genes identified as substantially different between the primary discovery set of patients and the control group. Comparisons among individuals with and without pathogenic/likely pathogenic variants were performed using 2-sided χ^2 or Fisher exact tests for categorical variables and Mann-Whitney U tests for continuous variables (eg, age). We used 2-sided exact binomial tests and logistic regression models to compare the frequencies of pathogenic/likely pathogenic variants between patients and individuals in the ExAC group only for the selected genes identified as substantially different between the primary discovery set of patients and individuals in the control group who had comparable whole-exome sequencing performed at the National Cancer Institute. We compared overall survival between patients carrying pathogenic/likely pathogenic variants and individuals without pathogenic/likely pathogenic variants for all cancer-susceptibility genes and the *TP53* gene using adjusted Cox proportional hazards regression models and estimated hazard ratios (HRs) and 95% CIs. All data were analyzed from June 1, 2017, to July 1, 2019.

Results

Among 1244 patients with osteosarcoma, the mean (SD) age at diagnosis was 16 (8.9) years (age range, 2-80 years), and 684 patients (55.0%) were male (eTable 1 in the Supplement). Our primary analyses were based on patients and individuals in the control group with whole-exome sequencing data jointly called that yielded comparable quality-control measures and coverage (Figure 1; eFigure 1 in the Supplement).

We assessed the frequency of pathogenic/likely pathogenic variants in 238 cancer-susceptibility genes in the discovery set of patients and the control group. Overall, 281 of 1004 patients with osteosarcoma (28.0%; 95% CI, 22.7%-33.2%) had a pathogenic/likely pathogenic variant in a gene of interest, which was significantly higher than the frequency observed in the control group (128 of 1062 individuals [12.1%]; 95% CI, 6.4%-17.7%; Fisher exact $P = 1.3 \times 10^{-18}$; **Figure 2**A, Figure 2B, and **Figure 3**; eTable 6 in the Supplement). The pathogenic/likely pathogenic frequency among European patients with osteosarcoma was also higher compared with the frequency among individuals in the ExAC group (2527 individuals [9.3%]; 95% CI, 8.2%-10.5%; Fisher exact $P = 2.3 \times 10^{-53}$; Figure 2A and Figure 2B; eTable 6 in the Supplement). Patients with pathogenic/likely pathogenic variants were significantly younger (mean [SD] age, 15.3 [7.2] years; age range, 2-61 years) than patients without pathogenic/likely pathogenic variants (mean [SD] age, 16.9 [10.2] years; age range, 2-80 years; Mann-Whitney U $P = .02$; **Figure 4**; eFigure 2 in the Supplement).

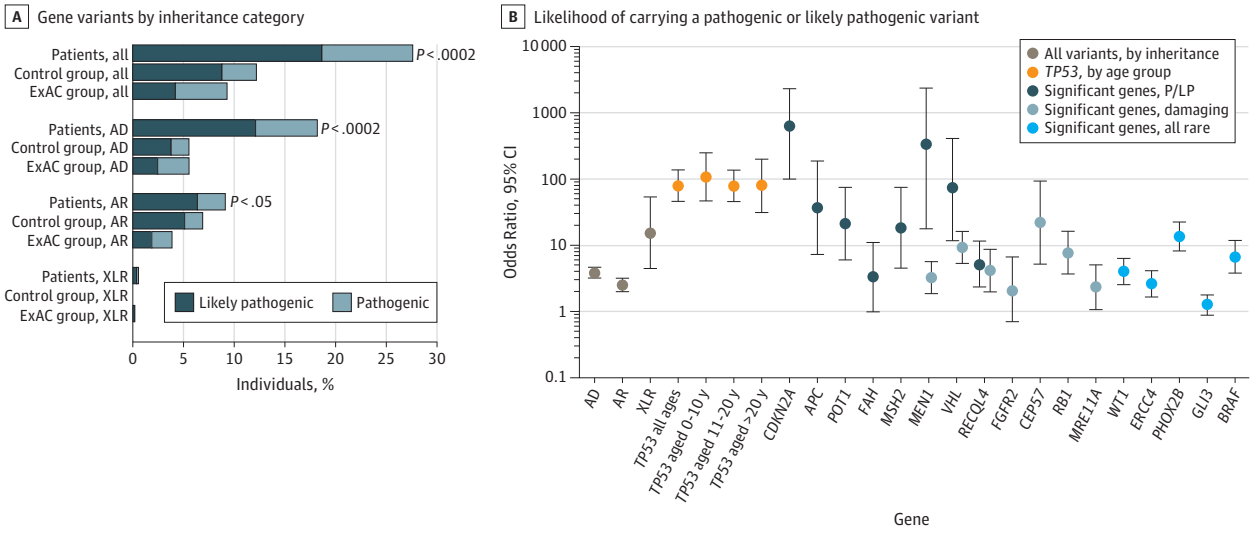
Among 364 patients with osteosarcoma subtype information, cancer-susceptibility genes with pathogenic/likely pathogenic variants were less common in those with surface subtypes (3 of 22 patients [13.6%]) vs conventional subtypes (104 of 342 patients [30.4%]; eTable 8 in the Supplement). A pathway enrichment analysis[52,53] of the 101 cancer-susceptibility genes with pathogenic/likely pathogenic variants indicated enrichment in DNA repair pathway genes (Fisher exact $P = 3.4 \times 10^{-28}$; eFigure 3 and eTable 9 in the Supplement).

Autosomal-Dominant Genes

Overall, 185 of 1004 patients (18.4%; 95% CI, 12.8%-24.0%) with osteosarcoma had a pathogenic/likely pathogenic variant in an autosomal-dominant or an autosomal-dominant and autosomal-recessive cancer-susceptibility gene, whereas the variant frequency was 56 individuals (5.3%; 95% CI, 0%-11.1%) in the control group and 1494 individuals (5.5%; 95% CI, 4.3%-6.6%) in the ExAC group (Figure 2A and Figure 2B; eTable 6 in the Supplement). The highest frequency of pathogenic/likely pathogenic autosomal-dominant cancer-susceptibility gene variants was found in patients aged 0 to 10 years (37 of 151 patients [24.5%]; Mann-Whitney U $P = .006$; Figure 4). The 732 European patients with cancer had a higher burden of pathogenic/likely pathogenic autosomal-dominant variants than the 994 European individuals in the control group (burden $P = 1.9 \times 10^{-16}$). This higher burden translated to a nearly 4-fold greater risk of carrying a pathogenic/likely pathogenic variant compared with the ExAC group (odds ratio [OR], 3.9; 95% CI, 3.3-4.6).

Eighteen patients (1.8%) had more than 1 pathogenic/likely pathogenic autosomal-dominant variant compared with 4 individuals (0.4%) in the control group (Fisher exact $P = .002$). No significant difference was observed in overall patient survival for those carrying any pathogenic/likely pathogenic variant or an autosomal-dominant pathogenic/likely pathogenic variant compared with patients without these variants (Cox [adjusted for age, sex, and tumor location] $P = .55$ for all genes and $P = .34$ for autosomal-dominant genes) in the subset of 407 patients for whom outcome data was available.

Figure 2. Frequency of Rare Pathogenic or Likely Pathogenic Germline Variants in Cancer-Susceptibility Genes



A, Includes 1004 patients in the discovery set, 1062 individuals in the control group, and 27 173 individuals in the ExAC group. Genes with both AD and AR inheritance patterns and variants in genes with unknown inheritance are grouped with AD genes. B, Includes genes with a significantly higher frequency of pathogenic/likely pathogenic, damaging, or rare variants in 732 European patients compared with 27 173 individuals in the ExAC group. *P* values represent European patient-control group burden tests, with $P < .0002$ significant at the Bonferroni threshold. AD indicates autosomal-dominant genes; AR, autosomal-recessive genes; ExAC, Exome Aggregation Consortium database; P/LP, pathogenic/likely pathogenic; and XLR, X-linked or Y-linked recessive inheritance pattern genes.

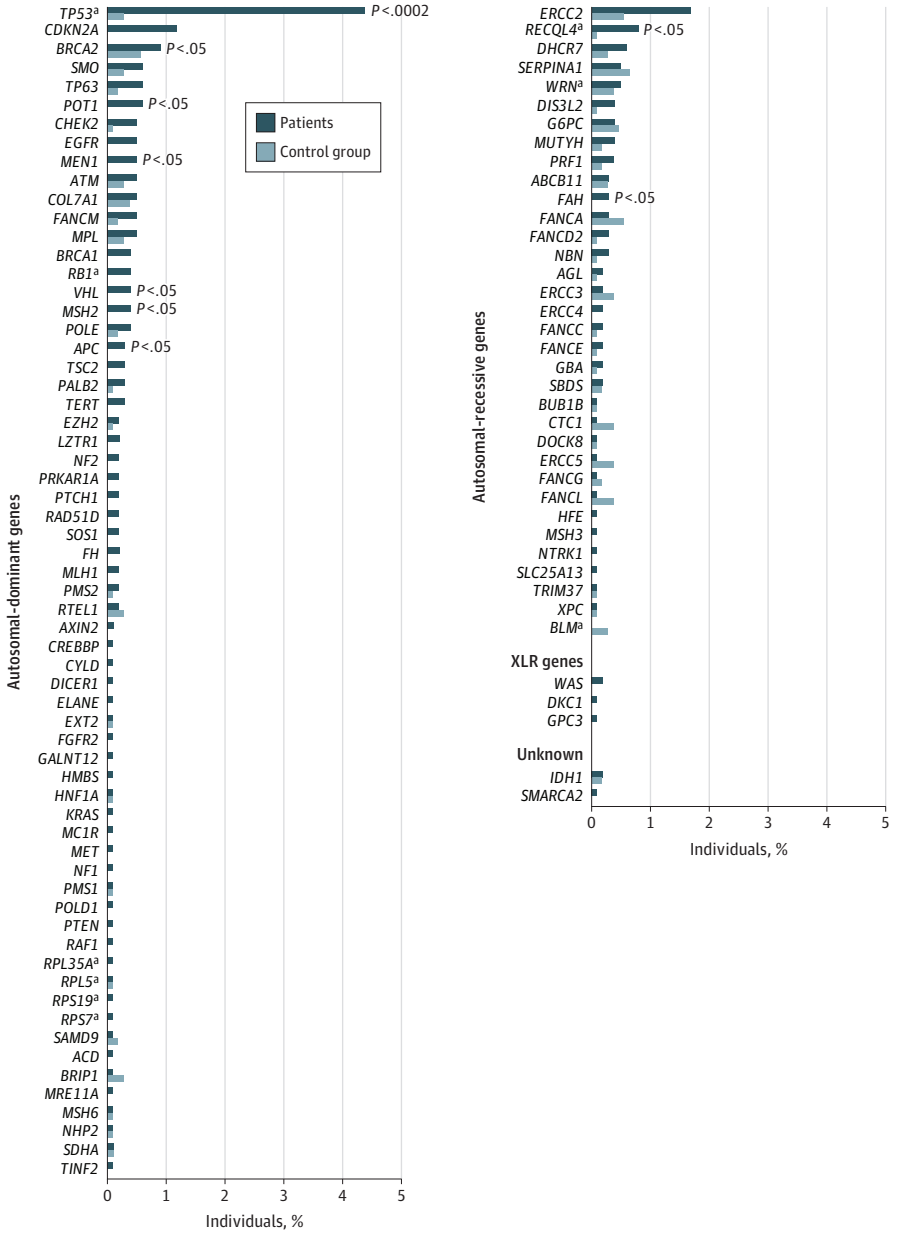
Pathogenic/likely pathogenic variants in the *TP53* gene were the most frequent of all autosomal-dominant genes (44 of 1004 total patients [4.4%]; 30 of 732 European patients [4.1%]) and substantially higher than those observed in the control group (3 of 1062 total individuals [0.3%]; 3 of 994 European individuals [0.3%]; burden $P = 3.2 \times 10^{-8}$) and the ExAC group (27 individuals [0.1%]; Fisher exact $P = 9.0 \times 10^{-44}$; Figure 2B and Figure 3; eTable 7 and eTable 10 in the Supplement). This finding is consistent with a previous study,[22] which included 360 patients who were also in the current study. Analyses restricted to European patients who did not participate in the previous study found that 32 of 644 patients (5.0%) had a pathogenic *TP53* variant.

All pathogenic/likely pathogenic *TP53* variants were observed in patients younger than 30 years at diagnosis, with the exception of 1 patient, who was aged 39 years at diagnosis (Mann-Whitney U $P = .05$; eFigure 2 and eTable 8 in the Supplement). Patients aged 0 to 10 years (n = 151) had the highest estimated likelihood of carrying a *TP53* pathogenic/likely pathogenic variant (OR, 108; 95% CI, 47-247; Figure 2B and Figure 4). Patients with a pathogenic/likely pathogenic *TP53* variant were more likely to have osteosarcoma of the axial skeleton (χ^2 $P = .001$), and the data suggested that patients with *TP53* pathogenic/likely pathogenic variants were more likely to have metastases at diagnosis (χ^2 $P = .06$; eTable 8 in the Supplement). In the subset of patients with outcome data, an adjusted Cox proportional hazards model indicated that patients carrying a *TP53* pathogenic/likely pathogenic variant had significantly worse overall survival compared with patients without these variants (HR, 2.2; 95% CI, 1.2-4.0; Cox $P = .009$). These variants occurred in several functional domains, including the DNA-binding domain (subregion-based burden[54] $P = 1.5 \times 10^{-6}$; eFigure 4A in the Supplement), which is consistent with previous studies.[55-59]

The gene *CDKN2A* (OMIM 600160) had the second highest frequency of pathogenic/likely pathogenic variants in the patients with osteosarcoma (12 of 1004 total patients [1.2%]; 8 of 732 European patients [1.1%]) compared with no pathogenic/likely pathogenic variants among individuals in the control group (burden $P = 3.1 \times 10^{-3}$) and the ExAC group (Fisher exact $P = 2.2 \times 10^{-13}$; Figure 2B and Figure 3; eTable 7 in the Supplement). Individuals with a *CDKN2A* pathogenic/likely pathogenic variant were younger (mean [SD] age, 12.9 [4.4] years) than patients without pathogenic/likely pathogenic variants (mean [SD] age, 6.9 [10.2] years; Mann-Whitney U $P = .03$). Notably, the youngest patients (aged 0-10 years) had the highest frequency of these variants (3 of 151 patients [2.0%]; Figure 4). The *CDKN2A* variants mapped to sites that were somatically mutated in bone cancers[58] (eFigure 4B in the Supplement). Five additional autosomal-dominant genes (*MEN1* [OMIM 613733], *VHL* [OMIM 608537], *POT1* [OMIM 606478], *APC* [OMIM 611731], and *MSH2* [OMIM 609309]) had a significantly higher pathogenic/likely pathogenic burden in European patients compared with European individuals in the control group (Figure 3; eTable 7 in the Supplement).

We compared the frequency of pathogenic/likely pathogenic variants among individuals in the ExAC group and observed that the risk of carrying a pathogenic/likely pathogenic variant in genes *MEN1*, *VHL*, *POT1*, and *APC* was elevated in European patients with osteosarcoma after a Bonferroni adjustment (Figure 2B; eTable 7 in the Supplement). Fifty-five additional autosomal-dominant genes had pathogenic/likely pathogenic variants in 1 or more patients (each were present

Figure 3. Frequency of Pathogenic or Likely Pathogenic Variants



Frequency of pathogenic or likely pathogenic variants in the 1004 patients and 1062 individuals in the control group. *P* values represent European patient-control group burden tests, with $P < .0002$ significant at the Bonferroni threshold.

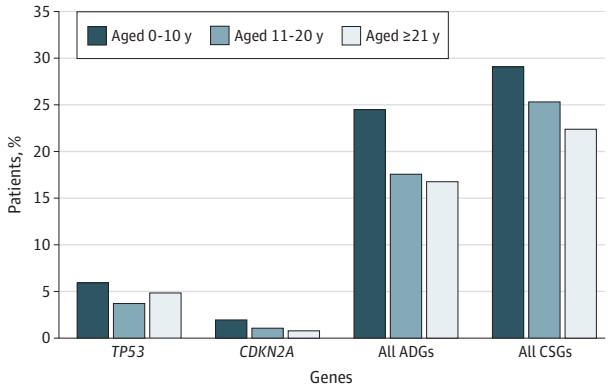
in 0.1%-0.6% of patients; Figure 3; eTable 10 in the Supplement). Most of the specific variants observed in patients were absent in individuals in both the control group and the ExAC group as well as other public databases (the 1000 Genomes Project and the Exome Sequencing Project).

In addition, 316 patients (25.4%) with osteosarcoma had a rare variant of uncertain significance that was predicted to be damaging in silico in an autosomal-dominant gene, in the absence of another pathogenic/likely pathogenic autosomal-dominant variant. Altogether, 545 patients (43.8%) had at least 1 pathogenic variant, likely pathogenic variant, or variant of uncertain significance that was predicted to be damaging in silico in an autosomal-dominant gene. The European patients had more variants of uncertain significance that were predicted to be damaging in silico in the genes *RB1* (OMIM 614041) and *VHL* (OMIM 608537) compared with individuals in the control group after adjustment for multiple testing (eTable 7 in the Supplement).

Autosomal-Recessive Genes

A total of 92 of 1004 patients (9.2%; 95% CI, 3.3%-15.1%) had a pathogenic/likely pathogenic variant in 33 autosomal-recessive genes, which is higher than that of the control group (72 of 1062 individuals [6.8%]; burden $P = .03$) and the ExAC group (1041 individuals [3.8%]; Fisher exact $P = 2.6 \times 10^{-13}$; Figure 2A and Figure 2B; eTable 6 in the Supplement). All autosomal-recessive gene variants were present as single heterozygotes, with the exception of 1 patient aged 13 years who

Figure 4. Frequency of Rare Pathogenic or Likely Pathogenic Variants



All ADGs include genes *TP53* and *CDKN2A*. A total of 151 patients were aged 0 to 10 years, 698 patients were aged 11 to 20 years, and 125 patients were aged 21 years and older. ADG indicates autosomal-dominant gene and CSG, cancer-susceptibility gene.

had osteosarcoma with 2 *RECQL4* (OMIM 603780) pathogenic/likely pathogenic variants; we were unable to phase the variants. The gene *RECQL4* had the highest frequency of pathogenic/likely pathogenic variants in European patients with osteosarcoma (7 of 732 patients [1.0%]) compared with European individuals in the control group (1 of 994 individuals [0.1%]; burden $P = .02$; Figure 2B and Figure 3; eFigure 4C and eTable 7 in the Supplement). One *RECQL4* variant was previously reported in a patient with Rothmund-Thomson syndrome and osteosarcoma (c.2476C>T, p.Arg826*).[60] Several other autosomal-recessive genes had more pathogenic/likely pathogenic variants in patients than in the control group but were not significantly associated (Figure 2B and Figure 3; eTable 7 and eTable 10 in the Supplement).

We observed a preponderance of male patients (4 of 1004 patients [0.4% of the total patients and 0.7% of the 540 male patients in the discovery set]) who carried a pathogenic/likely pathogenic variant in an X-linked cancer-susceptibility gene (*DKC1* [OMIM 300126], *GPC3* [OMIM 300037], or *WAS* [OMIM 300392]) compared with no individuals in the control group and 7 individuals (0.03%) in the ExAC group (OR, 15.5; 95% CI, 5-53; Fisher exact $P = 4.4 \times 10^{-9}$; Figure 2; eTable 6 in the Supplement).

Known Osteosarcoma Syndrome Genes

Genes associated with cancer-predisposing syndromes associated with the occurrence of osteosarcoma were also associated with sporadic osteosarcoma or patients unselected for family history. We identified that 6.5% of the patients had a pathogenic/likely pathogenic variant in 1 of the following syndromic genes: *RB1, RECQL4, RPL35A* (OMIM 180468), *RPL5* (OMIM 603634), *RPS19* (OMIM 603474), *RPS7* (OMIM 603658) , *TP53,* and *WRN* (OMIM 277700). A total of 2.2% of patients had a pathogenic/likely pathogenic variant in a syndromic gene without *TP53*. A total of 19.6% of patients had either a pathogenic/likely pathogenic variant in any autosomal-dominant gene or an osteosarcoma-associated autosomal-recessive syndrome gene.

Replication of Findings

Two independent patient data sets were used to evaluate the frequency of pathogenic/likely pathogenic variants in the cancer-susceptibility genes; set 1 comprised 100 patients with whole-exome sequencing data, and set 2 comprised 140 patients with targeted sequencing data. The overall prevalence of pathogenic/likely pathogenic variants in the replication sets (28 patients [28.0%] in set 1 and 38 patients [27.1%] in set 2; eTable 6 in the Supplement) was consistent with the carrier rates observed in our larger discovery set of 1004 patients with osteosarcoma (281 patients [28.0%]).

Of note, pathogenic/likely pathogenic variants in specific genes identified in our discovery set were also identified to have pathogenic/likely pathogenic variants in the 240 total patients in the replication sets; these genes were *TP53* (13 patients [5.4%]), *MEN1* (1 patient [0.4%]), *MSH2* (1 patient [0.4%]), *FAH* (OMIM 613871; 4 patients [1.7%]), *RECQL4* (8 patients [0.8%]), *DKC1* (1 patient [0.4%]), and *WAS* (1 patient [0.4%]; eTable 10 and eTable 11 in the Supplement).

Candidate Gene Rare-Variant Burden

To explore whether unidentified germline genetic associations with osteosarcoma existed, we evaluated rare variants in 736 candidate genes, which included 140 genes previously associated with osteosarcoma and 596 somatically altered genes (eTable 3 in the Supplement).

Burden tests of in silico-predicted damaging variants (minor allele frequency ≤0.005) and all rare variants (minor allele frequency ≤0.01) did not identify an association with the evaluated genes (eTable 12 in the Supplement). One exception was observed; the gene *ATRX* (OMIM 300032) had a higher rare-variant burden in European patients (28 of 732 patients [3.8%]) compared with European individuals in the control group (18 of 994 individuals [1.8%]). One variant was pathogenic (c.6532C>T, p.Arg2178Trp, in 1 male patient; absent in the control group) and was previously reported to be pathogenic for alpha-thalassemia X-linked (ATR-X) intellectual disability syndrome in a patient with ATR-X syndrome who also developed osteosarcoma.[61,62]

Discussion

We report that 28.0% of patients with osteosarcoma had a pathogenic/likely pathogenic variant in a cancer-susceptibility gene, with 18.4% of those variants in an autosomal-dominant gene; to our knowledge, this frequency is higher than previously reported for any other pediatric cancer.[23-25,63] The highest carrier frequency was observed in the youngest patients, with 24.5% of patients aged 0 to 10 years carrying a pathogenic/likely pathogenic variant in an autosomal-dominant gene. An additional 25.4% of the total patients had an in silico-predicted damaging variant of uncertain significance in an autosomal-dominant cancer-susceptibility gene. We confirmed previous observations of a high frequency of germline *TP53* pathogenic/likely pathogenic variants in patients with osteosarcoma[22,49] with double the sample. These data suggest that germline *TP53* pathogenic/likely patho-

genic variants are associated with a younger age at diagnosis, an axial tumor location, and worse survival.

Previous reports of smaller numbers of patients with osteosarcoma have suggested enrichment of pathogenic/likely pathogenic variants in other autosomal-dominant cancer-susceptibility genes.[24,25] We similarly identified pathogenic/likely pathogenic variants in the genes *RB1*, *APC*, *MSH2*, and *PALB2* (OMIM 610355) . We additionally report that 6.5% of patients with osteosarcoma unselected for family history had a pathogenic/likely pathogenic variant in a gene associated with a cancer-predisposing syndrome that is associated with osteosarcoma.

This study identified several new candidate osteosarcoma-susceptibility genes that are worthy of additional study, including *CDKN2A*, *MEN1, VHL, POT1, APC*, *MSH2*, and *ATRX*. Notably, *CDKN2A* had the second highest frequency (1.2%) of pathogenic/likely pathogenic variants in patients with osteosarcoma and has not been associated with pediatric cancer; however, it has been associated with melanoma and pancreatic cancer.[64-66] A germline variant located 150 kilobases upstream of *CDKN2A* has been associated with the risk of canine osteosarcoma,[67] which has biologic similarity to human osteosarcoma.[68] Somatic *CDKN2A* loss is an important somatic event in human osteosarcomas.[48,49,69,70] Four of 6 of the *CDKN2A* pathogenic/likely pathogenic variants (p.Asp125His, p.Gly101Trp, p.Ile49Ser, and p.Ile49Thr) observed in the patients with osteosarcoma have been previously associated with a predisposition for melanoma or pancreatic cancer.[71-74]

The X-linked cancer-susceptibility genes have not been previously associated with osteosarcoma and were identified in both the discovery and replication patient sets. We report 2 patients with pathogenic *DKC1* variants (c.1223C>T and c.-142C>G) that are known to cause dyskeratosis congenita,[75,76] which is associated with a high risk of select solid tumors[77,78] but has not been previously associated with osteosarcoma.[79] We identified *WAS* loss-of-function mutations, which are associated with Wiskott-Aldrich syndrome and have previously been associated with lymphoma susceptibility but not with osteosarcoma. Our data further associate osteosarcoma with rare variants in *ATRX*, which has been reported to have somatic driver mutations in osteosarcoma.[48] Osteosarcoma has been reported in 5 children with the rare ATR-X genetic disorder, which is associated with heterozygous pathogenic germline variants in *ATRX*.[62,80,81] One of these previously reported patients with ATR-X syndrome developed osteosarcoma[61,62] and had a worse outcome, which is comparable with the osteosarcoma patient who had the same *ATRX* variant.

Strengths and Limitations

A strength of our study is that, to our knowledge, the 1244 patients with osteosarcoma in our analysis represent the largest set of patients with a single solid pediatric cancer to be evaluated for cancer-susceptibility gene pathogenic variants to date and consequently provide more precise pathogenic/likely pathogenic carrier prevalence estimates. The use of internal individuals without cancer who were jointly called with the patients improved the whole-exome sequencing quality-control measures.

The limitations of our study include the inability to assess family history, the incomplete data on important clinical variables from all centers, and the use of ExAC whole-exome sequencing data, which could not be directly used for discovery analyses or burden testing owing to distinct biases associated with its accumulation of data from many sources. In addition, 284 of the 1004 patients in the discovery set were derived from the Childhood Cancer Survivor Study, which could have resulted in survival bias for this subset. Notably, patients in the Childhood Cancer Survivor Study had a lower carrier frequency of *TP53* pathogenic/likely pathogenic variants compared with all other patients (2.8% vs 5.0%, respectively; Fisher exact $P = .17$).

Conclusions

We report that an estimated 28.0% of patients with osteosarcoma carried a rare germline pathogenic/likely pathogenic variant in a cancer-susceptibility gene, and more patients carried likely damaging variants in autosomal-dominant cancer-susceptibility genes. We confirm known associations and identify new genes that provide insight into the biology of osteosarcoma. Our findings have important implications for the genetic testing of patients, especially younger patients, who are newly diagnosed with osteosarcoma because these patients were more likely to have a potentially clinically relevant disease-associated pathogenic/likely pathogenic variant. We acknowledge that our estimates, particularly those based on in silico analyses, may be high because functional studies are required to prove pathogenicity.

Our data underscore the high frequency of potentially actionable cancer risk variants in patients with osteosarcoma, suggesting a need for further preventive and early detection strategies as well as a consideration of cascade genetic testing for the patient and the entire family.[82-84] We note that individuals harboring Li-Fraumeni syndrome-associated *TP53* mutations benefit from active screening, which could translate into improved outcomes.[85,86] Further studies are needed to refine our observations and identify optimal approaches to genetic testing and counseling for patients with osteosarcoma.

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