Case Report
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# Genetic Analysis of Osteosarcoma Cells in a 9-year-old Boy: Genes Involved in Cell Cycle Control

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**Abstract**

**Objective.** This study describes mutations of genes that stimulate and regulate cell growth, programmed cell death, DNA repair, and cell growth suppression in a boy with osteosarcoma. **Case Report.** We report a case of bone sarcoma in a 9-year-old boy with possible familial predisposition. In our patient, only a subset of tumor cells expressed the ATRX protein, which is known to control the expression of several genome regions. The function of the p53 protein, which acts as a transcription factor that regulates the DNA damage repair response, cell cycle progression, and apoptosis pathways, is lost in 40-50% of malignant cells. Retinoblastoma was positive in the predominant subset of tumor cells. Deletion is found on chromosome 9, cytoband 9p21.3, where the genes for CDKN2A and CDKN2B are located. Neoplastic cells were SATB2-positive in a substantial subset, with nuclear staining. The SATB2 protein is a DNA-binding protein involved in transcriptional regulation and chromatin remodeling. Chromosomal losses of 8p and 19q11-q13.43 were also found. These regions contain several tumor suppressor genes, including *NKX3.1*, whose reduced expression correlates with 8p loss in high-grade tumors. Although there was no known cancer syndrome in the family, the maternal grandfather had a similar tumor requiring amputation. **Conclusion.** Chromosomal instability is a hallmark of osteosarcoma and is characterized by heterogeneous and extensive genetic complexity. Various numerical and structural genomic rearrangements have been described in cancer cells. However, there is little consistent genetic change to understand the etiopathogenesis of this aggressive tumor.

**Key Words:** Osteosarcoma ▪ Regulation ▪ Genes ▪ Mutation ▪ Tumorigenesis.

## Introduction

Osteosarcoma (OS) is one of the most common primary bone tumor in children and adolescents, although its incidence is very low (1, 2). The timing of tumor diagnosis in patients coincides with the developmental growth spurt that occurs in this patient population (3), indicating a possible role for the growth hormone-insulin-like growth factor axis in the development and progression of osteosarcoma (4). On average, 4.4 cases of osteosarcoma are diagnosed per million children per year (5, 6). Statistical data show that this number has remained unchanged over the past few decades, while the introduction of multi-agent chemotherapy in the 1980s significantly reduced mortality (7). About 80% of patients present with grossly localized disease (8). Surgical resection following induction chemotherapy is the standard for local control of osteosarcoma. Systemic therapy with high-dose methotrexate, including adriamycin, cisplatin, and ifosfamide (MAP), demonstrated a five-year survival rate of about 60% (9). If the tumor is resectable, radiation therapy is not applied as a first-line definitive treatment approach because osteosarcoma is not a radiosensitive disease. The primary localization is in the metaphysis of the long bone, most often in the femur, tibia, and humerus, although cases with multifocal lesions have also been described (10). The tumor is

derived from bone-forming mesenchymal cells. Histologically, the presence of different malignant mesenchymal cells that produce the bone stroma characterizes this mesenchymal tumor. Several histological subtypes of osteosarcoma have been defined, including osteoblastic, chondroblastic, fibroblastic, and telangiectatic (11). Although the origin of the tumor cells is uncertain, scholars believe that the malignant transformation occurs in osteoblasts or preosteoblasts (12, 13). The results of recent research suggest that malignant transformation occurs at the level of multipotent mesenchymal stem cells that differentiate into bone-differentiation lineages (14, 15).

Although osteosarcoma is a sporadic disease, in a small number of cases, it occurs as a component of a hereditary cancer syndrome, which includes Li-Fraumeni syndrome, retinoblastoma, Rothmund-Thomson syndrome, and Bloom's and Werner's syndromes, with individuals inheriting germline inactivating mutations of the respective genes (16, 17). Genetic analysis of osteosarcoma cells in the 9-year-old boy presented here discusses a possible mechanism of tumorigenesis and a possible familial predisposition.

## Materials and Methods

Neoplastic tissue was obtained from the right tibial mass by a fluoroscopy-guided core biopsy and a core needle biopsy. Tissues were formalin-fixed and paraffin-embedded. Cytospins of the right distal tibia mass cyst fluid were stained with hematoxylin and eosin (H&E) and Wright-Giemsa. The cell block was stained with H&E. The histologic preparations were reviewed by the responsible pathologist and found to be adequate in terms of the quality of fixation, processing, microtomy, and H&E staining. Appropriate positive and negative controls for special stains, immunohistochemistry, and/or in situ hybridization showed the expected reactivity. Immunohistochemical testing and special stains, as applicable, were performed, and the performance characteristics were developed by the Anatomic Pathology Laboratory at St. Jude Children's Research Hospital (SJCRH) in Memphis, United States of America. Immunostaining for SATB2 was performed at Mayo Clinic Laboratories and reviewed at SJCRH. All tumor specimens were evaluated histopathologically for diagnostic purposes and to assess specimen adequacy.

The Archer VariantPlex Custom Panel assay is used to identify variants within the selected target region(s) only. A negative Archer VariantPlex targeted sequencing test result does not rule out the presence of variants at a level below the sensitivity of detection. This test will not detect variants in areas outside the targeted genomic regions, nor will it detect copy number alterations and translocations. The test is not intended for minimal residual disease testing. This test evaluates variants in both tumor and germline tissue(s) and may not be able to distinguish between somatic and germline variants. Only pathogenic or likely pathogenic variants are reported, and only canonical splice sites (exon +/- 2 bp neighboring intronic regions) are evaluated. The test does not report variants categorized as being of uncertain clinical significance, benign, or likely benign.

### *Analyzed Genes*

| Gene symbol | Reference sequence | Target exons |
|---|---|---|
| *RB* | NM_000321 | 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 |
| *TP3* | NM_000546 | 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 |

*Tech: LK.*

### *Analyte Specific Reagent Notification*

The Pathology Laboratory at SJCRH developed this test and determined its performance characteristics. The test has not been cleared or approved by the U.S. Food and Drug Administration (FDA). The use of Analyte Specific Reagents does not require FDA approval [21CFR809.30].

### *Fluorescence in Situ Hybridization (FISH)*

Target Probe CDKN2A: Locus 9p21 (laboratory developed). Control Probe: Locus 9q31.2

(laboratory developed). Target Probe CDK4: Locus 12q14 (Empire Genomics) orange. Control Probe: Locus CEP 12 (Empire Genomics) green. Scoring Method: Manual. Images captured and processed by GenASIs software, ASI (Applied Spectral Imaging).

## History of Present Illness

A 9-year-old boy presented for evaluation of a 5.5 cm × 3 cm × 3 cm bone mass in the distal right tibia that had grown over the preceding month. The patient and his family provided the history. The boy had developed right ankle pain and swelling three months prior. His primary care physician referred him to orthopedics. The boy was found to have a lytic lesion on XR that was thought to be a bone cyst (Figure 1).

An MRI was obtained (Figure 2), and a biopsy was recommended. A routine pre- and post-contrast MRI of the right tibia and fibula was performed. No definite diffusion restriction was identified, although diffusion images were limited due to signal loss in the presence of calcified/ossified tissue. Out-of-phase imaging showed no definite signal loss within the tumor, suggesting the absence of microscopic fat.

The heterogeneously enhancing, expansile lesion in the distal metadiaphysis increased in size over the interval. It measured 2.9 cm × 2.8 cm axially. The cortex showed thinning, and marrow edema extended to the adjacent tibial diaphysis. The lesion appeared to have multiple cystic components without appreciable fluid-fluid levels. There was no appreciable soft tissue component. The lesion was highly suspicious for telangiectatic osteosarcoma.

According to outside hospital records, a lack of insurance prevented the scheduling of a biopsy. The patient was placed in a boot, but pain and swelling continued to worsen. Due to severe pain, the family called 911, and the emergency room evaluated the patient. The MRI and XR were repeated and showed interval growth of the right distal tibial mass. Orthopedics was consulted and referred the boy for further evaluation due to the concerning findings on imaging. The patient was using Motrin and Tylenol, but the pain sometimes

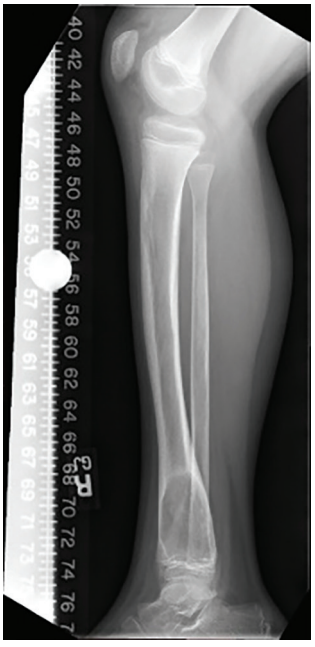

Figure 1. XR of the tibia/fibula right. A lytic lesion within the distal tibia primary, involving the metaphysis and abutting the physis.

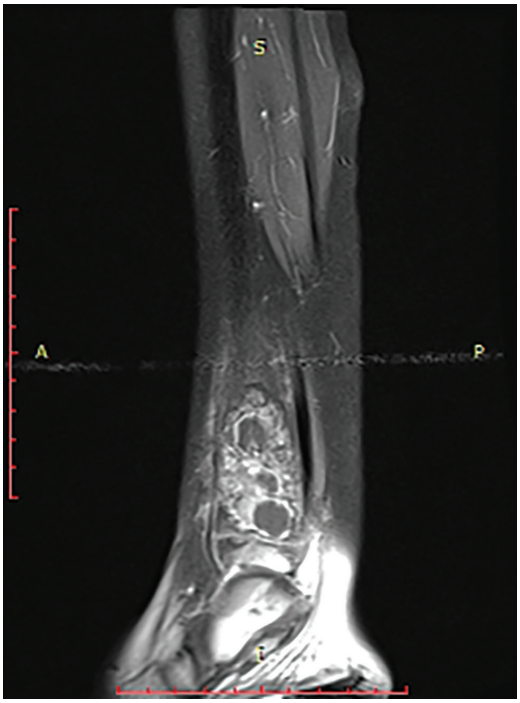


Figure 2. MRI of the right tibia/fibula.

awakened him from sleep. He was given a morphine prescription, which helped him rest better. He denied any trauma (prior to or since the onset of pain). He had no fevers, night sweats, weight loss, bony pain, fatigue, or malaise, nor did he experience changes in the color of the foot, numbness, or tingling. Imaging demonstrating an enlarging cystic lesion raised concern for telangiectatic osteosarcoma, while aneurysmal bone cyst (ABC) was also possible. Giant cell tumor was also considered, as air-fluid levels were not visualized on the MRI. He was started on a nurse practitioner training program (NPTP) with MAP chemotherapy. Following the first 5 weeks of MAP, imaging revealed no obvious evidence of disease progression, and the patient exhibited clinical evidence of response with reduced pain and localized soft tissue swelling. He developed significant hearing loss after the first cycle, and cisplatin was held; he received week 6 doxorubicin alone and was due for week 9 high-dose methotrexate (HDMTX). A month later, the right distal tibial mass underwent a biopsy. After surgery, due to hearing loss, the therapy was changed to an OS99 regimen with carboplatin, ifosfamide, and doxorubicin. The patient reported less pain and was using crutches or a wheelchair and avoiding bearing weight.

### *Perinatal, Social, and Family History*

The boy was born after a full-term, uncomplicated pregnancy. He was breastfed, had normal early childhood development, and lived with his mother and father, two brothers, and two sisters. To date, he had received early childhood vaccines, but the family was not certain whether he was "up to date". He had not received COVID-19 or flu vaccines. The boy did not suffer from varicella or herpes simplex (fever blisters). There were no medical conditions in the family. His four siblings were healthy. His mother and father, who are cousins, were also healthy and without any medical problems. There were no genetic disorders or cancers that ran in the family. The patient's maternal grandfather had "this tumor" in his leg in childhood, which resulted in the amputation of his ankle and foot.

## Results

A CT of the chest (Figure 3) showed clear lungs and no pleural or pericardial effusions. There was no adenopathy in the chest. The visualized portions of the thyroid and upper abdomen were unremarkable. There was no evidence of pulmonary metastatic disease.

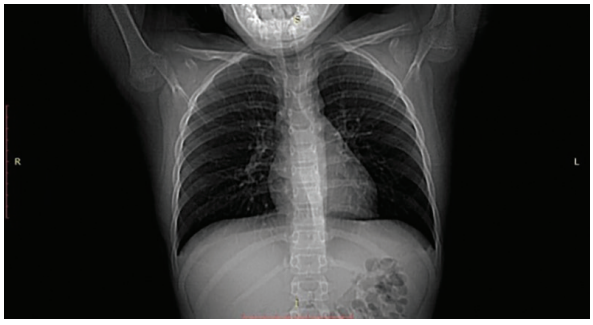

Figure 3. A chest CT revealed a hazy ground glass opacity along the minor fissure in the lateral segment of the right middle lobe.

Positron Emission Tomography (PET) imaging (Figures 4A and 4B) showed an expansile lesion of the distal right tibia, demonstrating significant hypermetabolic activity. This is nonspecific as hypermetabolic activity may be seen with giant cell tumors, aneurysmal bone cysts, and sarcoma. Hypermetabolic right inguinal and iliofemoral lymph nodes were noted. These were indeterminate and may have been reactive/inflammatory, especially considering the recent biopsy. However, metastatic disease could not be excluded.

The pathology of the biopsy sections revealed a hypercellular neoplasm composed of large cells with pleomorphic nuclei, abundant eosinophilic cytoplasm, and indistinct cell borders (Figure 5). Many tumor cells contained multiple large, hyperchromatic nuclei. Other tumor cells had smaller nuclei with granular chromatin and one to several small nucleoli. Frequent mitoses were present, including markedly enlarged, atypical mitoses. Occasional apoptotic cells and small areas of necrosis were seen, comprising less than 5% of the tumor. Frequent admixed multinucleated osteoclast-type giant cells were seen. Small amounts of loose fibromyxoid tissue were noted in some

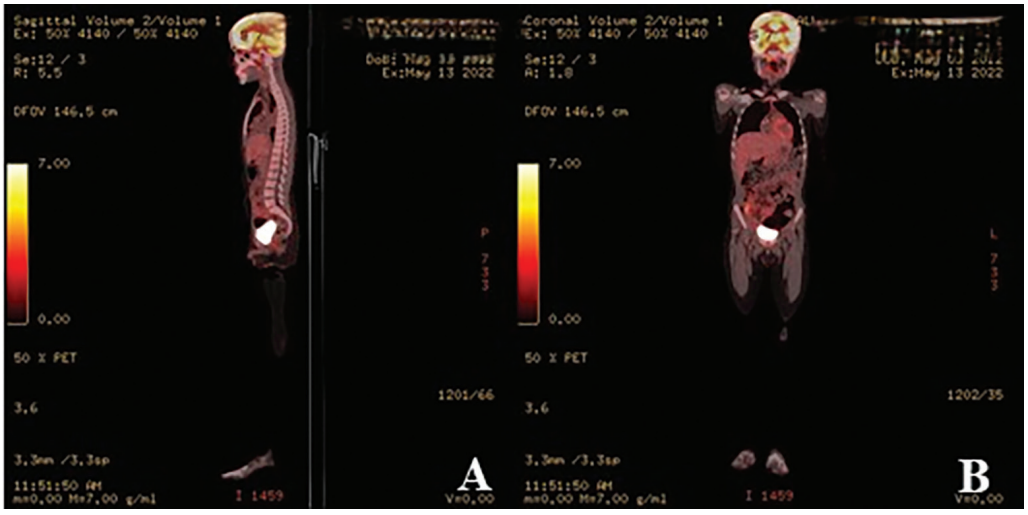


Figure 4. Body PET (Figures 4A and 4B) showed significant hypermetabolic activity of the distal right tibia and right inguinal and iliofemoral lymph nodes.

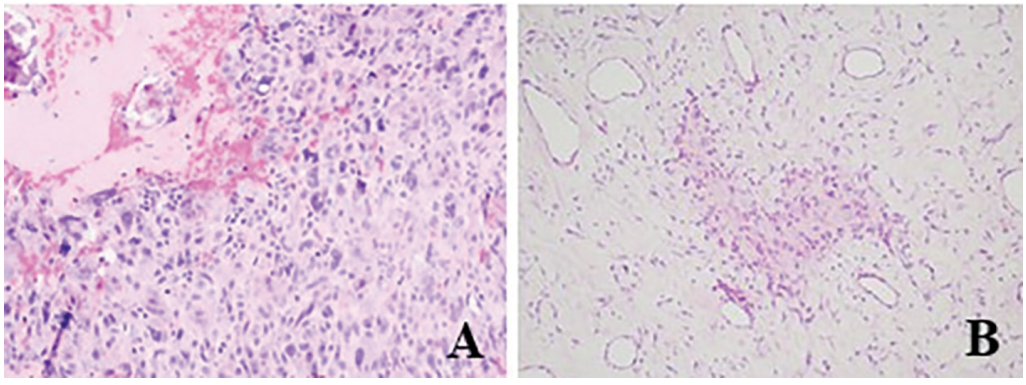


Figure 5. Malignant neoplasm, most consistent with high-grade osteosarcoma with cystic features and frequent multinucleated cells (A). Histologic response to induction chemotherapy (B).

sections. The osteoid matrix was not widely seen, but a partially mineralized matrix with cellular atypia consistent with neoplastic bone was noted focally. The performed Trichrome stain was weakly positive in the fibromyxoid tissue and positive in thin strands and small amounts of collagenous tissue admixed with tumor cells. Cytospins showed occasional large, atypical cells, including mononuclear and multinucleated forms, against a background of numerous red blood cells, scattered acute and chronic inflammatory cells, occasional macrophages, and occasional osteoclast-type giant cells (not shown).

***Immunostaining***

- CD68: Negative in tumor cells; positive in frequent mononuclear histiocytes; positive in frequent multinucleated osteoclast-type giant cells.
- ATRX: Positive in a predominant subset of tumor cells (retained). Although the specific function of the ATRX protein is unknown, studies suggest that it helps regulate the activity (expression) of other genes through a process known as chromatin remodeling. ATRX controls the expression of several genomic regions.
- p53: Positive in a subset - approximately 40 to 50% - of tumor cells.

- Retinoblastoma: Positive in a predominant subset of tumor cells (retained).
- SATB2 was positive in a substantial subset of lesional cells, with nuclear staining.

RNA transcriptome sequencing (RNA-Seq) was performed on sections and did not detect recurrent fusion transcripts. Sequencing of the whole exome was attempted on the tissue but could not be performed due to insufficient nucleic acid yield.

### *Fluorescence in Situ Hybridization (FISH)*

*CDKN2A* – Positive for homozygous deletion of *CDKN2A* in the 200 evaluable nuclei. The following patterns of deletion were observed [TC (21%) (cutoff = 48%); C (20.5%); CC (22.5%) (cutoff = 8%); 3C (6%); TCC (6%) (cutoff = 25%)]. The following additional signal pattern was observed [TTC (2%)]. *CDK4* – Negative for amplification of *CDK4* in the 200 evaluable nuclei. Approximately 26% of the nuclei showed 3 to 8 signals of both target and control [3×(13%) (cutoff = 16%); 4×(7%); 5-8× (6%)]. A gain of one *CDK4* signal was observed in 7% of the nuclei [3T2C (5%) (cutoff = 9%); 4T3C (2%)] along with a relative loss of one *CDK4* signal being observed in 2.5% of the nuclei [2T3C (1.5%); 3T4C (1%)]. The following additional signal patterns were observed [TC (16%); TCC (4.5%); TTC (5.5%)].

### *Methylation Array-Based Copy Number Analysis*

Copy number analysis was performed on recut sections using a methylation array, yielding the following results (Table 1).

Table 1. Large-Scale DNA Copy Number Variations

| Gene | Alteration | Cytoband |
|---|---|---|
| *CDKN2A* | Deletion | 9p21.3 |
| *CDKN2B* | Deletion | 9p21.3 |

*Chromosomal losses:* 8p, Chromosome 9, 17p13.1 (segment includes the *TP53* gene), 19q11-q13.43; *Chromosomal gains*: No large-scale chromosomal gains. Targeted sequencing analysis was positive for the following likely pathogenic variant of the *TP53* gene (Table 2).

Table 2. Targeted Sequencing Analysis of the *TP53* Gene

| Alteration<br>*TP53* P278S | HGVS<br>Nomenclature | Allele Frequency<br>Approximately 40% |
|---|---|---|
| NM_000546:<br>c.832C>T;<br>p.Pro278Ser | - | - |

### *Molecular Pathology Final Report*

Targeted sequencing analysis performed on this patient's sample was positive for the following likely pathogenic variant: *Alteration HGVS Nomenclature Allele Frequency.* TP53 P278S NM_000546: c.832C>T; p.Pro278Ser ~40%. *Comments*. Since tumor-only tests cannot reliably distinguish between somatic and germline alterations, correlation with clinical data, genetic counseling, and germline testing may be recommended if clinically indicated.

## Discussion

Our patient was a 9-year-old boy. Differential diagnosis for primary bone lesions in this age group is wide and includes osteosarcoma, Ewing sarcoma, giant cell tumor, osteoblastoma, eosinophilic granuloma, aneurysmal bone cyst, chondromyxoid fibroma, osteomyelitis, simple bone cyst, osteoid osteoma, and fibrous dysplasia. Based on location, progression, and multicystic appearance on MRI, the leading differentials were aneurysmal bone cyst and telangiectatic osteosarcoma. Giant cell tumor was ruled out because CD68 staining was negative in tumor cells and positive in frequent mononuclear histiocytes and frequent multinucleated osteoclast-type giant cells. Very rarely, lung metastases are detected in patients concurrently with a diagnosis of the primary tumor (18, 19), a condition known as early metachronous osteosarcoma (MOS), for which there appears to be a male prevalence. In our patient, no evidence of pulmonary metastatic disease was found. The lungs were otherwise clear, with no nodules to

suggest pulmonary metastatic disease. There were no pleural or pericardial effusions and no adenopathy in the chest. Hypermetabolic lesion activity of the distal right tibia is nonspecific, as this may be seen with giant cell tumors, aneurysmal bone cysts, and sarcoma. Pathohistological examination described a poorly differentiated, high-grade (grade 3) osteosarcoma with 5% necrosis. The morphologic features and the suggestion of blood and fibrin-filled cystic spaces raised the possibility of telangiectatic osteosarcoma. Telangiectatic osteosarcoma is a rare subtype of the disease, accounting for 2-12% of cases (20).

ATRX staining was positive in the predominant subset of tumor cells (retained). The ATRX protein controls the expression of several genomic regions through a process known as chromatin remodeling. ATRX mutations have been shown to cause diverse changes in the pattern of DNA methylation, which may provide a link between chromatin remodeling, DNA methylation, and gene expression in developmental processes. Inherited mutations of the *ATRX* gene are associated with X-linked mental retardation (XLMR) syndrome, most often accompanied by alpha-thalassemia (ATR-X) syndrome (21). Acquired mutations in *ATRX* have been reported in several human cancers, including osteosarcomas (22). A subset of tumor cells (approximately 40-50%) were positive for TP53. Our results showed that most tumor cells had lost the segment of chromosome 17 (17p13.1), a segment that includes the tumor suppressor *TP53* gene. Loss-of-function *TP53* mutations occur in 75% of osteosarcoma cases (23). The p53 protein acts as a transcription factor that regulates DNA damage repair response, cell cycle progression, and apoptosis pathways (24). Mutation of p53 was found in essentially all tumor types. In Li-Fraumeni syndrome (LFS), characterized by a germline mutation of the *TP53* gene, the risk of osteosarcoma is much higher (25). Retinoblastoma (Rb) was positive in the predominant subset of tumor cells (retained) of our patient. Rb plays a regulatory role in the $G_1$-to-S cell cycle transition by binding to E2F family transcription factors in the absence of mitogenic stimuli. When the function of this protein is lost, this cell checkpoint is lost (26). pRb is one component in a cell-cycle control pathway that includes the p16 (encoded by the *CDKN2A* gene) and cyclin-dependent kinase 4 (cdk4, encoded by the *CDK4* gene) proteins. *CDKN2A*, also known as cyclin-dependent kinase inhibitor 2A, is a gene located in humans at chromosome 9, band p21.3. It is ubiquitously expressed in many tissues and cell types. The gene codes for two proteins, including the INK4 family member p16 (or p16INK4a) and p14arf.

Analysis was negative for amplification of *CDK4*; a gain of one *CDK4* signal was observed in 7% of the nuclei, and a relative loss of one *CDK4* signal was observed in 2.5% of the nuclei. However, on chromosome 9, cytoband 9p21.3, where the genes for *CDKN2A* and *CDKN2B* are located, deletion was found. This agrees with the findings that tumor suppressor *CDKN2A* was inactivated in osteosarcomas that lack *RB* mutations and that the p16-pRb cell-cycle control pathway was deregulated in a large number of high-grade osteosarcomas (27). One study has reported a constitutional inversion at chromosome 9p11-9q12 in a patient, along with non-clonal balanced translocations in the tumor (28), and a familial occurrence of telangiectatic osteosarcoma in cousins, but without any apparent hereditary components (29). Special AT-rich sequence-binding protein 2 (SATB2) is also known as DNA-binding protein. SATB2 is a protein encoded by the *SATB2* gene in humans. It is a DNA-binding protein that specifically binds nuclear matrix attachment regions and is involved in transcriptional regulation and chromatin remodeling. Although not specific to osteosarcoma, SATB2 is a marker of osteoblastic differentiation in benign and malignant mesenchymal tumors (30). Lesional cells in our patient were SATB2 positive in a substantial subset, with nuclear staining. Chromosomal losses 8p and 19q11-q13.43 were also found in our patient. Several tumor suppressor genes are located on chromosome 8p. One of these genes is *NKX3.1*, whose reduced expression correlates with 8p loss in high-grade tumors (31). Osteosarcomas exhibit karyotypes with an unusually high degree of aneuploidy and structural rearrangements. Frequent structural alterations at

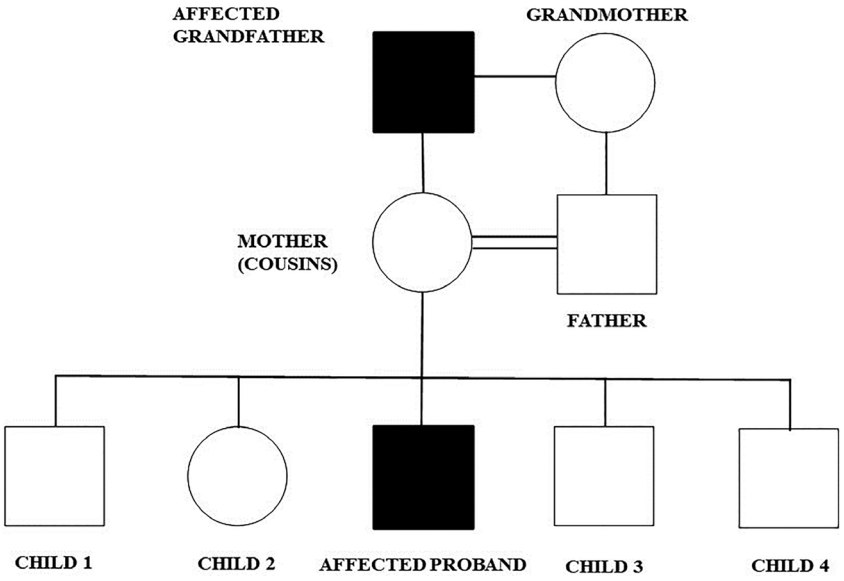


Figure 6. Possible hereditary predisposition.

chromosome bands or regions 19q11-13 were reported (32). In prostate cancer, research indicates that 19 regions contain susceptibility loci that regulate tumor aggressiveness (33).

Although there was no known cancer syndrome in the family, the maternal grandfather had a similar tumor requiring amputation. The parents were cousins, raising the possibility of inherited mutations by 40% (Figure 6). The family was advised to undergo a genetic analysis in order to detect a possible predisposition to malignant diseases.

## Conclusion

Cancer is a multistage process characterized by the accumulation of epigenetic alterations and the mutation of genes that regulate cell growth, apoptosis, DNA repair, and tumor-suppressor genes. Genetic changes can be inherited, making a person predisposed to developing neoplasia. The accumulation is associated with neoplasia risk and can be utilized for cancer risk diagnosis. Chromosomal instability is a hallmark of osteosarcoma and is characterized by heterogeneous and extensive genetic complexity. The field of mutations is highly complex and differs significantly between tumors. Unlike other sarcomas, osteosarcomas do not exhibit genetic translocations; instead, we find widespread and heterogeneous abnormalities in the number and structure of chromosomes, illustrating the numerous DNA alterations that can occur in cancer. In sarcoma cells, DNA-related/chromatin remodeling is not evidence for transformation into a cancerous phenotype. In order for a tumor to spread and metastasize, tumor cells acquire six hallmarks of cancer during their development (typically by mutations in the relevant genes): limitless replicative potential, tissue invasion and metastasis, insensitivity to anti-growth signals, self-sufficiency in growth signals, evading apoptosis, and sustained angiogenesis. Therefore, this study examined only a subset of genes that frequently mutate in patients with osteosarcoma. The authors hope that discovering cancer biomarkers will lead to the development of targeted therapies.

**What Is Already Known on This Topic:**

*Cancer of the bones and joints is a rare genetic disease accounting for approximately 20% of all benign and malignant bone neoplasia and 2% of pediatric cancers. The majority of osteosarcoma (OS) cases are sporadic but occur at increased rates in individuals with Paget's disease of bone, after therapeutic radiation, and in certain cancer predisposition syndromes. Although some subtypes exhibit characteristic genetic features and biological behaviors, the molecular basis for each subtype remains poorly understood. The etiological factors and pathogenetic mechanisms underlying OS development are complex, but significant progress has been made toward understanding its causes. The efforts made over the past few decades have focused on identifying so-called 'driver' mutations present in cases of inherited predisposition, as well as in sporadic OS. Cancer-causing genes (often called driver genes or drivers) contain driver mutations, which confer a proliferative advantage to cancer cells, leading to tumor clone outgrowth (34).*

**What This Study Adds:**

*So-called 'driver' mutations, including tumor suppressor genes p53, Rb, RECOL4, BLM, and WRN, play a critical role in developing OS. The molecular basis of OS is not well understood, so studying driver genes and their interaction in OS development will eventually advance preclinical investigations into new therapeutic strategies and drugs.*

**Acknowledgments:** We thank the patient, the parents, and the physicians for their participation in this study. The parents provided written consent for the use of the medical documentation and the publication of a paper. In our institution, the Institutional Review Board (IRB) also functions as the Ethical Committee. The IRB number for this work is 012025-001.

**Authors' Contributions:** Acquisition of data: AT and DJ; Conception, interpretation and analysis of data: DJ, AT and SD; Drafting the article: DJ; Approved final version of the manuscript: DJ, AT and SD.

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