

Identification of Genomic Alterations in Chemotherapy-Related Genes: A Preliminary Study in Three Thai Adolescents with Fatal Osteosarcoma at Maharaj Nakorn Chiang Mai Hospital

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ABSTRACT

OBJECTIVE Osteosarcoma (OS) is the most common primary bone malignancy among adolescents and young adults. It is characterized by a high mortality rate, though it remains a relatively rare disease overall. The treatment protocols can be complex and challenging. We conducted a comparative genomic variation study to identify genes involved in the metabolism of chemotherapy drugs that are affected by various genomic alterations.

METHODS This study analyzed the germline and tumor genomes of three OS patients who responded poorly to chemotherapy, developed lung metastases, and ultimately succumbed to the disease. Genes of interest were identified through a systematic review of apoptosis, autophagy, necroptosis, and chemotherapy-related databases. Whole-genome sequencing (WGS) revealed deleterious single nucleotide polymorphisms (SNPs), copy number variations (CNVs), and structural variations (SVs) in genes linked to cancer and chemotherapy. Protein association network analyses were used to highlight both shared and unique biological processes associated with OS in these patients.

RESULTS Pathogenic SNPs were identified exclusively in patients P1 and P3. Patient P1 exhibited a missense mutation in the *PIK3CD* gene and a splice donor site mutation in the *TP53* gene, while patient P3 carried a non-sense mutation in the *MLH1* gene. The tumor genome of patient P1 showed extensive CNVs, whereas those of patients P2 and P3 displayed fewer CNV regions. *TBX4* and *PPMID* were common genes affected in both patients P1 and P2. SVs were detected in all three tumor genomes, impacting different key genes involved in platinum drug resistance and DNA repair. Notably, all three patients shared SVs in the *TDG* and *SPECC1* genes. All identified mutated genes were correlated with clinical features of the patients.

CONCLUSIONS This study highlights the potential of genomic analysis to improve the diagnosis, treatment, and management of OS. Insights into therapy resistance mechanisms and key genomic alterations offer opportunities for developing targeted therapies and predictive biomarkers. These findings support the advancement of precision oncology in OS, as a means of paving the way for personalized treatment approaches to improve patient outcomes.

KEYWORDS osteosarcoma, genome, single nucleotide polymorphisms, copy number variations, structural variations, chemotherapy

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INTRODUCTION

Osteosarcoma (OS) is the most common malignant bone tumor; it is marked by the deposition of immature osteoid matrix (1). Its prevalence varies by age, ethnicity, and genetics, with a bimodal age distribution peaking in children and adolescents and again in the elderly (2). The 5-year survival rate is approximately 60–70% (3), but the prognosis worsens if the tumor shows less than 90% necrosis after treatment (4). Treatments include surgery, chemotherapy, and immunotherapy (3). While the pathogenesis of OS remains unclear, chromosomal abnormalities have been linked to increased susceptibility to the disease (2, 3, 5).

Genomic instability plays a critical role in tumor formation. Chromothripsis, in particular, results from the imperfect repair of fragmented chromosomes caused by clustered DNA double-strand breaks, occurring in a single event rather than gradually (6). Normally, this catastrophic rearrangement triggers apoptosis, but if apoptosis fails, the misrepaired genetic material can drive tumor development (6). Chromothripsis has been identified in OS, with studies reporting its presence in a significant number of cases (7, 8).

Chromothripsis plays a significant role in tumor development and progression by causing fragmented and rearranged chromosomes. These rearrangements can deactivate tumor suppressor genes, amplify oncogenes, and result in other genomic alterations which contribute to the cancer phenotype. Common rearrangements include structural variations (SVs) and copy number variations (CNVs). Examples include translocations impacting tumor suppressor genes like *TP53*, *RB1*, and *WWOX*, as well as CNVs such as copy number gains in *DK4* and *CCNE1* and copy number losses in *CDKN2A* and *TP53*. Additionally, *TP53* mutations are linked to early onset and higher metastatic rates in OS (9, 10). In addition, rs7591996 has been linked to increased OS risk (11). Abnormal expression of the anti-apoptotic *AKT1* gene and pro-apoptotic *BAX* gene (12), as well as autophagic and necroptotic genes like *BECN1* (13) and *RIPK1* (14), has also been observed in OS.

Platinum-based chemotherapy works by creating DNA crosslinks, leading to cytotoxicity and apoptosis. However, tumor resistance to platinum drugs can arise through mechanisms such as altered drug transport, enhanced DNA repair,

modified survival pathways, pleiotropic changes, and tumor microenvironment alterations. For example, increased expression of cisplatin export modulators *ATP7A* and *ATP7B* contributes to resistance (15). Previous reports have identified 74,880 single nucleotide variants and indels, along with common CNVs, such as gains and losses in specific chromosomal regions. High expression of *PTPRQ* has been linked to poor prognosis (16). Thus, exploring CNVs in chemotherapy-related genes could potentially help develop personalized treatment strategies.

This study addresses the complexity of OS, a rare bone cancer with diverse genetic alterations that impact cell death and chemotherapy response. By analyzing genomic data from tumor and normal samples, the research aimed to identify somatic and germline mutations, focusing on structural changes in the tumor genomes of adolescents with fatal OS and poor chemotherapy outcomes, to advance understanding and treatment strategies.

METHODS

Patients

Three OS patients, all of whom had been treated at Maharaj Nakorn Chiang Mai Hospital in Chiang Mai, Thailand, were identified as P1, P2, and P3 (Table 1). Both P1 and P3 were male patients, while P2 was a female patient. All three underwent surgeries and received chemotherapy as part of their treatment. As shown in Table S1, the treatment for P1 lasted 843 days, during which time the patient received chemotherapy twice. Table S2 shows that patient P2 was scheduled to receive chemotherapy 8 times over an 836-day treatment period. The treatment for patient P3 lasted 358 days with 2 scheduled chemotherapy sessions, as shown in Table S3. Tissue/tumor biopsy specimens from the patients were collected during their first surgeries. The tissue specimens and their whole blood samples were stored at the Tissue Bank of Musculoskeletal Science and Translational Research Center, Department of Orthopaedics, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. Toward the end of their treatment, all three patients developed lung metastases, and all succumbed to the disease. The whole blood and tumor tissue specimens collected from the patients were extracted for

Table 1. Patients' characteristic data

Parameter	Detail
Age	15.33 years (range 11–20 years)
Gender, n (%)	
Male	2 (66.67)
Female	1 (33.33)
Osteosarcoma type, n (%)	
Conventional type	3 (100.00)
Stage at the time of diagnosis, n (%)	
IIb	3 (100.00)
Chemotherapy, n (%)	
DOX+CPT	2 (66.60)
DOX+CPT+HDMTX	1 (33.30)
Tumor necrotic percentage	64% (range 42–85%)
Metastasis status, n (%)	
Yes	3 (100.00)
No	0 (0.00)
Recurrence status, n (%)	
Yes	2 (66.67)
No	1 (33.33)
Survival duration	678 days (357–842 days)
Status, n (%)	
Death	3 (100.00)
Alive	0 (0.00)

DOX, doxorubicin; CPT, camptothecin; HDMTX, high-dose methotrexate.

germline and somatic genomic DNA for Whole Genome Sequencing (WGS), respectively.

Systematic review

A list of genes of interest was created by reviewing articles available in public databases, referencing articles (12–14, 17–60) with keywords relevant to the biological processes of interest: apoptosis, autophagy, and necroptosis. The article selection scheme, depicted in Figure 1, resulted in the selection of 63 articles. From these articles, a list of 57 genes of interest was compiled, as shown in Table S4. The gene list included PharmGKB-curated genes involved in the pharmacodynamic and pharmacokinetic pathways of chemotherapy administered to the patients (Table S1, S2, and S3), such as the Platinum pathway (pharmacokinetics/pharmacodynamics), Etoposide pathway, Ifosfamide pathway, Methotrexate pathways (pharmacokinetics, antimetabolite, and cancer), and Doxorubicin pathways (pharmacokinetics and cancer) (61–65). The gene list also encompassed genes with mutations detected in OS or cancer patients (5, 9, 10, 16). In total, 1,794 genes of interest were identified in this study, as detailed in Table S5.

Genomic DNA extraction

Tumor tissue and matched normal genomic DNA samples were extracted from whole blood and OS tissue biopsy specimens, respectively, using a modified salting-out method, as described in Supplementary Data Doc. S1.

Whole Genome Sequencing

Genomic DNA samples extracted from both the matched normal genome (white blood cells, WBC) and corresponding OS tissue met the requirements for WGS and were sent to Macrogen Korea for sequencing. WGS for the germline genomic DNA was conducted at a 30X read depth, while that of the tissue genomic DNA was performed at a 60X read depth. An Illumina NovaSeq 6000 system (Illumina, Inc., San Diego, CA, USA) was used as the sequencing platform.

Secondary NGS data analysis

WGS data from the tumor and the matched normal genomes of the three patients were analyzed using the Genome Analysis Toolkit (GATK) Best Practices (66) or single-sample short variant discovery. The human reference genome hg38 was utilized throughout the pipeline. The analysis was conducted on a Google Cloud Compute server using a custom script written in the Workflow Description Language (WDL) and managed by Cromwell (67). The pipeline includes paired-end read alignment using BWA-MEM (68). The alignment files were subsequently sorted by chromosome and by base quality score recalibration (BQSR). Known sites of indels in the human reference genome hg38 were excluded to enhance the accuracy of variant calling. Local computer servers were employed for less resource-intensive tasks, e.g., variant selection, SVs detection, and CNV estimation.

Tertiary NGS data analysis

Since the extracted variation data contained both pathologic and common variants, the common genomic variants detected or reported in both the patients and the genome database of the 1,000 Genomes Project (69) were filtered out. Notably, the 1,000 Genomes Project includes data from the Dai ethnic group in China, whose genetic background is relatively close to that of the majority of the population in Thailand (70). The variants were

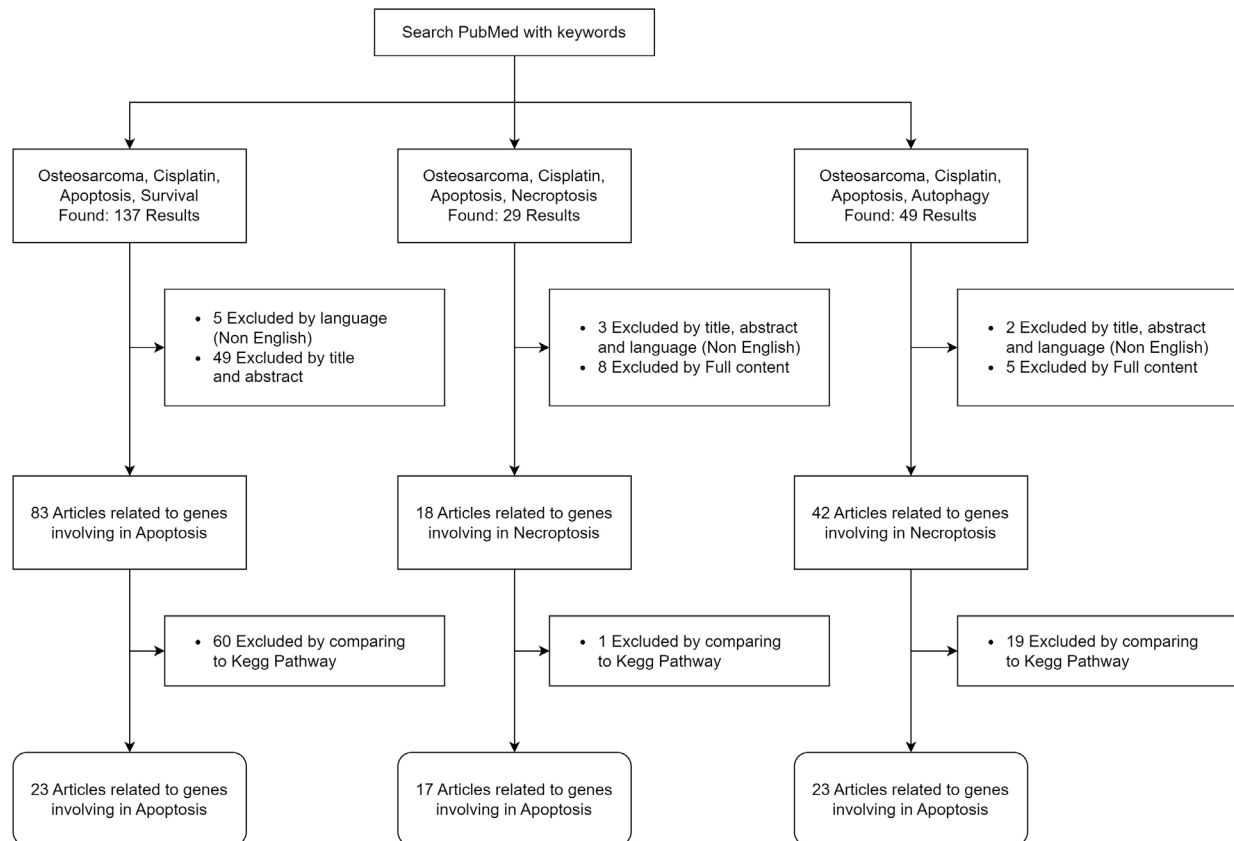


Figure 1. A selection scheme for identifying relevant PubMed articles

then annotated using the Ensembl Variant Effect Predictor (VEP) (71) as well as VarSome (72), and reported in a text-formatted file with clinically relevant information (73).

SVs in the patient dataset were identified using Manta on a binary alignment file (BAM) of a tumor genome with a matched normal genome and scored using a somatic variant model (74). CNV analysis was performed using Control-FREEC (75) on a BAM file generated from a tumor genome with a matched normal genome. The analysis provided genome-wide copy number estimates for each chromosome using a sliding window approach with a window size of 50,000 base pairs (bp). Copy number values were represented as log₂ ratios to identify genomic gains and losses. For autosomes, a log₂ ratio greater than 1.5 was classified as a copy number gain, while a log₂ ratio below 0.5 indicated a copy number loss. For the X and Y chromosomes, a log₂ ratio greater than 0.75 signified a gain, and a log₂ ratio below 0.25 represented a loss. These thresholds were used to distinguish between normal and amplified or deleted genomic regions.

Data visualization and STRING analysis

SVs detected in OS genomes were visualized and reported using Circos (76). STRING (77) analyses were conducted on various lists of genes/proteins to identify their interactions and find relevant Gene Ontology (GO) terms, biological pathways, and associated diseases.

Statistical analysis

STRING analysis incorporating statistical measures, including the false discovery rate (FDR) and interaction score, were used to identify significant protein-protein interactions, pathways, and related biological processes. Interactions with an FDR of less than 0.05 were deemed statistically significant (77).

Ethics approval and consent to participate

The collection and use of the tissue specimens for the present study were approved by the Research Ethics Committee of the Faculty of Medicine, Chiang Mai University (FAC-MED-2563-07135). All subjects supplied written informed consent for participation in compliance with the Declaration of Helsinki.

RESULTS

Pathogenic mutations in key genes associated with osteosarcoma

The analysis revealed several germline and somatic mutations linked to the genes of interest in patients P1 and P3, as outlined in Table 2. Patient P1 was found to have a missense mutation (rs746943903) in the Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Delta (*PIK3CD*) gene, essential for immune response. This mutation was heterozygous in the germline but homozygous in the tumor genome, and it was assessed to have a moderate impact on the gene's function. Additionally, the tumor genome of P1 uniquely presented a homozygous somatic mutation at a splice donor site in the *TP53* gene, identified by the identifiers COSV52762386, COSV52883623, and COSV53123147. Although this mutation does not alter an amino acid, it was deemed to have a high impact and was associated with Li-Fraumeni syndrome (78). Furthermore, Patient P3's germline and tumor genomes harbored a highly pathogenic nonsense mutation (rs1575481212) in the MutL Homolog 1 (*MLH1*) gene. The *MLH1* protein is crucial for DNA mismatch repair and safeguarding against DNA damage.

Copy number variation in tumor genomes of the patients

CNV is frequently detected in tumors. The genomic regions affected by the resulted DNA

imbalance may contain genes encoding essential proteins, whose altered dosage subsequently leads to oncogenesis (9, 16, 79, 80). The CNV analyses of all three tumor genomes revealed several 50,000-bp genomic regions of individual chromosomes demonstrated copy number alterations (Figures 2A – C).

Further investigations identified several affected genomic locations and genes that play important roles in chemotherapy (Table S6). As shown in Figure S1A, patient P1 exhibited copy number alterations in 6,362 regions, each spanning 50,000 base pairs (bp), including 6,275 regions with copy number gains and 87 regions with copy number losses. Among the 1,794 genes of interest, copy number gains were observed in 289 genes, whereas copy number losses were observed in 4 genes (e.g., *HLA-A*, *NF2*, *RB1*, and *WWOX*). Interestingly, both gain and loss of copy numbers were observed in the tumor suppressor and apoptotic gene WW Domain Containing Oxidoreductase (*WWOX*). Gain of copy numbers was observed in the chr16:78150001-78300000 and chr16:78350001-78450000 regions of *WWOX*, whereas loss of copy numbers was observed in the chr16:79150001-79500000 region of the gene. A complete list of genes affected by CNVs in patient P1 is presented in Table S7. The tumor genome of P1 demonstrated a copy number loss in the region of chromosome 13 (chr13:48,450,001-48,500,000), which overlaps the last 30,000 bp of

Table 2. A list of the germline and somatic mutations affecting the genes of interest.

	Patient		
	P1		P3
Gene symbol	<i>PIK3CD</i>	<i>TP53</i>	<i>MLH1</i>
Mutation/genotype	Germline/HET Somatic/HOM	Somatic/HOM	Germline/HET Somatic/HOM
SNP	rs746943903	COSV52762386 COSV52883623 COSV53123147	rs1575481212
Chr.	1	17	3
GRCh38 change	g.9721793G>A	g.7687376C>A	g.37014522C>A
Impact	Pathogenic Moderate	Pathogenic Strong	Pathogenic Very Strong
Consequence	Missense	Splice donor	Nonsense
Coding DNA change	<i>PIK3CD</i> :c.1988G>A	<i>TP53</i> :c.-29+1G>T	<i>MLH1</i> :c.768C>A
Protein change	p.Arg663His	p.?	p.Cys256Ter
RefSeq transcript ID	NM_005026.5	NM_000546.6	NM_000249.4
Ensembl Transcript ID	ENST00000377346.9	ENST00000269305.9	ENST00000231790.8

Genotypes: HET, Heterozygous; HOM, Homozygous.

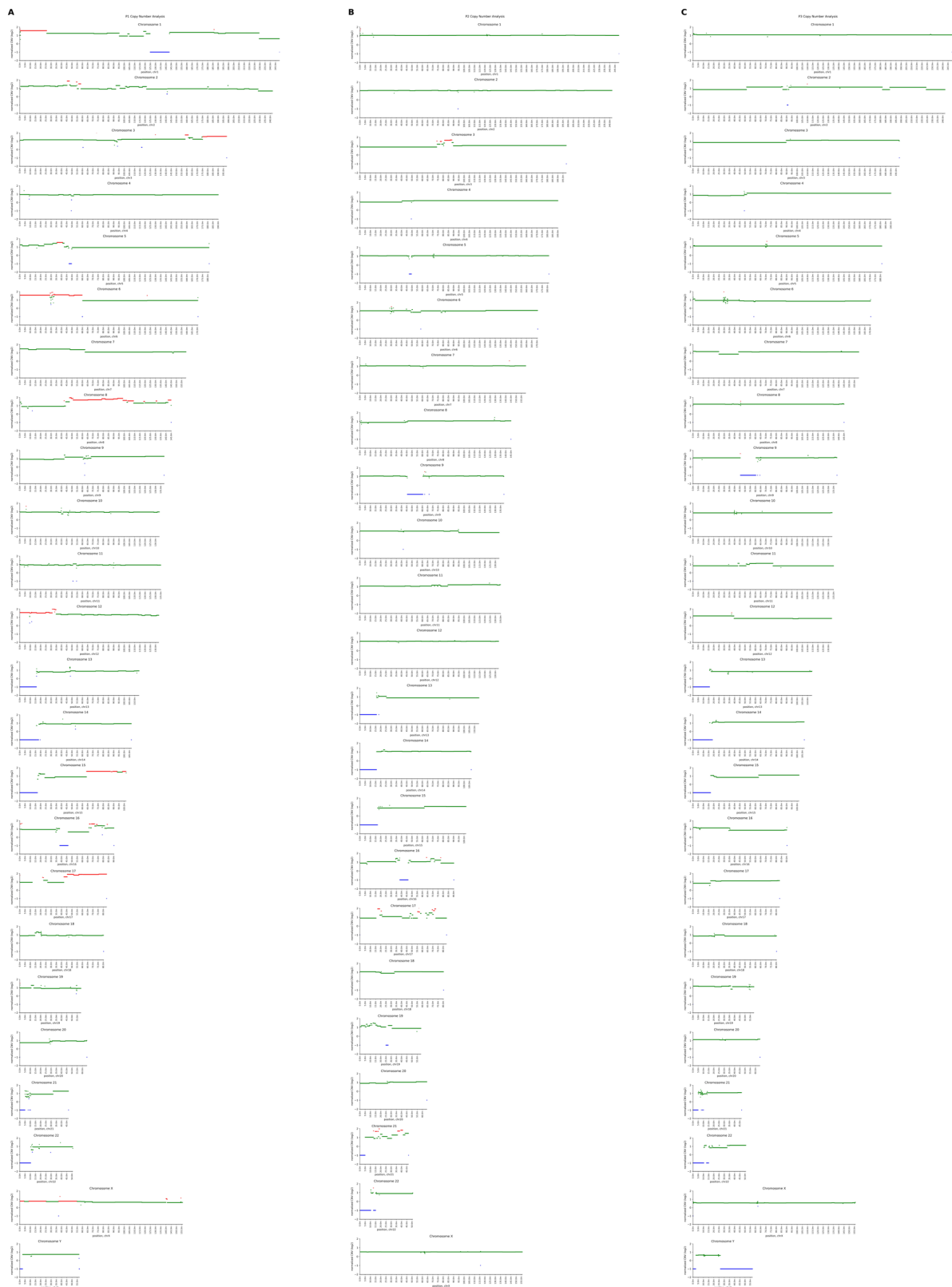


Figure 2. A comprehensive visualization of CNVs detected in the tumor genomes of patients (A) P1, (B) P2, and (C) P3 is presented. The log2 ratios reported by Control-FREEC were plotted, with the Y-axis representing the log2 ratio of copy number and the X-axis denoting the genomic positions within each chromosome. Each data point corresponds to the log2 ratio of copy number for a specific genomic window spanning 50,000 bps. Data points are color-coded for clarity: green indicates regions with a normal copy number, red represents regions with copy number gains, and blue signifies regions with copy number losses. This color scheme facilitates the identification of significant CNV events and highlights genomic regions with structural variations in the tumor genomes analyzed

the known tumor suppressor gene *RB1*. Furthermore, a copy number loss in *WWOX* is likely to affect the regulation of cell apoptosis in response to stress. Regarding platinum-based drug detoxification genes (61) in P1, *MPO* and *NQO1* exhibited gains of copy number. Additionally, copy number gains in *MSH2* and *PMS2* are likely to impact mismatch repair, whereas gains in *POLH* and *POLM* could potentially affect DNA replication. The P1 tumor genome also exhibited copy number gain of several protein-coding genes, e.g., *ABCC3*, *NQO1*, and *SLCO1B1*, which belong to the doxorubicin, etoposide, and methotrexate pathways (62, 64, 65, 81). Additionally, gains of copy number were observed in other genes belonging to the Doxorubicin pathway, including *ABCC5*, *ABCC9*, *ALDH3A1*, *GSTA1*, *GSTA2*, *MSH2*, and *MTHFR* (65). In the Methotrexate pathway, the genes that demonstrated gains in copy number included *BIRC5*, *GGH*, *MTHFR*, *MTHFS*, *SHMT1*, *SLCO1A2*, and *SLCO1B3* (64, 81).

As illustrated in Figure S1B, patient P2 exhibited copy number alterations in 821 regions, each 50,000 base pairs (bp) in size, including 742 regions with copy number gains and 79 regions with copy number losses. Specifically, copy number gains were observed in 31 genes, while a copy number loss was noted in *KIR2DL1*. A comprehensive list of genes affected by CNVs in patient P2 can be found in Table S7. The P2 tumor genome exhibited gains of copy number for DNA Topoisomerase II Alpha (*TOP2A*) and Serine Hydroxymethyltransferase 1 (*SHMT1*). *TOP2A* is a recognized target of the chemotherapeutic agents doxorubicin and etoposide (62, 65). On the other hand, *SHMT1* can indirectly reduce the effectiveness of methotrexate (64, 81, 82). Notably, P2 showed copy number gains in *MPO*, *NQO1*, and *SOD1*, genes implicated in reducing the intracellular concentration of platinum drugs, as documented in previous studies (61). However, the log₂ ratios of tumor-to-normal copy number for these genes were below 0.585, specifically, *MPO* (0.527), *NQO1* (0.551), and *SOD1* (0.405). This reduction is attributed to the presence of normal bone marrow cells in the tumor tissue of P2.

As depicted in Fig. S1C, patient P3 was found to have copy number alterations in 11 regions, each spanning 50,000 base pairs (bp), which included 9 regions with copy number gains and 2

with copy number losses (Table S6). Interestingly, these alterations did not impact any genes associated with platinum-based drug metabolism or DNA repair mechanisms. Additionally, several regions of the P3 tumor genome could have clearly demonstrated CNVs, likely due to the diluting effect of normal bone marrow cells. In summary, copy number variation underlines the complexity and individuality of tumor genomes, as demonstrated across different patients (Figure 2).

Structural variations in tumor genomes of the patients

SV is a key process responsible for oncogenesis. The resulting genomic rearrangements, deletions, and/or amplifications can affect genomic regions ranging from a few kilobases to entire chromosomes and can subsequently lead to deleterious gene dosage alterations (8, 83–90). Analyses of SV have revealed unique patterns of genomic rearrangements in three different tumor genomes (Figure 3). The details of all SVs detected in these tumor genomes are documented in Table S8. SV has also been observed in a small number of genes with significant roles in chemotherapy.

As shown in Table 3, the patient P1 tumor genome harbored 17 genes affected by chemotherapy. The P2 tumor genome included 19 genes, whereas the P3 genome had only 7 genes. Notably, the P1 tumor genome was burdened with genomic rearrangements of genes classified as tumor suppressors, e.g., *MSH2*, *NF2*, *RB1*, and *WWOX*. SV also impacted genes involved in DNA repair, for example, *ACTL6A*, *MSH2*, *SPIDR*, and *TRIP12*. An intrachromosomal translocation of *STAT3*, a gene known to be involved in cell growth and apoptosis, was also observed.

In the patient P2 tumor genome (Table 4), structural alterations of genes caused by the DNA damage and repair mechanism were observed, including the deletion of *ASCC3*, the translocation of *CLOCK*, the duplication and translocation of *BABAM1*, the translocation of *BRIP1*, and the deletion of the *RAD51C* gene. The latter three genes also play significant roles in homologous recombination. Interestingly, SVs were also observed in genes playing vital roles in cell adhesion, e.g., *DSCAM*, *FREM2*, *ICAM1*, *ITGA3*, and *PRKCA* (91, 92).

Despite having fewer genes affected by genomic alterations (Table 5), the patient P3 tumor

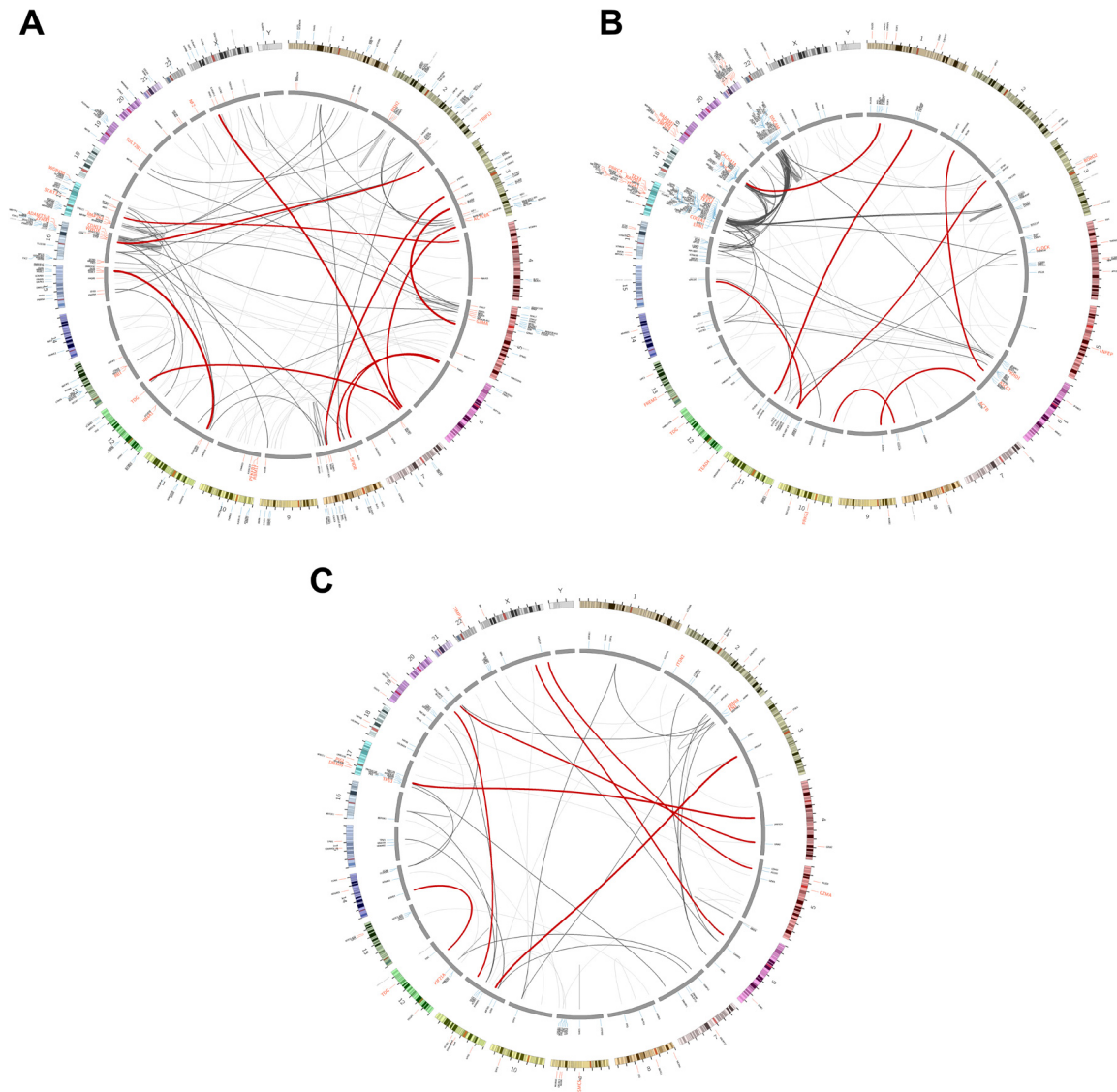


Figure 3. The CIRCOS plots visualize SVs detected in the tumor genomes of patients (A) P1, (B) P2, and (C) P3. The outer circle (first circle) depicts the cytoband of the human reference genome (GRCh38 or hg38), including the names and genomic locations of genes affected by SVs located on the plus strand. The second circle displays the names and genomic locations of genes affected by SVs on the minus strand, complementing the information provided in the outer circle. The regions harboring genes with significant roles in chemotherapy response are annotated in red. SVs, namely translocations, deletions, and duplications, are represented by lines. The red lines represent SV events that pass the filter, whereas the black and gray lines represent those that did not pass the filter. SVs, including translocations, deletions, and duplications, are depicted with lines connecting relevant genomic regions. The red lines indicate SV events that meet the predefined criteria and thus pass the filter. In contrast, the black and gray lines represent SV events that do not meet these criteria

genome exhibited an interchromosomal translocation of the tumor suppressor gene *TP53*, as well as translocations involving *ERBB4*, *FN1*, *TIMP3*, and *KIF21A*. A deletion in the *PIGL* gene was also detected in the tumor genome. These altered genes are associated with the biological pathway proteoglycan in cancer (93-96).

Functional protein association networks analysis of the affected genes

After identifying the commonly affected cancer genes in tumor genomes, their potential involvements in cancer risk, cancer development and progression, and cancer treatments were subsequently investigated for functional protein association networks. There were 312 affected genes in the P1 tumor genome. The P2 and P3

Table 3. Genes with significant roles in chemotherapy and types of SV were observed in tumor genomes of P1. Only SVs that have met the predefined criteria and thus passed the filter were included.

Gene	Description	Type	Interaction
ABHD2	Abhydrolase domain containing 2, acylglycerol lipase	Duplication	Intrachromosomal
ACTL6A	Actin like 6A	Translocation	Intrachromosomal
ADAMTS18	ADAM metalloproteinase with thrombospondin type 1 motif 18	Translocation	Intrachromosomal
CDH13	Cadherin 13	Translocation	Intrachromosomal
MAP2K4	Mitogen-activated protein kinase kinase 4	Translocation	Intrachromosomal
MSH2	MutS homolog 2	Duplication	Intrachromosomal
NF2	NF2, moesin-ezrin-radixin like (MERLIN) tumor suppressor	Deletion Translocation	Intrachromosomal Intrachromosomal
NR4A1	Nuclear receptor subfamily 4 group A member 1	Deletion Translocation	Intrachromosomal Interchromosomal
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	Duplication	Intrachromosomal
RB1	RB transcriptional corepressor 1	Translocation	Intrachromosomal
RBM17	RNA binding motif protein 17	Duplication	Intrachromosomal
SPIDR	Scaffold protein involved in DNA repair	Translocation	Interchromosomal
STAT3	Signal transducer and activator of transcription 3	Translocation	Intrachromosomal
SULT2B1	Sulfotransferase family 2B member 1	Duplication	Intrachromosomal
TRIP12	Thyroid hormone receptor interactor 12	Translocation	Interchromosomal
WWOX	WW domain containing oxidoreductase	Translocation Translocation Deletion	Interchromosomal Intrachromosomal Intrachromosomal
ZFXH3	Zinc finger homeobox 3	Translocation Duplication	Intrachromosomal Intrachromosomal

Table 4. Genes with significant roles in chemotherapy and types of SV were observed in tumor genomes of P2. Only SVs that have met the predefined criteria and thus passed the filter were included.

Gene	Description	Type	Interaction
ACTB	Actin beta	Duplication	Intrachromosomal
ASCC3	Activating signal cointegrator 1 complex subunit 3	Deletion	Intrachromosomal
BABAM1	BRISC and BRCA1 A complex member 1	Duplication Translocation	Intrachromosomal Intrachromosomal
BRIP1	BRCA1 interacting helicase 1	Translocation	Interchromosomal
CACNA1A	Calcium voltage-gated channel subunit alpha1 A	Duplication Deletion	Intrachromosomal Intrachromosomal
CLOCK	Clock circadian regulator	Translocation	Interchromosomal
COL1A1	Collagen type I alpha 1 chain	Translocation	Interchromosomal
DSCAM	DS cell adhesion molecule	Translocation	Interchromosomal
ERN1	Endoplasmic reticulum to nucleus signaling 1	Translocation Translocation Deletion	Intrachromosomal Interchromosomal Intrachromosomal
ICAM1	Intercellular adhesion molecule 1	Translocation	Interchromosomal
ITGA3	Integrin subunit alpha 3	Translocation	Interchromosomal
PKHD1	PKHD1 ciliary IPT domain containing fibrocystin/polyductin	Deletion	Intrachromosomal
PRKCA	Protein kinase C alpha	Deletion Translocation	Intrachromosomal Intrachromosomal
RAD51C	RAD51 paralog C	Deletion	Intrachromosomal
ROBO2	Roundabout guidance receptor 2	Translocation	Intrachromosomal
TBX4	T-box transcription factor 4	Deletion	Intrachromosomal
TEAD4	TEA domain transcription factor 4	Duplication	Intrachromosomal
TEX14	Testis expressed 14, intercellular bridge forming factor	Translocation	Interchromosomal
TNFSF9	TNF superfamily member 9	Duplication	Intrachromosomal

Table 5. Genes with significant roles in chemotherapy and types of SV were observed in tumor genomes of P3. Only SVs that have met the predefined criteria and thus passed the filter were included.

Gene	Description	Type	Interaction
FN1	Fibronectin 1	Translocation	Interchromosomal
KIF21A	Kinesin family member 21A	Translocation	Interchromosomal
		Translocation	Intrachromosomal
PIGL	Phosphatidylinositol glycan anchor biosynthesis class L	Deletion	Intrachromosomal
SMC5	Structural maintenance of chromosomes 5	Translocation	Intrachromosomal
TIMP3	TIMP metalloproteinase inhibitor 3	Translocation	Interchromosomal
TP53	Tumor protein p53	Translocation	Interchromosomal

tumor genomes carried 51 and 7 affected genes, respectively. These affected genes, identified in the tumor genome of each patient, were then subjected to STRING analysis (77). The functional enrichment analysis of protein-protein interaction networks for patient P1, P2, and P3, detailed in Tables 6, 7, and 8 respectively, identified common biological processes or pathways impacted across all patients. These include DNA damage and repair mechanisms, apoptosis (hsa04210), platinum drug resistance (hsa01524), and cancer pathways (hsa05200). Specifically, abnormal osteoclast differentiation (hsa04380) and cell death were linked to patient P1, while the tumor genome of P3 showed a correlation with proteoglycans in cancer (hsa05205), suggesting roles in cancer proliferation, cell-cell adhesion loss, angiogenesis, and metastasis. STRING analyses suggest copy number and/or SVs significantly influence OS pathogenesis, despite varying mutation burdens.

DISCUSSION

In this study, we identified pathogenic germline and somatic mutations in patients P1 and P3. Patient P1 exhibited a missense mutation in the *PIK3CD* gene, presented as a heterozygous germline mutation which evolved into a homozygous somatic mutation. Additionally, P1 exclusively possessed a somatic mutation at a splice donor site in the *TP53* gene. On the other hand, Patient P3 was found to have a nonsense mutation in the *MLH1* gene. This gene is a key component of the DNA mismatch repair mechanism, crucial for DNA damage tolerance. Notably, this nonsense mutation was observed in both the germline and tumor genomes of P3. Nonetheless, the OS genome of each patient showed a greater extent

of genome rearrangements. The tumor genome of patient P1 was particularly notable for its extensive CNVs, which could lead to drastic genetic imbalances. In contrast, the tumor genomes of patients P2 and P3 demonstrated relatively moderate levels of CNVs, suggesting varied patterns of genetic alterations across different individuals. Furthermore, SVs were detected in all three tumor genomes, affecting key genes involved in mechanisms of platinum drug resistance and DNA repair. Such alterations can significantly influence the effectiveness of chemotherapy treatments and the overall prognosis of patients. Importantly, the mutated genes identified in this study were also correlated with clinical features of the patients, suggesting a direct link between specific genetic alterations and the manifestation of the disease.

OS is a primary cancer characterized by high rates of metastasis and mortality (97–99). The survival rates for OS patients who develop metastases vary significantly, reflecting the variations in severity of the disease and the effectiveness of available treatments. According to the American Cancer Society, the five-year relative survival rate for OS with distant metastases, such as those to the lungs, is approximately 24% (100). OS patients with pulmonary metastases have a five-year survival rate of 20–40%. Patients with a countable number of pulmonary lesions may have survival rates up to 40%, whereas those with recurrent OS face a five-year survival rate of 28% or less (101, 102). These statistics highlight the dire prognosis faced by patients with metastatic OS and underscore the critical need for ongoing research and improvements in therapeutic strategies. Despite its low incidence, this cancer can affect individuals of all age groups (98, 103). In Thailand,

Table 6. The result of the functional enrichment analysis of protein-protein interaction networks for osteosarcoma patient P1. Pathways or biological processes with an FDR of less than 0.05 are reported.

Biological process (gene ontology)			
GO-term	Description	Strength	FDR
GO:0006281	DNA repair	1.03	6.15e-51
GO:0006302	Double-strand break repair	1.1	9.52e-26
GO:0051726	Regulation of cell cycle	0.59	4.11e-18
GO:0043067	Regulation of programmed cell death	0.52	1.21e-17
GO:0000725	Recombinational repair	1.15	2.76e-17
GO:0010941	Regulation of cell death	0.49	1.92e-16
GO:0000724	Double-strand break repair via homologous recombination	1.13	1.56e-15
GO:0006282	Regulation of DNA repair	0.94	7.94e-15
GO:0008219	Cell death	0.54	2.22e-14
GO:0012501	Programmed cell death	0.55	2.33e-14
GO:0042770	Signal transduction in response to DNA damage	1.03	3.39e-13
GO:0007049	Cell cycle	0.5	5.17e-13
GO:0097190	Apoptotic signaling pathway	0.8	1.58e-12
GO:0000077	DNA damage checkpoint signaling	1.06	4.17e-11
GO:0036297	Interstrand cross-link repair	1.25	2.03e-08
GO:0006301	Post-replication repair	1.28	7.00e-08
GO:2000779	Regulation of double-strand break repair	0.9	7.26e-08
GO:0000731	DNA synthesis involved in DNA repair	1.21	2.38e-07
GO:2001233	Regulation of apoptotic signaling pathway	0.62	1.31e-06
GO:0001649	Osteoblast differentiation	0.79	2.70e-05
GO:0048705	Skeletal system morphogenesis	0.64	0.00017
GO:0000729	DNA double-strand break processing	1.22	0.00099
GO:0051302	Regulation of cell division	0.59	0.0051
GO:0033688	Regulation of osteoblast proliferation	0.98	0.0229
GO:2000819	Regulation of nucleotide-excision repair	0.98	0.0229
GO:0006298	Mismatch repair	0.95	0.0270
GO:0006289	Nucleotide-excision repair	0.76	0.0361
KEGG pathways			
Pathway	Description	Strength	FDR
hsa05200	Pathway in cancer	0.65	4.87e-11
hsa01524	Platinum drug resistance	1.09	9.20e-09
hsa04210	Apoptosis	0.88	6.61e-08
hsa04110	Cell cycle	0.86	8.94e-07
hsa03430	Mismatch repair	1.16	0.00025
hsa04380	Osteoclast differentiation	0.65	0.0023
hsa03410	Base excision repair	0.78	0.0398

Table 7. The result of the functional enrichment analysis of protein-protein interaction networks for osteosarcoma patient P2. Pathways or biological processes with an FDR of less than 0.05 are reported.

Biological process (gene ontology)			
GO-term	Description	Strength	FDR
GO:0006302	Double-strand break repair	1.31	1.04e-06
GO:0006281	DNA repair	1.01	5.46e-06
GO:0008219	Cell death	0.62	0.0094
GO:0000077	DNA damage checkpoint signaling	1.2	0.0191
GO:0010941	Regulation of cell death	0.48	0.0387
KEGG pathways			
Pathway	Description	Strength	FDR
hsa01524	Platinum drug resistance	1.48	0.00016
hsa04210	Apoptosis	1.11	0.0072
hsa05200	Pathways in cancer	0.69	0.0175

Table 8. The result of the functional enrichment analysis of protein-protein interaction networks for osteosarcoma patient P3. Pathways or biological processes with an FDR of less than 0.05 are reported.

Biological process (gene ontology)			
GO-term	Description	Strength	FDR
GO:0006302	Double-strand break repair	1.63	0.0229
KEGG pathways			
Pathway	Description	Strength	FDR
hsa01524	Platinum drug resistance	2.1	7.64e-06
hsa05205	Proteoglycans in cancer	1.65	0.00020
hsa05200	Pathways in cancer	1.23	0.0023
hsa04210	Apoptosis	1.52	0.0184

however, the highest incidence of OS is observed during the first 25 years of life (98). The three OS patients whose genomes were investigated in this study aptly represent this affected demographic. All three developed primary OS at a young age (average age 15.33 years, range 11-20 years) and had a poor prognosis, as indicated by the average tumor necrotic percentage being low (64%, range 42-85%), with a low degree of necrosis associated with a poor prognosis (104). Additionally, they responded poorly to chemotherapy, as evidenced by metastasis even after treatment. Furthermore, they exhibited poor survival outcomes (average 678 days, range 357-842 days) following a combination of surgery and chemotherapy. Their clinical histories seem to correlate well with resistance to platinum-based drugs, primarily arising from CNVs and SVs.

This investigation identified a nonsense mutation in *MLH1* gene in patient P3. The mutation was heterozygous in the germline genome of P3, but became homozygous in the tumor genome. This mutation may have increased the risk of OS or contributed to oncogenesis due to compromised DNA mismatch repair (105-107) and DNA damage protection mechanisms (108, 109). A homozygous splice-site donor mutation in *TP53* detected in patient P1 could have acted synergistically with earlier mutations in cancer progression through abnormal splicing of *TP53* transcripts (110-113). Despite having a few deleterious SNPs, the patients demonstrated a significant degree of genomic alterations as evidenced by CNVs and SVs. However, the patterns of these alterations varied among the patients.

An important genomic alteration observed in the P1 tumor genome was the copy number loss of

RB1, which is a known contributor to poor prognosis in OS patients (10, 114). The tumor genome also carried copy number gains in several genes associated with resistance to Doxorubicin, Etoposide, and Methotrexate, specifically *ABCC3*, *MPO*, *NQO1*, and *SLCO1B1* (62, 64, 65, 81). In addition, the observed copy number gain of *SOX9* has been associated with a lower survival rate and poor chemotherapy response (115). The tumor genome exhibited SVs in genes associated with tumor suppression or DNA repair, namely, *RB1* (tumor suppressor), *NF2* (tumor suppressor), *WWOX* (tumor suppressor), *MSH2* (tumor suppressor and DNA repair), *ACTL6A* (DNA repair), *SPIDR* (DNA repair), and *TRIP12* (DNA repair). These genes are also collectively classified as negative regulators of cellular processes (GO:0048523).

Patient P2's history of undergoing multiple rounds of chemotherapy directed our investigation towards potential gene mutations that could result in resistance to chemotherapy. Copy number gains in *TOP2A* and *SHMT1* have been associated with reduced efficacy of Doxorubicin, Etoposide, and Methotrexate (62, 64, 65, 81, 82). Furthermore, the P2 tumor genome has demonstrated SVs in several genes associated with DNA damage, DNA repair, and homologous recombination, specifically *ASCC3* (DNA damage/repair), *CLOCK* (DNA damage/repair), *BABAM1* (DNA damage/repair and homologous recombination), *BRIPI* (DNA damage/repair and homologous recombination), and *RAD51C* (DNA damage/repair and homologous recombination). Notably, SVs in genes involved in cell adhesion (GO:0007155), e.g., *DSCAM*, *FREM2*, *ICAM1*, *ITGA3*, and *PRKCA*, could contribute to the cancer's aggressive behavior, necessitating intensive chemotherapy

treatments. Abnormal cell adhesion mechanisms contribute to oncogenesis and metastasis by disrupting normal cell-cell and cell-ECM interactions, enhancing tumor cell motility and invasion, altering interactions with the tumor microenvironment, and enabling evasion of immune surveillance (92, 116–119). These changes affecting patient P2 may have collectively facilitated the detachment of cancer cells from the primary tumor and their eventual spread to distant organs, leading to metastasis.

Patient P3, who had the poorest survival rate, exhibited interchromosomal translocations of the *TP53*, *ERBB4*, *FN1*, *TIMP3*, and *KIF21A* genes, along with a deletion in the *PIGL* gene. These genetic alterations are associated with the proteoglycan pathway in cancer, which facilitates tumor cell growth and migration by enabling interactions between proteoglycans in the tumor microenvironment and various growth factors and enzymes. The disruption of the proteoglycan pathway likely contributes to tumor progression and invasion (93–96), leading to the patient's untimely death.

Chromothripsis, marked by dozens to hundreds of DNA double-strand breaks in a localized chromosomal region in a single event, leads to faulty reassembly and complex genomic rearrangements (7, 8, 120). This accelerates tumor evolution through rapid genomic changes and mutation accumulation (79, 121, 122). Such alterations, especially in chemotherapy-related genes observed in three fetal OS patients, correlate with poor chemotherapy response and abnormal cell-cell adhesion. Comprehensive analysis of pathogenic SVs and CNVs could inform targeted cancer therapies to improve patient outcomes.

An estimated 30% of cancers possess known pathogenic SVs which are valuable for diagnosis and treatment (123–129). Moreover, 853,218 CNVs have been identified across 10,729 tumor samples belonging to 32 cancer types curated in The Cancer Genome Atlas (TCGA) database (130). Both SVs and CNVs disrupt genomic integrity, disturb gene expression, and ultimately lead to cancer development (80, 131). These large-scale genomic alterations can conveniently be observed via NGS. While Illumina's short-read sequencing technology has been the backbone of genomics because of its high accuracy and cost-effective-

ness, PacBio and Oxford Nanopore's long-read sequencing technologies offer superior capabilities in detecting SVs in the human genome (132–135). These advantages make long-read sequencing more suitable for comprehensive genomic studies focused on SVs, complex genomic regions, and complete genome assembly. However, the choice of sequencing technology may depend on specific project goals, budget, and the balance between the need for depth, accuracy, and read length.

This study has several limitations. First, the small sample size of three patients limits the generalizability of the findings to the broader OS patient population. Second, the WGS approach focused solely on genetic variations, without exploring gene expression or regulatory mechanisms such as epigenetics. Furthermore, the analysis relied on gene annotations from published data, which may not fully capture population-specific variants unique to the Thai population. Addressing these limitations in future research will require larger and more diverse cohorts, the integration of multi-omics approaches, and population-specific analyses to achieve a more comprehensive understanding of genomic alterations in OS and their clinical implications.

CONCLUSIONS

This study underscores the critical role of genomic alterations in driving poor chemotherapy responses and fatal outcomes in OS. The findings reveal a convergence of genetic disruptions that collectively contribute to therapy resistance, tumor progression, and metastasis, highlighting the need for precision oncology approaches in OS treatment. Key genomic alterations include CNVs and SVs in tumor suppressor genes (e.g., *RB1*, *NF2*, and *MSH2*), DNA repair genes (e.g., *BRIP1*, *RAD51C*, and *TRIP12*), and genes involved in chemotherapy resistance (e.g., *TOP2A*, *ABCC3*, and *SOX9*). Disruptions in cell adhesion pathways further exacerbated disease progression by enabling tumor cell detachment, migration, and metastasis. Additionally, genomic changes impacting the proteoglycan pathway facilitated tumor cell growth and invasion, contributing to treatment failure. These findings demonstrate how a combination of genomic instability, resistance mechanisms, and tumor microenvironment alterations can undermine chemotherapy efficacy and accel-

erate disease progression in OS. While the study provides valuable insights for developing targeted therapies and predictive biomarkers, limitations such as the small sample size and focus on genetic alterations necessitate further investigation. Future research should integrate multi-omics approaches, larger cohorts, and population-specific analyses to enhance understanding of OS and improve patient outcomes.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to report.

ADDITIONAL INFORMATION

Author contributions

N.N.: investigation, data validation, data curation, methodology, writing-original draft; S.W.: investigation, software, data validation, data curation, methodology, visualization, writing-original draft; P.T.: sample preparation, DNA extraction, NGS, writing – review and editing; P.C.: conceptualization, supervision, writing – review and editing; D.P.: resources, conceptualization, supervision, writing – review and editing; S.T.: conceptualization, supervision, methodology, funding acquisition, writing original draft, writing – review and editing. N.N. and S.W. contributed equally to this work and share first authorship. All authors have read and approved the final manuscript.

Patient consent for publication

Not applicable.

Data availability statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Supplementary materials

Doc. S1 A genomic DNA extraction protocol

Figure S1 The CIRCOS plots provide a comprehensive visualization of CNVs detected in the tumor genomes of patients (A) P1, (B) P2, and (C) P3. The plots organize genomic information across multiple layers from the outermost to the innermost circle. The outer circle (first circle) depicts the cytoband of the human reference genome (GRCh38 or hg38), including the names and genomic locations of genes of interest located on the plus strand. The second circle displays the names and genomic locations of genes of interest on the minus strand, complementing the information provided in the outer circle. The third circle illustrates the CNV profiles spanning across different chromosomes. Each dot within this circle represents a 50,000-bp genomic interval, with color coding to indicate the type of copy number variation: green dots indicate copy number gains, red dots indicate copy number losses, and gray dots denote regions with no copy number alteration.

Table S1. A timeline showing the treatments, chemotherapies, and important events of patient P1 at Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand

Table S2. A timeline showing the treatments, chemotherapies, and important events of patient P2 at Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand

Table S3. A timeline shows the treatments, chemotherapies, and important events of patient P3 at Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand

Table S4. A list of 57 genes of interest obtained from the systematic review

Table S5. A list of 1,794 genes of interest in this study

Table S6. Copy number variations observed in each chromosome in tumor genomes of (A) P1, (B) P2, and (C) P3

Table S7. A list of the selected genes of interest affected by CNVs in tumor genomes of all three patients

Table S8. Structural variations detected in the tumor genomes of patient P1, P2, and P3

Supplementary data are available at <https://doi.org/10.6084/m9.figshare.25514878.v2> (136)

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