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RESEARCH ARTICLE

A pilot exome sequencing study suggests that germline variants influence methotrexate-induced toxicities in pediatric patients with localized osteosarcoma

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Abstract

Introduction: Osteosarcoma (OS) is a rare pediatric cancer for which therapeutic approaches, including chemotherapy and surgery, show a wide interindividual variability in patient response, both in terms of adverse events and therapy efficacy. There is growing evidence that this individual variable response to therapies is also influenced by inherited genetic variations. However, the results obtained to date in these pediatric cancers have been contradictory and often lack validation in independent series. Additionally, these studies frequently focused only on a limited number of polymorphisms in candidate genes.

Methods: In order to identify germline coding variations associated with individual differences in adverse events occurrence in pediatric patients affected by localized OS, we carried out an exome-wide association study in 24 OS patients treated with methotrexate, cisplatin, and doxorubicin, using the SNP-Set (Sequence) Kernel Association Test (SKAT), optimized for small sample size.

Results: Gene sets significantly associated (FDR < .05) with neutropenia and hepatotoxicity induced by methotrexate were identified. Some of the identified genes map in loci previously associated with similar phenotypes (e.g., leukocyte count, alkaline phosphatase levels).

Conclusion: Further studies in larger series and with functional characterization of the identified associations are needed; nonetheless, this pilot study prompts the relevance of broadly investigating variants along the whole genome, to identify new potential pharmacogenes, beyond drug metabolism, transport, and receptor candidate genes.

KEYWORDS
chemotherapy, pediatric cancer, pharmacogenomics, polymorphisms, SKAT-O

Abbreviations: FDR, false discovery rate; OS, osteosarcoma; pQTL, protein quantitative trait locus; SKAT, SNP-Set (Sequence) Kernel Association Test; SNP, single nucleotide polymorphism.

1 | INTRODUCTION

Osteosarcoma (OS) is the most common primary malignant bone tumor, and it occurs mainly in children and adolescents.[1] It is classified as an orphan disease as the overall incidence is 0.2–3/100,000 population a year (0.8–11/100,000 people aged 15—19 years).[2] Despite its rarity in the population as a whole, it is reportedly one of the most common solid tumors in adolescence. Its incidence peaks between 10 and 30 years of age, and males are affected more than females, with a ratio of 1.6:1.[3–6] The sites most often involved are the metaphysis of the distal femur (42%), the proximal tibia (19%), and the proximal humerus (10%). About 70—80% of patients with OS present with localized disease, while 15—20% have macroscopic evidence of metastases at diagnosis.[7–9] The lung is the most common site of metastases, followed by bone. Patients with metastases or recurrent disease continue to carry a very poor prognosis, with 5-year survival rates ranging between 13 and 40%.[10–12]

With the introduction of neoadjuvant chemotherapy, 5-year survival rates for patients with localized tumor have improved from 15 to 70%.[13–16] The current treatment strategy for newly diagnosed OS is based on a combined approach including surgical removal of the primary tumor and systemic pre- and postoperative multidrug chemotherapy based on doxorubicin, cisplatin, and high-dose methotrexate, plus leucovorin (the so-called MAP regimen, considered the gold standard), with or without ifosfamide. OS biology is complex: chromosomal aneuploidy, increased number of mutations, copy number variation and structural variation, intratumoral heterogeneity, genomic instability, kategesis, and chromotripsis are typical features of OS.[17–20] A growing number of studies have evaluated common genetic variants—single nucleotide polymorphism (SNPs)—with the aim of better understanding the OS etiology and the pharmacogenomic (in the latter analyzing SNPs associated with drug response and toxicity, with the goal to modify and improve chemotherapeutic approaches). These studies frequently focused only on a limited number of polymorphisms in candidate genes and the results obtained to date are contradictory and often lack validation in independent series.

Here, we performed a pilot exome sequencing study aimed at investigating the genetic predisposition to develop toxicities after chemotherapy. The analyses were performed collapsing variant information at gene level, using Optimal SNP-set Kernel Association Test (SKAT-O).[21]

2 | METHODS

2.1 | Patient series, clinical data, and biological material

This study analyzed exome data from genomic DNA taken from peripheral blood samples from 24 pediatric patients with OS at the Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy. The ethics committee approved the study protocol, and parents or patients provided written informed consent to the use of their biological samples and data for the research purposes of this study. Clinical data for each patient were collected regarding sex, age at diagnosis, tumor histology and site, administered drugs, and reported toxicities (evaluated in accordance with the Common Terminology Criteria for Adverse Events, CTCAE, version 5.0). A peripheral blood sample was taken from each patient, stored at 4°C, and immediately processed for genomic DNA extraction, using the DNeasy Blood & Tissue Kit (Qiagen). DNA was quantified with Qubit 2.0 Fluorometer (Thermo Fisher Scientific), using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) and then stored at −20°C.

2.2 | Exome library preparation, sequencing, and variant calling

Exome libraries from genomic DNA were prepared using Ion AmpliSeq™ Exome RDY kit (Thermo Fisher Scientific) on a Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific), following manufacturer instructions. During library preparation, each library was labeled with a unique DNA barcode (Ion Xpress Barcode Adapters; Thermo Fisher Scientific) to allow for library pooling during the sequencing procedure. Libraries were quantified by quantitative PCR with the Ion Library TaqMan Quantitation Kit (Thermo Fisher Scientific). Template preparation was done using an Ion Chef Instrument (Thermo Fisher Scientific) and the sequencing, on Ion 550 Chips, was performed using an Ion S5 XL sequencer (Thermo Fisher Scientific). Ion Torrent Suite software (Thermo Fisher Scientific) was used to analyze raw sequencing data and to automatically align sequencing reads to the reference genome (version hg19) to produce the BAM files. Samples that did not achieve a target base coverage at 20× ≥90% were re-run and BAM files were merged using Samtools.[22] Variant calling and annotation were carried out with Ion Reporter Software using the AmpliSeq Exome Hi-Q single sample (Germline) v. 5.16 workflow (Thermo Fisher Scientific).

2.3 | Statistical analyses

Logistic regressions between toxicities and clinical variables (age, sex, for all toxicities, and adjuvant methotrexate treatment, just for methotrexate-induced toxicities) were performed in R environment.

All patients' genotype data matrix was obtained by merging vcf files using bcftools[22] and imported into PLINK v. 1.9 software.[23] With this software, we carried out principal component analysis and prepared datasets suitable for subsequent analyses.

SKAT-O analysis (with small-sample size kurtosis adjustment) was performed using the SKAT package[21] in R environment. For each phenotype of toxicity, first, a null model was created with age, sex, and the first four principal components as covariates. Just for the analysis with the methotrexate-induced neutropenia phenotype,

we carried out another analysis with an additional covariate, to account for possible differences due to the methotrexate adjuvant treatment, received only by a subset of patients. To adjust for multiple testing, the false discovery rate (FDR) was calculated using the Benjamini–Hochberg method,[24] and an FDR < .05 was set as the significance threshold. The manhattan function of the qqman package in R was used to draw the Manhattan plot. For each statistically significant gene set identified by SKAT, we extracted the analyzed variants, and we used them in a logistic regression model with the toxicity phenotype and age and sex as covariates, using PLINK software.

Candidate SNP association study was carried out on 25 coding variants (Table S1), selected among those already reported in the Pharmacogenomics Knowledge Base (https://www.pharmgkb.org/; accessed on May 2022) as significantly associated, in pediatric patients, with response to treatment with methotrexate, cisplatin, or doxorubicin. Logistic regression analyses between the phenotypes and the genotypes of the 25 SNPs were done using PLINK.

2.4 | Functional annotation and gene prioritization

The list of gene sets significantly associated with tested toxicity phenotypes were loaded on WEB-based Gene SeT AnaLysis Toolkit[25] to perform an over-representation analysis against the OMIM disease phenotype database, KEGG and Reactome pathways databases (accessed in September 2022). Results were considered statistically significant at FDR < .05. Top significant gene sets were searched in the Open Target Genetics Portal (accessed in September 2022[26,27]) to prioritize them. Additionally, STRING protein query and enrichment analysis in Cytoscape v3.9,[28] using default parameters, were performed, starting from the top-significant gene sets, with Bonferroni adjusted $p < .1$.

3 | RESULTS

3.1 | Patient characteristics and experienced toxicities

Twenty-four OS patients with a median age of 13 years were included. Clinical information of the 24 recruited patients is summarized in Table 1. They were predominantly males (79%) and affected by an osteoblastic OS (71%), mostly localized in the femur (54%). All patients received standard preoperative chemotherapy treatment with methotrexate, doxorubicin, and cisplatin (the so-called MAP regimen). Fifteen patients received these same drugs, after surgery, as adjuvant treatment, whereas the remaining nine received one cycle of doxorubicin and four cycles of high dose ifosfamide (15 g/m^2 over 5 days) and mifamurtide.

During chemotherapy cycles, patients suffered different types of toxicities. In Table 2, we reported the numbers of patients who experienced toxicity of grade 3 or higher during treatment with the indicated drugs. Methotrexate induced hepatotoxicity (AST/ALT > 5 × ULN) in most of the patients (88%), neutropenia in about half of the patients, and thrombocytopenia in 29% of the patients. Treatment with doxorubicin and cisplatin caused neutropenia in all but one patient, thrombocytopenia and anemia in 88 and 54% patients, respectively. Additionally, doxorubicin also induced mucositis in a quarter of our patients. The number of patients who were administered ifosfamide was quite small: all of them experienced neutropenia following ifosfamide treatment and only one patient suffered thrombocytopenia. Other types of toxicity were not taken into account, due to their low frequency in our small patient series, for instance only one patient experienced G1 cardiotoxicity and another patient G1 ototoxicity. We decided to analyze only the associations between genotypes and the phenotypes of toxicity highlighted in bold in Table 2, but first of all, we performed preliminary logistic regressions between each phenotype and age at diagnosis and sex, in order to test whether any of these two clinical characteristics were associated with the observed toxicities. Only a significant association was observed between the age at diagnosis and anemia after treatment with doxorubicin and cisplatin (OR = 0.5; $p = .015$), indicating that older patients had a lower risk of developing anemia after this treatment than younger patients. In addition, no significant differences were observed, in toxicity after methotrexate treatment, between patients who received it only in neoadjuvant setting, compared with those who were treated with this drug also after surgery.

TABLE 1 Patient characteristics and clinical information.

Characteristic	Total n = 24
Age at diagnosis, years, median (range)	13 (10–19)
Sex, n (%)	
Female	5 (21)
Male	19 (79)
Tumor localization	
Femur	13 (54)
Humerus	5 (21)
Tibia	6 (25)
Tumor histology	
Chondroblastic	1 (4)
Osteoblastic	17 (71)
Osteo-condroblastic	2 (8)
Teleangectatic	3 (13)
Not available	1 (4)
Neoadjuvant treatment, n (%)	
Methotrexate, doxorubicin, and cisplatin	24 (100)
Adjuvant treatment, n (%)	
Methotrexate, doxorubicin, and cisplatin	15 (62)
Doxorubicin and high-dose ifosfamide	9 (38)

TABLE 2 Toxicities experienced by patients with OS, included in the study.

Toxicity (≥G3), *n* (%)	Chemotherapy		
	Methotrexate (*n* = 24)	Doxorubicin and cisplatin (*n* = 24)	Ifosfamide (*n* = 9)
Neutropenia	**11 (46)**	23 (96)	9 (100)
Thrombocytopenia	**7 (29)**	**21 (88)**	1 (11)
Anemia	1 (4)	**13 (54)**	0 (0)
Hepatotoxicity	**21 (88)**	1 (4)	0 (0)
Mucositis	1 (4)	**6 (25)**	0 (0)

Phenotypes analyzed with SKAT-O are in bold.

TABLE 3 Summary of coverage analyses from exome sequencing.

	Median (range)
Number of mapped reads	37,511,572 (32,225,471–53,146,443)
Mean coverage depth, fold	98 (82–140)
Target base coverage at 20×	94% (92–96%)
Uniformity of base coverage	93% (91–95%)

3.2 | SKAT-O analysis identified gene sets associated with neutropenia and hepatotoxicity induced by methotrexate

The whole germline exome of the enrolled patients was sequenced at a mean 100× coverage. A summary of coverage analyses is reported in Table 3. For all patients, we obtained at least 90% target base coverage at 20×. A median of 95% reads mapped on target and a median uniformity of base coverage of 93% was achieved. Germline variants were called for each patient and merged in a unique dataset, suitable for the subsequent analyses.

SKAT-O analyses were performed on 14,972 gene sets containing 114,564 variants using dichotomous phenotypes of each toxicity (in bold in Table 2). In the models, age, sex, and the first four principal components were added as covariates. Neutropenia induced by methotrexate treatment was significantly associated (FDR < .05) with 280 gene sets including a total of 1354 variants (Figure 1 and Table S2). A logistic regression between methotrexate-induced neutropenia phenotype and genotypes of these 1354 variants, did not point to any FDR-significant single variant (not shown). The results of SKAT-O analysis with even methotrexate adjuvant setting as covariate, did not differ much from those obtained from the model without this covariate: indeed, 294 gene sets were identified (FDR < .05), 200 of which were among the 280 gene sets resulted in the first analysis (in bold in Table S2).

Only two gene sets resulted significantly associated with hepatotoxicity caused by the same drug (Table S2). Almost significant results (FDR < .10) for 15 gene sets were also observed in the analysis with mucositis phenotype, experienced by patients after doxorubicin and cisplatin treatment. No significant gene sets were associated with the other toxicity phenotypes.

Functional annotation of the 280 gene sets associated with methotrexate-induced neutropenia did not identify any significant enriched pathway. On the other hand, we found a significant enrichment (FDR = .016, enrichment score = 81.2) of the OMIM disease phenotype "susceptibility to asthma" (# 600807), which included *TNF* and *PLA2G7* genes. The top significant gene set identified included variants in *IGFBP7* genes, that, according to Open Target Genetic Platforms, is associated with the "leukocyte count" phenotype (association score = 0.127), since several intergenic variants in *IGFBP7* locus were found associated with this phenotype in some genetic studies.[29–33] Additionally, the top fourth significantly associated gene set, *CRYL1*, was another interesting finding, since a polymorphism in this gene (i.e., rs7989332) was previously reported as associated with neutropenia/leukopenia in chemotherapy-treated patients, although it did not reach genome-wide significance.[34] Regarding the two gene sets significantly associated with hepatotoxicity after methotrexate, in Open Target Genetics, we found *CCDC69* gene maps in a locus previously associated with serum alkaline phosphatase levels,[35] a marker of liver functionality, among several other phenotypes. *FGD5* gene, instead, maps in a locus, whose lead variant is rs56164115, associated with protein levels in liver, that is, it is a liver protein quantitative trait locus (pQTL[36]).

Finally, we loaded the 156 most significant gene sets (Bonferroni-adjusted $p < .1$) in the stringApp of Cytoscape to draw a protein-protein interaction network (PPI enrichment p value: .00941). We observed that the protein coded by 49 of these genes was connected by 59 edges in a protein–protein interaction network (Figure S1). A functional enrichment analysis of these nodes indicated an enrichment in mitochondrial proteins (Table S3).

3.3 | Association study of candidate SNPs in known pharmacogenes

In addition to the exome-wide association study with SKAT-O, we also carried out logistic regression between the genotypes of 25 selected coding variants, in known pharmacogenes already reported in PharmGKB as associated with toxicities in pediatric patients treated with

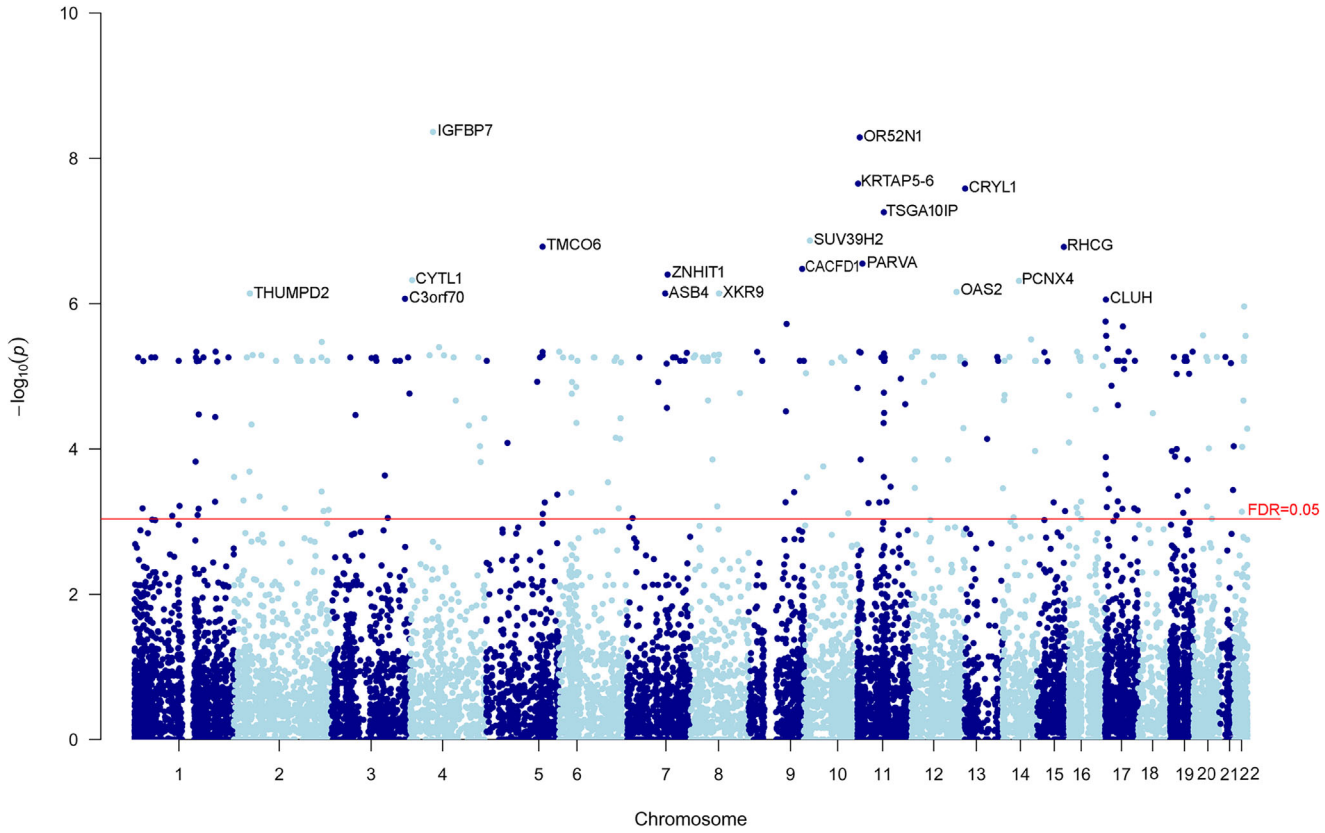

FIGURE 1 Gene-based association testing results obtained from SKAT-O analysis with the methotrexate-induced neutropenia phenotype. Red horizontal line, FDR = .05.

methotrexate, cisplatin, and doxorubicin (Table S1). No significant results, at FDR < .05, were observed. However, for three variants (i.e., rs1045642, rs8667, and rs1979277) in *ABCB1*, *ATF5*, and *SHMT1* genes respectively, we found associations (at nominal p value < .05) with mucositis induced by treatment with cisplatin and doxorubicin (rs1045642, OR = 0.055, p = .037) or with neutropenia caused by methotrexate treatment (rs8667, OR = 0.11, p = .034; rs1979277, OR = 5.3, p = .040).

4 | DISCUSSION

In this preliminary study on 24 OS pediatric patients, we sequenced patients' whole exome and used the SKAT-O method to explore the possibility that common and rare germline gene variants might modulate toxicities induced by chemotherapy. We found significant associations for two types of toxicity, that is, the methotrexate-induced neutropenia and hepatotoxicity. Regarding the first phenotype, we observed that some variants in the identified genes (e.g., *IGFBP7* and *CRYL1*) have been previously reported to be associated with several phenotypes, among which, for instance, there are leukocyte count[29–33] and neutropenia/leukopenia in chemotherapy-treated patients[34] that might be related, to some extent, to our toxicity phenotype. We also found a significant enrichment of our gene sets in the OMIM disease phenotype named "susceptibility to asthma," with *TNF* and *PLA2G7* genes. Of note, it has been reported that TNF inhibitors can cause neutropenia (reviewed in Andrès et al. 2019); therefore, it would be interesting to understand whether the variants in the TNF gene set might have a deleterious effect on the encoded protein, such as an inhibitor effect, and thus they might predispose patients to neutropenia after methotrexate treatment. Of course, this speculation should be verified in a larger patient series and functionally validated.

Only two gene sets significantly associated with hepatotoxicity induced by methotrexate treatment, that is, *CCDC69* and *FGD5* genes. The first one maps in a locus that was previously associated with a marker of liver functionality, serum alkaline phosphatase levels,[35] among several other phenotypes. The *FGD5* gene, instead, maps in a liver pQTL, whose lead variant is rs56164115[36] indicating that variants in this gene modulate its expression in the liver. However, to the best of our knowledge, the function of *FGD5* gene in this tissue has not yet been elucidated.

A protein–protein interaction network analysis of the protein coded by the gene sets, significantly associated with methotrexate-induced neutropenia, indicated an enrichment of mitochondrial proteins. Thus, variants in the genes coding for these proteins might affect mitochondrial function. Several studies showed that some common adverse effects of methotrexate therapy (e.g., Refs. 37–40) including a cytotoxic effect on human blood lymphocytes,[41] can be due to a mitochondrial dysfunction. Further studies are also needed to understand the

functional effect of the variants in the gene sets coding for mitochondrial proteins and their potential role in predisposition to methotrexate-induced neutropenia.

Overall, it is interesting that for at least one phenotype of toxicity we did find several statistically significant results with SKAT-O. This algorithm allows us to consider both common and rare variants and to identify associations at gene level, instead of looking at any single variant, one by one. Nonetheless, due to the very limited sample size, we are perfectly aware that the results herein obtained are preliminary and require further investigation in a wider patient series and, if validated, they might be helpful for a better management of the toxicity and administration of methotrexate.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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