Role of pharmacogenetics of drug-metabolizing enzymes in treating osteosarcoma

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Introduction: Drug-metabolizing enzymes (DMEs) biotransform several toxins and xenobiotics in both tumor and normal cells, resulting in either their detoxification or their activation. Since DMEs also metabolize several chemotherapeutic drugs, they can significantly influence tumor response to chemotherapy and susceptibility of normal tissues to collateral toxicity of anticancer treatments.

Areas covered: This review discusses the pharmacogenetics of DMEs involved in the metabolism of drugs which constitute the backbone of osteosarcoma (OS) chemotherapy, highlighting what is presently known for this tumor and their possible impact on the modulation of future treatment approaches.

Expert opinion: Achieving further insight into pharmacogenetic markers and biological determinants related to treatment response in OS may ultimately lead to individualized treatment regimens, based on a combination of genotype and tumor characteristics of each patient.

Keywords: collateral toxicity, drug-metabolizing enzymes, drug resistance, genetic polymorphisms, osteosarcoma, tailored therapy

1. Introduction

Drug-metabolizing enzymes (DMEs) are responsible for biotransformation of several toxins and xenobiotics in both tumor and normal cells, resulting in either their detoxification or their activation. Since DMEs also metabolize several chemotherapeutic drugs, they can significantly influence tumor response to chemotherapy, as well as susceptibility of normal tissues to collateral toxicity of anticancer treatments.

The group of DMEs includes several members, which are classified as Phase I or Phase II enzymes. Genes encoding for these enzymes show relevant interindividual and intraindividual variability, deriving from both genetic polymorphisms and other alterations, which lead to differential expression or variable enzymatic activity and inducibility [1,2]. In tumors, these variations have important consequences for either detoxification of chemotherapeutic drugs or activation of anticancer prodrugs. For these reasons, DMEs have been studied in several human neoplasms, but data specifically relating to high-grade osteosarcoma (OS) are still incomplete.

OS is a rare neoplasm (120 – 150 new cases/year in Italy) mainly affecting children and young adolescents, for whom it is still a life-threatening disease [3,4]. Conventional first-line drug treatment for OS is based on neoadjuvant chemotherapy with doxorubicin, methotrexate, cisplatin and, in more recent regimens, ifosfamide [3-6].

In addition to these, the chemotherapeutic drugs which are most commonly used for the second and further lines of treatment for relapsed OS patients are gemcitabine, Taxotere (docetaxel), vinca alkaloids, etoposide and trabectedin [6-16].

As shown in Table 1, several DMEs are involved in detoxification of the drugs used for OS treatment. Moreover, efficacy of ifosfamide (a nonspecific alkylating...
1.1 Methods of data searching and selection

A systematic literature search was performed in PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) using standard gene nomenclature for DMEs to identify the impact of their polymorphisms on chemotherapeutic treatment, with particular regard to OS and other sarcomas. Review and full-length articles were included, whereas data published only in conference abstracts were not considered.

In addition to this, the key terms ‘polymorphism’, ‘collateral toxicity’, ‘drug response’, ‘pharmacogenetic’, ‘osteosarcoma’ and ‘sarcoma’ were used to refine the results of the previous searches in order to better focus on studies revealing clinically relevant associations. The PharmGKB website (http://www.pharmgkb.org) was consulted for each gene variant considered for the present review in order to implement the knowledge regarding clinical annotations. Information about gene functions in relation to the metabolism of chemotherapeutic drugs was also extracted by searching genes in OMIM (http://www.ncbi.nlm.nih.gov/omim/?term=*) or in specific databases (http://bioinformatics.charite.de/super-cyp/index.php?site=cyp_snps&species=Homo_sapiens; where '*' stands for ‘gene name’).

2. Drug-metabolizing enzymes

2.1 DMEs involved in the detoxification of chemotherapeutic drugs used in OS treatment

The currently used treatment protocols for OS patients are based on different classes of chemotherapeutic drugs. As mentioned above, the most effective agents used in the first-line treatment of patients newly diagnosed with conventional OS are doxorubicin (synonymous for adriamycin, a topoisomerase II targeting agent, which inhibits DNA replication and transcription), methotrexate (a folate structural analog, which inhibits the synthesis of nucleotides), cisplatin (which directly targets and damages DNA) and ifosfamide (which damages DNA by adding methyl or alkyl groups onto nucleotide bases).

In addition to these, other drugs are used for relapsed OS patients, who are presently treated with different regimens including agents that affect mitosis through interference with formation or breakdown of the mitotic spindle (vinca alkaloids) or stabilization of microtubules (taxanes, as docetaxel), topoisomerase II inhibitors (etoposide) or DNA-damaging drugs (trabectedin) [6-16].

In general, drug metabolism mainly occurs through two distinct consecutive phases named ‘Phase I’ and ‘Phase II’, although this order is not exclusive [17]. Phase I reactions are most commonly described as ‘functionalisation’ and include oxidations, reductions and hydrolysis [18,19]. Phase II reactions are usually defined as ‘conjugation’ and include glucuronidation, sulfonation, acetylation, methylation and glutathione (GSH) or glycine/glutamine conjugation [20,21].

All these reactions are catalyzed by DMEs, which therefore play a key role in the activation and/or detoxification of cytotoxic agents [22]. The most relevant DMEs which metabolize the drugs used for OS treatments are listed in Table 1 and include several members of the CYP superfamily, glutathione-S-transferases (GSTs) and uridine diphospho-glucuronosyltransferases (UGTs). All these enzymes are present and active in both normal and tumor cells and can be affected by a relevant genetic variability, which can lead to differences in treatment response and susceptibility to chemotherapeutic collateral toxicity.

Most of these genetic variations are single nucleotide polymorphisms (SNPs), the identification of which is rapidly increasing, a trend that will probably lead to a better understanding of the observed variability in efficacy and toxicity of anticancer drugs in cancer patients [1]. Based on the
Concerning anticancer agents, CYPs are involved not only in their detoxification but also in activation of prodrugs, making them therapeutically effective [23].

As shown in Table 1, several CYP family members can detoxify doxorubicin, cisplatin, vinca alkaloids, taxanes and trabectedin, all of which are used for OS treatment.

Ifosfamide is also metabolized by several CYP enzymes, resulting in either activation or detoxification [28,29]. In fact, to become therapeutically active, ifosfamide must be metabolized in the liver through 4-hydroxylation reactions which are mostly catalyzed by CYP3A4 and CYP3A5 but also involve CYP2A6, CYP2B1, CYP2B6, CYP2C8, CYP2C9 and CYP2C19 (Table 1) [2]. On the other hand, CYP3A4 and CYP3A5 are also responsible for inactivation of ifosfamide-derived metabolites [2].

Substrate specificity of each CYP can considerably overlap with that of other enzymes of the same superfamily; thus, one drug can be metabolized by one or more CYP enzymes [1].

### 2.3 Phase II enzymes

Phase II DMEs play an important role in the transformation of pharmacologically active compounds and xenobiotics to more easily excretable forms [21]. Phase II DMEs generally detoxify cytotoxic drugs and, therefore, alterations that reduce their metabolic activity may lead to the appearance of toxic effects [21].

The Phase II enzymes that are mostly involved in the metabolism of OS drugs belong to the GSTs and UGTs families.

GSTs are ubiquitous intracellular enzymes that catalyze the conjugation of GSH to many exogenous and endogenous compounds [30]. There are at least three gene families encoding for GSTs: the cytosolic (or soluble) GSTs, which belong to the seven families α, μ, π, θ, σ, ζ and ω [30]; the mitochondrial GSTs, also referred as κ-class GSTs [31] and the membrane-associated proteins involved in eicosanoid and GSH metabolism (the so called MAPEG) [32,33].

Several OS specific drugs are substrates of different GSTs, which catalyze their functional inactivation and detoxification [34-36]. Some of these drugs may also become substrates for ATP-binding cassette (ABC) transporters after conjugation with GSH and, therefore, be actively exported out of the cell [37,38].

It is therefore evident that metabolism of anticancer agents by GSTs is related to drug resistance and collateral toxicity. Among GST isoenzymes, GSTP1 has retained much attention because of its enhanced expression in many tumors and cancer cell lines [39]. In OS, higher GSTP1 expression has been associated with acquired resistance to cancer drugs and worse event-free survival [40].

UGTs are other dominant players of Phase II drugs detoxification reactions. UGTs belong to a superfamily of proteins, which uses UDP-glucuronic acid as a substrate to inactivate several molecules, including steroids, bile acids and numerous chemotherapeutic agents [1,41,42]. Products of Phase I reactions
mediated by CYP-dependent monooxygenases are major substrates of UGTs, which are membrane-bound enzymes, predominantly associated with the endoplasmic reticulum [41].

In humans, 22 UGTs classified into five subfamilies, 1A, 2A, 2B, 3A and 8, have been described [42,43]. Most UGTs are mainly expressed in the liver, the main organ for drug metabolism and the intestine. Glucuronidation by UGTs leads to more hydrophilic metabolites that are subsequently excreted via the kidneys or the bile and the gut depending on their residual hydrophobicity and affinity to transport proteins, thus influencing drug clearance. On the other hand, in case of bioactivation by glucuronidation, drugs can become more active or even toxic [42].

3. Pharmacogenetics of Phase I enzymes

3.1 CYP polymorphisms

Genetic variability in CYP genes may significantly contribute to differences in drug pharmacokinetics, which can be responsible for adverse drug reactions and/or for drug resistance development [28,44]. Moreover, substantial evidence suggest that genetic polymorphisms within the CYP genes can affect drug disposition and, therefore, on treatment tumor response [23,28]. As summarized in the next paragraph, there is evidence derived from studies on other human tumors which indicates that polymorphisms affecting CYP genes involved in the metabolism of OS chemotherapeutic drugs may have clinical relevance. For example, CYP2B6*2, CYP2B6*8, CYP2B6*9, CYP2B6*4 variant alleles have been reported to be associated with response to doxorubicin-cyclophosphamide therapy and with a worse outcome in breast cancer patients [49].

The CYP1B1-4326C > G (432LeuVal) polymorphism emerged as possible predictive marker of response to docetaxel and clinical outcome in castration-resistant prostate cancer patients [46].

3.2 Evidence reported for OS

Among the numerous studies that have investigated CYP enzymes because of their frequent occurrence in tumor specimens, their ability to undergo induction and to detoxify a variety of chemotherapeutic drugs, few findings have also been reported for OS.

Dhaini et al. [47] assessed the expression of five major CYP isoenzymes (CYP1A1, CYP1A2, CYP1B1, CYP3A4 and CYP3A5) by qualitative and quantitative immunohistochemistry in pretreatment tumor samples obtained from 18 OS patients. About 83% of these samples were positive for CYP1A1, CYP3A4 and CYP3A5, and 67% were positive for CYP1A2, and CYP1B1. Although this study did not provide information on the catalytic activity of the enzymes or on the presence of CYP polymorphisms, it suggested that CYPs can be expressed in OS tumor cells.

Other studies performed in different series of OS tumor samples revealed expression of all genes encoding the CYPs listed in Table 1, further supporting the evidence of the presence of these enzymes in OS cells (supplementary Table S1).

Caronia et al. [48] analyzed 366 SNPs affecting genes involved in the metabolism or transport of drugs used in OS chemotherapy, including 7 CYPs (CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4, CYP3A5). Unfortunately, the results obtained on CYP gene polymorphisms were not reported and discussed in detail, probably because no significant associations were revealed as it was the case for the two ABC transporter genes ABCB1 and ABCC3, which were extensively described in this manuscript.

Recently, Hagleitner et al. [49] analyzed polymorphisms of 54 genes involved in cisplatin and doxorubicin pathways in a total of 177 patients with OS. They identified variants of five different genes (FasL, MSH2, ABCC5, CASP3 and CYP3A4), which were associated with 5-year progression-free survival. Since the CYP3A4 (rs4646437) variant had a positive effect on survival, the authors suggested that it might result in a low enzyme expression and therefore in less resistance to therapy.

In addition to these findings, in musculoskeletal sarcomas it has been indicated that CYP polymorphisms may influence drug sensitivity and side effects [50,51], suggesting that pharmacogenetic analysis of these genes may provide useful information for improving the predictability of patients’ tumor response and toxicity profile. However, the impact of CYP pharmacogenetics for OS drug treatment response and prognosis needs to be further explored and defined.

4. Pharmacogenetics of Phase II enzymes

4.1 Glutathione-S-transferases

Several studies have demonstrated that altered GST’s catalytic activities caused by genetic polymorphisms are linked to cancer susceptibility and prognosis [2,52]. Most human GSTs harbor SNPs and several studies have documented clinically relevant germline polymorphisms in the GSH system in cancer patients, which have been correlated to prognosis and response to therapy [1,2]. However, there are also studies which have documented an inconsistent relationship between tumoral GST status and response to therapy [2].

Polymorphisms reported in GSTs are primarily single nucleotide variations (SNVs) and, less frequently, deletions. Four different alleles have been described for GSTP1 (*A, *B, *C and *D), which cause amino acid substitutions, thus encoding proteins with different ability to metabolize anticancer agents (for review, see Ref. [53]). For GSTM3, the *B allele has a 3bp deletion in intron 6 which generates a recognition site for the transcription factor YY1 [54]. Both genes among others have been reported to be associated with platinum-induced ototoxicity [55].

For GSTA1, two alleles, GSTA1*A and GSTA1*B, as consequence of SNVs in the promoter region have been described. The GSTA1*B allele is associated with reduced promoter activity, thereby reducing its expression [53].

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Complete homozygous deletion of the GSTM1 and GSTT1 genes result in the null genotype and the absence of the encoded enzymes. Their frequencies are ethnicity-dependent [56,57]. The GSTM1 null genotype ranges from 39 to 62% in Europeans and 33 to 63% in Asian populations, whereas the GSTT1 null genotype ranges between 10 and 21% in Europe and between 16 and 64% in Asia. Higher frequencies have been reported in China, Japan and Korea. Patients harboring GSTM1/GSTT1 null or GSTA1/GSTP1 genotypes, which are associated with reduced enzymatic activity, should therefore experience higher effective dose and activity of chemotherapeutic drugs being substrates of these enzymes. Accordingly, some studies reported either higher therapy efficacy in these patients, which actually showed longer survival probabilities or more severe therapy-related toxicity, ending in a shorter survival due to collateral adverse effects of treatment (for review, see Ref. [11]). Like for other genes, the clinical impact of GST polymorphisms must therefore be carefully estimated inside each tumor type and in relation to the characteristics of single or homogeneous chemotherapeutic regimens.

### 4.2 Evidence reported for OS

Expression of genes encoding for several GSTs has been reported in different series of OS tumor samples, indicating that they are expressed in OS cells (supplementary Table S2). Moreover, GST germline polymorphisms have also been studied in OS patients. So far, nine studies have analyzed deletions of the GSTT1 (Table 2) and GSTM1 genes (Table 3), seven studies have analyzed the GSTP1 Ile105Val polymorphism due to the GSTP1*D allele (Table 4), followed by three papers including the GSTM3*B deletion (Table 5).

### Table 2. Studies reporting germline GSTT1 deletion polymorphism in patients with OS.

| Country (number of OS patients) ethnicity | Method | Drugs | GSTT1 | Ref. |
|------------------------------------------|--------|-------|-------|------|
| Germany (39 pediatric tumors; 27 OS)     | PCR, RFLP | Pre-op and Post-op: MAP and ifosfamide | No associations | [59] |
| US (171 pediatric tumors; 12 OS)         | PCR-based | | Non-null alleles with increased risk for OS | [58] |
| CAU (88%), HISP (11%), AA (1%)           | | | | |
| Brazil (80; 32 metastatic patients at diagnosis) | Multiplex-PCR, RFLP | Pre-op and Post-op: carboplatin, cisplatin, doxorubicin, ifosfamide and mesna | Nonmetastatic patients: Non-null allele with higher toxicity | [63] |
| Null allele with chondroblastic histotype | | | Metastatic patients | |
| Null allele with worse OVS | | | | |
| China (110 patients with OS)             | Taqman PCR | | Null allele with increased risk of OS, especially for patients aged < 15 years | [61] |
| UK (58 patients with OS; 6 metastatic)   | PCR-RFLP Multiplex-PCR for GSTT1 and GSTM1 Illumina 610-Quad SNP array | Pre-op and Post-op: MAP | Null allele with shorter EFS | [65] |
| CAU (41, 71%), AC (8, 14%), IA (9, 15%) | | | | |
| China (159 patients with OS; 24 metastatic) | Taqman PCR | Cisplatin, doxorubicin, carboplatin, ifosfamide | Null allele with better survival rates but not with EFS | [64] |
| China (146 patients with OS; 27 metastatic) | 384-well Sequenom MassARRAY platform | Pre-op: MAP Post-op: MP or ADV | No associations | [68] |
| China (162 patients with OS; 23 metastatic) | 384-well Sequenom MassARRAY platform | Pre-op: MAP Post-op: MP or ADV | No associations | [66] |
| China (186 patients with OS; 38 metastatic) | 384-well Sequenom MassARRAY platform | Pre-op.: MAP Post-op.: MP or ADV | No associations | [67] |

Information regarding the number of patients with metastases at diagnosis and the drugs used for treatment are reported in the table when they were provided in the publication.

AA: African-American; AC: African-Caribbean; ADV: actinomycin D vincristine; CAU: Caucasian; EFS: Event-free survival; HISP: Hispanic; IA: Indian/Asian; MAP: Methotrexate, adriamycin, cisplatin; MP: Methotrexate, cisplatin; OS: Osteosarcoma; OVS: Overall survival; PCR: Polymerase chain reaction; Post-op: Post-operative; Pre-op: Pre-operative; RFLP: Restriction fragment length polymorphism; SNP: Single nucleotide polymorphism.
Among these, two studies analyzed pediatric tumors including few patients with OS [58,59]. One recent meta-analysis reanalyzed and discussed all four polymorphisms including six already-published case-control studies [60]. Copy number analysis of GSTT1 and GSTM1 was performed in one study but data did not correlate with clinical parameters [48].

An increased risk of OS was seen in pediatric patients when carrying the GSTM1 and/or GSTT1 gene [59]. In a large Chinese study, however, the GSTM1 and/or GSTT1 null allele was associated with higher risk of developing OS [61]. For GSTT1, this association was even more evident in patients aged < 15 years, but unfortunately p values were not provided throughout the whole paper. On the other hand, a recently published genome-wide association study (GWAS) did not identify a GST-related higher risk of OS, indicating that the results mentioned above may derive from genetic differences related to the patient’s ethnicity [62].

The GSTM1 null allele was reported to be associated with increased relapse in nonmetastatic OS patients, and with poor survival in metastatic patients [63]. Regarding GSTT1, the non-null allele was associated with poor survival in metastatic patients, whereas patients with both GSTM1 null allele and GSTT1 non-null alleles showed worst survival. Interestingly, the GSTT1 null allele was associated with lower toxicity and chondroblastic histotype. This study was conducted in Brazil and is the only one, in which metastatic patients were analyzed separately. This fact might explain that the findings were not confirmed in any other studies. By contrast, associations between the GSTM1 and/or GSTT1 null genotypes and better survival rates but not event-free survival were reported in a recent Chinese study [64]. Interestingly, also association between

### Table 3. Studies reporting germline GSTM1 deletion polymorphism in patients with OS.

| Country (number of OS patients) ethnicity | Method | Drugs | GSTM1 | Ref. |
|----------------------------------------|--------|-------|-------|------|
| Germany (39 pediatric tumors; 27 OS)   | PCR, RFLP | Pre-op and post-op: MAP and ifosfamide | No associations | [59] |
| US (171 pediatric tumors; 12 OS)       | PCR-based | | Non-null alleles with increased risk for OS | [58] |
| CAU (88%), HISP (11%), AA (1%)         |        | | | |
| Brazil (80; 32 metastatic patients at diagnosis) | Multiplex-PCR, RFLP | Pre-op and post-op: carboplatin, cisplatin, doxorubicin, ifosfamide and mesna | Nonmetastatic patients: Null allele with increased relapse, especially to the lungs The presence of at least one allele with good response Metastatic patients: Null allele with worse OVS | [63] |
| China (110 patients with OS)            | Taqman PCR | | Null allele with increased risk for OS, especially for patients aged < 15 years | [61] |
| UK (58 patients with OS; 6 metastatic)  | PCR-RFLP | Pre-op and post-op: MAP | Null allele with treatment delay | [65] |
| CAU (41, 71%), AC (8, 14%), IA (9, 15%) | Multiplex-PCR for GSTT1 and GSTM1 Illumina 610-Quad SNP array | | | |
| China (159 patients with OS; 24 metastatic) | Taqman PCR | Cisplatin, doxorubicin, carboplatin, ifosfamide | Null allele with better survival rates but not with EFS | [64] |
| China (146 patients with OS; 27 metastatic) | 384-well Sequenom MassARRAY platform | Pre-op: MAP Post-op: MP or ADV | No associations | [68] |
| China (162 patients with OS; 23 metastatic) | 384-well Sequenom MassARRAY platform | Pre-op: MAP Post-op: MP or ADV | No associations | [66] |
| China (186 patients with OS; 38 metastatic) | 384-well Sequenom MassARRAY platform | Pre-op: MAP Post-op: MP or ADV | No associations | [67] |

Information regarding the number of patients with metastases at diagnosis and the drugs used for treatment are reported in the table when they were provided in the publication.

AA: African-American; AC: African-Caribbean; ADV: Actinomycin D vincristine; CAU: Caucasian; EFS: Event-free survival; HISP: Hispanic; IA: Indian/Asian; MAP: Methotrexate, adriamycin, cisplatin; MP: Methotrexate, cisplatin; OS: Osteosarcoma; OVS: Overall survival; PCR: Polymerase chain reaction; Post-op: Post-operative; Pre-op: Pre-operative; RFLP: Restriction fragment length polymorphism.
the GSTM1 null allele and treatment delay was described in Caucasian patients [65], whereas in the same study the GSTT1 null allele was associated with poor event-free survival. Most concordant results have been published regarding the GSTP1 Ile105Val (rs1695) polymorphism (Table 4).

Table 4. Studies reporting germline GSTP1 polymorphism in patients with OS.

| Country (number of OS patients) ethnicity | Method | Drugs | GSTP1 (rs1695) Ile105Val, A allele = wild-type, G allele = variant | Ref. |
|------------------------------------------|--------|-------|---------------------------------------------------------------|-----|
| Germany (39 pediatric tumors; 27 OS)    | PCR, RFLP | Pre-op and post-op: MAP and ifosfamide | No associations | [59] |
| UK (58 patients with OS; 6 metastatic)  | PCR-RFLP | Pre-op and post-op: MAP | AG heterozygotes associated with: Poor histological response (< 90% necrosis), Decreased EFS, Leucopenia, Cardiotoxicity | [65] |
| CAU (41, 71%), AC (8, 14%), IA (9, 15%) | Multiplex-PCR for GSTT1 and GSTM1 Illumina 610-Quad SNP array | | | |
| China (187 patients with OS; 38 metastatic) | PCR-RFLP | Pre-op and post-op: MAP + ifosfamide | Val/Val (= GG) associated with good response | [69] |
| China (159 patients with OS; 24 metastatic) | Taqman PCR | Cisplatin, doxorubicin, carboplatin, ifosfamide | Val/Val (= GG) associated with higher risk of death | [64] |
| China (146 patients with OS; 27 metastatic) | 384-well Sequenom MassARRAY platform | Pre-op: MAP Post-op: MP or ADV | GG allele associated with: Poor histological response (< 90% necrosis), Highest risk of death | [68] |
| China (162 patients with OS; 23 metastatic) | 384-well Sequenom MassARRAY platform | Pre-op: MAP Post-op: MP or ADV | GG allele associated with: Poor histological response (< 90% necrosis), Worse OS | [66] |
| China (186 patients with OS; 38 metastatic) | 384-well Sequenom MassARRAY platform | Pre-op: MAP Post-op: MP or ADV | GG allele associated with: Poor histological response (< 90% necrosis), Poorest OS and EFS | [67] |

Information regarding the number of patients with metastases at diagnosis and the drugs used for treatment are reported in the table when they were provided in the publication.

AC: African-Caribbean; ADV: Actinomycin D vincristine; CAU: Caucasian; EFS: event-free survival; IA: Indian/Asian; MAP: Methotrexate, adriamycin, cisplatin; MP: Methotrexate, cisplatin; OVS: Overall survival; OS: Osteosarcoma; PCR: Polymerase chain reaction; Pre-op: Pre-operative; Post-op: Post-operative; RFLP: Restriction fragment length polymorphism.

Although not revealed in previous single studies, a meta-analysis indicated an association between homozygous GSTM3*B and OS risk, which however is difficult to be precisely estimated since the p value of this correlation was not reported [60].

Different from the studies discussed above, which all analyzed germline variants, Goricar et al. recently assessed GSTP1 polymorphisms and GSTM1 and GSTT1 deletions in tumor tissue of 66 OS patients [70]. Deletions of either GSTM1 or GSTT1 were not associated with survival. Based on the evidence that the GSTP1 (rs1138272) variant was associated with both shorter event-free and overall survival and remained significant in multivariate analysis, authors suggested that this polymorphism may impact on response to cisplatin-based chemotherapy.

Despite the fact GST polymorphisms, which have shown to impact on clinical outcome in patients with different diseases, have been studied in OS patients for more than 10 years, it is still too early for drawing conclusions regarding their role in OS. Even a recent meta-analysis [60] could not overcome...
the limits inherent in small studies, which often lack statistically significant p values because of too small patient numbers or because it gives rise to false-positive correlations due to biased data. Regarding associations between GST polymorphisms and survival, none of the results obtained in single studies could be confirmed, whereas associations between GSTT1 null or GSTM3 homozygous variant genotype and increased risk of OS were confirmed [60]. Again, the significance of these findings is difficult to fully judge since the authors did not provide p values.

Some of the discordant results could be ascribed to the fact that different techniques were used in the papers included in the meta-analysis. For example, classical or multiplex polymerase chain reaction and restriction fragment length polymorphism analyses cannot distinguish between the homozygous and heterozygous non-null genotype for GSTM1 and GSTT1. Therefore, more refined analyses for these deletion polymorphisms in the future might reveal new associations previously not detected.

Insights gained from GWAS may help to overcome, at least partially, the discrepancies that emerge from candidate gene-driven approaches and to determine whether these are stable associations or are perhaps due to chance.

### 4.3 Uridine diphospho-glucuronosyltransferases

All UGT family members are highly polymorphic (http://www.pharmacogenomics.pha.ulaval.ca/cms/ugt_alleles/). Most extensive genetic variation exists in UGT1A and UGT2B genes [71,72], which have recently been confirmed by sequencing of UGT1A1, UGT2B7 and UGT2B15 in five different ethnic groups [73]. In general, polymorphisms and expression of UGTs have mainly been studied in normal tissues or in vitro experimental models because of their prevalent expression in liver and intestine. Their clinical relevance has recently been reviewed on the basis of existing knowledge on pharmacokinetic changes related to certain polymorphisms affecting UGT activity [42,74]. Concerning OS drugs, UGTs are mainly involved in the detoxification of doxorubicin, epirubicin and etoposide (Table 1). Similar to what has been described above regarding the other DMEs, the expression of several UGT1A, 2A and 2B genes has been found in different series of OS tumor samples, indicating that they are present in OS cells (supplementary Table S3), even if no data about their catalytic activity have been reported for this tumor so far.

Some good examples, including drugs used for OS, for how genetic variation of UGTs alters both drug response and toxicity have recently been reported [74,75]. In a group of 81 patients with NSCLC treated with irinotecan and cisplatin, the UGT1A1*6/*6 genotype proved to be significantly associated with higher toxicity and shorter overall survival [76]. In 78 patients with NSCLC treated with irinotecan with paclitaxel or gemcitabine, the UGT1A1*6 and UGT1A1*27 genotypes emerged as candidate predictors of grade 4 neutropenia [77]. In children with acute leukemia, treated with methotrexate, susceptibility to develop hyperbilirubinemia was associated with the UGT1A1*28 allele, most probably due to an enhanced drug-induced inhibition of UGT1A1 [78]. In African-American children suffering from acute lymphoblastic leukemia, homozygosity of the UGT1A1*28 allele showed a lower etoposide clearance [79].

The potential use of UGT polymorphisms for tailoring chemotherapy regimens in specific subgroups of patients, as has recently been suggested by Innocenti et al. [80], who indicated that the UGT1A1*28 genotype can be used

### Table 5. Studies reporting germline GSTM3 deletion polymorphism in patients with OS.

| Country (number of OS patients) ethnicity | Method | Drugs | GSTM3 (*B 3bp deletion in intron 6) | Ref. |
|-----------------------------------------|--------|-------|----------------------------------|------|
| Germany (39 pediatric tumors; 27 OS)    | PCR, RFLP | Pre-op and post-op: MAP and ifosfamide | Protective effect of the GSTM3*B allele in the group with normal hearing | [59] |
| US (171 pediatric tumors; 12 OS)        | PCR-based | | No associations | [58] |
| Brazil (80; 32 metastatic patients at diagnosis) | Multiplex-PCR, RFLP | Pre-op and post-op: carboplatin, cisplatin, doxorubicin, ifosfamide and mesna | Nonmetastatic patients: GSTM3 AB/BB associated with metastases at diagnosis Metastatic patients: GSTM3 AA genotype associated with worst survival (compared to AB/BB) | [63] |

Information regarding the number of patients with metastases at diagnosis and the drugs used for treatment are reported in the table when they were provided in the publication.

AA: African-American; CAU: Caucasian; HISP: Hispanic; OS: Osteosarcoma; PCR: Polymerase chain reaction; Pre-op: Pre-operative; Post-op: Post-operative; RFLP: Restriction fragment length polymorphism.
to individualize irinotecan treatment in patients with advanced solid tumors, will hopefully be extended in the near future.

5. Clinical relevance of DMEs in human tumors

5.1 Introduction and current state of art

DMEs are deeply involved in determining patients’ predisposition to adverse events, and variations in their expression and activity play a critical role not only in susceptibility to collateral toxicity but also in drug effectiveness [81]. Several studies have demonstrated that cancer cells have a unique metabolism compared to normal cells [82] and provided evidence that drug metabolism also occurs within tumors [44,83]. A promising new field of translation research is therefore aimed to take advantage of cancer cell specificity, in order to develop more effective therapeutic approaches.

The presence of DMEs within tumors can either positively or negatively influence the efficacy of chemotherapeutic agents, depending on the enzyme activity and the cytotoxic drug. Based on this, some possible therapeutic interventions have been suggested and explored.

For example, the enhanced expression of GSTP1 that has been described in several tumors, including OS, makes this enzyme a new promising candidate therapeutic target [38-40].

In order to take advantage of GSTP1 overexpression in cancer cells, two strategies have been performed [89]. The first one consisted in developing GSTP1 inhibitors to decrease the metabolism of active anticancer drugs that are inactivated by this enzyme or to overcome GSTP1-mediated drug resistance mechanisms [3,53]. In OS, we have demonstrated that targeting GSTs with 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol is a very efficient strategy to inhibit the in vivo growth of human OS cell lines exposed to cisplatin [40]. Unfortunately, this agent is not available for a clinical use.

The second strategy consisted in designing prodrugs activated by GSTP1 in order to target specifically or preferentially the tumor cells overexpressing this enzyme [2,3,53,84,85]. Following this strategy, Phase III studies have been performed in which genes encoding specific DMEs were delivered to tumor cells followed by a prodrug that was locally converted to a cytotoxin by the enzyme [86]. Other trials demonstrated that, by taking advantage of the high tumoral GSTs levels, use of the GST-activated nitrogen mustard TLK286 in combination with standard chemotherapeutic agents (including platinum, taxanes and anthracyclines) provided positive clinical results [38].

A similar rationale has been used for developing prodrugs, which are activated to cytotoxic agents by CYP enzymes endogenously expressed within the neoplastic cells, only or primarily at the site of the tumor because of peculiar environmental conditions (i.e., hypoxia), which are different from those present in normal tissues [23].

Another indication for taking advantage of the DME status in neoplastic cells derives from studies which demonstrated that tumor cells can lack expression of specific DMEs [87]. In these situations, it is therefore possible to assume that administration of anticancer drugs in patients with tumors lacking specific inactivating DMEs may prove useful in widening the therapeutic index of the currently used chemotherapeutics.

5.2 Future directions

Although cancer chemotherapy has largely improved over the past few decades, the goal of killing cancer cells without toxic side effects on normal cells has yet to be achieved. As described above, different strategies have been taken into account or are still explored with the aim to improve treatment efficacy without increasing or possibly decreasing collateral toxicity by taking advantage of differential DME characteristics between tumor and normal cells.

Possible future strategies may be based on delivering prodrugs and DMEs to tumor cells. In this respect, approaches in which an exogenous CYP gene and a prodrug activated by that enzyme are administered together to the site of the tumor have been explored [23,88].

For example, in a Phase I/II clinical study, CYP2B1 (microencapsulated in genetically modified allogeneic human embryonic kidney cells) was delivered to the tumor vasculature in 14 patients with inoperable pancreatic cancer treated with ifosfamide [89]. Thanks to the local activation of ifosfamide, in 4 patients tumors regressed after treatment and in the other 10, tumors remained stable. Moreover, in these patients, median survival was doubled and 1-year survival rate was increased three times in comparison with historic controls. However, despite these promising indications, several issues are required to be addressed before translating these strategies to clinical practice (reviewed in Ref. [23]).

As a second example, an experimental study can be considered in which the novel CYP3A-activated anticancer prodrug methoxyxmorpholinyl-doxorubicin (MMDX) was investigated [88]. The potentiation of MMDX activity is due to metabolic activation by liver-expressed CYP3A enzymes and activated MMDX retains activity against tumor cells with different mechanisms of resistance to classical anticancer agents [88]. This study investigated whether CYP3A4 may be used together with the prodrug MMDX for gene-directed enzyme prodrg therapy (GDEPT) applications by delivering the enzyme and the drug to tumor cells in vivo. Authors demonstrated that endogenous expression of CYP3A4 in tumor cells, and not hepatic CYP3A4 activity, is a key determinant of responsiveness to MMDX and suggested that CYP3A4 endogenous tumor expression in individual patients may serve as an important determinant of responsiveness to therapeutic efficacy of MMDX treatment in combination with CYP3A4 gene transfer [88]. Moreover, these findings provided proof of concept for the potential
CYP3A4 prodrug activation-based gene therapy, also in the context of a high liver CYP3A activity.

The clinical use of prodrugs is often limited by host toxicity associated with the systemic distribution of cytotoxic metabolites formed in the liver. There are however indications that these limitations may, at least in part, be circumvented by implementation of CYPs GDEPT approaches, such as those described above, aimed to increase tumor cell exposure to cytotoxic drug metabolites generated locally by a prodrug-activating CYP (reviewed in Ref. [90]).

In general, development of novel DME-based therapeutic approaches must take into account at least the major gene variants, which may influence patient’s response to these treatments. Hence, specific pharmacogenetic profiling of patients and information about the DME status in tumor cells will be absolutely necessary to identify those which could be eligible for these innovative treatments.

6. Conclusion

The clinical efficacy of presently used anticancer therapy is severely limited by the inability to accurately predict patients’ outcomes in terms of both tumor response and collateral toxicity. This lack of prediction has a great clinical significance because, since anticancer drugs have a narrow therapeutic index, individual differences in metabolic activities could easily either reduce treatment efficacy or result in severe toxic effects.

To date, pharmacogenetic studies on human tumors have mainly analyzed the effects of single polymorphisms on variation in treatment response and outcome. These studies have unfortunately very rarely provided clinically relevant information, which can be used to modulate cancer chemotherapy.

In the past decades, huge progress has been made in the characterization, expression, function and regulation of DMEs. Moreover, it has been clearly demonstrated that variability between tumor patients in the pharmacokinetics of cancer chemotherapeutics has important consequences for therapeutic efficacy and safety. This variability derives from different factors, including the genetic background of each individual and epigenetic mechanism affecting some DME genes [91,92], with a consequent influence on the pharmacokinetic and pharmacodynamic profile of anticancer drugs leading to differences in treatment response and/or development of severe toxicities. Pretreatment analysis of DME polymorphisms, which have been proven to be associated with differential enzymatic activities, may therefore predict patients’ predisposition to treatment-related toxic effects and/or drug resistance. However, the lack and sparsity of information on functional characteristics of many DME polymorphisms, especially in rare tumors as OS, indicate the need for future research on this topic in order to actually transfer this knowledge into clinic.

7. Expert opinion

The problem of adverse reactions to drugs, first of all to chemotherapeutic agents, is frequently underestimated. It is approximately calculated that these reactions are responsible for ~7% of all hospital admissions in Europe and for >100,000 death/year in the US [93,94]. Moreover, adverse reactions lead to additional pharmacologic treatments, with a progressively worsening of patients’ quality of life and a significant increase of costs in medical cares, therefore claiming for development of personalized treatment strategies.

Personalized medicine is particularly important and needed in oncology, since most clinically used anticancer drugs have a narrow therapeutic window and exhibits a large interindividual pharmacokinetic and pharmacodynamic variability, which can lead to treatment failure or severe toxicity. A large part of this interindividual variability derives from genetic polymorphisms affecting DME genes and, therefore, understanding how these genetic variations influence drug action and collateral toxicity in each tumor entity could be of great help in tailoring cancer therapy.

With the need to target and individualize anticancer therapies, alterations in drug metabolism due to specific gene polymorphisms must be considered as potential mechanisms of tumor chemotherapy resistance and/or elevated toxicity and, thus, factors to be taken into consideration for achieving better treatment results.

The availability of new genome-wide (microarray) technology may potentially enable the rapid characterization of DME genes within and between tumor types and in somatic tissues, as well as to document the relationships of polymorphisms with gene expression and activity inside each tumor and in each patient.

Despite there being a relevant amount of work done in the field of human cancer pharmacogenetics, the practical application of pharmacogenetics in clinical oncology is still very limited. This context is even more severe for rare tumors as OS, for which the presently available information is very scarce and incomplete.

Pharmacogenetic studies in OS patients encounter several limitations. First, the rarity of this tumor makes it difficult to collect adequate number of patients to perform wide-scale studies. This problem may be overcome by collecting cases and data from different countries inside international collaborative studies, as has recently been shown [62]. Moreover, analyzing the samples from different ethnic origin is a challenge to study the effect of polymorphisms not only in relation to the disease but also in relation to the different ethnicity.

Almost all pharmacogenetic studies on OS reported so far have investigated the effect of one or a few SNPs in specific genes at a time, following a candidate gene approach. In fact, the few studies performed in OS were based on a priori knowledge of polymorphisms and gene functions in relation to the drugs used in treatment protocols. This strategy can
produce informative data, but further implementation and validation of the obtained results are needed.

Like for other tumors, future researches based on the use of whole genome approaches, such as SNP arrays, may lead to the discovery of unknown associations between OS-related genetic factors and tumor features, patients’ susceptibility to collateral toxicity and clinical outcome. Finally, when a reliable set of knowledge about SNPs inside specific cohorts of patients will be achieved, it can be predicted that the study of genetic polymorphisms may provide useful prognostic markers for improving the current clinical results of both first-line and secondary treatments for OS.

Conventional neoadjuvant chemotherapy protocols have reached a survival plateau (60 – 65% probability of long-term survival) that has remained unchanged over the past 30 years. Moreover, a major, still unsolved problem is the dismal prognosis for patients with recurrent OS (< 20% long-term survival probability), for whom no standard effective regimens are yet available. Translational research is therefore required not only to identify targets and drugs for novel treatment modalities but also to indicate pharmacogenetic markers which can be used to personalize the currently available treatments.

Nowadays, dosages of chemotherapeutic drugs are calculated on the basis of patients’ body surface area, a parameter that is not directly correlated with drug pharmacokinetics, because this approach is based on the assumption that each individual is capable of metabolizing drugs with the same efficacy. Moreover, the current way of treating patients consists in giving the planned treatment to all patients and to adjust drug dosages during treatment in case of adverse events.

Achieving more insights into pharmacogenetic markers and biological determinants related to treatment response and prognosis in OS will ultimately lead to individualized treatment regimens, based on a combination of genotype and tumor characteristics of each patient. The possibility to modulate treatment on the basis of validated pharmacogenetic markers will significantly impact not only on the costs of cures, treatment response and clinical outcome but also on improvement of patients’ quality of life, a fact that is particularly important for tumors which, like OS, mainly affect young people with long life expectancies.

Under this perspective, collecting information on DME’s impact in OS may greatly help to reach these goals. Studies on DME gene polymorphisms in OS have indicated that they may have relevant clinical impact but the data reported so far, which all have been considered in this review, still need further confirmation. For these reasons, insights gained from GWAS may provide important indications that may help to improve OS therapy in the future.

Focusing on DME study in OS can also have a great relevance to address the optimal use of new cytotoxic agents which can be substrates of these enzymes. On the other hand, the possible influence of these novel agents on cellular DMEs in relation to their genetic variability will also need to be explored, to avoid possible antagonistic effects with the metabolism of classical cytotoxics with which these novel agents will probably be combined.

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Declaration of interest

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Supplementary material available online

Supplementary Table S1, S2 and S3

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