Analysis of prognostic factors, pharmacokinetic and pharmacogenetic examinations in children with osteosarcoma

PhD theses

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Introduction

Osteosarcoma is one of the most frequent primary malignant bone tumour occurring in childhood. Intensive chemotherapy had been introduced in its treatment in the 1970s, which significantly improved survival of the disease. Two third of children treated with osteosarcoma have permanently recovered since 1980. Cytostatic drugs of preoperative chemotherapy are doxorubicin, cisplatin, ifosphamide and methotrexate (MTX).

Objective of the treatment is elimination of the potential micrometastases as well as reduction of the initial tumour volume. The neoadjuvant chemotherapy is followed by a radical surgery. Development of analyses by different imaging techniques as well of prosthetizing techniques has ensured for orthopaedist a more precise preoperative planning and a broader scope of solutions. After the surgical procedure patients are classified into different risk groups based on the initial tumour size and the histological response given to the chemotherapy and a 6-8 months of medication follows. During the adjuvant treatment, in the standard group we apply previous drugs of preoperative chemotherapy, while in the high risk group new drugs, carboplatine and etopiside are being applied.

High-dose intravenous MTX is corner stone of treating osteosarcoma. Among all the tumours, MTX is used in the highest dosage in the case of osteosarcoma therapy, in dose of 12g/ m^2 body surface area, 4-12 times during a protocol. MTX belongs to the folic acid antagonist group of cytostatic drugs. Having penetrated into the cell it inhibits the cofactor production essential for the synthesis of nucleotides. It is transported into the cell by active transport, where glutamate groups are bound to it and the newly formed polyglutamated form will be able to hinder the *de novo* synthesis of pyrimidine by inhibiting the dihydrofolate-reductase and thymidylate-synthase enzymes. Despite its broad and long use it is still hard to predict effectiveness and toxicity of MTX. Severe hepato- and nephrotoxicity, myelosuppression, gastrointestinal- and neurotoxicity may also occur as its acute side-effects. Former analyses had shown a significantly better survival in case of high concentrations of MTX over 700 to 1000 μmol/l, so this level was regarded to be the recommended level. Occurrence of toxicity was linked to the prolonged excretion period of MTX, however subsequent analyses could not confirm these connections. It is vital to study more profoundly the correlation between the pharmacokinetics and the MTX-effects, to ensure the potential for a much more effective and safer application of MTX.

There are huge interindividual differences in the toxic symptoms during the MTX therapy as well as in the elimination of the drug. One of the reasons for that may be the polymorphisms of genes participating in the kinetics and action mechanism of the drugs, as they may influence and alter adsorption, distribution, metabolization and excretion of the drugs. MTX enters the cell through the molecule SLC19A1 (solute carrier family 19, member 1, its old name: reduced folate carrier 1). The molecule SLC19A1 works through a bidirectional anion exchange mechanism, without ATP-hydrolizing activity. The often observed polymorphism of this gene is the 80G>A, which causes an Arg27His exchange in the first transmembrane domain of the protein, thus causing an increased affinity for folic acid in the transporter molecule. So in case of genotype *SLC19A1* AA the MTX penetration into the cell is higher, which causes a better therapeutic response and at the same time a more frequent occurrence of toxicity. Within the cell the MTX is polyglutamated by the enzyme folylpolyglutamate-synthetase, and this active form is the one that enters the folic acid cycle. The polyglutamated folic acid derivatives enter the lysosome, where they are hydrolyzed into a monoglutamated form by the gamma-glutamyl-hydrolase (GGH). Tumour cells that have a high GGH enzyme level are antifolate-resistant, and the degree of resistance increases parallel with the increase of the GGH level. Promoter polymorphisms of this gene *-401C>T* (rs3758149) and *-124T>C* enhance the promoter activity and thus the expression of protein GGH. The increased GGH-level leads to a decreased accumulation of the polyglutamated MTX and MTX-resistance.

The free MTX exits the cell through the ABCC1-5 and ABCG2 transporters of the ATP-binding cassette family. The ABC-transporter family contains several ATP-binding transmembrane proteins, participating in the transport of xenobiotics. The ABCB1 works against adsorption of its substrates in the intestines and the blood vessels, performing the transport towards their lumen. In the liver cells and cells of renal tubules this transporter participate in extraction of its substrates through the gall bladder and the urine, and in forming blood-tissue barriers. ABCC1 mainly appears in cells with explicit barrier-function and in those in stage of cell division. The primary task of this protein is protection of cells against toxic effects and contribution to forming the barriers function. ABCC2 is mainly expressed on the apical surface of hepatocytes, and in the proximal tubules of kidney and the intestine as well. This transporter protein takes an important part in eliminating endogenous metabolites and xenobiotics and their metabolites too. Specific function of ABCC3 has not been clarified yet, but it definitely participates in biliary and intestinal excretion of organic anions. ABCG2 blocks the adsorption of toxic substances in the intestine and in the liver it

enhances their excretion into the gall, thus decreasing the amount of biologically accessible xenobiotics. Polymorphisms of genes encoding the transporter proteins may change the gene expression, as well as the substrate recognition, activity and function of transporters.

The Steroid and Xenobiotic Receptor gene (*NR1I2*, alias *SXR*) belongs to the nuclear receptor super family. Protein SXR activates transcription of many other genes which participate in the metabolism and secretion of potentially harmful xenobiotics, drugs and endogenous chemical compounds. It does not play a direct role in the MTX metabolism, and in development of drug resistance it participates at the regulation level of gene transcription.

Thesis Objectives

During my work I pursued the following objectives:

- To map the data related to children suffering from osteosarcoma in Hungary. To analyze overall survival (OS) and event-free survival (EFS). By use of retrospective data processing, to analyze the data based on the extent and localization of the disease, the histological subtype, the patients' age and gender, the type of surgery, the histological response of the tumour to the preoperative chemotherapy, as well as on the time of diagnosis.
- To examine correlations between survival, pharmacokinetics and toxicity of high dosage MTX infusion as the basic treatment of osteosarcoma, in infant patients with osteosarcoma. To analyse the correlations between prognostic and population parameters (gender, age, presence of metastasis, histological response and risk groups), pharmacokinetic parameters and occurrence of toxicity. To examine if there is any link between the pharmacokinetic parameters and toxicity of liver, kidneys and bone marrow. To examine how the occurrence of toxicity will change throughout the treatment. And besides, to check if the overall and the event-free survival will show any correlation with the pharmacokinetic parameters and the toxicity data.
- To examine pharmacokinetics of high dose MTX treatment and development of toxicity from the point of view of genetic background. To isolate the DNA from blood of children with osteosarcoma. To define the single-nucleotide polymorphisms (SNP) of genes playing an important role in the MTX metabolism (*ABCB1*, *ABCC1*, *ABCC2*, *ABCC3*, *ABCC10*, *ABCG2*, *GGH*, *SLC19A1*, *SXR*). To examine the correlations between the polymorphisms and different pharmacokinetic parameters and the occurrence of toxicity.

Methods

Our patient population consisted of 122 children diagnosed with osteosarcoma between 1988 and 2006 at the 2nd Department of Pediatrics of Semmelweis University. Their treatments were based on the COSS-86 and COSS-96 protocols, involving neoadjuvant and adjuvant chemotherapy and radical surgery as well. From the 122 patients' charts we collected retrospectively their age, gender, date of diagnosis, localization of the tumour and its histological subtype, occurrence of metastasis or local recurrence, its date, type of surgery, the histological response to the chemotherapy, classification of the patients in risk-groups; then examined the 5-year overall (OS) and event-free survival (EFS) in accordance with all these data.

For our pharmacokinetic analysis we collected the data, such as the serum drug levels measured in 6, 24, 36, 48 hours subsequent to the application of MTX, as well as the lowest overall protein, leukocyte and granulocyte numbers and the highest values of glutamic-pyruvate-transaminase (GPT), gamma-glutamyl-transferase (GGT), bilirubin and creatinine measured during the first week following the treatment. For the discretization necessary for the analysis we took into consideration the fact how frequent the occurrence of toxicity was. Many patients had liver and bone marrow toxicity subsequent to the treatment, so the clinically relevant question was which were the factors influencing appearance of severe, grade III and IV acute toxicity according to Common Terminology Criteria for Adverse Events (CTC) verion 3.0. The glomerular kidney toxicity characterized by creatinine levels occurred infrequently, so in this case we tried to examine the factors that influence appearance of toxicity. From the drug levels we calculated the area under the concentration-time curve (AUC_{0-48}), plasma clearance and according to the two-compartment excretion we calculated two half-lives of MTX excretion.

Parallel to the pharmacokinetic data-collecting, from the DNA isolated from the blood of children with osteosarcoma, we started genotyping the SNPs of genes coding proteins which play a vital role in the MTX metabolism. Based on the international literature, our team accomplished genotyping of altogether 46 single-nucleotide polymorphisms of 12 genes. During the genotyping we used methods of PCR-RFLP and Real-Time PCR, as well as mini-sequencing, Melting Point Analysis and GenomeLab SNPstream. Then we checked the individual SNPs of the examined population from the point of view of Hardy-Weinberg

equilibrium, and also made linkage studies in order to avoid redundant analyses. As a result, we tested the effects of altogether 29 polymorphisms of genes *ABCB1, ABCC1, ABCC2, ABCC3, ABCC10, ABCG2, GGH, SLC19A1* and *SXR* on the clinical data.

The overall and event-free survival of the patients were analysed with the Kaplan-Meier method, while the patient-groups created according to different aspects were compared with log-rank test. In order to decide whether there is a correlation between the occurrence of toxicity and the pharmacokinetic parameters, we applied paired sampled T-test in the case of normally distributed variables and the Mann-Whitney U-test in the case of abnormally distributed variables. We applied the chi-square (χ^2) test for determining relationship between categorical variables. Beyond the above mentioned frequentist statistic methods, during our pharmacokinetic analysis we also applied analyses based on Bayesian methods. The applied method was developed by our research team and it is a Bayesian network based Bayesian multilevel analysis of relevance (BN-BMLA).

Results

In 37 out of the 122 children occurred metastases, local recurrence, or both. In 15 cases of the 37 patients there were detected early metastases at the time of diagnosis, and unfortunately only 3 of them survived the disease. In our patient population late metastases were detected in 13 children. In all cases the metastasis was localized in the lungs, in 4 cases it appeared in the regional lymph nodes too, and in 8 cases the lung metastasis was accompanied by a local recurrence. Five of the 13 patients are long-term survivors. In 23 patients the tumour recrudesced locally and 18 of them died.

Among children with osteosarcoma treated at the 2nd Department of Paediatrics between 1988 and 2006 the 5-year overall survival was 68%, while the 5-year event-free survival was 61.5%. From the analyzed prognostic factors, development of metastasis, operability of the patient, histological response to neoadjuvant chemotherapy and the age at the time of diagnosis proved to be the factors with significant effects. In the group of patients without metastasis the overall survival was 79%, in case of late metastasis it was 41.6%, while in case of early metastasis detected at the time of diagnosis it was 16.6%. Patients, who had only biopsy but the tumour could not be surgically removed, all died. However, in patients treated with amputation or limb preservation surgery the difference in survival was not significant ($p=0.3$). Based on the histological response given to the neoadjuvant chemotherapy, a significant difference could be detected in survival of patients who reached a

minimum 90% tumour-cell necrosis level, and those who did not reach that level (p=0.018). Healing results of children under 14 are significantly better (p=0.008). No difference was found from the point of view of patients' survival related to the time of diagnosis (before or after 1996), their gender, the histological sub-type and also to the localization of the disease on the lower or upper limb, although its localization on torso was found a bad prognostic factor.

In our pharmacokinetic analysis we analyzed 989 MTX blocks of 98 patients out of this 122 children with osteosarcoma, founding significant correlations between the pharmacokinetic parameters and occurrence of toxicity. In patients suffering from severe hepatic toxicity the top concentration ($p=10^{-7}$) and the area under the 48-hour curve ($p=10^{-7}$) were significantly higher, while the MTX clearance proved to be lower ($p=5x10^{-5}$). There was a more frequent occurrence of nephrotoxicity subsequent to 24-hour MTX serum levels ($p=10^{-7}$). Occurrence of bone marrow toxicity was in correlation with the higher 24-hour MTX serum levels ($p=4x10^{-4}$) and the higher 48-hour MTX serum levels (p=0.001). There was no significant difference in the pharmacokinetic parameters and occurrence of toxicity from the point of view of gender, age, presence of metastasis and histological response. We found a significant difference, however, related to risk groups. In the high risk groups the area under the 48-hour curve was lower ($p= 10^{-6}$). In standard risk groups occurrence of liver toxicity was more frequent than in the high risk group (p = 0.0083).

The 48-hour MTX serum level according to the Cox proportional hazards model test shown a significant correlation to both the event-free (p=0.047) and the overall survival (p=0.008). In case of higher serum levels survival of the patients was better. Having examined the correlation of top concentration to survival, the difference was not significant in survival of patients with top concentration above and under 1000 μmol/l (p=0.061), although a tendency of better event-free survival results could be observed in case of an average top concentration above 1000 μmol/l. No correlation was found between occurrence of toxicity and the outcome of treatment.

In our pharmacology analysis we tested data of altogether 571 MTX blocks of 62 patients. First we looked for correlations between polymorphisms of genes *GGH* and *SLC19A1* and the MTX kinetics and toxicity. While in the case of *GGH*-401 C>T polymorphism there was no significant difference in the 24-hour MTX clearance (p=0.1), in the patient group with genotype *SLC19A1* 80AA the 24-hour MTX clearance was lower than in the group of patients carrying the *SLC19A1* 80G allele (p=0.04). MTX level values of the 48-hour serum were significantly lower in the group of patients with *GGH*-401 TT genotype

than in those carrying the C-allele (p=0.016), while in the case of *SLC19A1* 80G>A polymorphism no significant difference could be detected (p=0.53). These results are coherent with the pharmacokinetic role of the *SLC19A1* transporter molecule and the *GGH* enzyme, as *SLC19A1* takes part in the initial phase of the MTX kinetics, while the gamma–glutamyl-hydrolase acts during the so called cellular elimination phase. Occurrence of severe acute hepatic impairment was less frequent in the *GGH*-401TT group than in patients carrying the C allele (p=0.00061), and more frequent in *SLC19A1* 80AA homozygous patients than in those carrying the G allele (p=0.00245). We compared the occurrence of severe acute hepatotoxicity in SLC19A1 80AA and *GGH*-401CC/CT genotype patients with in other patients (p=0.00014) so consequently the previous difference between *SLC19A1* 80AA homozygous patients and in those carrying the G allele became more expressed when the protection of *GGH*-401TT genotype was not present in *SLC19A1* AA genotype patients.

In our univariate association analysis the presence of rare *ABCG2* rs2231142 (OR:4.2, p=0.037) allele associated to longer first half-lives of MTX levels ($T_{\alpha 1/2}$), moreover, the presence of SNPs *SXR* rs7643038 (OR:2.6, p=0.02), rs3814055 (OR:2.22, p=0.04) shown a positive correlation as well. In the presence of allelic variant *ABCB1* rs9282564 (OR:4.2, p=0.04) the AUC_{0-48} values were higher, while these values were lower in the case of allelic variant *ABCC3* rs4793665 (OR:0.24, p=0.03), as well as in the case of the *ABCC2* rs3740066 homozygous variant genotype (OR:0.096, p=0.01). The top MTX concentrations proved to be higher in the presence of polymorphism *ABCB1* rs9282564 (OR:8.8, p=0.02). The presence of allelic variants of *SXR* rs3732361, rs3814058, rs6785049 SNPs showed correlation with higher 48-hour MTX concentration values. Risk of hepatic toxicity was lower in the presence of rare alleles of *SXR* rs3732361, rs3814058, rs6785049 SNPs. Risk of bone marrow toxicity was higher in the case of mutation of *ABCC2* rs2273697 (OR:3.3, p=0.02), and lower in the case of presence of rare alleles of *ABCC2* rs3740066 (OR:0.4, p=0.02) and *SXR* rs3732361, rs3814058, rs6785049 SNPs.

Using the univariate BMLA method, we analysed whether the presence of the above mentioned 29 SNPs would affect the pharmacokinetic factors (AUC_{0-48}, top MTX level, $T_{\alpha 1/2}$), as well as the hepatic and bone marrow toxicity. In brackets, after the SNP, the posterior probability is given, which indicates the level of probability the models may confirm the certain relationship between the SNP and the analyzed variable. The AUC_{0-48} is much likely to be influenced by *ABCB1* rs9282564 (0.47), *ABCC3* rs4793665 (0.31), *GGH* rs3758149 (0.45) and by *SXR* rs3814058 (0.38). The top concentration is much likely to be influenced by *ABCB1* rs9282564 (0.7), *ABCC3* rs4793665 (0.6) and *SXR* rs3814058 (0.58).

Hepatic toxicity may be influenced by *ABCC1* rs246219 (0.6), while bone marrow toxicity may be affected by the presence of *ABCC2* rs717620 (0.56).

In a multiple target variable BMLA analysis, beyond all possible inner interrelations of the cluster of target variables (pharmacokinetic and toxicity data) and explanatory variables (age, gender, applied dose, duration of infusion and the 29 SNPs), we also examined which explanatory variables will have direct relevance for the cluster of target variables. A relevant effect for the target variable cluster was detected in the case of *ABCB1* rs928256 (0.6) SNP, which affected the area under the curve and the top concentration too according to the univariate analysis; as well as the *ABCC3* rs4793665 (0.48) and the *GGH* rs3758149 (0.41) SNPs, the influence of which on the area under the curve could be demonstrated with the univariate analysis too. *SXR* rs3814058 (0.44) may also influence the system of correlation of toxicity with pharmacokinetics, as using the univariate BMLA method it was found to be related to the pharmacokinetic data, while using the univariate association method it showed correlation with occurrence of toxicity.

Conclusions

Among the 122 children with osteosarcoma treated in the 2nd Department of Paediatrics between 1988 and 2006 the 5-year overall survival was 68%, while the event-free survival was 62%; which correspond to the best international data. Having analyzed the prognostic factors, our results showed that in infant osteosarcoma patients with a tumour localized in the limb and without metastases, treated with limb preservation surgery and chemotherapy, chances of survival in younger ages proved to be excellent. We have found that the event-free survival of osteosarcoma patients under 14 is significantly better compared to those over 14, which has proven to be a new observation in the international literature.

Our pharmacokinetic analysis shows that the risk of toxicity is higher with higher MTX exposure, but at the same time in case of higher MTX serum levels the survival was significantly better. There was no significant correlation between toxicity and survival. Although prolonged presence of MTX in the system may lead to severe side-effects, it is beneficial, as it promotes effectiveness of the treatment. Therefore, our results justify the high dose MTX therapy, and in case of patients with insufficiently high MTX exposure, defined by the area under the concentration-time curve, further increase in dose of MTX may even improve effectiveness of the treatment. In patients of standard risk groups we detected a higher area under the curve, lower MTX clearance and an increased hepatic toxicity, than in

the high risk group. A possible explanation for that may be that in a patient with higher metabolic sensitivity to MTX, the anti-tumour effect will also be stronger, which results in a better histological response; so following the operation these patients will be classified in the standard risk group. Based on our results, the age of patients at the time of diagnosis as well as the area under the 48-hour curve measured in the course of neoadjuvant MTX blocks, could both be taken into consideration when determining risk classification of osteosarcoma patients.

In the course of our pharmacogenetic analyses we found that the MTX elimination is faster and severe side-effects occur less frequently in patients with *GGH* -401TT genotypes than in 401CC/CT infants. In presence of allele *SLC19A1* 80A, severe side-effects occur more frequently; and the difference gets stronger in patients where the protective effect of genotype *GGH* -401TT is not present. In our further analysis we used 3 different statistic methods to find correlation between the genotype and the clinical data. SNPs *ABCB1* rs928256, *ABCC3* rs4793665, *GGH* rs3758149 and *SXR* rs3814058 proved to have relevant effect in both the univariate and the multivariate analyses.

Haplotype data based on the above described 4 SNPs and pharmacokinetic data would allow developing a population pharmacokinetic model, which could forecast the probable pharmacokinetic parameters individually prior to the MTX therapy, thus ensuring an individual medication dosage for each patient.

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