

Megalin genetic polymorphisms and individual sensitivity to the ototoxic effect of cisplatin

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Ototoxicity and nephrotoxicity are dose-limiting side effects of cisplatin. Megalin, a member of the low-density lipoprotein receptor family, is highly expressed in renal proximal tubular cells and marginal cells of the stria vascularis of the inner ear – tissues, which accumulate high levels of platinum–DNA adducts. On the assumption that the mechanisms of cisplatin-induced nephro- and ototoxicity involve megalin we analyzed the incidence of the non-synonymous single nucleotide polymorphisms (SNP) rs2075252 and rs4668123 in 25 patients who developed a distinct hearing loss during cisplatin therapy and in 25 patients without hearing impairment after cisplatin therapy. We found no association between cisplatin-induced ototoxicity and any allele of rs4668123 but observed a higher frequency of the A-allele of rs2075252 in the group with hearing impairment than in the group with normal hearing after cisplatin therapy (0.32 versus 0.14) ($\chi^2 = 5.83$, $P < 0.02$; odds ratio: 3.45; 95% confidence interval: 1.11–11.2) indicating that SNPs at the megalin gene might impact the individual susceptibility against cisplatin-induced ototoxicity.

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Introduction

Cisplatin is effectively used for the treatment of childhood malignancies, such as neuroblastomas, osteosarcomas and germ cell tumors. Unfortunately the use of cisplatin is limited by its nephro- and ototoxicity.^{1–3} While cisplatin-induced nephrotoxicity is successfully reduced by forced diuresis and by vigorous hydration regimens, cisplatin-induced hearing loss still remains a problem. The risk estimates for ototoxicity after cisplatin range from 23 to 54% depending on the criteria used and the diligence of the search.^{4–6} Cumulative dosage, age < 5 years, pre-existing renal dysfunction or sensorineural hearing loss and high rates of cisplatin delivery were identified as risk factors for cisplatin-induced hearing loss.^{4,5} Apart from these risk factors interindividual susceptibility to the ototoxicity induced by cisplatin was observed in clinical studies as well as in animal tests. These interindividual differences were not correlated to variations in peak plasma concentrations of cisplatin but were associated with a discrete and transient renal dysfunction.⁷

In spite of these facts the mechanisms of the cisplatin-induced ototoxicity are poorly understood so far. Cisplatin extensively binds to DNA and proteins.⁸ The amount of platinum–DNA adducts in tumor cells correlates well with the antitumor activity of cisplatin.⁹ After treatment of guinea pigs with cisplatin Thomas *et al.*¹⁰ measured the formation and persistence of platinum–DNA adducts in the nuclei of inner ear cells of guinea pigs using immunofluorescence

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staining of platinum–DNA adducts by monoclonal antibodies combined with quantitative image analysis. They observed a pronounced three- to fivefold accumulation of platinum–DNA adducts exclusively in the marginal cells of the stria vascularis as compared to other cell types of the cochlea. These results were consistent with the ^{195}M platinum labeling studies of Schweitzer *et al.*,¹¹ who reported a two- to threefold increase in platinum uptake in the stria vascularis as compared to the organ of Corti.^{11,12} Thomas *et al.*¹⁰ also detected a three- to fourfold higher platinum–DNA adduct level in proximal tubular cells as compared to other renal cells. They supposed that a membrane pump expressed in the marginal cells of the stria vascularis and the proximal tubular cells was responsible for the high accumulation of platinum–DNA adducts especially in these cells as compared to adjacent tissues. Megalin is a multiligand endocytotic receptor, which is expressed primarily in polarized cells like the marginal cells of the stria vascularis and the proximal tubular cells of the kidney. Megalin has been associated with the uptake of aminoglycosides, which are known like cisplatin for their nephro- and ototoxicity.¹³ Thus, the high expression of megalin in the proximal tubular cells of the kidney and the marginal cells of the stria vascularis in the inner ear might play a role in the high sensitivity of these organs to cisplatin-induced toxicity. Against the background of polymorphisms and genetic variants determining inter-individual variations in drug effects we analyzed whether non-synonymous single nucleotide polymorphisms (SNPs) at the megalin gene, which occur with a high frequency in Caucasians, might be related to an increased incidence of cisplatin-induced ototoxicity.

Results

The distribution of the non-synonymous SNPs rs2075252 and rs4668123 at the megalin gene was analyzed in 74

children, who received cisplatin for their cancer treatment. In order to exclude other risk factors for cisplatin-induced hearing loss only patients who were devoid of these risk factors were considered eligible for the comparison of the rs2075252 and rs4668123 distribution between patients with and without cisplatin-induced ototoxicity after treatment. Thus, only 50 patients out of 74 children were available for further analysis. These patients were classified according to the grading system for the early detection of cisplatin-induced bilateral high-frequency hearing loss (Muenster classification) (Table 1) into two groups. Group 0 comprised patients with no hearing impairment (hearing grade ≤ 1) ($n=25$) and group S contained patients with a hearing grade ≥ 2 after cisplatin therapy ($n=25$).¹⁴ The clinical data of patients in group S and 0 are summarized in Table 2. The results for the distribution of alleles and genotypes of the megalin gene are presented in Table 3.

Overall there were no significant differences in distribution of the alleles of both SNPs between the patients and the reference group (Table 3) (<http://www.hapmap.org>). However, the allele distributions of SNP rs2075252 in group 0 and S were significantly different. The A-allele frequency in group S (0.32) was higher than in the group 0 (0.14), the group of all patients (0.22), and the reference group (0.26). This represented a significant intergroup difference (0 versus S: $\chi^2 = 5.83$; $P < 0.02$). In addition the distribution of the rs2075252 genotypes differed between both groups. No A/A homozygotes were detected in group 0, while three A/A homozygotes were found in group S. According to Fischer's exact test this difference was not statistically significant. The distribution of the G/G genotypes versus the G/A + A/A genotypes was significantly different between group S and 0 ($\chi^2 = 4.14$, $P = 0.041$).

With respect to the power of this study with 25 cases and 25 controls and a minor allele frequency of 0.258 for the A-allele of rs2075252, there was 80 % power to detect an odds ratio (OR) of 5.2. As the OR in our study was close to 3.45, the magnitude of power was only 34 % for the analysis of

Table 1 Muenster classification for early detection of cisplatin-induced bilateral high-frequency hearing loss (14)

Bilateral hearing loss	Pedaudiological valuation according to WHO classification	Grade
≤ 10 dB at all frequencies	No considerable damage	0
> 10 to ≤ 20 dB at all frequencies or tinnitus	Questionable, commencing damage	1
Hearing loss ≥ 4 kHz, > 20 dB	Moderate damage	2
> 20 to ≤ 40 dB		2a
> 40 to ≤ 60 dB		2b
> 60 dB		2c
Hearing loss < 4 kHz; > 20 dB	Impairment compensable with hearing aid	3
> 20 to ≤ 40 dB		3a
> 40 to ≤ 60 dB		3b
> 60 dB		3c
Mean hearing loss < 4 kHz; ≥ 80 Db	Loss of function, compensable by cochlear implantation	4

* The results were obtained by pure-tone audiometry from both ears.

Table 2 Clinical data of the patients

	Group 0 (n = 25)	Group S (n = 25)
Mean age at therapy (range, years)	13,6 (5–19)	12,6 (6–22)
Sex (male/female)	13/12	14/11
Histology		
Osteosarcoma	16	22
Neuroblastoma	1	2
Medulloblastoma	4	1
Germ cell tumor	2	0
Teratoma	1	0
Testicle cancer	1	0
Grade of hearing loss after therapy (0/1/2/3)	20/5/0/0	0/0/15/10
Chemotherapy protocol		
COSS 82/86/91	1/1/14	0/4/18
Hit 91	4	1
MAKEI 89	3	0
MAHO-94	1	0
NB-90	1	2
Mean cumulative dose of cisplatin at the end of therapy (mg/m ²)	434	425
Cumulative dose of cisplatin on reaching grade ≥2 (mean, range, mg/m ²)	—	297 (120–480)

Table 3 Results of the Taqman SNP genotyping

Gene/SNP	Genotypes	References ^a		Patients		Group 0		Group S		p ^{b,c}	OR ^d (95% CI)
		n = 60	%	n = 74	%	n = 25	%	n = 25	%		
		n	%	n	%	n	%	n	%		
LRP2 rs 2075252 Glu4094Lys	GG	33	55.0	45	60.8	19	76.0	12	48.0		1.00 (Reference)
	AG	23	38.3	25	33.8	6	24.0	10	40.0	0.121	2.64 (0.65–11.1)
	AA	4	6.7	4	5.4	0	0.0	3	12.0	0.076 ^e	109 (0.68–7133)
	AG+AA	27	45.0	29	39.2	6	24.0	13	52.0	0.041	3.43 (0.88–13.8)
	A allele	0.258		0.223		0.120		0.320		0.016	3.45 (1.11–11.2)
		n = 60		n = 73		n = 24		n = 25			
		n	%	n	%	n	%	n	%		
LRP2 rs 4668123 Thr2872Ala	CC	32	53.3	41	56.2	15	62.5	13	52.0		1.00 (Reference)
	CT	22	36.7	26	35.6	9	37.5	7	28.0	1.000	0.89 (0.22–3.67)
	TT	6	10.0	6	8.2	0	0.0	5	20.0	0.049 ^e	126 (0.87–8211)
	CT+TT	28	46.7	32	43.8	10	41.7	12	48.0	0.458	1.54 (0.42–5.64)
	T allele	0.283		0.260		0.188		0.340		0.087	2.32 (0.80–6.30)

^aReference data were taken from <http://www.hapmap.org>.

^bCompared with the GG genotype for rs2075252 and the CC genotype for rs4668123.

^cχ² test for the distribution of either genotype or allele frequencies between group S and group 0.

^dOR, odds ratio; CI, confidence interval.

^eFischer's exact test for the distribution of GG and AA genotypes (rs2075252) and CC and TT genotypes (rs4668123) between group S and group 0.

A-allele distribution between both groups. In addition with a frequency of 0.45 for the A/G + A/A genotypes of rs2075252 and an OR of 3.43 the magnitude of power was only 55 % for the analysis of G/G and A/G + A/A genotype distribution.

The allele distributions of SNP rs4668123 in group 0 and S were not significantly different. The T-allele frequency in group S (0.34) was higher than in the reference group (0.28), the group of all patients (0.26) and the group 0 (0.21).

But this represented no significant intergroup difference (0 versus S: $\chi^2 = 2.92$; $P = 0.087$). The distribution of the T/T genotype of the rs4668123 SNP differed significantly between the both groups. There was an accumulation of T/T homozygotes in group S (0.20) compared to group 0 (0) (Fischer's exact test $P = 0.049$). The distribution of C/C genotypes and C/T + T/T genotypes did not differ significantly between group S and 0.

Remarkably there were neither T/T homozygotes of rs4668123 nor A/A homozygotes of rs2075252 in group 0 compared to group S including five T/T homozygotes and three A/A homozygotes. All A/A homozygotes of rs2075252 ($n = 3$) also were T/T homozygotes of rs4668123.

The results of the haplotype analysis are summarized in Table 4. The AT haplotype was noted in 23.8% of the cases and 9.9% of controls. However, this difference was not significant when the haplotypes were analyzed using Haploview (χ^2 : 3.472; $P = 0.0624$). Linkage analysis using the Haploview software resulted in a LOD score of 10.6 for rs2075252 and rs4668123.

Discussion

In tissues, highly susceptible to cisplatin toxicity, higher levels of platinum–DNA adducts were observed as compared to tissues less affected by cisplatin toxicity. The selective accumulation of cisplatin in the marginal cells of the stria vascularis of the cochlea and the proximal tubular cells of the kidney suggests the existence of a membrane pump or a receptor-mediated uptake of cisplatin. Megalin is highly expressed in the marginal cells of the stria vascularis and the proximal tubular cells. Mizuta *et al.*¹⁵ examined the localization of megalin using a post-embedding immunogold method in the rat cochlear duct. Labeling was seen predominantly on the apical membrane and subapical vesicles of strial marginal cells. Cells not stained by the antibody included outer hair cells, inner hair cells and supporting cells. Megalin is involved in the endocytosis of many different endogenous ligands, such as the binding proteins for retinol or vitamin D, and drugs as aminoglycosides. The transport of aminoglycosides is strongly linked to the endocytotic receptor megalin, which is highly expressed in the proximal tubular cells damaged by aminoglycosides. Aminoglycosides as well as cisplatin are well known for their nephro- and ototoxicity and both cisplatin and aminoglycosides show the same manifestation of organ toxicity. Cisplatin is a strong nucleophilic compound and can easily and reversibly react with low-molecular-weight proteins or oligopeptides. Today numerous polymorphisms are known, which modulate the function, expression and stability of proteins involved in drug metabolism, transport and elimination. Especially non-synonymous SNPs, which result in amino-acid exchanges, are likely to impact protein function. Supposing that megalin is involved in the transport

of cisplatin we analyzed whether two non-synonymous SNPs in the megalin gene were linked to the interindividual susceptibility to cisplatin-induced ototoxicity.

We found that the A-allele of rs2075252, the AG + AA genotype of rs2075252 and the T/T genotype of rs4668123 were linked with an increased risk of patients to develop hearing loss during cisplatin therapy. All A/A homozygotes of the SNP rs2075252 and all T/T homozygotes of the SNP rs4668123 were only detected in the group of patients who experienced cisplatin-induced ototoxicity. Moreover, all patients with an A/A genotype of rs2075252 displayed a T/T genotype of rs4668123. The AT haplotype was detected in 23.8% of cases and 9.8% of controls, which did not reach statistical significance. The low number of patients eligible for this study certainly influenced the association testing of the haplotype analysis as well as the power of test results. Thus, we acknowledge that the study is underpowered and requires validation.

Since apart from the marginal cells of the stria vascularis megalin is highly expressed in the proximal tubular cells of the kidney, the patients who experienced cisplatin-induced ototoxicity might have also suffered from cisplatin-induced nephrotoxicity. All patients received vigorous hydration along with their cisplatin infusions. Normal renal function was required for the application of cisplatin and renal function was monitored routinely before each course of cisplatin. However, since the introduction of vigorous hydration nearly abolished cisplatin-induced nephrotoxicity, renal function was hardly analyzed during or after the administration of cisplatin in these patients. Thus, it was impossible to analyze the distribution of the megalin SNPs rs2075252 and rs4668123 along with cisplatin-induced nephrotoxicity in this study.

The A-allele of the SNPs rs2075252, which occurred with a significantly higher frequency in patients who experienced cisplatin-induced ototoxicity, was not found in all patients, who were affected by cisplatin-induced hearing loss and who were devoid of other risk factors for ototoxicity. Apart from megalin other mechanisms of cisplatin uptake in the cells exist and megalin is not a prerequisite for cisplatin uptake. Despite of this so far exploratory analysis the reported results indicate that megalin might be linked to the transport of cisplatin or cisplatin adducts and that polymorphisms of the megalin gene in turn might be associated with patients' interindividual susceptibility against cisplatin-induced ototoxicity. Further studies on the role

Table 4 Results of the haplotype analysis by Haploview

	Haplotype frequency	Case, control ratios ^a	χ^2	P-value
GC	0.72	31.9:18.1; 39.7:10.3	3.02	0.08
AT	0.181	11.9:38.1; 4.9:45.1	3.47	0.06
GT	0.077	5.1:44.9; 4.3:45.7	0.08	0.78
AC	0.022	1.1:48.9; 1.1:48.9	0.00	0.99

^acase, control counts.

of megalin in cisplatin transport will clarify the role of megalin and mutated megalin in cisplatin transport and cisplatin-induced ototoxicity.

Patients and methods

Patients

Seventy-four patients, who were diagnosed with osteosarcomas ($n=38$), germ cell tumors ($n=2$), testicle cancers ($n=2$), hepatoblastoma ($n=1$), teratoma ($n=1$), neuroblastomas ($n=17$) and brain tumors ($n=13$), participated in the study. The study was approved by the institutional review board and all patients and/or their guardians gave their written informed consent to participate in the study. Cisplatin chemotherapy was applied according to the treatment protocols of the German Society of Pediatric Hematology and Oncology at the Department of Pediatric Hematology and Oncology of the University Children's Hospital of Muenster.

To exclude other risk factors for hearing loss the distribution of megalin SNPs rs2075252 and rs4668123 was only evaluated in patients, who had no hearing impairment before therapy (audiogram grade = 0), who were > 5 years at the time of therapy, who did not receive cranial irradiation or ototoxic medication in addition to cisplatin and who were devoid of renal impairment before cisplatin therapy.¹⁶ According to these criteria 22 out of 74 patients were excluded from further analysis; 14 patients were younger than 5 years, two patients have already suffered from renal insufficiency before start of cisplatin therapy and six patients have received cranial irradiation. Two patients, who had developed no hearing impairment, were also excluded from the study, as they have received cumulative cisplatin dosages of 80 and 120 mg/m², respectively. The risk of inducing a hearing impairment below a cumulative dose of 240 mg/m² is negligible.¹⁶ Since it could not be excluded that patients, who received a cumulative cisplatin dose below 240 mg/m², might have experienced cisplatin-induced ototoxicity at higher dosages, a cumulative dose of 240 mg/m² was considered eligible for patients without cisplatin-induced hearing impairment to enter the study. The characteristics of patients eligible for the study are summarized in Table 2.

The osteosarcoma patients received two cycles of cisplatin-based chemotherapy with 90–150 mg/m² cisplatin according to the COSS protocols COSS 82/86/91. The total cisplatin dose in these protocols ranged from 180 to 300 mg/m². The neuroblastoma patients were treated according to the NB 90 protocol with a continuous cisplatin infusion (40 mg/m²/day) over 96 h. On the basis of tumor staging patients received either two or four courses of cisplatin resulting in total dosages of 320 and 640 mg/m², respectively. In the MAHO 94 protocol standard chemotherapy consisted of four courses with cisplatin given at 20 mg/m² per day on 5 consecutive days (total cisplatin dose: 400 mg/m²). For high-risk patients the MAHO 94 protocol prescribes an additional salvage therapy with four courses of cisplatin

given at 20 mg/m²/day on 4 consecutive days (total cisplatin dose: 320 mg/m²). MAKEI 89 patients with incompletely resected immature teratomas grade 3 received three courses of cisplatin 20 mg/m²/day applied on days 1–5 (total cisplatin dose: 300 mg/m²). According to the HIT 91 protocol patients with medulloblastomas were randomized to receive either two courses of chemotherapy after surgery and before irradiation including 40 mg/m²/day cisplatin applied on 3 consecutive days or eight cycles of chemotherapy containing 70 mg/m²/day cisplatin for maintenance after surgery and irradiation (cumulative cisplatin dosages: 240 and 560 mg/m², respectively).

Grading system for the early detection of cisplatin-induced bilateral high-frequency hearing loss: Muenster classification

Before therapy, after every course of cisplatin and at the follow-up audiologic examinations were performed, including pure-tone audiometry via air and bone (in children younger than 3 years sound-field audiometry), tympanometry, and recording of transient click-evoked and distortion product otoacoustic emissions (TEOAE and DPOAE). If necessary, brain-stem audiometry was performed. The degree of hearing impairment was determined by using the 'Muenster classification' for early detection of cisplatin-induced bilateral high-frequency hearing loss (Table 1).¹⁴ A pure-tone audiogram of grade 0 was necessary to enter the study.

Genotyping

Ethylendiaminetetraacetic acid blood samples were obtained from all patients, who participated in the study. DNA was isolated from peripheral blood mononuclear cells according to standard research protocols. Genotyping was done using the validated genotyping assays C_16165996_10 for rs2075252 and C_3017531_1_ for rs4668123 designed and supplied by Applied Biosystems (Applied Biosystems GmbH, Darmstadt, Germany). We only chose these SNPs (rs2075252 and rs4668123) out of 757 SNPs reported for the megalin gene so far, since they were non-synonymous and occurred at frequencies which match those of cisplatin-induced hearing loss.^{4–6} The SNP rs2075252 is characterized by a G→A exchange in position 12384 of the megalin mRNA. This results in an amino-acid exchange of glutamic acid against lysine. The SNP rs4668123 results in an amino-acid exchange of threonine versus alanine at position 2872 of megalin protein because of the nucleotide transition A→G in position 8718 of the megalin mRNA. According to blast search both SNPs are located in a protein structure related to the A chain of the extracellular domain of the low-density lipoprotein receptor (<http://www.ncbi.nlm.gov/blast>).

The SNP genotyping assays used are based on the TaqMan PCR technology combined with SNP-specific fluorescent minor groove-binding probes.^{17,18} All assays were carried out according to the manufacturer's instructions. In brief, the PCR was carried out in a 96-well plate, each well containing 200 ng DNA, 12.5 μ l TaqMan Universal PCR Master Mix (Applied Biosystems), 1.25 μ l of probe and primer solution (Applied Biosystems) and 11.25 μ l distilled water. PCR was initiated at 95°C for 10 min, followed by 40

cycles of 92°C for 15 s and 60°C for 60 s. After PCR fluorescence was measured in an ABI Prism 7700 Sequence Detector (Applied Biosystems).

Because of missing material one male osteosarcoma patient (age: 15 years) was only genotyped for the SNP rs2075252 and not for the SNP rs4668123 (Table 3). This patient received cisplatin according to the COSS 91 protocol and experienced no hearing impairment (grade 0) after cisplatin therapy (cumulative dose of cisplatin: 480 mg/m²).

Statistics

Single-marker case-control differences were evaluated for both megalin variants rs2075252 and rs4668123 using the χ^2 test, if the total sum was >40 and each frequency >5. At frequencies <5 the Fisher's exact test was used. The level of statistical significance was set at $P < 0.05$. ORs and 95% confidence intervals for the two markers in the contingency tables were calculated. Statistical analysis was performed with the JUMBO statistical package (University of Muenster, Germany, <http://imib.uni-muenster.de>). Linkage and haplotype analysis was done using Haploview. This program takes one haplotype at a time and compares it between cases and controls.

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Duality of Interest

The authors confirm that there is no duality of interest.

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