

Translesion Polymerase Genes Polymorphisms and Haplotypes Influence Survival of Osteosarcoma Patients

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Abstract

Cytotoxic activity of most chemotherapeutic agents is based on their ability to induce DNA damage. Interstrand crosslinks are among the most detrimental forms of DNA damage as both DNA strands are affected. As translesion polymerases participate in their repair, they may be important for response to chemotherapeutic agents that induce such lesions, including commonly used cisplatin. Altered expression of translesion polymerase genes *REV1* and *REV3L* may modify sensitivity to cisplatin. As osteosarcoma patients are commonly treated with cisplatin-based chemotherapy, our aim was to investigate if *REV1* and *REV3L* polymorphisms influence survival of osteosarcoma patients treated with cisplatin-based chemotherapy. We determined the genotypes of common functional tag *REV1* and *REV3L* polymorphisms in 66 osteosarcoma patients. Cox regression was used for survival analysis. Carriers of at least one polymorphic *REV1* rs3087403 allele had significantly shorter EFS and overall survival (OS) ($p=0.004$; HR=3.79; 95%CI=1.53–9.35 and $p<0.001$; HR=4.44; 95%CI=1.92–10.27, respectively). Combination of *REV1* rs3087403 and *REV3L* rs462779 polymorphisms was also significantly associated with shorter OS ($p_{\text{trend}}<0.001$) and shorter EFS ($p_{\text{trend}}=0.003$). The results of this first study on polymorphisms in translesion polymerase genes in osteosarcoma suggest they could help predict outcome of cisplatin-based chemotherapy in osteosarcoma patients.

Introduction

CYTOTOXIC ACTIVITY OF MANY CHEMOTHERAPEUTIC AGENTS is based on the ability to induce DNA damage in rapidly dividing tumor cells (Woods and Turchi, 2013). Different mechanisms of action of individual therapeutics lead to formation of different types of DNA damage. Several different DNA repair pathways are involved in response to chemotherapy-induced DNA damage and can affect both the cytotoxic activity in tumor cells or toxicity in normal cells (Woods and Turchi, 2013).

Cisplatin is one of the most commonly used antitumor agents. The bifunctional electrophilic platinum compounds covalently bind to DNA and form DNA adducts, including intra- and interstrand crosslinks (ICLs), thus inhibiting DNA replication (Marsh et al., 2009). Intrastrand crosslinks are more common, but ICLs are more detrimental, as they compromise both strands and completely prevent DNA replication and transcription. Successful ICL repair is thus essential for genome stability and cell survival (Enoiu et al., 2012; Sharma and Canman, 2012) and requires interplay of different DNA repair pathways (Ho and Scharer, 2010). Apart from nucleotide excision repair and homologous re-

combination repair, translesion (TLS) polymerases have a crucial role in this process.

TLS polymerases polymerase zeta (Pol ζ) and DNA polymerase REV1 (DNA directed) (REV1) are involved in both replication-independent and replication-dependent mechanisms of ICL repair, as they enable the bypass of ICLs and restoration of one of the DNA strands (Ho and Scharer, 2010). Pol ζ , whose catalytic subunit is encoded by the *REV3L* gene, is involved in the extension step of TLS synthesis, while REV1 coordinates TLS synthesis through interactions with Pol ζ and other TLS polymerases, but also has a deoxycytidyltransferase activity (Ho and Scharer, 2010).

Differences in expression of either *REV1* or *REV3L* have been associated with modified sensitivity to cisplatin (Doles et al., 2010; Lin et al., 2006), but the role of single nucleotide polymorphisms (SNPs) in these genes has not been explained yet. Some studies have shown that these SNPs may modify cancer susceptibility (He et al., 2008; Pan et al., 2012; Sakiyama et al., 2005; Varadi et al., 2011; Zhang et al., 2013). The influence on response to cisplatin treatment was previously unknown, so in our recent studies we have used a haplotype-based approach to select common functional *REV1* and *REV3L* tag SNPs in the coding region or 3'

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untranslated region that may influence enzyme function on expression. We have shown that they influence cisplatin-based treatment outcome in malignant mesothelioma (Gorincar et al., 2014a). However the role of genetic variability of TLS polymerases in treatment of other cancers remains to be elucidated.

Cisplatin-based chemotherapy is used also in treatment of osteosarcoma, a bone malignancy that mostly affects adolescents and has a very low incidence of approximately 3 patients per million people per year (Ritter and Bielack, 2010). Current chemotherapy protocols include cisplatin in combination with doxorubicin, high-dose methotrexate, and/or ifosfamide (Quartuccio et al., 2013). Such multiagent chemotherapy significantly improved prognosis of the disease (Ritter and Bielack, 2010), but survival rates have not improved since the start of chemotherapy use, and poor outcome, local relapse, or metastases remain a challenge in as much as half of osteosarcoma patients (Chou and Gorlick, 2006; Salinas-Souza et al., 2010).

Apart from clinical characteristics, interindividual differences in treatment outcome may be attributed to genetic variability of mechanisms involved in response to chemotherapeutic agents and some polymorphisms have already been associated with response to methotrexate or cisplatin (Biason et al., 2012; Caronia et al., 2009; Gorincar et al., 2014b; Hao et al., 2012; Yang et al., 2012), but the role of genetic variability in TLS polymerases has so far not been investigated in osteosarcoma. The aim of the present study was to evaluate the influence of polymorphisms and haplotypes in TLS polymerase genes on the survival of osteosarcoma patients treated with cisplatin-based chemotherapy.

Materials and Methods

Patients

Slovenian osteosarcoma patients diagnosed between 1990 and 2008 were eligible for inclusion in our retrospective study. We included all osteosarcoma patients who were treated with cisplatin-based chemotherapy at the Department of Hematology and Oncology, University Children's Hospital, Ljubljana, Slovenia, or at the Institute of Oncology, Ljubljana, Slovenia, and had sufficient formalin fixed, paraffin embedded (FFPE) material for DNA extraction and data from medical records available. The study was approved by the Slovenian Ethics Committee for Research in Medicine and was carried out according to the Declaration of Helsinki.

Assessment of treatment outcome

Demographic, clinical, and treatment parameters were obtained from the medical records. The primary outcome of interest was survival of osteosarcoma patients. Event-free survival (EFS) was defined as time from the beginning of treatment to disease recurrence, development of metastases, or death. Overall survival (OS) was defined as time from the beginning of treatment to death. If disease recurrence, development of metastases, or death did not occur to the time of the analysis, we censored patients at the date of the last follow-up.

DNA extraction and genotyping

Hematoxylin and eosin-stained sections of each FFPE sample obtained at surgery were examined by an experienced

pathologist to confirm the diagnosis and select areas representative of tumor or normal tissue. Genomic DNA was isolated from two to three cores (1 mm in diameter) of histologically confirmed normal tissue using QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Gorincar et al., 2014). Genotyping of *REV1* rs3087403 (p.Val138Met), rs3087386 (p.Phe257Ser), and rs3087399 (p.Asn373Ser) and *REV3L* rs462779 (p.Thr1224Ile), rs455732 (p.Val1430=), rs3204953 (p.Val3064Ile), rs465646 (c.*461C>T) tag SNPs (Gorincar et al., 2015) was performed using KASPar assay. Genotyping was repeated in 20% samples to check for genotyping accuracy.

Statistical analyses

Median and interquartile range or frequencies were used to describe the distribution of continuous and categorical variables, respectively. Deviation from Hardy-Weinberg equilibrium (HWE) was calculated with the standard chi-square test. Cox proportional hazards model was used in survival analysis to calculate hazard ratio (HR) and the 95% confidence interval (CI). A dominant genetic model was used in all statistical analyses. All statistical analyses were carried out by IBM SPSS Statistics, version 19.0 (IBM Corporation, Armonk, NY, USA). Thesias software was used for haplotype analysis (Tregouet and Garelle, 2007) as previously described (Erculj et al., 2012): the most frequent haplotype was used as reference and only haplotypes with frequencies above 5% were included in the analyses. Benjamini-Hochberg false discovery rate (FDR) was used to account for multiple comparisons (Benjamini and Hochberg, 1995) and *p* values less than 0.010 were considered significant after correction.

Results

Patients' characteristics

A total of 66 osteosarcoma patients treated with cisplatin-based chemotherapy were included in our study. Thirty-five (53.0%) were male. Patients' median age was 17.5 (13.8–33.3) years. The majority of patients had osteoblastic osteosarcoma located in the extremities [59 (89.4%) and 57 (86.4%), respectively]. Median tumor size was 9.5 (7.5–11.5) cm. Regarding stage, 18 (27.3%) patients had stage 1 osteosarcoma, 36 (54.5%) stage 2, and 10 (15.2%) stage 3, while the information was missing for two patients. Most patients, 38 (57.6%), were treated with multiagent chemotherapy that included both cisplatin and methotrexate, while 28 (42.4%) were receiving only combination of cisplatin and doxorubicin. Only four (6.1%) patients were not surgically treated and among the rest, ten (15.1%) patients were receiving adjuvant chemotherapy.

All patients were genotyped for *REV1* and *REV3L* SNPs. Their minor allele frequencies (MAFs) are presented in Table 1. All of the genotype frequencies were in HWE except *REV3L* rs455732 ($p=0.014$), which was excluded from further analyses.

Survival analysis

The median follow-up time for osteosarcoma patients in our study was 143.0 (109.1–205.6) months. A total of 39 (59.1%) patients experienced an event and 33 (50.0%) had

TABLE 1. ASSOCIATION OF INVESTIGATED POLYMORPHISMS WITH EVENT-FREE AND OVERALL SURVIVAL IN OSTEOSARCOMA PATIENTS

Gene	Polymorphism	MAF	Genotype CT+TT	Event-free survival		Overall survival	
				HR (95% CI)	p^a	HR (95% CI)	p^a
REV1	rs3087403	0.24	CC	Reference	0.004	Reference	< 0.001
	p.Val138Met		CT+TT	3.79 (1.53–9.35)		4.44 (1.92–10.27)	
	rs3087386	GG	Reference	Reference			
	p.Phe257Ser ^b	GA+AA	1.19 (0.42–3.39)	1.96 (0.72–5.33)			
REV3L	rs3087399	0.45	TT	Reference	0.744	Reference	0.189
	p.Asx373Ser		TC+CC	1.38 (0.59–3.26)		0.87 (0.41–1.82)	
	rs462779	TT	Reference	Reference			
	p.Thr1224Ile	TC+CC	2.49 (0.98–6.36)	2.60 (1.18–5.74)			
REV3L	rs3204953	0.14	GG	Reference	0.998	Reference	0.265
	p.Val3064Ile		GA+AA	0.99 (0.40–2.48)		1.56 (0.71–3.51)	
	rs465646	AA	Reference	Reference			
	c.*461C>T	AG+GG	1.33 (0.46–3.83)	1.50 (0.66–3.44)			

^a p less than 0.010 was considered statistically significant after correction for multiple comparisons; ^bdata missing for 1 patient. CI, confidence interval; HR, hazard ratio; MAF, minor allele frequency; OR, odds ratio.

died by the time of the analysis. The median EFS was 27.2 (10.9–136.3) months, while the median OS was 56.5 (25.4–140.7) months. Several clinical characteristics were associated with survival. Older patients had shorter EFS and OS ($p=0.003$; HR=1.02; 95%CI=1.01–1.04; and $p=0.001$; HR=1.03; 95%CI=1.01–1.05, respectively). Patients receiving multiagent chemotherapy including cisplatin and methotrexate had longer EFS and OS ($p<0.001$; HR=0.27; 95%CI=0.14–0.52; and $p=0.002$; HR=0.33; 95%CI=0.16–0.66, respectively) compared to patients treated only with the cisplatin and doxorubicin combination. Treatment with methotrexate was significantly correlated with age, as pediatric patients were more often treated with methotrexate according to the COSS protocols. Higher stage was also associated with shorter EFS and OS ($p<0.001$; HR=3.04; 95%CI=1.78–5.19; and $p=0.003$; HR=2.31; 95%CI=1.34–3.98, respectively). The size of the tumor was only associated with shorter EFS ($p=0.033$; HR=1.17; 95%CI=1.01–1.24). Due to correlations between clinical variables, only stage and age were included in the final multivariable model for OS, while the model for EFS was adjusted for stage, age, and tumor size.

The association of investigated polymorphisms with EFS and OS after adjustment for clinical variables is presented in Table 1. Carriers of at least one polymorphic REV1 rs3087403 or REV3L rs462779 allele had significantly shorter OS (HR=4.44; 95%CI=1.92–10.27; $p<0.001$ and HR=2.60; 95%CI=1.18–5.74; $p=0.018$, respectively). Polymorphic REV1 rs3087403 was also associated with significantly shorter EFS (HR=3.79; 95%CI=1.53–9.35; $p=0.004$; Fig. 1).

Since REV1 and Pol ζ interact during DNA repair, we also evaluated the combined effect of REV1 rs3087403 and REV3L rs462779 SNPs on survival of osteosarcoma patients. The combination of both SNPs was also significantly associated with shorter OS ($p_{\text{trend}}<0.001$) and patients with at least one polymorphic allele for each SNP had even shorter OS (HR=7.52; 95%CI=2.14–26.43). The same combination of SNPs in these two genes also conferred to shorter EFS ($p_{\text{trend}}=0.003$) and individuals with at least one polymorphic

allele for each SNP had much shorter EFS (HR=5.12; 95%CI=1.64–15.98). All these associations except the influence of REV3L rs462779 on OS remained significant after adjustment for multiple comparisons ($p<0.010$).

Haplotype analysis

As more than one SNP was investigated in most of the genes, we studied their combined effect using haplotype analysis. Four haplotypes with frequencies above 5% could explain approximately 88% of the REV1 variability (Table 2). Compared to the reference REV1 CAT haplotype, patients with the TGT haplotype had shorter OS, but the difference was not statistically significant ($p=0.058$). Four common

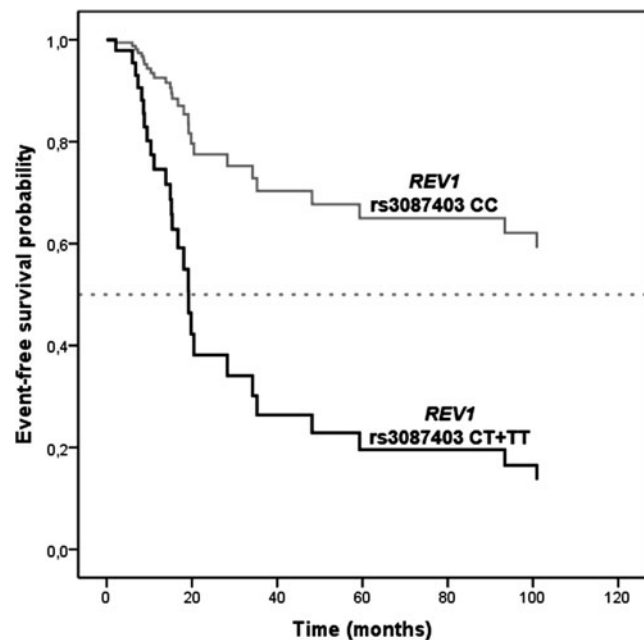


FIG. 1. The influence of REV1 rs3087403 on event-free survival of osteosarcoma patients.

TABLE 2. THE INFLUENCE OF HAPLOTYPES ON SURVIVAL IN OSTEOSARCOMA PATIENTS

Gene	Haplotype	Estimated frequency	Event-free survival		Overall survival	
			HR (95 % CI)	<i>p</i> ^a	HR (95 % CI)	<i>p</i> ^a
<i>REV1</i> ^b	CAT	0.45	Reference		Reference	
	TGT	0.18	1.58 (0.83–3.00)	0.161	1.91 (0.98–3.71)	0.058
	CGC	0.14	1.49 (0.77–2.91)	0.239	1.20 (0.59–2.44)	0.624
	CGT	0.11	0.73 (0.31–1.71)	0.465	0.54 (0.21–1.38)	0.197
<i>REV3L</i> ^c	TGA	0.65	Reference		Reference	
	TAA	0.14	1.11 (0.62–2.00)	0.731	1.61 (0.85–3.05)	0.142
	CGG	0.08	2.98 (0.96–9.26)	0.059	3.27 (1.20–8.85)	0.020
	TGG	0.06	0.67 (0.24–1.86)	0.446	0.79 (0.21–2.96)	0.715

^a*p* less than 0.010 was considered statistically significant after correction for multiple comparisons; ^bThe SNPs are ordered from the 5'- to 3'-end as follows: rs3087403, rs3087386, and rs3087399; ^cThe SNPs are ordered from the 5'- to 3'-end as follows: rs462779, rs3204953, and rs465646.

CI, confidence interval; HR, hazard ratio.

haplotypes could explain around 93% of the *REV3L* variability. Carriers of the *REV3L* CGG haplotype had shorter OS and EFS (*p*=0.059 and *p*=0.020, respectively), but the influence did not remain significant after correction for multiple comparisons.

Discussion

We present the results of the first study investigating SNPs in TLS polymerase genes and outcome of cisplatin-based treatment of osteosarcoma, showing that *REV1* SNPs influenced both EFS and OS.

TLS polymerases have recently been described as important factors in DNA repair. But even though TLS polymerases might alter chemosensitivity to cisplatin due to their involvement in ICL repair (Makridakis and Reichardt, 2012), there is little information about the role of the genetic variability of *REV1* and *REV3L* that encode two of the most important TLS polymerases. Only a handful of studies have previously studied *REV1* and *REV3L* SNPs, and even those have focused mostly on cancer susceptibility (He et al., 2008; Pan et al., 2012; Sakiyama et al., 2005; Varadi et al., 2011; Zhang et al., 2013). Both genes are very polymorphic and to cover most of the genetic variability within *REV1* and *REV3L* genes, we have previously used a tag SNP approach to select putative functional SNPs with MAF above 5% in the coding or 3' untranslated region that could influence enzyme activity or expression (Goricar et al., 2014a).

In our recent study of cisplatin-treated malignant mesothelioma patients, we showed for the first time that *REV1* and *REV3L* SNPs might contribute to interindividual differences in response to cisplatin (Goricar et al., 2014a). In the present study, polymorphic *REV1* rs3087403 was associated with shorter survival of osteosarcoma patients. *REV1* facilitates bypass of cisplatin-induced DNA damage (Lange et al., 2011) and decreased expression of *REV1* increases genomic instability and sensitivity to cisplatin (Ho and Schärer, 2010; Lin et al., 2006). To the best of our knowledge, the non-synonymous *REV1* rs3087403 (p.Val138Met) was not investigated in any previous study except in our study on malignant mesothelioma treatment, where it was associated with hematological toxicity. However, other *REV1* SNPs have been associated with modified lung cancer and cervical cancer risk (He et al., 2008; Sakiyama et al., 2005). Osteo-

sarcoma patients with at least one polymorphic *REV3L* rs462779 allele tended to have shorter OS. It was also shown that loss of Pol ζ increases genomic instability and that decreased *REV3L* expression increases sensitivity to cisplatin (Doles et al., 2010; Ho and Schärer, 2010). Studies have reported that *REV3L* rs462779 (p.Thr1224Ile) is associated with decreased breast cancer risk (Varadi et al., 2011), but increased colorectal cancer risk (Pan et al., 2012). Moreover, one study also observed that breast cancer patients with a polymorphic *REV3L* rs462779 allele had shorter time from surgery to an event (Varadi et al., 2011).

In our recent study on malignant mesothelioma, the *REV3L* rs462779 polymorphism conferred longer survival after cisplatin-based chemotherapy. The observed differences of the impact of *REV1* and *REV3L* SNPs in different cancer types could partly be explained by differences in chemotherapy combinations and doses used for treatment of a particular malignancy. For example, compared to patients with malignant mesothelioma, patients with osteosarcoma had considerably different toxicity profiles as other chemotherapeutic agents also contribute to occurrence of adverse events. Moreover, other clinical factors might also contribute to differences in treatment response.

Because studies have shown that *REV1* serves as a scaffold for other TLS polymerases (Tissier et al., 2004) and directly interacts with *REV3L* (Sharma et al., 2012), we also investigated the combined effect of both *REV1* rs3087403 and *REV3L* rs462779 SNPs, associated with survival in single SNP analysis. Combination of *REV1* rs3087403 and *REV3L* rs462779 polymorphic alleles was associated with further decrease in EFS and OS. In haplotype analysis, patients with the *REV1* TGT haplotype tended to have shorter OS, while patients with the *REV3L* CGG haplotype tended to have shorter OS and EFS. These results were not adjusted for stage due to the small number of patients with late stage of disease. This could in part explain why the observed results that did not withstand the correction for multiple comparisons.

Although our study brings novel and interesting results, it is limited by the small sample size. This is mainly due to the fact that osteosarcoma is a very rare cancer, so large cohorts are difficult to obtain, especially in small populations. However, an advantage of our study was that all patients were from an ethnically homogenous population (Vidan-Jeras et al., 1998). Another limitation of our study is the fact that the

patients were not all treated according to the same protocol. Even though cisplatin represents a cornerstone in osteosarcoma treatment, other chemotherapeutic agents contribute to treatment outcome, and validation studies should be limited to patients treated according to a single protocol.

Conclusions

The results of this first study investigating TLS polymerase genes in osteosarcoma identified TLS polymerase polymorphisms as a novel genetic factor associated with survival of osteosarcoma patients. Further studies are needed to validate our results and explore the potential role of TLS polymorphisms in personalization of osteosarcoma treatment.

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Abbreviations Used

CI = confidence interval
EFS = event-free survival
FDR = false discovery rate
FFPE = formalin fixed, paraffin embedded
HR = hazard ratio
HWE = Hardy–Weinberg equilibrium
ICL = interstrand crosslink
MAF = minor allele frequency
OS = overall survival
Pol ζ = TLS polymerases polymerase zeta
REV1 = DNA polymerase REV1 (DNA directed)
SNP = single nucleotide polymorphism
TLS = translesion