

## HOMOLOGOUS RECOMBINATION REPAIR POLYMORPHISMS AND THE RISK FOR OSTEOSARCOMA

### POLIMORFIZAM GENA ODGOVORNIH ZA REPARACIJU DNK HOMOLOGNOM REKOMBINACIJOM I RIZIK ZA POJAVU OSTEOSARKOMA

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#### Summary

**Background:** DNA repair mechanisms are essential for maintaining genome stability, and genetic variability in DNA repair genes may contribute to cancer susceptibility. Our aim was to evaluate the influence of polymorphisms in the homologous recombination repair genes *XRCC3*, *RAD51*, and *NBN* on the risk for osteosarcoma.

**Methods:** In total, 79 osteosarcoma cases and 373 controls were genotyped for eight single nucleotide polymorphisms (SNPs) in *XRCC3*, *RAD51*, and *NBN*. Logistic regression was used to determine the association of these SNPs with risk for osteosarcoma.

**Results:** None of the investigated SNPs was associated with risk for osteosarcoma in the whole cohort of patients, however, in patients diagnosed before the age of thirty years *XRCC3* rs861539 C>T and *NBN* rs1805794 G>C were associated with significantly decreased risk for osteosarcoma ( $P=0.047$ , OR=0.54, 95% CI=0.30–0.99 and  $P=0.036$ , OR=0.42, 95% CI=0.19–0.94, respectively). Moreover, in the carriers of a combination of polymorphic alleles in both SNPs risk for osteosarcoma was decreased even more significantly ( $P_{\text{trend}}=0.007$ ). The risk for developing osteosarcoma was the lowest in patients with no wild-type alleles for both SNPs ( $P=0.039$ , OR=0.31, 95% CI=0.10–0.94).

**Conclusions:** Our results suggest that polymorphisms in homologous recombination repair genes might contribute

#### Kratak sadržaj

**Uvod:** Mehanizmi reparacije DNK su neophodni za održavanje stabilnosti genoma i genetska varijabilnost u genima koji učestvuju u ovim procesima može doprineti riziku za pojavu kancera. Naš cilj je bio da procenimo uticaj polimorfizama u genima odgovornim za reparaciju DNK mehanizmom homologne rekombinacije, *XRCC3*, *RAD51* i *NBN*, na rizik za pojavu osteosarkoma.

**Metode:** Ukupno 79 uzoraka bolesnika sa osteosarkomom i 373 kontrola bilo je genotipizirano na osam pojedinačnih polimorfizama (SNP-ova) u genima *XRCC3*, *RAD51* i *NBN*. Logistička regresija korišćena je za određivanje povezanosti ovih SNP-ova sa rizikom za osteosarkom.

**Rezultati:** Nijedan od istraživanih SNP-ova nije bio povezan s rizikom za osteosarkom u čitavoj kohorti pacijenata, međutim, kod bolesnika mlađih od trideset godina kod kojih je dijagnostikovano osteosarkom, polimorfizmi *XRCC3* rs861539 C>T i *NBN* rs1805794 G>C su bili povezani sa znatno smanjenim rizikom za osteosarkom ( $P=0,047$ , OR=0,54, 95% CI=0,30–0,99 i  $P=0,036$ , OR=0,42, 95% CI= 0,19–0,94, redom). Osim toga, nosioci kombinacije polimorfnih alela za ove SNP-ove imali su još značajnije smanjen rizik za osteosarkom ( $P_{\text{trend}}=0,007$ ). Rizik za razvoj osteosarkoma bio je najniži kod bolesnika koji su bili homozigoti za normalne (wt) alele za oba SNP-a ( $p=0,039$ , OR=0,31, 95% CI=0,10 do 0,94).

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List of abbreviations: 95% CI, 95% confidence interval; DSB, double strand break; FFPE, formalin fixed, paraffin embedded; HRR, homologous recombination repair; OR, odds ratio; SNP, single nucleotide polymorphism; *XRCC3*, X-ray repair cross-complementing protein 3.

to risk for osteosarcoma in patients diagnosed below the age of thirty years.

**Keywords:** osteosarcoma, risk, DNA repair, polymorphism

## Introduction

Osteosarcoma is the most common bone malignancy, but is still a relatively rare tumor, with the incidence of around 3 patients per 1 000 000 people per year (1). Osteosarcoma occurs primarily among adolescents and young adults, with a peak of the incidence around 16 years, however, there is also a second peak of the incidence in elderly patients (2). It often occurs at the sites of rapid bone growth and studies have shown that osteosarcoma risk increases in taller children (3, 4). The etiology of the disease is not well explained. Osteosarcoma might be associated with rapid bone proliferation, but exposure to environmental agents can also contribute to susceptibility to osteosarcoma, especially in adults (1). The incidence of osteosarcoma is also higher in some hereditary disorders with germline alterations of tumor suppressor genes and DNA repair pathways (1, 3).

DNA repair mechanisms are essential for maintaining genome stability. Double strand breaks (DSBs) are one of the most detrimental forms of DNA damage because both strands of DNA are damaged (5). DSBs can form as a result of exposure to radiation or various chemotherapeutic agents, but are also produced endogenously, for example, during DNA replication (6). Homologous recombination repair (HRR) is one of the mechanisms involved in DSB repair (7, 8) and genetic variability of HRR genes has already been associated with cancer risk (9, 10). Several enzymes participate in HRR, among them also NBN, RAD51, and X-ray repair cross-complementing protein 3 (XRCC3). NBN is involved in DSB recognition, while RAD51, with help from mediator proteins like XRCC3, catalyses the strand transfer between the damaged region and the undamaged homologous chromatid and therefore enables resynthesis of the damaged region (11).

Single nucleotide polymorphisms (SNPs) in HRR genes have been associated with modified cancer susceptibility in various cancer types (9, 10), but no study reported on association with osteosarcoma. Nevertheless, evidence that variability in DNA repair mechanisms plays a role also in this cancer came from studies reporting that some SNPs in other DNA repair genes, especially *MDM2*, influenced risk for osteosarcoma (3, 12–14).

We have previously shown that selected tag SNPs in *NBN*, *RAD51*, and *XRCC3* genes influence the levels of DNA damage in healthy individuals (15).

**Zaključak:** Naši rezultati pokazuje da bi polimorfizam gena odgovornih za reparaciju DNK mehanizmom homologne rekombinacije mogao doprineti riziku za pojavu osteosarkoma kod bolesnika kod kojih je bolest dijagnostikovana pre tridesete godine života.

**Ključne reči:** osteosarkom, rizik, reparacija DNK, polimorfizam

Our aim was to determine whether *NBN*, *RAD51*, or *XRCC3* tag SNPs modify risk for developing osteosarcoma in Slovenian patients.

## Materials and Methods

### Subjects

We obtained the data on all the patients with histologically confirmed osteosarcoma, diagnosed between 1990 and 2008, from The Cancer Registry of Slovenia. Only patients that were treated at the Department of Hematology and Oncology, University Children's Hospital, Ljubljana, Slovenia, or at the Institute of Oncology, Ljubljana, Slovenia, and had available sufficient formalin fixed, paraffin embedded (FFPE) material for DNA extraction were included in our study. Unrelated healthy Slovenian blood donors between the ages of 18 and 65 years were used as the control group (15). The study was approved by the Slovenian Ethics Committee for Research in Medicine and was carried out according to the Declaration of Helsinki.

### DNA isolation and genotyping

Hematoxylin and eosin-stained sections of each FFPE sample were examined by an experienced pathologist to confirm the diagnosis and to select areas representative of tumor and areas representative of normal tissue. Two – three cores (1 mm in diameter) of histologically confirmed normal tissue were obtained from each specimen for DNA extraction using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Genotyping of tag SNPs in *XRCC3* (rs861539 C>T, rs1799794 A>G), *RAD51* (rs1801320 G>C, rs1801321 G>T, rs12593359 T>G), and *NBN* (rs1805794 G>C, rs709816 C>T, rs1063054 A>C) was carried out using a fluorescence-based competitive allele-specific (KASPar) assay (KBiosciences, Herts, UK) according to the manufacturer's instructions as previously described (15).

In the control group, DNA isolation from peripheral blood leukocytes and genotyping for the selected tag SNPs were performed during the previous study (15).

### Statistical analysis

Chi-squared test or Fisher's exact test were used for the comparison of categorical data distribution, while nonparametric Mann–Whitney U-test was used for comparison of numerical data. Logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) to examine the associations of selected SNPs with the risk for developing osteosarcoma. Dominant genetic model was used in all analyses. All statistical tests were two-sided and the level of significance was set to 0.050. All statistical analyses were carried out by IBM SPSS Statistics, version 19.0 (IBM Corporation, Armonk, NY, USA).

Incidence rates were calculated per 1 million inhabitants for five-year age groups. Data on all osteosarcoma cases between 1990 and 2008 were obtained from The Cancer Registry of Slovenia (16) and population data of all the inhabitants in Slovenia during the same period were obtained from the Statistical Office of the Republic of Slovenia (17).

### Results

Based on data from The Cancer Registry of Slovenia, 118 osteosarcoma patients were diagnosed in Slovenia between 1990 and 2008. Among them, 79 (66.9%) fulfilled all the inclusion criteria and were included in the study along with 373 healthy controls. The characteristics of the subjects are presented in Table I.

Osteosarcoma patients were significantly younger than healthy controls ( $P < 0.001$ ), but no differences regarding gender were observed ( $P = 0.252$ ).

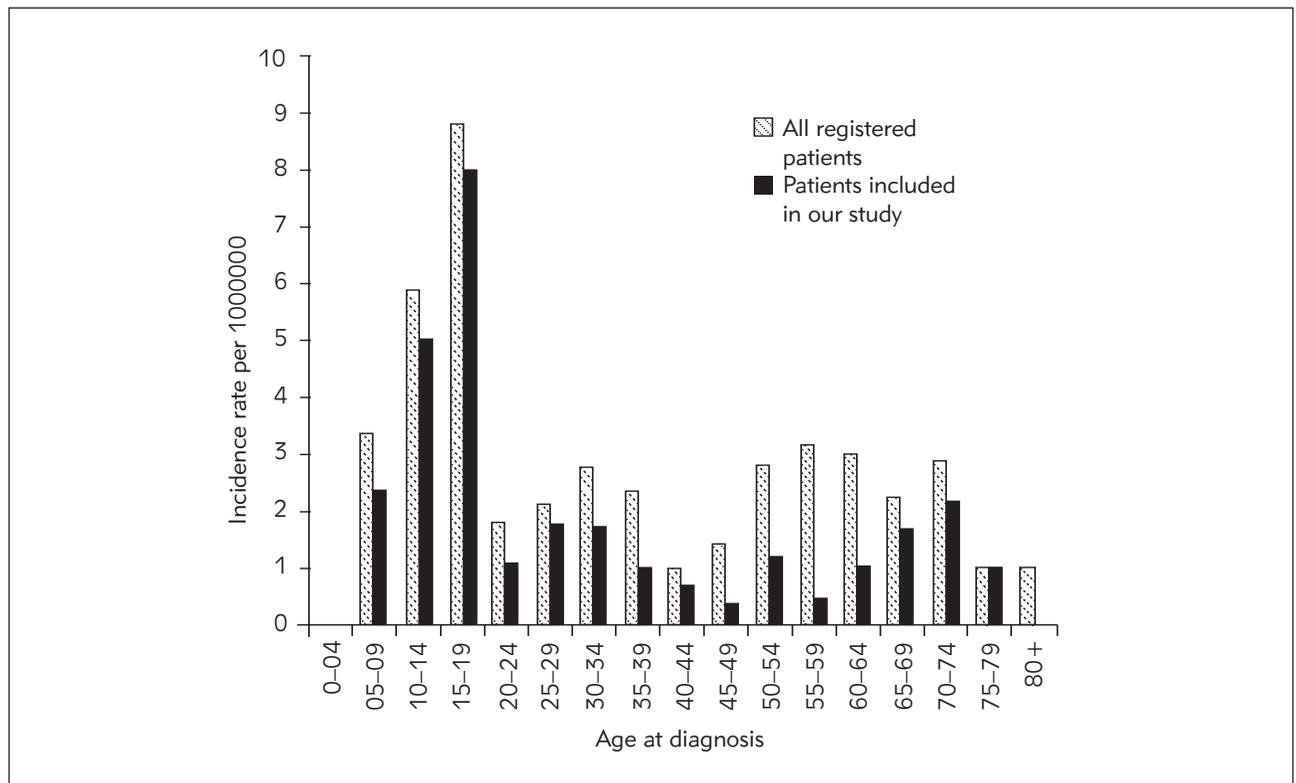
In total, 48 (60.8%) patients were younger than 30 years at the time of diagnosis. Two peaks in the average incidence rates of osteosarcoma were observed in our sample (Figure 1). The first peak occurred in the age group between 15 and 19 years and the second between 70 and 75 years. In comparison, data for all the patients diagnosed in Slovenia between 1990 and 2008 also showed the first peak in incidence rate in the age group between 15 and 19 years, however, the second increase of incidence rate started already after the age of 50 years (Figure 1).

We analyzed the risk for osteosarcoma in all patients, but also separately in osteosarcoma patients younger than 30 years at the time of diagnosis. The patients younger than 30 years did not differ from the whole group regarding gender ( $P = 0.855$ ) or histological type ( $P = 0.403$ , calculated for osteogenic osteosarcoma against other histological types). In four patients (5.1%), osteosarcoma developed as the secondary malignancy, but these patients were all older than 30 years.

No significant differences in the genotype frequency distribution of the investigated SNPs were observed when all the osteosarcoma cases were compared to healthy controls (Table II). However, patients diagnosed with osteosarcoma before the age of 30 differed significantly from healthy controls regarding the genotype distribution of XRCC3 rs861539 C>T ( $P = 0.047$ ) and NBN rs1805794 G>C ( $P = 0.036$ ) and the respective polymorphic XRCC3 rs861539 T and NBN rs1805794 C alleles were significantly associated with decreased osteosarcoma risk (OR=0.54, 95% CI=0.30–0.99 and OR=0.42, 95% CI=0.19–0.94, respectively) (Table II).

**Table I** The characteristics of controls and osteosarcoma patients.

Characteristic		Controls (%)	All cases (%)	Cases <30 years (%)
		N=373	N=79	N=48
Age	Median (interquartile range), years	30 (23–53.5)	19 (15–38)	15.5 (12.3–18.0)
Gender	Male	219 (58.9)	40 (51.9)	26 (54.2)
	Female	153 (41.1)	37 (48.1)	22 (45.8)
Osteosarcoma histological type	Osteogenic		70 (88.6)	47 (97.9)
	Chondroblastic		2 (2.5)	1 (2.1)
	Fibroblastic		3 (3.8)	
	Teleangiectatic		1 (1.3)	
	Not defined		3 (3.8)	
Primary/secondary malignancy	Primary		75 (94.9)	48 (100.0)
	Secondary		4 (5.1)	



**Figure 1** Average incidence rates of osteosarcoma in Slovenia between 1990 and 2008.

**Table II** Association of *XRCC3*, *RAD51*, and *NBN* SNPs with risk for osteosarcoma.

Gene	Polymorphism	Genotype	Controls (%)	All patients			Patients <30 years		
				Cases (%)	OR (95% CI)	P	Cases (%)	OR (95% CI)	P
<i>XRCC3</i>	rs1799794 A>G c.-316A>G	AA	247 (66.2)	47 (59.5)	1.29 (0.78–2.14)	0.315	27 (56.3)	1.53 (0.83–2.80)	0.175
		AG+GG	126 (33.8)	31 (39.2)			21 (43.8)		
	rs861539 C>T p.Thr241Met	CC	153 (41.0)	39 (49.4)	0.71 (0.44–1.16)	0.174	27 (56.3)	0.54 (0.30–0.99)	0.047
		CT+TT	220 (59.0)	40 (50.6)			21 (43.8)		
<i>RAD51</i>	rs1801320 G>C c.-98G>C	GG	304 (81.5)	69 (87.3)	0.58 (0.27–1.21)	0.143	41 (85.4)	0.65 (0.26–1.58)	0.337
		GC+CC	69 (18.5)	9 (11.4)			6 (12.5)		
	rs1801321 G>T c.-61G>T	GG	133 (35.7)	25 (31.6)	1.20 (0.71–2.01)	0.497	18 (37.5)	0.92 (0.50–1.72)	0.802
		GT+TT	240 (64.3)	54 (68.4)			30 (62.5)		
	rs12593359 T>G c.*502T>G	TT	103 (27.6)	22 (27.8)	0.99 (0.58–1.70)	0.966	12 (25.0)	1.14 (0.57–2.29)	0.702
		TG+GG	270 (72.4)	57 (72.2)			36 (75.0)		
<i>NBN</i>	rs1805794 G>C p.Glu185Gln	GG	33 (8.8)	11 (13.9)	0.60 (0.29–1.25)	0.170	9 (18.8)	0.42 (0.19–0.94)	0.036
		GC+CC	340 (91.2)	68 (86.1)			39 (81.3)		
	rs709816 C>T p.Asp399=	CC	139 (37.3)	29 (36.7)	1.02 (0.62–1.69)	0.926	18 (37.5)	0.99 (0.53–1.84)	0.975
		CT+TT	234 (62.7)	50 (63.3)			30 (62.5)		
	rs1063054 A>C c.*1209A>C	AA	163 (43.7)	38 (48.1)	0.78 (0.47–1.27)	0.315	22 (45.8)	0.81 (0.44–1.51)	0.508
		AC+CC	210 (56.3)	38 (48.1)			23 (47.9)		

Furthermore, when the combined effect of both *XRCC3* rs861539 C>T and *NBN* rs1805794 G>C was investigated in patients diagnosed before the age of 30 years, osteosarcoma risk was further decreased ( $P_{\text{trend}}=0.007$ ). The risk was the lowest in patients with no wild-type alleles for both SNPs ( $P=0.039$ ,  $OR=0.31$ ,  $95\% CI=0.10-0.94$ ). The combination of both SNPs also showed a trend, albeit insignificant, towards decreased risk for developing osteosarcoma regardless the age at diagnosis ( $P=0.072$ ;  $OR=0.70$ ;  $95\% CI=0.47-1.03$ ), although individual *XRCC3* rs861539 C>T and *NBN* rs1805794 G>C SNPs were not significantly associated with osteosarcoma in the whole cohort.

## Discussion

In the present study, we observed that SNPs in HRR genes influence the risk for developing osteosarcoma in adolescents and young adults. Significantly decreased risk for osteosarcoma was observed for *XRCC3* rs861539 CT and TT genotypes and *NBN* rs1805794 GC and CC genotypes, and the risk decreased even more in the carriers of polymorphic alleles in both genes. On the other hand, none of the investigated SNPs were associated with risk for osteosarcoma in the whole cohort of patients.

We included in our study almost all the pediatric and adolescent osteosarcoma patients diagnosed in Slovenia, because most of them are treated at University Children's Hospital, Ljubljana. On the other hand, we could not include all the adult cases, possibly because they were diagnosed and treated only surgically in other hospitals and were not admitted to the Institute of Oncology, Ljubljana.

Osteosarcoma incidence usually has a bimodal age of distribution, with the first peak among adolescents and a second peak in adults older than 60 years (2). Most patients are diagnosed before the age of 30 years, with a peak of the incidence around 16 years (18). We also observed the highest osteosarcoma incidence among adolescents between 15 and 19 years. The bimodal incidence distribution might be associated with different disease etiology in younger and older osteosarcoma patients. Environmental exposure to radiation, alkylating agents, or other factors that may contribute to osteosarcoma most likely plays an important role in older patients (1), where osteosarcoma can present as a secondary malignancy (2), which we also observed in our study. On the other hand, genetic factors probably contribute more to osteosarcoma in younger patients, where it often coincides with rapid bone growth (2). Genetic variation in DNA repair mechanisms leading to differences in DNA repair capacity and level of unrepaired DNA damage could influence risk for osteosarcoma, especially considering the fact that a higher osteosarcoma incidence was observed in hereditary disorders with

alterations in DNA repair pathways. We therefore separately analyzed osteosarcoma in patients younger than 30 years at the time of diagnosis, and significantly decreased risk for osteosarcoma was observed for carriers of at least one polymorphic *XRCC3* rs861539 T or *NBN* rs1805794 C allele.

Both SNPs, *XRCC3* rs861539 T or *NBN* rs1805794 C allele, are non-synonymous and could therefore affect protein structure or function. Both SNPs were associated with decreased levels of DNA damage, detected using the comet assay in our previous study, suggesting that both polymorphisms may affect the DNA repair capacity of these enzymes (15). To our knowledge, no previous studies investigated SNPs in HRR genes in osteosarcoma. The results of studies investigating the associations of *XRCC3* rs861539 C>T with susceptibility to other cancer types were often inconclusive, so several meta-analyses were published in the last few years, either examining susceptibility to just one or several cancer types (9, 19–22). The results differed even among meta-analyses, but most have detected increased breast or bladder cancer susceptibility in the carriers of polymorphic alleles (9, 19, 22), while lung or skin cancer susceptibility was decreased (9, 19, 20).

Fewer meta-analyses have been published regarding the influence of *NBN* rs1805794 G>C (10, 23–25). Increased overall cancer susceptibility (10) and increased bladder cancer susceptibility were reported (25), while the previously reported decreased breast cancer susceptibility (23) was no longer significant in a more recent meta-analysis (24). Other single studies observed no influence on acute lymphoblastic leukemia susceptibility (26), but susceptibility to acute myeloid leukemia was decreased (27). In our ongoing study, the polymorphic *NBN* rs1805794 C allele was also associated with decreased susceptibility to pediatric B-cell acute lymphoblastic leukemia. The most studied SNPs regarding osteosarcoma susceptibility are those in the *MDM2* gene. *MDM2* is a negative regulator of the tumor suppressor p53, and several studies, including a recent meta-analysis, have shown that *MDM2* SNPs influence osteosarcoma susceptibility (3, 13, 14). *MDM2* also contributes to the regulation of DSB repair and it was shown that it directly binds *NBN* (28), suggesting that our observation of *NBN* rs1805794 G>C modifying osteosarcoma susceptibility is biologically plausible.

The discrepancies between the results of different studies suggest that individual SNPs contribute only a small part to overall cancer susceptibility. Indeed, one of the meta-analyses concluded that while *XRCC3* SNPs definitely modify cancer susceptibility, they do not represent the most important risk factor (19). Interplay of more SNPs is more likely. This is also supported by our observation that, compared to a polymorphism in a single gene, the combination

of *XRCC3* rs861539 C>T and *NBN* rs1805794 G>C SNPs has a larger protective effect on osteosarcoma susceptibility in adolescents and young adults.

As osteosarcoma is a rare form of cancer, our study was performed on a relatively small group of patients, thus our results need to be confirmed in a larger study on osteosarcoma in adolescents. However, different observations for the influence of SNPs were reported for different populations and the advantage of our study is that both patients and controls were recruited in a geographic area with an ethnically homogeneous population (29).

Although our study focused only on genes within HRR, SNPs in other DNA repair pathways have also been associated with osteosarcoma risk. The polymorphic allele of nucleotide excision repair gene *XPD* rs1799793 was associated with decreased risk of developing osteosarcoma (12), while various *FANCM* SNPs were associated with increased risk (3).

In conclusion, our results support the observations that DNA repair polymorphisms, specifically SNPs in the HRR genes *XRCC3* and *NBN*, might contribute to risk for developing osteosarcoma in children, adolescents and young adults.

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### Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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