Association of *XRCC1* gene polymorphisms with the susceptibility and chromosomal aberration of testicular germ cell tumors

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Abstract. It is known that many genomic and genetic alterations caused by aging or environmental factors are responsible for cancer development and progression. XRCC1 is involved in the repair of DNA single-strand breaks formed by exposure to ionizing radiation and alkylating agents. The objective of this study was to investigate the association of genomic alterations and the susceptibility of testicular germ cell tumors with XRCC1 polymorphisms. Two polymorphisms of *XRCC1*, Arg194Trp and Arg399Gln, were genotyped in 83 patients with testicular germ cell tumors (TGCT) and 87 male controls. Allelic imbalances (AI) were evaluated using 4 microsatellite markers in a subgroup of 50 patients. Patients with at least one Gln allele of the Arg399Gln polymorphism had an increased risk of TGCT than those with the Arg/Arg genotype (aOR=1.775, 95% CI=1.045-3.016, P=0.034). Furthermore, the increased risk associated with the Gln allele against the Arg homozygote was more strongly observed in patients with pure seminoma (aOR=2.242, 95% CI=1.149-4.374, P=0.018) or with metastasis (aOR=2.481, 95% CI=1.267-4.862, P=0.008). In the Arg194Trp polymorphism, there was no significant difference in the genotype distribution between TGCT patients and the controls. In AI analysis, the frequency of AI was significantly higher in tumors with at least one Gln allele than those with the Arg/Arg genotype in D13S317 (P=0.010) and in a combination of 4 markers (0.51±0.32 vs 0.32±28, P=0.028). Our results suggest that the Gln allele of the *XRCC1* Arg399Gln polymorphism may genetically modify the development and progression of TGCT through genomic instability.

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Introduction

Testicular germ cell tumor (TGCT) is the most common solid malignancy among young males (1,2). The incidence of TGCT has been increasing worldwide, although there is a marked difference in the incidence, with a higher rate in Scandinavian countries, Germany and Switzerland, and a lower rate in Africa and Asia (3). In recent studies, Finnish men who emigrated to Sweden had a risk of TGCT that is reportedly compatible with that of men in Finland despite a 4x lower incidence ratio in the Finnish than the Swedish population (4,5). These findings suggest that the risk of TGCT is possibly determined by environmental exposure early in life. Several risk factors for TGCT, including an undescended testis (cryptorchism), history of TGCT, and infertility, have been reported (6). Although little is known about the etiology of TGCT, *in utero* exposure to diethylstilbestrol or endogenous estrogens, and early exposure to viruses or chemical substances are hypothesized to be causes of the increased incidence and geographic differences (7-10). Geographic differences in the incidence of TGCT may be modified by genetic factors. This hypothesis is supported by the observation that the relative risk of TGCT is 7- to 10- and 4-fold higher in the brothers and sons of TGCT patients, respectively (11). Recent epidemiological studies have revealed that many genetic polymorphisms are associated with the development and progression of malignant tumors. However, most studies failed to find an association between the risk of TGCT and genetic polymorphisms including androgen receptor, estrogen receptor, detoxification enzyme, and several DNA repair genes (12-15), although only a few studies found an association between polymorphisms and the risk of TGCT (16).

It is known that many genomic and genetic alterations caused by aging or environmental factors are responsible for cancer development and progression. Various chromosomal aberrations (17,18) or gene mutations (19,20) in TGCTs have been reported and these accumulative genetic alterations are considered to be implicated in the tumorigenesis of TGCT. Chromosomal aberrations, which are detected by PCR-based analysis of allelic imbalance (AI), *in situ* fluorescent hybridization (FISH), or comparative genomic hybridization (CGH), are induced by DNA damage possibly due to single or double-strand breaks (21). In TGCTs, many chromosomal

Table I. Association of *XRCC1* polymorphisms with the susceptibility of testicular germ cell tumors.

	No. of genotype (%)			
Arg194Trp	Arg/Arg	Arg/Trp + Trp/Trp	aOR (95% CI)	P-value
Normal control (n=89)	44 (49.4)	36+9 (50.6)	1.000 (ref.)	
TGCT (n=85)	43 (50.6)	36+6 (49.4)	0.902 (0.568-1.434)	0.664
	No. of genotype (%)			
Arg399Gln	Arg/Arg	Arg/Gln + Gln/Gln	aOR (95% CI)	P-value
Normal control (n=89)	52 (58.4)	35+2 (41.6)	1.000 (ref.)	
TGCT (n=85)	37 (43.5)	42+6 (56.5)	1.831 (1.077-3.010)	0.025

alterations, including loss of chromosomes 4, 5, 11, 13q, 17p and 18q, and gain of chromosomes 7, 8, 12, 15q and 21q, have been described. The rate of chromosomal alteration may be modulated by the capacity of DNA repair proteins, such as the X-ray repair complementing defective in Chinese hamster 1 X-ray repair cross complementing protein (XRCC) 1 as a base excision repair protein or XRCC3 as a homologous double-strand break repair protein (21).

The *XRCC1* gene has three single nucleotide polymorphisms (SNPs), Arg194Trp, Arg280His, and Arg399Gln, in exons 6, 9 and 10, respectively. Although the functional significance of these polymorphisms is not fully understood, several studies have shown the association of XRCC1 function and the Arg399Gln polymorphism. The Arg399Gln polymorphism is located in the BRCA1 carboxyl-terminal (BRCT)-I domain which binds to poly(ADP-ribose) polymerase 1 (PARP-1) activated after DNA damage, and the 399Gln allele is reportedly associated with reduced DNA repair function, p53 mutations, and an increased frequency of sister chromatid exchanges (22-24). The Arg194Trp polymorphism is located in the proliferating cell nuclear antigen (PCNA) binding region (4) and is suggested to alter the DNA repair capacity of XRCC1 (25). Several epidemiological studies have demonstrated the association of *XRCC1* polymorphisms with the risk of many cancers, including head and neck, lung, and breast cancers (26-28). However, some other studies have contradicted this association of *XRCC1* polymorphisms with DNA repair functions (22,29) or with cancer susceptibility (30-32).

In this study, we examined the association of *XRCC1* polymorphisms with the risk and clinicopathological features of TGCT and analyzed AIs to assess the association with *XRCC1* polymorphisms and chromosomal alterations.

Materials and methods

Subjects and tissue samples. We studied 89 male controls and 85 patients with pathologically diagnosed primary TGCT treated at Akita University Medical Center or Kyoto University Hospital. The testicular tumors comprised 44 seminomas and 41 non-seminomatous germ cell tumors. The mean ages (± SD) of the patients and control were 35.2±12.0 (range, 2-64) and 33.3±13.8 (range, 23-63) years, respectively. Control subjects were recruited among students of Kyoto University or healthy subjects attending a health 'check-up'. No significant difference in the mean age was found between the patients and control (P=0.327). This study was approved by the Institutional Review Board (the Ethics Committee) of the Akita University School of Medicine, Akita, Japan and the Kyoto University Graduate School of Medicine, Kyoto, Japan. Written informed consent was obtained from all subjects for the use of their DNA from peripheral blood and resected specimens, and clinical information. Pathological diagnosis and clinical stage were determined according to modified WHO and TNM classifications, respectively (33,34). These clinicopathological data are summarized in Table I. Blood samples were collected from each subject and DNA was extracted using a QIAamp Blood Kit (Qiagen, Hilden, Germany). In 50 patients, DNA was extracted from tumors by the standard method with proteinase K digestion followed by phenol-chloroform extraction.

Genotyping of XRCC1 gene polymorphisms. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) was used to detect the *XRCC1* Arg194Trp and Arg399Gln polymorphisms. The primer sets were as follows: *XRCC1* Arg194Trp forward, 5'-ATGCTTGGCCAGTTCCG TGTGAAG-3', and reverse, 5'-CACCTGGGGATGTCTTGT TGATCC-3'; *XRCC1* Arg399Gln forward, 5'-TCCTCCACC TTGTGCTTTCT-3', and reverse, 5'-AGTAGTCTGCTGGC TCTGGG-3'. PCR was carried out in a 15 μl aliquot containing ~25 ng of genomic DNA, 12 pmol of each primer, 2.5 μl of 10X buffer solution, 20 nmol/μl each of dATP, dCTP, dGTP, and dTTP, and 1 U of Taq polymerase (Ampli-Taq Gold DNA polymerase, PE Applied Biosystems, Branchburg, NJ, USA). Initial denaturation at 94˚C for 10 min was followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at 57˚C for Arg194Trp and at 61˚C for 30 sec for Arg399Gln, and extension at 72˚C for 30 sec, with a final extension at 72˚C for 7 min. The products obtained by overnight digestion with *Alu*I or *Nci*I were electrophoresed on 3.0% agarose gels, respectively. The PCR products of 100 bp and 517 bp, for the Arg194Trp and the Arg399Gln polymorphisms respectively, contain *Alu*I and *Nci*I restriction sites and digestion of the PCR products results in two fragments of 76 and 24 bp and

Table II. Association of *XRCC1* polymorphisms with pathological type and clinical stage.

	No. of genotype (%)			
Arg194Trp	Arg/Arg	Arg/Trp + Trp/Trp	aOR (95% CI)	P-value
Normal control (n=89)	44 (49.4)	36+9 (50.6)	1.000 (ref.)	
Pure seminoma (n=44)	21 (38.6)	20+3 (61.4)	0.992 (0.554-1.773)	0.977
Mixed GCT (n=41)	22 (47.4)	16+3 (52.6)	0.841 (0.470-1.504)	0.560
Stage I (n=38)	14 (36.8)	20+4 (63.2)	1.338 (0.751-2.385)	0.323
Stage II-III (n=42)	26 (61.9)	14+2 (38.1)	0.635 (0.345-1.166)	0.143
	No. of genotype (%)			
Arg399Gln	Arg/Arg	Arg/Gln + Gln/Gln	aOR (95% CI)	P-value
Normal control (n=89)	52 (58.4)	35+2 (41.6)	1.000 (ref.)	
Pure seminoma (n=44)	17 (38.6)	23+4 (61.4)	2.242 (1.149-4.374)	0.018
Mixed GCT (n=41)	20 (47.4)	19+2 (52.6)	1.365 (0.696-2.677)	0.365
Stage I (n=38)	20 (52.6)	16+2 (47.4)	1.444 (0.724-2.878)	0.297
Stage II-III (n=42)	14 (33.3)	25+3 (66.7)	2.550 (1.303-4.990)	0.006

385 and 132 bp with a Trp and Gln allele, respectively. Several samples were directly sequenced using PCR primers and Dye Terminator Sequencing Kit version 1.0 (PE Applied Biosystems) on an ABI PRISM 310 auto-sequencer to confirm the results of PCR-RFLP for each polymorphism.

Analysis of allelic imbalance (AI). AI was analyzed in 4 microsatellite markers, D11S1392, D13S317, TP53, and D18S538, which reportedly showed frequent AI using paired DNA obtained from tumor and peripheral blood in a subgroup of 50 patients (18). The nucleotide sequences of forward and reverse primers used for PCR were 5'-TTGCATCCATACG GAAAGTC-3' and 5'-ACATCTGAGACTTGTAGTAGAAG GC for D11S1392, 5'-ACAGAAGTCTGGGATGTGGA-3' and 5'-GCCCAAAAA6ACAGACAGAA-3' for D13S317, 5'-AGGATACTATTCAGCCCGAGGTG-3' and 5'-ACTGCCA CTCCTTGCCCCATTC-3' for TP53, and 5'-AAGCTGAGT GAGCCAATACC-3' and ATTCTCCAGACAAATAATA CC-3' for D18S538. Each forward primer was labeled with HEX (a fluorescent dye). The PCR reaction was carried out in a final volume of 15 μl containing 20 ng of genomic DNA, 5 pmol of each primer, 0.2 mM of each dNTP, 1.0 mM of $MgCl_2$, and 0.5 unit of Ampli-Taq Gold DNA polymerase (Perkin-Elmer, Branchburg, NJ). Initial denaturation at 94˚C for 10 min was followed by 26 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C, and extension at 72˚C for 30 sec, with final extension at 72˚C for 7 min. One μl of the PCR product and 0.5 μl of the size marker [GS-ROX400-HD (Applied Biosystems, Foster, CA)] were diluted to 20 μl with HiDi formamide loading buffer and heat-denatured at 95˚C for 5 min. The mixture was then analyzed using an ABI PRISM 310 automated DNA sequencer (Applied Biosystems) and GeneScan (version 3.1) (Applied Biosystems). AI was determined by measuring the signal imbalance between the opposing alleles and defined as the presence of AI when one signal peak in a PCR product from tumor DNA was less than 70% of that from the corresponding peripheral blood DNA.

Statistical analysis. All data were analyzed by SSPS version 11.0J software (SSPS Inc., Chicago, IL). Hardy-Weinberg equilibrium analyses were performed to compare the observed genotype frequencies with those expected using the χ^2 test. The age-adjusted odds ratio (aOR) and 95% confidence interval (CI) for the relative risk of prostate cancer in each genotype were determined by multiple logistic regression analysis with the inclusion of an age factor. The relationship between each genotype and the AI status was analyzed by χ^2 analysis. A probability of <0.05 was required for statistical significance.

Results

Association of XRCC1 polymorphisms with the risk of testicular germ cell tumors. The genotype distribution and allele frequency of *XRCC1* polymorphisms are summarized in Table I. The genotype distribution in each polymorphism was in Hardy-Weinberg disequilibrium. The genotypes using peripheral blood DNA were completely identical with those using DNA obtained from corresponding tumor tissue, thus demonstrating the absence of the somatic alteration of *XRCC1*. To evaluate the risk of TGCT according to *XRCC1* polymorphisms, logistic regression analysis was employed with age adjustment at diagnosis. There was no significant association between the Arg194Trp polymorphism and the risk of TGCT (aOR=0.902, 95% CI=0.568-1.434, P=0.664). When the subjects were divided into two groups according to the presence of the Gln allele of the Arg399Gln polymorphism, Gln allele carriers had a significantly increased risk of TGCT compared to non-Gln allele carriers (aOR=1.831, 95%CI=1.077-3.010, P=0.025).

Table III. Association of *XRCC1* polymorphisms with allelic imbalances in four loci.

Locus	Allelic imbalance	Arg194Trp			Arg399Gln		
		Arg/Arg	Arg/Trp + Trp/Trp	P-value	Arg/Arg	Arg/Gln + Gln/Gln	P-value
Overall							
D11S1392	-	12 (66.7)	14 (58.3)		11 (64.7)	15 (60.0)	
	+	6 (33.3)	10 (41.7)	0.750	6 (35.3)	10 (40.0)	1.000
D13S317	-	9 (50.0)	8 (36.4)		12 (66.7)	5 (22.7)	
	+	9 (50.0)	14 (63.6)	0.523	6 (33.3)	17 (77.3)	0.010
TP53	-	15 (71.4)	15 (60.0)		16 (76.2)	14 (56.0)	
	+	6 (28.6)	10 (40.0)	0.538	5 (23.8)	11 (44.0)	0.217
D18S538	-	10 (62.5)	10 (47.6)		10 (58.8)	10 (50.0)	
	+	6 (37.5)	11 (54.1)	0.508	7 (41.2)	10 (50.0)	0.743
Pure seminoma							
D11S1392	-	4 (50.0)	9 (60.0)		5 (62.5)	8 (53.3)	
	+	4 (50.0)	6 (40.0)	0.685	3 (37.5)	7 (46.7)	1.000
D13S317	-	5 (55.6)	6 (46.2)		8 (80.0)	3 (25.0)	
	+	4 (44.4)	7 (53.8)	1.000	2 (20.0)	9 (75.0)	0.030
TP53	-	10 (83.3)	11 (68.8)		11 (91.7)	10 (62.5)	
	+	2 (16.7)	5 (31.3)	0.662	1 (8.3)	6 (37.5)	0.184
D18S538	-	6 (66.7)	7 (53.8)		6 (75.5)	7 (50.0)	
	+	3 (33.3)	6 (46.2)	0.674	2 (25.5)	7 (50.0)	0.380

When TGCT patients were stratified by pathological type and clinical stage, there were no significant differences between the pure seminoma and mixed germ cell tumor, and between early stage (stage I) and advanced stage (stage II-III) TGCT in each polymorphism (data not shown). However, when compared with control subjects, a significantly increased risk associated with the Gln allele of the Arg399Gln polymorphism was observed in patients with pure seminoma (aOR=2.242; 95% CI=1.149-4.374; P=0.018) or advanced stage (aOR=2.550; 95% CI=1.303-4.990; P=0.006), whereas the risk was not significant in those with mixed germ cell tumors (aOR=1.365; 95% CI=0.696-2.677; P=0.365) or early stage (aOR=1.444; 95% CI=0.724-2.878; P=0.431) (Table II). There were no associations between the Arg194Trp polymorphism and either tumor type or clinical stage (data not shown).

Association of XRCC1 polymorphisms with allelic imbalance. The association between *XRCC1* polymorphisms and AI is summarized in Table III and a representative AI case of each locus is shown in Fig. 1. The number of informative cases (heterozygotes at tested loci) was 42 (84.0%), 40 (80.0%), 46 (92.0%), and 37 (74.0%) and AI was observed in 16 (38.1%), 23 (57.5%), 16 (34.8%), and 17 (45.9%) cases for D11S1392, D13S317, TP53, and D18S538, respectively. When all analyzed cases were included, the presence of the Gln allele of the Arg399Gln polymorphism had a significant association with the AI of D13S317 (P=0.010). Although the tumors showing AI had a tendency toward an increased number of Gln allele carriers in other loci, there were no statistical significances. The Arg194Trp polymorphism was not associated with the AI of any loci. The association between the AI of each locus and the polymorphisms in a subgroup of pure seminoma was analyzed. The presence of the Gln allele of the Arg399Gln polymorphism was only significantly associated with the AI of D13S317 (P=0.030), although the tumors showing AI only had a tendency toward an increased number of Gln allele carriers in other loci. The Arg194Trp polymorphism was not associated with the AI of any loci in the subgroup of pure seminoma. The mean AI frequency of the four loci analyzed in this study was compared between tumors with Arg/Arg genotype and those with Arg/Gln or Gln/Gln genotype. Tumors with at least one Gln allele had a significantly higher frequency of overall AI than those with the Arg/Arg genotype (0.50±0.33 vs 0.21±0.13, P=0.018) when all cases were included (Fig. 2A). In the subgroup of pure seminoma, the frequency of AI in tumors with the Gln allele showed a significantly higher frequency than those with the Arg/Arg genotype (0.51±0.32 vs 0.32±0.28, P=0.028) (Fig. 2B).

Discussion

Previous epidemiological studies have demonstrated various risk factors of TGCT. Among these factors, cryptorchism, carcinoma *in situ*, and *in utero* exposure to estrogens are

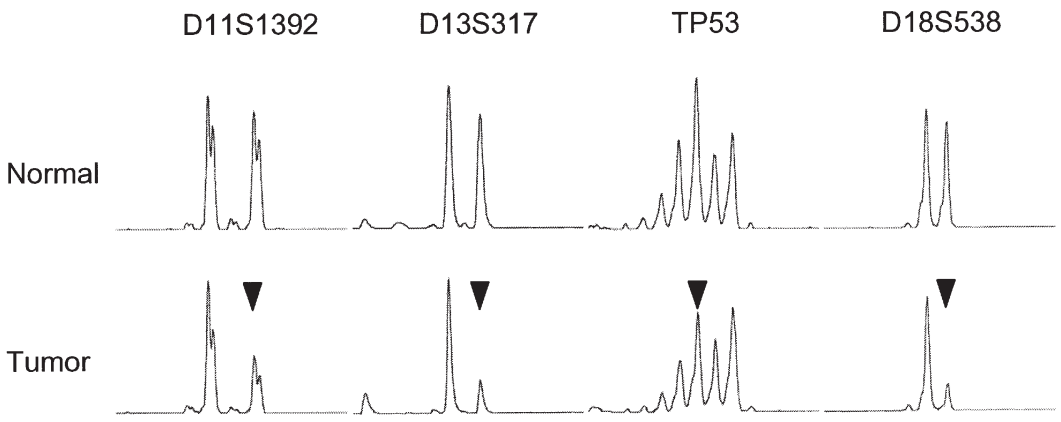

Figure 1. Representative AI cases of each microsatellite region, D11S1392, D13S317, TP53, and D18S538. A black triangle indicates the allele whose signal intensity is less than 70% compared to that of the corresponding allele in normal tissue.

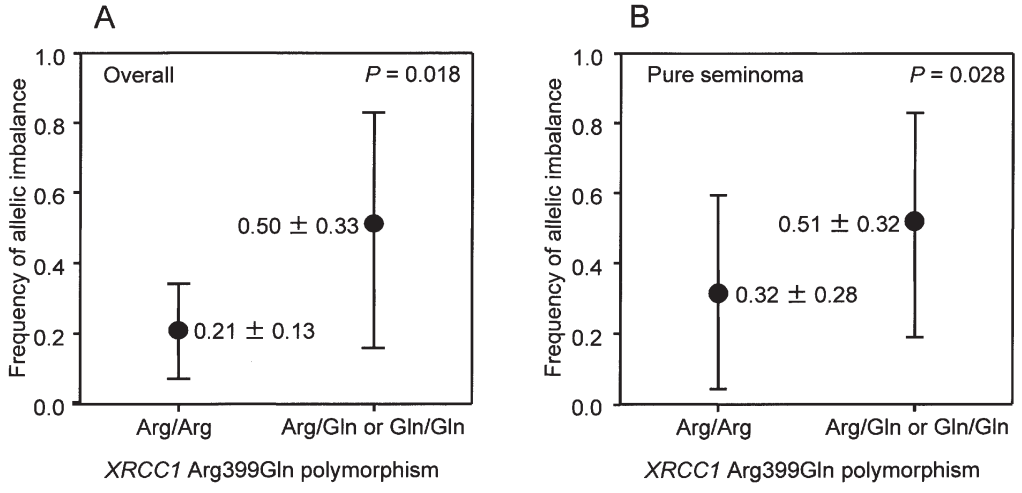

Figure 2. The frequency of AI in each group of XRCC1 genotypes in all cases of TGCT (A) and in cases of pure seminoma (B) (mean ± SD). The frequency of AI in tumors with the Gln allele of the Arg399Gln polymorphism showed a significantly higher frequency than those with the Arg/Arg genotype in each group.

considered consistent risk factors of TGCT (35-37), whereas exposure to chemical materials, electromagnetic fields, or maternal or personal smoking, which possibly induce DNA damage, have demonstrated conflicting results (38-43). Ochratoxin A, a natural food contaminant, has been reported to have a possible association with TGCT development (10). Animal experiments demonstrated that the consumption of ochratoxin A or its *in utero* transfer induces addicts in testicular DNA, which may cause further DNA damage (44). Among genetic factors, polymorphisms involved in the hormonal environment are most likely related to TGCT, but DNA repair gene polymorphisms may also affect the development of TGCT through the functional differences of those genes against the exposure to risk materials.

Several studies have assessed the association of genetic polymorphisms with the risk of TGCT. However, only a few polymorphisms, including *androgen receptor CAG* repeats, *glutathione S-transferase P1* Ile105Val, and *bcl10 G13T* and *C24G* polymorphisms, are reported to influence the susceptibility, phenotype, or progression of the tumor (15,16,45). Recently, Laska *et al* reported that nucleotide and base excision repair genes, *XPD, ASE-1, ERCC1, XRCC3, OGG1* and *XPC*, were not related to the risk of TGCT in the Danish population (14). Meanwhile, there are many studies investigating the association between *XRCC1* polymorphisms and cancer risk; however, these studies show conflicting results in various cancers. For example, in breast cancer, Kim *et al* (28) reported that a significantly higher risk was associated with the Gln/Gln genotype of the Arg399Gln polymorphism, while other reports observed no association between the polymorphism and breast cancer risk. In cancers of the lung (27), head and neck (26), and non-melanoma skin (46), the Arg/Arg genotype was shown to be associated with cancer risk, but all other reports failed to prove the association in these cancers. Meanwhile, the Arg194Trp polymorphism showed a similar tendency among various cancer types. Although most studies did not find an association between the polymorphism and cancer susceptibility, the Trp/Trp or Arg/Trp genotype tends toward a lower risk of each cancer. Hu *et al* (47) recently conducted a meta-analysis for *XRCC1* polymorphisms and revealed that the Trp allele of the Arg194Trp polymorphism was associated with a significantly lower risk when all tumor types were included. However, there was no association between the Arg399Gln

polymorphism and overall cancer risk. In the same study, they found an effect of the Arg399Gln polymorphism on cancer risk in some ethnicities, suggesting that racial or environmental factors influence the significance of the polymorphism in particular types of cancer. In this study, we demonstrated that the *XRCC1* Arg399Gln polymorphism may be associated with susceptibility to TGCTs and, especially, to seminoma. Since the sample size of our study is small, a further study with a larger number of TGCT patients is needed to confirm the results.

The AI of tumor suppressor genes (TSGs) is believed to be the key step to carcinogenesis in various cancers and frequent chromosomal deletions have been identified on particular chromosomal regions in TGCT which possibly harbor important TSGs (17,18,48). Rothe *et al* previously analyzed AI at 25 chromosomal loci in 76 TGCTs using the microdissection technique (18). Our results for AI analysis showed that each frequency of AI at the 4 loci was almost concordant with the previous result and the slightly higher frequencies observed in our data may be caused by differences in the detecting method or setting the threshold for determining AI. Of the four loci selected in this study, D11S1392, TP53, and D18S538 harbor *wilms tumor 1 (WT1)*, *tumor protein p53 (TP53)*, and *deleted in colorectal carcinoma (DCC)* respectively, and no definite TSG has been identified for D13S317 (13q31). Since the association between these TSGs and tumor characteristics or clinical significance is unknown, further studies are needed to elucidate the issue. In this study, tumors with at least one Gln allele of the *XRCC1* Arg399Gln polymorphism had a significantly higher frequency of overall AI than those with the Arg/Arg genotype. A decreased activity of DNA repair function due to the polymorphism may induce frequent chromosomal aberrations. Further studies are required to delineate the biological role of the Arg399Gln polymorphism in relation to chromosomal stability and DNA repair.

The AI of nucleotide excision repair genes has also been reported in ovarian, colon, and lung cancers (49). However, the frequency of AI varied among cancer types and no association of AI with an impact on carcinogenesis or cancer progression has been clarified. In regard to *XRCC1*, CGH analysis in TGCTs showed no large deletion on 19q13 where the gene is mapped (17). In this study, the genotype was completely identical between peripheral blood DNA and tumor tissue DNA, suggesting that at least no apparent deletions existed in the *XRCC1* polymorphic loci.

In conclusion, these results suggest that the *XRCC1* Arg399Gln polymorphism may affect the susceptibility and progression of TGCT through effects on chromosomal stability. Further characterization of the relationships between polymorphisms of DNA repair genes and chromosomal aberrations may allow a better understanding of how the polymorphisms affect cancer development and progression.

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