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Original contribution

Seminoma component of mixed testicular germ cell tumor shows a higher incidence of loss of heterozygosity than pure-type seminoma☆,☆☆

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Summary Using analysis of allelic loss (loss of heterozygosity [LOH]), we previously reported a putative progression pathway from germ cell neoplasia in situ (GCNIS) to seminoma and then to embryonal carcinoma in mixed-type testicular germ cell tumors. To identify the genetic backgrounds related to the progression of nonseminomatous germ cell tumor, patterns of LOH were studied in seminoma components in mixed tumors (18 cases), pure seminomas (20 cases), and coexisting GCNIS lesions. Each tumor was assessed for LOH at 22 polymorphic loci located on 12 chromosomal arms: 3q, 5q, 6p, 9p, 10q, 11p, 12p, 12q, 13q, 17p, 17q, and 18q. For all informative loci, the frequency of LOH in seminoma components in mixed tumors was significantly higher than that in pure seminomas (32% [96/302 loci] versus 19% [60/323 loci], $P < .0001$). The frequency of LOH in GCNIS lesions was not significantly different between the 2 tumor groups. The frequencies of LOH at chromosomes 6p and 10q were significantly higher in seminoma components in mixed tumors than in pure seminomas ($P = .020$ and $P = .0041$, respectively). Immunohistochemical analysis demonstrated a close association between the allelic status of the 10q23 locus and levels of phosphatase and tensin homolog deleted from chromosome 10 protein expression in seminoma ($P = .00051$). These data indicate that the seminoma, which has a potential to progress to nonseminomatous germ cell tumor, already exhibits several genetic changes including allelic losses of 6p and 10q, unlike pure seminoma.

1. Introduction

Adult testicular germ cell tumor (TGCT) is the most frequent malignant solid tumor among men aged 15 to 45 years, and rates of TGCT incidence have been increasing in the past 30 years [1]. Clinicopathologically, TGCTs are divided into 2 entities: seminomas and nonseminomatous germ cell tumors (NSGCTs). NSGCTs are further distinguished by variable

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composition of the histologic elements: embryonal carcinoma, choriocarcinoma, yolk sac tumor, and teratoma. Seminoma in its pure form (ie, tumor of one histologic type) accounts for approximately 50% of all TGCTs, whereas more than 70% of the rest exhibits a mixture of more than one histologic components (ie, seminoma and/or NSGCT) and are called mixed tumors [2]. Despite advances in the management of TGCT, a small group of patients with late relapse or tumors refractory to chemotherapy show poor prognosis [3,4]. Tumors with NSGCT components are more likely to be metastatic at presentation, and those in advanced stages confer worse prognosis than pure seminomas at an equivalent stage of the disease [4]. This poor prognosis is attributed in part to lack of knowledge of the tumorigenesis and progression of NSGCTs.

Seminoma and the various forms of NSGCT originate from a common noninvasive precursor lesion, histologically referred to as germ cell neoplasia in situ (GCNIS) [5,6]. Several studies have shown that NSGCTs develop when a GCNIS cell or a seminoma cell becomes reprogrammed to an embryonal carcinoma cell, which is the neoplastic counterpart of the human embryonal stem cell [7,8]. As its normal counterparts, it is comparable to the reprogramming of murine and human primordial germ cells to embryonal stem cells [9-11]. By analyzing the pattern of allelic loss, we previously suggested the progression pathway from GCNIS to seminoma, then to embryonal carcinoma in TGCTs with a mixture of seminoma and embryonal carcinoma components (the so-called linear progression model) [12]. Although little is known what causes the seminoma cell to be reprogrammed to the embryonal carcinoma cell, a few previous studies have reported molecular heterogeneity of testicular seminomas. For example, using hierarchical clustering based on complementary DNA expression array and protein profiling, one report noted that a subset of pure seminomas exhibits a molecular profile that is more similar to that of embryonal carcinomas than it is to that of conventional seminomas [13]. Taken together our previous report [12], seminoma components in mixed tumors, unlike pure seminomas, may already show a molecular status related to their progression to NSGCT. From this viewpoint, it could be reasonable to investigate and compare detailed molecular genetic status between pure seminomas and seminoma components in mixed tumors.

In this study, we extracted DNA from tumor cells of the 18 seminoma components in mixed tumors, 20 pure seminomas, and coexisting GCNIS lesions using laser microdissection. Then, we performed polymerase chain reaction (PCR)–based loss of heterozygosity (LOH) analyses using 22 polymorphic markers located on 12 chromosomal arms, on which frequent allelic losses have been reported in TGCTs [14-18]. We compared allelic statuses among these histologic components of TGCTs to determine whether (1) the overall frequencies of LOH between seminoma components in mixed tumors and pure seminomas, and between GCNIS lesions in mixed tumors and those in pure seminomas are significantly different or not; (2) LOH events on specific chromosomal loci are related to the progression of NSGCT; and (3) these LOH events affect the loss of protein expression, which is coded by the candidate tumor suppressor gene located on the chromosomal loci examined. Such information would allow us to clarify a part of the molecular basis of tumor cell reprogramming in TGCTs and identify earlier (ie, that has been observed in GCNIS and/or seminoma) the molecular genetic changes to predict the progression to NSGCT.

2. Materials and methods

2.1. Cases enrolled and histologic components analyzed

Based on the criteria of the World Health Organization [1], a total of 38 patients with TGCTs were identified upon reviewing files from the Department of Laboratory Medicine, National Defense Medical College Hospital, Tokorozawa, Japan. Of these, 18 had been diagnosed with seminoma and 1 or more NSGCT components (cases 1-18, mixed tumors) and 20 had only seminomas (cases 19-38, pure seminomas). All these patients underwent primary surgery between 1988 and 2017, and none had undergone preoperative chemotherapy or radiation therapy. Pathological staging of the disease was performed according to the American Joint Committee on Cancer system [19]. From this cohort of 38 patients, 32 had coexisting GCNIS lesions (cases 1-12 and 19-38). NSGCT components observed in mixed tumors were embryonal carcinoma in cases 1, 3-7, 10, and 12-16; yolk sac tumor in cases 1, 2, 4, 9, 13, 17, and 18; choriocarcinoma in cases 2, 9, and 17; and teratoma in cases 2, and 8-12. These NSGCT components were not further analyzed in this study because we focused on seminoma components and coexisting GCNIS lesions. Matched normal DNA was isolated from tumor-free epididymal tissues. The research protocol was approved by the ethics committee of the National Defense Medical College, Tokorozawa, Japan.

2.2. Tissue microdissection and DNA extraction

All specimens were formalin-fixed and paraffin-embedded and cut into 10-μm-thick sections using a microtome. Sections of polyethylene naphthalate foil slides were deparaffinized and stained with hematoxylin and eosin as described previously [12]. Specific cells were selected and microdissected using a Leica LMD 6000 system in accordance with the manufacturer's instructions (Narishige Micromanipulator; Leica, Wetzlar, Germany; Fig. 1), which is described previously [12].

2.3. Selection of polymorphic markers

Twenty-two polymorphic markers, located on 12 chromosomal arms, were selected on the basis of the following criteria: (1) the markers were localized on regions in which frequent LOH events have been reported in adult TGCTs [14-18] and (2) the expected sizes of the amplified fragments

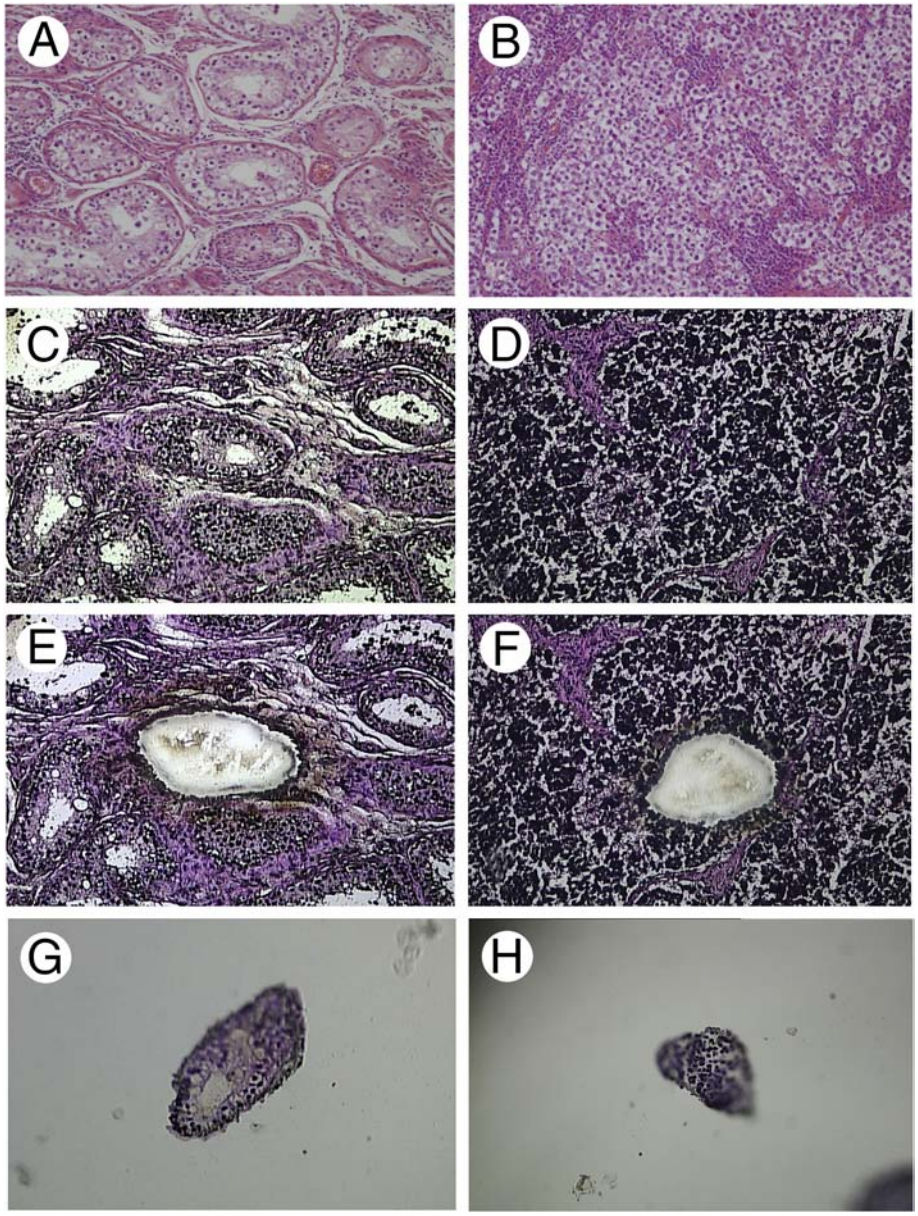

Fig. 1 Images of hematoxylin-eosin–stained sections and laser microdissection of tumor cells from a GCNIS lesion and a seminoma. Microscopic findings of a GCNIS lesion (A) and seminoma (B). Images of pre-microdissection and post-microdissection of a GCNIS lesion (C, pre-microdissection; E, post-microdissection) and a seminoma (D, pre-microdissection; F, post-microdissection). GCNIS (G) and seminoma (H) cells in a collecting tube. Hematoxylin-eosin stain, original magnification ×200.

were less than 250 base pairs for reliable amplification of template DNA.

Pairs of forward and reverse primers of 22 oligonucleotide polymorphic markers corresponding to the sequences retrieved from the Genome Database (http://www.ncbi.nlm.nih.gov/genome/) were synthesized commercially by PerkinElmer (Applied Biosystems, Foster City, CA). The chromosomal regions (markers) were as follows: 3q27 (D3S1262), 3q27-qter (D3S1265), 3q29 (D3S1311), 5q21-22 (RH65746, G32214), 5q35.1 (D5S1402), 6p21.2 (D6S276, D6S2036), 9p21-22 (D9S162, D9S171), 10q23.2 (D10S608), 10q23.3 (D10S541), 11p13 (D11S3501), 12p13.3 (G33070, SHGC34900), 12q22-23 (D12S1074), 13q14.2 (D13S153), 17p13.1 (D17S786), 17q12.31 (RH102656), 18q21-21.3 (D18S846), and 18q21.3 (D18S867, D18S1056). The 5′ ends of the forward primers were labeled with carboxyfluorescein.

2.4. DNA amplification and allelic pattern analysis

PCR amplification was performed in 25 μL reaction mixture containing 100 ng of DNA, 0.24 pmol/μL of each primer, 0.63 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 200 μM of each dNTP, and 4 mM $MgCl_2$, using a GeneAmp PCR system 9600 (Applied Biosystems). Aliquots of the PCR products were then mixed with size standards

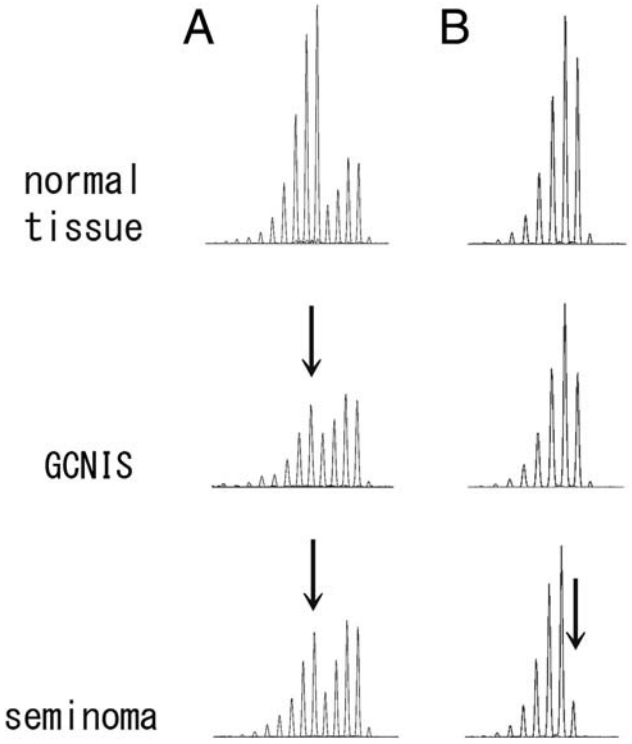

Fig. 2 LOH in the components of GCNIS and seminoma. Two examples of electrophoretograms showing allelic losses are shown. A, Pure seminoma, case 16. Both GCNIS and seminoma showed relatively decreased height of shorter alleles on the left (arrows) at D17S786. B, Mixed tumor, case 9. The GCNIS retained heterozygosity; however, the seminoma components showed relatively decreased height of shorter alleles on the right (arrow) at D3S1311. According to the definition given in the text, these were considered to be LOH.

and formamide, denatured, and run on an ABI 3500 automated capillary electrophoresis DNA sequencer (Applied Biosystems). The allelic products were assessed for peak height and peak area using GeneMapper software (version 5; Applied Biosystems).

DNA samples from noncancerous tissue specimens with 2 different amplified bands were defined as informative cases for LOH analysis (Fig. 2). The presence of LOH was determined in accordance with the manufacturer's criteria. LOH was considered to exist if the ratio of peak heights calculated by the following formula was less than 0.5 or greater than 1.5: (peak height of the affected allele [allele A] of the tumor × peak height of the unaffected allele [allele B] of normal cells)/(peak height of allele A of normal cells × peak height of allele B of tumor cells; Fig. 2). Questionable results were demonstrated at least twice with equivalent results. Results were considered noninformative when the normal tissue was homozygous, when the tissue lysate failed to be amplified, or when the results could not be interpreted unambiguously.

2.5. Immunohistochemistry

Expression of p21 and phosphatase and tensin homolog deleted from chromosome 10 (PTEN) protein in seminoma components in mixed tumors and pure seminomas was analyzed using immunohistochemistry according to the manufacturer's instructions. Briefly, deparaffinized sections were subjected to antigen retrieval with Target Retrieval Solution High pH (Dako, Glostrup, Denmark) for 30 minutes at 95°C and treated with 6% hydrogen peroxide for 5 minutes to inhibit endogenous peroxidase activity. Then, sections were incubated at 4°C overnight with primary antibodies against p21 (12D1, rabbit monoclonal, dilution 1:50; Cell Signaling Technology, Danvers, MA) and PTEN (138G6, rabbit monoclonal, dilution 1:200; Cell Signaling Technology). The tissue sections were then treated with a dextran polymer reagent combined with secondary antibodies and peroxidase (Dako) for 30 minutes at room temperature. Specific antigen-antibody reactions were visualized with 0.2% diaminobenzidine tetrahydrochloride and hydrogen peroxide. Nonneoplastic tonsil tissue was used as a positive control. Sections that were not treated with the primary antibody were used as negative controls.

Nuclear and both nuclear/cytoplasmic immunoreactivities were considered for p21 and PTEN expression, respectively. According to the scoring system described by previous reports [20-22], p21 and PTEN immunoreactivity was assessed based on the predominant staining intensity in the tumor (component) and the fraction of positively stained tumor cells. Intensity of immunoreactivity was classified into the following 4 categories: nonstaining (score 0), weak (score 1), moderate (score 2), and strong (score 3). The fraction of positively stained tumor cells was estimated using a 4-tiered scale (0-10%, 1; 11%-50%, 2; 51%-80%, 3; and >80%, 4). The overall score of each tumor was determined by multiplication of the staining intensity and the positive fraction score. Each tumor was scored twice independently by each of the 2 pathologists (K. M. and H. T.) with subsequent reconciliation of scored values. Any discrepancies between the assessments were resolved by conferring over a multiviewer microscope.

Table 1 Comparison of clinicopathological variables of mixed germ cell tumor and pure seminoma

Variables	Mixed tumor (n = 18)	Pure seminoma (n = 20)	*P*
Age (y), mean (range)	32 (20-52)	40 (28-52)	.00095
Tumor location (left/right)	8/10	14/6	.19
Size of tumor (mm), mean (range)	65 (13-160)	56 (26-90)	.61
Pathological stage [a] (I/II/III)	11/2/5	16/2/2	.35

[a] Staged according to the American Joint Committee on Cancer system [19].

Table 2 LOH at 22 polymorphic chromosomal loci in 18 seminomas and coexisting 12 GCNIS lesions in 18 testicular mixed germ cell tumors

Markers/ Chromosome	Case no. and histologic component																		Case with LOH/ informative cases (%)	
	1		2		3		4		5		6		7		8		9															
	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	10 G	S	11 G	S	12 G	S	13 S	14 S	15 S	16 S	17 S	18 S	G	S
D3S1262/3q	○	○	–	○	○	○	○	●	⊚	⊚	○	○	○	○	○	●	●	●	○	●	●	●	●	●	⊚	○	–	⊚	–	–	3/10 (30)	6/12 (50)
D3S1265/3q	○	○	–	–	○	○	●	–	●	●	●	●	●	●	⊚	⊚	●	–	⊚	⊚	●	●	○	○	●	⊚	○	○	●	–	6/9 (66)	6/11 (55)
D3S1311/3q	○	○	⊚	⊚	○	○	○	●	●	●	○	●	○	○	●	●	○	●	○	○	○	○	⊚	⊚	●	●	●	○	–	●	2/10 (20)	9/15 (60)
RH65746/5q	●	●	○	○	○	○	–	○	○	○	○	●	●	●	○	○	○	●	○	○	○	○	○	○	○	●	○	●	–	–	2/11 (18)	6/16 (38)
G32214/5q	○	○	–	○	○	○	○	○	○	○	○	●	○	–	○	○	○	●	○	○	○	○	–	–	○	–	○	○	○	–	0/10 (0)	2/14 (14)
D5S1402/5q	○	○	–	–	○	○	○	○	●	●	●	–	○	○	○	○	○	○	–	○	○	○	○	●	○	–	–	–	○	○	2/10 (20)	2/13 (15)
D6S276/6p [a]	○	○	⊚	⊚	⊚	⊚	○	●	○	○	○	●	⊚	–	–	⊚	○	○	●	●	○	○	○	○	○	○	–	–	–	○	1/8 (13)	4/11 (36)
D6S2036/6p [a]	○	○	○	○	○	○	○	●	○	○	○	●	○	●	○	●	○	●	○	●	●	●	○	●	○	○	○	○	○	○	1/12 (8)	8/18 (44)
D9S162/9p	⊚	⊚	○	○	⊚	⊚	○	○	○	○	–	–	○	–	○	–	–	–	○	○	⊚	⊚	⊚	⊚	⊚	–	–	–	○	–	0/6 (0)	0/4 (0)
D9S171/9p	⊚	⊚	⊚	⊚	⊚	⊚	○	●	⊚	⊚	○	○	⊚	–	⊚	–	–	⊚	○	○	○	○	○	○	⊚	⊚	–	⊚	–	⊚	0/5 (0)	1/5 (20)
D10S608/10q [a]	○	●	○	–	○	○	○	●	⊚	⊚	○	●	○	○	○	●	○	○	○	●	●	●	○	○	●	●	○	–	●	–	1/11 (9)	9/14 (64)
D10S541/10q [a]	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	–	○	○	○	○	○	○	–	–	○	○	○	○	○	○	0/11 (0)	0/16 (0)
D11S3501/11p	○	○	–	○	○	○	○	●	○	○	●	●	⊚	⊚	●	●	⊚	⊚	○	○	–	○	–●		○	○	–	⊚	⊚	⊚	2/7 (29)	4/12 (33)
G33070/12p	○	○	●	●	○	○	○	○	○	○	○	○	○	○	●	●	–	●	○	●	○	○	○	○	●	○	○	○	●	●	2/11 (18)	7/18 (39)
SHGC34900/ 12p	○	○	○	○	●	●	○	○	○	○	○	○	○	○	○	●	○	○	○	○	○	○	●	●	○	○	○	●	●	○	2/12 (17)	5/18 (28)
D12S1074/12q	○	○	○	●	○	○	○	○	○	●	○	○	●	●	○	○	–	–	○	○	○	○	○	○	○	●	–	○	●	●	1/11 (9)	6/16 (38)
D13S153/13q [a]	○	○	○	–	○	○	○	●	○	○	○	○	○	○	–	○	○	○	○	●	⊚	⊚	⊚	⊚	●	●	●	●	●	○	0/9 (0)	7/15 (47)
D17S786/17p	⊚	⊚	○	○	○	○	○	○	○	○	○	○	–	○	○	○	–	–	○	●	○	–	○	●	●	○	●	○	–	○	0/9 (0)	4/14 (29)
RH102636/17q	○	○	○	○	○	○	○	○	○	○	–	○	○	○	○	○	○	○	⊚	⊚	○	○	○	○	●	○	○	⊚	●	○	0/10 (0)	2/16 (13)
D18S846/18q	○	○	–	–	○	○	–	○	○	○	–	–	○	○	–	⊚	–	–	○	○	–	○	○	○	●	⊚	○	●	●	○	0/6 (0)	3/13 (23)
D18S867/18q	○	○	●	–	○	○	○	●	●	●	⊚	⊚	○	○	–	○	○	○	○	○	–	–	–	○	○	⊚	⊚	○	●	○	2/8 (25)	3/13 (23)
D18S1056/18q	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	○	○	○	○	○	●	○	○	○	○	○	0/12 (0)	2/18 (11)
Total																															27/208 (13)	96/302 (32)

Abbreviations. ●, LOH; ○, retention of heterozygosity; ⊚, noninformative (homozygous); −, noninformative (not determinable); G, GCNIS; S, seminoma.

[a] Statistically significant differences in the frequency of LOH at combined loci of 6p, 10q, and 13q were noted between GCNIS lesions and seminoma components ($P < .05$, each).

2.6. Statistical analysis

Statistical analyses were performed using R software (R Core Team and Foundation for Statistical Computing, Vienna, Austria, version 3.4.2). The mean age, mean tumor size, and overall score of p21/PTEN immunoreactivity between pure seminoma and mixed tumor were compared using the Student *t* test, Welch *t* test, or Mann-Whitney *U* test. The χ^2 test or Fisher exact test was used to compare tumor location, pathological stage of disease, and the frequencies of LOH. Differences with $P < .05$ were considered statistically significant.

3. Results

3.1. Comparison of clinicopathological variables of mixed tumor and pure seminoma

Clinicopathological characteristics of the analyzed cases are shown in Table 1. The mean (range) age of the patients with mixed tumor was 32 (20 to 52) and 40 (28 to 52) years, respectively; a statistically significant difference between the 2 groups was observed ($P = .00095$). There was no significant difference in tumor location, the mean size of tumor, and pathological stage between patients with mixed tumor and those with pure seminoma.

3.2. Allelic status of mixed tumors

The allelic status in 18 cases with mixed tumors is summarized in Table 2. Allelic losses on one or more chromosomal loci were detected in all 30 histologic components examined (ie, 12 GCNIS lesions and 18 seminoma components in mixed tumors). In all cases, there was no chromosomal locus that showed LOH in a seminoma component but retained heterozygosity in coexisting GCNIS. When all the 22 polymorphic markers were studied and the informative cases were combined, the total frequency of LOH events in seminoma components was significantly higher than that in GCNIS lesions (32% [96/302] versus 13% [27/208], $P < .0001$; Table 2). With respect to the frequency of LOH on each of the 22 chromosomal loci, compared with GCNIS lesions, seminoma components showed significantly high rates of LOH at 2

Table 3 LOH at 22 polymorphic chromosomal loci in 20 pure seminomas and coexisting intratubular GCNIS lesions

Markers/ Chromosome	Case no. and histologic component																				Case with LOH/ informative cases (%)	
	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15		16		17		18		19		20			
	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S
D3S1262/3q	○	○	○	○	–	–	–	–	○	○	○	○	–	–	●	●	○	○	○	●	○	○	○	○	–	–	●	●	●	●	⊚	⊚	–	–	○	○	○	○	●	●	4/14 (29)	5/14 (36)
D3S1265/3q	○	○	○	○	–	–	–	–	●	●	–	–	●	–	○	●	○	●	○	●	○	●	○	○	–	–	○	●	●	●	–	–	○	○	○	○	○	○	–	–	3/14 (21)	7/13 (54)
D3S1311/3q	○	○	○	○	–	–	–	–	○	○	⊚	⊚	●	●	○	○	○	●	●	●	⊚	⊚	○	●	–	–	⊚	⊚	○	●	○	○	○	○	○	○	○	○	○	○	2/14 (14)	5/14 (36)
RH65746/5q	⊚	⊚	○	○	–	–	–	⊚	●	●	○	○	⊚	⊚	○	●	○	○	○	●	⊚	⊚	○	○	⊚	⊚	○	○	○	○	○	○	●	●	⊚	⊚	○	○	●	●	3/13 (23)	5/13 (38)
G32214/5q	○	○	○	○	○	○	–	–	○	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	–	○	○	○	○	○	○	○	○	○	○	○	○	○	○	0/19 (0)	1/18 (8)
D5S1402/5q	–	–	●	●	–	–	–	–	○	○	○	○	○	○	–	–	○	○	○	○	–	–	○	○	–	–	○	○	○	○	○	○	○	○	○	○	○	–	○	○	1/14 (7)	2/16 (13)
D6S276/6p	○	○	○	○	○	○	○	○	○	○	⊚	⊚	○	●	⊚	⊚	○	○	○	○	○	○	⊚	⊚	–	–	○	○	⊚	⊚	○	○	○	○	○	●	⊚	⊚	○	○	0/14 (0)	2/14 (14)
D6S2036/6p	–	–	○	○	○	○	○	○	○	●	⊚	⊚	–	○	○	○	○	○	●	●	○	○	○	○	–	–	○	○	–	–	○	○	○	○	–	○	○	○	○	○	1/14 (7)	2/16 (13)
D9S162/9p	–	–	⊚	⊚	⊚	⊚	⊚	⊚	○	○	⊚	⊚	○	○	⊚	⊚	○	○	⊚	⊚	⊚	⊚	○	○	–	–	⊚	⊚	○	○	⊚	⊚	⊚	⊚	⊚	⊚	○	○	–	–	0/6 (0)	0/6 (0)
D9S171/9p	⊚	⊚	○	○	⊚	⊚	⊚	⊚	○	○	○	○	⊚	⊚	○	○	⊚	⊚	–	●	⊚	⊚	⊚	⊚	–	–	⊚	⊚	○	○	○	○	⊚	⊚	⊚	⊚	⊚	⊚	⊚	⊚	0/6 (0)	1/7 (14)
D10S608/10q	–	–	●	●	○	○	○	○	○	○	–	–	○	○	–	–	○	○	○	○	○	○	○	○	–	–	○	○	○	○	○	○	○	○	○	○	○	○	○	○	1/16 (6)	1/16 (6)
D10S541/10q	○	○	○	○	○	○	○	○	–	–	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	0/19 (0)	0/19 (0)
D11S3501/11p	–	–	○	●	○	○	–	–	○	○	○	○	○	○	○	●	○	●	○	●	○	○	○	○	–	–	○	○	○	○	○	○	○	○	–	–	○	○	○	○	0/16 (0)	4/16 (25)
G33070/12p	○	○	●	●	○	○	○	○	○	○	–	○	○	○	●	●	○	○	○	○	○	○	○	○	○	○	●	●	○	○	○	●	○	○	–	–	○	●	–	–	3/17 (18)	5/18 (28)
SHGC34900/12p	○	○	○	○	–	–	○	○	○	●	–	○	●	●	○	○	○	○	○	○	–	○	○	○	–	–	○	○	●	●	⊚	⊚	○	○	○	○	○	○	–	–	2/14 (14)	3/16 (19)
D12S1074/12q	○	○	○	○	○	○	○	○	○	●	–	–	○	○	–	○	○	●	○	○	○	○	–	–	○	○	○	○	○	○	●	○	○	●	○	●	–	–	○	○	0/16 (0)	4/17 (24)
D13S153/13q	⊚	⊚	○	●	○	○	○	○	●	●	○	○	–	–	○	○	○	○	○	○	○	○	○	○	–	–	○	○	○	○	○	●	○	●	○	●	○	●	○	○	1/17 (6)	5/17 (29)
D17S786/17p	○	○	●	●	⊚	⊚	○	○	○	○	–	–	○	○	○	○	○	○	○	●	–	–	●	●	○	○	○	○	●	●	⊚	⊚	○	○	○	○	⊚	⊚	○	–	3/15 (20)	4/14 (29)
RH102636/17q	⊚	⊚	○	○	○	○	○	○	○	●	○	○	○	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊚	⊚	○	○	0/18 (0)	2/18 (11)
D18S846/18q	–	–	○	○	○	○	○	○	○	●	○	○	○	○	⊚	⊚	○	○	○	○	○	○	○	○	○	○	○	○	○	○	⊚	⊚	–	–	–	–	○	○	–	–	0/13 (0)	1/13 (8)
D18S867/18q	○	○	○	○	○	○	○	○	○	○	–	–	⊚	⊚	○	○	○	○	○	○	–	–	○	○	○	○	○	○	●	●	–	–	○	○	○	●	⊚	⊚	⊚	⊚	1/14 (7)	2/14 (14)
D18S1056/18q	○	○	○	○	○	○	–	–	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	–	–	–	–	○	○	○	○	0/17 (0)	0/17 (0)
Total																																									25/320 (8)	60/323 (19)

Abbreviations: ●, LOH; ○, retention of heterozygosity; ⊚, noninformative (homozygous); −, noninformative (not determinable); G, GCNIS; S, seminoma.

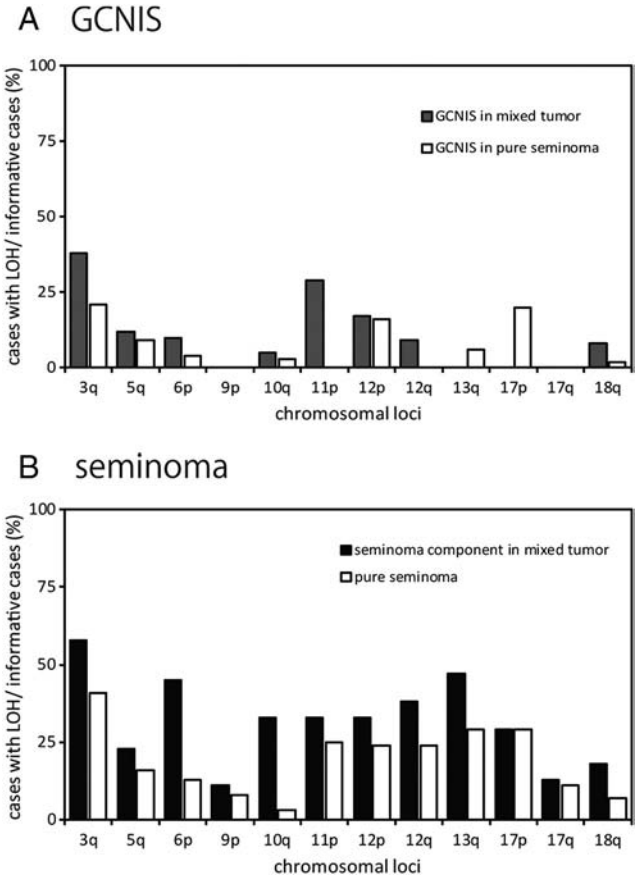

Fig. 3 Comparison of the frequency of LOH on specific chromosomal loci: GCNIS in mixed tumor versus GCNIS in pure seminoma (A) and seminoma component in mixed tumor versus pure seminoma (B). Each bar indicates the percentage of cases with LOH among all informative cases.

combined loci on 6p (10% versus 45%, $P = .024$), 2 combined loci on 10q (5% versus 33%, $P = .021$), and 13q (0% versus 47%, $P = .022$).

3.3. Allelic status of pure seminomas

The allelic status in 20 cases with pure seminomas is summarized in Table 3. For a combination of all the markers and all cases with pure seminomas, the frequency of allelic loss was 8% (25/320) and 19% (60/323) for all informative loci in GCNIS lesions and seminomas, respectively (Table 3). There was a statistically significant difference in the frequencies of LOH events between GCNIS lesions and seminomas ($P = .00013$). There was no statistically significant difference in the frequency of LOH on each chromosomal locus between seminomas and GCNIS lesions.

Table 4 Overall immunoreactivity scores for p21 and PTEN in seminomas

Component	Average of immunohistochemical score		
	Seminoma with LOH	Seminoma without LOH	*P*
p21 [a]	0.28 (n = 11)	0.40 (n = 24)	.93
PTEN [a]	1.2 (n = 10)	4.1 (n = 27)	.00051

[a] Allelic status on chromosome 6p and 10q was noninformative in 3 cases and 1 case, respectively.

3.4. Comparison of the frequencies of LOH between GCNIS in mixed tumors and GCNIS in pure seminomas

When all polymorphic markers were studied and the informative cases were combined, the total frequency of LOH events in GCNIS lesions was 13% (27/208) in mixed tumors and 8% (25/320) in pure seminomas (Tables 2 and 3). There was no statistically significant difference in the frequency of the LOH events between GCNIS lesions in mixed tumors and pure seminomas. No significant difference was detected in the frequency of LOH on each chromosomal locus between GCNIS lesions in mixed tumors and those in pure seminomas (Fig. 3A).

3.5. Comparison of the frequencies of LOH between the seminoma component in mixed tumors and pure seminomas

For a combination of all the markers and all informative cases, the frequency of allelic losses in seminoma components in mixed tumors was significantly higher than that in pure seminomas (32% [96/302] versus 19% [60/323], $P < .0001$; Tables 2 and 3). With respect to each chromosomal locus, seminoma components in mixed tumors showed statistically higher frequencies of allelic loss than did pure seminomas at 2 combined loci of 6p (45% versus 13%, $P = .020$) and 2 combined loci of 10q (33% versus 3%, $P = .0041$; Fig. 3B).

3.6. Expression of p21 and PTEN protein in seminomas

To investigate whether the observed variable allelic patterns on 6p and 10q among seminomas could be correlated with protein expression, immunohistochemical staining for p21 and PTEN protein (ie, protein products of the candidate genes located in the used polymorphic markers on 6p and 10q, respectively) was performed. Results of p21 and PTEN immunohistochemistry are summarized in Table 4. The allelic status of chromosome 6p and 10q was noninformative in 3 cases (cases 24, 31, and 33) and 1 case (case 24), respectively. Therefore, p21 and PTEN protein expression was assessed in a total of 35 and 37 cases, respectively. The representative immunohistochemical images of seminomas are shown in Fig. 4. There was no statistically significant difference in the average of p21 immunoreactivity score between seminomas with (n = 11) and without (n = 24) allelic loss on 6p21.2 (0.28 versus 0.40, $P = .93$). On the other hand, the PTEN immunoreactivity score was significantly lower in

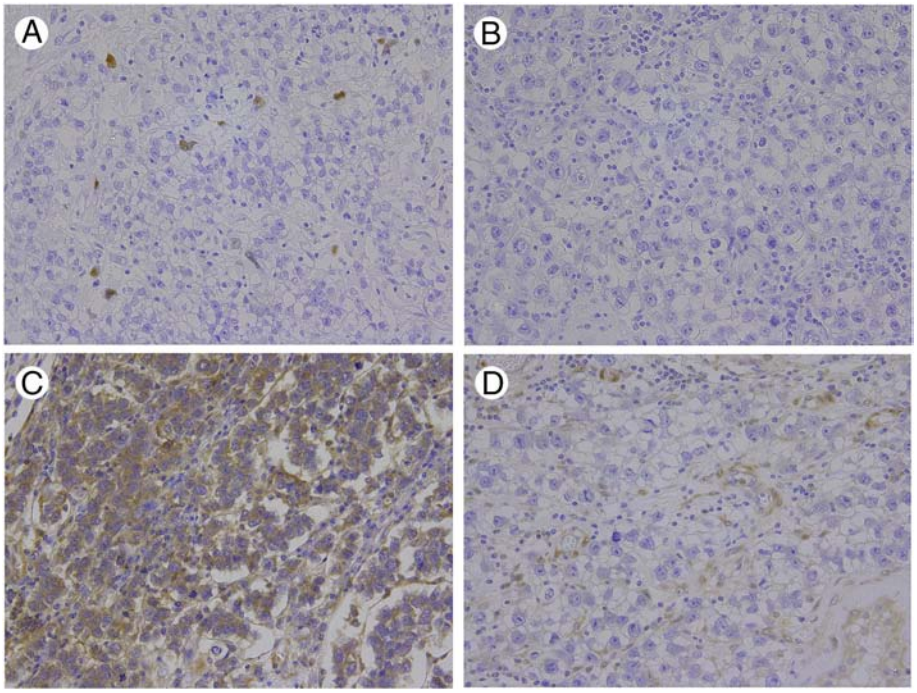

Fig. 4 Representative immunohistochemical data for p21 and PTEN expression in seminomas. A pure seminoma with focal (A) and no (B) immunoreactivity for p21. C, A pure seminoma section with diffuse and strong immunoreactivity for PTEN. D, A seminoma component in a mixed tumor showing loss of PTEN expression. Note the retention of PTEN immunoreactivity in surrounding stromal cells and vascular endothelium. Immunoperoxidase stain, original magnification ×400.

seminomas showing LOH on 10q23 (n = 10) than that in seminomas without LOH on 10q23 (n = 27; 1.2 versus 4.1, P = .00051).

4. Discussion

By analyzing the pattern of allelic loss, we recently provided a genetic evidence of the linear progression pathway in TGCTs with a mixture of seminoma and embryonal carcinoma components and indicated several specific chromosomal loci that might be related to their progression [12]. In the present study, the overall frequency of LOH in seminoma components of mixed tumors was significantly higher than that in pure seminomas, and allelic losses on specific chromosomal loci of 6p and 10q mainly contributed to the difference. These results suggest that seminoma cells that have a "fate" to progress to embryonal carcinoma cells (ie, seminoma components in mixed tumors) already exhibit some genetic changes unlike those observed in pure-type seminomas.

A few previous studies have demonstrated the molecular heterogeneity in testicular seminomas showing a generally uniform morphology [13,23-25]. Hofer et al [13] performed hierarchical clustering based on complementary DNA expression array analysis and a refined protein expression of 8 pure seminomas and 4 pure embryonal carcinomas and identified 2 groups: the first consisting solely of seminomas and the other of seminomas and embryonal carcinomas. Prior studies based on immunohistochemistry against methylation of CpG dinucleotides at position 5 of deoxycytidine residues have reported that seminoma is relatively more CpG hypomethylated than NSGCTs [23-25]. However, in their study, some pure seminomas showed high levels of DNA methylation, similar to those observed in NSGCTs [23-25]. Given the putative linear progression from seminoma to embryonal carcinoma in a subset of TGCTs [12], these data suggest that investigation of molecular profiles of pure seminomas might play a role in identifying high-risk tumors for NSGCT progression.

In our study, there was no significant difference in the total frequency of LOH between GCNIS lesions in mixed tumors and pure seminomas. A recent study using gene expression analysis using quantitative reverse-transcription PCR revealed that *PIWIL1/2/4* and *DDX4* genes, from the PIWI family, which are key factors in germ cell development, were concertedly expressed in GCNIS lesions adjacent to NSGCTs but were down-regulated in those adjacent to seminomas [26]. They also indicated that DNA methylation levels of long interspersed nucleotide factor-1 (LINE-1) promoters coincided in this pattern and concluded that these molecular changes could take place of neoplastic transformation into seminoma, unlike NSGCT, and be used as diagnostic biomarkers for the 2 types of TGCTs [26]. However, their data were relatively limited (ie, specimens from 7 seminomas and 15 NSGCTs were studied), and mixed tumors with or without seminoma components had not been distinguished in the study. Interestingly,

hypomethylation of LINE-1 has been reported to be associated with chromosomal instability in several human malignant tumors including prostate [27] and colon adenocarcinoma [28] and gastrointestinal stromal tumors [29]. To elucidate an earlier change of development of TGCTs, further molecular genetic investigation of a larger number of GCNIS lesions categorized by histologic types of adjacent tumors (ie, pure seminomas or mixed tumors with/without NSGCT) will be necessary.

With respect to specific chromosomal loci, in the present study, LOH on 3q27-qter and 3q29 was not rare (25%-30%) in GCNIS lesions adjacent to mixed tumors and pure seminomas, and often (approximately 50%) occurred in seminomas of both tumor groups. Faulkner et al [18] observed frequent LOH at 3q27-q28 in pure seminomas and precursor GCNIS cells, which is in accordance with our findings. This result suggests that allelic losses at this locus could be commonly associated not only with tumorigenesis of GCNIS but also with the development of invasive seminoma regardless of the fate of progression to NSGCT.

LOH on 6p21.2 and 10q23 was rarely found in GCNIS, whereas high incidence of LOH on these loci was observed in seminoma components in mixed tumors, unlike pure seminomas. *p21* and *PTEN* are representative tumor suppressor genes located at 6p21.2 and 10q23, respectively. Immunohistochemically, seminomas consistently showed loss of p21 protein expression regardless of the allelic status on 6p21.2 locus. On the other hand, the loss of PTEN protein expression in seminomas was significantly correlated with LOH on 10q23 locus. The overall frequencies of p21 and PTEN protein expression in seminomas correspond with those reported in previous studies [21,22], and to our knowledge, this is the first report to compare these protein expression levels between seminoma components in mixed tumor and pure seminomas. Although inactivation of tumor suppressor genes is generally identified by LOH typing at polymorphic chromosomal loci [30,31], LOHs are only surrogate markers for the gene itself and caused by several conditions including deletion, gene conversion, mitotic recombination, or loss of a chromosome segment. In addition, effective alterations of the subsequent second copy of genes such as point mutation and methylations could not be detected by the present allelotyping analysis. Because PTEN is reported to be loss or mutated in several cancers and might prove to be a putative therapeutic target that antagonizes phosphatidylinositol 3-kinase signaling [32], the functional analysis of PTEN deficiency in testicular TGCTs needs to be performed in the near future.

In summary, the present data demonstrate the following: (1) seminoma components in mixed tumors show significantly higher overall frequency of LOH compared with pure seminomas; (2) there is no significant difference of the allelic loss frequency (both overall and each specific chromosomal locus) among GCNIS lesions with or without adjacent NSGCTs; (3) several chromosomal regions seem to undergo earlier genetic changes as a common event (ie, tumorigenesis of GCNIS: 3q) and possibly specific events related to the NSGCT progression (ie, development of seminoma: 6p and 10q); and (4) some of these LOHs are associated with loss of protein expression (ie, PTEN). Identification of specific genetic changes, especially earlier events that are responsible for the progression of NSGCT, needs to be explored further.

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