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Equivalent Parental Distribution of Frequently Lost Alleles and Biallelic Expression of the *H19* Gene in Human Testicular Germ Cell Tumors

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Epigenetic alterations such as genomic imprinting might play an important role in human tumorigenesis, in addition to specific genetic alterations. To clarify the role of genetic and/or epigenetic alterations in the tumorigenesis of testicular germ cell tumors (GCTs), we analyzed 40 primary and 3 metastatic testicular GCTs with regard to specific chromosomal losses and their parental origin. A high incidence of loss of heterozygosity (LOH) was demonstrated on chromosomes 1p, 3p, 11p, and 17p: 9/19 (47%), 18/39 (46%), 13/40 (33%) and 20/36 (56%), respectively. However, there was no correlation between the frequency of LOH on any chromosome and clinicopathological features. Regarding the parental origin of the lost allele at these chromosomes, preferential loss was not demonstrated in this study. To clarify the imprinting status in GCTs, we analyzed the allele-specific expression of the *H19* gene, which is paternally imprinted on chromosome 11p. All of 11 tumors without LOH at this locus showed biallelic expression of *H19*. Based on previous work demonstrating the biallelic expression of *H19* in primordial germ cells and spermatogonia in the mouse germ line, these results suggest that the biallelic expression of *H19* in testicular GCTs reflects the characteristics of the original germ cells in which the imprinting marking has been erased and not established, rather than loss of imprinting during tumorigenesis. It is also possible that a failure to re-establish the imprinting might be an initial event which leads to testicular GCTs.

Key words: Testicular germ cell tumor — Loss of heterozygosity — Parental origin — Imprinting — *H19*

The accumulation of specific gene alterations, such as the activation of proto-oncogenes and the silencing of suppressor genes, is indispensable for human tumorigenesis.[1] In addition, epigenetic alterations might play an important role in tumorigenesis. The degree of DNA methylation is altered in a variety of human cancers, and this might influence the transcriptional regulation of genes.[2] Several tumor suppressor genes, such as *Rb*,[3] *VHL*[4] and *MTS1/CDKN2/(p16)*,[5] are suppressed by aberrant methylation of the promoter region.

Genomic imprinting is considered to be a kind of epigenetic modification that regulates the differential expression of particular genes according to parent-of-origin-specific molecular marking. So far, the human *IGF2*, *H19* and *SNRPN* genes are known to be regulated in this manner.[6] Parent-of-origin-specific modification has important roles in tissue differentiation. A murine pronuclear transplantation study has shown that paternal or maternal genomes have crucial roles in extraembryonic or embryonic differentiation, respectively.[7] Genomic imprinting is also implicated in human tumorigenesis. The maternal allele is preferentially lost in several types of childhood tumors.[8–10] LOI, represented by the expression of a normally silenced parental allele, has been identified at *IGF2* and/or *H19* in several tumors such as Wilms' tumor,[11] rhabdomyosarcoma,[12] hepatoblastoma,[13] choriocarcinoma,[14] and lung cancer.[15]

In GCTs, cytogenetic studies have revealed several types of structural abnormalities, such as duplication, triploidy or deletion, in several chromosomes.[16] In particular, isochromosome i(12p) was shown at a frequency of over 60%.[17,18] Molecular genetic studies have demonstrated LOH of 11p alleles in about 40% of cases,[19,20] and a high frequency of LOH was also found on many other chromosomal arms.[21] However, specific tumor suppressor genes and genetic alterations involved in the tumorigenesis of testicular GCTs have not yet been identified. To determine the genetic and epigenetic alterations implicated in tumorigenesis and the differentiation of testicular GCTs, we performed an LOH study on 1p, 3p, 11p and 17p and determined the parental origin of the lost allele. Furthermore, we investigated the allele-specific expression of *H19*, an imprinted gene on chromosome 11p, in tumor tissues.

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The abbreviations used are: LOI, loss of imprinting; GCT, germ cell tumor; LOH, loss of heterozygosity; RFLP, restriction fragment length polymorphism; RT-PCR, reverse transcriptase polymerase chain reaction.

MATERIALS AND METHODS

Samples We analyzed 40 primary and 3 metastatic tumors from 42 patients with testicular GCT treated at our institution or at other community hospitals. Specimens of about 5 mm in diameter were cut in half, then one was frozen immediately and stored at −70°C until DNA extraction. The other was fixed with buffered formalin for routine histopathological examination to make a pathological diagnosis and to exclude samples with a significant amount of contaminating normal or necrotic tissue. Control DNA was extracted from the heparinized peripheral blood of each patient. If the parents were alive and gave informed consent to participate in this study, DNA was also prepared from their peripheral blood. Histological diagnosis and clinical stage were determined according to modified WHO[22] and TNM classifications,[23] respectively. The clinicopathological features of testicular tumors examined are summarized in Table I.

Table I. Clinicopathological Summary

Total cases		42[a]
Age	3–64 (mean 36)	
TNM stage	T 1	25
	T 2–4	17
	N 0	22
	N 1–4	20
	M 0	33
	M 1	9
Pathology	Seminoma	25
	NSGCT[b]	13
	Seminoma with NSGCT	4

a) Primary and metastatic tumors were analyzed in one case.
b) NSGCT, non-seminomatous germ cell tumor.

DNA isolation, Southern blotting and LOH determination Tissue samples were mechanically homogenized and incubated overnight at 50°C in tissue lysis buffer (4 *M* urea, 200 m*M* NaCl, 0.5% sarcosyl, 10 m*M* EDTA, 100 m*M* Tris-HCl) in the presence of 0.5 mg/ml proteinase K, pH 7.5. DNA was extracted with phenol/chloroform as described.[24] Normal DNA was similarly extracted from leukocyte pellets.

For Southern blotting, 8 μg of DNA was digested with 100–150 units of restriction endonucleases overnight under appropriate reaction conditions. The digested DNA was resolved by electrophoresis on 1.0% agarose gel, then transferred to nylon membrane. Hybridization, autoradiography and the determination of LOH were conducted as described.[24] Briefly, after measuring signal intensities using a densitometer (LKB Ultrascan XL laser), LOH was defined as a reduction in the signal intensity of one allele from tumor DNA by more than 30% compared with the corresponding signal intensity from normal DNA. When LOH was marginal according to the above criteria, the degree of normal tissue contamination was determined histologically, and the results were confirmed after calculating the amount of DNA

Table II. Chromosomal Loci Tested for Loss of Heterozygosity in Testicular Germ Cell Tumor

Locus symbol	Probe	Restriction enzyme	Map location	Allelic loss/ informative (%)
D1S57	pYNZ 2	*Taq* I	1p35–32	9/19 (47)
			chromosome 1p	9/19 (47)
D3S2	pHF12–32	*Msp* I	3p21	10/23 (43)
D3S4	B67	*Msp* I	3pter–q21	11/23 (48)
D3S32	pEFD145.1	*Taq* I	3p	8/23 (35)
THRB (*erbAβ*)	pBH302	*Hind* III	3p24.1–p22	8/13 (61)
		Msp I	3p24.1–p22	11/22 (50)
			chromosome 3p	18/39 (46)
HRAS	PT24–C3	*Taq* I	11p15.5	3/10 (30)
CALCITONIN	pEMBL36	*Taq* I	11p15.2–15.3	6/15 (40)
INSULIN	pHINS6.0	*Taq* I	11p15.5	2/7 (29)
HRAS	pT24–C3	*Msp* I	11p15.5	6/15 (40)
D11S16	p32–1	*Msp* I	11p13	11/24 (46)
IGF2[a]		*Apa* I	11p15.5	6/20 (30)
H19[a]		*Rsa* I	11p15.5	4/17 (24)
			chromosome 11p	13/40 (33)
D17S1	pHF12–2	*Msp* I	17p13	8/18 (44)
D17S30	pYNZ22	*Taq* I	17p13.3	17/29 (59)
D17S31	pMCT35.1	*Msp* I	17p13.1	10/17 (59)
			chromosome 17p	20/36 (56)

a) Determined by PCR-RFLP.

loaded on each lane by comparing the X-ray films of Southern analysis with the same membrane re-hybridized with several probes on multiple chromosomal arms. Four chromosomal arms which have been reported to show a high incidence of LOH in testicular GCTs were examined. The polymorphic probes, corresponding locus symbols, locations and the restriction endonucleases used are listed in Table II.

PCR-RFLP analysis and allele-specific expression of the *H19* gene For the LOH analysis of the *IGF2* or *H19* loci, we performed PCR-RFLP using *Apa* I or *Rsa* I polymorphisms within the *IGF2* or *H19* genes, respectively. PCR amplification for the *H19* polymorphic site consisted of 35 cycles (94°C for 1 min, 62°C for 1 min, 72°C for 1 min) after the initial denaturation step (94°C for 4 min). The primers were: H1(sense), 5′-AACAACCCTCACC-AAAGGCC[15] and AH1(antisense), 5′-TCGGAGCTT-CCAGACTAG. Those for *IGF2* PCR amplification were as described.[12] After digestion with restriction enzymes, PCR products were resolved by electrophoresis on 2.0% NuSieve gels, then LOH was determined by visualizing PCR bands stained with ethidium bromide.

To determine the allele-specific expression of *H19*, total RNA was extracted from frozen tissues using an Ultraspec RNA kit (BIOTECX Laboratories, Inc., Houston, TX). The cDNA was synthesized using First Strand cDNA synthesis kits (Pharmacia Biotech AB, Uppsala, Sweden) and amplified with primers H1 and AH1. The PCR conditions were the same as those in the PCR-RFLP study. After digestion with *Rsa* I, the amplified products were resolved by electrophoresis on 2.0% NuSieve gels and the results were interpreted after ethidium bromide staining.

RESULTS

The results of LOH study We studied LOH in 43 testicular tumor specimens from 42 cases. The specimens included 40 primary tumors and 3 retroperitoneal lymph node metastases. None of the cases had a history of chemotherapy or radiotherapy before high orchiectomy. Over half of the cases were histologically diagnosed as pure seminoma and clinically determined as early-stage tumor without metastasis (Table I). The results of an LOH study on 4 chromosomal arms (1p, 3p, 11p, 17p), which were lost at a high frequency according to previous cytogenetic or molecular analyses of testicular tumors,[16, 19, 21] are shown in Table II. The primary and metastatic tumors from case 22, showing the same histology, were analyzed and the same LOH results were found on each chromosome. The overall frequencies of allelic loss at chromosomes 1p, 3p, 11p, and 17p were 47% (9/19), 46% (18/39), 33% (13/40), and 56% (20/36), respectively. A representative Southern hybridization and PCR-RFLP analysis demonstrating allelic losses are shown in Fig. 1. The allelic losses of 4 chromosomal arms in each individual are shown in Table III. With respect to the relationship between clinicopathological parameters and the frequency of LOH, no significant difference was demonstrated between seminoma and non-seminoma or low and high stage (data not shown). A specific relationship among the LOH of the 4 chromosomal arms was not demonstrated.

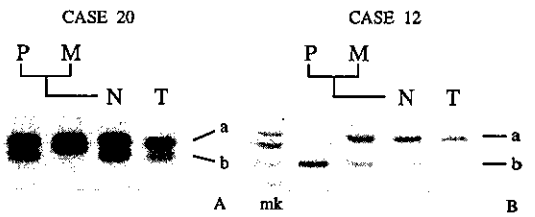

Fig. 1. Loss of heterozygosity at chromosome 11p and the origin of the lost allele in testicular cancers. Both alleles (a and b) were present in normal DNA, indicating that these cases are informative at the locus examined. A, Southern blot hybridized with the *HRAS* probe, indicating paternal allele loss. B, PCR-RFLP analysis at *IGF2/Apa* I site, indicating paternal allele loss. P, paternal DNA; M, maternal DNA; N, normal DNA; T, tumor DNA; mk, size marker.

Determination of the parental origin of the lost allele In 27 cases, the normal DNA of both or one of the parents was obtained, and the parental origin of the lost alleles was determined in 7 cases with 1p LOH, 10 with 3p LOH, 9 with 11p LOH and 13 with 17p LOH. The origin of the lost allele was determined by comparing the polymorphic DNA profile of patients with that of their parents (Fig. 1). The results are summarized in Table IV. The distribution was equivalent with respect to the parental origin of the lost alleles regardless of histopathological type. In the same individual, the parental origin of the absent allele on different chromosomal arms was random but that of different loci on the same chromosomal arms was identical (Table III).

Allele-specific expression of *H19* In the cases heterozygous at the *H19* polymorphic site, 14 tumors and 6 matched normal testes were examined for the expression status of the gene. RT-PCR detected *H19* expression in all normal and tumor specimens examined. All normal testes showed monoallelic expression of *H19* and the maternal origin of the expression was determined in 4 cases (Fig. 2). In contrast, all tumors with retention of heterozygosity at the *H19* locus showed biallelic expression (Table V). The other 3 tumors, cases 3, 21 and 34, which showed LOH at this site, expressed *H19* monoallelically. The maternal origin of the expression was determined in 1 of the 3 tumors with LOH. In case 21, in

Table III. Loss of Heterozygosity on 1p, 3p, 11p and 17p and the Parental Origin of the Lost Allele

Case No.	Histology[b]	TNM classification			Allelic loss on each chromosome[a]			
		T	N	M	1p	3p	11p	17p
1	Seminoma	1	0	0	—	—	—	◑
2	Seminoma	2	2	0	—	◌	◌	◌
3	Seminoma	1	0	0	—	◌	◑P[c]	◑P
4	Seminoma	1	0	0	—	◑M	◌	◑M
5	Seminoma	1	0	0	◌	◑	◌	◌
6	Seminoma	2	0	0	—	◌	◌	◌
7	Seminoma	2	0	0	—	◌	◌	◌
8	Seminoma	1	0	0	—	—	◌	◑
9	Seminoma	1	0	0	◌	◑M	◌	◌
10	Seminoma	1	0	0	—	◑	◑	◌
11	Seminoma	2	0	0	◑P	◌	◌	◑M
12	Seminoma	2	0	0	◑M	◌	◑P	◑M
13	Seminoma	1	0	0	—	◑P	◑M	◑P
14	Seminoma	1	0	0	◌	◑	◑	◑
15	Seminoma	1	0	0	—	◑P	◌	—
16	Seminoma	1	1	0	—	◌	◌	◌
17	Seminoma	2	3	0	—	◌	◑	◑
18	Seminoma	1	3	0	◑	◌	◌	—
19	Seminoma	1	0	0	—	◌	◌	◌
20	Seminoma	4	3	0	◌	◑P	◑P	◑P
21	Seminoma	4	3	0	◑M	◑P	◑M	◑M
22	Seminoma	2	2	0	◑P	◌	—	◑M
23	Seminoma	3	2	0	◌	◌	◌	◌
24	Seminoma	1	4	1	◌	◑	◑	◑
25	Anaplastic seminoma	2	0	0	◑M	◑M	◌	◌
26	EC	2	2	0	—	◌	◌	—
27	EC	1	4	1	—	◑M	◑P	◑M
28	EC	1	4	1	◌	◌	◌	—
29	YST	3	0	0	—	◌	◌	◑P
30	YST	2	3	1	—	◌	◌	—
31	YST	1	4	1	◌	◑	◑M	◑P
32	EC, Te	3	0	0	◑	—	◌	◑M
33	EC, Te	1	0	0	—	◌	◌	◌
34	EC, Te	1	0	1	—	◑P	◑P	◌
35	Te	1	0	0	◑P	◑	◌	◌
36	Te	1	3	0	◌	◌	◌	◌
37	EC, YST	2	3	0	—	◑M	◑M	◌
38	EC, YST, Te	1	4	0	—	◌	◌	—
39	Seminoma, EC	1	3	1	◌	◑	◌	◌
40	Seminoma, EC, Te	1	0	0	◑M	◑	◌	◑
41	Seminoma, EC, Te	2	4	1	—	◌	◌	◑
42	Seminoma, YST	1	2	1	—	◌	◌	◑M

a) ◌, retention of heterozygosity; ◑, loss of heterozygosity; —, not informative.
b) EC, embryonal carcinoma; YST, yolk sac tumor; Te, teratoma.
c) P, paternal allele loss; M, maternal allele loss.

which the lost *HRAS* allele was maternally derived, it is likely the *H19* is expressed from the paternal allele, whereas the parental origin could not be determined in case 34.

DISCUSSION

In this study, we have demonstrated a high frequency of LOH at 4 chromosomal arms in testicular GCTs; 47% at 1p, 46% at 3p, 33% at 11p and 56% at 17p. These results are consistent with those of other molecular studies[19-21] and support the notion that putative tumor suppressor genes with important roles in the tumorigenesis and development of testicular GCTs are located on these chromosomal arms. Three tumor suppressor genes involved in human carcinogenesis have been assigned to these chromosomal arms; *VHL* at 3p25, *WT1* at 11p13 and *p53* at 17p13. Although a mutational anal-

Table IV. Parental Origin of the Lost Allele in Testicular Germ Cell Tumor

Histology	1p		3p		11p		17p	
	P[a]	M	P	M	P	M	P	M
Seminoma	2	3	4	3	3	2	3	5
NSGCT	1	0	1	2	2	2	2	2
Seminoma with NSGCT	0	1	0	0	0	0	0	1
Total	3	4	5	5	5	4	5	8

a) P, paternal allele loss; M, maternal allele loss.

Table V. Allele Specific Expression of *H19* in Testicular Germ Cell Tumor

Patient No.	Histology	Informativity	Expression[a] (origin)[b]
3	Seminoma	–/b	Mono(m)
6	Seminoma	a/b	Bi
10	Seminoma	a/b	Bi
11	Seminoma	a/b	Bi
15	Seminoma	a/b	Bi
18	Seminoma	a/b	Bi
19	Seminoma	a/b	Bi
21	Seminoma	–/b	Mono (p)[c]
28	EC	a/b	Bi
30	YST	a/b	Bi
32	EC, Te	a/b	Bi
34	EC, Te	–/b	Mono
39	Seminoma, EC	a/b	Bi
42	Seminoma, EC	a/b	Bi

a) Mono, monoallelic expression; Bi, biallelic expression.
b) p, paternal expression; m, maternal expression.
c) The origin was estimated from the result of LOH analysis at the *HRAS* locus.

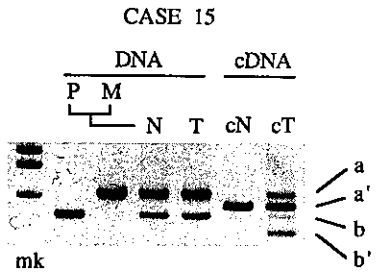

Fig. 2. Expression of *H19* in testicular tumors determined by means of RT-PCR with RFLP analysis at the *H19/Rsa* I site. The *Rsa* I digested and undigested bands of the DNA specific products were designated alleles a and b respectively. Those of the cDNA specific products were designated alleles a′ and b′ since the PCR primers were designed containing the 80 bp intron. PCR with normal and tumor DNA showed both alleles (a and b), indicating the retention of heterozygosity. From the polymorphic profile of both digested parental DNAs, the a and b bands were of maternal and paternal origin, respectively. RT-PCR showed biallelic expression (a′ and b′) in the testicular tumor and maternal monoallelic expression (a′) in the normal testis. The a and b bands in RT-PCR were products amplified from contaminating DNA. P, paternal DNA; M, maternal DNA; N, normal DNA; T, tumor DNA; cN, normal cDNA; cT; tumor cDNA; mk, size marker.

ysis of *VHL* gene in testicular GCTs has not been reported, it is suggested that the involvement of *WT1*[20] and *p53*[25] inactivation is unlikely in the tumorigenesis of testicular GCTs. Considering that frequent LOH at other loci on these chromosomal arms has been demonstrated in several human cancers, other tumor suppressor genes might be involved in the tumorigenesis of testicular GCTs.

It is notable that there was no correlation between the frequency of LOH on the 4 examined chromosomal arms and clinical data, such as clinical stage (Table III), tumor weight, or value of tumor marker (data not shown). In addition, we failed to show either accumulated allelic losses related to the tumor progression of testicular GCTs or a positive relationship between the histopathological differentiation and LOH at any chromosomal arm examined. A recent molecular study demonstrated that the *NRAS* mutations were equally prevalent in seminomatous and nonseminomatous GCTs.[26] In addition, cytogenetic analysis of testicular GCTs has shown that isochromosome 12p was the most common cytogenetic anomaly, regardless of the histopathological characteristics.[16] These data indicate that testicular GCTs with a variety of histopathological features originate from normal germ cell progenitors through a common genetic pathway, at least in the early stage of tumorigenesis. The number of GCTs in our study was too small to determine the specific chromosomal regions for LOH associated with histopathological features of malignant potential of GCTs. However, our LOH data suggest that the genetic events leading to testicular tumorigenesis are more complex than in the case of other human solid tumors derived from somatic cells. A combination of chromosomal losses accompanied with the functional inactivation of multiple tumor suppressor genes may be more important in testicular GCTs than a specific sequential loss of tumor suppressor genes.

Genomic imprinting is a phenomenon characterized by the differential expression of genes arising from parent-of-origin-specific epigenetic changes. Several lines of evidence suggest that epigenetic modifications have important roles both in tissue differentiation and in human carcinogenesis. Indeed, murine pronuclear transplantation experiments have shown that both paternal and

maternal genomes are indispensable in normal extraembryonic or embryonic differentiation, respectively.[7] In addition, it has been proven that hydatidiform mole, a neoplasm of placental elements, arises from abnormal pregnancy resulting in an androgenetic genome.[27] In contrast, ovarian teratoma, a benign tumor consisting of embryonic elements, originates from parthenogenetic ovarian germ cells.[28] These findings suggested that an epigenetic phenomenon may influence the differentiation of male GCTs. This study, however, did not show non-equivalent distribution of lost alleles with regard to their parental origin, which is normally taken as indirect evidence for the involvement of genomic imprinting in the tumorigenesis. A previous study by Lothe *et al.* demonstrated that testicular GCTs showed a preferential loss of the paternally derived 11p.[19] Because the number of analyzed tumors, including our study, is too small to demonstrate a skewed distribution of the lost allele, it is necessary to test more testicular GCTs.

There might be two explanations for the equivalent distribution of the lost alleles with regard to their parental origin. The first is that important genes for testicular tumorigenesis on the examined chromosomes are not imprinted. The second is that tumor cells simply represent the epigenetic status of germ cell progenitors whose imprinting has been erased and not yet re-established. Our findings of allele-specific *H19* expression support the latter possibility. We demonstrated that *H19* was expressed monoallelically from the maternal allele in normal testes, suggesting the maintenance of *H19* imprinting. This is the first report describing the monoallelic expression of *H19* in normal human testes. A recent study using the mouse germ line has demonstrated that 4 imprinted genes (*H19*, *Igf2r*, *Igf2* and *Snrpn*) are biallelically expressed in primordial germ cells and spermatogonia, whereas the postpartum testis or their somatic component showed monoallelic expression of *H19* and *Igf2*.[29] It was suggested that inherited imprinting may be erased by the time of genital ridge colonization of germ cells and new imprinting may not be established until late gametogenesis. They also demonstrated that the *H19* message is undetectable late in gametogenesis, indicating the establishment of new imprinting at this stage. Based on these findings, we speculate that the monoallelic *H19* message detected in the normal human testes was derived from non-germ cells, whereas the biallelic message from germ cells in normal testes was not detected because of the relatively low level of expression. If this is true, our findings, that all of 11 tumors without LOH showed biallelic expression of the *H19* gene, indicate that testicular GCTs originate from germ cells before the establishment of new paternal imprinting rather than that *H19* imprinting is relaxed during tumorigenesis. Furthermore, a failure to re-establish the imprinting might be an initial event which leads to testicular GCTs. Consistent with this notion, case 21 with loss of the maternal *H19* allele showed *H19* expression from the retained paternal allele. This particular tumor might have originally shown biallelic *H19* expression followed by loss of the maternal *H19* allele. However, we could not exclude the possibility that the timing of the erasure or the re-establishment of genomic imprinting differs between human and mouse spermatogenesis.

Several studies have demonstrated that *H19* is expressed from the paternally derived allele in androgenetic human tissues such as hydatidiform mole or pregnancy-associated choriocarcinoma, suggesting the relaxation of *H19* imprinting.[30] Testicular choriocarcinoma was not included in this study due to the rarity of pure choriocarcinoma among testicular GCTs. The biallelic expression of the *H19* gene might be a common characteristic of testicular GCTs regardless of histopathology. This notion is consistent with study by van Gurp *et al.*, who demonstrated biallelic *H19* expression in all of 12 testicular GCTs without LOH.[31] However, this might conflict with the notion that *H19* has potential tumor suppressor activity in an embryonal tumor cell line, as reported by Hao *et al.*[32] We assume that a role of the *H19* gene as a tumor suppressor gene would be unlikely in testicular GCTs. The same situation has been found in human lung cancer, in which *H19* expression was enhanced and associated with loss of *H19* imprinting.[13]

In this study, we could not demonstrate that an epigenetic alteration influences the histopathological differentiation of testicular GCTs. However, a study of DNA methylation of human imprinted genes in testicular GCTs would be of interest, because gamete-specific methylation of a specific site of imprinted genes and its alteration associated with imprinting status have been demonstrated in normal human tissues and in several human tumors.[33–36] Such studies are under way using the same tumor samples in our laboratory. There are many unresolved issues regarding the roles or molecular mechanisms of genomic imprinting. A detailed molecular genetic study of testicular GCTs might provide important clues to address these issues.

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