

# Yolk sac tumor but not seminoma or teratoma is associated with abnormal epigenetic reprogramming pathway and shows frequent hypermethylation of various tumor suppressor genes

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Germ cell tumors (GCTs) are thought to arise from primordial germ cells (PGCs) that undergo epigenetic reprogramming: erasure of the somatic imprint in the genital ridge, and re-establishment of the sex-specific imprint at gametogenesis in the developing gonad. Previous studies suggested that GCTs show epigenetic patterns reflecting the reprogramming process of PGCs; however, epigenetic alterations of imprinted genes and their relationship with the methylation status of tumor suppressor genes (TSGs) have not been comprehensively studied. We analyzed the methylation status of the *H19* and *SNRPN* differential methylated regions (DMRs) and the promoter region of 17 TSGs, and the expression status of *H19*, *IGF2* and *SNRPN* in 45 GCTs, and found that 25 and 20 were in the normal and abnormal reprogramming pathways, respectively, defined on the basis of the methylation status of the two DMRs and the anatomical tumor site. The methylation pattern of the *H19* and *SNRPN* DMRs was total erasure in seminomas, mostly physiological in teratomas, and various in yolk sac tumors. There were no correlations between the methylation status of the *H19* DMR and mono- or biallelic expression of *H19* or *IGF2*. Furthermore, we found that yolk sac tumors had a higher number of methylated TSGs than seminomas ( $P < 0.001$ ) teratomas ( $P = 0.004$ ) or other childhood tumors. While TSG methylation was known to have prognostic implications in various cancers, it did not affect the outcomes of patients with yolk sac tumor, suggesting that mechanisms of TSG methylation may be different between yolk sac tumor and other cancers. (*Cancer Sci* 2009; 100: 698–708)

Childhood germ-cell tumor (GCT) is a rare malignant neoplasm of the gonad or the extra-gonadal site, occurring at an incidence of 2.4 per million children and representing approximately 1% of cancers diagnosed in persons younger than 15 years.<sup>(1)</sup> In contrast, testicular GCT in men between the ages 20–40 is the most common cancer with an incidence of 8–10 per 100 000 in some European countries.<sup>(2)</sup> GCT is a unique tumor, thought to arise from primordial germ cells (PGCs) that undergo epigenetic reprogramming.<sup>(3)</sup> Genomic imprinting is the phenomenon that the paternal and maternal sets of chromosomes have different functionality in mammals, due to parental-specific epigenetic modification of the genome. This modification in mouse PGCs starts with imprint erasure on embryonic day 10.5 and is completed with imprint reestablishment at gametogenesis.<sup>(4–6)</sup>

The *H19* gene is located in 11p15.5 and encodes non-coding RNA, and the gene *SNRPN* (small nuclear ribonucleoprotein associated polypeptide N) located in 15q11–13 and encodes

SmN, a protein involved in spliceosomes.<sup>(7,8)</sup> In normal tissues, while paternal *H19* is not expressed due to methylation of the *H19* differentially methylated region (DMR), maternal *H19* is expressed due to the unmethylation of *H19* DMR. Likewise, while paternal *SNRPN* is expressed due to the unmethylation of *SNRPN* DMR, maternal *SNRPN* is not expressed due to the methylation of *SNRPN* DMR. It has been suggested in several studies that the pattern of *H19* and *SNRPN* methylation in GCTs can be used as a marker of gametic development of PGC giving rise to the tumor.<sup>(9–12)</sup> The methylation status of these two DMRs was studied in childhood GCTs, and the results suggested that the methylation status of DMR of *H19* or *SNRPN* might reflect preservation of the physiologic imprinting erasure in PGCs.<sup>(10–12)</sup> These studies analyzed the methylation status of either *H19* DMR or *SNRPN* DMR in GCTs using various methods, including Southern blot with a methylation-sensitive enzyme, combined bisulfite restriction assay (COBRA) and the methylation-sensitive single-nucleotide primer extension method, and did not determine the relationship of the methylation statuses of the two DMRs in the same GCTs.

Methylation of the promoter region of a tumor suppressor gene (TSG) has been regarded as an important epigenetic change in the development of various cancers.<sup>(13)</sup> The methylation status of many TSGs has been studied mainly in adult testicular GCTs, and these studies showed that non-seminomas had a higher incidence of promoter methylation in many TSGs than seminomas.<sup>(14,15)</sup> Furthermore, Smiraglia *et al.* reported that seminomas show almost no CpG island methylation, in contrast to non-seminomas that show CpG island methylation at a level similar to other solid tumors by restriction landmark genomic scanning.<sup>(16)</sup> It remains unknown whether childhood non-seminoma has a high incidence of methylated TSGs similar to adult non-seminoma, whether dysgerminoma, histologically equivalent to seminoma developing in the ovary, share a low incidence of TSG methylation, and whether childhood yolk sac tumor has a similar incidence of TSG methylation to other childhood solid tumors.

To explore the epigenetic reprogramming process of 45 GCTs, we examined the methylation status of DMR of two imprinted genes, *H19* and *SNRPN*, and the relationship between the DMR methylation and the expression status of *H19*, *IGF2*

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and/or *SNRPN*. Furthermore, we evaluated the methylation status of the promoter region in 17 TSGs, and examined differences in the numbers of methylated TSGs between the two groups of tumors classified by clinical, pathological and epigenetic characteristics. We also clarified the relationship between the reprogramming pathway and the clinical, pathological and epigenetic findings.

## Materials and methods

**Patients and samples.** Tumor tissues were obtained from 38 children and seven adults with GCT. There were 23 males and 22 females, ranging in age from 1 month to 42 years with a median age of 8 years and 7 months (Table 1). In all tumors, the diagnosis of GCT was made by pathologists at each institution according to the classification proposed by Woodward *et al.* (2004) and/or the Japanese Pathological Society.<sup>(2,17)</sup> The pathologist in each institution verified that each sample for molecular genetic analysis contained 70% or more tumor cells. Tumor tissues, which were obtained at Saitama Cancer Center or sent from other hospitals, were immediately frozen after resection or on arrival at the center. The study design was approved by the ethics committee of Saitama Cancer Center. Histologically and clinically, GCTs are subdivided into seminoma (called seminoma when occurring in the testis, dysgerminoma when occurring in the ovary or dysgenetic gonads, and germinoma when occurring in the brain) and non-seminoma, including teratoma, yolk sac tumor, embryonal carcinoma and choriocarcinoma.<sup>(2,17)</sup> There were four seminomas, five dysgerminomas and 36 non-seminomas, including 27 yolk sac tumors, 25 of which occurred in children, five teratomas and four immature teratomas. The representative histology was used for seven yolk sac tumors and one immature teratoma that showed more than one histological criterion (Table 1). Thirty-five tumors originated from the gonad (ovary, 20; testis, 15), and 10 from extra-gonadal sites (mediastinum, 5; the sacrococcygeal region, 2; retroperitoneum 1; stomach 1; and pineal body, 1). Data on outcomes were available for all 45 patients, four (Nos. 5, 9, 33 and 42) of whom died of the disease, and the others were alive at the last follow-up.

**Bisulfite treatment and combined bisulfite restriction assay (COBRA) of the CTCF6 binding site at *H19* DMR and the 5'-untranslated region of *SNRPN* DMR.** Genomic DNA from tumor samples was treated with sodium bisulfite, and we performed COBRA to determine the methylation status of the CCCTC-binding factor binding site 6 (CTCF6) binding site at *H19* DMR, as described previously.<sup>(18)</sup> In addition, we also evaluated the methylation status of the 5'-untranslated region of *SNRPN* DMR.<sup>(19)</sup> *MluI* and *CfoI* were used as restriction enzymes to evaluate the methylation status of *H19* DMR and *SNRPN* DMR, respectively. The intensity of methylated and unmethylated bands was examined by a fluoro-image analyzer, FLA-3000G (Fujifilm, Tokyo, Japan). Two methylated bands represented *H19* DMR; 197 bp and 82 bp, and only the larger band was evaluated because the smaller band was too faint to be evaluated in all samples. Likewise, two methylated bands represented *SNRPN* DMR; 175 bp and 65 bp, and only the larger band was evaluated. The experiments were performed three times, and the mean value of the DNA methylation percentages was calculated.

**Loss of heterozygosity (LOH) analysis of *IGF2* and *SNRPN*.** High-resolution single-nucleotide polymorphism (SNP) array, Affymetrix Mapping 250K-Nsp array (Affymetrix, Santa Clara, CA, US), was used to analyze chromosomal aberrations, including 11p15.5 and 15q11-13 where *IGF2* and *SNRPN* reside, respectively. Genomic DNA in 7/45 tumors was assayed according to the manufacturer's protocol, and the genomic status of *IGF2* and *SNRPN* was determined using copy number analyzer for GeneChip® (CNAG) software as previously described (Table 1).<sup>(20,21)</sup> In addition, the *ApaI* polymorphic site in exon 9 of *IGF2* was used

to evaluate heterozygosity or homozygosity in 42 tumors, as described previously,<sup>(19)</sup> and the *HpaII* polymorphic site in intron 1 of *SNRPN* was also used to evaluate heterozygosity or homozygosity in 38 tumors using the polymerase chain reaction (PCR)-primers as follows; 5'-GGCGACAGTGGGTATTGG-3' and 5'-CAGAAATGCGGTGGAATCCTT-3'.

**Expression analysis of *H19*, *IGF2* and *SNRPN* and allelic expression analysis of *H19* and *IGF2*.** Reverse transcription (RT)-PCR analysis was performed in 12 tumors, the RNA of which was available. Primers used for *H19* were 5'-TACAACCACTGCACTACCTG-3' and 5'-TGGAAATGCTTGAAGGCTGCT-3', those for *SNRPN* were 5'-CCACCAGGCATTAGAGGTCCAC-3' and 5'-GCAG-AATGAGGGAACAAAAAGCTC-3', and those for *IGF2* were reported previously.<sup>(18,22,23)</sup> The *RsaI* polymorphic site in *H19* and the *ApaI* polymorphic site in *IGF2* were used to evaluate allelic expression of respective genes. PCR products were analyzed by electrophoresis in a 2.0% agarose gel and stained with ethidium bromide (Sigma, St Louis, MO, US).

**Methylation-specific PCR (MSP) analysis of various TSGs.** The methylation status of the promoter region in 17 genes was analyzed by MSP, as previously reported.<sup>(15,24)</sup> The genes examined were *RASSF1A*, *HOXA9*, *RUNX3*, *CASP8*, *SCGB3A1*, *SFRP2*, *DCR2*, *RASSF5*, *RASSF2A*, *BLU*, *SFRP5*, *HOXB5*, *p16INK4A*, *SFRP1*, *p14ARF*, *RIZ1* and *SFRP4*. The methylation status of 12 of the 17 genes was also examined in 20 Wilms tumors to compare the number of methylated TSGs between childhood yolk sac tumors and Wilms tumors. The primer sequences and their location in the original genomic sequences were reported elsewhere.<sup>(15,24)</sup> CpG genome™ Universal Methylated DNA (Chemicon International, Temecula, CA, US) and normal lymphocyte DNA were used as controls for completely methylated and unmethylated templates, respectively. PCR products were run on 2% agarose gels and visualized after staining with ethidium bromide.

## Results

**Combined bisulfite restriction assay of the CTCF6 site in *H19* DMR and *SNRPN* DMR.** COBRA showed that the mean methylation percentage  $\pm$  standard deviation (SD) of seven lymphocyte samples was  $49.0 \pm 1.6\%$  at *H19* DMR and  $40.1 \pm 5.7\%$  at *SNRPN* DMR. Forty-five tumors showed a mean methylation percentage of CTCF6 ranging from 1.7% to 77.9% and that of *SNRPN* DMR from 0.4% to 86.0%. We chose a cut-off value of <33.3% methylation, between 33.3% and 66.6% methylation, and >66.6% methylation as definitions of hypomethylated, somatically methylated, and hypermethylated states, respectively, as proposed by Sievers *et al.*<sup>(12)</sup> *H19* DMR and *SNRPN* DMR were hypomethylated in 26 and 26 tumors, somatically methylated in 16 and 15 tumors, and hypermethylated in three and four tumors, respectively (Table 1 and Fig. 1).

**Normal and abnormal reprogramming pathways defined by the methylation status of *H19* DMR and *SNRPN* DMR and the anatomical site in germ cell tumors.** Normal mouse PGCs originate from proximal epiblast cells of embryonic day 6.5 (E6.5) egg cylinder, and migrate into the genital ridge.<sup>(4-6)</sup> PGCs before entering the genital ridge retain parental imprints (somatic methylation); that is, while the paternal and maternal *H19* DMRs are methylated and unmethylated, respectively; this epigenotype is abbreviated to (MU) in the present study, and the paternal and maternal *SNRPN* DMRs are unmethylated and methylated, respectively (UM) (Fig. 2).<sup>(9-12)</sup> When PGCs enter the genital ridge, parental imprints are erased (*H19* DMR/*SNRPN* DMR; UU/UU). PGCs maintain the erased imprint in the ovary or testis, and parental imprints are re-established when PGCs proceed to the meiosis I stage of gametogenesis in female (UU/MM) and to the premeiotic stage in male (MM/UU). Embryologic studies reveal that PGCs actively migrate along the

Table 1. Clinical characteristics and genetic and epigenetic status of *H19* and *SNRPN* regions in 45 germ cell tumors

| Patient number                | Age/sex | Primary site    | Pathology                        | % methyl <i>H19</i> DMR | 11p15 SNP | <i>Apal</i> site | % methyl <i>SNRPN</i> DMR | 15q11 SNP | <i>HpaII</i> site | Epigenotype <i>H19/SNRPN</i> | Reprogram. pathway | Methylated TSGs |
|-------------------------------|---------|-----------------|----------------------------------|-------------------------|-----------|------------------|---------------------------|-----------|-------------------|------------------------------|--------------------|-----------------|
| Extra-gonadal tumors (n = 10) |         |                 |                                  |                         |           |                  |                           |           |                   |                              |                    |                 |
| Teratomas (n = 5)             |         |                 |                                  |                         |           |                  |                           |           |                   |                              |                    |                 |
| 1                             | 2 m/F   | Sacrococcygeal  | Teratoma                         | 44.7                    | ND        | ND               | 41.8                      | ND        | Homo              | MU/UM                        | Normal             | 0               |
| 2                             | 5 m/F   | Mediastinum     | Teratoma                         | 66.2                    | ND        | ND               | 41.2                      | ND        | Hetero            | MU/UM                        | Normal             | 0               |
| 3                             | 13 y/M  | Mediastinum     | Teratoma                         | 36.0                    | ND        | Hetero           | 41.4                      | ND        | ND                | MU/UM                        | Normal             | 9               |
| 4                             | 5 y/M   | Pineral body    | Immat. teratoma                  | 2.8                     | ND        | Homo             | 0.4                       | ND        | ND                | UU/UU                        | Abnormal           | 1               |
| 5                             | 14 y/M  | Mediastinum     | Immat. teratoma                  | 39.7                    | ND        | Hetero           | 27.7                      | ND        | Homo              | MU/UU                        | Abnormal           | 1               |
| Yolk sac tumors (n = 5)       |         |                 |                                  |                         |           |                  |                           |           |                   |                              |                    |                 |
| 6                             | 10 m/F  | Sacrococcygeal  | Yolk sac tumor                   | 65.5                    | ND        | Homo             | 35.8                      | ND        | Hetero            | MU/UM                        | Normal             | 8               |
| 7                             | 2 y/M   | Stomach         | Yolk sac tumor                   | 16.1                    | ND        | Hetero           | 45.3                      | ND        | Hetero            | UU/UM                        | Abnormal           | 6               |
| 8                             | 10 y/M  | Mediastinum     | Yolk sac tumor                   | 10.0                    | ND        | ND               | 1.7                       | ND        | ND                | UU/UU                        | Abnormal           | 7               |
| 9                             | 13 y/M  | Mediastinum     | Yolk sac tumor                   | 12.5                    | ND        | Homo             | 6.7                       | ND        | Homo              | UU/UU                        | Abnormal           | 2               |
| 10                            | 11 m/M  | Retroperitoneum | Yolk sac tumor                   | 67.3                    | ROH       | Homo             | 34.6                      | ROH       | ND                | MM/UM                        | Abnormal           | 1               |
| Gonadal tumors (n = 35)       |         |                 |                                  |                         |           |                  |                           |           |                   |                              |                    |                 |
| Teratomas (n = 4)             |         |                 |                                  |                         |           |                  |                           |           |                   |                              |                    |                 |
| 11                            | 13 y/M  | Testis          | Immat. teratoma /choriocarcinoma | 1.7                     | ND        | Hetero           | 1.5                       | ND        | Hetero            | UU/UU                        | Normal             | 2               |
| 12                            | 4 y/F   | Ovary           | Immat. teratoma                  | 10.8                    | UPD       | Homo             | 84.7                      | UPD       | Homo              | UU/MM                        | Normal             | 1               |
| 13                            | 9 y/F   | Ovary           | Teratoma                         | 3.1                     | ROH       | Homo             | 86.0                      | ROH       | Homo              | UU/MM                        | Normal             | 0               |
| 14                            | 20 y/F  | Ovary           | Teratoma                         | 39.4                    | ND        | Hetero           | 52.7                      | ND        | ND                | MU/UM                        | Abnormal           | 0               |
| Yolk sac tumors (n = 22)      |         |                 |                                  |                         |           |                  |                           |           |                   |                              |                    |                 |
| 15                            | 18 m/M  | Testis          | Yolk sac tumor                   | 17.5                    | ND        | Homo             | 14.7                      | ND        | Hetero            | UU/UU                        | Normal             | 3               |
| 16                            | 18 m/M  | Testis          | Yolk sac tumor                   | 14.0                    | ND        | Hetero           | 23.7                      | ND        | ND                | UU/UU                        | Normal             | 2               |
| 17                            | 2 y/M   | Testis          | Yolk sac tumor                   | 15.0                    | ND        | Hetero           | 8.6                       | ND        | Homo              | UU/UU                        | Normal             | 5               |
| 18                            | 9 y/F   | Ovary           | Yolk sac tumor /dysgerminoma     | 20.2                    | ND        | Homo             | 5.4                       | ND        | Homo              | UU/UU                        | Normal             | 5               |
| 19                            | 13 y/F  | Ovary           | Yolk sac tumor                   | 20.2                    | ND        | Hetero           | 12.9                      | ND        | Hetero            | UU/UU                        | Normal             | 8               |
| 20                            | 14 y/F  | Ovary           | Yolk sac tumor /teratoma         | 29.0                    | ND        | Homo             | 6.7                       | ND        | Hetero            | UU/UU                        | Normal             | 6               |
| 21                            | 38 y/M  | Testis          | Yolk sac tumor. /embryonal ca.   | 7.4                     | ND        | Homo             | 2.2                       | ND        | Homo              | UU/UU                        | Normal             | 0               |
| 22                            | 6 y/F   | Ovary           | Yolk sac tumor /immat. teratoma  | 12.9                    | ROH       | Homo             | 81.4                      | ROH       | Homo              | UU/MM                        | Normal             | 1               |
| 23                            | 11 y/F  | Ovary           | Yolk sac tumor                   | 19.1                    | ROH       | Homo             | 70.6                      | ROH       | Homo              | UU/MM                        | Normal             | 7               |
| 24                            | 9 y/F   | Ovary           | Yolk sac tumor                   | 15.9                    | ND        | Homo             | 53.1                      | ND        | Homo              | UU/UM                        | Abnormal           | 8               |
| 25                            | 6 m/M   | Testis          | Yolk sac tumor                   | 38.7                    | ND        | Hetero           | 17.4                      | ND        | Homo              | MU/UU                        | Abnormal           | 7               |
| 26                            | 11 m/M  | Testis          | Yolk sac tumor                   | 41.8                    | ND        | Hetero           | 19.2                      | ND        | Homo              | MU/UU                        | Abnormal           | 6               |
| 27                            | 18 m/M  | Testis          | Yolk sac tumor                   | 36.4                    | ND        | Hetero           | 17.8                      | ND        | Hetero            | MU/UU                        | Abnormal           | 6               |
| 28                            | 18 m/M  | Testis          | Yolk sac tumor                   | 42.0                    | ND        | Homo             | 29.3                      | ND        | Homo              | MU/UU                        | Abnormal           | 3               |
| 29                            | 2 y/M   | Testis          | Yolk sac tumor                   | 39.4                    | ND        | ND               | 11.5                      | ND        | Homo              | MU/UU                        | Abnormal           | 8               |
| 30                            | 1 m/M   | Testis          | Yolk sac tumor                   | 57.5                    | ND        | Homo             | 39.4                      | ND        | Homo              | MU/UM                        | Abnormal           | 6               |
| 31                            | 9 y/F   | Ovary           | Yolk sac tumor                   | 33.6                    | ND        | Hetero           | 48.7                      | ND        | Homo              | MU/UM                        | Abnormal           | 5               |
| 32                            | 11 y/F  | Ovary           | Yolk sac tumor                   | 34.1                    | ND        | Homo             | 40.3                      | ND        | Homo              | MU/UM                        | Abnormal           | 3               |
| 33                            | 14 y/F  | Ovary           | Yolk sac tumor                   | 43.7                    | ND        | Homo             | 58.5                      | ND        | Hetero            | MU/UM                        | Abnormal           | 4               |
| 34                            | 18 m/F  | Ovary           | Yolk sac tumor /dysgerminoma     | 77.9                    | ROH       | Homo             | 35.1                      | ROH       | Hetero            | MM/UM                        | Abnormal           | 7               |
| 35                            | 6 y/F   | Ovary           | Yolk sac tumor                   | 72.8                    | ROH       | Homo             | 56.3                      | ROH       | Homo              | MM/UM                        | Abnormal           | 9               |

Table 1. Continued

| Patient number    | Age/sex | Primary site | Pathology                                            | % methyl H19 DMR | 11p15 SNP | Apal site | % methyl SNRPN DMR | 15q11 SNP | HpaII site | Epigenotype H19/SNRPN | Reprogram. pathway | Methylated TSGs |
|-------------------|---------|--------------|------------------------------------------------------|------------------|-----------|-----------|--------------------|-----------|------------|-----------------------|--------------------|-----------------|
| 36                | 33 y/M  | Testis       | /immat. teratoma<br>Yolk sac tumor<br>/embryonal ca. | 47.9             | ND        | Homo      | 37.7               | ND        | Homo       | MU/UM                 | Abnormal           | 1               |
| Seminomas (n = 9) |         |              |                                                      |                  |           |           |                    |           |            |                       |                    |                 |
| 37                | 5 m/F   | Ovary        | Dysgerminoma                                         | 22.3             | ND        | Hetero    | 12.5               | ND        | Hetero     | UU/UU                 | Normal             | 0               |
| 38                | 8 y/F   | Ovary        | Dysgerminoma                                         | 28.2             | ND        | Homo      | 14.9               | ND        | Homo       | UU/UU                 | Normal             | 0               |
| 39                | 8 y/F   | Ovary        | Dysgerminoma                                         | 23.4             | ND        | Homo      | 13.4               | ND        | Hetero     | UU/UU                 | Normal             | 0               |
| 40                | 11 y/F  | Ovary        | Dysgerminoma                                         | 15.5             | ND        | Hetero    | 4.5                | ND        | ND         | UU/UU                 | Normal             | 7               |
| 41                | 13 y/F  | Ovary        | Dysgerminoma                                         | 9.7              | ND        | Homo      | 9.2                | ND        | Homo       | UU/UU                 | Normal             | 0               |
| 42                | 31 y/M  | Testis       | Seminoma                                             | 14.8             | ND        | Homo      | 5.7                | ND        | Hetero     | UU/UU                 | Normal             | 0               |
| 43                | 34 y/M  | Testis       | Seminoma                                             | 9.5              | ND        | Hetero    | 10.2               | ND        | Hetero     | UU/UU                 | Normal             | 0               |
| 44                | 38 y/M  | Testis       | Seminoma                                             | 12.1             | ND        | Hetero    | 31.8               | Hetero    | ND         | UU/UU                 | Normal             | 0               |
| 45                | 42 y/M  | Testis       | Seminoma                                             | 9.4              | ND        | Hetero    | 3.1                | ND        | Hetero     | UU/UU                 | Normal             | 0               |

% methyl., % methylation; Epigenotype, see text; Reprogram., Reprogramming; TSGs, tumor suppressor genes; m, months; y, years; F, female; M, male; ND, not done; ROH, retention of heterozygosity; UPD, uniparental disomy; Homo, homozygosity; Hetero, heterozygosity; Immat., immature; ca, carcinoma; DMR, differential methylated region; SNP, single-nucleotide polymorphism.

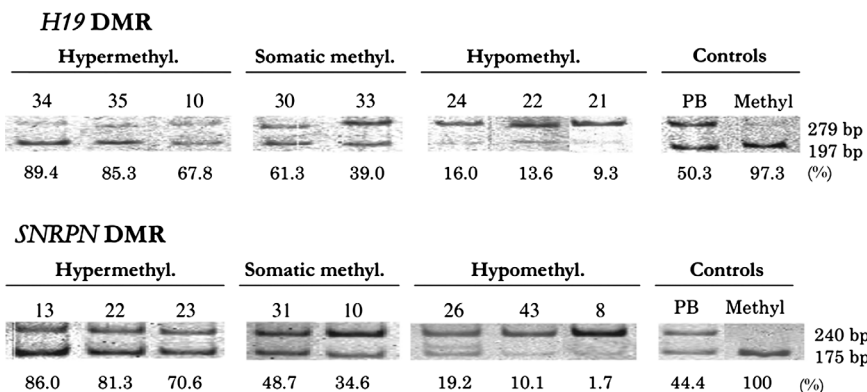
mesentery to the posterior hindgut, in very close proximity to the coccyx, before they enter the genital ridges;<sup>(4-6)</sup> therefore, tumors located in the extra-gonadal sites, including the sacrococcyx, mediastinum, retroperitoneum and pineal body, are postulated to have arisen from PGCs that had yet to reach the genital ridge and to erase their imprint. Thus, we assume that GCT cells originating from the extra-gonadal site retain parental imprints (MU/UM), whereas GCT cells originating from the gonad show an erased imprint (UU/UU) or re-established imprint (MM/UU or UU/MM).<sup>(3-6)</sup>

Of 10 tumors originating from the extra-gonadal site, four showed a normal somatic imprint status, MU/UM, and the other six showed various abnormal imprint statuses. Likewise, of 35 gonadal tumors, 21 showed the normal imprint status of UU/UU (17 tumors with imprint erasure) or UU/MM (4 ovarian tumors with re-establishment of the maternal imprint), and 14 showed various abnormal imprint statuses (Table 2).

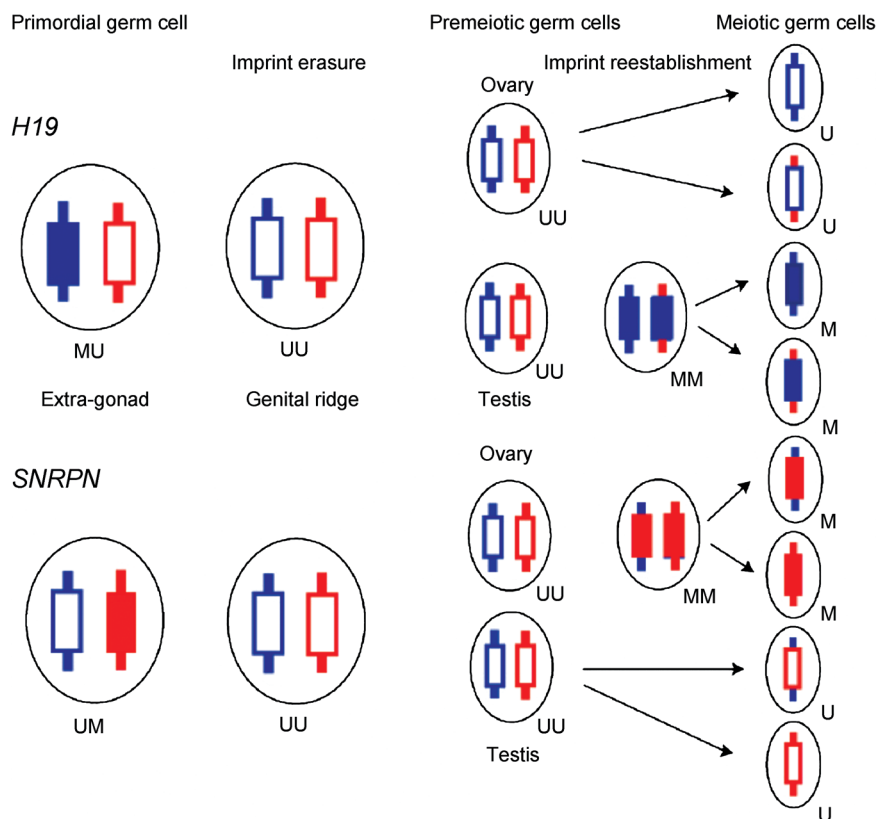
**LOH analysis of seven tumors with hypermethylated H19 DMR or SNRPN DMR.** SNP array analysis was performed in three tumors with hypermethylated H19 DMR and four with hypermethylated SNRPN DMR. Of the three tumors, one developed from the extra-gonadal site of a boy and two from the gonadal site of two girls showing heterozygous H19-IGF2 alleles, indicating aberrant methylation of the maternal allele (Table 1). Of the remaining four tumors, all developed from the ovary, three showed heterozygous SNRPN alleles, indicating physiological methylation of the maternal allele, SNRPN MM (Fig. 2), and one showed uniparental duplication of the maternal SNRPN allele. ApaI polymorphism analysis showed heterozygosity of the IGF2 locus in 18 tumors and homozygosity in 24 tumors. Of the 18 tumors, eight had the MU epigenotype in H19 DMR, and 10 had the UU epigenotype, indicating that the UU genotype was caused by demethylation of the paternal H19 DMR but not by duplication of the maternal H19 DMR. Likewise, HpaII polymorphism analysis showed heterozygosity of the SNRPN locus in 16 tumors and homozygosity in 22 tumors. Of the 16 tumors, five had the UM epigenotype and 11 had the UU epigenotype, indicating that the UU epigenotype was caused by demethylation of maternal SNRPN DMR but not by duplication of paternal SNRPN DMR. Thus, it was proved that uniparental disomy was not the cause of the MM/UM, UU/MM, or UU/UU epigenotype in the majority of GCTs. SNP analysis demonstrated that one immature teratoma (No. 12) with the UU/MM epigenotype had uniparental disomy of both IGF2-H19 and SNRPN regions, indicating that duplication of the uniparental H19 DMR or SNRPN DMR may cause the UU/MM epigenotype in a minority of GCTs.

**Expression analysis of H19, IGF2 and SNRPN and allelic expression analysis of H19 and IGF2.** The results of RT-PCR analysis are shown in Table 3 and Fig. 3. The expression status of each gene was defined as 1+ or 2+. Tumors with the H19 MU epigenotype expressed 2+ IGF2 mRNA more frequently than tumors with the H19 UU epigenotype (P = 0.0133), whereas tumors with the H19 MU epigenotype and those with the H19 UU epigenotype expressed 2+ H19 mRNA with a similar frequency (P = 0.1982). Tumors with the SNRPN UU epigenotype tended to express 2+ SNRPN mRNA more frequently than tumors with the UM or MM SNRPN epigenotype (P = 0.0668). The reasons of the correlation between the H19 MU DMR and IGF2 2+ expression is presently unknown.

Of four tumors with the heterozygous RsaI site, three and one showed monoallelic and biallelic expression, respectively, of H19. Two of three tumors with the H19 UU DMR and one with H19 MU DMR showed the monoallelic expression. Of six tumors with the heterozygous ApaI site, three and three showed monoallelic and biallelic expression, respectively, of IGF2. All three tumors (Nos. 27, 43 and 45) with the monoallelic expression simultaneously showed a faintly stained opposite band. Two tumors with the monoallelic expression and one with the



**Fig. 1.** Examples of the methylation status of the CCCTC-binding factor binding site 6 (CTCF6) binding site of *H19* differential methylated region (DMR) and *SNRPN* DMR analyzed by a combined bisulfate restriction assay (COBRA). Bisulfate-modified polymerase chain reaction products were digested with *MluI* or *CfoI*. Upper 279 bp and lower 197 bands in the upper lane show unmethylated and methylated fragments containing the CTCF6 binding site, respectively, and upper 240 and lower 175 bands in the lower lane show unmethylated and methylated fragments containing *SNRPN* DMR, respectively. PB, lymphocyte DNA showing normal somatic methylation; Methyl. control methylated DNA. Numbers above lanes indicate the tumor number. The percentage of methylated DNA calculated from the bands above is shown below each lane. The tumors were classified as hypermethylated (>66.6%), somatically methylated (33.3–66.6%), and hypomethylated (<33.3%).



**Fig. 2.** Model of *H19* and *SNRPN* imprint resetting in the human germline. Primordial germ cells (PGCs) before entering the genital ridge retain a somatic methylation pattern showing the paternal imprint at *H19* differential methylated region (DMR) and the maternal imprint at *SNRPN* DMR (*H19* DMR/*SNRPN* DMR is shown as MU/UM). When PGCs have entered the genital ridge, both homologs are unmethylated and the imprint is erased (UU/UU). Imprint is re-established when both homologs of *H19* DMR in male PGCs are methylated (MM) at premeiotic stage in the testis and both homologs of *SNRPN* DMR in female PGCs are methylated (MM) at meiosis in the ovary. M, methylated DMR; U, unmethylated DMR.

biallelic expression had the *H19* UU DMR, whereas one tumor with the monoallelic expression and two with the biallelic expression had the *H19* MU DMR. Thus, there seems to be no correlation between the methylation status of *H19* DMR and the allelic expression status of *H19* and *IGF2*.

**MSP analysis of various TSGs in germ cell tumors.** We examined the methylation status of 17 TSGs in 45 GCT samples by MSP, and found hypermethylation of *RASSF1A* in 25 (55.6%), *HOXA9* in 24 (53.3%), *RUNX3* in 22 (48.9%), *CASP8* in 16 (35.6%), *SCGB3A1* in 16 (35.6%), *SFRP2* in 16 (35.6%), *DCR2* in 15 (33.3%), *RASSF5* in eight (17.8%), *RASSF2A* in five (11.1%), *BLU* in three (6.7%), *SFRP5* in two (4.4%), *HOXB5* in

one (2.2%), *p16INK4A* in one (2.2%) and *SFRP1* in one (2.2%) (Table 4 and Fig. 4). No tumors showed hypermethylation of *p14ARF*, *RIZ1* and *SFRP4*.

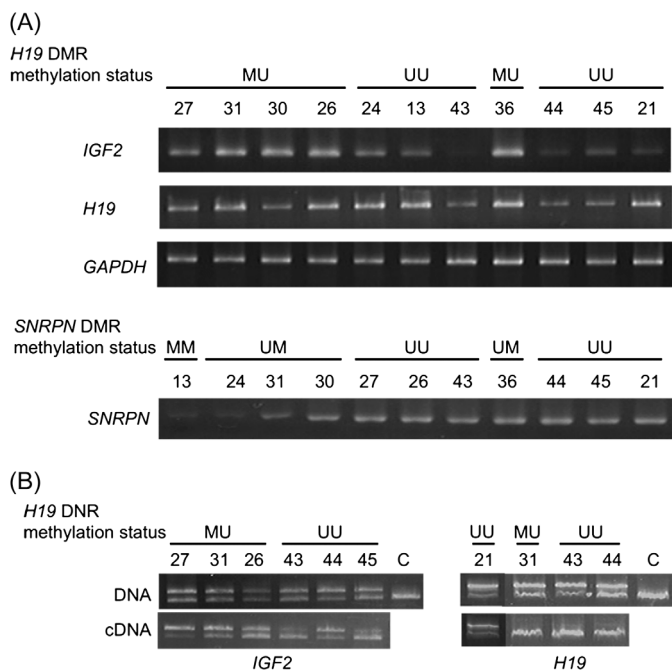
We compared the numbers of methylated TSGs between childhood yolk sac tumors and other childhood solid tumors (Table 4).<sup>(25–28)</sup> The incidences of *CASP8* and *DCR2* methylation were similarly high in childhood yolk sac tumors, Wilms tumors and neuroblastomas, although the incidences were lower in hepatoblastomas. The incidences of *RASSF1A*, *RUNX3* and *SFRP2* methylation were higher in childhood yolk sac tumors than in hepatoblastomas ( $P = 0.0020$ ,  $<0.0001$  and  $<0.0001$ ) and Wilms tumors ( $P = 0.0272$ ,  $<0.0001$  and  $0.0040$ ). *HOXA9*

**Table 2. Epigenotype and normal and abnormal reprogramming pathways in extra-gonadal and gonadal germ cell tumors**

| Epigenotype                             | Extra-gonad (n = 10)                | Gonad (n = 35)                                    |
|-----------------------------------------|-------------------------------------|---------------------------------------------------|
| <i>H19</i> DMR/ <i>SNRPN</i> DMR        |                                     |                                                   |
| Normal reprogramming pathway (n = 25)   |                                     |                                                   |
| MU/UM                                   | 4 (M 1: Tera 1; F 3: Tera 2, YST 1) |                                                   |
| UU/UU                                   |                                     | 17 (M 9: YST 4, Sem 4, Tera 1; F 8: Dys 5, YST 3) |
| UU/MM                                   |                                     | 4 (F 4: Tera 2, YST 2)                            |
| Abnormal reprogramming pathway (n = 20) |                                     |                                                   |
| MM/UM                                   | 1 (M 1: YST 1)                      | 2 (F 2: YST 2)                                    |
| UU/UU                                   | 3 (M 3: YST 2, Tera 1)              |                                                   |
| MU/UM                                   |                                     | 6 (M 1: YST 1; F 5: YST 4, Tera 1)                |
| MU/UU                                   | 1 (M 1: Tera 1)                     | 5 (M 5: YST 5)                                    |
| UU/UM                                   | 1 (M 1: YST 1)                      | 1 (F 1: YST 1)                                    |

MU/UM represents methylated paternal *H19* allele and unmethylated maternal *H19* allele/unmethylated paternal *SNRPN* allele and methylated maternal *SNRPN* allele. Please also see text and Fig. 2.

M, male; F, female; Tera, teratoma; YST, yolk sac tumor; Sem, seminoma; Dys, dysgerminoma; DMR, differential methylated region.



**Fig. 3.** (A) The upper lanes show examples of *IGF2* and *H19* expression in germ cell tumors with various methylation statuses of *H19* differential methylated region (DMR) (CCCTC-binding factor binding site 6 [CTCF6]). The lower lanes show examples of *SNRPN* expression in germ cell tumors with various methylation statuses of *SNRPN* DMR. (B) Allelic expression analysis of *IGF2* and *H19* mRNA. Electrophoretic patterns of genomic DNA polymerase chain reaction (PCR) or reverse transcription-PCR after *Apal* digestion for *IGF2*, and after *RsaI* digestion for *H19*. Biallelic expression of *IGF2* was found in Nos. 31, 26 and 44, and monoallelic expression of *IGF2* with a faintly stained opposite band was found in Nos. 27, 43 and 45. Three tumors (Nos. 31, 43 and 44) showed monoallelic expression of *H19*, and one (No. 21) showed biallelic expression of *H19*. c, control.

and *SGGB3A1* methylation was more frequent in yolk sac tumors than in hepatoblastomas ( $P < 0.0001$  and  $< 0.0001$ ), Wilms tumors ( $P < 0.0001$  and  $0.0777$ ) and neuroblastomas ( $P < 0.0001$  and  $P = 0.0040$ ). In summary, both the number of methylated TSGs and the incidence of TSG methylated tumors were higher in childhood yolk sac tumor than in other childhood solid tumors.

**Association between epigenetic reprogramming pathways and clinical, pathological, and epigenetic findings (Table 5).** There were no differences in age, sex and the primary site between tumors in

the normal pathway and those in the abnormal pathway by chi-square test. In contrast, seminomas and teratomas were more frequent in the normal pathway than yolk sac tumors ( $P = 0.013$ ). Furthermore, tumors with the UU/UU epigenotype and tumors with no methylated TSG were more frequent in the normal pathway than tumors with the other epigenotypes ( $P = 0.0007$ ) and tumors with 1–9 methylated TSGs ( $P = 0.0009$ ), respectively.

**Differences in the number of methylated TSGs between two groups of tumors classified by clinical, pathological and epigenetic characteristics (Table 5).** All seven adult tumors had no methylated TSGs. There were no differences in the numbers of methylated TSGs between tumors in males and females, and between tumors developed in the gonad and in the extra-gonadal site. In contrast, yolk sac tumors had a higher number of methylated TSGs than seminomas ( $P < 0.001$ ) or teratomas ( $P = 0.004$ ) by Student's *t*-test. Tumors with the UU/UU epigenotype and tumors in the normal pathway had a lower number of methylated TSGs than tumors with other epigenotypes ( $P = 0.048$ ) and tumors in the abnormal pathway, respectively ( $P = 0.042$ ).

In 27 yolk sac tumors, there were no significant differences in the number of methylated TSGs between 22 gonadal tumors and five extra-gonadal tumors, between 10 tumors in the normal reprogramming pathway and 17 tumors in the abnormal pathway, and between nine tumors with the UU/UU epigenotype and 18 tumors with other epigenotypes.

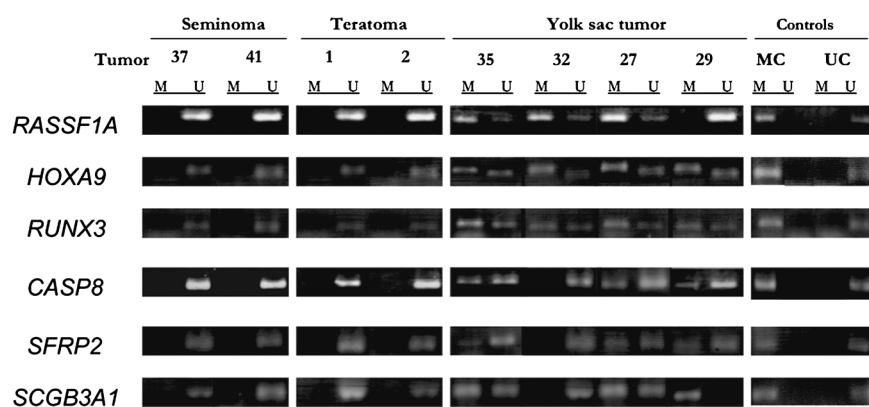
On the basis of the methylation status of the *H19* and *SNRPN* DMRs and the numbers of methylated TSGs, we epigenetically characterized GCTs into three groups; seminomas with total erasure of *H19* and *SNRPN* DMR methylation and few methylated TSGs; teratomas with mostly physiological methylation and demethylation of the *H19* and *SNRPN* DMRs and few methylated TSGs, and yolk sac tumors with various methylation statuses of the *H19* and *SNRPN* DMRs and a high incidence of methylated TSGs.

**1p36 deletion and the methylation status of *RUNX3* and *RIZ1*.** SNP array analysis showed hemizygous deletion of the 1p36 chromosomal region in 3/7 tumors; the deletion region of 1p36 was 15.5–37.2 Mb in No. 23, 0–31.7 Mb in No. 34 and 0–35.6 Mb in No. 35 (National centre for Biotechnology Information [NCBI] v36.2). Because *RUNX3* and *RIZ1* are located in the 25.1 Mb and 13.9 Mb regions (NCBI v36.2), respectively, all three tumors lost the *RUNX3* locus and 2/3 lost the *RIZ1* locus. All three tumors with 1p36 deletion had *RUNX3* methylation but no *RIZ1* methylation, whereas the other four tumors without 1p36 deletion had neither *RUNX3* methylation nor *RIZ1* methylation.

**Table 3. Methylation status of *H19* and *SNRPN* DMRs and total and allelic expression of *H19*, *IGF2* and *SNRPN***

| Patient Number | Pathology                    | <i>H19</i> DMR | <i>H19</i> mRNA | <i>RsaI</i> site | <i>H19</i> allelic expression | <i>IGF2</i> mRNA | <i>Apal</i> site | <i>IGF2</i> allelic expression | <i>SNRPN</i> DMR | <i>SNRPN</i> mRNA |
|----------------|------------------------------|----------------|-----------------|------------------|-------------------------------|------------------|------------------|--------------------------------|------------------|-------------------|
| 13             | Teratoma                     | UU             | 2+              | Homo             | Not done                      | 2+               | Homo             | Not done                       | MM               | 1+                |
| 15             | Yolk sac tumor               | UU             | 2+              | Homo             | Not done                      | 2+               | Homo             | Not done                       | UU               | 2+                |
| 21             | Yolk sac tumor /embryonal Ca | UU             | 1+              | Hetero           | Biallelic                     | 1+               | Homo             | Not done                       | UU               | 2+                |
| 24             | Yolk sac tumor               | UU             | 2+              | Homo             | Not done                      | 1+               | Homo             | Not done                       | UM               | 1+                |
| 26             | Yolk sac tumor               | MU             | 2+              | Homo             | Not done                      | 2+               | Hetero           | Biallelic                      | UU               | 2+                |
| 27             | Yolk sac tumor               | MU             | 2+              | Homo             | Not done                      | 2+               | Hetero           | Monoallelic <sup>†</sup>       | UU               | 2+                |
| 30             | Yolk sac tumor               | MU             | 2+              | Homo             | Not done                      | 2+               | Homo             | Not done                       | UM               | 2+                |
| 31             | Yolk sac tumor               | MU             | 2+              | Hetero           | Monoallelic                   | 2+               | Hetero           | Biallelic                      | UM               | 2+                |
| 36             | Yolk sac tumor /embryonal Ca | MU             | 2+              | Homo             | Not done                      | 2+               | Homo             | Not done                       | UM               | 2+                |
| 43             | Seminoma                     | UU             | 1+              | Hetero           | Monoallelic                   | 1+               | Hetero           | Monoallelic <sup>†</sup>       | UU               | 2+                |
| 44             | Seminoma                     | UU             | 1+              | Hetero           | Monoallelic                   | 1+               | Hetero           | Biallelic                      | UU               | 2+                |
| 45             | Seminoma                     | UU             | 1+              | Homo             | Not done                      | 1+               | Hetero           | Monoallelic <sup>†</sup>       | UU               | 2+                |

UU/MM/MU/UM, see the legend for Table 2; Homo, homozygosity; Hetero, heterozygosity; Ca, carcinoma; DMR, differential methylated region; <sup>†</sup>Monoallelic, a faint opposite band is visible.



**Fig. 4.** Examples of methylation status using methylation-specific polymerase chain reaction (PCR). PCR products of methylated or unmethylated *RASSF1A*, *HOXA9*, *RUNX3*, *CASP8*, *SFRP2* and *SCGB3A1* from germ cell tumors are shown. M, methylated products; U, unmethylated products.

**Methylation status of TSGs and the outcome of patients.** Of 45 patients who were followed from 3 to 278 months with a median of 97 months, only four died of the disease. Of these four patients, two children with yolk sac tumor had *HOXA9* and *CASP8* methylation; one of the two had *RASSF1A* methylation, one child with immature teratoma had *DCR2* methylation and one adult with seminoma had no methylation of 17 TSGs. Conversely, 24 of 25 patients with *RASSF1A* methylation, 14 of 16 patients with *CASP8* methylation, and 14 of 15 patients with *DCR2* methylation were alive. Thus, there was no correlation between TSG methylation and the outcome in patients with GCT in the present study, although studies including a large number of deceased patients may be necessary to get a definite conclusion.

## Discussion

Primordial germ cells undergo erasure of the somatic-like epigenetic pattern, and are transformed into the sex-specific pattern of mature germ cells during the epigenetic reprogramming process.<sup>(4-6)</sup> Previous studies suggested that GCTs, thought to originate from PGCs, show epigenetic patterns reflecting the developmental process of PGCs.<sup>(9-12)</sup> In the present series of 45 childhood and adult GCTs, 25 and 20 tumors showed the epigenetic patterns thought to reflect normal and abnormal reprogramming pathways, respectively (Table 2).

Kawakami *et al.*<sup>(29)</sup> showed that the patterns of methylation of the promoter and CTCF-binding site upstream of *H19* were

thoroughly unmethylated in testicular GCTs, and proposed that the patterns of methylation of *H19* in testicular GCTs showed erasure of methylation. The present study, which showed the UU/UU epigenotype in all four seminomas and in all five dysgerminomas, confirmed the findings reported by Kawakami *et al.*<sup>(29)</sup> and furthermore indicated a novel evidence that dysgerminoma, that is, the counterpart of seminoma in females, also retained unmethylated *H19* and *SNRPN* DMRs. Thus, we present data that seminoma developing in the testis and dysgerminoma developing in the ovary share not only the same morphological features but also the same epigenotype, indicating that both may be derived from PGCs in the same reprogramming process.

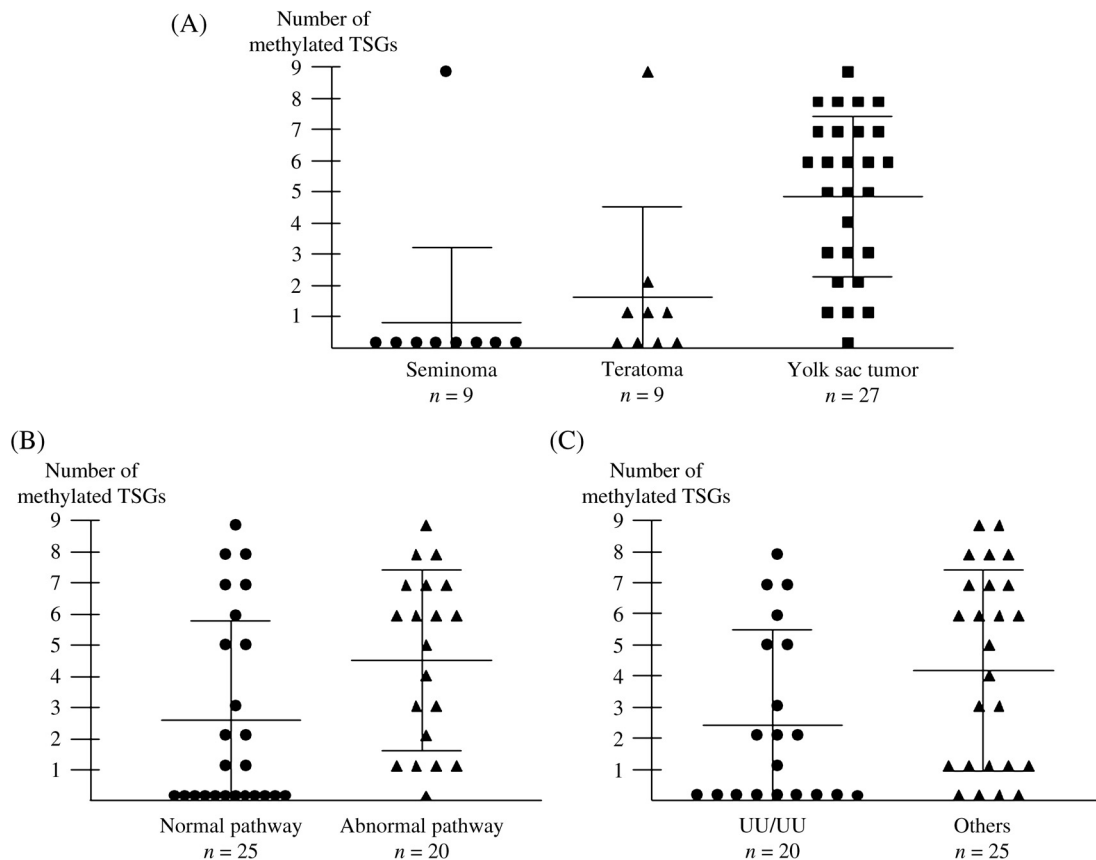
All four tumors with hypermethylated *SNRPN* DMR (MM) originated from the ovary, and 3/4 retained heterozygosity in *SNRPN* alleles. These findings suggest that PGCs that entered into female gametogenesis and underwent biparental methylation of *SNRPN* may have transformed to teratoma and yolk sac tumor (Fig. 2). Bussey *et al.* stated that *SNRPN* methylation pattern in germ cell tumors reflects primordial germ cell development.<sup>(11)</sup> Other investigators also reported hypermethylation of *SNRPN* in some benign and immature ovarian teratomas.<sup>(9,10)</sup> While three extra-gonadal tumors showed the UU/UU epigenotype, which is the normal imprint status of gonadal tumors, suggesting that aberrant imprint erasure may have occurred in PGCs before entering the genital ridge, and five ovarian and one testicular tumors showed MU/UM, which is the normal imprint status of extra-gonadal tumors, suggesting that incomplete imprint erasure

**Table 4. Tumor suppressor genes and the incidence of methylated seminoma, non-seminoma, childhood yolk sac tumor, hepatoblastoma, Wilms tumor and neuroblastoma examined by methylation-specific polymerase chain reaction**

| Gene            | Seminoma                 |                                            | Non-seminoma              |                                            |         | Childhood solid tumors     |                                                            |         |                                                              |         |                                            |         |
|-----------------|--------------------------|--------------------------------------------|---------------------------|--------------------------------------------|---------|----------------------------|------------------------------------------------------------|---------|--------------------------------------------------------------|---------|--------------------------------------------|---------|
|                 | Present study<br>(n = 9) | Lind et al. 2006 <sup>15</sup><br>(n = 20) | Present study<br>(n = 36) | Lind et al. 2006 <sup>15</sup><br>(n = 35) | P-value | Yolk sac tumor<br>(n = 25) | Hepatoblastoma                                             |         | Wilms tumor                                                  |         | Neuroblastoma                              |         |
|                 |                          |                                            |                           |                                            |         |                            | (n = 20 <sup>†</sup> , 39 <sup>‡</sup> , 97 <sup>§</sup> ) | P-value | (n = 20 <sup>¶</sup> , 39 <sup>††</sup> , 40 <sup>‡‡</sup> ) | P-value | (n = 56 <sup>§§</sup> , 70 <sup>¶¶</sup> ) | P-value |
| <i>RASSF1A</i>  | 1 (11%)                  | 0 (0%)                                     | 24 (67%)                  | 10 (29%)                                   | 0.001   | 21 (84%)                   | 43 <sup>§</sup> (44%)                                      | 0.0020  | 21 <sup>†</sup> (54%)                                        | 0.0272  | 39 <sup>†</sup> (70%)                      | 0.2768  |
| <i>HOXA9</i>    | 1 (11%)                  | 0 (0%)                                     | 23 (64%)                  | 9 (26%)                                    | 0.003   | 22 (88%)                   | 0 <sup>†</sup> (0%)                                        | <0.0001 | 0 <sup>†</sup> (0%)                                          | <0.0001 | 0 <sup>†</sup> (0%)                        | <0.0001 |
| <i>RUNX3</i>    | 1 (11%)                  | 0 (0%)                                     | 21 (58%)                  | 1 (3%)                                     | <0.001  | 20 (80%)                   | 0 <sup>†</sup> (0%)                                        | <0.0001 | 4 <sup>†</sup> (20%)                                         | 0.0002  | 0/45                                       |         |
| <i>CASP8</i>    | 0 (0%)                   |                                            | 16 (44%)                  |                                            |         | 14 (56%)                   | 15 <sup>§</sup> (16%)                                      | <0.0001 | 17 <sup>§</sup> (43%)                                        | 0.2891  | 39 <sup>†</sup> (56%)                      | 0.9803  |
| <i>SCGB3A1</i>  | 1 (11%)                  | 0 (0%)                                     | 15 (42%)                  | 19 (54%)                                   | 0.287   | 13 (52%)                   | 0 <sup>†</sup> (0%)                                        | <0.0001 | 5 <sup>†</sup> (25%)                                         | 0.0777  | 15 <sup>†</sup> (21%)                      | 0.0040  |
| <i>SFRP2</i>    | 1 (11%)                  |                                            | 15 (42%)                  |                                            |         | 13 (52%)                   | 0 <sup>†</sup> (0%)                                        | <0.0001 | 2 <sup>†</sup> (10%)                                         | 0.0040  |                                            |         |
| <i>DCR2</i>     | 1 (11%)                  |                                            | 14 (39%)                  |                                            |         | 11 (44%)                   | 0 <sup>†</sup> (0%)                                        | <0.0001 | 6 <sup>†</sup> (30%)                                         | 0.5137  | 31 <sup>†</sup> (44%)                      | 0.9803  |
| <i>RASSF5</i>   | 1 (11%)                  |                                            | 7 (19%)                   |                                            |         | 7 (28%)                    | 0 <sup>†</sup> (0%)                                        | 0.012   | 6 <sup>§</sup> (15%)                                         | 0.3390  | 0/45                                       |         |
| <i>RASSF2A</i>  | 0 (0%)                   |                                            | 5 (14%)                   |                                            |         | 5 (20%)                    | 0 <sup>†</sup> (0%)                                        | 0.0561  | 2 <sup>†</sup> (10%)                                         | 0.4367  | 0/45                                       |         |
| <i>BLU</i>      | 0 (0%)                   |                                            | 3 (8%)                    |                                            |         | 3 (12%)                    | 0 <sup>†</sup> (0%)                                        | 0.2424  | 0 <sup>†</sup> (0%)                                          | 0.2424  | 5/45                                       |         |
| <i>SFRP5</i>    | 0 (0%)                   |                                            | 2 (6%)                    |                                            |         | 1 (4%)                     | 0 <sup>†</sup> (0%)                                        | 0.3906  | 0 <sup>†</sup> (0%)                                          |         | 0/45                                       |         |
| <i>HOXB5</i>    | 0 (0%)                   | 0 (0%)                                     | 1 (3%)                    | 5 (13%)                                    | 0.107   | 1 (4%)                     | 0 <sup>†</sup> (0%)                                        | 1       | 0 <sup>†</sup> (0%)                                          | 1       | 0/45                                       |         |
| <i>P161NK4A</i> | 0 (0%)                   |                                            | 1 (3%)                    |                                            |         | 0 (0%)                     | 0 <sup>†</sup> (0%)                                        | 1       | 4 <sup>§</sup> (10%)                                         | 0.2706  | 0/45                                       |         |
| <i>SFRP1</i>    | 0 (0%)                   |                                            | 1 (3%)                    |                                            |         | 1 (4%)                     | 0 <sup>†</sup> (0%)                                        | 1       | 0 <sup>†</sup> (0%)                                          | 1       | 0/45                                       |         |
| <i>P14ARF</i>   | 0 (0%)                   |                                            | 0 (0%)                    |                                            |         | 0 (0%)                     | 0 <sup>†</sup> (0%)                                        | 1       | 6 <sup>§</sup> (15%)                                         | 0.0743  | 0/45                                       |         |
| <i>RIZ1</i>     | 0 (0%)                   |                                            | 0 (0%)                    |                                            |         | 0 (0%)                     | 0 <sup>†</sup> (0%)                                        | 1       | 0 <sup>†</sup> (0%)                                          | 1       | 0/45                                       |         |
| <i>SFRP4</i>    | 0 (0%)                   |                                            | 0 (0%)                    |                                            |         | 0 (0%)                     | 0 <sup>†</sup> (0%)                                        | 1       | 0 <sup>†</sup> (0%)                                          | 1       | 0/45                                       |         |

<sup>†</sup>, <sup>‡</sup>, <sup>§</sup>Total number of tumors examined in Ref. 22; <sup>¶</sup>total number of tumors examined in this study; <sup>††</sup>total number of tumors examined in Ref. 26; <sup>‡‡</sup>total number of tumors examined in Ref. 27; <sup>§§</sup>total number of tumors examined in Ref. 25; <sup>¶¶</sup>total number of tumors examined in Ref. 28.





**Fig. 5.** Numbers of methylated tumor suppressor genes (TSGs) in 45 germ cell tumors. The large and small horizontal bars indicate the mean number of methylated TSGs and the mean number  $\pm$  standard deviation (SD), respectively. (A) The number of methylated TSGs was higher in yolk sac tumors than in seminomas ( $P < 0.001$ ) or teratomas ( $P = 0.004$ ) by Student's *t*-test. (B, C) The number of methylated TSGs was lower in tumors in the normal reprogramming pathway than in tumors in the abnormal pathway ( $P = 0.042$ ), and in tumors with the UU/UU epigenotype than in tumors with other epigenotypes ( $P = 0.048$ ).

may have occurred in PGCs in the ovary. Thus, the present study showed that half of PGCs giving rise to GCT underwent developmentally regulated erasure and resetting of imprinted genes, and that the other half of PGCs underwent inappropriate timing of imprint erasure or aberrant retention of the imprint. In regard to histology, while all seminomas and most teratomas were in the normal reprogramming pathway, the majority of yolk sac tumors were in the abnormal pathway.

In regard to the relationship between the methylation status of *H19* DMR and *H19* expression, tumors with somatically methylated *H19* DMR (MU) showed a higher amount of *IGF2* mRNA than tumors with hypomethylated *H19* DMR (UU). Furthermore, the methylation status of *H19* DMR was not correlated with monoallelic or biallelic expression of *H19* or *IGF2* (Table 3). These findings are not consistent with the enhancer completion model in somatic cells, that is, *IGF2* and *H19* promoters compete on the same chromosome for a shared enhancer, and access of the maternal *IGF2* allele to this enhancer is blocked by *H19* DMR when unmethylated, because of the insulator activity of CTCF binding to unmethylated *H19* DMR.<sup>(30,31)</sup> Jaenisch suggested that DNA methylation has no role in non-somatic lineages such as embryonic stem (ES) cells, PGCs and cleavage embryo.<sup>(32)</sup> The present and previous studies suggest that the hypothesis is applicable to GCT.<sup>(29)</sup>

In a study of 55 primary adult testicular GCTs, Lind *et al.*<sup>(15)</sup> reported that non-seminomas were significantly more often methylated than seminomas, and that the three most frequently methylated genes among this subtype were *SCGB3A1* (54%), *RASSF1A* (29%), and *HOXA9* (26%). Our data not only confirmed the higher incidence of TSG methylation in non-seminomas

than in seminomas but also revealed that the incidences of *RASSF1A* (67%), *HOXA9* (64%) and *RUNX3* (58%) methylation were much higher in the present series of non-seminomas, mostly occurring in children, than in the previous series of adult testicular non-seminomas, although there was no difference in the incidence of *SCGB3A1* methylation between the two series of non-seminomas (Table 4). In addition, a high incidence of *RUNX3* methylation in infant testicular yolk sac tumors and absence of *RUNX3* methylation in adult testicular non-seminomas were reported previously.<sup>(33)</sup> The present findings that all three childhood yolk sac tumors with 1p36 loss had *RUNX3* methylation but no *RIZ1* methylation, and that all four yolk sac tumors or teratomas with no 1p36 loss had neither *RUNX3* nor *RIZ1* methylation. The biological significance of *RUNX3* methylation should be elucidated.

Recently, Calvanese *et al.* reported that some of the genes frequently hypermethylated in cancer are also frequently hypermethylated in human embryonic stem cells, and proposed a hypothesis that the aberrant methylation of some TSGs in cancer should be understood as a defect in establishing an unmethylated promoter during differentiation, rather than as an anomalous process of de novo hypermethylation.<sup>(34)</sup> Yolk sac tumors had more frequent TSG methylation and were more frequent in the abnormal reprogramming pathway than seminomas or teratomas. Furthermore, the incidence of methylated TSGs was higher in childhood yolk sac tumors than in other childhood tumors. Nevertheless, methylation of TSGs did not affect the outcome of children with yolk sac tumors, although prognostic implication of methylated TSGs has been reported in many childhood and

**Table 5. Association between two or three groups of tumors classified by clinical, pathological and epigenetic factors and reprogramming pathways**

|                               |                | Number of tumors | Normal reprogramming pathway (n = 25) | Abnormal reprogramming pathway (n = 20) | P-value <sup>†</sup> | Mean number of methylated TSGs ± SD | P-value <sup>‡</sup> |
|-------------------------------|----------------|------------------|---------------------------------------|-----------------------------------------|----------------------|-------------------------------------|----------------------|
| Age (years)                   | 0~14           | 38               | 20                                    | 18                                      |                      | 4.05 ± 3.09                         |                      |
|                               | 15~42          | 7                | 5                                     | 2                                       | 0.437                | 0 (0)                               | Not calculated       |
| Sex                           | Male           | 23               | 10                                    | 13                                      |                      | 3.30 ± 2.98                         |                      |
|                               | Female         | 22               | 15                                    | 7                                       | 0.172                | 3.59 ± 3.45                         | 0.766                |
| Primary site                  | Gonad          | 35               | 21                                    | 14                                      |                      | 3.43 ± 3.12                         |                      |
|                               | Extra-gonad    | 10               | 4                                     | 6                                       | 0.446                | 3.50 ± 3.57                         | 0.951                |
| Pathological classification   | Seminoma       | 9                | 9                                     | 0                                       |                      | 0.78 ± 2.33                         | <0.001               |
|                               | Teratoma       | 9                | 6                                     | 3                                       |                      | 1.56 ± 2.88                         |                      |
|                               | Yolk sac tumor | 27               | 10                                    | 17                                      | 0.013                | 4.96 ± 2.61                         |                      |
| Epigenotype                   | UU/UU          | 20               | 17                                    | 3                                       |                      | 2.40 ± 2.85                         |                      |
|                               | Others         | 25               | 8                                     | 17                                      | 0.0007               | 4.24 ± 3.28                         | 0.048                |
| Reprogramming pathway         | Normal         | 25               | NA                                    | NA                                      |                      | 2.56 ± 3.22                         |                      |
|                               | Abnormal       | 20               | NA                                    | NA                                      |                      | 4.50 ± 2.91                         | 0.042                |
| The number of methylated TSGs | 0              | 14               | 13                                    | 1                                       |                      | NA                                  |                      |
|                               | 1~8            | 31               | 12                                    | 19                                      | 0.0009               | NA                                  |                      |

<sup>†</sup>Chi-square test; <sup>‡</sup>Student's *t*-test; SD, standard deviation; TSGs, tumor suppressor genes; SD, standard deviation; Epigenotype, see text; NA, not applicable.

adult cancers.<sup>(13,25,28)</sup> The number of methylated TSGs seems to be not correlated with specific methylation patterns of *H19* and *SNRPN* DMRs in yolk sac tumors, although the number of the tumors with specific methylation patterns of the two DMRs may be too small to draw a conclusion. The high incidence but no prognostic significance of methylated TSGs in childhood yolk sac tumor may be explained by a hypothesis, that is, hypermethylation of TSGs may reflect epigenetic characteristics of PGCs giving rise to yolk sac tumor, but not the aberrant promoter methylation occurring in the tumorigenic process of various other cancers.<sup>(34)</sup> The present findings are also consistent with the hypothesis proposed by Jaenisch who suggested that DNA methylation has no role in PGCs.<sup>(32)</sup> In contrast, the low incidence

of methylated TSGs in seminoma and teratoma suggests that TSGs in these tumors may be repressed by the Polycomb group of proteins but not by the promoter DNA methylation. In conclusion, the present study of epigenetic reprogramming pathways and TSG methylation in seminoma, teratoma and yolk sac tumors disclosed distinct epigenetic characteristics in each of the three histological subtypes of GCT.

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