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ORIGINAL ARTICLE

Cell-to-cell variation of chromosomal number in the adult testicular germ cell tumors: a comparison of chromosomal instability among histological components and its putative role in tumor progression

Kosuke Miyai[1,2] · Keiichi Ito[3] · Kuniaki Nakanishi[2] · Hitoshi Tsuda[1]

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Abstract

By allelotyping analysis, we previously reported a putative progression pathway from germ cell neoplasia in situ (GCNIS) to seminoma, then to embryonal carcinoma in mixed-type testicular germ cell tumors (TGCTs), and detected that loss of heterozygosity events in seminoma components in mixed tumors were more frequent than those in pure seminomas. To elucidate a role of chromosomal instability in the progression of non-seminomatous germ cell tumor (NSGCT), we performed fluorescence in situ hybridization with centromeric probes for chromosomes 1, 7, 8, 12, 17, and X on a cohort of 52 TGCT cases with 103 histologically distinct components: 39 GCNIS lesions (16 and 23 in tumors with and without NSGCT components, respectively), 39 seminomas (27 as pure seminomas and 12 in mixed tumors), and 25 embryonal carcinomas. On a total component basis, both the mean copy number per tumor cell nucleus and the deviations from the modal number of all chromosomes examined significantly increased from GCNIS to seminoma, then to embryonal carcinoma with few exceptions. Seminoma components in mixed tumors showed a significantly greater extent of chromosomal instability in chromosomes 8 and 12 than pure seminomas, whereas no statistically significant difference was observed between GCNIS lesions with and without NSGCT components. These results suggest that not only aneuploidy, but also the cell-to-cell variation of chromosomal number is a sensitive indicator of chromosomal instability and would be implicated in the progression of NSGCT.

Keywords Testis · Germ cell tumor · Chromosomal instability · Fluorescence in situ hybridization

Introduction

Adult testicular germ cell tumor (TGCT) is the most frequent malignant solid tumor among men aged 15–45 years, with increasing incidence in the past 30 years [1]. Clinicopathologically, TGCTs are divided into two entities: seminomas and non-seminomatous germ cell tumors (NSGCTs) consisting of embryonal carcinoma, yolk sac tumor, choriocarcinoma, and teratoma. Seminoma in its pure form (i.e., tumor of one histological type) accounts for approximately 50% of all TGCTs, whereas over 70% of the rest exhibit a mixture of more than one histological components (i.e., seminoma and/or NSGCT) and are called mixed tumors [2]. Despite advances in the management of TGCT, a small group of patients with NSGCTs, especially those with embryonal carcinoma components, are more likely to be metastatic at presentation, and those in advanced stages confer worse prognosis than pure seminomas at an equivalent stage of the disease [3, 4]. This poor prognosis is attributed in part to the lack of knowledge on the progression of NSGCTs.

Seminoma and NSGCTs originate from a common non-invasive precursor lesion, histologically referred to as germ cell neoplasia in situ (GCNIS) [5, 6]. Several studies have shown that NSGCTs develop when a GCNIS cell or a seminoma cell becomes reprogrammed to an embryonal

✉ Kosuke Miyai
mykusu228@nifty.com

[1] Department of Basic Pathology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan

[2] Department of Laboratory Medicine, National Defense Medical College, Tokorozawa, Saitama 359-8513, Japan

[3] Department of Urology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan

carcinoma cell, which is the neoplastic counterpart of the human embryonal stem cell [7, 8]. Then, the embryonal carcinoma cells are likely to differentiate into other NSGCT components [7]. By analyzing the pattern of allelic loss, we previously suggested the progression pathway from GCNIS to seminoma, then to embryonal carcinoma in TGCTs with a mixture of seminoma and embryonal carcinoma components [9], and detected that loss of heterozygosity (LOH) events in seminoma components in mixed tumors were more frequent than those in pure seminomas [10]. To detect what causes GCNIS/seminoma cells to be reprogramed to NSGCT, it appears to be effective to investigate and compare the molecular genetic changes among the histological components separately for the tumor groups (i.e., pure seminoma, mixed tumor consisting of seminoma and NSGCT, and tumor consisted only NSGCT components).

Cytogenetic, comparative genomic hybridization, and allelic imbalance studies have reported that TGCTs are consistently aneuploid and show several chromosomal aberrations common to both seminoma and NSGCTs, including gain of material from chromosomes 1, 7, 8, 12, 17, 21, and X and loss of material from chromosomes 4, 5, 11, 13, 18, and Y [11–16]. Among these, isochromosome 12p or other type of 12p amplification is known as a hallmark of TGCTs. Although these aneuploidy/chromosomal aberrations are firm as evidence related to the development of TGCTs, aneuploidy is basically a "state" and not an underlying "rate" of chromosomal instability which reflects a continuing cellular defect. From a viewpoint of evaluating the relative status of chromosomal instability that is implicated in the multistep progression of TGCT, it would be more adequate to investigate the cell-to-cell variations of chromosomal gain or loss.

In the present study, we performed the histological review of surgically resected specimens from 52 primary TGCTs and selected a total of 103 histologically distinct components, including GCNIS, seminomas, and embryonal carcinomas. We performed fluorescence in situ hybridization (FISH) with centromeric probes for chromosomes 1, 7, 8, 12, 17, and X (i.e., the chromosomes for which copy numbers have been reported to increase in adult TGCTs [11–16]). We analyzed the absolute chromosomal numbers and cell-to-cell variation of chromosomal numbers with the aim of answering the following questions: (1) whether the extent of chromosomal instability increases along the progression from GCNIS to seminoma, then to embryonal carcinoma; (2) whether chromosomal instability is commonly observed in the various chromosomes examined; and (3) whether the status of chromosomal instability is significantly different between seminoma components in mixed tumors and pure seminomas, and between GCNIS lesions with and without adjacent NSGCT components. Such information would allow us to evaluate the quantitative status of chromosomal instability among histological components of TGCTs and its role in the tumor progression.

Materials and methods

Cases

Fifty-two cases of primary TGCT with seminoma and/or embryonal carcinoma components were identified from the files of the Department of Laboratory Medicine, National Defense Medical College Hospital, Tokorozawa, Saitama, Japan. All the cases were surgically resected specimens obtained from 1987 to 2010, and the age of patients ranged from 18 to 79 (mean 33.2) years. All the pathology specimens were reviewed in our institution and the tumors were classified according to the World Health Organization criteria [1]. Pathological tumor staging was assessed according to the American Joint Committee on Cancer (AJCC) system [17]; 33, 5, and 14 cases were staged I, II, and III, respectively.

Consequently, of the 52 tumors, 27, 12, and 13 cases were classified as pure seminomas, mixed tumors (i.e., mixture of seminoma and embryonal carcinoma), and NSGCT-only tumors, respectively. Moreover, a total of 119 histologically distinct components were identified in the 52 cases: 39 GCNIS lesions (23 in pure seminomas, 7 in mixed tumors, 9 in NSGCT-only tumors), 39 seminomas (27 as pure seminomas and 12 in mixed tumors), 25 embryonal carcinomas (12 in mixed tumors and 13 as components of NSGCT-only tumors), 1 choriocarcinoma (as a component of NSGCT-only tumor), 6 yolk sac tumors (3 in mixed tumors and 3 as components of NSGCT-only tumors), and 9 teratomas (2 in mixed tumors and 7 as components of NSGCT-only tumors). Choriocarcinoma, yolk sac tumor, and teratoma components were not analyzed further in this study because we focused on the tumor progression to embryonal carcinoma which is likely to differentiate into other three NSGCT components [7]. The cases enrolled and histological components examined are summarized in Table 1. The research protocol was approved by the Ethics Committee of the National Defense Medical College, Tokorozawa, Japan.

FISH analysis

We selected formalin-fixed, paraffin-embedded tissue blocks containing the areas in which histological diagnosis for tumor components was performed. These tissue blocks were then cut into 4.5-μm-thick sections and subjected to FISH analysis. CEP1, 7, 8, 12, 17, and X SpectrumOrange DNA probes (Abbott Molecular, Des Plaines, IL, USA) were used for the FISH analysis by the same method as that of the PathVysion DNA probe kit (Abbott Molecular), as described previously [18]. Hybridization was performed between the denatured probes and denatured DNA in tissue block sections at 37 °C for 14–18 h. The sections were counterstained with 4,6-diamidino-2-phenylindone (DAPI).

Table 1 Cases enrolled and distinct histological components analyzed in this study

Tumor type	No. of cases	No. of histological components examined		
		GCNIS	SE	EC
Pure seminoma	27	23	27	–
Mixed tumor	12	7	12	12
NSGCT-only tumor	13	9	–	13
Total	52	39	39	25

EC embryonal carcinoma, *GCNIS* germ cell neoplasia in situ, *NSGCT* non-seminomatous germ cell tumor, *SE* seminoma

Representative images of FISH are shown in Fig. 1. The number of fluorescence signals from CEP1, 7, 8, 12, 17, and X probes in 100 interphase tumor cell nuclei was counted independently by two observers (KM and HT). The mean chromosomal number per tumor cell nucleus for each tumor component was calculated as the average of *CEP* signals (hereafter referred to as "number"). For the purpose of elucidating the cell-to-cell variation of chromosomal numbers, the modal chromosomal number was determined in each tumor component, and the fraction (percentage) of cells whose chromosomal number was different from the mode (hereafter referred to as "extramodal fraction") was calculated according to previous reports [19, 20]. As a control, non-neoplastic testicular tissue (seminiferous tubules containing non-neoplastic spermatocytes) was used. When the judgments by the two observers differed for a tumor, they counted further to a total of 40 nuclei and reached consensus by discussion.

Statistical analysis

Statistical analyses were performed using R software (version 3.4.2, R Core Team and Foundation for Statistical Computing, Vienna, Austria). The number and the extramodal fraction of chromosomes among the tumor components examined were compared using Student's t test, Welch's t test, or Mann-Whitney's U test. The correlation between the tumor pathological stage and the number and the extramodal fraction of chromosomes was assessed using the one-way ANOVA test or the Kruskal-Wallis test. Differences with $P < 0.05$ were considered to be statistically significant.

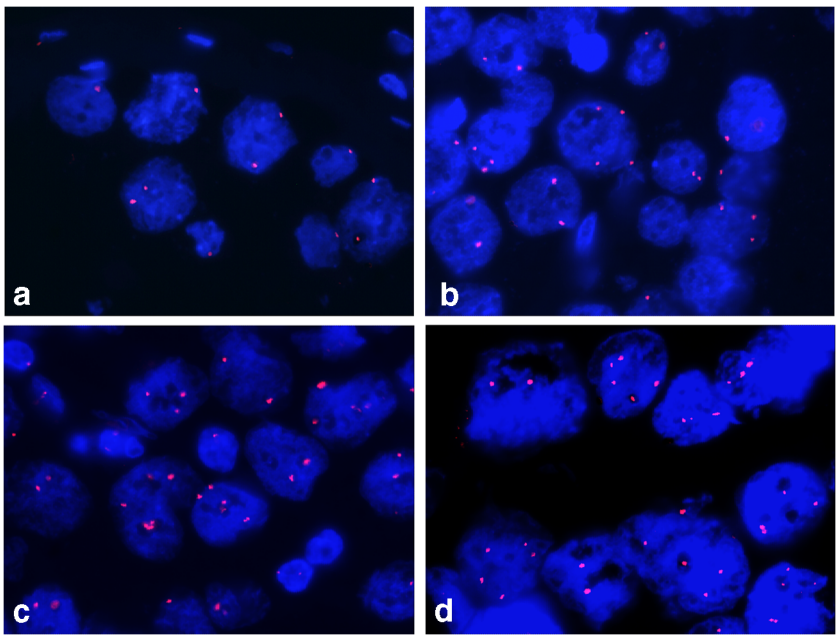

Fig. 1 Status of copy number of chromosome 8 determined by fluorescence in situ hybridization (FISH) in histological components of testicular germ cell tumors. **a**, **b** A case of **b** pure seminoma and **a** adjacent germ cell neoplasia in situ (GCNIS). One or two red signals indicating a chromosomal number are noted in neoplastic cells of GCNIS, whereas two to four signals are noted in seminoma cells. **c**, **d** A case of mixed tumor with a mixture of **c** seminoma and **d** embryonal carcinoma components, both of which show two to seven red signals indicating highly variable cell-to cell copy numbers of chromosome 8. DAPI-counterstained interphase nuclei are shown for each specimen (original magnification × 1000)

Results

Results of the number and the extramodal fraction of chromosomes examined are summarized in Tables 2 and 3, respectively. There was only one GCNIS lesion showing the discrepancy in counting of chromosome 8 between two observers. They reached consensus when they counted further to a total of 40 nuclei. No seminoma and embryonal carcinoma components showed the discrepancy in counting the chromosomes examined.

Chromosome 1

The number [mean ± standard error (SE)] of chromosome 1 was 2.8 ± 0.048, 3.7 ± 0.10, and 4.2 ± 0.17 in GCNIS lesions, seminoma, and embryonal carcinoma components, respectively (Table 2). Significant statistical differences in the mean number of chromosome 1 were observed between GCNIS lesions and seminoma components ($P < 0.0001$), and between seminoma and embryonal carcinoma components ($P = 0.0066$). The extramodal fraction (mean ± SE) of chromosome 1 was 51.2 ± 0.78%, 58.6 ± 1.3%, and 66.4 ± 1.5% in GCNIS lesions, seminoma, and embryonal carcinoma components, respectively (Table 3), with significant differences between GCNIS lesions and seminoma components ($P < 0.0001$), and between seminoma and embryonal carcinoma components ($P = 0.00031$).

Chromosome 7

The number (mean ± SE) of chromosome 7 was 2.3 ± 0.043, 3.1 ± 0.11, and 3.1 ± 0.084 in GCNIS lesions, seminoma, and embryonal carcinoma components, respectively (Table 2). The extramodal fraction (mean ± SE) of chromosome 7 was 48.7 ± 1.2%, 56.3 ± 1.0%, and 58.7 ± 1.3% in GCNIS lesions, seminoma, and embryonal carcinoma components, respectively (Table 3). There were significant differences in the number and the extramodal fraction of chromosome 7 between GCNIS lesions and seminoma components (each, $P < 0.0001$).

Chromosome 8

The number (mean ± SE) of chromosome 8 was 2.2 ± 0.043, 3.1 ± 0.11, and 3.1 ± 0.079 in GCNIS lesions, seminoma, and embryonal carcinoma components, respectively, with a significant statistical difference between GCNIS lesions and seminoma components ($P < 0.0001$; Table 2). The extramodal fraction (mean ± SE) of chromosome 8 was 47.4 ± 1.1%, 56.5 ± 0.82%, and 60.7 ± 1.1% in GCNIS lesions, seminoma, and embryonal carcinoma components, respectively (Table 3), with significant differences between GCNIS lesions and seminoma components ($P < 0.0001$), and between seminoma and embryonal carcinoma components ($P = 0.0030$).

Table 2 The number of chromosomes per nucleus in each distinct histological component

	The mean number of chromosomes per nucleus					
	Chr. 1	Chr.7	Chr. 8	Chr. 12	Chr. 17	Chr. X
GCNIS total (n = 39)	2.8	2.3	2.2	2.4	2.1	1.7
with NSGCT (n = 16)	2.8	2.2	2.3	2.4	2.1	1.7
without NSGCT (n = 23)	2.8	2.3	2.2	2.4	2.1	1.7
Seminoma total (n =39)	3.7	3.1	3.1	3.7	2.5	2.0
with NSGCT (n =12)	3.8	3.0	3.0	4.1	2.4	2.0
without NSGCT (n = 27)	3.6	3.2	3.1	3.6	2.5	2.0
Embryonal ca. (n = 25)	4.2	3.1	3.1	4.2	2.7	2.2

ca. carcinoma, *chr.* chromosome, *GCNIS* germ cell neoplasia in situ, *NSGCT* non-seminomatous germ cell tumor

*$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$

Table 3 The fraction of tumor cells with extramodal chromosomal numbers in each distinct histological component

	The mean of extramodal fraction[a] of chromosomes examined (%)					
	Chr. 1	Chr.7	Chr. 8	Chr. 12	Chr. 17	Chr. X
GCNIS total (n = 39)	51.2	48.7	47.4	51.4	47.4	45.3
with NSGCT (n = 16)	54.0	48.5	48.6	52.3	49.1	46.3
without NSGCT (n = 23)	48.5	48.8	46.5	50.3	46.3	44.6
Seminoma total (n =39)	58.6	56.3	56.5	60.1	52.4	46.1
with NSGCT (n =12)	61.6	55.9	59.4	64.4	52.7	44.7
without NSGCT (n = 27)	57.1	56.4	55.1	58.1	52.2	46.7
Embryonal ca. (n = 25)	66.4	58.7	60.7	66.2	57.2	52.0

ca. carcinoma, *chr.* chromosome, *GCNIS* germ cell neoplasia in situ, *NSGCT* non-seminomatous germ cell tumor

*$P < 0.01$; **$P < 0.001$; ***$P < 0.0001$

[a] The percentages of cells whose chromosome number is outside the mode

Chromosome 12

The number (mean ± SE) of chromosome 12 was 2.4 ± 0.045, 3.7 ± 0.12, and 4.2 ± 0.12 in GCNIS lesions, seminoma, and embryonal carcinoma components, respectively (Table 2). The extramodal fraction (mean ± SE) of chromosome 12 was 51.4 ± 1.0%, 60.1 ± 1.1%, and 66.2 ± 0.89% in GCNIS lesions, seminoma, and embryonal carcinoma components, respectively (Table 3). Significant statistical differences in the number and the extramodal fraction of chromosome 12 were observed between GCNIS lesions and seminoma components (each, $P < 0.0001$), and between seminoma and embryonal carcinoma components ($P = 0.0075$ and $P < 0.0001$, respectively).

Chromosome 17

The number (mean ± SE) of chromosome 17 was 2.1 ± 0.026, 2.5 ± 0.043, and 2.7 ± 0.058 in GCNIS lesions, seminoma, and embryonal carcinoma components, respectively (Table 2). The extramodal fraction (mean ± SE) of chromosome 17 was 47.4 ± 0.89%, 52.4 ± 1.0%, and 57.2 ± 1.1% in GCNIS lesions, seminoma, and embryonal carcinoma components, respectively (Table 3). There were significant statistical differences in the number and the extramodal fraction of chromosome 17 between GCNIS lesions and seminoma components (each, $P < 0.0001$), and between seminoma and embryonal carcinoma components ($P = 0.0020$ and $P = 0.0091$, respectively).

Chromosome X

The number (mean ± SE) of chromosome X was 1.7 ± 0.028, 2.0 ± 0.031, and 2.2 ± 0.054 in GCNIS lesions, seminoma, and embryonal carcinoma components, respectively, with a significant statistical difference between GCNIS lesions and seminoma components ($P < 0.0001$), and between seminoma and embryonal carcinoma components ($P = 0.012$; Table 2). The extramodal fraction (mean ± SE) of chromosome X was 45.3 ± 0.81%, 46.1 ± 1.0%, and 52.0 ± 1.5% in GCNIS lesions, seminoma, and embryonal carcinoma components, respectively (Table 3), with significant differences between seminoma and embryonal carcinoma components ($P = 0.0014$).

Comparison between GCNIS lesions with and without adjacent NSGCT components

With respect to the number and the extramodal fraction of chromosomes examined, no statistically significant difference was observed between GCNIS lesions with and without adjacent NSGCT components (Tables 2 and 3).

Comparison between seminoma components in mixed tumors and pure seminomas

The number of chromosome 12 (mean ± SE) was 4.1 ± 0.24 in seminoma components in mixed tumors and 3.6 ± 0.12 in pure

seminomas, with a statistically significant difference ($P = 0.039$; Table 2). The extramodal fraction of chromosomes 8 and 12 (mean ± SE) was 59.4 ± 1.0% and 66.4 ± 1.5% in seminoma components in mixed tumors and 55.1 ± 1.0% and 58.1 ± 1.3% in pure seminomas, respectively (Table 3). There were significant differences in the mean fraction of chromosomes 8 and 12 between seminoma components in mixed tumors and pure seminomas ($P = 0.0051$ and $P = 0.0063$, respectively). No other significant difference in the number and the extramodal fraction of chromosomes was detected between seminoma components in mixed tumors and pure seminomas.

Correlation between tumor pathological stage and the number and the extramodal fraction of the chromosomes examined

No significant correlations between the tumor pathological stage and the mean number and the mean extramodal fraction of the chromosomes were detected (Table 4).

Discussion

It has been considered that chromosomal instability is an integral component of the pathogenesis of human neoplasia and the structure of each chromosome can be highly variable (aneuploid) [21, 22]. In some multi-stage developmental models of human malignancy, such as the esophageal adenocarcinoma (from Barrett's esophagus to dysplasia, then to invasive adenocarcinoma) [23] and the vulvar squamous cell carcinoma (from vulvar intraepithelial neoplasia to squamous cell carcinoma in situ, then to invasive squamous cell carcinoma) [24], the chromosomal instability and copy number alteration have been reported to be rare in their early stages but more frequent and higher in their later stages. By analyzing the pattern of allelic loss, we previously provided a genetic evidence for the linear progression pathway in TGCTs with a mixture of seminoma and embryonal carcinoma components [9]. In the present study, both the mean number and the mean extramodal fraction of the chromosomes examined gradually increased from GCNIS to seminoma, then to embryonal carcinoma. With few exceptions,

Table 4 Correlation of the mean number and the extramodal fraction of chromosomes with pathological stage of cases

1) Patients with pure seminoma ($n = 27$)						
Pathological stage[a]	The mean chromosomal number in seminomas					
	Chr. 1	Chr. 7	Chr. 8	Chr. 12	Chr. 17	Chr. X
I ($n = 21$)	3.5	3.3	3.2	3.6	2.5	2.0
II ($n = 2$)	3.7	3.0	2.9	3.2	2.4	1.9
III ($n = 4$)	3.7	2.6	3.2	3.5	2.5	2.0
P value	0.74	0.21	0.62	0.66	0.90	0.45
Pathological stage	The mean extramodal fraction of chromosomes in seminomas					
	Chr. 1	Chr. 7	Chr. 8	Chr. 12	Chr. 17	Chr. X
I	56.4	57.1	55.1	57.5	52.5	46.8
II	56.5	52.5	56.0	59.5	58.5	43.0
III	60.1	54.8	55.0	60.0	55.5	48.0
P value	0.68	0.55	0.90	0.77	0.085	0.67
2) Patients with embryonal carcinoma components ($n = 25$)						
Pathological stage	The mean chromosomal number in embryonal carcinomas					
	Chr. 1	Chr. 7	Chr. 8	Chr. 12	Chr. 17	Chr. X
I ($n = 12$)	4.4	3.2	3.1	4.1	2.7	2.2
II ($n = 3$)	5.1	3.5	3.2	4.6	3.0	2.5
III ($n = 10$)	3.8	2.9	3.1	4.2	2.6	2.1
P value	0.11	0.067	0.98	0.51	0.22	0.34
Pathological stage	The mean extramodal fraction of chromosomes in embryonal carcinomas					
	Chr. 1	Chr. 7	Chr. 8	Chr. 12	Chr. 17	Chr. X
I	66.6	58.4	62.3	66.5	57.1	51.5
II	72.5	59.0	61.3	67.0	61.7	56.7
III	64.9	59.0	58.7	65.7	56.0	51.0
P value	0.42	0.98	0.59	0.88	0.28	0.51

Chr. chromosome

[a] Pathological tumor staging was assessed according to the American Joint Committee on Cancer system [17]

significant statistical differences in the mean number and the extramodal fraction of the chromosomes were observed between GCNIS and seminoma and between seminoma and embryonal carcinoma (Tables 2 and 3). These results demonstrate that the chromosomal instability of various chromosomes is commonly observed in the histological components of adult TGCTs and could be related to each step of the progression to NSGCT.

It is generally believed that tetraploidization is crucial in the development from fetal primordial germ cells to GCNIS cells, and then a non-random net loss of chromosomes renders the GCNIS cells aneuploid in the postpubertal testis [25, 26]. In addition, previous studies using karyotyping analysis, comparative genomic hybridization, and allelic imbalance analysis have reported that several chromosomal aberrations including gain of material from chromosomes 1, 7, 8, 12, 17, 21, and X and loss of material from chromosomes 4, 5, 11, 13, 18, and Y were detected during the progression from GCNIS to invasive TGCTs [11–16], which is compatible with the present data. On the other hand, NSGCTs have fewer copies of chromosomes 7, 15, 19, and 22 than seminomas, and only chromosome 17 has more copies in NSGCTs and seminomas [12, 15]. In the present study, embryonal carcinoma showed significantly higher mean copy numbers for chromosomes 1, 12, and X, in addition to chromosome 17, compared with seminoma. There was no significant difference in the mean copy number of chromosome 7 between embryonal carcinoma and seminoma. This discrepancy would mainly arise from the difference in the tumor cohort: most of the previous studies analyzed NGSCTs as a mixture of several histological components (i.e., embryonal carcinoma, yolk sac tumor, choriocarcinoma, and teratoma).

The presented data also indicated an increased rate of chromosomal instability during the progression of NSGCT, by analyzing the cell-to-cell variation of chromosomal numbers. Although there was no significant difference in the mean number of chromosome 8 between seminoma and embryonal carcinoma, the mean extramodal fraction of chromosome 8 was significantly higher in embryonal carcinoma than that in seminoma, suggesting that the cell-to-cell variation of chromosomal number is also a sensitive indicator of chromosomal instability. To our knowledge, there is only one previous study that investigated this type (i.e., cell-to-cell variation of chromosomal number) of chromosomal instability in various histological components of adult TGCTs. Using interphase cytogenetics in combination with immunohistochemistry, Looijenga et al. [27] investigated the number distribution of chromosomes 1, 12, and 15 in 11 GCNIS lesions, 6 seminomas, and 5 NSGCTs. Although the deviation from the modal number of chromosomes appeared to increase in NSGCTs compared to seminomas, they presented the figures of the distribution of chromosomal numbers but did not quantify the relative status of the chromosomal number variation [27].

In our study, there was no significant difference in the mean number and extramodal fraction of the chromosomes examined between GCNIS lesions with and without adjacent NSGCT components. A recent study using gene expression analysis by quantitative reverse-transcription polymerase chain reaction revealed that *PIWIL1/2/4* and *DDX4* genes, from the PIWI family which are key factors in germ cell development, were concertedly expressed in GCNIS lesions adjacent to NSGCTs, but were downregulated in those adjacent to seminomas [28]. They also indicated that the DNA methylation levels of long interspersed nucleotide factor-1 (LINE-1) promoters coincided with this pattern and concluded that these molecular changes could occur during the neoplastic transformation into seminoma, unlike NSGCT, and can be used as diagnostic biomarkers for the two types of TGCTs [28]. However, their data were relatively limited (i.e., specimens from 7 seminomas and 15 NSGCTs were studied) and mixed tumors with or without seminoma components had not been distinguished in the study. Interestingly, the hypomethylation of LINE-1 has been reported to be associated with chromosomal instability in several human malignant tumors including prostate [29] and colon adenocarcinoma [30] and gastrointestinal stromal tumors [31]. To elucidate an early change (i.e., events already detected in GCNIS and/or seminoma) in the development of TGCTs, further molecular genetic investigation of a larger number of GCNIS lesions categorized by histologic types of adjacent tumors (i.e., pure seminomas or mixed tumors with/without NSGCT) will be necessary.

We recently reported that the overall frequency of LOH in seminoma components of mixed tumors was significantly higher than that in pure seminomas and the allelic losses on specific chromosomal loci of 6p and 10q mainly contributed to the difference [10]. In the present study, the mean number of chromosome 12 and the mean extramodal fractions of chromosomes 8 and 12 in seminoma components in mixed tumors were significantly higher than those in pure seminomas. Given the evidence that the progression to embryonal carcinoma is likely to occur after the development of invasive seminoma in mixed tumors [9], these results suggest that seminoma cells which have a potential to progress to embryonal carcinoma cells (i.e., seminoma components in mixed tumors) already exhibit some genetic changes unlike those observed in pure-type seminomas. A few previous studies have demonstrated the molecular heterogeneity in testicular seminomas showing a generally uniform morphology [32–35]. Hofer et al. [32] performed hierarchical clustering based on complementary DNA expression array analysis and a refined protein expression of 8 pure seminomas and 4 pure embryonal carcinomas, and identified two groups: the first consisting solely of seminomas and the other of seminomas and embryonal carcinomas. Prior studies based on immunohistochemistry against methylation of CpG dinucleotides at position 5 of deoxycytidine residues ($5^{m}C$) have reported that seminoma is relatively more CpG hypomethylated than NSGCTs [33–35]. However, in their study, some pure seminomas

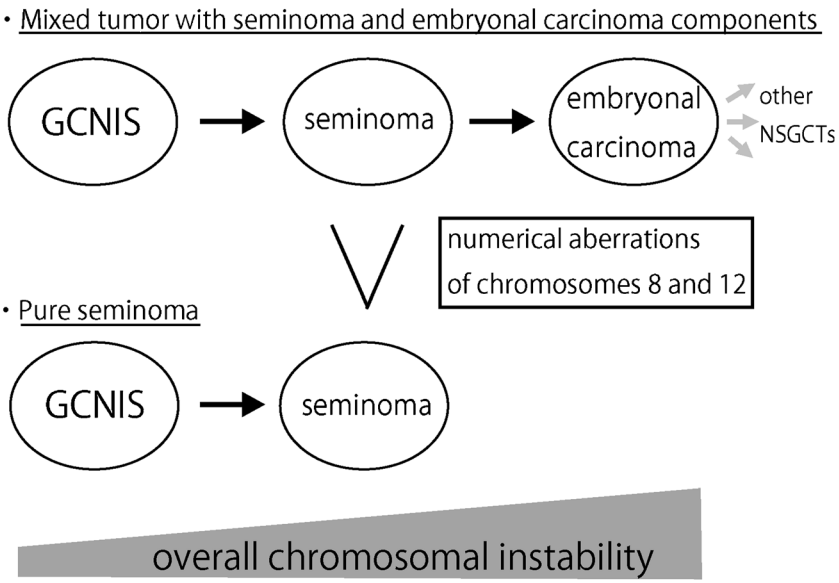

Fig. 2 Schematic summary of the present study. Both the mean chromosomal copy number and the deviation from a modal chromosomal number increase along the progression from germ cell neoplasia in situ (GCNIS) to seminoma, and then to embryonal carcinoma. These types of chromosomal instability are commonly observed in various chromosomes examined. In addition, seminoma components in mixed tumors exhibit a greater extent of numerical aberrations of chromosomes 8 and 12 than pure seminomas. NSGCT nonseminomatous germ cell tumor

showed high levels of DNA methylation, similar to those observed in NSGCTs [33–35]. Given the putative linear progression from seminoma to embryonal carcinoma in a subset of TGCTs [9], these data suggest that the investigation of molecular profiles of pure seminomas might play a role in identifying high-risk tumors for NSGCT progression.

There are several limitations in this study. First, we investigated a relatively small number of the samples which consist of GCNIS, seminoma, and embryonal carcinoma. Although in the pilot data, choriocarcinoma, yolk sac tumor, and teratoma components showed similar chromosomal status to embryonal carcinoma components (data not shown), the sample size of these three histological components was too small to analyze the statistical significance. Further study focused on the relationship between embryonal carcinoma and other NSGCT components is needed in near future. Another caveat of our study was the exclusion of chromosomes 4, 5, 11, 13, 18, and Y for which copy numbers have been reported to decrease in adult TGCTs [11–16]. Although our method using FISH has a relative difficulty in evaluating loss of chromosomes (i.e., only chromosomal number of 0 or 1 is regarded as "loss"), the selection bias cannot be avoided completely. Finally, there is no available patients' outcome data to compare with the status of chromosomal instability, which decreases the clinical significance of this study.

The main results of the present study are summarized as a schema in Fig. 2. Our studies demonstrate the following: (1) both the mean chromosomal copy number and the deviation from a modal chromosomal number increase along the progression from GCNIS to seminoma, and then to embryonal carcinoma; (2) these types of chromosomal instability are commonly observed in various chromosomes examined; and (3) seminoma components in mixed tumors show a greater extent of chromosomal instability than pure seminomas, whereas there is no significant difference in the status of chromosomal instability between GCNIS lesions with or without adjacent NSGCT components. The identification of specific genetic changes, especially the early events that are implicated in the progression of NSGCT, need to be explored further.

Authors' contributions K.M. collected the clinical patient data, examined histopathological findings, performed the experiments (FISH), evaluated the FISH data, analyzed the data, participated in the study design, and wrote the manuscript. K.I. assisted in clinical data acquisition, histopathological examination, and revised the manuscript. K.N. stimulated the study, provided the reference pathology for all the samples used, and revised the manuscript. H.T. conceived the study, analyzed the data, and drafted and revised the manuscript.

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Conflict of interest The authors declare that there is no conflict of interest.

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