Association between Large-scale Genomic Homozygosity without Chromosomal Loss and Nonseminomatous Germ Cell Tumor Development

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Abstract

The genotype of a tumor determines its biology and clinical behavior. The genetic alterations associated with the unique embryonal morphology of nonseminomatous subtypes of testicular germ cell tumors remain to be established. Using single nucleotide polymorphism microarray analysis, we found in all of the 15 nonseminomas analyzed, large-scale chromosomal homozygosities, most of which were not associated with relative chromosome loss. This unusual genotype, distinguishing nonseminoma from seminomas and other human tumors, may be associated with the special embryonal development morphologic transition of this malignancy. Based on these genetic data, we hypothesized a new potential origin of nonseminomas through sperm fusion. Nonrandom involvement of certain chromosomes also suggests that genes on these chromosome regions may play an important role in nonseminoma development. (Cancer Res 2005; 65(20): 9137-41)

Introduction

Testicular germ cell tumors (TGCT) are the most common malignancy in young men and the incidence is increasing (1). There are two main morphologic subtypes of TGCT: seminomas, a monomorphic tumor similar to undifferentiated germ cells and nonseminoma, with multiple cell lineages from all three embryonic layers as well as extraembryonic tissues resembling abnormal embryonal development at different stages (1, 2). Although many genetic studies have been carried out in TGCT and characteristic genetic changes identified, the development pathway of TGCT is still debatable and little is known of the genetic alterations associated with the unique morphologic phenotype of nonseminoma (1–3). Cells of seminoma are morphologically similar to those of intratubular germ cell neoplasias unclassified (IGCNU) and undifferentiated germ cells (primordial germ cells). However, a simple chromosome loss from either IGCNU or seminoma is insufficient to explain the morphologic transition relevant to embryonal development. Using single nucleotide polymorphism (SNP) microarray analysis (4), we studied the genotype and genomic copy number changes in both seminoma and nonseminoma samples to gain a better insight into the morphologic transition of nonseminoma. Unexpectedly, we found in nonseminomas, many large homozygous chromosome regions (HCR) not associated with chromosome loss or deletion that may account for the unique phenotype of nonseminoma.

Materials and Methods

Primary samples and cell lines. All primary tumor and control samples were collected directly after surgical operation with ethical approval and patient consent. Twelve nonseminoma, seven seminoma primary tumor samples, and seven morphologically nonmalignant testicular tissues from 21 adult individuals were analyzed. The histopathologic subtype of each nonseminoma was known; and four of the nonseminoma samples were from mixed tumors with small areas of seminoma component (Table 1). To confirm the tumor subtypes and tumor cell proportion of the samples, a consultant histopathologist (D.B.) reviewed the histopathologic diagnosis of each case and examined frozen sections directly adjacent to the tissue from which the DNA was extracted. For the tumor biopsies, only samples containing a majority of tumor tissues were used for this study and all the seminomas and most of the nonseminomas contained pure tumor lesions. Three nonseminoma cell lines GCT27, 833K, and SUSA were also studied. They were cultured in RPMI 1640 with 10% FCS.

10K GeneChip assay. DNA was extracted using standard phenol-chloroform techniques. The GeneChip mapping assay protocol (Affymetrix, Inc., Santa Clara, CA) was used to produce the 10K SNP array results following the manufacturer's instruction. The protocol was adapted such that the purification of PCR product was done using the Ultrafree-MC filtration column (Millipore, Billerica, MA). Signal intensity data were analyzed by the GeneChip DNA analysis software, which used a model algorithm to generate SNP calls (4, 5). We used a custom-designed computer program to display SNP calls and determine regions of homozygosities as HCRs (≥98% homozygosities in a minimum region of 50 contiguous SNPs). This software was also used to display the relative genomic gains and losses.

Fluorescence *in situ* hybridization analysis. Fluorescence *in situ* hybridization (FISH) was carried out using a standard protocol with slight modification. Labeled centromere probes were bought from Abbot Diagnosis (Dartford, Kent, United Kingdom). Touch-imprinted slides were made by touching the slide surface with small tissue biopsies and then fixed with 3:1 methanol/acetic acid solution. Slides were denatured at 72°C for 2 minutes and hybridized overnight with denatured probes in 10 μL hybridization buffer (2× SSC, 50% formamide, 10% dextran sulfate). Cells were counterstained with 4′,6-diamidino-2-phenylindole, and images were captured using an Olympus fluorescent microscope equipped with a cold charge-coupled device camera controlled by a computer software Macprobe v4.3 (Applied Imaging, Newcastle upon Tyne, United Kingdom). Signals from 50 cells were counted for each sample.

Results and Discussion

Using 10K SNP array analysis, we determined the DNA genotype status of the 12 nonseminoma, seven seminoma primary tumor

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Table 1. Clinicohistopathologic data of all the primary samples

Case no.	AAD	Histopathology diagnosis
RH53	27	EC + T + YS
93	29	EC + YS + CC
RH39	24	EC + YS
82*	26	EC + YS
RH9	30	EC + T + YS
RH48	41	EC + SE
105	27	YS + T + SE
94	23	EC + YC + SE
88*	54	EC + SE + T + YS
RH30	23	EC
29	29	EC
RH26	30	EC
9	51	SE
33	23	SE
74*	39	SE
86	51	SE
87*	49	SE
90	26	SE
92*	51	SE
2	33	Normal tissue from patient with hematoma in testis
38	24	Fibrous tissue after chemotherapy of SE

Abbreviations: AAD, age at diagnosis; EC, embryonal carcinoma; T, teratoma; YS, yolk sac tumor; CC, coriocarcinoma; SE, seminoma.
*Adjacent morphologic nontumor samples were also analyzed.

samples, and seven morphologically nonmalignant testicular tissues as well as the three nonseminoma cell lines. Surprisingly, we observed in the nonseminomas, including both primary tumors and cell lines, many large-scale homozygous SNP domains, each extending over large cytogenetic regions up to the full length of the chromosomes, appearing as HCRs. In contrast, fewer similar HCRs were found in the seminomas and the majority of them occurred in two of the seven samples. To quantify the HCRs more precisely, they were defined as regions of contiguous SNP calls with $\geq$98% homozygosities (minimum region of 50 SNPs). Using these criteria, we detected 108 HCRs excluding the sex chromosome X in the 15 nonseminomas, including 49 in the eight pure nonseminomas, 19 in the four mixed tumors, and 40 in the three cell lines. However, only 18 HCRs were detected in the seven seminomas and none in the non–tumor testis samples (Fig. 1). The difference in HCR frequencies between nonseminoma and seminoma is statistically significant (P = 0.005, t test). As a control, we further analyzed 24 nonmalignant bone marrow samples using the same criteria and no HCRs were found on the autosomes (data not shown). When the signal intensities of these HCRs were calculated against the average signal intensity of each individual case to identify relative genomic gains and losses, we observed HCRs without a relative reduction of chromosome copy numbers in each of the nonseminomas. Only two seminomas, both of them are typical ones, had more than one chromosome associated with HCRs; and all the HCRs in one of them were associated with genomic losses (Fig. 1). The frequencies of samples with multiple HCRs between nonseminomas (15 of 15) and seminomas (2 of 7) are statistically different (P = 0.0018, Fisher's exact test).

In this study, we had three seminomas and two nonseminomas for which paired, adjacent, and morphologically nonmalignant tissue was available (Table 1). None of the HCRs seen in the tumor samples were found in the adjacent morphologically normal tissues (Fig. 1) on the relevant chromosomes, indicating that the abnormalities were acquired during tumor development. For each pair, we compared tumor with the adjacent normal sample, analyzing the loss-of-heterozygosity (LOH) status in parallel with the signal intensity ratio along the chromosomes. There were many large chromosome regions showing complete LOH but no significant reduction of signal intensity ratios in the two nonseminomas (Fig. 2). All the data suggest that those HCRs not associated with relative genomic losses may indicate an important mechanism for nonseminoma development. Although HCRs exist in other tumors, such lesions are detected at a low frequency and are usually associated with chromosome losses or deletions (4–8).

It is known that nonseminomas generally have fewer chromosomes than seminomas (1–3, 9, 10). Therefore, the difference in HCR frequency between nonseminomas and seminomas seems to fit the generally accepted concept that TGCTs develop from tetraploid IGCNUs into seminoma and then nonseminoma, following stepwise overall chromosome loss (1–3, 9, 10). However, if these HCRs were simply generated by loss of two chromosomes of one parental origin, we should expect to see two or fewer copies of chromosomes over the HCRs. This conflicts with the DNA ploidy data that nonseminomas are generally hypotriploid (1–3, 9, 10). To address this issue, we further investigated chromosome copy numbers in all the nonseminoma samples (except two nonseminomas, 88T and RH9T, for which no frozen tissue was available) and the two seminomas with multiple HCRs.

Interphase FISH was done using chromosome 1, 4, 8, 10, 15, X, and Y centromere probes to determine the exact copy numbers of these chromosome regions. The copy numbers of other chromosome regions and cell ploidy were calculated by the combination of FISH data with relative copy number gain and loss generated by signal intensity analysis of the SNP array results normalized against the five apparently normal samples (data not shown). For the six RH cases and cell lines for which chromosomal comparative genomic hybridization analysis data were available (11), the chromosome gain and loss profile from SNP analysis correlated well with chromosomal comparative genomic hybridization results. FISH results showed that TGCT cells were heterogeneous in that each chromosome had variable copy numbers in different cells. Therefore, we calculated the average chromosome copy number for each chromosome for all cases as shown in Table 2. Interestingly, copy number data showed generally less genomic copies of the HCRs in the seminoma samples than in the nonseminomas (Fig. 1; Table 2). In the nonseminomas with FISH data, two thirds of the HCRs had an average chromosome copy number of >2.5 and many of them no less than 3. As 1p loss and LOH of this chromosome region were frequently detected in many human tumors including TGCTs (3, 11, 12), two bacterial artificial chromosome clones, RP11-466K16 on 1p36.13 and RP11-312B08 on 1p36.31, were used to detect their copy numbers in samples RH53T, 93T, RH39T, and RH30T of which HCRs occurred on 1p (Fig. 1). We saw a mixture of three and two signals in a cell with three slightly dominant in all the samples except RH53T. In RH53T cells, two copies of RP11-312B08 and a mixture of two and four copies of RP11-466K16 were found. Therefore, simply a loss of two chromosomes from

tetraploid cells cannot explain the HCRs with more than two copies of chromosomes. For about two thirds of the HCRs not spreading to the whole chromosomes, we did not find a relative genomic copy number loss in HCRs compared with the heterozygous chromosome regions on the same chromosomes. This further supports the contention that these HCRs are not generated simply by chromosome loss or deletion.

From the above data, there must be a mechanism other than simply chromosome losses leading to multiple homozygous chromosomes during the development of nonseminomas in adult men. Based on the tetraploid IGCNU origin of TGCTs, this can be explained by loss of one parental chromosome and duplication of the remaining parental chromosome, although this event is generally rare in tumorigenesis. Although the overall mutation rate is low, it was found in embryonal stem cells that chromosome loss/reduplication leading to uniparental chromosomes was the predominant genetic alteration caused most likely by chromosome nondisjunction (13). Mitotic recombination can also lead to chromosome regional HCRs without copy number alterations (5). A further alternative pathogenetic mechanism we proposed here, which would generate multiple HCRs without chromosome loss, is cell fusion involving a sperm. The multiple HCRs can be explained by a sperm fusing with a meiosis I four-chromatid stage cell and subsequent chromosome loss during the development of IGCNUs, seminomas, and nonseminomas. A subgroup of nonseminoma with several HCRs might also be generated by a sperm cell fusing with a two-chromatid stage post-meiosis I cell. In either case, the random recombination of chromosomes within these two cells can eventually lead to three chromosomes of the same parental origin. It is proven that primordial germ cells have the potential to develop into either sperm or oocyte in response to different stimulating factors, irrespective of whether they are from male (XY) or female (XX; ref. 14). It is well known that the development of TGCT is influenced by risk factors in fetal life (9) when primordial germ cells differentiate into gonocytes and spermatogonia (2). If spermatogenesis-stimulating factors are attenuated in regions where local abnormality of the male fetal gonad exists, some primordial germ cells may develop into abnormal spermatocytes, which acquire certain oocyte-specific gene expression permitting sperm penetration (14). This explains the significantly increased risk of TGCTs in phenotypic female but genetically male individuals (22%; ref. 1). Cell fusion or abnormal fertilization is believed to lead to clonal proliferation in human diseases with a similar phenotype to nonseminomas (e.g., ovarian teratomas and trophoblastic diseases; refs. 15–18). In both cases, uniparental chromosomes are frequently generated by recombination of two or more sets of chromosomes from haploid cells of a same parental origin (15, 17, 18).

Although further investigations are required to understand the mechanism leading to the multiple HCRs in adult nonseminomas, the phenomenon of multiple HCRs fits well with the embryonal

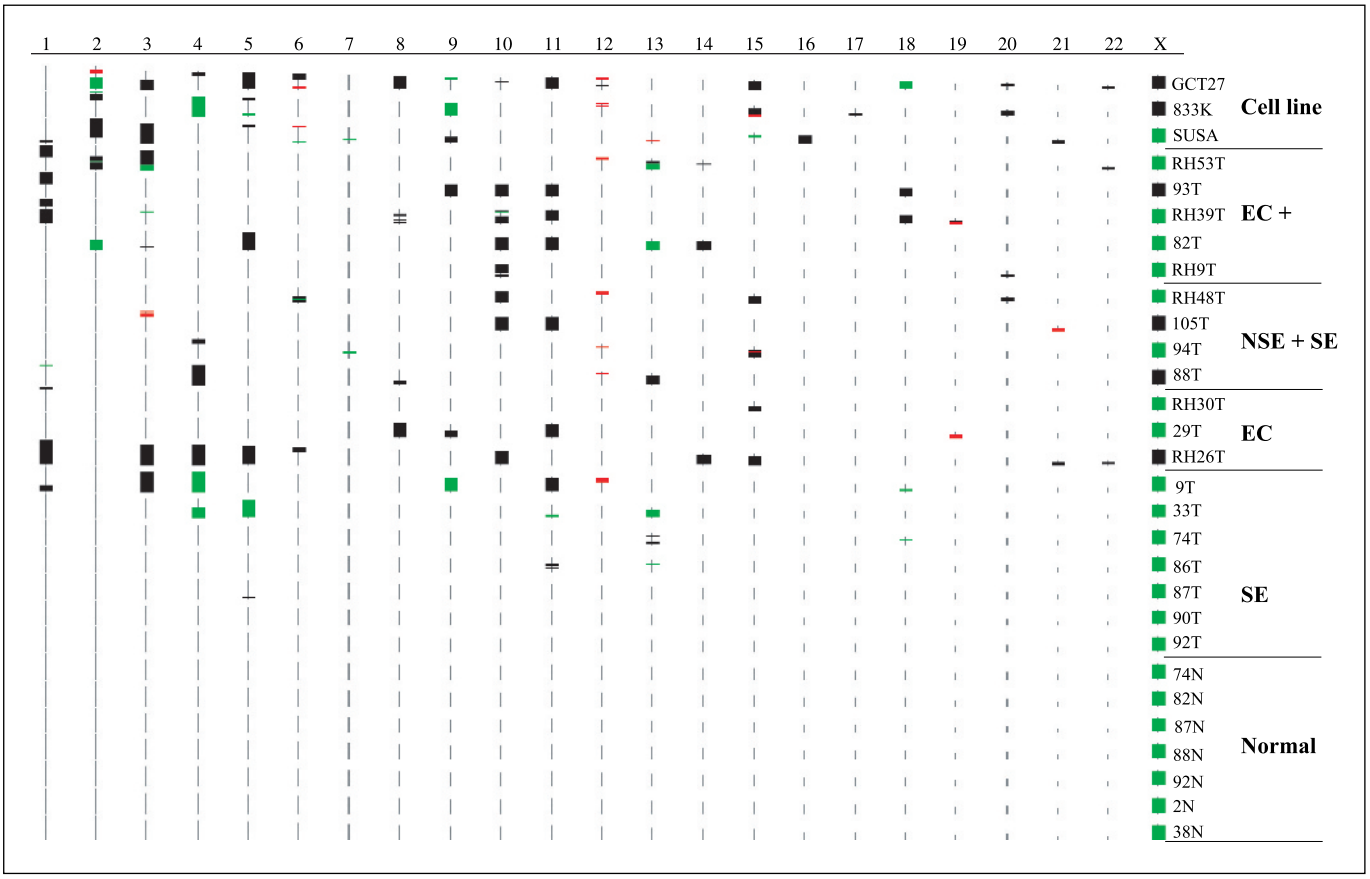

Figure 1. A summary of the HCRs (chromosome regions with ≥98% homozygosities in a minimum region of 50 contiguous SNPs) on each chromosome for each sample analyzed by SNP array. *Black boxes,* HCRs without significant chromosome copy number changes (signal intensity ratio compared with case average, between 0.75 and 1.25); *green boxes,* HCRs with relative chromosome copy number loss (signal intensity ratio, <0.75); *red boxes,* HCRs with relative chromosome copy number gains (signal intensity ratio, >1.25). Case numbers + T (*right*) mean tumor sample from those cases; and case numbers + N are morphologically nontumor tissues. Abbreviations: EC, embryonal carcinoma; EC+, mixed tumor with embryonal carcinoma and other nonseminoma subtypes; NSE, nonseminoma; SE, seminoma.

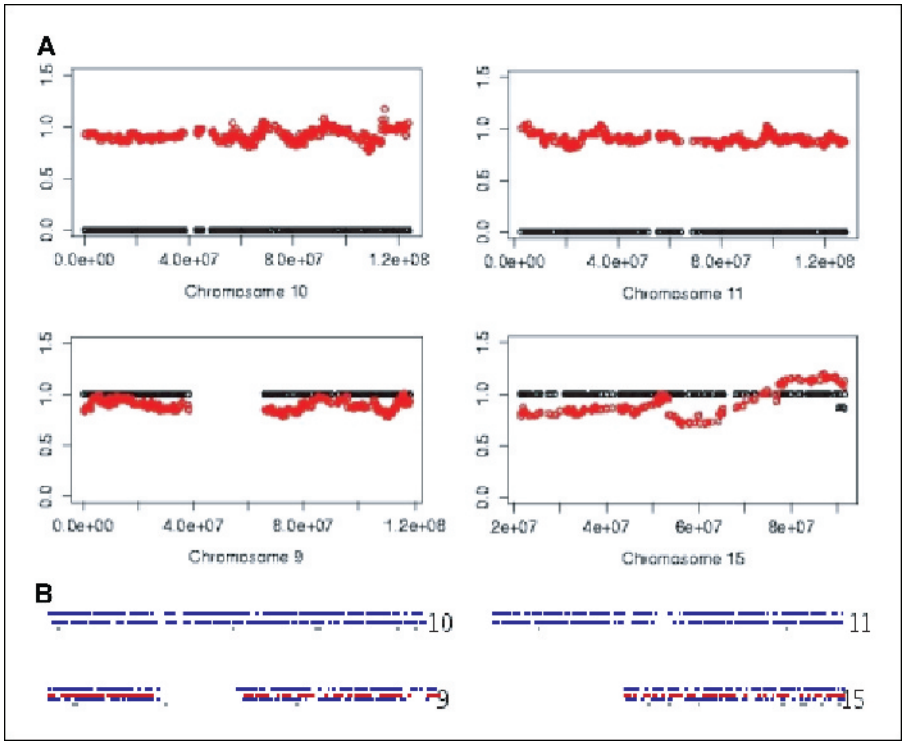

Figure 2. Examples of HCRs without chromosome loss shown by SNP analysis in case 82. *A,* ratio of the number of heterozygous to homozygous calls was calculated in a running window of 20 Mbp and the LOH score (*black*) was generated by dividing the above ratio in the tumor sample by the same ratio in the adjacent morphologically nontumor sample. The signal score (*red*) was calculated as the ratio of the mean signal, in a running window of 20 Mbp, between tumor and the adjacent normal sample. We show that LOH of the whole of chromosomes 10 and 11 is not accompanied by signal intensity decrease, but for a control chromosome without HCR, a similar signal intensity for chromosome 9 occurs without LOH and in normal situation, a slight signal intensity variation (along chromosome 15) did not routinely cause LOH. *B,* chromosomal SNP genotype patterns for the four chromosomes presented in (*A*). For each chromosome: *two blue lines,* either AA or BB homozygous calls; *red line,* AB heterozygous calls; and *gray line,* SNPs not called (*bottom*). Red lines (AB heterozygous calls) are present in chromosomes 9 and 15 but absent in chromosomes 10 and 11. The relative signal intensity data of chromosomes 10 and 15 correlated well with the FISH data shown in Table 2.

development–like morphology of nonseminomas. Multiple uniparental chromosomes occur in human tumors. Interestingly, such genotypes are mainly associated with abnormal embryonal development, as seen in ovarian teratomas or trophoblastic diseases (15, 17, 18). Therefore, it seems that HCRs may be the genetic basis for nonseminoma morphology transition. This finding not only associated HCRs with nonseminoma development but also provided useful genetic information for studying embryonal

Table 2. The copy numbers of chromosome 1, 4, 8, 10, 15, X, and Y centromeres and ploidy status of the 13 nonseminomas and two seminoma samples with multiple HCRs

Cases	Ploidy status*	Chr. 1, ave. (var.)	Chr. 4, ave. (var.)	Chr. 8, ave. (var.)	Chr. 10, ave. (var.)	Chr. 15, ave. (var.)	Chr. X, ave. (var.)	Chr. Y, ave. (var.)
GCT27	Hypotriploid	2 (1, ***2***)	2.5 (1, ***2, 3***, 4)	2.8 (2, ***3***)	—	3 (2, ***3***, 4)	3 (1, 2, ***3***, 4)	1 (***1***, 2)
833K	Hyperdipoid	3 (***3***)	2.2 (1, ***2***, 3)	2.8 (1, 2, ***3***)	—	2.1 (1, ***2***, 3)	2 (***2***)	0 (***0***)
Susa	Hypotriploid	3 (2, ***3***)	2.6 (***2, 3***, 4)	2.3 (***2***, 3, 4)	—	1.2 (***1***, 2, 3)	2 (1, ***2***)	1 (0, ***1***, 2)
RH53T	Hypotriploid	3 (1, 2, ***3***, 4)	2.5 (1, 2, ***3***)	2.6 (***2, 3***, 4)	2.6 (1, ***2***, 3, ***4***, 5)	2.2 (1, ***2***, 3, 4)	1.5 (1, ***2***)	0 (***0***)
93T	Hyperdipoid	2.5 (1, ***2, 3***, 4)	2 (1, ***2***, 3, 4)	2.8 (1, 2, ***3***, 4)	2.4 (1, ***2, 3***)	2 (1, ***2***)	1.5 (1, ***2***, 3)	1.5 (0, ***1, 2***)
RH39T	Hypotriploid	3 (2, ***3***, 4)	2.4 (1, ***2, 3***, 4)	2.5 (***2, 3***)	2.8 (1, 2, ***3***, 4)	2.5 (1, ***2, 3***)	1.2 (***1***, 2)	1.5 (***1, 2***, 3)
82T	Hypotriploid	3 (2, ***3***, 4)	3 (1, 2, ***3, 4***)	3 (2, ***3***, 4)	2.7 (1, 2, ***3***, 4)	2.2 (1, ***2***, 3)	1.9 (1, ***2***, 3)	0 (***0***)
RH9T	Hyperdipoid	2.5 (1, ***2, 3***, 4)	2.2 (***2***, 3)	2.1 (***2***, 3)	2.3 (1, ***2***, 3, 4)	2 (1, ***2***, 3)	1.1 (***1***, 2)	1.2 (***1***, 2, 3)
RH48T	Triploid	3.2 (2, ***3***, 4, 5)	3 (***2, 3***, 4)	3 (2, ***3***, 4)	2.8 (1, 2, ***3***, 4)	3 (1,2, ***3***, 4)	2 (1, ***2***, 3)	2.2 (1, ***2***, 3)
105T	Hypotriploid	3 (2, ***3***, 4)	2.5 (***2, 3***, 4)	3 (2, ***3***, 4)	2.6 (***2, 3***, 4)	3 (2, ***3***, 4, 5)	3 (2, ***3***, 4)	2 (1, ***2***, 3)
94T	Hypotriploid	3 (2, ***3***, 4, 5)	3.3 (1, 2, ***3, 4***)	2.3 (1, ***2***, 3)	2.5 (1, ***2, 3***, 4)	2.8 (2, ***3***)	1.1 (***1***, 2)	1.2 (***1***, 2)
RH30T	Hypotriploid	2.7 (***2, 3***, 4, 5)	2.5 (1, ***2, 3***, 4)	2.5 (1, ***2, 3***)	2.7 (***2, 3***, 4)	2.5 (1, ***2, 3***, 4)	1.8 (***1***, 2, 3)	1.1 (***1***, 2)
29T	Hypotriploid	3 (2, ***3***, 4)	2.5 (***2, 3***, 4, 5)	2 (1, ***2***, 3)	2.5 (1, ***2, 3***, 4)	2.6 (1, ***2, 3***)	2 (1, ***2***, 3)	1 (0, ***1***)
9T	Hypotriploid	2.5 (1, ***2***, 3, 4)	1.9 (1, ***2***, 3)	3 (***2, 3, 4***)	2.2 (1, ***2***, 3, 4)	3.2 (1, 2, ***3***, 4, 5)	1.4 (***1***, 2, 3)	1.8 (1, ***2***)
33T	Hyperdipoid	2.5 (1, ***2, 3***, 4)	2.4 (1, ***2***, 3, 4, 5)	2.3 (1, ***2, 3***)	2.2 (1, ***2***, 3, 4)	2.3 (1, ***2***, 3, 4)	1.2 (***1***, 2)	1 (0, ***1***, 2)

NOTE: Numbers in bold italics represent the signals from common (>30%) cell clone(s). Numbers underlined indicate these chromosomes contained HCRs in those cases except the sex chromosomes. The top three samples (GCT27, 833K, and Susa) are nonseminoma cell lines. The bottom two samples (9T and 33T) are seminomas.

Abbreviations: Chr., chromosome; ave., average; var., variants.

*The ploidy status was calculated using both relative chromosomal gain and loss information from SNP array analysis and the centromere copy numbers from FISH analysis.

development using TGCT as a model. There are many potential mechanisms that may lead to multiple HCRs in two of the seminomas but not the others, although extensive future work is needed to reveal the particular one involved. We show here that HCRs are nonrandomly associated with some chromosomes. We detected a high frequency of HCRs associated with chromosomes 1p (6 of 15), 10 (8 of 15), 11 (6 of 15), and 15 (7 of 15), whereas only few small HCRs of chromosomes 7 (2 of 15), 16 (1 of 15), 17 (1 of 15), and 19 (2 of 15; Fig. 1). High frequency of LOH on 1p, 11, and 15 was detected previously (12, 19), and loss of 15 has been associated with nonseminoma development (20). Gain of 12p is a characteristic genetic change of TGCTs (1–3). Although overrepresentation of 12p was detected in all the tumor samples in this study, interestingly, HCRs of this region were found predominantly in nonseminomas (Fig. 1). Genes, particularly those imprinted, on these frequently involved chromosomes may be important in promoting nonseminoma development.

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