

Novel genomic aberrations in testicular germ cell tumors by array-CGH, and associated gene expression changes

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Abstract. *Introduction:* Testicular germ cell tumors of adolescent and young adult men (TGCTs) generally have near triploid and complex karyotypes. The actual genes driving the tumorigenesis remain essentially to be identified. *Materials and Methods:* To determine the detailed DNA copy number changes, and investigate their impact on gene expression levels, we performed an integrated microarray profiling of TGCT genomes and transcriptomes. We analyzed 17 TGCTs, three precursor lesions, and the embryonal carcinoma cell lines, NTERA2 and 2102Ep, by comparative genomic hybridization microarrays (array-CGH), and integrated the data with transcriptome profiles of the same samples. *Results:* The gain of chromosome arm 12p was, as expected, the most common aberration, and we found *CCND2*, *CD9*, *GAPD*, *GDF3*, *NANOG*, and *TEAD4* to be the therein most highly over-expressed genes. Additional frequent genomic aberrations revealed some shorter chromosomal segments, which are novel to TGCT, as well as known aberrations for which we here refined boundaries. These include gains from 7p15.2 and 21q22.2, and losses of 4p16.3 and 22q13.3. Integration of DNA copy number information to gene expression profiles identified that *BRCC3*, *FOS*, *MLL11*, *NES*, and *RAC1* may act as novel oncogenes in TGCT. Similarly, *DDX26*, *ERCC5*, *FZD4*, *NME4*, *OPTN*, and *RBI* were both lost and under-expressed genes, and are thus putative TGCT suppressor genes. *Conclusion:* This first genome-wide integrated array-CGH and gene expression profiling of TGCT provides novel insights into the genome biology underlying testicular tumorigenesis.

Keywords: Array-CGH, comparative genomic hybridization, gene expression, microarray, testicular germ cell tumor

1. Introduction

Testicular germ cell tumor (TGCT) is the most common malignancy among adolescents and young adult men in Western industrialized countries, and the incidence has increased dramatically over the past fifty

years [5,8]. TGCT develops from precursor lesions called intratubular germ cell neoplasia (IGCN, alias carcinoma *in situ*; ref. [44]), believed to originate from primordial germ cells during fetal life [45]. TGCT is histologically classified into seminomas and nonseminomas, and nonseminomas are further subdivided into embryonal carcinomas, choriocarcinomas, yolk sac tumors, and teratomas [31].

The TGCT genome [reviewed in ref. 47] is generally hypo- to hyper-triploid [7,10,34]. Extra genomic material of chromosome arm 12p, often in the form of isochromosomes [4], is present in virtually all of

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these tumors [40,52], regardless of histological subtypes. The application of comparative genomic hybridization to metaphase spreads (chromosomal CGH) has enabled genome-wide analysis of DNA copy number aberrations [16] at a resolution level of 5–10 Mbp. Several reports of chromosomal CGH of the TGCT genome have revealed many recurrent copy number gains and losses, and despite the morphological and transcriptional distinctions between seminoma and nonseminoma, they share many of the same genomic aberrations, although frequencies may vary [21,23,29,35,41,54,55].

Transcriptional profiling of TGCT has revealed major differences at the gene expression levels between all the histological subgroups [15,19,20,48,49], indicating that the selective pressure on the different subtypes may as well enforce different genetic aberrations. However, this has not been reflected at the gross genome level, as reported by previous chromosomal CGH studies [47].

DNA microarrays are useful for measuring both the gene expression [43] and DNA copy number levels (array-CGH; [25,50]) in a genome-wide high-resolution scale. Integration of these two high-throughput technologies on the same set of samples gives a possibility to identify the genetic mechanisms affecting expression level changes of the target genes. In this report, we present the integrative results of genome and transcriptome profiling of a series of TGCTs, IGCN, and cell lines. Within commonly altered genomic regions, we identify key genes that show both the altered DNA copy number and gene expression, and thus are putative oncogenes or tumor suppressor genes.

2. Material and methods

2.1. Sample set

Altogether, 22 testicular germ cell neoplasms were analyzed, including 17 TGCTs, 2 TGCT cell lines (NTERA2 and 2102Ep, refs. [1,2]), and 3 pre-malignant IGCN. All samples were from different individuals. The TGCT tissue samples were selected in order to contain a single histological subtype each, as judged from examination of HE stained sections by a reference pathologist (ref. [48], author V.M.A.), and to cover the known histological subtypes (3 seminomas, 5 embryonal carcinomas, 1 choriocarcinoma, 4 yolk sac tumors, and 4 teratomas). The IGCN samples were from areas adjacent to invasive tumors. HE stained sec-

tions of these three samples demonstrated absence of malignant cells, and that about 100, 50, and 10% of the seminiferous tubuli were filled with IGCN cells.

Frozen tissue samples were ground in liquid N₂, and subsequently transferred to two tubes. DNA was obtained from one sample part by the phenol/chloroform extraction principle, and RNA was obtained from the other identical part by using the TRIzol[®] reagent (Invitrogen, Carlsbad, CA, USA).

2.2. CGH microarrays

DNA copy number analyses on cDNA microarrays were performed as previously reported [38,60]. Briefly, DNA samples were digested over night by AluI and RsaI (Sigma-Aldrich, St. Louis, MO, USA) and purified by phenol/chloroform extraction and ethanol precipitation. Five micrograms of fragmented DNA were labeled using the RadPrime DNA Labeling System (Invitrogen) in the presence of Cy3-dUTP for tumors/cell lines and Cy5-dUTP (Amersham Biosciences, Piscataway, NJ, USA) for normal reference (pool of DNA isolated from normal lymphocytes of two healthy males). Labeled DNA was then purified using Microcon YM-30 centrifugal filter units (Millipore, Billerica, MA, USA) and 50 μ g human Cot-1 DNA (Invitrogen), 100 μ g yeast tRNA (Invitrogen), and 20 μ g of each of pd(A)₄₀₋₆₀ and pd(T)₁₂₋₁₈ (Amersham Biosciences) were added before a new round of spin-concentration/purification. SSC and SDS (final concentrations of 3.4 \times and 0.3%, respectively) were added, and the total mixture was placed onto cDNA microarrays with 12557 unique cDNA clones (Agilent Human 1; G4100AM; Agilent Technologies, Palo Alto, CA, USA) and incubated at 65°C over night in a sealed humidified chamber. After the hybridization, microarray slides were washed for 2 min in each of 0.1% SDS, 0.5 \times SSC/0.01% SDS, and 0.06 \times SSC solutions.

2.3. Data processing, statistics and quality control measurements

The fluorescence intensities at the targets were detected by a confocal laser scanner (Agilent Technologies), and resulting images were processed using the Feature Extraction software (version 6.1.1.1, Agilent Technologies). This included defining the spots, measuring intensities, flagging spots with inadequate measurements, subtracting local background, and locally weighted scatter plot smoothing (LOWESS) dye-

normalization. For spots that were not flagged as having inadequate measurements, ratios (sample over reference) of the processed intensities were used for further analysis.

The gene annotation of the cDNA clones on the array was updated according to the TIGR Resourcerer,¹ and UniGene identifiers were used to extract chromosome and base pair positions according to the UCSC Genome Browser.² Subsequently, 10142 cDNA clones were positioned and ordered along the genome sequence, giving an average spacing of 303 kb throughout the human genome. There was a median of 283 cDNA clones per sample not meeting the quality control criteria. These are for the respective samples treated as missing values in the data set.

CGH-Plotter, a MATLAB toolbox for array-CGH data analysis [3], was utilized for finding the gains and losses within the data. The moving median window was set to move across five clones, and the constant used when the number of changes is calculated was set to three. Gains and losses were scored when log-2 of the resulting dp value were >0.1 and <-0.07 , respectively. An additional cut-off for genomic amplification was set at 0.3.

A self versus self-hybridization was used as a control to measure the technical variability of the non-altered regions in the genome. With the current settings, this led to identification of 12 clones (2 regions) of false positives among the 10142 clones (0.1% false discovery rate). This is in sharp contrast to the average of 2664 positive clones in the invasive TGCT samples and 594 in the IGCN samples. A dye swap control hybridization (reverse labeling of cell line and reference DNA) of the NTERA2 was also performed with satisfactory results, as the average difference between their processed log₂-values was 0.03.

2.4. Integration of genomic aberrations and gene expression data

RNAs from the same sample set were hybridized on Agilent Human 1A 22k oligo microarrays. The transcriptome profiles have been published elsewhere [48], and the raw data can be obtained from the Gene Expression Omnibus at the National Center for Biotechnology Information website³ (accession num-

ber GSE1818). For each oligo, the expression values were divided by the median of the three normal samples included in that data set. For oligos that were present as replicates on the microarray, we used median expression values for further analyses. Missing values were imputed by use of the k nearest neighbors algorithm ($k = 10$; J-Express v.2.0, MolMine, Bergen, Norway). The matching genomic positions for the DNA copy number and gene expression were retrieved using CGH Plotter v2⁴ to integrate the DNA and RNA level data on 12K cDNA and 22K oligo microarrays.

To evaluate the influence of DNA copy number aberrations (gains and losses) on gene expression, weights were calculated with previously reported statistical methods [14]. Briefly, for each gene, separate calculations were performed for genes underexpressed due to DNA copy number loss and for genes overexpressed due to DNA copy number gain. The difference between the median expression values in samples with and without aberrations were divided by the sum of their standard deviations. In the case of gains, the weight w_G , was calculated for each gene as follows:

$$w_G = [m_{G1} - m_{G0}] / [\sigma_{G1} + \sigma_{G0}],$$

where m_{G1} , σ_{G1} , and m_{G0} and σ_{G0} denote the means (m) and standard deviations (σ) for the expression levels for samples with gain (G1) and without gain (G0), respectively. For association between DNA copy number loss and low gene expression, the weight w_L was calculated as follows:

$$w_L = [m_{L0} - m_{L1}] / [\sigma_{L1} + \sigma_{L0}].$$

To assess the statistical significance of each weight, 10 000 random permutations of the label vector (random assignment of 0 and 1) were performed. The probability that a gene had a larger or equal weight by random permutation than the original weight was denoted by α . A low α (<0.05) indicates a significant association between gene expression and DNA copy number aberrations.

We set three criteria for genes to be considered as gained and overexpressed, or as lost and underexpressed in TGCT. First, genes had to have α -values <0.05 . Secondly, the genes had to be located within regions of gain or loss seen in at least 25% of the samples. Thirdly, from these, only genes with more than

¹TIGR Resourcerer version 11.0 (December 2004), URL: <http://pga.tigr.org/tigr-scripts/magic/r1.pl>

²UCSC, May 2004 assembly, URL: <http://genome.ucsc.edu/>

³URL: <http://www.ncbi.nlm.nih.gov/geo/>

⁴URL: http://www.cs.tut.fi/~bsmg/cgh_plotter.html

two-fold different mean expression values, as compared to the normal testicular tissues, were considered for the eventual list of putative target genes for TGCT.

2.5. Statistical analysis of target genes on chromosome arm 12p

Statistical analysis of 12p target genes was performed differently because DNA copy number gain of 12p was present in all cancer samples, and because calculation of α -values requires a group of samples without the genetic aberration. Therefore, a two class unpaired Significance Analysis of Microarrays (SAM; ref. [58]) was performed comparing expression values in the 21 samples found to have gain of 12p by array-CGH with the three normal testis samples. A false discovery rate of 0.05 was set as cut-off, and further, we restricted the genes of interest only to include those with more than three-fold higher average expression in the tumors than in the normal testis.

2.6. Gene expression validation by quantitative RT-PCR

For reverse transcriptase polymerase chain reaction (RT-PCR), of the candidate genes *FOS*, *MLLT11*, and *RAC1*, and the reference gene *GUSB*, new first-strand cDNA was synthesized from 5 μ g total RNAs of the same sample set in a randomly primed polymerization using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). To quantify the respective mRNA levels, ten ng of this cDNA was used as template in real time PCR, utilizing the 7900HT System (TaqMan; Applied Biosystems). Probe and primer sets were pre-designed and ordered from Applied Biosystems (*FOS*, Hs00170630_m1; *MLLT11*, Hs00199111_m1; *RAC1*, Hs01588892_g1; and *GUSB*, 4322171) and each gene was analyzed in triplicate for all samples. The quantitative expression levels were measured against a standard curve generated from dilutions of cDNA from the human universal reference RNA (Stratagene). In order to adjust for the possibly variable amounts of cDNA input in each PCR, we normalized the expression quantity of the target genes with the quantity of the endogenous control *GUSB*. To facilitate the comparison between the TaqMan and oligo microarray transcriptome data, changes in gene expression relative to the normal tissues was calculated by dividing the median of the three replicates by the median expression from the normal samples.

3. Results

The DNA copy number gains and losses were identified by array-CGH from 22 testicular germ cell neoplasms, including 17 TGCTs, 2 TGCT cell lines, and 3 IGCN (precursor lesions). Both gains and losses were identified within all testicular DNA samples. Genome-wide frequencies and locations of gains and losses are summarized in Fig. 1.

Gain from chromosome arm 12p was seen in all 19 TGCTs (including all invasive tumors and the two embryonal carcinoma cell lines), and in two of the three IGCN samples. The further most commonly gained regions were of the whole or from parts of chromosomes X (79%), 21 (74%), 7 (58%), 8, and 14 (42%). The most commonly lost regions were from chromosomes 22 (68%), 4, 5, 13 (47%), 16, and Y (42%). Many of these gains and losses span large chromosomal regions, or even whole chromosomes. But still, within this list of the most common aberrations, we identified the smallest regions of overlapping gains, defined to include breakpoints detected in at least three samples, at 7p11.2-ter (0–55.91 Mbp), 12p11.22-ter (0–28.00 Mbp), 21q21.3–22.2 (29.64–39.75 Mbp), and losses at 4p16.3 (0–0.66 Mbp), 5p14-ter (0–27.07 Mbp), 13q14.3-ter (52.52–114.15 Mbp), 16p13.3 (0–3.10 Mbp), 22q13.31-ter (45.25–49.48 Mbp) (Table 1).

We considered the regions of DNA copy number gain to be distinct high-level amplifications if at least three samples exceeded three times the gain detection threshold. In addition to 11 samples with high-level amplifications at 12p, five samples had overlapping amplifications at 21q22.2 (38.60–39.75 Mbp), four at 17p13.3 (1.27–1.34 Mbp), and three in each of 6p21.1 (43.15–43.30 Mbp), 7p15.2 (26.91–26.97 Mbp), 19q13.32 (53.31–53.77 Mbp), and Xp22.1-q13.1 (18.81–70.29 Mbp).

As a crude comparison of DNA copy number changes to gene expression data, we first looked into the whole chromosome arms with the highest frequency of gains and losses, and compared expression levels of the therein-located genes. We found overexpression (mean log₂ value within tumor samples >0.5) of 35% of the genes located on the generally gained chromosome arm 12p. This is significantly more than the frequency of 13% overexpressed genes on the genome-wide level (Fisher's exact test, $p = 5 \times 10^{-16}$). Conversely, we found reduced expression (mean log₂ < -0.5) of 37% of the genes located on the generally lost chromosome arm 13q, as

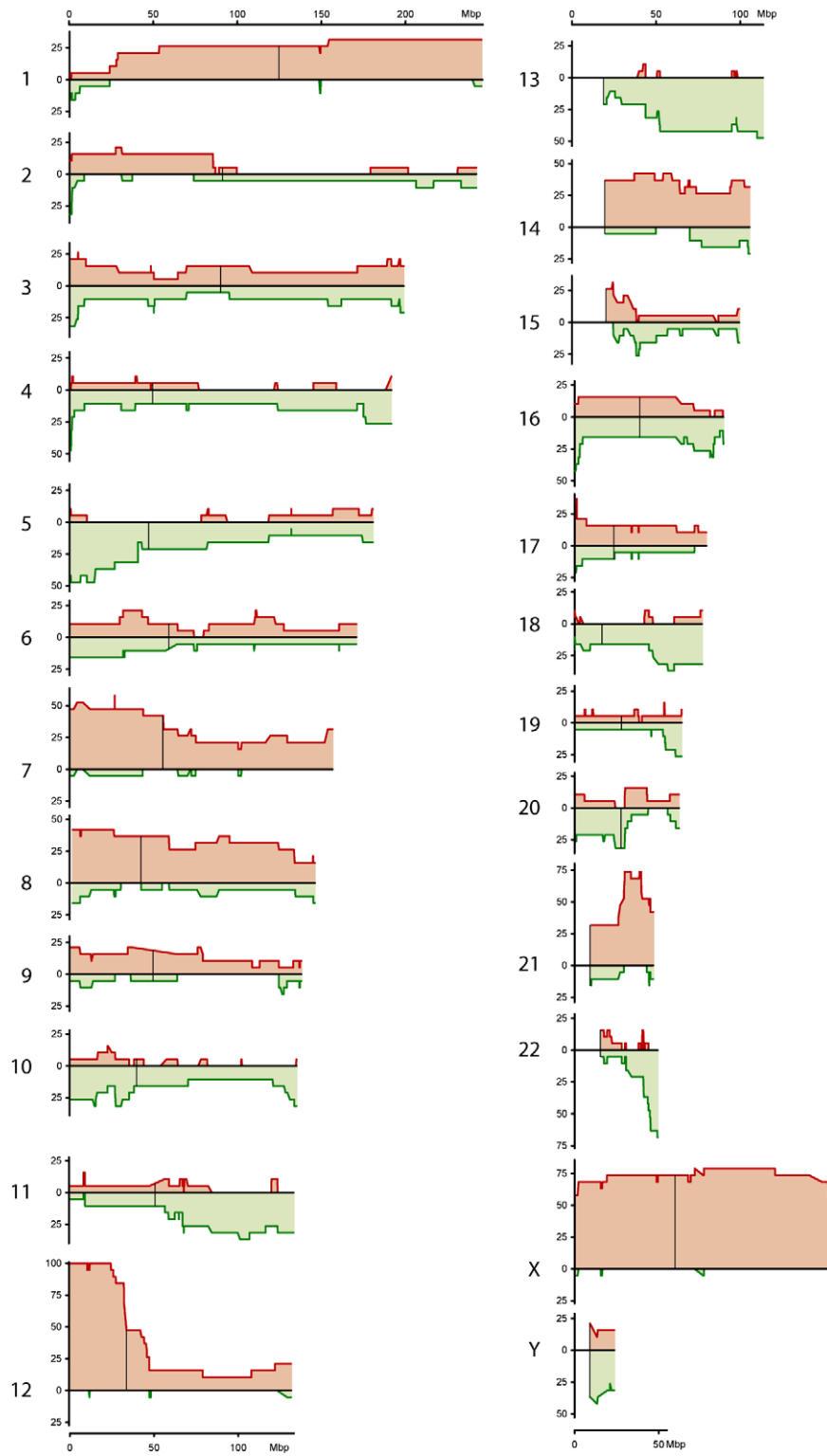


Fig. 1. The testicular germ cell tumor genome. Percentages of DNA copy number gains (red) and losses (green) from 19 testicular malignancies (17 TGCTs and 2 TGCT cell lines) are shown. Vertical black lines indicate centromere positions.

Table 1

Smallest regions of overlapping gains and losses. These genomic regions were identified by showing DNA copy number changes in more than 40 percent of the samples and being limited by break points in at least three tumors at each side. For each region, genes with DNA copy number associated expression are listed

Cytogenetic map position	Pct.	Mbp from <i>p</i> -telomere	Genes overexpressed in samples with gain/ underexpressed in samples with loss
Gains			
7p11.2-ter	58	0–55.91	<i>C1GALT1</i> , <i>CCT6A</i> , <i>DFNA5</i> , <i>ECOP</i> , <i>IGFBP3</i> , <i>IMP-3</i> , <i>LSM5</i> , <i>NDUFA4</i> , <i>RAC1</i> , <i>RP9</i> , <i>SEC61G</i> , <i>SNX10</i>
12p11.22-ter*	100	0–28.00	<i>CD9</i> , <i>CCND2</i> , <i>CGI-04</i> , <i>CGI-141</i> , <i>DERA</i> , <i>DRPLA</i> , <i>FLJ20696</i> , <i>FLJ22662</i> , <i>GDF3</i> , <i>KCNJ8</i> , <i>KLRB1</i> , <i>KLRG1</i> , <i>LDHB</i> , <i>LEPREL2</i> , <i>MGST1</i> , <i>MLF2</i> , <i>NANOG</i> , <i>PHC1</i> , <i>TEAD4</i>
21q21.2-22.2	74	29.64–39.75	<i>SFRS15</i>
Losses			
4p16.3	47	0–0.66	
5p14-ter	47	0–27.07	<i>LOC133957</i>
13q14.3-ter	47	52.52–114.15	<i>CLN5</i> , <i>DACH1</i> , <i>ERCC5</i> , <i>KCTD12</i> , <i>UCHL3</i> , <i>UGCGL2</i>
16p13.3	42	0–3.10	<i>NME4</i>
22q13.31-ter	68	45.25–49.48	

*The chromosome 12p genes were selected through different criteria as the α -values can not be calculated for genes where there are no samples without DNA copy number aberrations.

compared to 18% of the genes genome-wide (Fisher's exact test, $p = 4 \times 10^{-12}$).

To identify genes which expression correlates to the DNA copy number status of the same samples, a stringent three-step statistical selection was performed. There were 88 genes identified to be over-expressed in samples with aCGH gain, and similarly, 46 genes were identified showing under-expression with concomitant DNA copy number loss (Supplementary Table 1). Genes among these that are located with the herein identified smallest regions of overlapping gains and losses are listed in Table 1. Three of the amplified and overexpressed genes, *FOS*, *MLL11*, and *RAC1*, were validated by real-time RT-PCR with satisfactory results (Table 2). Although the quantitative RT-PCR expression values had a slightly different scale as compared to the microarray data, the same association of higher expression in samples with array-CGH gain was seen for the three genes.

Criteria for detecting high-level expression of genes on chromosome arm 12p are outlined in the methods section, and among the resulting 16 genes, *CCND2*, *CD9*, *GAPD*, *GDF3*, *NANOG*, and *TEAD4* had the highest fold over-expressions (Table 3). A dissection of the gene expression levels of 12p-genes in TGCT is illustrated in Fig. 2.

From the analyzed IGCN precursor lesions, no genomic region was altered in all three samples. Table 4 contains aberrations seen in two of the three IGCN,

along with the target genes whose median expression in the IGCN was more than two-fold changed accordingly as compared with the median expression in the three normal testis samples.

4. Discussion

In the current study, we have integrated genome profiles with the transcriptome profiles of primary testicular cancers of germ cell origin. Thereby, we identified novel gene targets with changes in expression which are associated to, and may be caused by, DNA copy number changes. Using array-CGH technology to map the genomic aberrations in TGCT in detail, we identified several novel loci of common DNA copy number gains and losses. Previous genomic data obtained by chromosome-based techniques are restricted by the low resolution of the metaphase chromosomes. Furthermore, the chromosome-CGH method do not give reliable results at the telomere regions, which often lead to underscoring of common changes in these chromosomal locations. Several of the herein detected DNA copy number gains and losses have not previously been detected, most likely due to these technical limitations.

The previously reported DNA copy number aberrations in TGCT, obtained by chromosome-based methods, are in all essence gross gains and losses span-

Table 2

Validation of *FOS*, *MLLT11*, and *RAC1* as over-expressed genes in samples with array-CGH gain. The rank values indicate the sort order of the samples based on the expression values (lowest rank numbers for samples with highest expression). Expression values from both real-time RT-PCR and oligo microarray analyses are relative to the expression level in normal testis

	<i>FOS</i>		<i>MLLT11</i>		<i>RAC1</i>	
	microarray	RT-PCR	microarray	RT-PCR	microarray	RT-PCR
Average rank						
gain	8.4	7.4	6.7	6.8	10.1	11.3
no gain	15.3	15.5	18.2	18.2	18.9	17.7
Median expression						
gain	7.9	13.9	4.9	11.1	8.4	2.3
no gain	1.2	1.2	1.1	2.1	1.6	1.4

Table 3

Significantly overexpressed genes on chromosome arm 12p

Symbol	Gene name	d-score	Fold change	Cytoband	Mbp from pter
<i>TEAD4</i>	TEA domain family member 4	2.39	5.15	12p13.33	2.94
<i>CCND2</i>	cyclin D2	2.70	6.66	12p13.32	4.25
<i>CD9</i>	CD9 antigen (p24)	2.25	8.38	12p13.31	6.21
<i>GAPD</i>	glyceraldehyde-3-phosphate dehydrogenase	2.02	12.19	12p13.31	6.52
<i>MLF2</i>	myeloid leukemia factor 2	1.96	3.43	12p13.31	6.73
<i>LEPREL2</i>	leprecan-like 2	2.07	3.16	12p13.31	6.81
<i>GDF3</i>	growth differentiation factor 3	1.61	6.22	12p13.31	7.73
<i>NANOG</i>	Nanog homeobox	1.50	4.16	12p13.31	7.83
<i>KLRB1</i>	killer cell lectin-like receptor subfamB, memb1	1.98	3.32	12p13.31	9.64
<i>FLJ22662</i>	hypothetical protein FLJ22662	1.74	4.03	12p13.1	14.55
<i>DERA</i>	2-deoxyribose-5-phosphate aldolase homolog	2.38	3.28	12p12.3	16.08
<i>MGST1</i>	microsomal glutathione S-transferase 1	1.75	3.86	12p12.3	16.41
<i>GOLT1B</i>	golgi transport 1 homolog B	2.27	3.31	12p12.1	21.55
<i>LDHB</i>	lactate dehydrogenase B	1.88	3.23	12p12.1	21.68
<i>KCNJ8</i>	potassium inwardly-rectifying ch, subfamJ, memb8	2.04	3.75	12p12.1	21.81
<i>CGI-04</i>	CGI-04 protein	2.23	3.22	12p11.21	32.79

Table 4

Genetic changes in intratubular germ cell neoplasia. DNA copy number changes seen in at least two of the three IGCN are shown together with their associated gene expression changes

Cytogenetic map position	Mbp from <i>p</i> -telomere	Gained and overexpressed/lost and underexpressed genes
Gains		
12pter-q12	0–45.90	<i>CIR</i> , <i>CIS</i> , <i>CCND2</i> , <i>CLEC2</i> , <i>CLECSF8</i> , <i>DERA</i> , <i>ELKS</i> , <i>FLJ20696</i> , <i>FLJ22662</i> , <i>GRCC9</i> , <i>KCNA1</i> , <i>KCNJ8</i> , <i>KLRB1</i> , <i>KLRF1</i> , <i>KRAS2</i> , <i>LAG3</i> , <i>MGP</i> , <i>NANOG</i> , <i>PIK3C2G</i> , <i>PPFIBP1</i> , <i>SSPN</i> , <i>TEAD4</i>
15q11-13.1	0–27.20	<i>GABRB3</i>
21q21.3-22.11	29.83–33.56	<i>GRIK1</i>
Xp11.23-ter	0–46.81	<i>ACE2</i> , <i>ATP6AP2</i> , <i>EFHC2</i> , <i>EGFL6</i> , <i>PPEF1</i> , <i>RAI2</i> , <i>S100G</i> , <i>SAT</i>
Losses		
4p16.3	0–0.66	
10q26.2-ter	129.14–135.48	<i>C10orf92</i> , <i>VENTX2</i>
18p11.32	0–0.15	

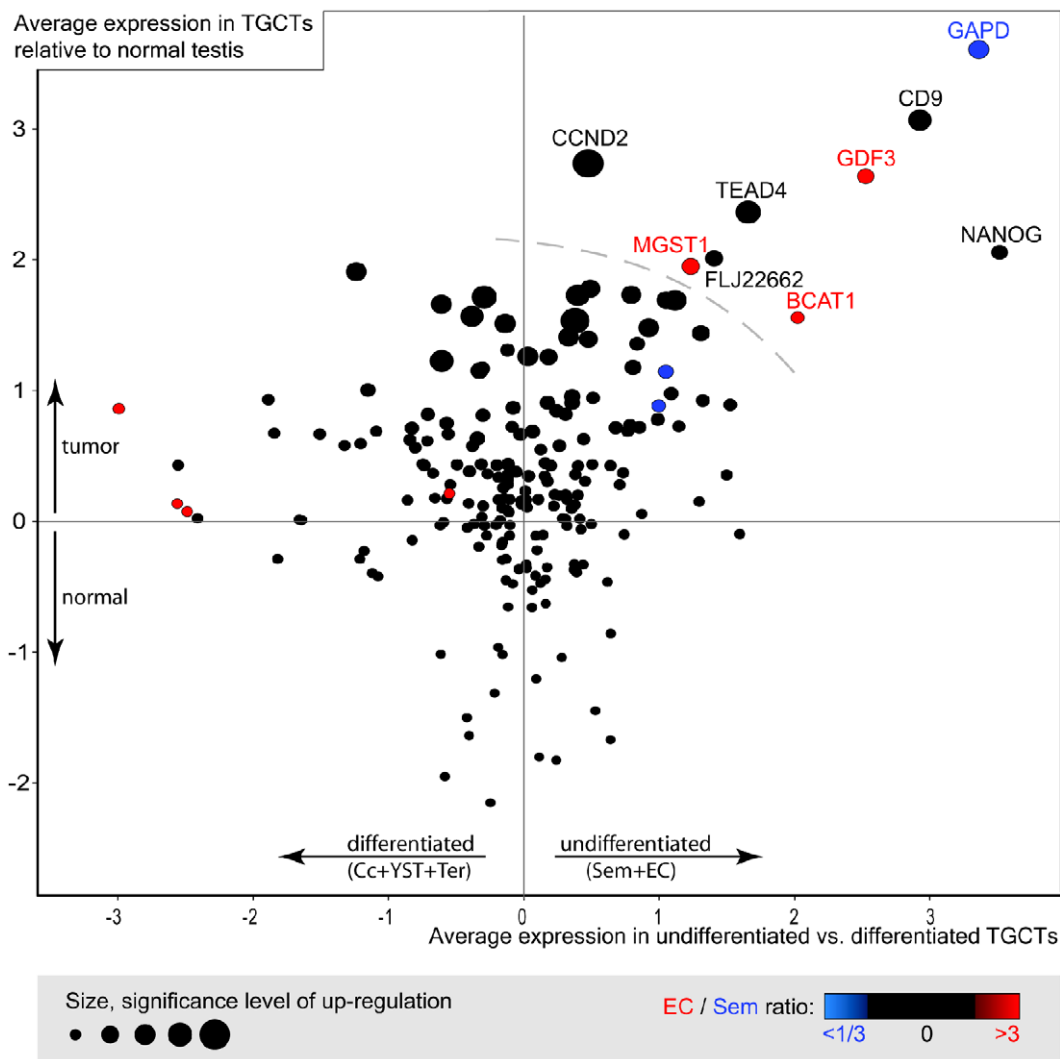


Fig. 2. Over-expression of genes located on chromosome arm 12p. Genes located in the upper part of the chart have higher expression in TGCTs than in normal testicular tissues. The significance of the association is implicated by the size of the circles (the bigger the circle, the more significant the association). Genes in the right part of the figure have a higher expression in undifferentiated stem cell-like histological subtypes (seminomas and embryonal carcinomas) than in more differentiated types (choriocarcinoma, yolk sac tumor, and teratoma). Our hypothesis is that the genes in the upper-right corner are likely to be target “driving genes” of the 12p-amplifications of TGCTs.

ning several chromosomal bands, or even entire chromosomes [47]. This was also the case in the current data set in which gains of whole or parts of chromosomes 7, 8, 12, 14, 21, and X, and losses from whole or parts of 4, 5, 13, 16, 22, and Y were the most frequent aberrations. However, in some tumors, these aberrations covered smaller regions, and due to the improved resolution of array-CGH compared to chromosome-based CGH, we were able to narrow down some of these regions and describe their smallest regions of overlapping gains and losses in TGCT. These include for example the smallest region of overlapping gains

at 21q21.2-22.2, and overlapping losses at 4p16.3 and 22q13.31-ter.

In addition to these mostly gross chromosomal changes, we discovered frequent losses of several small subtelomeric regions. These regions have not been described previously because chromosomal CGH does neither have the sufficient resolution nor provide reliable results close to the telomeres. One explanation may be that the subtelomeric losses occur due to telomere shortening. However, these aberrations were not randomly distributed across the telomeres, since sequences close to the 4p-telomere were lost in 47% of

the malignancies, whereas several other subtelomeric regions were retained in all samples (Fig. 1).

One recent study has also used array-CGH to detect aberrations in TGCT [27]. In that study, 11 samples, mainly seminomas, were analyzed on 5k cDNA microarrays, and there were no accompanying expression data. Two previous reports of combined DNA and RNA level measurements in TGCT limited their analyses to genes located on chromosome arm 12p [6, 61]. Thus, to our knowledge, the present study is the first genome-wide analysis with integration of both the DNA and RNA level information in TGCT.

We identified 88 genes that were both frequently gained and overexpressed in a correlated manner. Similarly, 46 genes with DNA copy number loss and under-expression were identified. Through gene set analyses,⁵ the enriched categories for the gained and overexpressed genes included the terms “proto-oncogene” (including the genes *BRCC3*, *FOS*, *MLLT11*, and *RAC1*), “NF- κ B cascade” (*BCL10*, *ECOP*, *GOLT1B* (alias *GCT2*; ref. [6]), *NFKBIA*, and *TSPAN6*), and “morphogenesis” (*ATN1*, *BMP4*, *CD9*, *CYR61*, *IGFBP3*, *IMP-3*, *LMNA*, *NES*, *NRXN3*, *RAC1*, *RPL10A*, *TEAD4* and *TPD52*). Similarly, for the lost and under-expressed genes, the enriched categories included “disease mutation” (*CLN5*, *CRYAB*, *ERCC5*, *FZD4*, *PTS*, and *RBI*) and “DNA binding” (*DACH1*, *ERCC5*, *GTF2F2*, *HSFY2*, *PRKRIR*, *RBI*, and *ZFP28*).

The proto-oncogenes *FOS* and *RAC1* were both gained and over-expressed in our data, suggesting that increased activity of these down-stream RAS effectors may be a selective advantage for malignant germ cells. This adds to the growing evidence of the RAS pathway as an ubiquitously and ectopically stimulated pathway in TGCT. Although rare, activating mutations of *KRAS* have been reported by us and others [32,33,39]. Also, the effector *RASSF1A* has been shown inactivated by an epigenetic mechanism in a significant subgroup of TGCT [13,17,22]. Furthermore, the up-stream of RAS tyrosine kinase receptor *KIT* have been found mutated in TGCT, typically in bilateral cases [18,24,56]. *KIT* has also been found amplified in some TGCTs, preferably in the seminoma subtype [26]. Amplifications at this locus were not detected within the current sample set. Finally, the overexpression of *GRB7* in TGCT [28, 46,49], which protein binds both *KIT* and *RAS* [57, 59], suggests an ubiquitous deregulation of this pathway.

A gene such as *RBI* has previously been suggested to play a role in the etiology of testicular tumorigenesis through its loss of expression and location on the frequently lost chromosome 13 [37,51]. Although *RBI* appeared on the current list of jointly deleted and under-expressed genes, it is located outside the identified smallest region of overlapping losses, in that five samples displayed losses restricted distally to *RBI*. The DNA excision repair gene *ERCC5* is an interesting candidate among the concomitantly lost and under-expressed genes within this region.

DNA copy number gain of the chromosome arm 12p was detected in all malignant samples, and because this data set includes all histological subtypes of TGCT, this is indicative of 12p gain as an early tumorigenic event. But, again, because 12p gain is not ubiquitously present in all the precursor lesions [36, 42,55, and current data], we believe that having extra 12p-copies would at least also give selective advantage for cells of the histological subtypes thought to originate as derivatives from IGCN, that is, the undifferentiated histological subtypes seminoma and embryonal carcinoma. The more differentiated subtypes of TGCT (choriocarcinomas, yolk sac tumors, and teratomas) are believed to be derivatives of the undifferentiated embryonal carcinomas. Thus, having extra 12p-copies does not necessarily be of similar selective advantage after the tumor cells differentiate. Genes that are either highly over-expressed and/or having higher expression in undifferentiated than in differentiated TGCTs, are marked out in the Fig. 2. The latter include *BCAT1*, *CD9*, *FLJ22662*, *GAPD*, *GDF3*, *NANOG*, and *TEAD4*. Among these, *BCAT1*, *GDF3*, and *MGST1*, are most highly expressed in embryonal carcinomas, whereas *GAPD* is most highly expressed in seminomas. *CCND2* is the most highly overexpressed gene at 12p that do not have differentiation-specific expression levels. The latter is in agreement with a recent study reporting ubiquitous over-expression of *CCND2* in all histological subtypes of TGCT [20].

The 12p amplifications, seen in virtually all TGCTs, are generally a result of isochromosomes of the 12p chromosome arm [4], although 12p-amplifications also occur through other mechanisms [40,52]. In the present array-CGH data set, we detected uniform amplification levels along the length of the 12p chromosome arm in all samples. However, a few cases have been reported with a smaller amplified region at 12p12.1 [30,53,61] or with increased levels of amplification at the 12p13 chromosome band [12,23,40]. Out of the nine highlighted genes in Fig. 2, only *BCAT1* is local-

⁵Database for Annotation, Visualization and Integrated Discovery 2.0 (DAVID 2.0), URL: <http://niaid.abcc.ncifcrf.gov/>

ized within the 12p12.1 region, whereas *CCND2*, *CD9*, *FLJ22662*, *GAPD*, *GDF3*, *NANOG*, and *TEAD4* are all localized in the distal region of 12p. We have previously shown by both *in vivo* and *in vitro* studies that three of these genes, *CD9*, *GDF3*, and *NANOG*, are highly expressed in undifferentiated TGCTs, but are significantly downregulated as embryonal carcinomas differentiate [48]. Furthermore, all these three genes were implicated in two recent studies [11,20] which concluded that there is a cluster of stem cell-related genes at 12p13 whose down-regulated gene expression is associated to differentiation in TGCT. Together, this speaks for the importance of the pluripotency-related 12p13 genes, both playing roles in the initiation of the TGCT development through their over-expression, and in the subsequent differentiation steps of TGCT cells through their silencing.

Genomes of human embryonic stem cells have also been found to contain DNA copy number aberrations. Interestingly, these were extra copies of the chromosome arms 12p and 17q [9]. The fact that net gain of 12p sequences is a pathognomonic marker for germ cell tumors and that gain of 17q is also common in TGCT [23,49] is yet another line of evidence for these stem-cell specific genes being the target genes on the 12p amplification – as well as evidence for GCT as a caricature-model for early embryogenesis. In the present series, 17q gain was detected in five of the 22 samples. This frequency is lower than in our previous study using chromosomal CGH (22/33 samples), a discrepancy which may be coincidental in small samples series. Five samples in the present series were overlapping with the previous study, and none of these showed 17q gain in neither of the studies.

In summary, we have pinpointed many novel regions of DNA copy number aberrations in the TGCT genome. These non-random genomic aberrations are likely to give the tumor cells selective advantage, and thus, the affected genes that are also altered in expression levels, are strong candidates to play roles in the molecular etiology of TGCT. These genes include the proto-oncogenes *FOS* and *RAC1*, showing both DNA copy number gain and over-expression in TGCT. We also provide evidence for the importance of amplification and over-expression of 12p13 genes, both in the initiation and differentiation of TGCT.

Supplementary material

Supplementary tables are available on:
www.cellularoncology.org/CLOvol28.htm

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