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Genome-wide analysis of genetic alterations in testicular primary seminoma using high resolution single nucleotide polymorphism arrays ☆

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ABSTRACT

Testicular germ cell tumors (TGCT) represent the most common malignancy among young males. To our knowledge no comprehensive Copy Number Variation (CNVs) studies of TGCT using high-resolution Single Nucleotide Polymorphism (SNP) array have been performed. By a genome-wide analysis of CNV and loss of heterozygosity (LOH) in 25 primary seminomas, we confirmed several previously reported genomic alterations and discovered eight novel genomic alterations including amplifications and homozygous deletions. Moreover, a comparison of genomic alterations of early and late stage seminoma identified CNVs that correlate with progression, which included deletions in chromosomes 4q, 5p, 9q, 13q and 20p and amplifications in chromosomes 9q and 13q. We compared previously perform Affymetrix expression analysis in a subset of samples and found robust correlation between expression and genomic alterations. Furthermore, high correlations (40–75%) were observed between CNV by SNP analysis and quantitative PCR. Our findings may lead to better understanding of TGTC's pathogenesis.

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1. Introduction

Testicular germ cell tumors (TGCT) represent the most common malignancy among young Caucasian males between the ages of 15 to 40 years and account for the highest cancer related cause of death in this age group [1]. The incidence of testicular cancer has substantially increased in the USA (www.cancer.gov). White men are five times more likely than African American men to develop testicular cancer, thus indicating a genetic predisposition for this disease. In the United States, approximately 8400 newly diagnosed testicular cancer cases and 380 deaths are expected in 2009.

Based on their degree of differentiation and histological characteristics TGCT can be classified into seminomas (SE) and non-seminomas (NS), as well as combinations of these [2,3]. SE resembling primordial germ cells and NS composed of neoplastic tissues exhibit somatic, embryonal or extra-embryonal differentiation [4]. SE and NS are thought to arise from pre-invasive carcinoma *in situ* (CIS), also referred to as intra-tubular germ cell neoplasia (IGCN) unclassified. Details on the pathogenesis of SE and NS were reviewed previously [5,6]. SE represent approximately 40% of diagnosed cases [7]. Up to 32% of patients with SE clinical stage I can relapse if no adjuvant treatment is given. Nevertheless, the cure rate in patients with stage I SE is almost 100% and can be achieved with three accepted strategies: adjuvant radiotherapy, single-agent carboplatin chemotherapy or a surveillance strategy. Around 28% of the patients that present with seminoma have regional or distant metastasis (http://seer.cancer.gov/statfacts/html/testis.html), of those with distant metastasis, the survival rate in 5 years is around 70%.

There are four main types of NS tumors: embryonal carcinoma, yolk sac tumor, immature or mature teratoma, and choriocarcinoma. These tumor types are often seen together in various combinations, referred to as mixed TGCTs, which may also include a SE component [8].

Several studies have provided insight into the progression of TGCTs although few studies have identified the underlying genes involved in the pathogenesis of testicular cancer [5,6]. Over-representation of chromosome 12p is common in TGCTs but is not seen in CIS, suggesting a critical role for this amplification in TGCT progression [9]. A progressive genomic instability correlating to the stage of the disease has also been previously described including gains of DNA material from whole chromosomal arms of 1q, 7p, 7q, 8p, 8q, 12p, 12q, 14q, 15q, 21q, and 22q

and losses of 4p, 4q, 5p, 5q, 11p, 11q, 13q, and 18q [10,11]. In the case of SE, gain of 12p is the most striking alteration observed [12]. A recent study demonstrated that the loss of PTEN expression marks the transition from noninvasive IGCN to invasive cancer, being PTEN expression retained in IGCN, the presumed precursor lesion of germ cell tumors, and lost in tumors [13].

DNA copy number alterations and/or changes in allelic ratios are hallmarks of different subsets of neoplasia [14]. In the past, various techniques such as Comparative Genomic Hybridization (CGH) arrays, microsatellite analysis and Fluoresence in situ hybridization (FISH) were used to identify novel genomic alterations, such as amplification, deletions, and translocations, however these techniques had limitations which included the inability to detect loss of heterozygosity (LOH) that were not directly a result of deletions and a small detection range (1–20 kb). The introduction of FISH overcame these limitations of conventional cytogenetic analysis, however this technique was not high throughput and required probes with high specificity [15]. CGH arrays were the first improved high throughput assays that facilitated the analysis of genome wide localization of chromosomal imbalances without prior knowledge of specific regions of genomic damage [16]. They also provided the ability to investigate highly fragmented DNA often obtained from formalin fixed tissues [17]. In addition, detection of amplifications less than 1 Mb were relatively high, however the resolution of regional deletions, especially homozygous ones, remained limited [18]. Furthermore, CGH has limits of definition for small losses and cannot distinguish between paternal and maternal recombination events [19,20].

Microsatellite instability has been well established as a mechanism for the development of many diseases including cancer [21]. Microsatellite analysis is beneficial in elucidating microsatellite instability, gene dosage and kinship. This technique can be tedious and uses relatively large amounts of DNA. Another important limitation was the occurrence of "null alleles" in which the microsatellite primers failed to amplify in PCR reactions, due to point mutations [22]. Furthermore, PCR failure occasionally results in heterozygous individuals being scored as homozygous. The introduction of single nucleotide polymorphism (SNP) array technology allows for combined detection of both copy number and LOH information throughout the genome at greater resolution than previous techniques [23,24]. In addition, this technology is high throughput and permits the fine mapping of copy number changes with a range of 30–900 kb [24].

Although testicular cancer is highly responsive to cisplatin, 10–20% of metastatic patients will not achieve a complete remission after the first treatment, due to incomplete response or tumor relapse [25]. The sensitivity of TGCTs to chemotherapeutic drugs may lay in the susceptibility of germ cells to apoptosis which makes this cancer type an ideal tumor type model to investigate and understand the molecular determinants of chemotherapy sensitivity of solid tumors. Attempts to develop personalized medicine treatments for solid tumors could benefit of better understanding of the biology and genetic alterations in TGCTs. The availability of microarray-based high-resolution SNP analysis allows a reproducible and rapid determination of genome-wide allelic changes such as genomic instability and LOH from a single DNA sample. This technique has been employed for SNP genotyping in various human cancers [23,26–28] and in this report, we present the first data using the SNP microarray mapping technique to characterize SE DNA samples from 25 patients, the most common subtype of testicular cancer.

2. Materials and methods

2.1. Sample collections

We used collected samples from the Erasmus MC — University Medical Center Rotterdam (Daniel den Hoed Cancer Center), Department of Pathology, The Netherlands. The use of tissues for scientific purposes included in this study were approved by an institutional review board (MEC 02.981 and CCR2041), and the samples were used according to the "Code for Proper Secondary Use" (FMWV) (version 2002). Briefly, primary tumor and peripheral blood samples were collected from 25 patients undergoing surgical resection of testicular SE (Table 1). Two samples were excluded for further analysis due to low call rate. Tumor samples were promptly frozen at −80 °C after initial gross pathological examination. DNA was isolated from tumor tissue and lymphocyte pellets by standard SDS/proteinase K digestion followed by phenol and chloroform extraction and ethanol precipitation. Approval for research on human subjects was obtained from The Johns Hopkins University Institutional Review Boards. This study qualified for exemption under the U.S. Department of Health and Human Services policy for protection of human subjects [45 CFR 46.101(b)].

2.2. SNP mapping assay

Genomic alterations in tumor samples were genotyped using 250 K Nsp1 SNP arrays (Affymetrix). A detailed protocol is available on the Affymetrix web page (http://www.affymetrix.com/support/technical/manuals.affx). Briefly genomic DNA from tumor and paired lymphocyte were restriction digested with Nsp1, ligated to the adaptor, and amplified by polymerase chain reaction (PCR) using a single pair of primer. After purification of PCR products with the MinElute 96 UF PCR purification kit (Qiagen), amplicons were quantified. 100 µg of PCR amplified DNA was digested with DNase I to sizes ranging from 200 to 1000 bp. Fragmented PCR products were labeled with biotin and hybridized to the array. Arrays were then washed on Affymetrix fluidics stations. The bound DNA was fluorescently labeled using streptavidin–phycoerythrin conjugates and scanned using Gene Chip Scanner 3000. Hybridization, scanning and raw analysis were performed by the Microarray Core facility at Johns Hopkins School of Medicine.

2.3. Data analysis

CEL files containing intensity value for each of the probes on the NspI 250 K chips were generated using GeneChip Operating Software (Affymetrix Inc., USA). The allelic intensity of each SNP from the GeneChip Operating Software was measured using the GeneChip Genotyping

Table 1
Clinical and pathological characteristics of the seminoma cancer patients (N = 25).

Sample no.	All loci	Normal	Tumor	Stage	Age
SE1	95.22%	96.94%	93.49%	1	30
SE2	94.44%	96.14%	92.73%	1	31
SE3	97.26%	98.68%	95.83%	1	60
SE4	96.74%	96.65%	96.83%	1	45
SE5	96.05%	95.86%	96.24%	1	30
SE6	88.43%	93.29%	83.56%	1	28
SE7	91.21%	91.61%	90.80%	1	47
SE8	86.13%	84.91%	87.34%	1	45
SE9	88.04%	82.49%	93.59%	1	38
SE10	97.16%	98.64%	95.68%	1	29
SE11	96.03%	94.94%	97.11%	1	42
SE12	96.23%	97.81%	94.65%	1	60
SE13	95.25%	93.87%	96.62%	1	29
SE14	95.81%	93.52%	98.09%	1	30
SE15	93.02%	95.34%	90.70%	1	32
SE16	70.15%	75.72%	64.58%	1	39
SE17	95.38%	93.42%	97.33%	1	33
SE18	78.52%	78.52%	78.51%	1	26
SE19	88.23%	92.28%	84.18%	2	32
SE20	94.08%	96.60%	91.56%	2	32
SE21	92.33%	90.98%	93.67%	2	39
SE22	97.09%	97.46%	96.71%	2	39
SE23	89.63%	95.45%	83.80%	3	40
SE24	96.68%	95.44%	97.91%	2	39
SE25	96.68%	95.44%	97.91%	2	48

analysis software (GTYPE v4.0) and the genotypes calls (AA, AB or BB) were done using BRLMM-P algorithm for each of the SNPs.

For further analyses, we utilized the copy number analysis protocol from Partek Genomic Suite™ (ver 6.4., http://www.partek.com/partekgs). Briefly, the CEL files were imported onto copy number analysis pipeline and subsequently normalized to baseline reference intensities using 270 HapMap samples to infer the paired allele ratio for each of the samples (for both tumor and normal samples). Additionally, for better inference of copy number estimates, we used genomic smoothing in order to reduce variance in the array data. Hidden Markov models (HMM) assuming diploid copy for normal samples were employed to infer the copy number estimates in tumor samples. Delineating copy number change and its boundaries can often be challenging. To guard against detection of false positive CNVs arising due to inherent microarray "noise", we utilized a genomic segmentation algorithm (requiring a minimum of 10 probes) to characterize isolated islands of significantly higher or lower intensity ratios into cancer specific copy number alteration regions. A threshold of ≥ 3 and ≤ 1.25 was used to categorize altered regions as copy number gains (amplification) and copy number losses (deletions), respectively. Student's t-tests were performed to identify stage specific (stage 1 vs. stage II + III) copy number altered regions.

For LOH analysis, we utilized the algorithm incorporated in dChip software [12]. The LOH events were estimated with regard to informative markers in the different chromosomes, and an average probability was calculated based on prevalence of LOH events across all our samples. As we had matched normal DNA available from all our samples, a matched pair analysis (based on SNP by SNP genotypes) was employed for the detection of LOH regions.

For further analysis in our regions of interest, we performed a fine-scale mapping of the copy number altered regions to identify specific regions of allelic imbalance (Fig. 1). Each of the genotypes was compared and regions/SNPs showing somatically altered genotypes (based on paired SNP by SNP comparison) were identified and annotated for the presence of cancer associated genes (e.g., tumor suppressor genes, oncogenes etc.).

Annotation information for SNPs and genomic locations were carried out using the human genome build 17 (hg17) and the widely used and available online databases (Netaffyx: http://www.affymetrix.com; Ensembl: http://www.ensembl.org; UCSC: http://genome.ucsc.edu).

2.4. Affymetrix expression array

Affymetrix arrays were performed in a subset of SE samples (from Table 1) for gene expression profiling per the manufacturer's instruction in our previous study [29]. We used Affymetrix GeneChip Human Genome U133A plus 2.0 Arrays containing >22,000 probe sets for the analysis of >18,400 transcripts, which include ~14,500 well-characterized human genes. Biotinylated RNA probe preparation and hybridization were previously described [30].

2.5. Quantitative PCR validation

SNP array data was validated by real-time quantitative PCR using the ABI Prism 7900HT Sequence Detection System [31] and Roche SYBR green kit. Genes FAT3, EDNRB1 and ALKBH8 were chosen from deleted areas and gene RASSF8 is chosen from amplified area. Primers were designed by using Primer3 and the human genome reference assembly (UCSC version hg17, based on the National Center for Biotechnology Information build 35). All samples were run in triplicate. Copy number alterations were assessed by relative quantification methods that compensate for differences in target and reference amplification efficiencies. The reference tissue used for the copy number calculations was from the same individual from whom the tumor sample was taken. Primer sequences and PCR cycling conditions are detailed in Supplemental Table 1.

The number of copies corresponding to the gene of interest was determined by real-time quantitative PCR with a 7900HT Sequence detector (Perkin-Elmer Applied Biosystems). Briefly, PCRs were carried out in a reaction volume of 10 µl using FAST SYBR Green (Qiagen). 20 ng of DNA was used in each real-time PCR reaction. The conditions used for amplification were: one cycle of 95 °C for 3 min, followed by 50 cycles of 95 °C for 15 s and 58 °C for 1 min. Reactions were performed in triplicate and the average of the threshold cycle values was calculated. DNA content was normalized to that of Line-1 — a repetitive element for which copy numbers per diploid genome are similar in normal or neoplastic human cells [32,33]. Changes in copy number were calculated as: $2^{(Dt - Dline) - (Nt - Nline)}$ as published previously [33], where Dt is the mean threshold cycle number for experimental primer in DNA extracted from tumor cells, Dline is the mean threshold cycle number for Line-1 primer in DNA extracted from tumor cells, Nt is the threshold cycle number in reference DNA, and Nline is the threshold cycle number for Line-1 primer in reference DNA.

3. Results

3.1. Identification of novel copy number alterations in seminoma

We examined tumor and paired normal lymphocyte DNA from 25 patients diagnosed with a primary testicular SE with a 250 K Nsp1 platform array for genomic alterations. Clinico-pathological and demographic information of these patients are provided in Table 1. Initially, we identified 1810 potential copy number (CN) altered regions in our cohort using SNP array. The putative CN altered regions were further filtered based on number of probes (≥ 10) and Hidden Markov Model (HMM) segmentation to delineate CN altered regions and plausible gene candidates which included RASSF8, ALKBH8, EDNRB1 and FAT3 (Supplementary Fig. 1).

The signal intensity of each SNP probe along the chromosomes was detected and normalized with normal tissue to calculate the genome-wide distribution of DNA copy number alterations. The average signal intensity for normal tissues was 2.0. Cutoffs of copy number boundaries used were <1.5 and >2.6 for deletions and amplifications respectively. A heat map depicting genome-wide CN profile in all SE samples is shown in Fig. 1. The frequency of imbalances shown at the top of the figure depicts CN intensity ratio (log2 ratio) for all samples on a representative color gradient scale. Compared to normal lymphocyte DNA, SE samples show wide spread copy number gains and losses involving all chromosomes. Detectable gains in chromosomes 2, 3, 7, 8, 12 and X and losses in 4, 5, 11, 13, 18 and 20 were observed (Fig. 1, red = gain; blue = loss).

Inferred copy number changes were observed by fine mapping on chromosome 13q (deletion; ~40%), which encodes EDNRB1 (Fig. 2A, upper panel) and 11q (FAT3) (Fig. 2B, upper panel). Chromosome 12p indicated high level amplification (~60% of our samples) and encodes for RASSF8 gene (Fig. 2C, upper panel). Both deletion of 11q and gain of 12p are known to be altered in testicular cancer [34,35]. We also performed fine mapping of our novel amplification region in chromosome 2 (Supplemental Fig. 2). Samples 16, 17, 19 and 21 all indicated amplification in tumor compared to normal. In addition, we identified a novel deletion in chromosomal region 20p12. In general, allelic imbalance was more common in SE stages II–III than in stage I tumors. An overview of genomic alterations in SE found in SNP array analysis in our study is shown in Table 2. Novel genomic alterations discerned in this study are on chromosomal arms 2q14, 2q22, 2q23, 2q24, 2q32 and 20p11–12 harboring $\geq 17\%$ frequency of copy number gains (Table 3). Candidate genes found in regions 2q14–35 included ERCC3, XIRP2, IKZF2 and CRYGC. IKZF2 is a stress related gene that has been shown to be aberrantly expressed in various lymphomas and leukemia [36]. Candidate genes found in 20q11–12 included ESF1, C20orf7, SEL1L2, and MACROD2. ESF1 has been shown to be required for 18S rRNA synthesis in S. cerevisiae [37].
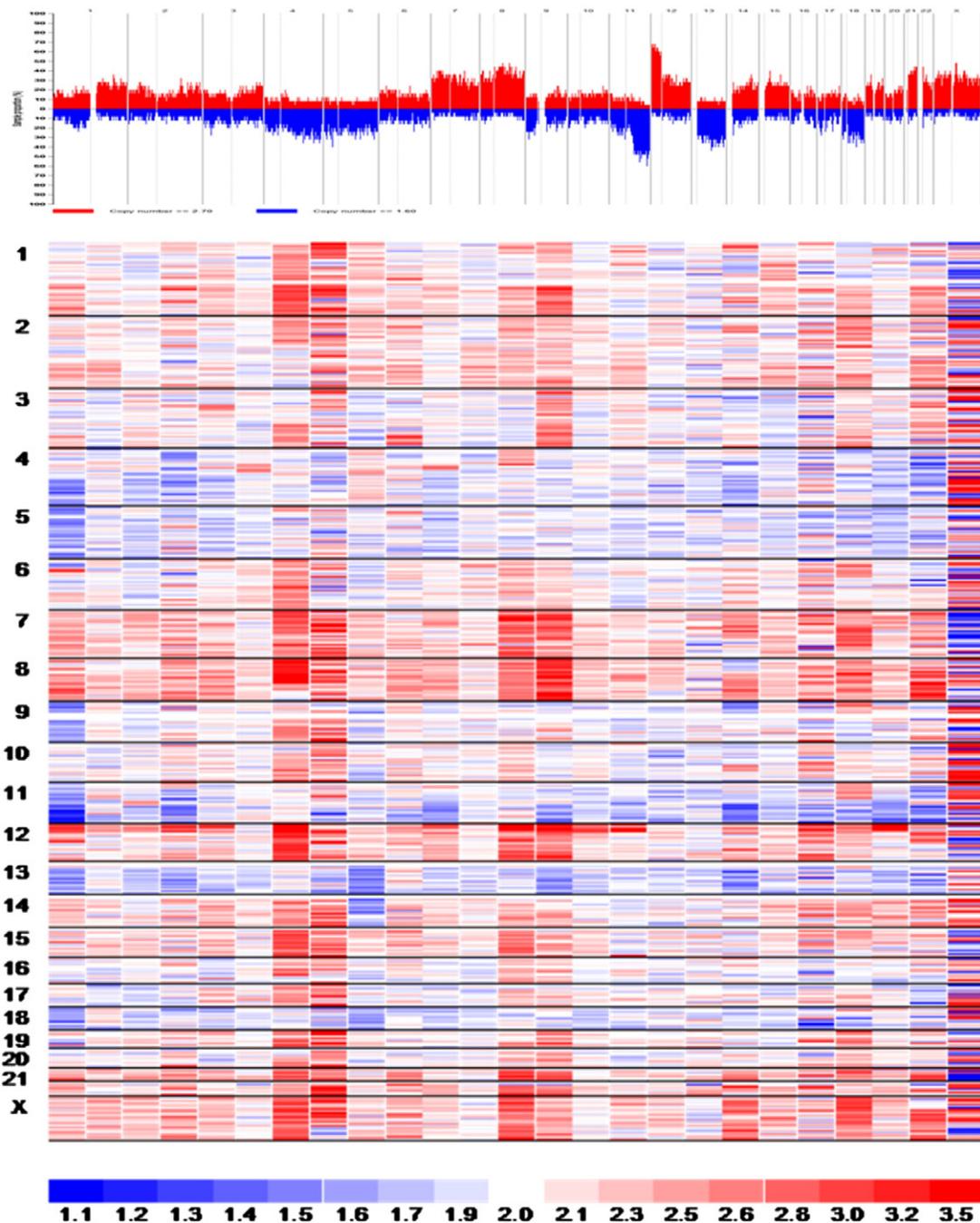
Fig. 1. Genome-wide distribution of DNA copy number alterations in seminoma. Copy number (CN) alterations are depicted in color gradients corresponding to DNA; CN gains (amplifications — red) and CN losses (deletions — blue). The top panel shows frequency distribution of all the CN changes across all 23 chromosomes. The bottom panel shows the intensity plots showing each of the samples.

3.2. Validation of CNV

For validation of SNP array based data, *RASSF8*, *FAT3*, *ALKBH8* and *EDNRB1* were selected for validation from 77 cancer specific somatic CN altered regions. The chromosomal locations of the later genes are on 12p12, 11q14.3, 11q22.3 and 13q22.3 respectively. The frequencies of genomic alterations of these regions by SNP array analysis are shown in Table 2. The number of copies corresponding to *RASSF8*, *FAT3*, *ALKBH8* and *EDNRB1* locus were examined by Quantitative-PCR (QPCR). Representative amplification curves for EDNRB, FAT3 and RASSF8 are shown in lower panel of Fig. 2 A, B and C respectively. *ALKBH8*, *EDNRB1* and *FAT3* copy number losses (<1.5) were detected in at least 39% of all 23 samples. The relative findings of the SNP array compared to the

QPCR were at least 40% consistent (Table 4). Homozygous deletions (HD, less than <0.5 copy) by both the SNP array and QPCR methods were found for *ALKBH8* (21%) and *FAT3* (36%) in our samples, suggesting that these genes are potential tumor suppressor genes (TSGs) for SE and need to be explored in further studies.

3.3. Comparison of copy number alterations and expression of related genes

After confirming the SNP data with QPCR, we compared these genetic alterations with Affymetrix expression array data that was undertaken by a separate study [29]. Consistent findings were observed for genes *FAT3*, *RASSF8* and *ALKBH8* (Supplementary Fig. 3).
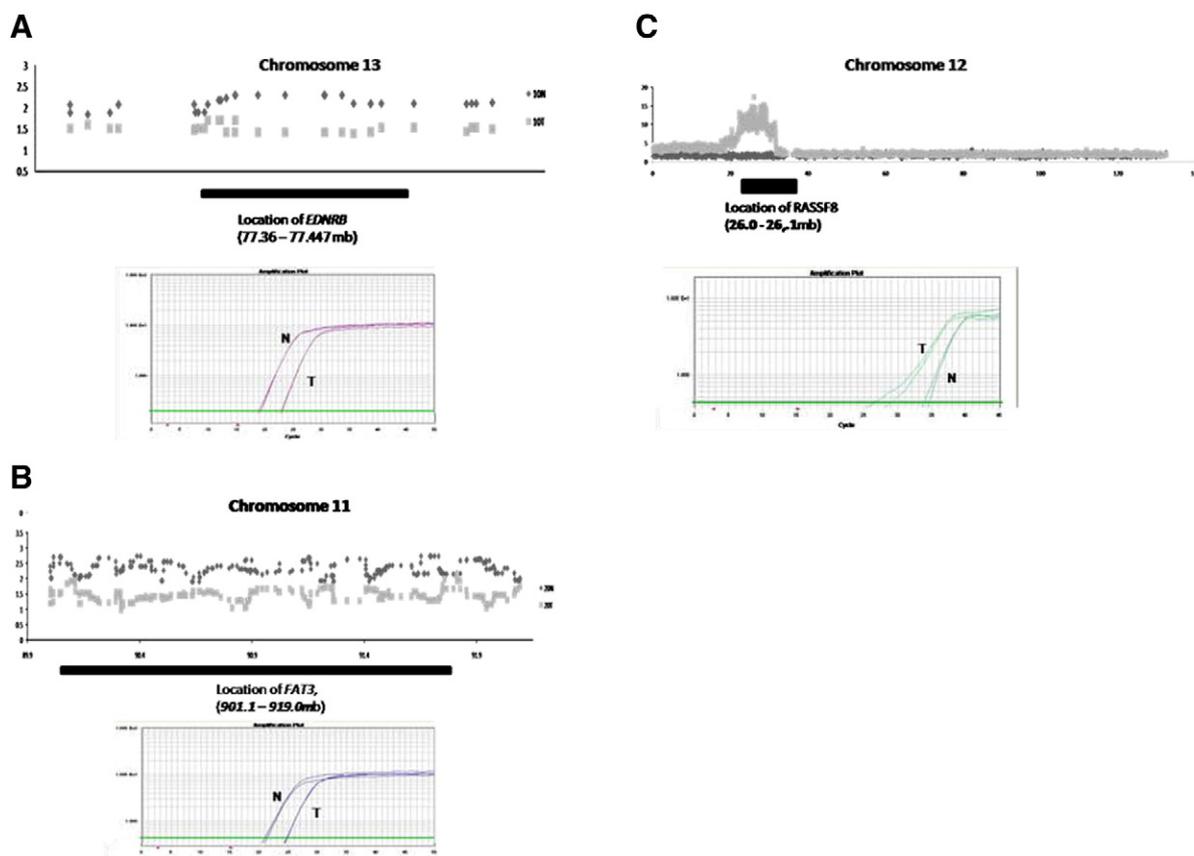
Fig. 2. Fine mapping on significant Copy Number (CN) alterations in seminoma tumors. (A). Inferred copy number of stage 1 matched normal-tumors pair showing hemizygous deletions of *EDNRB* (chromosome 13 band q22.3). The dark and light grey shaded points are inferred copy number estimates for matched normal and tumor samples, respectively. (B). Inferred copy number changes (deletions) on chromosome 11 band q14.3 encompassing the putative tumor suppressor gene *FAT3*. (C). High level amplification on the short arm of chromosome 12, a region previously reported to be highly amplified in seminoma.

3.4. Genome wide imbalances correlated with progression

Although many chromosomal alterations in testicular cancer have been identified, no data exist correlating these changes to disease progression. Our data suggest that CN deletions in chromosomes 4, 5, 9, 13, and 20 and amplifications of chromosomes 9 and 13 associated with advanced stage (stages II/III vs. stage I) of the disease and details are found in Table 5. A deletion of chromosome 13q13.3 which encodes for *DCLK1* and *SOHLH2* was altered in 20% of the samples. SOHLH2 is a germ cell specific transcription factor that may be a

Table 2
Cancer specific genomic alterations (amplifications and deletions) seen in seminoma samples (N = 23). The plausible candidates that may play a role in testicular carcinogenetic pathway are listed here.

Region	10-15%	15-20%	20-25%	>25%	Candidate Genes	CN change
1q42.13		▉			PRSS38,JMJD4	
2q14.3			▉		CYP27C1,ERCC3,IWS1	
2q35-36.1				▉	STK16, ABCB6, DES	
2q34			▉		IKZF2	Amplification
7q21.11	▉				MAGI2	
8q24.12		▉			NOV,ENPP2	
12p12.1			▉		RASSF8	
18q21.33	▉				SERPINB3,SERPINB4	
21q21.1					BTG3	
1p31.3			▉		DIRAS3,SFRS11,MIR186	
1q31.1			▉		TPR	
2p21			▉		PRKCE	
4q21.3		▉			MAPK10	
4q12	▉				KIT	
5q12.1			▉		MAST4	
9q21.13				▉	TMC1	Deletion
11q14.3				▉	FAT3	
11q22.3		▉			ALKBH8	
13q32.1			▉		ABCC4	
13q14.11				▉	ENOX1	
13q22.3			▉		EDNRB1	
13q21.32			▉		PCDH9	
13q31.3			▉		GPC5	

Table 3
Novel alterations of chromosomal regions in seminoma.

Region	CNV Alteration	Frequency	Genes	Candidate gene function	Samples
2q24.1	amp	13%	UPP2	Folliculogenesis, hormone secretion regulation,	6, 16, 17, 20, 21, 24
2q14.3	amp	26%	CYP27C1, ERCC3, MAP3K2	CYP27C1 — only expressed in testis; ERCC3 — DNA repair enzyme NER	6, 16, 17, 19, 21, 24
	amp	22%	MKI67IP, TSN	TSN — chromosomal translocations and regulation of RNA expression	16, 17, 19, 21, 24
2q22.1	amp	17%	THSD7B	Unknown	16, 17, 21, 24
2q23.3	amp	17%	NEB, ARL5A	NEB — Calcium/CaM regulation	16, 17, 21, 24
		26%	XIRP2	DNA repair	6, 17, 19, 20, 21, 24
2q32.1	amp	22%	ELF2P4, FSIP2	FSIP2 — spermatocyte development	6, 17, 19, 21, 24
		26%	SLC23A3, ABCB6	STK16 — involved in VEGF expression regulation, ABCB6 — drug transporter ATP dependent, expressed in testis and linked with breast cancer	16, 17, 19, 20, 21, 24
2q33.3	amp	26%	CRYGEP1, CRYGC, CRYGB, CRYGA	Stress response	6, 17, 19, 20, 21, 24
2q34	amp	26%	IKZF2	Aberrant expression in Hodgkins and non-Hodgkins lymphoma	6, 16, 17, 19, 21, 24
20p12.1	del	13%	ESF1, C20orf7, SEL1L2, MACROD2	ESF1 — pre-RNA processing	3, 15, 16

critical regulator of early germ cell development [38]. Interestingly, most of the genetic alterations that correlated with advance stage disease related to deletions.

3.5. LOH

Paired DNA samples from the same patient allowed us for the identification of regions of LOH in the tumor samples. Alterations associated with copy number losses and gains were found to be associated with LOH in 12 chromosomal regions. Frequencies of LOH regions are detailed in Table 6. Detectable LOH regions were identified on 2p15, 2q21, 2q32, 2q35, 3p26, 4q13, 5q21, 8q11, 9q21, 11q2, 13q1 and 18q12.2 (Table 6). Interestingly, we identified 30 homozygous deletions (HD) in the only stage III SE analyzed. The most important HDs centered on chromosome 4 (deleted gene: *UNC5C*), chromosome 6 (deleted gene: *NKAIN2*), chromosome 8 (deleted gene: *CSMD1*) and chromosome 12 (deleted gene: *IPO8*). We also detected 43 focal amplifications, although no alteration was common among our samples. Supplemental Fig. 4 shows heat map inferred LOH calls based on dChip software analysis using the paired tumor and normal samples.

4. Discussion

Prior studies have investigated genomic alterations in testicular cancer by means of CGH and microsatellite marker analysis in both testicular cell lines and tissue samples, however to our knowledge; no large scale study has been performed on SE using high resolution SNP arrays. Gene amplifications and deletions are one of the major mechanisms of oncogene activation and TSG inactivation in cancer development, respectively. The goal of our study was to identify new genomic regions of alterations by analyzing 25 SEs with matched normal DNA by using a 250 K *Nsp*1 array. We hypothesized that using a highly sensitive technique such as Affymetrix 250 K *Nsp*1 array; we could detect small unidentified regions that may have been undetectable using previous techniques. These newly discovered regions could in turn provide valuable information on specific genes that are deregulated throughout the different stages of SE. This information could be a useful avenue to understand the biology of SE and also help with the discovery of biomarkers such as for early detection and therapy response prediction. Using paired samples we found chromosomal copy number gains and losses to be in agreement with previous findings [39,40]. In addition, we identified eight novel regions of copy number gains (2q14.3, 22.1, 23.3, 24.1, 32.1, 33.3, and 34) and losses (20p12.1). However, the biologic importance of the genes positioned in these altered regions remains to be determined. Copy number losses for 20p were recently reported in colorectal cancer, Barrett's adenocarcinoma and neuroblastoma [24,41,42].

Allelic losses of chromosome 2q were previously reported in TGCT cell lines [40], albeit we found gain in this region. The inconsistency may be due to uses of cell lines vs. a primary tumor tissue specimens and different technique and stringent conditions were used to define losses and gains.

Although we did not perform any formal correlation study between CNV and mRNA expression, by our initial comparison analysis among CNV and mRNA expression we observed that expression and CNV correlate for several genes like *FAT3*, *RASSF8* and *EDNRB1*. Comprehensive analysis of CNV and mRNA expression may identify a panel of promising genes and those may represent the "driver genes" among a sea of "passenger genes" that were aberrant. Other confounding factors include epigenetic alteration such as methylation and miRNA expression that may have a significant contribution to the steady-state gene expression levels even in the setting of gene copy number changes. This hypothesis can be tested when methylation and miRNA expression data on the same samples become available; a study currently undertaken.

Table 4
Analysis of *ALKBH8*, *FAT3*, *EDNRB1* and *RASSF8* copy number: The number of copies corresponding to *ALKBH8*, *FAT3*, *EDNRB1* and *RASSF8* locus was determined by real-time quantitative PCR. The % correlation between the SNP array and QPCR is indicated at the bottom of the table. The total number of altered samples was divided by the total number of samples to determine the % of deletions (blue) and amplifications (red). NA = QPCR not available. A) For deleted region the correlation between SNP analysis vs. QPCR were 75%, 71% and 75% for *ALKBH8*, *FAT3* and *EDNRB1* respectively B) For amplified region the correlation between SNP analysis vs. QPCR was 40% for *RASSF8* locus.

(A) Deleted region				(B) Amplified region	
Sample ID	ALKBH8	FAT3	EDNRB1	Sample ID	RASSF8
SE2	0.457	NA	0.5697	SE2	1.3
SE5	0.388	1.341	0.67102	SE3	NA
SE6	0.858	3.04	1.431	SE5	1.01
SE8	0.962	0.42	1.085	SE6	4.14
SE10	2.28	0.506	1.128	SE8	0.63403
SE12	2.09	NA	1.996	SE10	0.523
SE15	0.805	0.686	0.6743	SE12	1.94
SE16	4.29	0.806	1.427	SE15	3.183
SE17	1.803	0.377	1.094	SE16	1.62
SE18	0.885	0.584	1.052	SE17	1.943
SE19	1.33	1.875	1.244	SE18	4.11
SE20	2.116	0.6565	2.416	SE19	1.57
SE22	NA	2.3	NA	SE23	1.151
SE23	0.599	0.1524	0.968	SE24	2.49
SE24	1.105	0.0247	NA		
Consistency	75%	71%	75%	40%	

Between SNP array and QPCR analysis.

Table 5
Genomic alterations that correlate with progression seen in seminoma samples (N = 23). The plausible candidates that may play a role in testicular carcinogenetic pathway are listed here.

Chr	Cytoband	% samples	# of markers	Genomic alteration	Transcribed genes
4	4q35.1	16	15	Deletion	MGC45800
5	5p15.1	16	19	Deletion	ZNF622, FAM134B
9	9q31.3	16	105	Deletion	ACTL7A, ACTL7B, IKBKAP, C9orf6, ACRP, CTNNAL1, C9orf5, CS266556, AL390170, C9orf4, AX747119, EPB41L4B, PTPN3
9	9q22.31	16	18	Amplification	FAM120A, PHF2
9	9q33.1	16	30	Deletion	TLR4
9	9q21.31	16	10	Deletion	ENSG00000218149
9	9q21.33	16	10	Deletion	NTRK2
13	13q13.3	20	14	Deletion	DCLK1, SOHLH2
13	13q34	16	6	Deletion	ARHGEF7
13	13q22.3	16	51	Deletion	SCEL, SLAIN1, EDNRB1, BX647243, AK090854
13	13q22.2	16	35	Deletion	TBC1D4, COMMD6, MST076, UCHL3
13	13q12.13	12	16	Amplification	ATP8A2
13	13q22.2	12	5	Deletion	LMO7
20	20p12.1	16	19	Deletion	ESF1, C20orf7, SEL1L2, MACROD2
20	20p11.21	12	16	Deletion	NXT1

We confirmed our SNP array data by quantitative PCR. Several genes from altered regions were randomly selected for validation study. These genes include *FAT3* (chromosome 11q), *BTG3* (chromosome 21q), *ALKBH8* (chromosome 11q), *RASSF8* (Chromosome 12p) and *EDNRB1* (chromosome 13q). Frequency of deletion of *FAT3* is more by QPCR as compared to SNP array analysis, most likely due to it being less specific than the array which incorporates more than one primer set for each SNP. Loss of *FAT3* expression in lung adenocarcinomas has been reported previously [43]. Amplifications in copies of the *RASSF8* locus (>3) were seen in 31% of the samples, making this alterations less consistent with SNP array. Previous studies reported contradictory results on *RASSF8*, one study found higher amount of *RASSF8* RNA in plasma and cell-bound fraction of patients with breast cancer compared with patients with benign tumors and healthy controls [44] while another group reported that *RASSF8* gene transcript levels were approximately seven-fold-lower in lung adenocarcinomas as compared to normal lung tissue [45]. To understand clearly the relationship between amplified and deleted areas found by our analysis and function of relevant genes in SE further studies using immunohistochemistry and/or RT-PCR need to be performed. Discrepancies of copy number variations between SNP analysis and QPCR may have many reasons; the most likely explanations are software noise of both assays and different threshold

levels for cutoff. To understand the exact consistency between SNP and QPCR analysis, other methods like FISH are required.

FAT3 is the human homolog of a tumor suppressor gene in *Drosophila*. It has been suggested that *FAT3* protein plays an important role in axon fasciculation and modulation of the extracellular space surrounding axons during embryonic development [46]. The protein encoded by *EDNRB* is a G protein-coupled receptor which activates a phosphatidylinositol–calcium second messenger system, and this gene has been shown by our group to be epigenetically altered in oral cancer [47]. *ALKBH8* is a DNA repair molecule and has been correlated with bladder cancer progression [48]. *RASSF8* is reported as a lung cancer tumor suppressor gene that regulates cell–cell adhesion and actin cytoskeleton organization, inhibiting cell growth, playing a role in the regulation of Wnt and NF-KB signaling pathways [49].

It is very important to elucidate the biological relevance of newly identified potential TSGs and oncogenes in the pathogenesis of SEs. However, a major limitation for studies of SEs is the lack of relevant cell lines. To date there is only one cell line (TCam-2) that has been characterized to be SEs [50]. There are no available normal cell lines that represent the tissues from where SEs developed, however there is a mixed normal cell line called Hs.1.Tes. We performed RT-PCR experiments to determine if the normal mixed cell line showed any differences in expression compared to the one SE cell line (TCam-2). We were unable to see any obvious mRNA expression differences of our amplified or deleted genes in the TCam-2 and available NE cell lines (data not shown), thus making functional studies not possible at this moment. Interestingly, a recent study in TGCT comparing genomic alterations and expression profiling found that a number of regions of copy number changes did not always correlate with expression [40].

Genomic instability has been correlated with progression in SEs [51]. Our data suggest that CN deletions in chromosomes 4, 5, 9, 13, and 20 and amplifications of chromosomes 9 and 13 correlate with advanced stage of disease. Few studies have identified the underlying genes involved in the pathogenesis of testicular cancer and identifying the key genes critical for the pathogenesis of SE will be explored within a larger sample group. Gain of chromosome 12p is suggested to be a crucial event for the development of invasive disease. Genes on chromosome 12 that have been suggested to be important for the pathogenesis of TGCT include *SOX5*, *JAW1* and *K-RAS*, among others [52]. We found amplifications of chromosomal regions 12p12.1 harboring *RASSF8*. 28% of our samples had amplifications of this region. Interestingly, regions 12p11.2 through 12p12.1 were previously identified as an amplified region supporting our findings [51].

LOH and HDs provide valuable information for identifying TSGs. We found 30 HDs in the only stage III sample (SE23), those have not been previously shown to play a role in the initiation of SE development. HD of tumor suppressor *DCC* on chromosomal region 18q in SE has also been suggested to be associated with TGCT progression [53]. The candidate tumor suppressors identified here

Table 6
Genomic location and associated genes of detectable loss of heterozygosity (LOH) regions in seminoma samples (probability threshold of 0.20).

Cytoband	Start	End	Size (in Kb)	No. of SNP in LOH area	N (%)	Gene	Sample IDS
2p15	63519189	63779097	259.908	11	6/23(26)	LOC51057,MDH1	15,21,2,5,6,8
2q21.3	135735952	135961986	226.034	11	5/23(22)	RABGAP1, ZRANB3	19,21,3,9,17
2q21.3	136647841	136744005	96.164	5	5/23(26)	CXCR4	19,21,8,9,20
2q32.2	189653052	189708407	55.355	7	5/23(26)	(COL3A1)	19,21,8,16,20
2q35	219353217	219456470	103.253	7	6/23(26)	BCSI1L, STK36,TTLL4	19,6,8,16,17,20
3p26.3	416553	511456	94.903	11	5/23(22)	CHL1	11,4,7,18,20
4q13.3	75661840	75693143	31.303	5	5/23(22)	AREG	15,21,8,16,17
5q21.3	109207920	10925834	50.714	5	5/23(22)	MAN2A1	1,21,6,16,18
8q11.21	50816259	50990529	174.27	20	7/23(30)	SNTG1	11,19,21,23,2,16,20
11q24.1	122255254	122305361	50.107	5	5/23(22)	C11orf63, LOC79864	10,3,8,16,18
13q12.3	28134226	28185843	51.617	12	6/23(26)	POMP, SLC46A3	15,21,8,16,17,18
18q12.2	31481579	31580034	98.455	8	5/23(22)	GALNT1	1,22,6,9,16

include *UNC5C, CSMD1,* and *IMP8.* The loss of UNC5C expression has been observed in human colorectal cancer and is suggested to be a selective advantage for tumor progression [54]. *CSMD1* is a candidate TSG in breast, upper aerodigestive tract, prostate, ovary and bladder cancer [55,56]. Further studies including sufficient number of stage III tumors are essential to select candidate HD regions and to study the biological function of related genes harboring in these area.

A major limitation in our study is the only one stage III tumor that was available to us; therefore we could not determine whether the homozygous deletions we found were representative of advanced stage. However, our results provide important insight of chromosomal instability relative to tumor progression. MiRNAs are often known to exist in fragile genomic regions. Notably, one of our identified candidates, IMP8 (chromosome 12) is required for binding of Ago proteins to a variety of mRNA targets and that depletion of Imp8 interferes with miRNA-guided gene silencing [57]. Other HDs are likely to point to the location of as yet unknown and probably uncharacterized SE TSGs. Oncogenes can be identified by focal amplifications and we detected 43 focal amplifications events in eight of our samples. However, due to our limited sample size, we were unable to detect common focal amplification. Analysis of additional samples will further justify these focal amplifications and functional studies of the genes located in these focal amplifications may be the fertile avenue to understand the biology of SEs.

SEs are radio and chemo-sensitive tumors and mostly curable at each stage [25]. This sensitivity makes this tumor type an excellent model for studying the mechanism of chemotherapy drug resistance [58]. Relapse poses a major challenge in treatment of different kinds of cancer, mainly due to the lack of response to cisplatin-based chemotherapy. The molecular mechanisms underlying this drug resistance have not been fully elucidated but extensively studied. Some genetic aberrations have been suggested as a potential pathway to drug resistance. It has been reported that chromosomal amplifications of regions 1q, 2p, 7q, 9q, 15q, 16q and 20q are associated with cisplatin resistance in TGCT [59,60]. Interestingly, most of the novel alterations in our study were found on chromosome 2q which encode many genes involved in drug transport and DNA excision repair. The drug transporter ATP dependent gene (*ABCB6*) is located in genomic regions 2q35–36 which we report to have a SE specific amplification. This gene has been reported to have copy number gains that were associated with cisplatin drug resistance in A549 lung cancer cells [61]. Another gene of interest is the *ERCC3,* a DNA repair gene, which we found to have copy number gains. Gene expression profiling has linked TGCT resistance to cisplatin and high level expression of DNA repair genes including *ERCC1, XPA, ERCC2* and *ERCC3* [60,62]. Correlation with response to cisplatin was not possible given the fact that only one patient in our sample set was treated after surgical removal of the primary tumor with the agent (SE14), therefore, we will have to monitor this patient for longer periods of time to determine if relapse occurs and what the consequence will be. In addition, many ovarian carcinomas are resistant to cisplatin or become resistant after recurrence. We will explore the potential of identifying key genes that could be correlated with sensitivity or resistance, by the comparison of SNP array data from testicular and ovarian cancers. Although these are different tissue types and have differential expressions of many genes due to tissue specificity, we hypothesize that there is a key common component that determines the ability of the cancer cell to survive in the presence of toxic chemicals.

In summary, our study provides new insight into the genetic alterations associated with SE. We found several novel copy number amplifications and deletions of chromosome 2. We also identified several chromosomes associated with progression of SE including deletions of chromosomes 4, 5, 9, 13, and 20 and amplifications of chromosomes 9 and 13. In addition, we discovered chromosomal regions of LOH which could be critical for the development of SE;

however these findings need further analysis and functional characterization of any of the newly identified potential TSGs and oncogenes need to be performed in future studies. 30 homozygous deletions were found in our stage III sample and may contain potential important TSG; however a combined functional study including gene re-expression and immunohistochemistry remains to be performed.

Here, we used high-density whole-genome SNP arrays (Affymetrix 250 K *Nsp*1 array), with an average inter-SNP distance of 5.8 kb to define a comprehensive allelotype of SE based on LOH and copy number changes. We identified several novel areas of amplification and deletion. It has been suggested that these areas of amplification and deletion may harbor relevant TSGs and oncogenes, respectively. Further study will elucidate the relevance of these gains and losses with different clinical parameters including drug resistance and overall outcome of SE patients.

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