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Exome-wide Sequencing Shows Low Mutation Rates and Identifies Novel Mutated Genes in Seminomas

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Abstract

Background: Testicular germ cell tumors are the most common cancer diagnosed in young men, and seminomas are the most common type of these cancers. There have been no exome-wide examinations of genes mutated in seminomas or of overall rates of nonsilent somatic mutations in these tumors.
Objective: The objective was to analyze somatic mutations in seminomas to determine which genes are affected and to determine rates of nonsilent mutations.
Design, setting, and participants: Eight seminomas and matched normal samples were surgically obtained from eight patients.
Intervention: DNA was extracted from tissue samples and exome sequenced on massively parallel Illumina DNA sequencers. Single-nucleotide polymorphism chip-based copy number analysis was also performed to assess copy number alterations.
Outcome measurements and statistical analysis: The DNA sequencing read data were analyzed to detect somatic mutations including single-nucleotide substitutions and short insertions and deletions. The detected mutations were validated by independent sequencing and further checked for subclonality.
Results and limitations: The rate of nonsynonymous somatic mutations averaged 0.31 mutations/Mb. We detected nonsilent somatic mutations in 96 genes that were not previously known to be mutated in seminomas, of which some may be driver mutations. Many of the mutations appear to have been present in subclonal populations. In addition, two genes, *KIT* and *KRAS*, were affected in two tumors each with mutations that were previously observed in other cancers and are presumably oncogenic.
Conclusions: Our study, the first report on exome sequencing of seminomas, detected somatic mutations in 96 new genes, several of which may be targetable drivers. Furthermore, our results show that seminoma mutation rates are five times higher than previously thought, but are nevertheless low compared to other common cancers. Similar low rates are seen in other cancers that also have excellent rates of remission achieved with chemotherapy.

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Patient summary: We examined the DNA sequences of seminomas, the most common type of testicular germ cell cancer. Our study identified 96 new genes in which mutations occurred during seminoma development, some of which might contribute to cancer development or progression. The study also showed that the rates of DNA mutations during seminoma development are higher than previously thought, but still lower than for other common solid-organ cancers. Such low rates are also observed among other cancers that, like seminomas, show excellent rates of disease remission after chemotherapy.

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1. Introduction

Testicular germ cell tumors (TGCTs) are the most common cancer in young men and occur predominantly in Western populations. Striking characteristics of TGCTs are their early stem-cell origins [1] and extreme sensitivity to cisplatin-based chemotherapy or radiotherapy, which renders them highly curable [2,3]. However, rates of TGCT incidence have increased in most populations studied in the last several decades [4]. This strongly suggests that environmental factors contribute to TGCTs [5]. Nevertheless, their etiology is still poorly understood. It has been hypothesized that the mutation rates of tumor types correlate with resistance to treatment, and thus that the sensitivity of seminomas to treatment is indicative of low mutation rates [6]. However, little is known about the roles of somatic mutations in the development of TGCTs in general and seminomas in particular. In this study we investigated previously undescribed genetic events that may drive the development of seminomas.

Seminomas are typically approximately triploid and almost all have amplifications involving chromosome arm 12p [7]. Although functional studies have not definitively identified the driver gene or genes on 12p [8], *KRAS* is likely to be one such gene, as it is a well-established oncogene located in a minimum overlapping amplification region, and it sometimes undergoes activating point mutations in TGCTs [9]. Furthermore, cultured seminoma cells with codon-12 *KRAS* mutations exhibited suppressed apoptosis and enhanced survival [10].

The largest study of point mutations in TGCTs to date examined 518 kinases in 13 tumors. This study found one mutation (in the *STK10* gene) in a total of 17.7 Mb examined across all 13 tumors combined, corresponding to a rate of 0.06 mutations/Mb (95% confidence interval [CI] 0.003–0.37) [11].

Other than *KRAS*, the only oncogene known to recurrently undergo somatic point mutations in seminomas is *KIT*, which is often mutated, amplified, and overexpressed [12]. Somatic activating *KIT* mutations occur in ~16% of seminomas (95% CI 12–22%) [13]. Indeed, mutations occur more often in *KIT* than in *KRAS* [10,14]. In addition, the presence of *KIT* mutations and amplifications in precursor lesions suggests that *KIT* is a key contributor to tumor initiation [15].

In summary, little is known about specific driver genes mutated in seminomas or the overall rate of somatic point mutations in these tumors. To investigate these questions, we undertook exome sequencing and analysis of copy number alterations in eight seminomas and matched normal DNA.

2. Materials and methods

2.1. Patient samples

Patient samples and clinical information (Supplementary Table 1) were obtained from patients who had surgery for testicular cancer at the Spectrum Health Hospital, Grand Rapids, MI, USA. Informed consent was obtained from all subjects, and the study was approved by the Institutional Review Boards of the Van Andel Research Institute, Grand Rapids, MI, USA (Protocol #011228BT), Spectrum Health, Grand Rapids, MI, USA (IRB# 2002-087), and the National University of Singapore (NUS-IRB Reference Code 11-192E).

2.2. Single-nucleotide polymorphism microarrays and ASCAT analysis

The Supplementary methods provide details regarding the use of ASCAT (http://heim.ifi.uio.no/bioinf/Projects/ASCAT/) to simultaneously estimate the proportion of malignant cells present in a tumor and the integer copy numbers of chromosomes in the tumor genomes. ASCAT estimated that seven of the eight tumors were approximately triploid, which is consistent with previous studies of seminoma karyotypes and therefore lends confidence to the ASCAT analyses.

2.3. Subclonality analysis

We used two approaches to assess whether a somatic mutation was likely to be subclonal, that is, not present in all the malignant cells of a tumor. In the first approach, we performed a statistical test to check if the proportions of reads with mutations were significantly lower than expected based on the conservative model that the somatic mutation was present on only one chromosome (using prop.test in R, Supplementary Tables 2 and 3). To determine the expected proportion of reads with the mutation, we used ASCAT estimates of the chromosome copy number at the mutated site and of the proportion of malignant cells in the tumor. In the second approach we used the ABSOLUTE software [16] to determine the probability that each mutation was clonal (Fig. 1, Supplementary Fig. 1). We considered that a variant was subclonal if this probability was <0.5.

3. Results

We sequenced the exomes of eight nonmetastatic seminomas to an average mapped depth of 318 reads in targeted

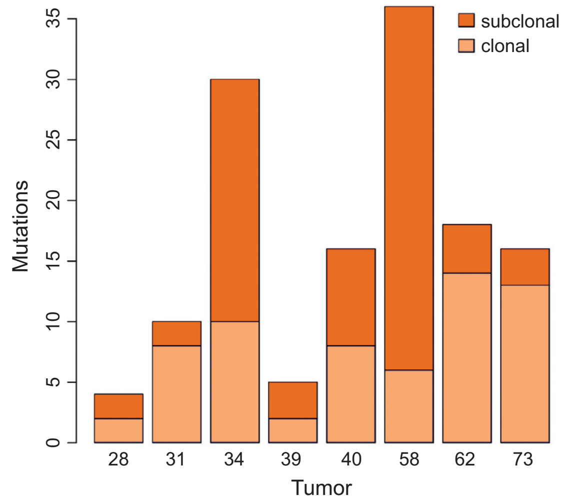

Fig. 1 – Clonal composition of somatic mutations in each sample. Each bar indicates the number of clonal and subclonal mutations for one tumor as inferred using ABSOLUTE.

regions; we sequenced the exomes of matched nonmalignant samples to an average mapped depth of 92 reads (Supplementary Table 4). On average, 98% of targeted bases were covered by more than ten reads in the tumor samples. Coverage of receptor tyrosine kinases and genes in the PI3K/AKT/mTOR and RAS/RAF/MAPK pathways was generally very good: the median read depth in tumors for each group of genes was >240 (Supplementary Fig. 2 and Supplementary Tables 5–7). After read mapping, we used three variant callers to maximize sensitivity (Supplementary methods) and visually inspected the candidate variants in Integrative Genomics Viewer (http://www.broadinstitute.org/igv/). Nonsilent variants that passed visual inspection were validated by Sanger sequencing or sequencing on an Ion Torrent Personal Genome Machine (Supplementary methods and Supplementary Table 8). Overall validation rates were 83.2% for single-nucleotide substitution (SNS) mutations and 42.1% for microindels (small insertions or deletions).

Supplementary Table 2 lists the validated somatic nonsilent variants found in the eight tumors, together with their characteristics.

The eight tumors bore a total of 90 somatic nonsilent SNSs, two double substitutions, eight coding microindels, and 35 silent mutations. On average, there were 12.5 somatic nonsilent mutations per tumor (median 11.5, range 2–27, Table 1 and Supplementary Table 2) and four somatic silent mutations per tumor (median 3, range 1–13, Table 1 and Supplementary Table 3). The rate of nonsynonymous mutations in the exome averaged 0.31 mutations/Mb (median 0.3, range 0.05–0.69). There was a weak association between patient age and the number of mutations (Pearson correlation coefficient 0.71, $p = 0.047$), although this was not significant after considering multiple hypothesis testing.

In the tumors we studied, only the oncogenes *KIT* and *KRAS* [10,12,14,17] were affected by nonsilent mutations in more than one tumor. *KRAS* harbored the G12V mutation and was amplified in two tumors. In one of these, *KRAS* was present in ten copies, of which we estimate that one bore the mutation (Supplementary Table 9). In the other tumor, *KRAS* was present in six copies, of which we estimate that two bore the mutation (Supplementary Table 9).

KIT harbored the mutations N822K and L576P in one tumor each, and each mutation has been reported previously in various types of tumor. N822K was reported previously in seminomas [11,15,17], melanomas, and gastrointestinal stromal tumors, and L576P was reported in TGCTs, melanomas, gastrointestinal stromal tumors, and breast and thymic carcinomas (http://cancer.sanger.ac.uk/cosmic). In the seminoma we studied, the L576P mutation was in a highly amplified region (more than eight copies) that was also subject to loss of heterozygosity. We estimate that all eight copies bore the mutation (Supplementary Table 9). Notably, this tumor also had the lowest level of 12p amplification (only 1.44 times the average ploidy).

More than half of the somatic mutations detected were present in very low proportions of the reads (<15%, Supplementary Tables 2 and 3). Two independent analyses indicated that many of these mutations were present in only a subset of the malignant cells of the tumors. The first

Table 1 – Summary of somatic mutations in each tumor (capture target size 37.81 Mb)

Tumor	NSYN SNVs	Splice-site SNVs	Micro-indels	NSYN mutations		Nonsilent mutations	Silent mutations		Total somatic mutations	
	(*n*)	(*n*)	(*n*)	(*n*)	(*n*/Mb)	(*n*)	(*n*)	(*n*/Mb)	(*n*)	(*n*/Mb)
28	2	0	0	2	0.053	2	2	0.053	4	0.106
31	7	1	0	7	0.185	8	2	0.053	10	0.264
34	20	1	6	26	0.688	27	3	0.079	30	0.793
39	3	0	1	4	0.106	4	1	0.026	5	0.132
40	12	0	1	13	0.344	13	3	0.079	16	0.423
58	20	3	0	20	0.529	23	13	0.344	36	0.952
62	13	0	0	13	0.344	13	5	0.132	18	0.476
73	10	0	0	10	0.264	10	6	0.159	16	0.423
Average	10.88	0.63	1	11.88	0.314	12.5	4.38	0.116	16.88	0.446
Median	11	0	0	11.5	0.304	11.5	3	0.079	16	0.423

NSYN = nonsynonymous; SNVs = single-nucleotide variations.

analysis compared the actual read counts of each mutation to the minimum that would be expected based on (1) the proportion of malignant cells in the tumor sample and (2) the chromosomal copy number at the mutation site, as described in Section 2. The second analysis used the ABSOLUTE software [16], which has also been used in several recent studies of subclonality [18,19]. ABSOLUTE detects subclonal heterogeneity based on the proportions of reads bearing somatic mutations and a sophisticated model that simultaneously estimates the proportion of malignant cells in the sample and chromosomal copy numbers across the genome. The model also takes into consideration sampling variation with respect to the true proportion of mutation-bearing reads. Given the more complete model of ABSOLUTE, we expected it to be more sensitive. Indeed, ABSOLUTE identified 72 mutations as subclonal, while the first method identified 46 (Fig. 1, Supplementary Tables 2 and 3, Supplementary Fig. 1). However, out of 46 somatic mutations estimated to be subclonal by the first method, 40 were also estimated to be subclonal by ABSOLUTE (Supplementary Tables 2 and 3). Thus, both methods support the conclusion that there were a substantial proportion of subclonal somatic mutations.

Besides *KIT* and *KRAS*, our genomic analysis revealed several genes with plausible driver roles in this tumor type. For example, the *CHD1* gene, which encodes a chromodomain helicase DNA-binding protein, was associated with both frameshift mutations and genomic deletions in our cohort (Supplementary Table 2), consistent with a tumor suppressor role. Interestingly, tumor-suppressive roles for *CHD1* have also been reported in prostate cancer, another male-organ–specific malignancy [20,21]. Another gene exhibiting a dual pattern of mutation and genomic loss was *MCC*, which has been implicated in tumor-suppressive roles in colorectal cancer [22]. Experimental work has shown that *MCC* can inhibit several cancer phenotypes, including cell cycle progression and oncogenic Wnt signaling [23,24]. A third gene exhibiting mutation/genomic loss was *SMARCA5*, a member of the SWI/SNF chromatin remodeling complex. Mutations in SWI/SNF complex members have been repeatedly observed in multiple tumor types [25,26], and such mutations may influence somatic patterns in gene expression and chromosomal instability in tumors. Supplementary Table 10 provides a more extensive list of nonsilent somatic mutations with potential driver functions. The mutations in some of these potential drivers appear to be subclonal; this has also been observed in other tumor types [18].

Several of the mutated potential driver genes may be therapeutically targetable. The products of two mutated genes, *CSNK2A* and *PIK3R2*, have functions in important enzymatic complexes (casein kinase 2 and class I phosphoinositol-3-kinases, respectively) that have attracted substantial interest as potential drug targets in cancer (Supplementary Table 10). In addition, *CDH17*, which was clonally mutated in one seminoma, has been implicated in gastric cancer progression [27] and may represent a potential therapeutic target, as *CDH17* knockdown in mice inhibited tumor growth [28]. Another potential target is *SETDB1*, a histone methyltransferase that may act as an oncogene in multiple tumor types [29,30] and that is downregulated by the histone methylation inhibitor DZNep [31].

Because seminomas arise from germ cells, three mutated genes are of interest because, to the best of our knowledge, their functions are germ-cell–specific. *GTF2A1L* encodes a component of a germ-cell–specific general transcription factor [32]. *DZIP3* encodes an E3 ubiquitin ligase with an RNA-binding domain and interacts with the DAZ protein, which is essential for normal spermatogenesis [33]. Finally, *SPATS1* (also known as *SRSP1*) is a serine-rich gene of largely unknown function with a rat homolog that is expressed during spermatogenesis [34].

Single-nucleotide polymorphism microarray data showed that the seminomas studied were typical with respect to copy-number alterations (Fig. 2, Supplementary Fig. 3). Seven of the eight tumors studied were approximately triploid, and all had amplifications involving 12p. As expected from previous studies, the region containing *KRAS*

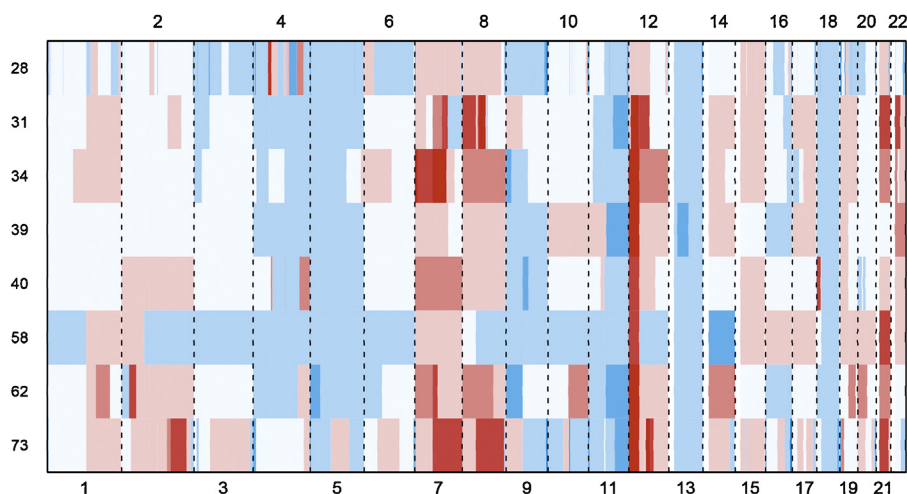

Fig. 2 – Summary of copy number alterations in the eight seminomas. The heat map shows the copy number values across the chromosomes for each tumor. White represents regions with copy number equal to the average ploidy of the tumor; red represents copy-number gain and blue represents copy-number loss.

was included in all 12p amplifications, and *KRAS* was highly amplified (copy number >2.5 times the average ploidy) in five out of the eight tumors. As mentioned above, two of the tumors with *KRAS* amplifications also had somatic mutations in this gene (Supplementary Table 2). Other large chromosomal aberrations found in two or more seminomas included copy-number gains involving 2q, 7, 8, 12q, 21q, 22q, and X (Fig. 2, Supplementary Table 11, Supplementary Fig. 3) and copy-number losses involving 3p, 4, 5, 9, 11q, 13q, 16q, and 18 (Fig. 2, Supplementary Table 12, Supplementary Fig. 3). Copy-number alterations involving these regions were detected previously in other cancer types (Supplementary Table 13). Regions of copy-number gain harbored eight nonsilent mutations (including those in *KIT* and *KRAS*), and regions of copy-number loss harbored ten nonsilent mutations (Supplementary Table 2). In addition, five mutations were found in regions with loss of heterozygosity, including one in *KIT* in a highly amplified region, as discussed above (Supplementary Tables 2 and 9).

4. Discussion

This study enlarges the list of genes known to be mutated in seminomas. In total, 98 distinct genes were affected by nonsilent mutations, and only two of these genes, *KIT* and *KRAS*, were previously implicated in TGCTs [10,12,14,17]. Thus, we detected nonsilent somatic mutations in 96 genes that were not previously known to be mutated in seminomas. As detailed above, the known functions of affected genes suggest that some of these mutations could be drivers.

The exome sequencing reported here also provides the first comprehensive estimate of the somatic mutation rates in seminomas. While higher than previous estimates based on very limited data [11], the mutation rates in seminomas are nevertheless much lower than the rates observed in many other cancers (Fig. 3) [35].

The low numbers of mutations in seminomas might be related to their sensitivity to therapy [6]. Most advanced-stage cancers are incurable, even with aggressive chemotherapy; although chemotherapy can lead to initial disease regression, the cancer invariably becomes resistant to drug treatment, and patients ultimately succumb to the disease. Seminomas are unique among solid-organ cancers in that even patients with widely metastatic or advanced-stage tumors usually achieve a complete clinical response and long-term remission after several cycles of combination chemotherapy [2,3]. Several other cancers with low mutation rates share this characteristic, even in advanced-stage disease. These include pediatric acute myeloid leukemia, pediatric medulloblastoma, and pediatric acute lymphoblastic leukemia (Fig. 3) [35–38].

If the sensitivity of seminomas to therapy is indeed related to their low numbers of somatic mutations, this connection might be explained by the theory that with a low number of somatic mutations, seminomas have little chance of harboring pre-existing clones with drug resistance mutations. By contrast, the low number of mutations may simply reflect the relatively young ages of the patients,

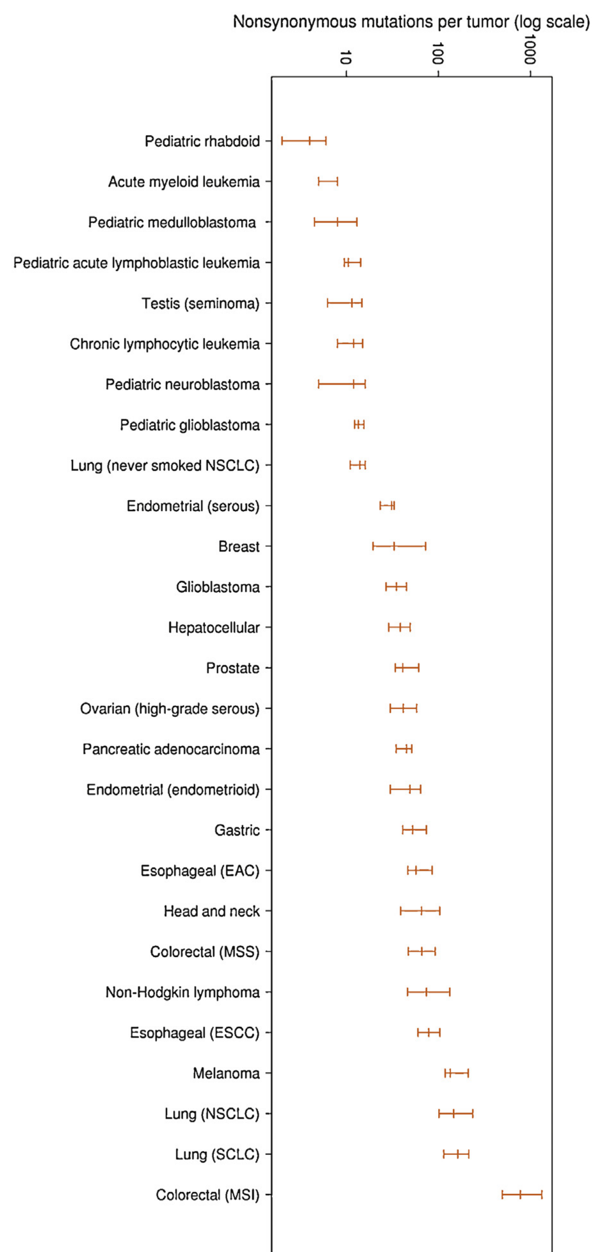

Fig. 3 – Comparison of the number of nonsynonymous mutations per tumor in seminomas and blood, pediatric, and adult solid tumors. Lower and upper horizontal bars indicate the first and third quartiles; middle bars indicate the median. The numbers of mutations in nonseminoma cancers are from Supplementary Table S1C of reference [35]. NSCLC = non–small-cell lung cancer; EAC = esophageal adenocarcinoma; MSS = microsatellite stable;ESCC = esophageal squamous-cell carcinoma; SCLC = small-cell lung cancer; MSI = microsatellite instability.

especially considering that the cells of origin are probably quiescent during childhood. The low numbers of mutations might also be a consequence of the stem-cell origins of seminomas, which might require fewer aberrations to develop an oncogenic or even metastatic phenotype. Although not investigated here, it is possible that relatively few genetic changes are required for metastasis of

seminomas, a possibility supported by the similarity of transcript profiles in primary and metastatic seminomas [39]. This may explain the vulnerability of even metastatic seminomas to therapy.

5. Conclusions

Our results, the first reported for exome sequencing of seminomas, identified 96 new genes harboring somatic mutations in seminomas; the mutations and known functions of some of these genes suggest that they could be drivers, of which several are potential drug targets. This study also provides the first comprehensive estimate of somatic mutation rates in seminomas. These rates are five times higher than previously estimated from very limited data, but are nevertheless low than those for other common solid-organ cancers. The low rates seen in seminomas are also observed among other cancers that share excellent rates of disease remission achieved with chemotherapy.

Microarray and read data are deposited at the European Genome-phenome Archive (http://www.ebi.ac.uk/ega, accession number EGAS00001000943).

Author contributions: Steven G. Rozen had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Drafting of the manuscript: Cutcutache, Suzuki, I.B. Tan, P. Tan, Teh, Rozen.
Critical revision of the manuscript for important intellectual content: Cutcutache, P. Tan, Teh, Rozen.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eururo.2014.12.040.

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