Runs of Homozygosity and Testicular Cancer Risk

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Abstract

Background

Testicular germ cell tumour (TGCT) is highly heritable but >50% of the genetic risk remains unexplained. Epidemiological observation of greater relative risk to brothers of men with TGCT

compared to sons has long alluded to recessively acting TGCT genetic susceptibility factors, but to date none have been reported. Runs of homozygosity (RoH) are a signature indicating underlying recessively acting alleles and have been associated with increased risk of other cancer types.

Objective

To examine if RoH are associated with TGCT risk.

Methods

We performed a genome-wide RoH analysis using GWAS data from 3,206 TGCT cases and 7,422 controls uniformly genotyped using the OncoArray platform.

Results

Global measures of homozygosity were not significantly different between cases and controls, and the frequency of individual consensus RoH were not significantly different between cases and controls, after correction for multiple testing. RoH at three regions, 11p13-11p14.3, 5q14.1-5q22.3 and 13q14.11-13q.14.13, were however nominally statistically significant at $P < 0.01$. Intriguingly, RoH200 at 11p13-11p14.3 encompasses *Wilms tumor 1* (*WT1*), a recognized cancer susceptibility gene with roles in sex determination and developmental transcriptional regulation, processes repeatedly implicated in TGCT etiology.

Discussion and Conclusion

Overall, our data does not support a major role in the risk of TGCT for recessively acting alleles acting through homozygosity, as measured by RoH in outbred populations of cases and controls.

Introduction

Testicular germ cell tumor (TGCT) is the most common cancer in young men with over 52,000 new cases diagnosed annually worldwide (Le Cornet et al. 2014). TGCT has a strong heritable basis, as evidenced by the 4 to 8-fold increased risk of TGCT seen in first-degree relatives of TGCT patients (Litchfield, Thomsen, et al. 2015; Hemminki and Li 2004; Swerdlow et al. 1997; McGlynn et al. 2005;

Kharazmi et al. 2015). Statistical analyses of heriability estimate that genetic factors may contribute to approximately half of all TGCT disease risk (Litchfield, Thomsen, et al. 2015).

Early linkage analyses in familial TGCT did not support existence of a major Mendelian TGCT susceptibility locus, but these studies were limited in power on account of modest sample sizes and the low prevalence of multiplex TGCT pedigrees (Crockford et al. 2006; Rapley et al. 2003; Rapley et al. 2000). More recently large-scale exome sequencing studies have also failed to identify rare high-penetrance susceptibility alleles, despite improved power compared to previous linkage analyses (Litchfield, Loveday et al. 2018). Nevertheless, neither analysis excludes the possibility that susceptibility genes/alleles for TGCT of lower frequency and/or more moderate effect size may exist. Indeed, very rare alleles in ciliary microtubule genes have recently been implicated through functional analyses in TGCT susceptibility in a minority of familial cases (Litchfield, Levy, Dudakia, et al. 2016).

However, collectively findings are consistent with advanced analyses of TGCT heritability, which have indicated that the genetic component of TGCT heritability is largely constituted by common variants. Recent genome-wide association studies (GWAS) have made substantial progress in exposition of this partition of heritability with 49 independent TGCT risk loci identified, together accounting for ~37% of the excess genetic risk of disease (Loveday et al. 2018; Wang et al. 2017; Litchfield et al. 2017; Litchfield, Levy, Orlando, et al. 2016; Litchfield, Shipley, and Turnbull 2015; Litchfield, Sultana, et al. 2015; Litchfield, Holroyd, et al. 2015; Koster et al. 2014; Ruark et al. 2013; Turnbull et al. 2010; Rapley et al. 2009; Kanetsky et al. 2009; Kristiansen et al. 2015; Schumacher et al. 2013; Turnbull and Rahman 2011). These TGCT susceptibility loci have provided invaluable insight into the biology of TGCT susceptibility, implicating as underlying mechanisms, widespread transcriptional dysregulation linked to developmental arrest of primordial germ cells, aberrant KIT-MAPK signaling and defective microtubule function (Litchfield et al. 2017). From these GWAS loci approximately half of the genetic

component of TGCT heritability has been accounted for, with heritability analysis indicating that the outstanding 'missing heritability' of TGCT is likely polygenic, with substantial contribution from common variation (Litchfield et al. 2017; Litchfield, Mitchell, et al. 2016; Litchfield, Thomsen, et al. 2015).

GWAS analysis has likewise made substantial impact in delineating the genetic architecture of many other common cancers but almost uniformly the reported susceptibility loci have been identified through analyses based on a log additive (multiplicative) model of inheritance, with little evidence generated for alleles acting recessively (Sud, Kinnersley, and Houlston 2017). This observation may be a reflection that GWAS is suboptimal in its ability to detect these alleles rather than an observation truly reflective of the underlying biology. In principle, it is entirely plausible that there may be an association between recessively acting disease alleles and susceptibility to cancer. Such a hypothesis is supported by observations reporting an increased burden of cancer in the offspring of consanguineous unions and in populations with a high degree of inbreeding (Bener et al. 2009; Lebel and Gallagher 1989; Shami, Qaisar, and Bittles 1991; Simpson et al. 1981; Assie et al. 2008). Furthermore, experimental inbreeding (e.g. backcrossing mice) has also been shown to increase tumor burden in mice (Demant 2003). In addition, uniparental disomy through dysregulated imprinting is a specific situation in which homozygosity can be directly associated with cancer (Henry et al. 1991). Of note, for TGCT, there has been a long-standing hypothesis that recessive (or X-linked) susceptibility factors are highly likely to be important, based on epidemiological data that siblings' relative disease risks are higher than parent-offspring risks (Hemminki and Li 2004; Kharazmi et al. 2015).

Homozygosity mapping provides a means of identifying recessive components of inheritance. It has been demonstrated that, on account of selective pressure, runs of homozygosity (RoH) occur at high frequency in outbred populations, the result of autozygosity (i.e. the co-location of two alleles at a

given locus originating from a common ancestor by way of non-random mating) (McQuillan et al. 2008; Ku et al. 2011). These RoH can be enriched for rare deleterious variants in homozygous form(Szpiech et al. 2013); multiple susceptibility loci have been reported for different diseases, identified through genome-wide analyses for RoH of SNP array data (reviewed in [(Ceballos et al. 2018)]).

Here, we sought to identify associations between homozygosity and TGCT risk through the characterization and comparison of genome-wide homozygosity measures and specific loci identified through consensus mapping of recurrent RoH in 3,206 TGCT cases vs 7,422 controls directly genotyped for 371,504 SNPs.

Methods

Sample description

TGCT cases (n=3,206) were ascertained via two UK studies: (1) a UK study of familial testicular cancer and (2) a systematic collection of UK TGCT cases. Case recruitment was via the UK Testicular Cancer Collaboration, a group of oncologists and surgeons treating TGCT in the UK. The studies were coordinated at the Institute of Cancer Research (ICR). Samples and information were obtained with full informed consent and Medical Research and Ethics Committee approval (MREC02/06/66 and 06/MRE06/41). All experiments were performed in accordance with relevant guidelines and regulations.

Control samples for the primary GWAS were all taken from within the UK. Specifically 2,976 cancer-free, male controls were recruited through two studies within the PRACTICAL Consortium: (1) the UK Genetic Prostate Cancer Study (UKGPCS) (age <65), a study conducted through the Royal Marsden NHS Foundation Trust and (2) SEARCH (Study of Epidemiology & Risk Factors in Cancer), recruited via

GP practices in East Anglia (2003-2009). 4,446 cancer-free female controls from across the UK were recruited via the Breast Cancer Association Consortium (BCAC).

GWAS

Genotyping was conducted using a custom Infinium OncoArray-500K BeadChip (OncoArray) from Illumina (Illumina, San Diego, CA, USA), comprising a 250K SNP genome-wide backbone and 250K SNP custom content selected across multiple consortia within COGS (Collaborative Oncological Gene-environment Study). OncoArray genotyping was conducted in accordance with the manufacturer's recommendations by the Edinburgh Clinical Research Facility, Wellcome Trust CRF, Western General Hospital, Edinburgh EH4 2XU.

The UK TGCT OncoArray dataset was filtered as follows: we excluded individuals with low call rate (<95%), with abnormal autosomal heterozygosity (>3 SD above the mean) or with >10% non-European ancestry (based on multi-dimensional scaling); we excluded SNPs with minor allele frequency <1%, a call rate of <95% in cases or controls or with a minor allele frequency of 1–5% and a call rate of <99%; and those deviating from Hardy-Weinberg equilibrium ($P > 10^{-12}$ in controls and 10^{-5} in cases). The final number of SNPs passing quality control filters was 371,504.

Bioinformatic and statistical analysis

Bioinformatic and statistical analyses were performed as previously described (Sud et al. 2015). Briefly, we detected RoH using PLINK v1.90 (Purcell et al. 2007), which moves a sliding window of SNPs across the entire genome. To allow for genotyping error or other sources of artificial heterozygosity (such as paralogous sequences) within a stretch of truly homozygous SNPs, 2% heterozygous SNPs were allowed in each window. This measure was implemented to prevent underestimation of the number and size of RoH. Default parameter values were employed (including allowing 5 missing calls per window), with the exception that we varied the parameter homozyg-snp

according to our heuristic preferences for defining the RoH as detailed below. Subsequent statistical analyses for comparison of frequencies of ROH were performed using packages available in R (version 3.4.1) with integration of results against genomic references executed using and custom written Perl code. Comparisons of global homozygosity measures between cases and controls were made using the Student t-test. Adjustment for multiple testing was based on the Bonferroni correction.

We used three metrics to investigate the selection pressure on each RoH. Integrated Haplotype Score (iHS) is based on Linkage disequilibrium (LD) surrounding a positively selected allele compared to background, providing evidence of recent positive selection at a locus (Voight et al. 2006). An iHS score >2.0 reflects that haplotypes on the ancestral background are longer compared to the derived allelic background. Episodes of selection tend to skew SNP frequencies in different directions and Tajima's D is based on the frequencies of SNPs segregating in the region of interest (Tajima 1989). Fixation index (Fst) measures the degree of population differentiation at a locus, taking values from 0 to 1.0(Holsinger and Weir 2009). iHS, D and Fst metrics were obtained from dbPSHP (Li et al. 2014).

Identification of Consensus RoH

In order to focus on more commonly occurring RoH and to empower our analysis to identify meaningful associations, only RoH in which 10 or more individuals shared the same RoH were retained for these analyses. The initial search for RoH was performed using PLINK (Purcell et al. 2007) with a specified length of 68 consecutive SNPs (homozyg-snp parameter). This RoH length was chosen (i) to be more than an order of magnitude larger than the mean haploblock size in the human genome (ii) without being so large as to be very rare. The likelihood of observing 68 consecutive chance events can be calculated as follows (Lencz et al. 2007). Mean heterozygosity in the samples was calculated to be 42%. Thus, given 371,504 SNPs and 10,628 individuals, a minimum length of 47 would be required to produce <5% randomly generated RoH across all subjects

$([1 - 0.42]^{47} \times 371{,}504 \times 10{,}628 < 0.05)$. A consequence of LD is that the SNP genotypes are not always independent, thereby inflating the probability of chance occurrences of biologically meaningless ROH. Analysis based on PLINK's pairwise LD SNP pruning function showed an approximate reduction of information compared to the original number of SNPs of 25%. Thus RoH of length 68 SNPs were used to approximate the degrees of freedom of 47 independent SNP calls.

Once all RoH of at least 68 SNPs in length were identified, these were pruned to only those RoH that occurred in more than 10 individuals. To ensure that a minimum length and minimum number of SNPs in each RoH was maintained, each individual's SNP data were recoded as one if the SNP was in an RoH for that individual and zero otherwise. Then, for each SNP, those SNPs with fewer than 10 individuals coded as one were recoded to zero before removing any ROH that due to this recoding were now less than the required number of SNPs in length. This process therefore resulted in a list of "consensus" ROH having a minimum of 68 consecutive homozygous SNP calls across 10 or more samples.

Data availability

Case GWAS data (PLINK binaries) are deposited at European Genome–phenome Archive [EGA] under accession code EGAS00001001836.

Results

We have previously implemented rigorous quality control measures to the UK TGCT OncoArray GWAS dataset[22], excluding samples and SNPs with poor call rates, SNPs with significant departure from HWE, and samples of non-European ancestry or with a sex discrepancy as inferred from the data. The final dataset included 10,628 individuals from the UK and of European ancestry, comprising 3,206 TGCT cases and 7,422 controls, all genotyped on the same platform. The final number of SNPs passing quality control filters was 371,504.

Across all samples (n=10,628), the total number of discrete autosomal RoH >1000 kb and comprising at least 68 consecutive SNPs as identified by PLINK was 137,833, with an average number of 12.97 RoH per individual, an average size of 1630.17 Kb per RoH per individual, and an average total length of the genome covered by RoH of 21,216.01 Kb per individual. These results are broadly similar to other studies using similar methodologies(Sud et al. 2015; Hosking et al. 2010; Thomsen et al. 2016; Thomsen et al. 2015). There was no significant difference in the average number, length per RoH, or total length of RoH per individual between TGCT cases and controls when compared using Student's T test (Table 1). Likewise, the cumulative distribution of RoH was broadly similar for TGCT cases and controls (Fig. 1).

Data indicate two different types of RoH shaped by different selective pressures, with the different types characterised by different run length(Pemberton et al. 2012). Small/intermediate sized RoH (<1.6 Mb) are shaped via serial migration as a result of decreasing population size, generating LD, reducing haplotype diversity and increasing chance pairing of identical haplotypes. Conversely, long RoH (>1.6 Mb) are generated through inbreeding. There was no difference in global homozygosity measures between TGCT cases and controls when performing these analyses on RoH separated into these size categories (Table 1).

We next identified a set of 319 consensus RoH (Supplementary Table S1), that is RoH that are present in at least 10 individuals. Eight of these consensus RoH had a frequency of greater than 25% across all individuals (Table 2). The vast majority of these common consensus RoH has been previously reported in other studies of RoH. For these RoH, selective pressure metrics are indicative of positive selection in Caucasian populations, and their locations are within genomic regions characterised by reduced numbers of structural variants and low recombination rates. The most frequently occurring RoH in our dataset (RoH164) has previously been identified as a site of selective

sweep in multiple studies(Voight et al. 2006; Wang et al. 2006; Williamson et al. 2007) and is frequently identified in studies of common consensus RoH. Importantly, previous reports of these RoH provide further validity of our approach.

Fig. 2 shows the correlation between the frequency of consensus RoH in TGCT cases and controls. No consensus RoH was exclusive to either group nor significantly associated with TGCT risk after correcting for multiple testing (i.e. $P < 0.0001$). Three consensus RoH demonstrated nominal associations with TGCT at a suggestive significance level ($P < 0.01$) (Table 3). Each of these regions showed highly significant values for three estimates of selective pressure (iHS_{max}, Tajimas' D_{max} , and Fay Wu's H), indicating that these regions may have been generated as the result of a selective sweep.

The RoH with the strongest evidence of association, RoH200, was identified in 5% of TGCT cases (n=148) and 3% of controls (n=243) ($P = 0.0009$; Table 3). It comprises 866 SNPs spanning 9 Mb of chromosome 11 and encompasses 52 genes/predicted transcripts, including *Wilms Tumor 1* (*WT1*), a developmental transcription factor involved in sex determination and establishment of the urogenital system, and with established oncogenic and proto-oncogenic roles in tumor formation. To further investigate a potential link between *WT1* and TGCT risk, we performed an association analysis of individual SNPs within 25 kb of WT1, considering only those with an info score > 0.8 and MAF > 0.01 (n=432). The strongest putative association was for a directly genotyped SNP, rs11031783, which maps to the non-coding *WT1 antisense RNA* (*WT1-AS*), OR = 1.18, $P = 0.0003$). This putative association warrants additional validation. Of note this region also contains two additional genes related to TGC tumorigenesis: *LGR4* and *FSHB*. LRG4 is involved in Wnt signalling and whilst variation in the FSH receptor has been implicated in TGCT susceptibility (Bang et al. 2018).

Discussion and Conclusion

In conclusion, our analyses demonstrate that levels of homozygosity are unlikely to play a substantial role in defining the risk of TGCT. Moreover, our findings suggest that existence of large numbers of recessive alleles that predispose to TGCT when unmasked by autozygosity is unlikely in outbred populations such as that of the UK. Therefore, from these analyses we are unable to provide explanation for epidemiological observation of the higher risks to siblings of cases than to other male family members. However, due to genome-wide testing and requisite correction for multiple-testing, this analysis certainly does not preclude existence of recessively acting disease alleles in TGCT risk; alternative analytic strategies will be needed to identify such alleles if they do exist. Though not statistically significant, the possible link between TGCT and an RoH hotspot that encompasses 11p.13 and *WT1* is an interesting observation that warrants further investigation.

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Authors' Contributions

C.T., C.L., and A.S. designed the study. Case samples were recruited by A.R., R.A.H. and through UKTCC. R.E., A.M.D., K.M., J.P., Z.K.-J., N.P. and D.F.E. supplied OncoArray control data, via the PRACTICAL Consortium. N.O. administrated genotyping of OncoArray case samples. D.D. coordinated all case sample administration and tracking. K.L., M.L., A.H. and P.B. prepared samples for genotyping experiments. C.T., R.S.H., A.S. and C.L. designed bioinformatics and statistical analyses. C.L., K.L. and M.L. performed bioinformatics and statistical analyses. C.L. drafted the manuscript with assistance from C.T., A.S., and R.S.H. All authors reviewed and contributed to the manuscript.

Disclosure of Interest

The authors have no conflicts of interest to disclose.

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Figure Legends

Fig. 1. Cumulative distribution of runs of homozygosity (RoH) in TGCT cases and controls. Data is presented in such a way that each data point represents the cumulative fraction (y-axis) of the samples with the corresponding minimum total length of the genome covered by RoH (x-axis), as determined from PLINK.

Fig. 2. Frequency of consensus runs of homozygosity (RoH) in TGCT cases versus controls. Consensus RoH were defined on the basis of being present in 10 or more individuals.

Tables

Table 1. Global Homozygosity Measures in TGCT Cases versus Controls

Measure	TGCT Cases (n=3206)	Controls (n=7442)	*P*
Any size			
Average number of RoH per individual	12.9	12.9	0.8
Average length per RoH per individual (Kb)	1,628.8	1,633.4	0.4
Average total length of RoH per individual (Kb)	21,346.9	21,159.5	0.2
< 1.6 Mb			
Average number of RoH per individual	8.4	8.3	0.7
Average length per RoH per individual (Kb)	1,256.5	1,255.9	0.8
Average total length of RoH per individual (Kb)	10,517.8	10,491.4	0.2
> 1.6 Mb			
Average number of RoH per individual	4.7	4.6	0.7
Average length per RoH per individual (Kb)	2,299.1	2,302.4	0.7
Average total length of RoH per individual (Kb)	10,945.2	10,781.3	0.7

RoH, runs of homozygosity. *P* calculated using Student's t-test.

Table 2. Consensus RoH with frequency > 25% in controls

RoH ID	Chr	Start (b37)	End	Length (Kb)	No. SNPs	Controls	Cases	*P*	Centromeric	iHS_{max}	Tajima D_{max}	Fst_{max}
ROH164	8	29737732	70143771	40,406.04	3286	34.6%	34.8%	0.9	Yes	3.16	4.80	0.30
ROH68	3	74113968	116444174	42,330.21	4272	34.0%	32.4%	0.1	Yes	3.27	5.02	0.25
ROH117	6	23594993	40225964	16,630.97	7636	32.4%	34.0%	0.1	No	3.17	4.80	0.27
ROH203	11	44966113	69074890	24,108.78	2174	30.6%	30.4%	0.9	Yes	3.91	4.48	0.24
ROH43	2	132755417	169516830	36,761.41	3352	27.4%	28.2%	0.4	No	3.24	4.99	0.46
ROH86	4	58139970	111262618	53,122.65	5794	26.0%	26.7%	0.5	No	3.25	5.10	0.33
ROH120	6	53377834	91107018	37,729.18	3378	25.8%	26.1%	0.8	Yes	3.31	4.67	0.26

RoH, runs of homozygosity. Chr, chromosome. iHS, D and Fst metrics were obtained from dbPSHP. *P* was calculated using Fisher's exact.

Table 3. Consensus RoH putatively associated with TGCT risk (P<0.01)

RoH ID	Chr	Start (b37)	End	Length (Kb)	No. SNPs	Controls	Cases	P	iHS_{max}	Tajima D_{max}	Fst_{max}
ROH200	11	24794324	33879547	9,085,223	866	3.3%	4.6%	0.001	4.05	4.91	0.25
ROH101	5	79191702	115939896	36,748,194	3520	22.2%	19.7%	0.005	3.46	4.62	0.31
ROH229	13	44234831	46712448	2,477,617	272	0.5%	0.1%	0.009	2.43	4.70	0.28

RoH, runs of homozygosity. Chr, chromosome. iHS, D and Fst metrics were obtained from dbPSHP. *P* was calculated using Fisher's exact.

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