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Endogenous DNA Damage and Risk of Testicular Germ Cell Tumors

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Running Head: DNA Damage and Testicular Cancer

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Abbreviations:

CDM	comet distributed moment
CI	confidence interval
CIS	carcinoma in-situ
FTC	Familial Testicular Cancer study
OR	odds ratio
OTM	Olive tail moment
USRT	U.S. Radiologic Technologists cohort
TGCT	testicular germ cell tumor

ABSTRACT

Testicular germ cell tumors (TGCT) are comprised of two histologic groups, seminomas and nonseminomas. We postulated that the possible divergent pathogeneses of these histologies may be partially explained by variable endogenous DNA damage. To assess our hypothesis, we conducted a case-case analysis of seminomas and nonseminomas using the alkaline comet assay to quantify single-strand DNA breaks and alkali-labile sites. The Familial Testicular Cancer study and the U.S. Radiologic Technologists cohort provided 112 TGCT cases (51 seminomas & 61 nonseminomas). A lymphoblastoid cell line was cultured for each patient and the alkaline comet assay was used to determine four parameters: tail DNA, tail length, comet distributed moment (CDM) and Olive tail moment (OTM). Odds ratios (OR) and 95% confidence intervals (95%CI) were estimated using logistic regression. Values for tail length, tail DNA, CDM and OTM were modeled as categorical variables using the 50th and 75th percentiles of the seminoma group. Tail DNA was significantly associated with nonseminoma compared to seminoma ($OR_{50th\ percentile}$=3.31, 95%CI: 1.00, 10.98; $OR_{75th\ percentile}$=3.71, 95%CI: 1.04, 13.20; *p* for trend=0.039). OTM exhibited similar, albeit statistically non-significant, risk estimates ($OR_{50th\ percentile}$=2.27, 95%CI: 0.75, 6.87; $OR_{75th\ percentile}$=2.40, 95%CI: 0.75, 7.71; *p* for trend=0.12) whereas tail length and CDM showed no association. In conclusion, the results for tail DNA and OTM indicate that endogenous DNA damage levels are higher in patients who develop nonseminoma compared with seminoma. This may partly explain the more aggressive biology and younger age-of-onset of this histologic subgroup compared with the relatively less aggressive, later-onset seminoma.

INTRODUCTION

The etiopathogenesis of testicular germ cell tumors (TGCT) and its distinct histologic subtypes, seminoma and nonseminoma, largely remains to be elucidated. Epidemiologic investigations, meanwhile, are being undertaken in an increasing number, a development piqued by the unexplained rise in incidence of this cancer over the past 40 years in Western Europe and the United States (1-3). Although some risk factors have been consistently associated with risk of TGCT, specifically cryptorchidism, prior history of TGCT and family history of TGCT (4), much of the epidemiologic evidence remains weak and contradictory (5). The current antithetical state of the TGCT literature may be partly attributable to the two main histologic groups having potentially divergent etiologies and natural histories (6, 7).

Accumulation of cellular DNA damage, the failure to repair such and the loss of the ability to undergo apoptosis are hallmarks of all cancers. Endogenous DNA damage, that present in the absence of exogenous mutagen challenges, is rate-limited by carcinogen-activation, carcinogen-detoxification and DNA repair efficiency (8, 9). Single-strand DNA breaks and alkali-labile sites, products of DNA metabolism processes including repair whose goal is to prevent genomic instability, can be quantified using the alkaline comet assay (10). The alkaline comet assay has suggested that higher levels of endogenous DNA damage are associated with diverse cancers, including those of the lung (11, 12), esophagus (13), breast (14-16), bladder (17) and ovary (18).

Although testicular seminoma and nonseminoma are thought to arise from a common precursor (19), it is postulated that they have divergent pathogeneses, which may be explained, partially, by variable levels of endogenous DNA damage. To assess our hypothesis, a case-case analysis of the two histologic subgroups of TGCT, seminoma and nonseminoma, was undertaken, using the alkaline comet assay to quantify endogenous DNA damage.

MATERIALS & METHODS

Study Design

Testicular germ cell tumor cases for this analysis were identified from two existing studies at the National Cancer Institute: the Familial Testicular Cancer (FTC) study and the U.S. Radiologic Technologists (USRT) study. The focus of the family-based FTC study is the identification of TGCT susceptibility genes (20). Families are eligible for enrollment if they have at least two confirmed cases of testicular cancer, or if they have a single bilateral case. For the current study, the FTC study provided samples from 72 family history positive unilateral TGCT cases, 6 family history positive bilateral cases and 18 family history negative bilateral cases. The USRT study is a cohort study of 143,517 radiologic technologists who were occupationally certified between 1926 and 1980 (21). Incident cancers in the cohort, including sixteen TGCTs, were ascertained through 2005. All cases of TGCT were unilateral and all provided samples to the current study. The FTC and USRT studies were approved the Institutional Review Board of the National Cancer Institute. Laboratory work conducted at Lawrence Livermore National

Laboratory (LLNL) has been approved annually by the LLNL Institutional Review Board.

Comet Analysis

An Epstein Barr virus transformed lymphoblastoid cell line was prepared from peripheral blood lymphocytes obtained from each subject. DNA samples were identified by a unique ID code, and investigators were blinded to case-group status. The methods used have been described in detail (Sigurdson et al., 2005). Briefly, cell lines were cultured in RPMI 1640 supplemented with 15% serum (Fetal Clone III, HyClone, Logan, Utah) and 2mM glutamine for 1 to 2 weeks prior to analysis. Viability was determined by Trypan blue dye exclusion. The alkaline comet assay was used to measure DNA damage in exponentially growing cells according to Singh et al. (10) with slight modifications. Images of 50 cells on each of two slides were captured and comet parameters determined using Komet4.0©: Image Analysis and Data Capture software (Kinetic Imaging, Ltd., Merseyside, England). The image analysis software generates many comet parameters but only four were retained and analyzed: tail DNA, tail length, comet distributed moment (CDM) and Olive tail moment (OTM). These are the four most common comet parameters reported in the literature, two of which (tail DNA and OTM) have recently been endorsed as the most quantitative and robust for epidemiologic studies (22). "Tail DNA" is the percent of DNA (fluorescence) in the tail. "Tail length" is the length of the tail in μm. CDM is the moment of fluorescence of the whole comet and does not distinguish between head and tail. OTM, or tail moment, is the product of the percentage of DNA in the tail (tail DNA) and the distance between the means of the head and tail fluorescence distributions. Both CDM and OTM are expressed in arbitrary units. All four parameters describe the amount of endogenous DNA damage; thus high values are thought to correspond to an increased cellular DNA strand breakage and/or alkali-labile sites.

Statistical analysis

The geometric mean of tail length, tail DNA, CDM and OTM of 100 randomly selected cells per subject was used as a summary measure to reduce the influence of outliers. Normality of the subject-specific summary measures was assessed for each comet parameter by Kolmogorov–Smirnov tests and visual inspection of quantile–quantile plots that were generated. Comet values did not deviate from normality. Analysis of variance (ANOVA) was used to compare means of comet values stratified by various factors, including date of cell culture, date of cell harvest, date of electrophoresis, date of image analysis, cell viability in culture, study from which sample originated, age of blood draw, age of TGCT diagnosis, history of cryptorchidism and TGCT laterality. Dates of cell culture, cell harvest, electrophoresis and image analysis were all highly correlated. If these variables were deemed to be confounding then the variable which produced the highest r^2 was retained in the fully adjusted model. The association between comet values and histologic group was evaluated by calculating odds ratios (OR) and 95% confidence intervals using logistic regression. The cluster option was used to estimate the standard errors and variance-covariance matrix, in order to account for any intra-group correlation caused by a subset of the familial cases being related (i.e., more than one affected member of a multiple case family was permitted into the data set).

Among the 24 bilateral TGCT cases, 8 men had discordant histology between the two affected testes. For analytic purposes, these individuals were coded as having nonseminoma based on the assumption that if endogenous DNA levels were different between the two histologic groups, they would more likely be higher in nonseminomas as they are more clinically aggressive (23-25) and arise at earlier ages (23, 26) than seminomas. Comet values for tail length, tail DNA, CDM and OTM were modeled as categorical variables using the 50th and 75th percentiles of the seminoma group as cutpoints. All models were adjusted for the potential confounders listed above and were evaluated by comparing comet value ORs with and without each factor in the model. If a covariate altered the risk estimate by >10% it was considered a confounder and retained within the model. When applicable, tests for linear trend in risk according to the medians of each category of a given ordered categorical variable were conducted to evaluate possible dose-response relationships. Statistical analyses were conducted with STATA 10 software (27). All tests were two sided, with $p<0.05$ defined as statistically significant. Coefficients of variation for the alkaline comet assay, as conducted at the LLNL using repeat samples, have been less than 15% (28).

RESULTS

This case-case analysis included 112 TGCT patients (51 seminomas and 61 nonseminomas). Of the 112, there were 52 individual cases, 27 related case-pairs and 2 related case-triads. The overall mean age at TGCT diagnosis for all study participants was 31.2 years; the mean age at diagnosis for seminoma (34.1 years) was later than nonseminoma (28.7 years). Mean age of blood draw was 42.5 years, which was an average of 10 years post-diagnosis. Six of the 51 seminoma patients (11.8%) and four of the 61 nonseminoma patients (6.8%) had a history of cryptorchidism.

The results of the logistic regression analyses are shown in the Table. Variables that affected a comet parameter's risk estimate were included in the model. These variables were date of cell harvest for tail DNA and for OTM and date of cell electrophoresis for CDM. Other variables tested as potential confounders, such as age at blood draw, age at TGCT diagnosis and history of cryptorchidism, did not alter the risk estimates and thus were not retained in the final models. Tail DNA was observed to be significantly associated with nonseminoma versus seminoma ($OR_{50th\ percentile}$=3.31, 95%CI: 1.00, 10.98; $OR_{75th\ percentile}$=3.71, 95%CI: 1.04, 13.20). The trend across the tail DNA estimates was positively correlated with nonseminoma (p=0.027). Although the results for OTM did not reach statistical significance, the estimates and trend were similar to that observed for tail DNA ($OR_{50th\ percentile}$=2.27, 95%CI: 0.75, 6.87; $OR_{75th\ percentile}$=2.40, 95%CI: 0.75, 7.71; p for trend=0.12). Tail length and CDM were not associated with TGCT histology. These results did not differ when the 16 USRT or the eight bilateral seminoma/nonseminoma cases were excluded from the analysis. The Figure shows boxplots of tail DNA and OTM stratified by histology.

DISCUSSION

To our knowledge this is the first study to quantify endogenous DNA damage in TGCT patients. The results for tail DNA and OTM indicate that endogenous levels of single strand DNA breaks and alkali-labile sites are higher in men who develop nonseminoma compared with seminoma. This result may partly explain the more biologically aggressive nature (23-25) and younger age at diagnosis (23, 26) of nonseminoma compared with the relatively less aggressive seminoma. This postulate is consistent with the evidence that nonseminoma may derive from seminoma via transitional genetic re-programming (19, 29, 30), a process that would theoretically be aided by increased genetic instability.

Quantitation of DNA damage in cultured lymphoblastoid cell lines, created using blood drawn on average 10 years post-diagnosis, is likely to represent an individual's capacity to limit DNA damage and its associated health consequences. Therefore, the increased levels of endogenous DNA damage observed in patients who developed nonseminoma, relative to those who developed seminoma, represent either a difference in the net rate of carcinogen metabolism and/or a difference in the ability to repair DNA.

The relationship between carcinogen metabolism and cancer risk is poorly understood, primarily due to the complexity of such metabolic interactions (31). Most studies have focused on either phase I (e.g. cytochrome P450s (CYP)) or phase II (e.g. *N*-acetyltransferase, glutathione S-transferase) gene variants, while studies analyzing TGCT risk have been few. One study has reported positive associations of *CYP3A4* -392G and *CYP3A5* 6986G and an inverse association of *CYP1A2* -163A with TGCT risk (32). A second study observed low *CYP1A2* activity to be associated with TGCT risk (33). Both of these studies found risk was unaltered when stratified by histologic group.

Although the mitotically dividing cells of the testes may have a limited DNA damage response (34), variation in DNA repair capacity could also explain the observed difference in levels of endogenous DNA damage between TGCT histologies. Presently, only two studies have investigated DNA repair gene variants in relation to TGCT risk. The first study found no association of single nucleotide polymorphisms of *XPD*, *ERCC1*, *XRCC3* and *OGG1* with risk of TGCT or either histologic type (35). However, this study only had 47% power to detect an OR of 2, an estimate which may be considered optimistic, especially given that a subsequently published genome-wide linkage study found no single major locus could account for familial aggregation of TGCT (36). The second study evaluated two polymorphisms of the *XRCC1* gene and found that having at least one Arg399Gln polymorphism increased TGCT risk (37). This association was stronger when restricted to patients with pure seminoma or metastatic disease.

The current literature regarding carcinogen metabolism and DNA repair in relation to TGCT risk, and its histologies, is still in its infancy, as is our understanding of the totality of these biologic processes with regard to cancer risk. As such, we cannot currently offer a specific explanation for the novel findings of differential levels of endogenous DNA damage by histology reported here.

It has been perceived that seminoma and nonseminoma have a similar etiopathogenesis and the epidemiological evidence is certainly consistent with this notion, insofar that they arise through a common precursor lesion (CIS) (19), have overlapping risk factors (38-

45) and, in many countries, share similar incidence trends over time (46). However, only a handful of studies investigating histologic differences have had a large number of cases (n>500) and many of the smaller studies may have lacked statistical power, increasing the chance of type two errors. Moreover, there is mounting epidemiologic evidence to suggest that a divergence occurs in the natural history of these histologies. Notable evidence includes cryptorchidism (6, 45, 47, 48), low birth weight (49, 50) and low birth order (43, 51) being predominantly associated with an increased risk of seminoma, while participation in specific sporting activities (6) and long gestational duration (52) appear more protective against seminoma relative to nonseminoma. In addition, risk factors primarily associated with an increased risk of nonseminoma include testicular trauma (6, 48), history of at least one sexually transmitted disease (6), younger age at shaving initiation (53) and short gestational duration (52), while later age of puberty may have a stronger protective effect against nonseminoma than seminoma (6, 40, 54). Although the literature is not congruent for any one of these histologic dissimilarities, the evidence is suggestive of a digression in the natural history and risk profile of these cancers, a hypothesis which is also indicated by divergent incidence rates for seminoma and nonseminoma in countries such as Italy (46) and the U.S. (55), and by the variable levels of endogenous DNA damage reported here.

Strengths of this analysis include: its case-case design, which mitigates the problem of reverse causality bias when using a cancer-free control group; the use of lymphoblastoid cell lines, damage levels of which are expected to reflect the net effect of endogenous processes of metabolism and DNA repair and are unlikely to reflect occupational, diet or other lifestyle variables; and its large size, relative to many other comet assay analyses. The main limitation, meanwhile, is the inherent selection bias of the retrospective study design caused by death of potential participants, although the cure rate of TGCT cases is exceedingly high as evidenced by a five-year survival rate of >97% (56), a fact which may assuage such concerns.

In summary, this study has found evidence for higher levels of endogenous DNA damage in patients who developed nonseminoma relative to seminoma. Replication of this finding should promote future studies to focus on the mechanism(s) underlying these differences.

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TABLE. An analysis of comet parameters in cell lines from men diagnosed with nonseminoma compared to seminoma (referent group)

Comet parameter	Nonseminoma vs. Seminoma				
	Seminoma (n)	**Nonseminoma (n)**	**Odds ratio**	**95% CI**	***p* value**
Tail DNA† (%)					
4.2 - 7.7	25	21	1.00	Referent	
7.8 - 9.4	13	19	3.31	1.00 - 10.98	0.050
9.5 - 13.7	13	21	3.71	1.04 - 13.20	0.043
p for trend			*0.039*		
Tail length (μm)					
12.1 - 32.1	25	29	1.00	Referent	
32.2 - 34.8	13	10	0.66	0.23 - 1.91	0.45
34.9 - 45.8	13	22	1.46	0.58 - 3.68	0.42
p for trend			*0.64*		
Comet distributed moment‡					
14.6 - 20.1	25	30	1.00	Referent	
20.2 - 21.7	13	16	0.95	0.40 - 2.27	0.91
21.8 - 27.7	13	15	0.85	0.30 - 2.38	0.75
p for trend			*0.75*		
Olive tail moment†					
1.0 - 1.8	25	23	1.00	Referent	
1.9 - 2.2	13	18	2.27	0.75 - 6.87	0.15
2.3 - 3.6	13	20	2.40	0.75 - 7.71	0.14
p for trend			*0.12*		

Comet parameters were divided into three categories using the 50th and 75th percentile of the seminoma distribution.

†adjusted for date of cell harvest

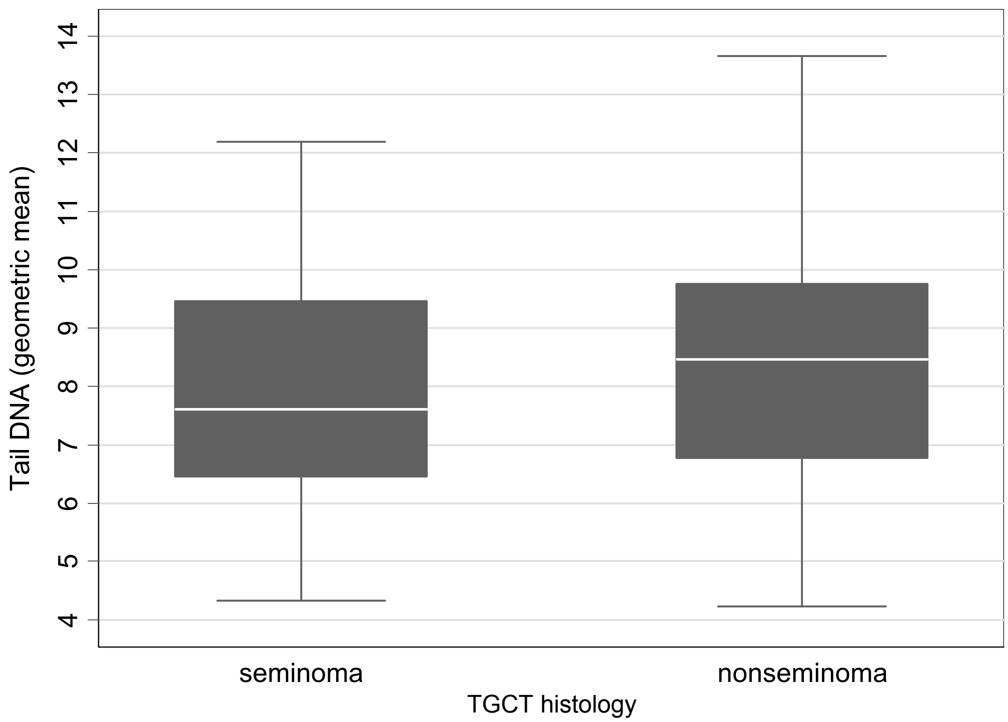
‡adjusted for date of electrophoresis

Figure legend

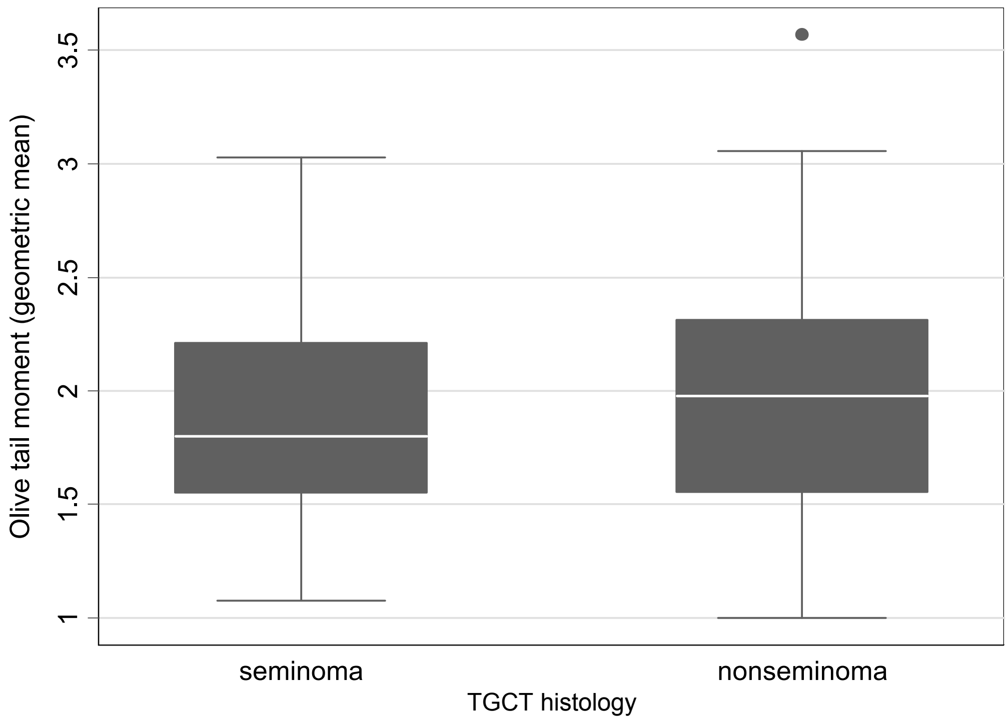
tail DNA; (b) Olive tail moment. Each boxplot represents the geometric means of the respective comet parameter of 100 cells for each individual within the nonseminoma or seminoma group. The boxplots display the median (thick line), interquartile range (lower and upper box borders), the 5th and 95th percentiles (error bars), and extreme individual values (●).

FIGURE. Boxplots of comet parameters by testicular germ cell tumor histology.

(a)



(b)



Olive tail moment (geometric mean)
1
1.5
2
2.5
3
3.5
seminoma
nonseminoma
TGCT histology