Short Communication

No Evidence for Differences in DNA Damage Assessed before and after a Cancer Diagnosis

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Abstract

The overwhelming majority of studies that have found increased cancer risk associated with functional deficits in DNA repair used a case-control design, in which measurements were made after cancer diagnosis. However, there are concerns about whether the cancer itself or cancer treatment affected the conclusions (reverse causation bias). We assessed the effect of cancer diagnosis among 26 breast cancer controls who had blood collected during 2001 to 2003 and again in 2005 to 2006 after being diagnosed with cancer. Using the alkaline comet assay, we quantified DNA damage in untreated lymphoblastoid cell lines. Comet distributed moment, olive tail moment, percentage of DNA in tail, and comet tail length were summarized as the geometric mean of 100 cells. For comet distributed moment, olive tail moment, tail DNA, and tail length, the proportions of women with before diagnosis values higher than after diagnosis were 65%, 50%, 50%, and 46%, respectively. We found no significant differences in the before or after diagnosis mean comet values. Median cut-points were determined from the before diagnosis distribution, and we used conditional logistic regression to calculate odds ratios (OR) and upper 95% bounds of the confidence intervals. ORs ranged from 0.6 to 0.9 with upper confidence interval bounds of 1.9 and 2.6, meaning biased ORs above 2.6 are unlikely. We found no evidence that reverse causation bias is an important concern in case-control studies using the comet assay applied to cell lines collected after cancer diagnosis. More work is needed to characterize the effect of cancer diagnosis on other phenotypic assays. (Cancer Epidemiol Biomarkers Prev 2008;17(4):990–4)

Introduction

Several studies have reported increased breast (1, 2), bladder (3), and multiple (4) cancer risks associated with higher DNA damage measured by the comet assay (5). However, the validity of the conclusions of these and similarly designed functional studies (reviewed in ref. 6) have been questioned because the results may be due to measuring a consequence, and not a cause, of cancer. A recent example of this bias, so called ''reverse causation,'' was suggested as one possible reason for the association between C-reactive protein levels and cancer in case-control, but not prospectively designed, studies (7). However, unlike C-reactive protein level measurements that are easy and economical to do, other phenotypic assays are time intensive and costly. Unless assays become simpler to perform or enormous resources are prioritized for prospective studies, such as DNA repair capacity and cancer outcome, it is unlikely that such studies would ever be conducted. Nested case-control studies in cohorts with prediagnostic samples are alternatives, but despite some successes (8), the reality is that stored frozen blood or cryopreserved buffy coats remain troublesome materials for assays that require cell stimulation and growth.

One approach to evaluating the reverse causation bias issue is to assess phenotype among samples from the same person collected before and after cancer diagnosis. If no material differences are found between measurements in the prediagnostic and postdiagnostic samples, then bias from reverse causation may be less concerning. We chose to evaluate single-strand DNA breaks and abasic sites of untreated lymphobastoid cell lines using the alkaline comet assay because we used this assay successfully in a previous study (4) and because cell lines do not contain interfering residual cellular debris from freezing. All of the study subjects were breast cancer controls from a genetic study of breast cancer among U.S. radiologic technologists (9, 10) that subsequently developed a first primary cancer.

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Materials and Methods

Study population. In 1982, the National Cancer Institute, in collaboration with the University of Minnesota and the American Registry of Radiologic Technologists, initiated a study of cancer incidence and mortality among 146,022 U.S. radiologic technologists, who were certified for at least 2 y between 1926 and 1982. The cohort members are predominantly female (73%), and their current mean age is 60 y. Specifics of the U.S. radiologic technologist cohort and survey methods have been published previously (11). The U.S. radiologic technologist genetics component has focused on breast (9, 10), papillary thyroid (12), and other selected cancer sites (4). This study has been approved annually by the human subjects review boards of National Cancer Institute, University of Minnesota, and, as applicable, Lawrence Livermore National Laboratory.

Cancer Confirmation and Recruitment. Cancers reported on the third cohort survey questionnaire given during 2005 to 2006 were confirmed by pathology or medical records. Women who had provided a peripheral blood sample for the breast cancer case-control study (9, 10) as controls and who had a newly diagnosed cancer at any site were asked for another blood sample. There were 33 controls who reported cancer diagnoses, of whom three refused and four were later found ineligible (cancer was found to have occurred before or <6 mo after blood collection or was denied by the physician), leaving 26 women for analysis. All of the women provided informed consent and updated cancer risk factor information.

Samples and DNA Damage Measurements. After the blood samples were processed at the Frederick Cancer Research and Development Center, the paired buffy coats (one prediagnosis and one postdiagnosis) were shipped to American Type Culture Collection, where they underwent Epstein Barr virus transformation, and a lymphoblastoid cell line was prepared. All samples were successfully transformed, cryopreserved, and shipped overnight to Lawrence Livermore National Laboratory. American Type Culture Collection and Lawrence Livermore National Laboratory investigators were blinded to sample status, and the cell lines were thawed and cultured in random order.

Cells were in exponential growth phase at the time of assessment of DNA damage. The alkaline comet assay was used to measure DNA damage according to Singh et al. (5) with slight modifications. Laboratory procedures and methods were the same as in our earlier study (4). In brief, images of 50 cells on each of two slides were captured, and comet variables were determined using Komet 4.0 Image Analysis and Data Capture software (Kinetic Imaging, Ltd.). Four comet variables were analyzed: ''tail DNA'' is the percentage of DNA fluorescence in the tail; ''tail length'' is the length of the tail, in micrometers, measured from the leading edge of the head; comet distributed moment (CDM) is the moment of fluorescence of the whole comet and does not distinguish head and tail; olive tail moment (OTM) is the percentage of tail DNA times the distance between the means of the tail and head fluorescence distributions, divided by 100. All four variables quantify endogenous DNA damage, and therefore, high values are thought to correspond to an increased amount of cellular DNA strand breakage and/or alkali-labile sites.

Table 1. Descriptive characteristics of the women who were cancer free at the time of blood collection and subsequently developed a cancer of any site (U.S. Radiologic Technologists Study)

Characteristic	*n* (%)
Race	
White	24 (92.3)
Black	2 (7.7)
Age at cancer diagnosis	
49-54	5 (19.2)
55-64	7 (26.9)
65-74	10 (38.5)
75-84	4 (15.4)
Cancer site	
Nonmelanoma skin cancer	13 (50.0)
Breast	7 (26.9)
Melanoma, kidney, bladder, endometrium	4 (15.4)
Non–Hodgkin's lymphoma, chronic lymphoctyic leukemia	2 (7.7)
Calendar year of cancer diagnosis	
2002	3 (11.5)
2003	8 (30.8)
2004	14 (53.8)
2005	1 (3.8)
Calendar year of first blood draw	
2001	9 (34.6)
2002	14 (53.8)
2003	3 (11.5)
Calendar year of second blood draw	
2005	20 (76.9)
2006	6 (23.1)
Years between first blood draw and cancer diagnosis	
0	4 (15.4)
1	13 (50.0)
2	8 (30.8)
3	1 (3.8)
Years between cancer diagnosis and second blood draw	
1	6 (23.1)
2	10 (38.5)
3	8 (30.8)
4	2 (7.7)
No. cancers reported among first degree relatives	
0	9 (34.6)
1	8 (30.8)
2	4 (15.4)
3	5 (19.2)

Statistical Analysis. We used the geometric mean of tail length, tail DNA, CDM, and OTM of 100 randomly selected cells per subject as a summary measure to reduce the influence of outliers. We assessed normality of the subject-specific summary measures for each comet variable and separately for prediagnosis and postdiagnosis groups by visual inspection of the quantile-quantile plots. Comet values did not deviate from normality. ANOVA and visual inspection were used to compare comet values by various factors, including cell viability in culture (determined by trypan blue dye exclusion), date the cells were scored, age at

Table 2. Descriptive statistics and test for differences in the means for the four comet variables by precancer or postcancer diagnosis

Cancer sites	*n*	Precancer diagnosis				Postcancer diagnosis				*P**	95% CI†
		Mean	SD	Min	Max	Mean	SD	Min	Max		
All cancers											
CDM	26	24.1	2.4	18.8	29.1	23.0	2.1	20.2	27.2	0.1	−0.3 to 2.3
OTM	26	3.0	0.7	1.5	4.1	2.8	0.7	1.4	3.8	0.4	−0.2 to 0.6
Tail DNA	26	11.3	2.5	5.9	15.9	10.8	2.1	5.4	13.7	0.4	−0.7 to 1.7
Tail length	26	37.4	4.4	21.9	44.4	36.4	5.6	19.8	48.0	0.4	−1.5 to 3.5
Nonmelanoma skin cancer											
CDM	13	24.3	1.9	22.2	28.2	23.5	2.5	20.2	27.2	0.5	−1.3 to 2.9
OTM	13	3.0	0.8	1.5	4.0	3.0	0.7	1.8	3.8	0.9	−0.6 to 0.7
Tail DNA	13	11.4	2.8	5.9	15.9	11.3	2.1	8.1	13.7	0.9	−1.9 to 2.0
Tail length	13	36.6	5.7	21.9	44.4	38.1	5.4	30.8	48.0	0.4	−4.8 to 2.0
Malignancies other than nonmelanoma skin cancer											
CDM	13	23.8	2.8	18.8	29.1	22.5	1.5	20.2	24.5	0.1	−0.5 to 3.1
OTM	13	2.9	0.6	1.9	4.1	2.6	0.6	1.4	3.6	0.3	−0.3 to 0.8
Tail DNA	13	11.2	2.2	8.5	15.6	10.3	2.0	5.4	13.6	0.3	−0.9 to 2.7
Tail length	13	38.1	2.4	34.1	43.0	34.6	5.6	19.8	39.7	0.1	−0.1 to 6.9
Breast cancers											
CDM	7	24.6	2.9	21.6	29.1	22.2	1.7	20.2	24.5	0.1	−0.4 to 5.3
OTM	7	2.9	0.6	2.3	3.7	2.4	0.7	1.4	3.6	0.2	−0.3 to 1.3
Tail DNA	7	11.2	1.9	8.7	13.4	9.7	2.5	5.4	13.6	0.2	−1.0 to 4.1
Tail length	7	38.4	3.1	34.1	43.0	33.3	6.8	19.8	39.0	0.1	−1.1 to 11.3

Abbreviations: min, minimum; max, maximum.
*From *t* test of means.
† CIs for difference between means.

the time of blood collection, time since cancer diagnosis to blood collection, tumor stage and grade, history of radiation treatment for cancer, and number of cancers in first-degree relatives.

For all cancers and various cancer subtypes (nonmelanoma skin cancer, breast cancer, etc.), we used paired *t* tests to compare the precancer and postcancer means of the subject-specific geometric mean comet values. The association between comet values and cancer risk was evaluated by calculating odds ratios (OR) and 95% confidence intervals (95% CI) based on conditional logistic regression, treating the prediagnostic group as matched controls. Comet values for tail length, tail DNA, CDM, and OTM were divided into two categories based on the median of the respective distribution in the entire prediagnosis group. Potentially confounding factors listed above (i.e., cell viability, calendar time the cells were scored, etc.) were evaluated by comparing comet value ORs when each factor was or was not included in the model. We found no meaningful change in the point estimates and therefore did not include any of these factors in the final model. All significance tests were two sided, and α was set at 0.05. SAS software (SAS Institute, release 8.02) was used for all analyses.

Results

Descriptive features of the 26 subjects are shown in Table 1. The women were nearly all White (92%), and most (65%) were diagnosed with cancer between the ages of 55 and 74 y. The majority of cancers were nonmelanoma skin cancer (50%). Others were breast (27%), hematolymphoproliferative (8%), melanoma (4%), endometrial (4%), kidney (4%), and bladder (4%). The time period from first blood collection to cancer diagnosis exceeded 1 year for 22 subjects (85%), and the time period between cancer diagnosis and second blood collection exceeded 2 years for 20 subjects (77%). We found no significant associations between comet values and the potentially confounding factors mentioned previously. In Table 2, descriptive and *t* test statistics are shown for the comet measures among samples collected before and after cancer diagnosis. None of the *t* tests were statistically significant, but three had *P* values of <0.1. All three showed higher mean comet values for prediagnosis assessed DNA damage than postdiagnosis. For CDM, OTM, tail DNA, and tail length, the proportions of women with values higher before than after were 65%, 50%, 50%, and 46%, respectively (data not shown).

We present ORs and 95% CIs for cancer diagnosis in relationship to comet variables categorized below and above the median in Table 3. The ORs for all cancers, nonmelanoma skin cancer, cancers other than skin, and breast cancer did not differ significantly between below and above median values of the comet variables. Among all the cancer groups, the highest upper 95% CIs ranged from 1.9 to 2.6, with an average of 2.2, and ORs did not increase with increasing comet variable values when divided into three categories (at the median and the 75th percentile; data not shown).

Discussion

We found no effect of an intervening cancer diagnosis on the levels of endogenous DNA damage, as measured by the comet assay in prediagnostic and postdiagnostic samples from the same individuals. Our finding is important because a key feature of a valid molecular biomarker is that it is unrelated to the host's cancer (13, 14) but measures some underlying cancer susceptibility. Examples of prediagnostic and postdiagnostic comparisons are rare, but do exist, such as

determining debrisoquine metabolism before and after lung tumor resection (15). Another important element is that the assay or phenotype measurement is unaffected by a known risk factor for the cancer of interest, such as cigarette smoking and lung cancer. In our previous study, we found little variation in comet assay outcomes with several factors, including age, survival time (among cases), radiation treatment, and reproductive history (4).

The advantage of using lymphoblastoid cell lines as opposed to primary lymphocytes is that they provide an infinite supply of cycling cells that can be used for additional types of assays. Mutagen sensitivity in primary lymphocytes and lymphoblastoid cell lines has been found to be correlated (16). Kennedy et al. successfully used lymphoblastoid cell lines to compare nucleotide excision capacity among sisters discordant for breast cancer and detected no association of this repair capacity with smoking in either controls or cases (17). Although damage levels in the cell lines are expected to reflect inherited DNA metabolism capabilities and, in contrast to peripheral blood lymphocytes, are unlikely to reflect occupational, diet, or other life-style variables, one cannot rule out the possibility that the transformed cells have acquired properties that affect relevance to normal tissues. So, due caution in interpretation of our results is advised.

Table 3. OR and 95% CIs for the four comet variables by all cancers, skin cancer, and breast cancer

Cancer sites	Cases (%)	Controls (%)	OR (95% CI)
All cancers (n = 26)			
CDM ≤ 23.7	16 (62)	13 (50)	1.0
CDM > 23.7	10 (38)	13 (50)	0.7 (0.2-1.9)
OTM ≤ 2.8	14 (54)	13 (50)	1.0
OTM > 2.8	12 (46)	13 (50)	0.9 (0.3-2.6)
Tail DNA ≤ 10.9	16 (62)	13 (50)	1.0
Tail DNA > 10.9	10 (38)	13 (50)	0.6 (0.2-2.0)
Tail length ≤ 37.8	15 (58)	13 (50)	1.0
Tail length > 37.8	11 (42)	13 (50)	0.8 (0.3-2.2)
Nonmelanoma skin cancers (n = 13)			
CDM ≤ 23.7	7 (54)	7 (54)	1.0
CDM > 23.7	6 (46)	6 (46)	1.0 (0.3-4.0)
OTM ≤ 2.8	6 (46)	5 (38)	1.0
OTM > 2.8	7 (54)	8 (62)	0.7 (0.1-4.0)
Tail DNA ≤ 10.9	6 (46)	7 (54)	1.0
Tail DNA > 10.9	7 (54)	6 (46)	1.5 (0.3-9.0)
Tail length ≤ 37.8	7 (54)	7 (54)	1.0
Tail length > 37.8	6 (46)	6 (46)	1.0 (0.2-5.0)
Malignancies other than nonmelanoma skin cancer (n = 13)			
CDM ≤ 23.7	9 (69)	6 (46)	1.0
CDM > 23.7	4 (31)	7 (54)	0.4 (0.08-2.1)
OTM ≤ 2.8	8 (62)	8 (62)	1.0
OTM > 2.8	5 (38)	5 (38)	1.0 (0.3. 4.0)
Tail DNA ≤ 10.9	10 (77)	6 (46)	1.0
Tail DNA > 10.9	3 (23)	7 (54)	0.2 (0.02-1.7)
Tail length ≤ 37.8	8 (62)	6 (46)	1.0
Tail length > 37.8	5 (38)	7 (54)	0.6 (0.1-2.5)
Breast cancers (n = 7)			
CDM ≤ 23.7	5 (71)	3 (43)	1.0
CDM > 23.7	2 (29)	4 (57)	0.3 (0.04-3.2)
OTM ≤ 2.8	5 (71)	4 (57)	1.0
OTM >2.8	2 (29)	3 (43)	0.5 (0.05-5.5)
Tail DNA ≤ 10.9	6 (86)	3 (43)	1.0
Tail DNA > 10.9	1 (14)	4 (57)	*
Tail length ≤ 37.8	5 (71)	3 (43)	1.0
Tail length > 37.8	2 (29)	4 (57)	0.3 (0.04-3.2)

NOTE: ORs were calculated using the Comet values from the prediagnostic samples as ''controls'' and the postdiagnostic samples as ''cases.'' Comet variables were divided into two categories at the median of the control distribution.

*Model would not converge because of single case in Tail DNA > 10.9 category.

Our sample size was relatively small; nevertheless, based on our study, it is unlikely that reverse causation bias would account for associations in excess of ~2.6-fold. Many of the cancer risk estimates associated with markers of DNA repair have exceeded 2.6-fold (6), which would not exclude the possibility of bias but may reduce concerns somewhat.

For 65% of study participants, the time between initial blood collection and cancer diagnosis was 1 year or less, with the maximum time period being only 3 years. As such, we cannot rule out the possibility of an occult cancer at the time of first blood draw. This is an issue that should be addressed by future studies with larger sample collections that could more definitively stratify or restrict the samples to evaluate the occult cancer scenario.

In summary, we found little evidence that reverse causation bias is an important concern in case-control studies using the Comet assay applied to lymphoblastoid cell lines collected after a cancer diagnosis. We have described a small collection, but it should be possible to use large respositories of prospectively collected lymphocytes to establish a larger set of prediagnosis and postdiagnosis lymphoblastoid cell lines that could be used to corroborate our finding and test additional assays in the future.

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