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DNA damage among thyroid cancer and multiple cancer cases, controls, and long-lived individuals

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Abstract

Variation in the detection, signaling, and repair of DNA damage contributes to human cancer risk. To assess capacity to modulate endogenous DNA damage among radiologic technologists who had been diagnosed with breast cancer and another malignancy (breast-other; n=42), early-onset breast cancer (early-onset, age ≤ 35; n=38), thyroid cancer (n=68), long-lived cancer-free individuals (hyper-normals; n=20) and cancer-free controls (n=49) we quantified DNA damage (single strand breaks and abasic sites) in untreated lymphoblastoid cell lines using the alkaline comet assay. Komet™ software provided comet tail length, % DNA in tail (tail DNA), comet distributed moment (CDM), and Olive tail moment (OTM) summarized as the geometric mean of 100 cells. Category cut-points (median and 75th percentile) were determined from the distribution among controls. Tail length (for ≥ 75% vs. below the median, age adjusted) was most consistently associated with the highest odds ratios in the breast-other, early-onset, and thyroid cancer groups (with risk increased 10-, 5- or 19-fold, respectively, with wide confidence intervals) and decreased risk among the hyper-normal group. For the other three Comet measures, risk of breast-other was elevated approximately three-fold. Risk of early-onset breast cancer was mixed and risk of thyroid cancer ranged from null to a two-fold increase. The hyper-normal group showed decreased odds ratios for tail DNA and OTM, but not CDM. DNA damage, as estimated by all Comet measures, was relatively unaffected by survival time, reproductive factors, and prior radiation treatment. We detected a continuum of endogenous DNA damage that was highest among cancer cases, less in controls, and suggestively lowest in hyper-normal individuals. Measuring this DNA damage phenotype may contribute to the identification of susceptible sub-groups. Our observations require replication in a prospective study with a large number of pre-diagnostic samples.

1. Introduction

There is considerable evidence that individual variation in the detection, signaling, toleration, and repair of DNA damage (from internal and external exposures and intrinsic instability of DNA) contributes to human cancer risk (reviewed in [1-6]). Several studies (with sample sizes in excess of 20 cases and 20 controls) have reported increased breast [7,8] and bladder [9] cancer risks associated with higher DNA damage measured by the comet assay [10]. In these studies elevated endogenous damage and post-mutagen challenge damage levels were associated with increased cancer risk. These results indicate that elevated endogenous damage is itself a risk factor, and are consistent with the notion that measures of endogenous damage are correlated with damage repair capability after an exogenous challenge.

We characterized risk of solid cancer associated with ability to limit DNA damage from endogenous DNA metabolic processes among persons with selected malignancies, compared to controls by quantifying single strand DNA breaks (SSB) and abasic sites of untreated cell lines using the alkaline comet assay. This assay provides a measure of the net effect of each cell's anti-oxidant and base excision repair pathway capacity to prevent and repair oxidative DNA damage, the major endogenous source of genomic instability [11-13]. In addition, we compared controls to individuals who were long-lived, cancer-free and reported no invasive cancer in first degree relatives. All the cases and controls were part of a larger, ongoing study of cancer and other health outcomes among radiologic technologists exposed through their occupation to low-dose radiation from medical sources.

2. Methods

2.1. Study population

In 1982, the U. S. 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 two years between 1926 and 1982. The cohort members are predominantly female (73%) and their current mean age is 55 years [14]. The study has focused on cancers of the thyroid gland (papillary histology) and the female breast as these tissues are thought to be particularly radiation sensitive [15].

2.2. Cancer confirmation and recruitment.

Cancer(s) reported on a study questionnaire were confirmed, and individuals were invited to provide a peripheral blood sample. At that time, for the rare persons with multiple cancers (including cancer of the breast and a second primary cancer), early-onset breast cancer (age 35 or younger) and papillary thyroid cancer, a portion of the sample was used to create EBV transformed lymphoblastoid cell lines. We sought to establish a lymphoblastoid cell line resource of approximately 40 to 60 individuals per cancer group. Approximately 50 cancer-free female controls were frequency matched on age (± 5 years) to the case groups. We also recruited 20 female radiologic technologists who were over 70 years of age, had never had any cancer (defined to include non-melanoma skin cancer), and had not reported any first degree relative with a cancer diagnosis at the time of blood collection. We referred to these individuals as "hyper-normal", akin to the naming convention used for similar selection strategies in related study designs [16] reasoning they may have a further decrease in background cancer risk compared to controls. All of the cases and controls provided informed consent and responded to a telephone interview that collected current cancer risk factors and family history of cancer. This study has been approved

annually by the human subjects review boards of the National Cancer Institute and the University of Minnesota. Studies conducted at Lawrence Livermore National Laboratory (LLNL) have been approved annually by the LLNL Institutional Review Board.

2.3. Samples

A lymphoblastoid cell line was prepared by Epstein Barr virus transformation of peripheral blood lymphocytes obtained from each subject. All fresh samples were successfully transformed and each line was cryopreserved. For this study, the samples were tracked by a unique ID code, and investigators were blinded to case-control and case-group status. Cell lines were cultured in RPMI 1640 supplemented with 15% serum (Fetal Clone III, HyClone, Logan, Utah) and 2mM glutamine prior to analysis. Aliquots of all cell lines were cryopreserved at LLNL. The period of culture prior to analysis varied among cell lines, from about 3 to 5 weeks, depending on the growth rate of the cell line and the proportion of viable cells during early culture. For quality assessment, replicate samples of five individuals were included, some in the same shipment, others in separate shipments. In addition, 16 cultures were repeated with personnel blinded to results of the first assay.

2.4. Measurement of DNA damage.

Cells were in exponential growth phase at the time of assessment of DNA damage. Viability was determined by Trypan blue dye exclusion. The alkaline comet assay was used to measure DNA damage according to Singh et al. [10] with slight modifications. Frosted end glass microscope slides were first coated with 1.0% (w/v) agarose and allowed to dry. Cells were suspended in 0.5% low melting point agarose and spread on each of two slides with a coverslip. After the agarose hardened, the coverslip was removed and slides were treated in the dark at 4°C with lysis buffer (1% Triton X-100, 10% DMSO, 89% stock lysing solution: 2.8M NaCl, 0.1M

Na_2EDTA, 0.01M Trizma Base) at least overnight, then rinsed in 0.4 M Tris, pH 7.5. Slides were then placed in the electrophoresis unit and covered with a fresh solution of 300 mM NaOH, 1 mM EDTA, final pH >13.0, for 60 min. A split, balanced slide layout design was used (one slide from each sample was in each of two rows, but in different electrophoresis runs) to attend to position effects and run to run variation. The slides were electrophoresed at 0.92V/cm with current adjusted to 300 mamps for 25 min. The slides were washed in 0.4M Tris, pH 7.5, placed for 5 min. in cold methanol and allowed to dry. Each slide was stained with ethidium bromide (2 μg/ml) for 5 min. Images of 50 cells on each of 2 slides were captured and comet parameters determined using Komet4.0©: Image Analysis and Data Capture software (Kinetic Imaging, Ltd., Merseyside, England). Four comet parameters were analyzed; the definitions used by Komet4.0 are: "Tail DNA" is the percent of DNA (fluorescence) in the tail. "Tail length" is the length of the tail in μm, measured from the leading edge of the head; Comet Distributed Moment (CDM), also referred to as comet moment (16), is the moment of fluorescence of the whole comet and does not distinguish head and tail; Olive Tail Moment (OTM) is the percentage of DNA in the tail (tail DNA) times the distance between the means of the tail and head fluorescence distributions, where "mean" is the profile center of gravity, divided by 100. Both CDM and OTM are expressed in arbitrary units. All four parameters describe the amount of 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.

2.5. 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 parameter and separately for

case and control groups by Kolmogorov-Smirnov tests and visual inspection of the 20 quantile-quantile plots that were generated. Comet values did not deviate from normality, except for CDM among the early-onset breast cancer and the breast cancer and other malignancy case groups and for tail length among the thyroid case group, even after logarithmic transformation. Analysis of variance (ANOVA) was used to compare means of comet values by various factors, including cell viability in culture (determined by trypan blue dye exclusion), cell shipment method (frozen cells or thawed and cultured before shipment), laboratory first handling the freshly collected blood sample (Frederick, MD or Manassas, VA), storage time, date the cells were scored, age of cases and controls at the time of blood collection, time since first cancer diagnosis to blood collection (cases only), history of radiation treatment for cancer (cases only), gender (thyroid cases only), selected reproductive variables (women only), and history of cancer in first degree relatives.

The association between comet values and cancer risk was evaluated by calculating odds ratios and 95% confidence intervals based on logistic regression. Comet values for tail length, tail DNA, CDM, and OTM were divided into three categories based on the median and 75th percentile of the respective distribution in the control group. All models were adjusted for age in three categories (40-54, 55-74, and 75 years or older), and potentially confounding factors listed above (i.e. cell viability, calendar time the cells were scored, etc.) were evaluated by comparing comet value odds ratios 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. Because there were no male controls, we also analyzed the thyroid group excluding the male cases (n=9). We found no consequential alteration in the point estimates and report the odds ratios with men included. Tests for trend were adjusted for age and based on the underlying continuous variable. All significance tests were two sided and α was set at 0.05. The Statistical Package for the Social Sciences version 12.0 (SPSS, Inc., Chicago, Il) was used for all analyses.

The coefficient of variation (CV) was calculated separately for each comet parameter [17] among 5 blinded replicate and 16 blinded repeated samples. The CVs for tail DNA were 11.8% and 14.8%, for tail length were 7.6% and 12.4%, for CDM were 2.6% and 6.8%, for OTM were 11.6% and 18.9% among the 5 replicates and 16 repeats, respectively. These CV values are comparable to results from a recent study in which a lymphoblastoid line was assayed by alkaline comet for baseline damage in 16 batches of assays; a CV of 22% was observed [7]. For a radioimmunoassay commonly used for epidemiological studies, a CV of less than 15% is considered acceptable [18].

3. Results

Individual comet results are presented in Table 1, as are the results of the 5 blinded replicates and the 16 blinded repeats. In Table 2 the arithmetic means and standard errors for the comet parameter tail length are shown by categories of selected demographic and descriptive characteristics. The means and standard errors for the comet parameters tail DNA and OTM by these characteristics were very similar to those for tail length and are not shown. Although the distributions of the CDM geometric means between case and control groups differed from those of the other parameter values (see Figure 1), the CDM values did not differ according to categories of the descriptive characteristics and are also not shown. Nearly all of the cases and controls were white (98.2%, data not shown) and female (95.9%). Comet values did not increase or decrease with age at the time of blood collection (Table 2) except for tail length among those with thyroid cancer, however, the means did not differ by age group ($P = 0.39$). Similarly, comet parameters did not differ by the number of years between cancer diagnosis and blood collection (cases only), radiation treatment for cancer (cases only), and selected reproductive variables (data not shown). Tail length tended to marginally increase in most groups as the number of first degree relatives with breast

cancer and the number of cancers within individuals increased (9 persons had three or more cancers, data not shown), but achieved only borderline statistical significance ($P = 0.07$). History of chemotherapy did not affect the means of the comet parameters, but the data obtained from medical records was incomplete (yes, n=13; no, n=16; unknown, n=118). The means and standard errors (parentheses) for tail length were 44.8 (1.7), 43.9 (2.8), and 45.0 (0.8) among those who did and did not have chemotherapy or their treatment status was not known, respectively ($P = 0.88$). We did not observe significant differences in comet parameters means for viability, sample shipment method, storage time, original laboratory, or calendar time over which the cells were scored.

Boxplots of the distributions of the geometric means of the comet parameters across 100 cells for each individual by case and control groups are shown in Figure 1. All the parameters, except CDM, tended to be lower in the normal controls than the cancer case groups, with the hyper-normal group having the lowest values of all the groups. Most comet parameters were highly correlated with each other ($r^2 \geq 0.49$, $P < 0.001$). However, CDM was not correlated with tail length among the normal controls, the hyper-normal controls, and the breast and other cancer cases.

We present age-adjusted odds ratios (OR) and 95% confidence intervals (CI) for cancer risk in relationship to comet parameter categories in Table 3, with cancer cases and hyper-normal controls compared to normal controls. As the values of the comet parameters increased, indicating increasing endogenous SSB and alkali labile DNA damage in the cells, the risks for breast and other cancer and thyroid cancer generally also increased. Longer tail length was consistently associated with increased cancer risk, with ORs of 10.1, 4.7, and 19.1 associated with values $\geq 75^{th}$ percentile vs. below the median for the breast and other cancer, early-onset breast cancer, and thyroid cancer groups, respectively, with wide confidence intervals. Risk for early-onset breast cancer was inversely associated with tail DNA and CDM, and was not related to OTM. The hyper-normal controls were at or below the median of the normal controls for all comet parameters except CDM.

4. Discussion

We found a generally consistent association of increased endogenous DNA damage, or more specifically, a reduced capacity to limit endogenous damage, and increased cancer risk. This association was most strongly indicated by higher values of the comet parameter tail length. Risk of breast and other cancer and thyroid cancer also increased with increased tail DNA and OTM or only OTM, respectively. In addition, we found suggestive evidence in three of the four comet parameters evaluated that lower levels of DNA damage were associated with being long-lived, cancer-free, and without a history of cancer among first degree relatives, supporting the notion of a graded capacity to control endogenous DNA damage. We consider detecting evidence of such a continuum unique because hospital- or clinic-based studies seldom possess a well-characterized cohort from which to select individuals with defined characteristics, such as longevity and first degree relatives who are cancer-free. It is likely the hyper-normal individuals were also relatively free of co-morbidities, as they were able to visit their doctor or clinic with a study venipuncture kit and mail the kit successfully. This suggests the hyper-normal participants were probably the "healthiest" and the best group for detecting such a continuum, if it exists.

Comet tail length showed the strongest positive association with cancer risk among the four DNA damage parameters evaluated. The tail length parameter increases with increased density of abasic sites and strand breaks due to higher mobility of regions of DNA. That tail length was optimal for discriminating among groups suggests that a better understanding of the mechanistic significance of comet parameters and underlying biology is needed. There are few studies comparing multiple comet parameters, and most treat cells with high doses of a damaging agent such as ionizing radiation that induces uniformally distributed damage. It is not known whether the lower response of tail length versus tail moment and tail DNA to damage induced up to 4 Gy [19] is

an appropriate reference for studies of endogenous damage, that is both quantitatively lower and qualitatively different due to preferential repair in transcribed regions of the genome. Our observations suggest tail length may be a sensitive indicator of low levels of inhomogeneously distributed unrepaired damage. It is appropriate to note, that of the four parameters, tail length is most subject to technical variation. Among all study groups, the three tail-related comet parameters (i.e. tail DNA, tail length, and OTM) were highly correlated. In the control, hypernormal, and breast and another cancer groups, CDM did not correlate with tail length. The correlation differences within study groups in comet parameters may indicate that the distribution of damage throughout the genome (between comet head and tail) is determined by multiple, independent processes, some operating throughout the genome, others not. Studies of the relationships between comet parameters and loss of specific DNA repair functions using cells with defined mutations of DNA repair genes would be useful.

Using post-diagnostic samples for assessing the predictive value of phenotype assays such as the Comet assay is less than ideal because assay outcome may be affected by disease status or treatment [20]. The study should be replicated with pre-diagnostic samples collected in a large prospective study. However, we found little variation in the comet assay outcome with several factors, including age, survival time (among cases), radiation treatment, and reproductive history. This is consistent with other investigations [7,8,21,22]. The lymphoblastoid cell lines provided an infinite supply of cycling cells that can be used for additional assays in the future, in which damage levels are expected to reflect endogenous processes of DNA metabolism and are unlikely to reflect occupational, diet or other lifestyle variables. One cannot rule out the possibility that the transformed cells have acquired properties that may affect relevance to normal tissues.

We evaluated several factors that could have affected comet measures in systematic ways, such as cell viability, storage time, etc. We did not find evidence that these factors introduduced

predictable variation in comet values. However, we did not randomly assay the lines nor did we assess "batch" effects. The closest proxy to "batch" was "cell scoring date", and we did not observe undue systematic variation over calendar time in the aggregate or by case or control groups.

In the future, studies of the level and specific nature of oxidative damage in unchallenged cells (as by Collins and colleagues [23,24]) would provide valuable knowledge about the variation among individuals, and contribute to development of hypotheses to test. As the environmental risk factors for these cancers are identified in case-control studies of the parent U.S. Radiologic Technologist cohort, challenge assays that measure the formation and repair of damage from exposure to related model mutagens, such as ionizing radiation or BPDE, may be appropriate [3].

Results of the comet assay, performed on un-challenged cells, may represent an individual's capacity to limit endogenous damage and its associated health consequences. This hypothesis is supported by several other studies in which higher background comet assay results were related to increased risk for cancer at different sites [7-9]. A corollary is that being cancer-free at advanced age might be associated with maintaining lower levels of endogenous damage. Analyses of such hyper-normal individuals have been limited generally because laboratory, clinical or hospital studies infrequently have a population base from which to select a hyper-normal group. However, enriching sample selection by various subject attributes may become more common as this design may offer gains in study power (reviewed in [25]). The ability to explore such concepts is a strength of the large U.S. Radiologic Technologist cohort utilized in this study.

In summary, we present results that indicate increased endogenous DNA damage may increase risk for multiple cancers (that include breast cancer) and thyroid cancer. Our conclusions are strengthened by the observation that decreased endogenous DNA damage was suggestively associated with being long-lived, cancer-free, and without a history of cancer in first degree relatives. Together these results indicate a continuum of DNA damage exists and may be detected

by phenotypic assays. If replicated in a large prospective study, relatively simple functional assays such as this could contribute to identification of persons with increased cancer susceptibility.

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Table 1. Comet data for each subject in the control and case groups by ascending values of tail length and quality control result values for the blinded replicates and blinded repeats.

ID number	Gender	Age at time of blood draw	Tail length	Tail DNA	Comet Distributed Moment	Olive Tail Moment	Group*
144	F	45	15.4	6.7	17.6	1.5	Control
273	F	57	17.7	6.1	17.9	1.4	Control
29	F	43	17.8	11.9	20.8	3.5	Control
25	F	60	17.9	6.6	18.5	1.6	Control
129	F	60	23.1	6.9	19.4	1.8	Control
110	F	66	23.5	6.4	17.1	1.4	Control
132	F	74	23.9	7.6	19.0	1.7	Control
74	F	53	24.2	10.1	19.9	2.6	Control
261	F	42	24.3	7.7	18.1	1.7	Control
216	F	76	26.3	8.4	22.0	2.2	Control
15	F	42	27.5	10.4	20.6	2.7	Control
43	F	69	27.7	10.0	20.8	2.7	Control
16	F	53	27.7	8.5	18.0	2.2	Control
134	F	90	27.9	9.3	19.8	2.1	Control
81	F	53	28.0	6.3	18.5	1.8	Control
34	F	82	28.3	7.7	20.5	2.1	Control
213	F	48	29.3	9.8	19.9	2.2	Control
35	F	81	31.5	10.0	19.9	2.6	Control
249	F	71	31.6	8.7	19.1	2.1	Control
79	F	51	32.3	9.0	16.2	1.9	Control
235	F	62	32.6	9.6	20.3	2.3	Control
233	F	65	33.0	10.3	22.7	2.8	Control
88	F	50	33.1	9.9	18.8	2.5	Control
68	F	61	33.3	9.2	19.5	2.4	Control
154	F	43	33.3	12.6	20.8	3.2	Control
101	F	68	33.6	7.7	20.5	2.1	Control
165	F	83	34.3	9.5	20.5	2.6	Control
195	F	71	34.5	8.3	21.3	2.3	Control
24	F	86	36.0	6.9	17.4	1.7	Control
92	F	74	36.2	10.0	19.5	2.4	Control
223	F	82	36.6	8.7	18.1	2.1	Control
50	F	46	37.0	10.5	17.6	2.4	Control
136	F	79	37.6	9.4	18.6	2.2	Control
151	F	71	38.6	11.2	21.3	3.0	Control
240	F	51	39.6	9.5	17.7	2.5	Control
214	F	61	41.9	11.7	23.0	3.0	Control
118	F	64	43.3	12.4	19.7	3.1	Control
3	F	46	45.4	9.9	18.7	2.5	Control
172	F	50	47.3	10.7	19.2	2.9	Control
231	F	71	47.3	11.8	18.9	2.8	Control
126	F	54	47.5	11.0	19.8	2.8	Control
52	F	84	48.5	9.6	19.5	2.5	Control
113	F	49	49.3	10.8	19.3	2.7	Control
38	F	64	49.4	9.9	20.1	2.6	Control
212	F	68	49.9	12.1	21.1	3.1	Control

95	F	74	50.1	9.3	18.8	2.5	Control
104	F	46	50.8	15.9	23.5	4.4	Control
178	F	65	53.7	10.0	18.6	2.6	Control
44	F	63	56.8	13.4	20.4	3.5	Control
148	F	89	10.2	5.9	17.8	1.3	H-Normal
255	F	72	12.4	8.3	21.1	2.3	H-Normal
122	F	77	19.9	6.1	18.4	1.5	H-Normal
185	F	89	21.3	8.4	22.2	2.4	H-Normal
269	F	78	21.6	6.2	20.1	1.5	H-Normal
67	F	71	26.4	8.5	21.2	2.3	H-Normal
2	F	70	26.5	6.3	17.4	1.4	H-Normal
141	F	78	26.8	10.3	21.5	2.5	H-Normal
215	F	72	28.1	7.6	18.8	1.9	H-Normal
133	F	70	28.4	8.1	20.8	2.1	H-Normal
45	F	75	30.9	7.4	17.6	1.8	H-Normal
149	F	70	31.1	8.1	18.4	1.9	H-Normal
251	F	71	31.5	9.4	22.9	2.4	H-Normal
150	F	85	31.6	6.4	17.6	1.6	H-Normal
245	F	73	32.0	8.0	21.9	2.0	H-Normal
180	F	81	33.5	6.8	17.0	1.5	H-Normal
202	F	74	35.6	8.5	21.2	2.1	H-Normal
107	F	70	40.1	12.3	22.8	3.1	H-Normal
219	F	80	51.2	11.5	20.4	3.0	H-Normal
119	F	79	51.9	9.3	18.7	2.4	H-Normal
112	F	67	18.7	6.3	19.5	1.6	Breast Plus
226	F	70	27.4	11.0	25.9	3.0	Breast Plus
194	F	76	29.2	8.0	20.5	2.0	Breast Plus
32	F	83	29.5	8.0	18.6	2.1	Breast Plus
250	F	67	29.8	9.3	21.7	2.3	Breast Plus
55	F	80	34.1	10.0	22.6	2.6	Breast Plus
218	F	57	34.9	9.2	18.2	2.4	Breast Plus
127	F	63	35.2	9.1	19.9	2.1	Breast Plus
140	F	70	35.5	10.6	22.7	2.8	Breast Plus
63	F	76	37.4	9.0	20.7	2.3	Breast Plus
152	F	63	38.0	10.8	19.5	2.8	Breast Plus
210	F	59	38.4	8.8	20.4	2.3	Breast Plus
143	F	76	39.3	8.7	21.2	2.3	Breast Plus
222	F	82	41.2	9.1	20.5	2.4	Breast Plus
49	F	49	41.2	13.8	25.5	3.6	Breast Plus
196	F	48	41.7	8.3	18.5	2.2	Breast Plus
70	F	82	43.1	9.1	20.0	2.6	Breast Plus
211	F	43	43.1	10.8	20.0	2.9	Breast Plus
183	F	69	44.4	10.2	18.4	2.4	Breast Plus
182	F	75	44.6	10.4	19.2	2.6	Breast Plus
189	F	45	44.6	9.7	19.9	2.6	Breast Plus
26	F	76	45.2	13.0	25.6	3.9	Breast Plus
174	F	47	46.4	10.9	19.2	2.9	Breast Plus
203	F	62	47.2	9.4	19.3	2.4	Breast Plus
197	F	59	47.7	11.3	20.8	3.0	Breast Plus
65	F	84	48.0	11.9	20.1	3.2	Breast Plus
130	F	77	48.3	9.8	19.1	2.5	Breast Plus

114	F	65	48.8	11.1	20.0	2.9	Breast Plus
84	F	63	49.6	9.6	20.5	2.7	Breast Plus
187	F	57	50.1	9.6	21.3	2.6	Breast Plus
40	F	64	50.2	11.2	20.3	2.9	Breast Plus
125	F	62	50.5	12.3	20.4	3.1	Breast Plus
206	F	72	51.2	10.3	18.9	2.7	Breast Plus
60	F	90	52.0	10.6	20.7	2.9	Breast Plus
80	F	74	52.3	13.7	23.3	3.8	Breast Plus
53	F	65	52.5	10.1	19.6	2.7	Breast Plus
236	F	58	52.9	9.7	18.9	2.7	Breast Plus
21	F	49	53.0	9.1	19.4	2.5	Breast Plus
76	F	48	56.9	12.5	21.3	3.4	Breast Plus
262	F	65	57.4	13.1	20.8	3.4	Breast Plus
94	F	70	57.7	12.8	22.1	3.7	Breast Plus
171	F	65	64.8	8.6	19.1	2.3	Breast Plus
167	F	48	28.8	7.5	17.8	1.8	Early onset
10	F	59	28.9	8.0	17.9	2.1	Early onset
14	F	67	31.1	7.3	18.9	1.8	Early onset
71	F	51	31.2	9.8	20.0	2.7	Early onset
153	F	58	31.9	8.9	18.3	2.4	Early onset
166	F	44	32.9	7.6	18.2	2.0	Early onset
77	F	64	33.1	8.3	18.2	2.1	Early onset
266	F	50	33.5	8.2	18.6	2.3	Early onset
164	F	62	33.8	8.1	18.5	2.1	Early onset
177	F	45	34.5	8.1	18.8	2.0	Early onset
200	F	49	34.7	8.9	18.2	2.3	Early onset
234	F	50	35.2	10.4	18.1	2.5	Early onset
83	F	47	37.2	7.4	16.8	1.9	Early onset
111	F	51	38.1	10.6	18.8	2.5	Early onset
28	F	42	39.7	8.7	18.8	2.3	Early onset
82	F	45	40.1	9.8	18.2	2.4	Early onset
274	F	57	40.5	9.2	18.5	2.3	Early onset
208	F	51	41.0	10.1	18.8	2.5	Early onset
254	F	48	41.8	10.6	20.2	2.7	Early onset
158	F	55	42.9	9.4	18.6	2.5	Early onset
169	F	45	44.5	9.3	20.0	2.7	Early onset
9	F	56	45.2	9.4	18.9	2.3	Early onset
237	F	49	45.8	10.2	18.9	2.5	Early onset
175	F	46	47.1	10.8	19.2	2.8	Early onset
173	F	70	48.4	9.9	18.6	2.7	Early onset
207	F	47	48.7	9.3	18.5	2.4	Early onset
252	F	60	48.8	10.1	18.7	2.6	Early onset
163	F	59	49.2	9.9	18.5	2.7	Early onset
8	F	49	50.4	10.0	19.4	2.9	Early onset
157	F	45	50.6	9.1	19.4	2.5	Early onset
267	F	55	50.7	11.9	20.7	3.3	Early onset
103	F	48	50.9	12.8	21.3	3.3	Early onset
264	F	59	51.8	10.2	20.2	2.9	Early onset
135	F	50	52.7	9.7	18.8	2.5	Early onset
191	F	67	53.3	9.5	23.0	2.8	Early onset
147	F	44	54.9	9.8	21.0	2.8	Early onset

6	F	46	55.9	10.2	19.7	2.8	Early onset
108	F	53	56.7	13.1	20.4	3.5	Early onset
51	F	53	26.8	7.6	18.0	1.8	Thyroid
75	F	42	29.9	7.0	16.2	1.4	Thyroid
46	F	53	30.7	7.2	17.9	1.7	Thyroid
256	F	49	32.9	7.7	18.0	2.0	Thyroid
272	F	48	33.1	8.0	17.6	1.7	Thyroid
105	F	54	33.2	6.9	17.7	1.6	Thyroid
17	F	63	34.1	8.7	18.7	2.4	Thyroid
271	F	52	34.5	8.6	19.6	2.0	Thyroid
225	M	57	34.7	8.8	18.8	2.1	Thyroid
176	F	48	35.1	7.0	18.2	1.7	Thyroid
109	F	56	36.9	7.6	17.1	1.8	Thyroid
61	F	42	37.2	6.9	18.1	1.6	Thyroid
263	F	54	38.7	7.8	18.7	1.9	Thyroid
42	F	41	39.6	8.6	18.9	2.3	Thyroid
217	F	66	39.8	8.9	19.0	2.3	Thyroid
85	F	58	39.8	7.7	18.4	2.1	Thyroid
209	F	56	40.2	8.4	18.5	2.0	Thyroid
72	F	49	40.3	9.0	19.5	2.2	Thyroid
162	F	61	40.8	7.3	18.5	2.2	Thyroid
159	M	44	42.3	9.1	19.8	2.5	Thyroid
247	M	71	43.8	11.4	21.1	3.1	Thyroid
7	F	66	45.2	10.7	20.2	3.0	Thyroid
33	F	53	45.4	9.4	17.8	2.3	Thyroid
23	F	53	45.7	12.2	21.0	3.1	Thyroid
87	F	63	45.7	10.8	19.1	2.8	Thyroid
19	F	48	46.4	12.0	20.5	3.3	Thyroid
224	F	44	46.7	10.1	19.6	2.8	Thyroid
78	F	63	47.0	11.3	20.6	2.9	Thyroid
181	F	46	47.0	11.0	21.9	2.9	Thyroid
259	F	51	47.4	10.1	19.3	2.7	Thyroid
260	F	71	47.5	10.8	20.1	2.8	Thyroid
241	F	74	47.6	10.8	21.5	2.9	Thyroid
270	F	54	47.7	9.8	20.4	2.6	Thyroid
275	F	74	48.0	11.8	17.8	2.9	Thyroid
193	F	49	48.5	8.4	17.3	2.1	Thyroid
1	F	60	48.7	10.2	19.0	2.6	Thyroid
98	F	48	49.3	9.7	19.5	2.6	Thyroid
131	F	55	49.4	9.2	19.2	2.6	Thyroid
246	F	66	49.4	11.1	20.0	2.8	Thyroid
117	F	90	49.7	10.4	21.5	2.8	Thyroid
160	F	62	50.2	9.7	18.3	2.4	Thyroid
186	F	51	50.2	9.7	18.3	2.4	Thyroid
99	F	70	51.3	9.8	20.0	2.6	Thyroid
248	F	58	51.5	9.5	20.2	2.7	Thyroid
161	F	68	51.7	12.7	20.7	3.2	Thyroid
137	F	57	51.9	9.7	19.2	2.6	Thyroid
239	F	45	52.0	8.6	21.2	2.5	Thyroid
242	F	65	52.0	9.6	20.3	2.6	Thyroid
229	F	53	52.3	11.2	20.0	3.0	Thyroid

22	F	45	52.4	9.1	19.1	2.6	Thyroid
86	F	45	52.4	9.0	20.4	2.5	Thyroid
258	F	54	52.6	10.8	21.5	3.1	Thyroid
18	M	47	53.0	11.0	21.4	3.0	Thyroid
11	F	55	53.2	12.0	21.7	3.4	Thyroid
227	F	56	53.2	11.6	20.6	3.0	Thyroid
253	M	56	53.4	9.8	20.9	2.8	Thyroid
27	F	52	53.7	9.1	18.7	2.5	Thyroid
13	M	82	53.8	11.4	20.4	2.8	Thyroid
64	F	43	53.9	11.9	22.0	3.4	Thyroid
31	F	78	54.2	12.1	22.1	3.2	Thyroid
48	F	69	54.4	9.8	18.2	2.6	Thyroid
12	M	66	54.7	11.0	19.9	2.9	Thyroid
73	F	60	55.4	11.7	20.0	3.0	Thyroid
37	M	51	55.7	11.0	19.0	2.8	Thyroid
220	F	54	56.3	12.9	20.3	3.2	Thyroid
30	F	54	56.7	10.4	20.5	2.9	Thyroid
66	M	69	60.5	10.5	20.2	2.9	Thyroid
41	F	50	61.7	14.5	20.4	3.7	Thyroid
A1	-	-	32.9	7.7	18.0	2.0	Replicate
A1	-	-	37.6	9.1	19.3	2.4	Replicate
B2	-	-	40.2	8.4	18.5	2.0	Replicate
B2	-	-	51.8	14.7	21.3	3.8	Replicate
C3	-	-	46.9	8.2	18.9	2.2	Replicate
C3	-	-	51.2	12.2	19.3	3.0	Replicate
D4	-	-	52.1	10.3	19.3	2.7	Replicate
D4	-	-	56.8	10.6	19.5	2.8	Replicate
E5	-	-	51.3	9.8	20.0	2.6	Replicate
E5	-	-	41.6	9.5	19.7	2.5	Replicate
R1	-	-	41.0	10.1	18.8	2.5	Repeat
R1	-	-	37.7	8.1	17.6	1.9	Repeat
R2	-	-	43.6	9.8	18.2	2.4	Repeat
R2	-	-	42.3	8.7	17.9	2.0	Repeat
R3	-	-	39.8	8.1	18.5	2.1	Repeat
R3	-	-	31.8	6.9	17.0	1.6	Repeat
R4	-	-	41.8	10.6	20.2	2.8	Repeat
R4	-	-	32.6	8.9	17.8	2.0	Repeat
R5	-	-	39.3	9.2	19.5	2.4	Repeat
R5	-	-	40.9	9.9	19.1	2.4	Repeat
R6	-	-	36.0	9.9	18.8	2.5	Repeat
R6	-	-	44.8	8.3	17.8	2.1	Repeat
R7	-	-	34.2	10.0	22.6	2.6	Repeat
R7	-	-	26.6	6.0	16.0	1.3	Repeat
R8	-	-	29.6	8.0	18.6	2.1	Repeat
R8	-	-	30.8	6.2	16.4	1.5	Repeat
R9	-	-	36.0	9.3	20.0	2.1	Repeat
R9	-	-	27.7	5.0	17.0	1.3	Repeat
R10	-	-	46.9	9.1	20.0	2.6	Repeat
R10	-	-	46.9	7.8	17.4	1.9	Repeat
R11	-	-	52.0	10.6	20.7	2.9	Repeat
R11	-	-	29.0	5.3	16.0	1.2	Repeat

R12	-	-	49.6	9.6	20.5	2.7	Repeat
R12	-	-	31.1	6.1	16.0	1.4	Repeat
R13	-	-	50.5	12.3	20.4	3.1	Repeat
R13	-	-	29.9	5.1	16.0	1.2	Repeat
R14	-	-	51.9	10.8	21.5	2.9	Repeat
R14	-	-	36.5	9.2	18.9	2.4	Repeat
R15	-	-	51.5	9.5	20.2	2.7	Repeat
R15	-	-	37.2	7.9	17.7	1.9	Repeat
R16	-	-	53.0	9.1	19.4	2.5	Repeat
R16	-	-	36.6	9.0	20.4	2.3	Repeat

*Group designations--Control: Cancer-free individuals, H-Normal: Hypernormal "controls" (were age 70 or older at the time of blood collection, had not reported a personal history of any cancer, and they had reported no invasive cancers in their first degree relatives at the time of blood collection, Breast Plus: Personal history of breast cancer and other type(s) of invasive cancer, Early onset: Diagnosed with breast cancer age 35 or younger, Thyroid: Personal history of thyroid cancer, Replicate: Five blinded replicate cell lines embedded in sample shipments, Repeat: 16 samples repeated, blinded as to results of earlier assays.

Table 2: Arithmetic means of the comet parameter tail length by selected characteristics of cancer case and control groups

Characteristic at time of blood draw (1997-2003)	Control groups				Case groups					
	Normal Controls (n=49)		Hyper-normal Controls (n=20)		Breast cancer and one or more other cancers (n=42)		Breast cancer diagnosed at age 35 or younger (n=38)		Thyroid cancer n=68	
	n	Mean (se*)	n	Mean (se)	n	Mean (se)	n	Mean (se)	n	Mean (se)
Age in years										
40-49	10	33.0 (4.0)	0	NA[†]	7	46.7 (2.3)	17	43.4 (2.0)	18	44.0 (1.9)
50-59	9	33.0 (3.4)	0	NA	5	44.8 (3.5)	15	42.0 (2.3)	27	46.4 (1.8)
60-69	14	37.1 (3.3)	0	NA	13	45.2 (3.4)	5	40.0 (4.6)	15	48.6 (1.8)
70-79	9	36.2 (2.9)	15	29.5 (2.3)	11	42.6 (2.9)	1	48.4 (--[‡])	6	48.7 (1.5)
80 or older	7	34.7 (2.6)	5	29.6 (6.8)	6	41.3 (3.4)	0	NA	2	51.7 (2.1)
Gender										
Female[§]	49	35.0 (1.5)	25	29.5 (2.3)	42	44.2 (1.5)	38	42.5 (1.4)	59	46.1 (1.0)
Male[‖]	0	NA	0	NA	0	NA	0	NA	9	50.2 (2.7)
Years between diagnosis of the first cancer and blood collection										
0-9	0	NA	0	NA	7	49.0 (2.4)	2	47.3 (7.6)	27	44.4 (1.7)
10-19	0	NA	0	NA	13	40.1 (2.9)	19	42.6 (1.9)	17	46.4 (2.1)
20-29	0	NA	0	NA	12	42.9 (2.3)	11	41.1 (2.7)	11	49.6 (2.0)
30 or more years	0	NA	0	NA	10	47.5 (3.2)	6	43.3 (3.7)	13	49.0 (1.4)
Number of breast cancers reported in first degree relatives										
None	41	34.0 (1.6)	20	29.5 (2.3)	35	44.4 (1.6)	25	42.5 (1.9)	55	46.5 (1.1)
One	7	37.8 (3.4)	0	NA	5	42.9 (4.5)	10	42.3 (2.3)	12	46.8 (2.0)
Two or more	1	56.8 (--)	0	NA	2	42.2 (2.9)	3	43.5 (3.8)	1	53.4 (--)
Reported radiation treatment for cancer (not including ^{131}Iodine; missing data not included)										
No	0	NA	0	NA	26	44.6 (2.0)	23	43.1 (1.7)	52	46.5 (1.2)
Yes	0	NA	0	NA	14	43.3 (2.0)	14	41.1 (2.4)	14	48.0 (1.7)

* SE - Standard Error
† NA - Not applicable
‡ Cell contained only one value, standard error cannot be computed
§ The differences in tail length means were borderline significantly different between controls and hypernormal controls (p=0.052); the means for all the case groups differed significantly from the mean for normal controls (p < or = 0.001).
‖ The difference in male and female mean tail length was not statistically significant (p=0.15).

Table 3. Associations between comet parameters and the case groups of breast and other cancer, early-onset breast cancer (diagnosed age 35 or younger), thyroid cancer, and hyper-normal controls, among US Radiologic Technologists

Case group and comet parameter*	Number of normal controls	Number of cases	Adjusted odds ratios[†] OR	95% CI	*P* for trend[‡]
Breast and other cancer					
Tail DNA					
1	24	16	1.0	Referent	
2	13	9	1.3	0.4-4.0	
3	12	17	3.0	1.0-8.8[§]	0.047
Tail length					
1	24	5	1.0	Referent	
2	13	13	4.4	1.2-15.6	
3	12	24	10.1	3.0-34.6	<0.001
CDM					
1	24	12	1.0	Referent	
2	13	13	1.7	0.6-5.0	
3	12	17	2.5	0.9-7.0	0.085
OTM					
1	24	14	1.0	Referent	
2	13	11	1.7	0.6-5.0	
3	12	17	3.1	1.1-9.1	0.035
Early-onset breast cancer					
Tail DNA					
1	24	19	1.0	Referent	
2	13	13	0.8	0.3-2.3	
3	12	6	0.4	0.1-1.2	(0.113)[‖]
Tail length					
1	24	7	1.0	Referent	
2	13	13	4.0	1.2-13.5	
3	12	18	4.7	1.5-14.8	0.010
CDM					

1	24	28	1.0	Referent	
2	13	6	0.4	0.1-1.4	
3	12	4	0.3	0.1-1.1	(0.048)
OTM					
1	24	16	1.0	Referent	
2	13	13	1.3	0.4-3.9	
3	12	9	0.8	0.3-2.5	(0.77)
Thyroid Cancer					
Tail DNA					
1	24	29	1.0	Referent	
2	13	14	0.8	0.3-2.1	
3	12	25	1.6	0.6-3.9	0.37
Tail length					
1	24	6	1.0	Referent	
2	13	15	6.1	1.8-21.3	
3	12	47	19.1	5.9-61.8	<0.001
CDM					
1	24	32	1.0	Referent	
2	13	19	1.3	0.5-3.2	
3	12	17	1.2	0.5-3.2	0.64
OTM					
1	24	23	1.0	Referent	
2	13	15	1.2	0.4-3.1	
3	12	30	2.6	1.1-6.6	0.040
"Hyper-normal" controls*					
Tail DNA					
1	24	17	1.0	Referent	
2	13	1	0.2	0.0-1.5	
3	12	2	0.4	0.1-2.6	(0.07)
Tail length					
1	24	15	1.0	Referent	
2	13	3	0.3	0.1-1.2	
3	12	2	0.3	0.1-1.5	(0.06)

CDM					
1	24	9	1.0	Referent	
2	13	2	0.3	0.0-1.7	
3	12	9	2.2	0.6-9.0	0.57
OTM					
1	24	16	1.0	Referent	
2	13	2	0.3	0.1-1.6	
3	12	2	0.4	0.1-2.3	(0.18)

* Comet parameters were divided into three categories at the median and 75th percentile of the control distribution. These cut-points were: Tail DNA (%), 9.6 and 10.5; Tail Length (μm), 33.3 and 44.0; Comet Distributed Moment (CDM; arbitrary units), 19.5 and 20.5; Olive Tail Moment (OTM; arbitrary units), 2.44 and 2.75.

† Adjusted for age at the time of blood collection using three categories: 40-54, 55-74, and 75 years or older

‡ p for trend adjusted for age and based on the continuous underlying variable

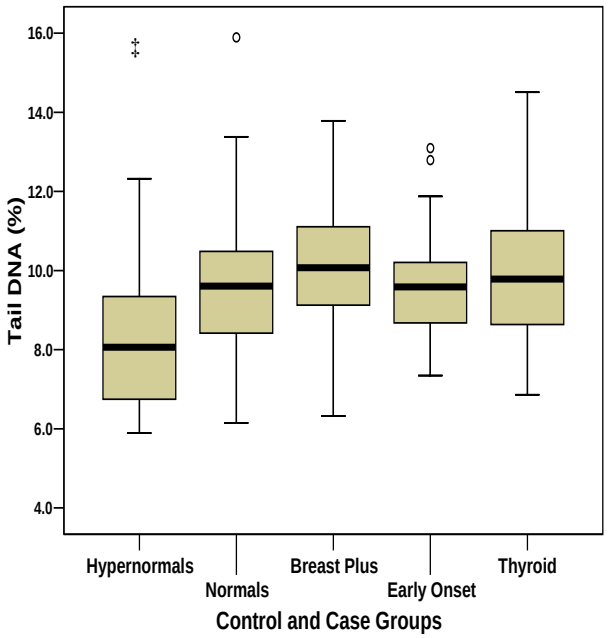
§ Confidence interval excludes 1.0, $P < 0.05$

‖ Parentheses indicate a negative beta coefficient for the trend test (negative slope)

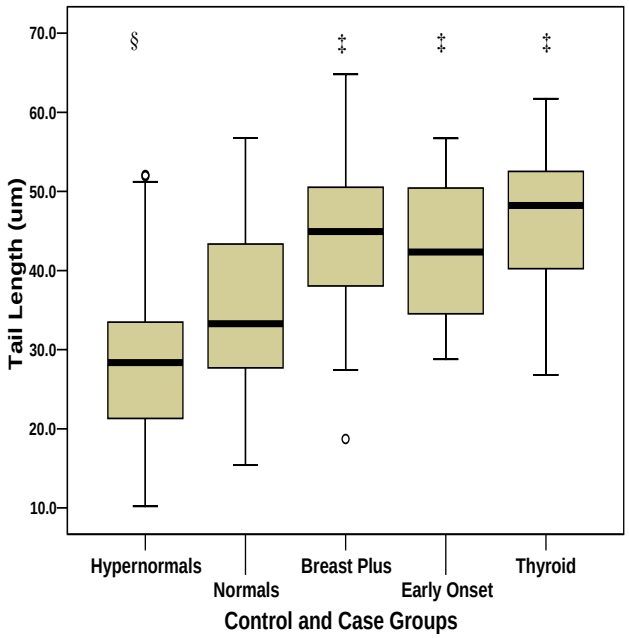
** "Hypernormal" controls were age 70 or older at the time of blood collection, had not reported a personal history of cancer (we also excluded those with non-melanoma skin cancer), and they had reported no invasive cancers in their family at the time of blood collection.

Figure 1. Boxplots* of comet tail DNA, tail length, comet distributed moment and Olive tail moment among cases and controls†, US Radiologic Technologist Study

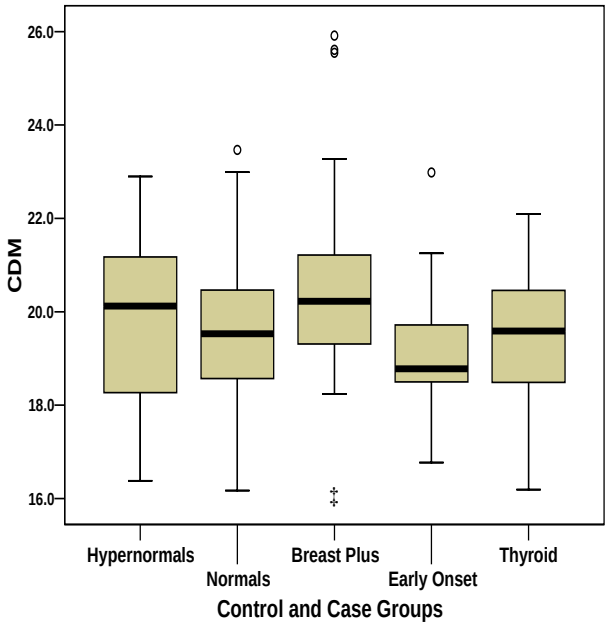
a. Tail DNA in %



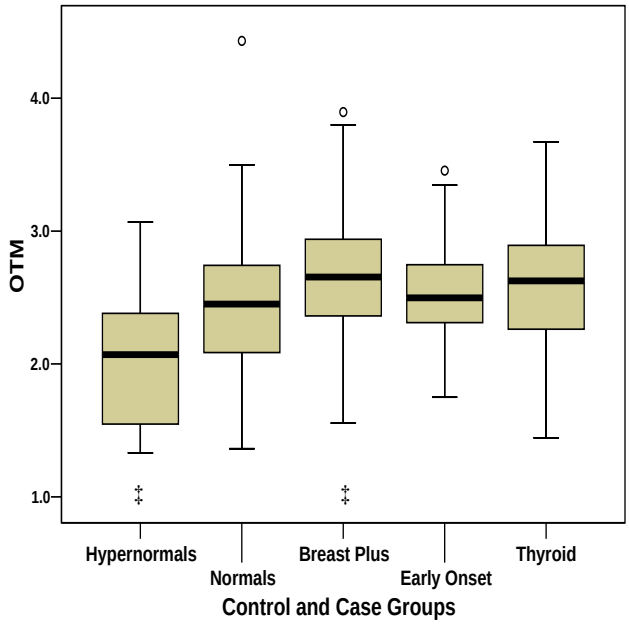
b. Tail Length in µm



c. Comet Distributed Moment (CDM)



d. Olive Tail Moment (OTM)



* Each boxplot represents the geometric means of the respective comet parameter of 100 cells for each individual within case or control 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 (o).

† Case and control groups are: Normal controls, n=49; Breast Plus (Breast cancer and other cancer), n=42; Early-onset (breast cancer diagnosed at age ≤ 35 years), n=38; Thyroid cancer, n=68; Hyper-normal controls (selected as long-lived, cancer-free, no cancer reported in first-degree relatives at blood collection), n=20.

‡ The mean for the indicated group is significantly different from the mean in controls ($p < 0.05$)
§ The mean for the indicated group is borderline significantly different from the mean in controls ($p=0.052$)