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Original article

Inter- and intra-tumoral heterogeneity in DNA damage evaluated by comet assay in early breast cancer patients

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ABSTRACT

There are no clinical tools to functionally assess degree of DNA damage in breast cancer. The comet assay is an accepted research tool for assessing DNA damage, however, most cancer studies have assessed lymphocytes as surrogate cells. The aim of this pilot study was to use the comet assay in early breast cancer directly in tumor tissue to compare DNA damage between and within traditionally defined subgroups, and to explore intra-tumoral heterogeneity. Scrapings of tumor and healthy breast tissue were obtained at primary surgery from 104 women. Comet assay was applied to quantitatively assess DNA damage, revealing substantial inter- and intra-subgroup variation. Marked intra-tumoral heterogeneity was evident across all subgroups. The degree of DNA damage for an individual could not be predicted by breast cancer subgroup. Comet assay warrants further study as a potential clinical tool for identification of tumoral DNA damage and ultimately, individualised use of DNA damaging therapy.

Introduction

Breast cancers with a high degree of innate DNA damage may be particularly vulnerable to treatments that further damage DNA or further inhibit DNA repair mechanisms. DNA damage appears critical, but not confined, to immunohistochemistry-defined triple negative breast cancer (TNBC). TNBC overlaps substantially but incompletely with molecularly defined basal-like breast cancer and BRCA1-mutation associated breast cancers.[1,2]

Several potential clinical tools have been employed to explore DNA damage and predict sensitivity to DNA damaging therapy in breast cancer. Promising results have emerged using immunohistochemical and molecular approaches.[3–6] Another promising tool is the comet assay.[7]

Comet is a gel electrophoresis assay which allows rapid detection and quantification of DNA strand breaks in individual cells.[7] Gel-embedded single cells are lysed to isolate nucleoids, which contain supercoiled loops of DNA linked to the nuclear matrix. Loops of DNA whose supercoiling is relaxed by a strand break can unwind and are able to extend under electrophoresis toward the anode. Cells with DNA damage appear as 'comets' under fluorescence microscopy due to tails of relaxed DNA loops extending from the nucleoid. The comet tail length and tail fluorescence reflect extent of damage. Tail length and intensity both increase linearly at low levels of DNA damage, beyond which, intensity continues to increase linearly without further increase in tail length. The standard comet assay provides information about DNA damage, while modified applications specify types of DNA damage and assess DNA repair capacity to external insults.[7–9]

The comet assay is simple, sensitive, rapid and inexpensive. It has been employed to investigate DNA damage and repair in different cell types in response to a range of DNA damaging agents.[7,10] In breast cancer, it has been applied in cell line assays and in clinical studies to assess tamoxifen and chemotherapy-induced DNA damage.[11–14] Most clinical comet analyses in cancer patients have used peripheral blood mononuclear cells (PBMC) as a tumor surrogate.

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PBMC are attractive for ease of collection however, such an approach assumes homogeneous DNA damage and repair ability between healthy PBMC and tumor. *In vitro* colon cancer studies reveal poor correlation in DNA repair activity between PBMC and tumor.[15]

We have used the comet assay to measure innate DNA damage in tumor and normal breast tissue from early breast cancer patients. We chose to work directly on tumor tissue rather than a surrogate cell. We chose to examine all breast cancer subtypes, not only TNBC. Our aim was to determine if a correlation exists between breast cancer subgroups and DNA damage. We hypothesised that (1) DNA damage would be greater in breast cancer compared with normal breast, (2) DNA damage would be seen predominantly but not exclusively in TNBC, and (3) not all TNBC would have substantial DNA damage.

Patients and methods

Patients with early breast cancer were recruited from the Breast Surgery Unit, Hospital of Prato, Italy between June 2008 and June 2010. The study received approval from the Institutional Ethical Committee. Patients provided written informed consent.

Tumor samples were obtained at the time of primary surgery. No patient had received neoadjuvant therapy. Tumors were assessed by a local pathologist and classified into 4 subgroups using immunohistochemical (IHC) markers and tumor grade.[16] See Table 1. IHC staining was carried out on glyoxal-fixed (Cell-Block, Bio-Optica) formalin embedded tumor samples using automated staining (Ventana Medical System Inc.).

Alkaline comet assays

Fresh tissue scrapings were collected from tumor and non-tumor healthy tissue within 20 min of surgical excision. Healthy tissue scrapings were collected as control, to assess iatrogenic DNA damage caused by the surgery, tissue hypoxia and scraping.

The Alkaline Comet Assay (CometAssay® Kit, Trevigen) was modified by the authors and applied as outlined: Single cells were separated by scalpel tissue scraping and collected in an ice-cold eppendorf tube containing phosphate buffered saline (0.5–1.0 mL, Dulbecco's phosphate buffered saline, Ca^{2+}/Mg^{2+} free, Euroclone). Cell aliquots (25 μL) were immersed in low melting point agarose (250 μL) and layered on slides precoated with normal melting point agarose.

Slides were incubated for 1 h at 4 °C in a freshly prepared lysis solution (CometAssay® Kit) to remove membranes and histones from the DNA. Nucleoids were then treated with alkaline buffer (pH 13, T 4 °C, CometAssay® Kit) for 40 min to allow denaturation and DNA unwinding.

Table 1
Thresholds for defining IHC positivity and tumor subgroups using IHC parameters and grade.

Thresholds for IHC parameter positivity	
• ER ≥ 10%	
• PgR ≥ 10%	
• HER2 3+ (or FISH HER2:CEP17 ratio ≥ 2.2)	
• Ki67 ≥ 20%	
• CK5/6 ≥ 1%	
Tumor subgroups	
Luminal A	ER+, PgR+, HER2−, Ki67−, and Grade 1 or 2
Luminal B	ER+ and ≥1 of PgR−, HER2+, Ki67+, and/or Grade 3
HER2+	ER−, PgR−, HER2+
TNBC	ER−, PgR−, HER2−
Subclassification of TNBC	
TNBC basal-like	ER−, PgR−, HER2−, CK5/6+
TNBC non basal-like	ER−, PgR−, HER2−, CK5/6−

Electrophoresis in an alkaline buffer (pH 13, T 4 °C, CometAssay® Kit) for 20 min allowed migration of loops of DNA whose super-coiling was relaxed by a strand break to migrate towards the anode. Slides were then soaked in a neutralization buffer (Tris base 400 mM pH 7.5) for 15 min, washed twice in distilled H_2O for 10 min and finally air dried to bring cells into a single plane to facilitate observation.

After staining with nucleic acid gel fluorochrome SYBR Green (CometAssay® Kit), single nucleoid migration profiles were analyzed with fluorescence microscopy (Leica DMRXA) connected to a camera (Leica DFC340 FX) and a computerized image analysis system (COMET IV software, Perceptive).

For each patient, approximately 100 tumor cells and up to 100 surrounding healthy cells were measured. Individual cells were selected randomly by the operator. For each cell, the software computed all major measurement parameters, including (1) Tail Length: distance from centre of nucleoid mass to distal tail end, (2) Tail Intensity (TI): relative fluorescence intensity of comet tail, a measure of percentage of DNA in the tail, and (3) Tail Moment (TM): essentially the product of tail length and TI. For our purposes TI and TM were selected to measure DNA fragmentation.

Cytochemical staining

To confirm the presence of healthy and tumor cells in the scrapings, cell samples were ethanol fixed, cytocentrifuged on positively charged slides (2500rpm, 5 min), stained with Papanicolau stain and reviewed by the pathologist.

Statistical analysis

For data analysis, TI and TM were used as markers of DNA damage. Tail length was used to calculate TM but was not used as a single parameter in further analysis as its linear correlation with DNA damage is only at low damage levels.

For each patient, 4 DNA damage markers were obtained from the raw TI and TM data: lr-meanTI: log [meanTI tumor cells/meanTI healthy cells], lr-medianTI: log [median TI tumor cells/median TI healthy cells], lr-meanTM: log [mean TM tumor cells/mean TM healthy cells], lr-medianTM: log [median TM cancer cells/median TM healthy cells]. The rationale for use of a ratio was to measure tumor DNA damage relative to DNA damage in healthy cells, thus incorporating iatrogenic DNA damage. The logarithm was used to normalise the raw data distribution.

Inter-subgroup analysis

Within each subgroup, the mean of the 4 previously defined markers was determined. ANOVA, Bartlett's test and Variance Ratio test were used to analyse the difference between subgroups by using modules in R (The R Foundation for Statistical Computing). To explore the degree of dispersion and the skewness of the data, box plots were constructed. Each plot was created by enclosing the subgroup median (single line) within 50% of values from 25th (Q1) to 75th (Q3) percentiles (boxed). The low whisker of the plot is defined as [Q1−1.5×(Q3−Q1)] while the high whisker is defined as [Q3 + 1.5×(Q3−Q1)]. Outliers lie outside the whiskers and were represented as points.

Intra-subgroup analysis

To describe DNA damage within subgroups, the distribution of the 4 DNA damage markers was divided arbitrarily into three groups of increasing DNA damage: low = lowest to 40th centile; medium = >40 up to 80th centile; and high = >80th centile. Each

patient was assigned as having low, medium or high DNA damage for each of the 4 markers.

Intra-tumor analysis

To explore the degree of intra-tumoral DNA damage within each patient, TI and TM were used. For brevity we report our analysis on TI. For each patient, the TI of each individual tumor cell was transformed to a log ratio using the mean TI of their healthy cells. Log TI ratios from all tumor cells of all patients were categorized into three groups using the same thresholds as for the intra-subgroup analysis: low = lowest to 40th centile; medium = >40 up to 80th centile; and high = >80th centile. For each patient, the frequency of tumor cells within low, medium and high DNA damage groups were expressed as a percentage of all tumor cells for that individual.

To assess if intra-tumoral heterogeneity in DNA damage was related to cell sampling procedures or, alternatively, if it was an innate tumor feature, we investigated DNA damage heterogeneity in non-cancer cells and compared results with those observed in cancer cells. Raw TI values (%) were used. We analyzed deviations (residuals) from patient mean TI values for healthy and tumor cells respectively. Residual box plots were presented according to the formula previously described.

When appropriate, statistical test of the null hypothesis was done at a significance level of 5%, two sided.

Results

One hundred and four early breast cancer patients were recruited; luminal A = 61, luminal B = 20, HER2 positive = 8, and TNBC = 15. In TNBC, 8 patients were non basal-like and 7 were basal-like.

The mean number of tumor cells analysed per patient was 95.2 (median = 100; range: 24–151). The mean number of healthy cells analysed per patient was 68.4 (median = 76.5; range: 5–101). Fig. 1 shows healthy (a) and cancer (b) cells stained with Papanicolau stain and healthy (c) and cancer (d) cells stained with SYBR Green.

Inter-subgroup analysis

Table 2 shows mean values and standard deviation of DNA damage markers by subgroup. Using ANOVA, subgroup means were significantly different from each other for 3 of 4 DNA damage parameters: lr-meanTI, lr-medianTI and lr-meanTM ($P = 0.01$, $P = 0.02$ and $P = 0.02$, respectively). The mean values of DNA fragmentation were lower in luminal A relative to other subgroups, however the study power is low for post hoc multiple comparisons. Within TNBC, basal-like tumors were not significantly different from non-basal like.

The subgroup variances (Bartlett-test) were significantly different from each other for 3 of the 4 DNA damage markers: lr-meanTI, lr-meanTM and lr-medianTM ($P = 0.03$, $P = 0.005$ and $P = 0.014$, respectively). Box plots of the 4 DNA markers show the distribution of DNA damage within subgroups (Fig. 2).

Luminal A consistently had the lowest median level of DNA fragmentation. TNBC had the highest median level of DNA fragmentation for 3 of 4 parameters. TNBC had the largest variability compared with other groups for 3 of 4 parameters: lr-meanTI, lr-meanTM and lr-medianTM ($P = 0.02$, $P = 0.003$ and $P = 0.003$, respectively) (Fig. 2). Of note, the lowest level of damage of all patients was detected in TNBC.

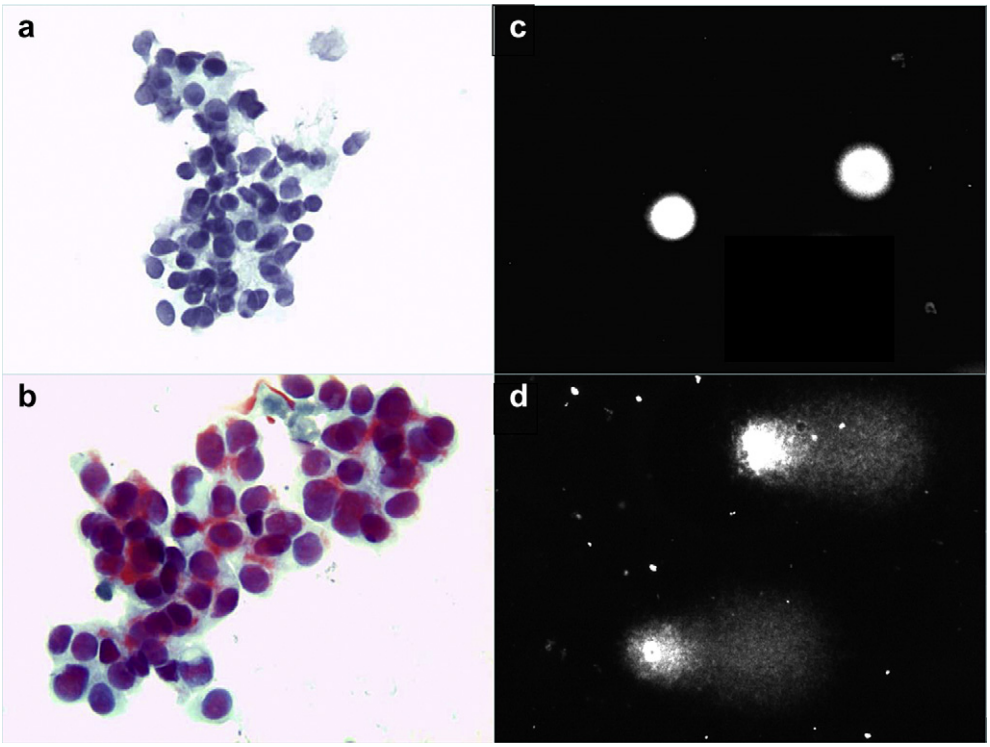

Fig. 1. Representative images. For each patient, the presence of healthy (a) and tumor (b) cells were confirmed on cell samples from healthy and tumor tissue scrapings. Using the COMET assay, cells with intact DNA appear as an oval nucleoid (c). Cells with DNA damage with strand breaks form a characteristic 'comet' due to a tail of relaxed DNA loops extending from the nucleoid towards the anode (d). For each cell, the COMET IV software (Perceptive) computed Tail Length (TL): the distance from the centre of the nucleoid to distal tail end; Tail Intensity (TI): the percentage of fluorescence in the tail compared with the nucleoid; and Tail Moment (TM): essentially a product of TL and TI.

Table 2
DNA damage markers in primary breast cancer tissue, divided by breast cancer subgroup.

IHC defined subgroups	DNA damage markers			
	lr-meanTI mean ± sd	lr-medianTI mean[a] ± sd	lr-meanTM mean ± sd	lr-medianTM mean[a] ± sd
Luminal A N = 61	0.33 ± 0.27	0.36 ± 0.34	0.53 ± 0.49	0.58 ± 0.57
Luminal B N = 20	0.54 ± 0.034	0.58 ± 0.37	0.97 ± 0.72	0.97 ± 0.77
HER2 positive *N* = 8	0.62 ± 0.38	0.73 ± 0.44	0.78 ± 0.53	0.93 ± 0.69
Triple negative *N* = 15	0.53 ± 0.48	0.60 ± 0.52	0.99 ± 0.98	1.07 ± 1.08
Triple negative subdivision				
Non-basal like *N* = 8	0.52 ± 0.58	0.58 ± 0.63	0.88 ± 1.02	0.98 ± 1.18
Basal-like *N* = 7	0.55 ± 0.37	0.61 ± 0.41	1.01 ± 0.98	1.17 ± 1.04

sd = standard deviation.
[a] Median values were averaged in order to apply ANOVA test.

Intra-subgroup analysis

Using categorized DNA damage markers, patients were defined accordingly as having high, medium or low DNA damage (Fig. 3) (For raw data, see Supplement Table). Within each IHC defined subgroup, DNA damage was heterogeneous. The majority of luminal A patients had low damage, but 5 patients (8%) had high damage by lr-meanTI and lr-medianTI, and 7 (11%) had high damage by lr-meanTM and lr-medianTM. In luminal B the majority had medium DNA damage. HER2 positive patients were concentrated in the medium-high DNA damage groups. Most TNBC cases had high damage, but 4 (27%) had low damage by lr-meanTI and lr-medianTI, and 5 (33%) had low damage by lr-meanTM and lr-medianTM.

Intra-tumor analysis

A certain degree of intra-tumor variability of DNA damage markers was observed. This intra-tumoral cellular diversity in degree of DNA damage was seen for TI and TM, in all 4 IHC defined subgroups (data not shown). Data is shown specifically for TI in TNBC. (Fig. 4).

Substantial inter-cellular heterogeneity in the degree of innate DNA damage in healthy tissue was also evident. The degree of DNA damage in healthy cells was consistently lower than that of tumor cells, across all subgroups. (Fig. 5a). The observed intra-tumoral heterogeneity could not be explained only by the sampling procedure, as the DNA damage induced by cell sampling procedure was constant across healthy and tumor tissue, and across subgroups. (Fig. 5b).

Discussion

Using the functional comet assay to assess intrinsic tumor DNA fragmentation, we show that the degree of DNA damage varies between IHC defined biological breast cancer subgroups, within subgroups and within individual tumors.

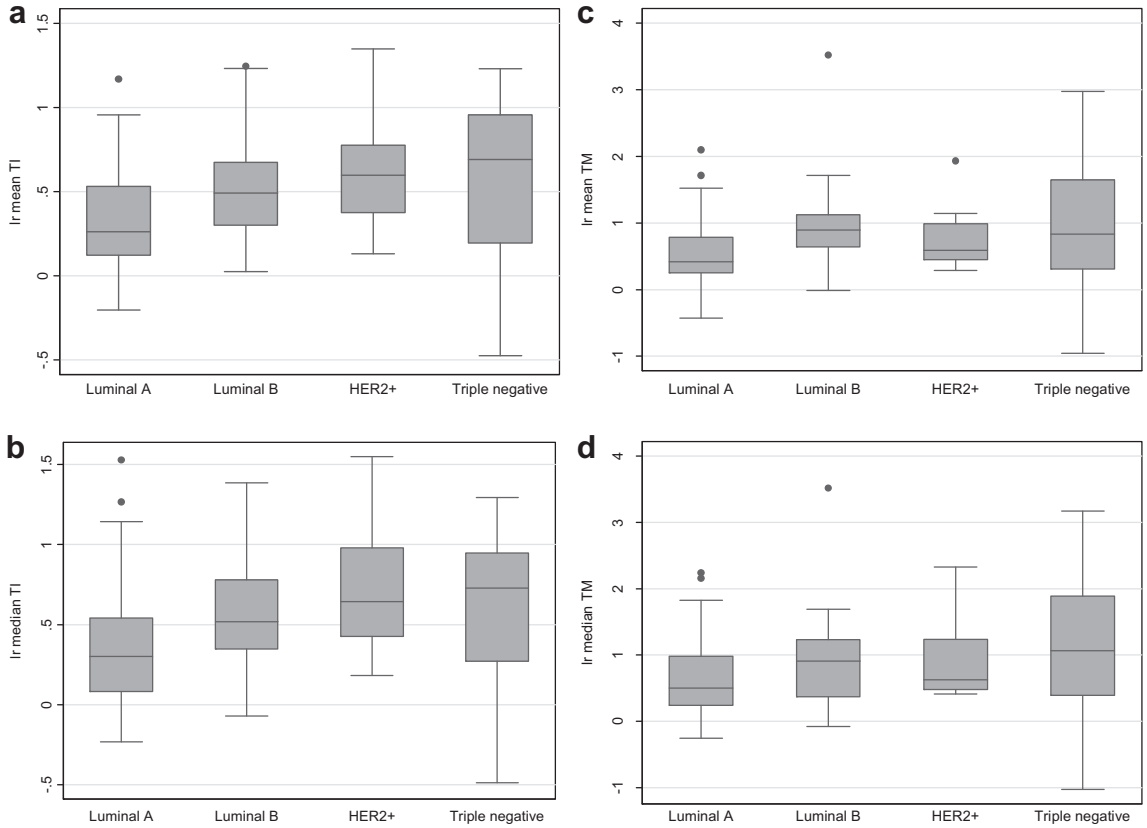

Fig. 2. DNA damage by Tail Moment (TM) and Tail Intensity (TI) in breast cancer subgroups. (a) lr-meanTI; (b) lr-medianTI; (c) lr-meanTM; (d) lr-medianTM.

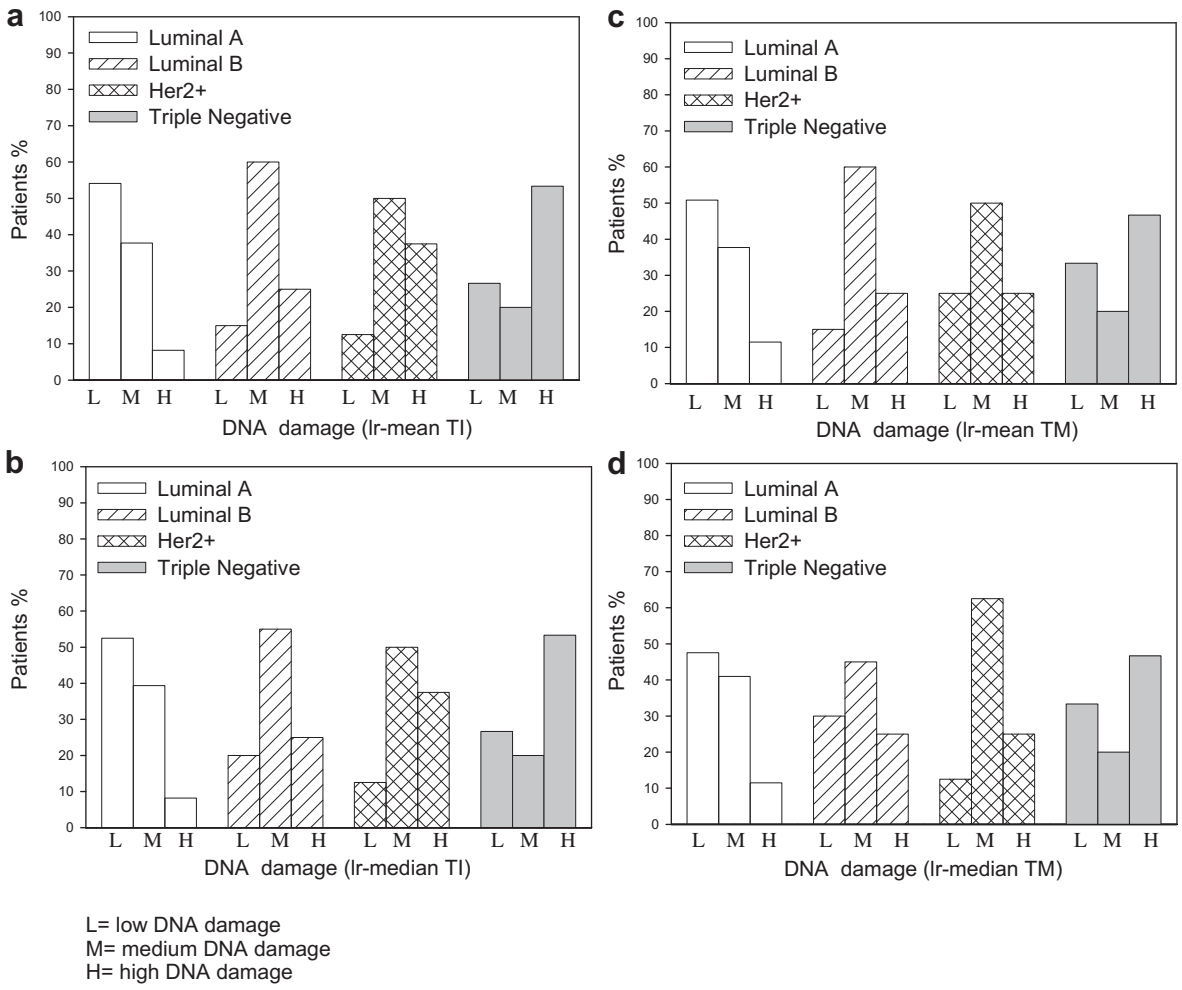

Fig. 3. DNA damage within IHC defined subgroups. (a) lr-meanTI; (b) lr-medianTI; (c) lr-meanTM; (d) lr-medianTM.

Significant differences were evident between biological subgroups. The highest median degree of DNA damage was observed in TNBC, while the lowest was observed in the luminal A subgroup. The difference between TNBC and luminal A was not statistically significant, however, the power for this type of analysis is low due to small patient numbers and unequal sample size between subgroups. Within TNBC cancer, limited by a small number of patients, no differential trends were seen between those with basal-like and non-basal like disease.

The inter-subgroup analysis allows exploration of differences in 'average DNA damage' between subgroups. However, substantial intra-subgroup variation limits the power of IHC defined subgroups to predict innate DNA damage for an individual. Some luminal A patients had high DNA damage, and some TNBC patients had low DNA damage. The greatest intra-subgroup variation was noted within TNBC, with a subgroup variance almost twice that of the other subgroups. As highlighted in Fig. 2, while the subgroup median level of DNA damage was highest in TNBC, not all individuals with TNBC had a high degree of damage. Indeed, the lowest mean level of fragmentation was in the TNBC subgroup.

Such variation within subgroups may in part explain the clinical experience of patients with luminal A 'good outlook' tumors relapsing despite appropriate adjuvant endocrine therapy, and conversely patients with 'poor outlook' TNBC tumors remaining disease-free in the long term in the absence of adjuvant systemic therapy.[17,18] Similarly, this biological heterogeneity may also explain diverse chemosensitivity within subgroups. A decade on from the pivotal publications of molecularly defined subgroups,[19] their prognostic power is proven but their predictive role remains unclear. Unlike ER for predicting response to endocrine therapy, or HER2 positivity for anti-HER2 therapy, the biological subgroups are not target specific. Functional assessment of treatment targetable pathways - independent of subgroup - may better guide therapeutic decision making. Some events may be across classes (e.g. DNA damage, topoisomerase IIα protein expression)[20] and some may be subclass specific (e.g. FGFR2 in TNBC).[21]

Intra-tumoral heterogeneity in degree of DNA damage was demonstrated, in all IHC defined subgroups. As patients in this study were naïve to systemic anti-tumor therapy, this is not a selective clonal pressure of treatment. Intra-tumoral heterogeneity has also been shown by distinct genomic subpopulations using multigene platforms[22] and HER2 positive clones in predominantly HER2 negative tumors.[23] The implications of such heterogeneity for outcomes are unclear. Minority clones may have critical prognostic and predictive implications. A potential reason for systemic treatment failure may, in part, be the assumption of homogeneous treatment sensitivity within an individual tumor.

A strength of the current study is direct assessment of tumor cells, rather than surrogate cells. Working directly on tumor requires fresh tumor scrapings. Potential surgical and scraping induced DNA damage was factored into the analyses by intra-patient comparison of tumor and healthy cells. Heterogeneity in

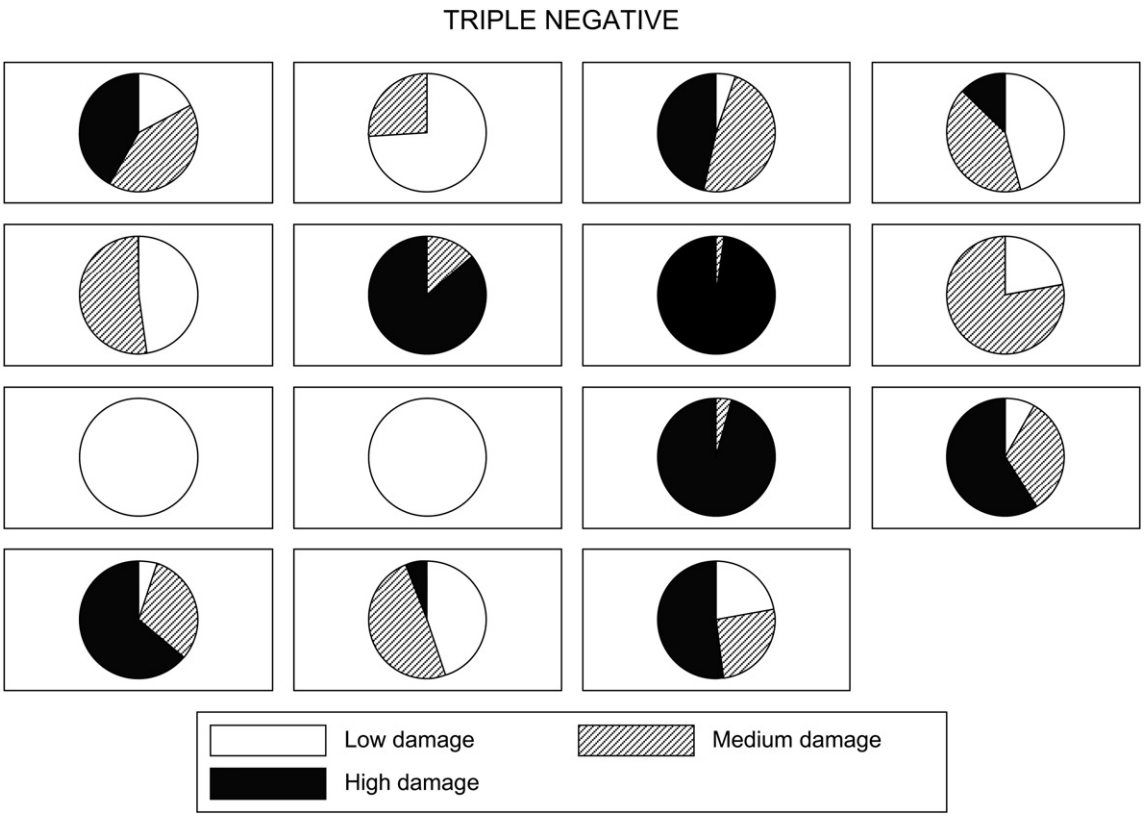

Fig. 4. Intra-tumoral DNA damage (determined by TI) in individuals with TNBC ($N = 15$). Each graph represents 1 patient with TNBC. For each patient, the frequency of tumor cells within low, medium and high DNA damage groups are expressed as a percentage of all tumor cells for that individual.

DNA damage in healthy tissue was expected and was demonstrated. Healthy cells had lower median DNA damage and narrower variance compared with tumor cells. Demonstration of dispersed but lower median level of damage in healthy tissue compared to tumor, across all subgroups, substantiated use of an individual's meanTI of healthy cells as a reference for their tumor cells. Sampling error was shown to be constant across all samples for both tumor and healthy tissue. This equivalence in sampling error allowed attribution of findings of heterogeneity to innate biological state, rather than being sampling related.

Therapy which further damages DNA and/or further inhibits cellular capacity for DNA damage repair may be particularly effective in cells with a high degree of innate DNA damage and/or reduced capacity for DNA damage repair. In this study we have adopted arbitrary thresholds for definition of low, medium and high DNA damage. Clinically meaningful thresholds of DNA damage indicative of likely increased sensitivity to DNA damaging therapy may emerge from ongoing *in vitro* studies which are assessing the potential of comet assay in predicting response to DNA damaging therapy.

A particular strength of comet assay, beyond its simplicity and low cost, is the functional analysis of DNA damage. Upstream molecular abnormalities linked to DNA damage are not necessarily translated into downstream impairments due to pathway crosstalk and downstream compensation, whereas comet is a downstream definitive assessment of fragmentation. DNA damage protein-profiling on tumor biopsies post neoadjuvant therapy has shown predictive value, however such a post-treatment biopsy is impossible in the post operative adjuvant setting in the absence of measurable disease. Comet offers promise as a DNA damage

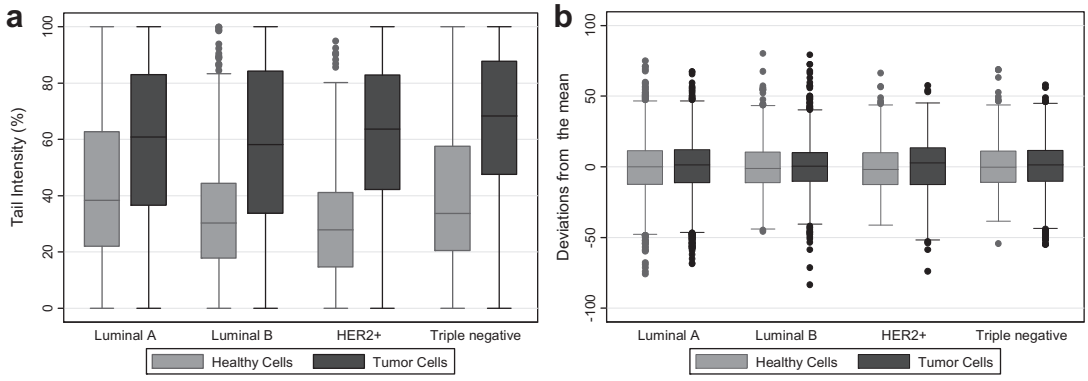

Fig. 5. (a) DNA fragmentation in healthy and tumor cells expressed as TI (%); (b) Sampling error in healthy and tumor cells expressed as deviations from the mean.

assessment tool at the time of surgery for primary breast cancer, using scrapings of the fresh tumor. A limitation of comet is that not all DNA repair dysfunction leads to comet detectable DNA fragmentation. However, the sensitivity may be enhanced by addition of lesion specific bacterial endonucleases which convert specific DNA lesions into detectable strand breaks.[7,24]

An ongoing challenge in the clinical translation of comet is the need to demonstrate its reliability and reproducibility. Reproducibility has been reported in studies using PBMC and epithelial cell suspensions, but not for tumor tissue scrapings.[25,26] Reproducibility was not explored in the current study: most tumors were small and not amenable to more than one scraping, and use of fresh tumor tissue at the time of surgery precluded the option of serial sampling which would be possible with PBMC or healthy tissue. To minimise variability in the current study, a single operator was employed to score nuclei and COMET IV software (Perceptive) was used to compute scores rather than visual comet scoring.

To conclude, breast cancer is a heterogenous disease and a 'one-size-fits all' approach for estimation of innate DNA damage is inappropriate. Substantial inter-subgroup differences in DNA damage exist. Intra-subgroup variation in degree of innate DNA damage limits the power of these subgroups to predict degree of DNA damage for an individual. Individualisation of DNA targeting therapy requires specific DNA assessment tools. Clearly validation studies, particularly correlating degree of innate damage with response to DNA damaging therapy and clinical follow up, are required. The functional comet assay is promising and warrants further study for its potential translation to the clinic.

Ethics

The study received approval from the Institutional Ethical Committee and patients provided written informed consent.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.breast.2012.02.007.

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