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Brief communication

Detection of genetic instability at *HER-2/neu* and *p53* loci in breast cancer cells using Comet–FISH

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Summary

A proportion of breast cancers acquire genetic alterations at 17q11.2–q12 (*HER-2/neu*), 20q13.2 (*ZNF217 gene*) and 17p13.1 (*p53*). We describe a unique technique (Comet–FISH) in which we documented relative genetic instability at *p53* and *HER-2/neu* gene loci within a panel of malignant breast cancer cell lines (MCF-7; MDA-MB-468 and CRL-2336). Furthermore, Comet–FISH data were consistent with preferential repair of the *p53* locus following gentoxic insult and suggest that this assay may be quite useful for the study of genetic instability.

Introduction

Few genetic biomarkers are available to easily identify individuals at risk for breast cancer or breast cancer progression [1–3]. Acquisition of genetic instability during breast cancer progression is common and the presence of non-random chromosome aberrations in breast cancer cells may reflect an underlying predisposition for chromosomal instability at specific gene loci [4]. The *BRCA1* and *BRCA2* genes are prototypes for the association between chromosomal instability, decreased fidelity of DNA repair and an increased risk for breast cancer [5, 6]. Other common genetic alterations associated with breast cancers include amplifications of *HER-2/neu* or *ZNF217* (located on 17q11.2–q12 and 20q13.2, respectively) and deletions or mutation in *p53* (located on 17p13.1). Mutation of *p53* and amplification of *HER-2/neu* are associated with increased cell proliferation, tumor grade and aneuploidy associated with an aggressive clinical course [7–11].

In this pre-clinical study, we hypothesized that genetic instability at specific gene loci, such as *p53* and *HER-2/neu*, might be a key characteristic of breast cancer progression. Indeed, using a unique combined Comet–FISH (fluorescent *in situ* hybridization) protocol, we observed increased instability specifically at *p53* and *HER-2/neu* loci, and not at *ZNF217*, in transformed breast cancer cell lines. The ease and rapidity of the Comet–FISH assay could make it useful in the clinical setting for assessing specific gene loci during tumor progression.

Materials and methods

The breast cancer cell lines used in this study were obtained from, and were characterized by, ATCC (VA; USA) and included: MCF-7 ($BRCA1^{+/+}$ and $p53^{WT/WT}$); MDA-MB468 ($BRCA1^{+/+}$ and $p53^{MT/WT}$); and CRL2336 ($BRCA1^{-/-}$ and $p53^{MT-/-}$). The GM1310B (lymphoblastoid) and AG11134 (normal mammary epithelial) cell lines were obtained from Coriell (NJ; USA). All cell lines were cultured as per the supplier's recommendations.

Cell irradiation was carried out as previously described [12] in logarithmically growing cultures under aerobic conditions using a ^{137}Cs source with a dose rate of 1.0 Gy/min (Gamma cell 40 research irradiator, MDS Nordion, Ontario, Canada). Cells were irradiated on ice. The doses of γ-radiation were selected based on previous experience with the comet assay: 2 and 10 Gy γ-radiation gives optimal DNA migration of alkali-labile DNA-ssbs and DNA-dsbs following alkaline and neutralysis, respectively [12, 13]. For selected experiments, logarithmically growing cells were also exposed to 100 μM hydrogen peroxide (H_2O_2) at room temperature for 5 min to induce DNA-ssbs.

The alkaline Comet assay was performed as previously described [12, 14]. Briefly, cells (approximately 1×10^4 cells per slide) were admixed with 75 μl of 0.5% low melting agarose at 37 °C and spread on a 1% agarose pre-coated slide. Slides were then placed in ice-cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, 10% DMSO, 1% Triton-X) and lyzed overnight. After lysis, the slides were placed in horizontal

electrophoresis tanks filled with alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA; pH 13.0) for 20 min, and then subjected to electrophoresis at 25 V/300 mA for a further 20 min. The lysis and electrophoresis for both neutral and alkaline assays were performed at 4 °C. After electrophoresis, the slides were washed (0.4 M Tris–HCl, pH 7.5; three washes of 5 min each), air-dried and stained with ethidium bromide (2 μg/ml) prior to scoring. For the neutral comet assay, initial steps of the protocol were similar save for additional incubation with Proteinase-K for 60 min at 37 °C and the use of neutral electrophoresis buffer (1 × TBE, pH 8.0). The relative amount of fragmented DNA contained within the Comet's tail, compared to the non-fragmented DNA within the Comet head, was determined by fluorescent image analysis (Northern Eclipse software) [12, 15]. Two hundred (200) consecutive Comets at random to quantify the parameters: 'relative % DNA in tail'; 'Comet tail length' and 'Olive tail moment' (the latter is similar to the normalized tail moment) [13]. At least three independent experiments were completed and the data are presented as the mean parameter values ±1 standard error (SE) of the mean. Differences in comet parameters for each cell line were inter-compared using the non-parametric Mann–Whitney test based on mean values from individual experiments.

For combined Comet–FISH assays, FISH probes were obtained from Vysis Inc (Spectrum Orange; Illinois; USA) and included *HER-2* (190 kb; 17q11.2–q12); *ZNF*217 (320 kb; 20q13.2) and *p53* (145 kb; 17p13.1). A 17-Alpha centromeric probe (Spectrum Green) was used as a reference probe. The commercial kit consists of a cocktail of *D17Z1*/*p53*/*HER-2* probes that were either Spectrum Green-labeled or Spectrum Orange-labeled. This required the use of counterstaining with Sybr® Green, propidium iodide or DAPI, dependent on the probe being scored in order to avoid confusion and increase clarity of signal. Exposure to high alkali during the alkaline Comet assay denatures DNA allowing for direct FISH as per manufacturer's recommendations [15]. For neutral Comet–FISH, the DNA was first chemically denatured (0.03 M NaOH for 2 min at room temperature) prior to probe hybridization. Scoring of Comet–FISH signals was carried out by visualizing the position of the fluorescent hybridization signal within the comet 'head and tail' profile: two hundred (200) consecutive Comets were scored at random. The relative distribution of FISH signals in the Comet head versus the Comet tail, were interpreted as *stable*, and *unstable*, genetic loci respectively. If signals were in both locations, these were classified as *labile* genetic foci.

Results

Initial alkaline and neutral Comet assays assessed DNA migration patterns for endogenous and IR-induced DNA breaks to determine the relative genetic instability within normal (GM1310B and AG1134) and malignant (MCF-7; MDA-MB468 and CRL2336) cell lines [12, 16, 17]. Following alkaline lysis, the breast cancer cell lines showed evidence of increased endogenous and IR-induced alkali-labile sites in comparison to normal cells (see Table 1). For every comet parameter tested, the mean values for the malignant cell lines were greater than the normal cell lines; but these differences were not

Table 1. Baseline and induced DNA damage in normal and malignant breast cancer cells[a]

Cell lines	Baseline DNA damage			DNA damage after 2 Gy radiation		
	% DNA in tail	Tail length	Olive tail moment	% DNA in tail	Tail length	Olive tail moment
(i) *Alkaline comet data*						
GM1310B	11.9 ± 2.8	21.3 ± 5.2	2.1 ± 0.5	33.1 ± 7.3	37.1 ± 4.5	8.1 ± 1.1
AG11134	12.5 ± 3.1	24.1 ± 7.2	1.9 ± 0.5	35.6 ± 6.7	39.6 ± 6.1	7.1 ± 1.4
MCF-7	18.3 ± 3.1	29.5 ± 5.3	3.0 ± 0.8	45.1 ± 8.2	48.1 ± 4.8	10.3 ± 2.1
MDA-MB468	16.9 ± 5.2	28.8 ± 4.1	3.1 ± 1.0	42.7 ± 3.2	49.6 ± 6.2	9.8 ± 1.2
CRL2336	19.2 ± 5.7	30.1 ± 5.2	3.0 ± 0.9	48.1 ± 6.6	51.2 ± 7.6	10.3 ± 2.9
p-value[b]	0.13	0.09	0.16	0.07	0.03	0.11
(ii) *Neutral comet data*						
GM1310B	10.1 ± 2.3	20.9 ± 1.4	0.8 ± 0.1	30.7 ± 5.6	36.2 ± 4.2	10.1 ± 2.7
AG11134	14.4 ± 2.1	19.2 ± 1.7	0.9 ± 0.2	28.8 ± 6.3	38.9 ± 5.1	10.4 ± 2.9
MCF-7	20.2 ± 4.2	31.7 ± 3.1	1.2 ± 0.4	38.1 ± 7.1	45.1 ± 6.1	15.2 ± 3.1
MDA-MB468	19.8 ± 4.8	30.9 ± 4.7	1.8 ± 0.5	42.4 ± 6.4	45.4 ± 5.3	15.5 ± 4.2
CRL2336	21.7 ± 4.9	28.7 ± 5.2	1.5 ± 0.4	40.8 ± 7.8	43.5 ± 6.1	14.6 ± 3.9
p-value	0.03	0.01	0.06	0.09	0.13	0.07

[a]All values are (mean of three independent experiments) ± (standard error of the mean).
[b]Comparison between malignant cell lines versus normal cell lines calculated using the Mann–Whitney test based on mean values for at least three individual experiments per cell line.

Table 2. Baseline genetic instability at *p53* and *HER-2* loci based on neutral Comet–FISH[a]

Cell lines	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
(i)*HER-2/neu*				
GM1310B	81	1	1	17
AG11134	87	2	0	11
MCF-7	56	11	10	23
MDA-MB468	19	20	43	18
CRL2336	14	18	50	18
(ii) *p53*				
GM1310B	81	4	0	15
AG11134	75	3	2	20
MCF-7	51	19	14	16
MDA-MB468	45	16	29	10
CRL2336	37	21	28	14

[a]Probe hybridization was optimized following alkaline or neutrallysis using the *ZNF217* probe. Hybridization of highly–detectable probe (320kb) occurred within 60–70% of alkaline comets and 80–90% of neutral comets. For the *ZNF217* locus, differences in hybridization efficiency between normal and cancer cell lines were not detected (data not shown). Initial FISH of cellular cystospins (400 cells per cell line) determined that AG1134, GM1310B, MCF-7 and MDA-MB468 cells had bi-allelic *p53* gene signals, whereas CRL-2336 was mono-allelic for *p53* (data not shown). The cell line CRL-2336 is known to have a loss of a wild-type *p53* allele. As the *p53* probe used in this study was about 170 kb size and covers not only the *p53* gene, but also the flanking regions on either side of the *p53* gene, this *p53* probe can give rise to two fluorescent signals in CRL-2336 cells. AG1134, GM1310B and MCF-7 cells had bi-allelic *HER-2* signals. Only 20% cells within the MDA-MB468 and CRL-2336 cell lines had bi-allelic *HER-2* signals, the remaining cells in the population with greater than two *HER-2* signals. All five cell lines were consistently bi-allelic for *ZNF217*.

statistically different (results for Mann–Whitney tests summarized in Table 1). A similar trend was observed in the endogenous and exogenous levels of DNA double-strand breaks using the neutral Comet assay. The differences in baseline endogenous DNA-double strand breaks between malignant and normal cells were significant or borderline-significant when assessed by the '% DNA in tail', 'Tail length' and 'Olive tail moment' comet analysis parameters (p-values ranging from 0.01 to 0.06; Mann–Whitney test). These results are consistent with previous data in which increased endogenous genetic instability was observed for malignant breast cells [18, 19].

We then used neutral Comet–FISH assay to interrogate the endogenous stability of *ZNF217, p53*, and *HER-2/neu* loci. These data are presented in Table 2 with representative images shown in Figure 1a. Less than 5% of *ZNF217*-FISH signals were observed to be in the tail of the comet in any of the five cell lines, consistent with chromosomal stability at this locus (data not shown). *HER-2/neu* or *p53* FISH signals were observed in the comet head in greater than 93% of FISH signal-positive cells in AG11134 or GM1310B cells, consistent with genetic stability at both loci in normal cells. In *WTp53*-expressing MCF-7 cells however, only 60–73% of FISH signal-positive cells had stable *HER-2/neu* and *p53* loci. This abnormal pattern increased in the *MTp53*-expressing MDA-MB468 and CRL2336 cell lines, in which only 17 or 23% of FISH signal-positive cells had stable *HER-2/neu* loci and 43 or 50% of FISH signal-positive cells had stable *p53* loci, respectively. There is a clear dichotomous difference pertaining to the level of unstable *HER-2/neu* and *p53* loci in the three malignant cell lines relative to the normal cell lines in Table 2.

In another series of neutral and alkaline Comet–FISH experiments, we assessed the location of *p53* and *HER-2/neu* signals over time following IR or H_2O_2-treatments, as a measure of repair of the allele-containing chromatin domain (see representative images in Figure 1b). FISH signals were scattered within the tail of the comet in the majority of AG1134 and MDA-MB468 cells immediately following irradiation or H_2O_2 exposure. Cells were then incubated for 15 or 60 min allowing for potential repair of DNA-ssbs and DNA-dsbs within 1 h following treatment during maximal repair. At 0 and 15 min following either treatment, multiple *p53* and *HER-2/neu* signals were observed in AG1134 and MDA-MB468 cells. After 60 min, both genetic loci were fully repaired in normal cells with no evidence of signal scatter in the comet tail. At the same time-point, the scattered *p53* signal resolved to solely two detectable signals in the tail of the MDA-MB468 cells, whereas the scattering of the *HER-2/neu* signals persisted (cf. Figure 1b(i) and (ii)). These data suggest that despite both loci being located on chromosome 17; there is preferential repair of the *p53* locus in both normal and malignant cells.

Discussion

The Comet assay has been extensively used for toxicological, mutational and DNA repair studies relating to cancer therapy response and carcinogenesis (reviewed in

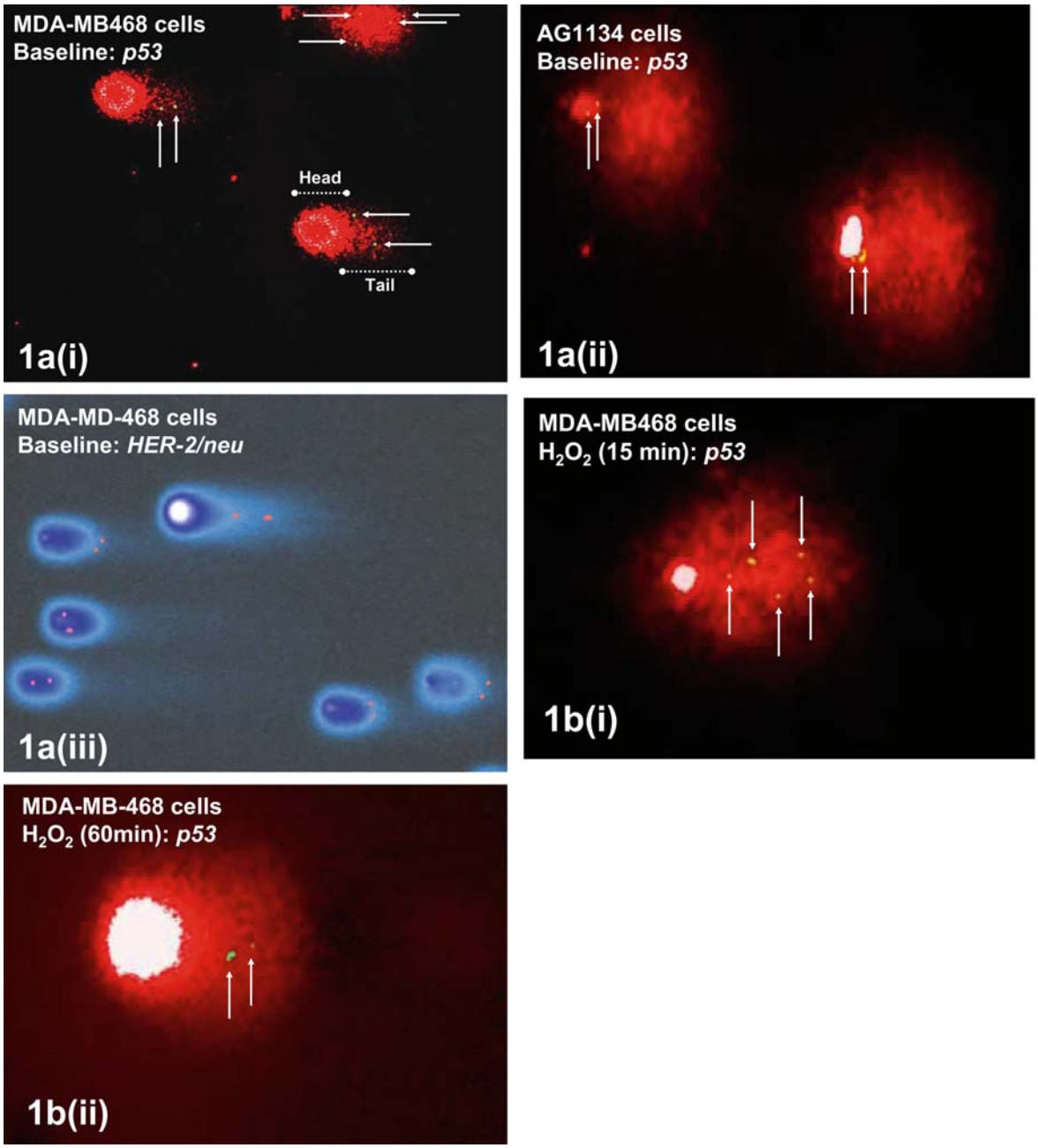

Figure 1. (a) Representative photomicrographs of neutral Comet–FISH for baseline *p53* and *HER-2/neu* signals in normal and malignant breast cells: (i) MDA-MB468 breast cancer cells prior to DNA damage showing baseline *p53* FISH signals in the tail of the comets. Also shown is a cell with multiple small baseline *p53* signals in the tail. The typical 'head' and 'tail' of the comet is demonstrated. (ii) AG11134 normal human mammary epithelial cells prior to DNA damage showing baseline *p53* FISH signals in the head of the comet. A similar observation was made for the location of *HER-2/neu* FISH signals in this cell line. (iii) MDA-MB468 breast cancer cells prior to DNA damage showing *HER-2/neu* FISH signals in the tail of the comets. Similar cells could be found which had *p53* signals within the comet tail. In each image above, the DNA is counterstained with either propidium iodide (red) or DAPI (blue). (b) In selected experiments, GM1310B and MDA-MB468 cells were exposed to 2 or 10 Gy (using a ^{137}Cs source at a dose rate of 1.0 Gy/min as described; [12] or 100 μM H_2O_2 for 5 min, to induce DNA double-strand (DNA-dsb) and single-strand (DNA-ssb), breaks, respectively. Representative photomicrographs of alkaline Comet–FISH for *p53* signals before and after DNA damage in MDA-MB48 breast cancer cells: (i) MDA-MB468 breast cancer cells at 15 min following exposure to 100 μM H_2O_2. Numerous *p53* signals are scattered throughout the tail of the comet. (ii) MDA-MB468 breast cancer cells at 60 min following exposure to 100 μM H_2O_2. Scattering of the *p53* FISH signals has resolved, leaving two discrete *p53* signals in the tail of the comet.

[13, 16, 20]. By combining the Comet assay with FISH, it is possible to determine gene-specific repair or endogenous genetic instability at specific gene loci. Utilizing neutral Comet–FISH, we were able to show that genetic instability occurs at both the *HER-2/neu* and *p53*, but not at *ZNF217*, loci in the breast cancer cell lines tested herein. We speculate that the instability of the *p53* and *HER-2/neu* loci in the breast cancer cell lines might be related to *p53* genotype: the two aneuploid and poorly differentiated cell lines expressing mutant p53 protein (MDA-MB468 and CRL2336) had increased instability at both loci in comparison with the more differentiated MCF-7 cells which express wild-type p53 protein. These data are consistent with clinical data in

which breast cancer that express mutant p53 proteins have increased chromosomal instability, resistance to cancer therapy and an increased propensity for distant metastases [1, 9].

The fact that *p53* and *HER-2/neu* sequences, but not *ZNF217* sequences, are specifically released to the tail region within fragmented DNA during Comet–FISH, suggests that the chromatin structures that underlie these two domains are particularly vulnerable [21]. These domains may contribute to breast tumor progression in the setting of genetic instability and clonal selection. Furthermore, the data in which *p53*, but not *HER-2/neu*, sequences are preferentially repaired following DNA damage in breast cancer cell lines is consistent with previous data using a bladder cancer cell line [17] and may relate to preferential repair of domains normally required for cell cycle checkpoint control [5, 17].

The Comet–FISH method has potential in the clinic setting given its ease and the rapid generation of genetic data. It may be amemable to patient-derived tissues (including peripheral blood lymphocytes, fine needle aspirates or core needle biopsies) to characterize breast cancer risk or progression by determining the relative instability in malignant or normal tissues [3, 22, 23]. The assay can be expanded to the use of multiple simultaneous genetic probes to determine complex relationships between re-arranged genetic loci following DNA damage. This may prove to be complementary to *BRCA1/BRCA2* mutational analyses as a functional assay for genetic instability[5, 6, 22]. Our preliminary studies are provocative and support further studies to test the utlility of this assay using primary lymphocyte or breast tissues as a means to predict risk of malignancy or progression.

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