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Alkaline single-cell gel electrophoresis (comet assay): a simple technique to show genomic instability in sporadic breast cancer

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Abstract

The alkaline comet assay was used to study the genomic instability of lymphocytes derived from untreated sporadic breast cancer patients (50 cases), and also following their *in vitro* irradiation up to 5 Gy. We compared the results (mean tail moment (MTM)) with a control population of 25 patients and with breast cancer patients who had been 'cured' of their disease, with a follow-up of 10 years or more (25 cases). At the basal level, 77.5% ($P<0.01$) of the untreated patients and 73.7% ($P<0.05$) of the 'cured' women had values higher than the basal cut-off level of 5.3, compared with only 44% of the controls. After *in vitro* irradiation, 83% of the untreated patients were above the cut-off value of 10.8 at the 5-Gy dose compared with only 48% of the controls ($P<0.01$). These results support the hypothesis that women affected by sporadic breast cancer have a constitutional genomic instability. The assessment of the prognostic value of this test could be of interest, particularly in women without axillary nodal involvement.

Keywords: Genomic instability; Sporadic breast cancer; Comet assay

1. Introduction

The notion of "genetic or genomic instability" is used to describe "the increased rate of acquisition of alterations in the mammalian genome, including chromosomal destabilisation, gene amplification and mutations following cellular exposure to DNA-damaging agents" [1]. The loss of stability of the genome in tumour cells is becoming widely accepted as one of the most important aspects of cancer [2]. It has been proposed that genomic instability could be a driving force behind multistep carcinogenesis [3]. Of more significance here is the correlation between chromosome instability and proneness to cancer: several syndromes exhibit chromosome instability. These well-documented syndromes include Fanconi Anemia, Ataxia Telangectasia, Werner and Bloom's syndromes [4,5]. Such diseases are characterised by high rates of spontaneous chromosomal breaks, unusual susceptibility to induction of breaks by clastogens, and a propensity for cancer development [6]. Genomic instability is also described in cases of hereditary cancers such as hereditary breast cancer which accounts for 5% of all breast cancer patients. In the basal state, the frequency of sister chromatid exchange and chromosome aberrations in the lymphocyte population of these untreated patients is higher than that observed in healthy individuals [7].

In genetic syndromes, as well as in hereditary breast cancer, increased genomic instability, whether inherent or induced by external agents, is a primary event leading to neoplastic transformation. To study genomic instability, the most extensively used biomarkers involve cytogenetic methods (evaluation of chromosomal aberrations and/or sister chromatid exchange in mitogen-stimulated cells) or biochemical and immunological techniques such as alkaline or neutral gel elution [8,9]. Nevertheless, these expensive and time-consuming techniques are usually limited to the proliferating cell

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population. Another faster method, the alkaline comet assay (single-cell gel electrophoresis), is able to show genomic instability [10]. This test detects DNA damage at the single-cell level. [11,12]. The use of this assay has increased in the past few years, especially in genotoxic testing and biomonitoring for many different applications [13,14]. This assay is much simpler and less expensive than the mutagen sensitivity assay and requires only a small number of cells [15].

In a preliminary study, we investigated 19 women with sporadic breast cancer sampled before chemotherapy and/or radiotherapy [16]. The results of this previous work showed that the lymphocytic DNA of patients is more damaged than in the controls, whether irradiated *in vitro* or not. Our hypothesis was that this baseline DNA damage could reflect a genomic instability in sporadic breast cancer. We present here, a new study including more patients, selected before and after surgery (to investigate the influence of this parameter on the results). In the present study, the comet assay was always performed before radiotherapy and/or chemotherapy. We compared the results obtained with a control population without cancer and also with results in a group of breast cancer patients 'cured' from their disease after a follow-up of at least 10 years.

2. Materials and methods

2.1. Patients and blood collection

All individuals were non-smokers and free from other pathology and were not on medication that is known to cause DNA damage.

The study population was composed of four groups:

Group 1: 25 women with metastasis-free breast cancer (aged 50–75 years) sampled before surgery.

Group 2: 25 women affected by breast cancer without metastasis involvement (aged 50–77 years) sampled 15 days after surgery and before radiotherapy and/or chemotherapy.

Group 3: 25 breast cancer patients (aged 52–81 years) treated by surgery and radiotherapy and followed-up for at least 10 years.

Group 4: 25 women volunteers (aged 49–74 years).

Table 1 shows the distribution (percentage and numbers) of groups 1, 2 and 3 according to the international TNM (Tumor Nodes Metastasis) classification of malignant tumours established by the International Union against Cancer (UICC):

T is defined as 1–4 according to the tumour size (T1 tumour size <2 cm, T2 between 2 and 4 cm, T3 >4 cm and T4 extension to adjacent structures), N for absence (N0) or presence (N1) of nodes metastasis and M for the presence or absence of metastasis [17].

Table 1
Distribution (percentage and numbers) of the population as a function of the TNM UICC classification.[a]

N	T				
	T0	T1	T2	T3	T4
N0	8% (2)	32% (8)	40% (10)	4% (1)	–
N1	–	–	12% (3)	–	4% (1)
Total G1	8% (2)	32% (8)	52% (13)	4% (1)	4% (1)
N0	12% (3)	52% (13)	32% (8)	–	–
N1	–	–	4% (1)	–	–
Total G2	12% (3)	52% (13)	36% (9)	–	–
N0	–	44% (11)	40% (10)	4% (1)	–
N1	–	–	12% (3)	–	–
Total G3	–	44% (11)	52% (13)	–	–

[a] The groups G1, G2 and G3 refer to metastasis-free breast cancer patients. G1, 25 patients sampled before surgery; G2, 25 patients sampled after surgery; G3, 25 'cured' patients.
UICC, International Union against Cancer.

2.2. Cell culture and irradiation

Mononuclear cells were separated from heparinised blood samples by centrifugation (Ficoll, 2000 rotations per minute (rpm), 20 min, 20 °C), washed in NaCl and resuspended in Roswell Park Memorial Institute (RPMI) 1640 containing 5% fetal calf serum. Lymphocytes were suspended at 3×10^5 cells/ml cultured in a 5% CO_2 incubator at 37 °C for 12 h. After 12 h, lymphocytes were exposed to cobalt-60 gamma rays at 1.8 Gy/min, with the dose ranging from 0.5 to 5 Gy. Following irradiation, lymphocytes were immediately subjected to the comet assay.

2.3. Comet assay

The single-cell gel electrophoresis (comet assay) was adapted from the method of Singh and colleagues reported in Ref. [13]. Lymphocytes were centrifuged and then embedded in 0.5% low-melting-point agarose at a final concentration of 10^4 cells/ml. 65 μm of this cellular suspension was then spread onto duplicate frosted slides that had previously been covered with 80 μl of 0.8% agarose as a basal layer. Slides were allowed to solidify for 10 min at 4 °C before being placed in lysis buffer for 1 h (NaCl 2.5 M, ethylene diamine tetraacetic acid (EDTA) 0.1 M, Tris 0.01 M, Triton X-1000 1%, dimethyl sulphoxide (DMSO) 10%, pH 10). After lysis, the slides were transferred into alkaline buffer for 40 min (EDTA 0.001 M, NaOH 0.3 M, pH 13) to allow the DNA to unwind before migration at 0.66 V/cm and 300 mA for 25 min. All these steps were performed in the dark. After neutralisation in Tris 0.4 M pH 7.4, slides were stored at 4 °C until analysis within the following 24 h. Before analysis, the slides were stained with ethidium bromide (20 μg/ml) and covered with a coverslip.

2.4. Analysis of results

The extent of DNA migration was analysed using a fluorescence microscope equipped with a camera and a computer-based image analysis system (Aphelion, ADCIS, Hérouville-Saint-Clair, France). The data are based on 50 randomly selected cells per sample, 25 cells from each of the two replicate slides. The mean tail moment (MTM ± 95% Confidence Interval (CI)) and the percentage of highly damaged cells or HDC (tail moment equal to 150, highest value corresponding to apoptotic cells) were used to express the results.

MTM is defined as the product of DNA in the tail and the mean distance of migration in the tail ([intensity in the tail comet/sum comet intensity]*[tail centre gravity−peak position]).

Statistical analysis was performed using the Student's *t*-test for the MTM and the Mann–Whitney test for the percentage of HDC.

The Receiver Operating Characteristic (ROC) analysis computes a cut-off point corresponding with the best accuracy of the test (minimal false-negative and false-positive results). In our study, the Medcalc computer displays the cut-off value of each population without irradiation and at an irradiation dose of 5 Gy and an interactive dot diagram. In a dot diagram, the data of both positive and negative groups (controls) are displayed as dots on vertical lines. A horizontal line indicates the cut-off point with the best sensitivity and specificity. The sensitivity is the probability that a test result will be positive when the disease is present (true-positive rate, expressed as a percentage). The specificity is the probability that a test result will be negative when the disease is not present (true-negative rate, expressed as a percentage).

To compare the controls with the studied populations in terms of the cut-off value, we used a contingency table with a Chi Square test.

3. Results

3.1. Description of the population

We used the Chi Square test to analyse the distribution of the population after surgery classified according to the TNM system (Table 1). This statistical test shows the homogeneity of the three studied populations. There is no statistically significant difference ($P = 0.61$) between the patients with breast cancer sampled before surgery (group 1), those sampled after surgery (group 2) and those cured at least 10 years after the initial treatment (group 3). 84, 88 and 96% of groups 1, 2 and 3, respectively, are classified as either T1N0/1 or T2N0/1, i.e. patients with a good prognosis.

3.2. MTM as a function of the in vitro irradiation dose

There is no statistically significant difference between the women with breast cancer sampled before surgery and those sampled afterwards (Table 2). These two groups were thus pooled to form a population of 50 individuals (population before supplementary treatment). This population (prior to receiving radiotherapy and/or chemotherapy) was compared with the control population and women 'cured' of breast cancer with at least 10 years follow-up (Table 3). For these three populations, the MTM increases in proportion to the dose of irradiation. Both with and without irradiation, the MTM of the 'new' patients with breast cancer is higher than values obtained for the controls. This difference is statistically significant under basal conditions and at doses of 2 and 5 Gy. However, there is no statistically significant difference between the average MTM of the controls and the average MTM of 'cured' patients, with or without irradiation.

3.3. Percentage of HDC as a function of the irradiation dose

The HDC are considered to be apoptotic cells. The percentage of HDC increases after irradiation at 5 Gy in all three studied populations (Fig. 1). The women with breast cancer (new patients) had a higher percentage of HDC than the controls (the result was statistically significant without irradiation and at a dose of 5 Gy: $P < 0.001$). By contrast, we observed no difference between the controls and the 'cured' patients.

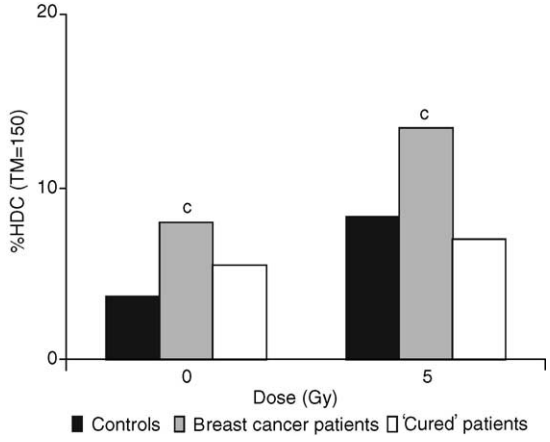

Fig. 1. Percentage of highly damaged cells (HDC) without irradiation and after 5-Gy irradiation of the lymphocytes. The breast cancer patients are 50 women sampled before therapy. The 'cured' patients are 25 others sampled 10 years after therapy. Values for the groups are compared by the Mann–Whitney test (c, $P < 0.001$). TM, tail moment.

Table 2
DNA damage detected with comet assay in lymphocytes of patients sampled before and after surgery

Dose (Gy)	Mean tail moment ±95% Confidence Interval (CI)	
	Before surgery	After surgery
0	14.13±2.33	15.16±2.23
0.5	15.62±2.50	11.81±2.09
1	13.78±2.27	13.71±2.11
2	16.34±2.48	22.48±2.25
5	23.66±2.94	24.79±2.43

Values of patients (25 in each group) are compared using the Student's *t*-test.

Table 3
DNA damage detected using the comet assay in the lymphocytes of 50 women sampled before therapy, 25 others 10 years after therapy and in 25 controls

Dose (Gy)	Mean tail moment±95% Confidence Interval (CI)		
	Controls	Before therapy	10 years after
0	7.74±1.64	14.67±1.66[b]	9.85±2.21
0.5	9.94±1.88	13.80±1.68	9.46±2.20
1	12.24±2.25	13.79±1.58	10.47±2.19
2	14.92±2.41	19.62±1.89[a]	11.87±2.38
5	17.09±2.40	24.60±2.14[a]	14.89±2.58

Values of each population are compared using the Student's *t*-test.
[a] $P<0.05$.
[b] $P<0.01$.

3.4. Statistics in terms of the cut-off values calculated by ROC analysis and individual variation of the MTM

Under basal conditions, the cut-off value calculated by ROC analysis is 5.3 for all the studied populations (Fig. 2). 77.5% ($P<0.01$) of the women before treatment and 73.7% ($P<0.05$) of the cured women have values higher than the basal cut-off, compared with 44% of the controls (Tables 4 and 5). After irradiation, whatever the dose, the percentage of patients before treatment showing values above the cut-off is higher than in the controls (Table 4). The sensitivity and specificity are optimal at a dose of 5 Gy, in this case yielding a cut-off value of 10.8: 83% of the patients with breast cancer yielded values above this cut-off, compared with 48% of the controls ($P<0.01$) (Table 4 and Fig. 3). With this same dose of irradiation, only 19.1% of the 'cured' women were above the cut-off value (equal to 22.6), compared with 40% of the controls (a non-significant difference) (Table 5 and Fig. 3).

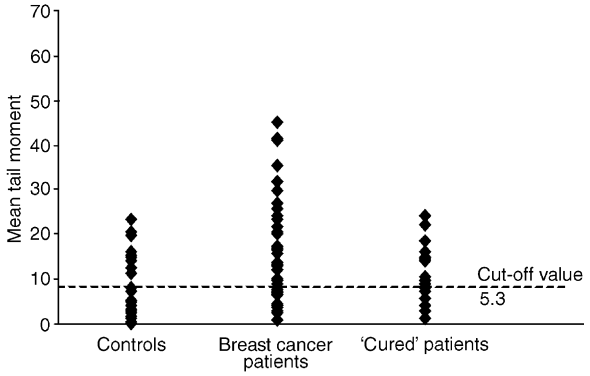

Fig. 2. Individual variation of the mean tail moment (MTM) under basal conditions. The 5.3 cut-off value refers to the controls, the new breast cancer patients (sampled before therapy) and the women 'cured' from their cancer followed-up for at least 10 years.

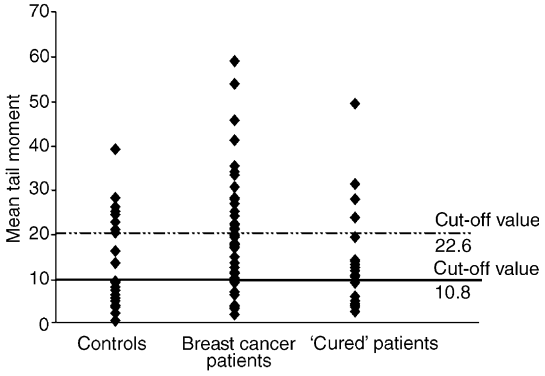

Fig. 3. Individual variation of the mean tail moment (MTM) at a dose of 5 Gy. The 10.8 cut-off value refers to the breast cancer patients (sampled before therapy) and the 22.6 cut-off value to women 'cured' from their cancer followed-up for at least 10 years.

4. Discussion

In this study, three groups of patients presenting with sporadic breast cancer were compared with a control group of individuals of the same age not presenting with any notable pathology thereby reducing potential confounding. Indeed, most biomonitoring studies involving the comet assay have not detected any age-related effect on the level of DNA damage [18,19]. The three breast cancer groups exhibited the same clinical profile according to the TNM classification. Most patients had a relatively early stage of disease, associated with a good prognosis. Irrespective of whether they had been irradiated *in vitro*, a comparison of the response of the patient's lymphocytes to the test before and after surgery failed to show any statistically significant differences (Table 2). We are therefore able to pool these two groups together.

Under basal conditions (without *in vitro* irradiation), the lymphocyte DNA of the untreated patients with breast cancer is more damaged compared with the controls. The patients exhibited a MTM and a percentage of HDC that was twice that of the controls (Table 3, $P<0.01$ and Fig. 1, $P<0.001$). Considering the inter-individual variation of the MTM as a function of the cut-off value (Table 4 and Fig. 2), we found 77.5% of

Table 4
Mean tail moment (MTM): percentage of individuals according to the cut-off value calculated by ROC analysis and compared by Chi Square test: 50 untreated breast cancer patients sampled before therapy and 25 controls

	0 Gy sensitivity = 78% specificity = 52%		0.5 Gy sensitivity = 64% specificity = 64%		1 Gy sensitivity = 86% specificity = 35%		2 Gy sensitivity = 63% specificity = 67%		5 Gy sensitivity = 83% specificity = 52%	
	Cut-off value MTM									
	< 5.3	> 5.3	< 9.1	> 9.1	< 4.3	> 4.3	< 13.4	> 13.4	< 10.8	> 10.8
Controls (%)	56	44	70	30	39.2	60.8	66.7	33.3	52	48
Untreated patients (%)	22.5[b]	77.5[b]	36.2[a]	63.8[a]	18.4	81.6	36.7[a]	63.3[a]	17[b]	83[b]

ROC, Receiver Operating Characteristic.
[a] $P < 0.05$.
[b] $P < 0.01$.

Table 5
Mean tail moment (MTM): percentage of individuals according to the cut-off value calculated by ROC analysis and compared by Chi Square test: 25 'cured patients' sampled at least 10 years after therapy and 25 controls

	0 Gy sensitivity = 78% specificity = 52%		0.5 Gy sensitivity = 75% specificity = 55%		1 Gy sensitivity = 25% specificity = 91%		2 Gy sensitivity = 13% specificity = 95%		5 Gy sensitivity = 76% specificity = 40%	
	Cut-off value MTM									
	< 5.3	> 5.3	< 7.5	> 7.5	< 1.2	> 1.2	< 19.6	> 19.6	< 22.6	> 22.6
Controls (%)	56	44	54.5	45.5	8.7	91.3	79.2	20.8	60	40
'Cured' patients (%)	26.3[a]	73.7[a]	25[a]	75[a]	9.5	90.5	90.5	9.5	80.9	19.1

ROC, Receiver Operating Characteristic.
[a] $P < 0.05$.

the patients were above this value compared with 44% of the controls ($P < 0.01$). Under basal conditions, the population of 'cured' women showed a higher MTM and percentage of HDC than the controls (Table 3 and Fig. 1), but this difference was not statistically significant. However, we observed that 73.7% of this group of patients had MTM values higher than the cut-off value, compared with 44% of the controls (Table 5) and this difference was statistically significant ($P < 0.05$).

Our hypothesis was that the comet assay allows us to show, especially in untreated sporadic breast cancer patients, a 'spontaneous' genomic fragility (instability) which can be considered as 'constitutional', i.e. present in all the somatic cells (in this case, peripheral blood lymphocytes) as well as in their descendant cells. This fragility is expressed as a very high level of DNA breaks, thus leading to a high percentage of very damaged lymphocytes corresponding to apoptotic cells [20]. This confirms the results of others who reported an increase in the numbers of deletions, breaks and gaps in the DNA of lymphocytes of patients with sporadic breast cancer. Such anomalies are described as markers of genomic instability [21,22]. This instability was still present in patients 'cured' from their breast cancer with the MTM value being higher in this group compared with controls. A larger number of 'cured' patients should be studied to see if this difference becomes significant. As for the new, untreated patients, we also observed a statistically difference between the percentage of 'cured' women with MTM values higher than the cut-off (5.3) compared with 'cured' women with lower values than the corresponding subsets in the control group.

So both our results, as well as data in the literature, argue in favour of a real genomic instability in women with sporadic breast cancer. This concept has already been proposed in cases of familial breast cancers. In these patients, the comet test reveals a genomic instability that is the result of a high frequency of sister chromatid exchange and chromosomal mutations at the lymphocyte level, whether the cells have been irradiated or not [23,24].

In this study, we also reported the results of the comet tests after the *in vitro* irradiation of the lymphocytes of patients with sporadic breast cancer. We showed an increase in the DNA breaks both in untreated patients as well as in the controls, but the lymphocytes of these patients did not display more radio-induced damage than the controls. Indeed, the linear regressions (MTM plotted as a function of irradiation dose) were parallel for the patients and the controls, with slopes of 2 and 1.8, respectively.

Environmental factors would therefore appear to have an enhanced impact on the genetic material in

these women [25,26]. It is well established that certain tissues or organs are specific targets of such factors. In breast cancer, for example, the role of oestrogens should be considered. In women, the mammary gland is particularly sensitive to these hormones. Oestrogens play a role in cell proliferation and also have an pro-oxidant effect on DNA [27,28]. A recent study has shown that prolonged exposure to oestrogens increases the risk of breast cancer [29]. Thus, women with a high level of oestradiol (>12 pmol/l) have a 2.07 times higher risk of developing a breast cancer than those with lower levels. Oestrogenic impregnation since puberty would cause an accumulation of DNA damage. We propose that this damage is conditioned by the existence, at the basal state, of a constitutional genomic instability of all the somatic cells.

It is accepted that this constitutional genomic instability, already described in cases of hereditary breast cancer, is related to somatic mutation in *BRCA1* or *BRCA2* [30]. A recent study has shown that these mutations might also be prognostic in cases of ovarian cancer. Indeed, patients carrying a *BRCA1* or *BRCA2* mutation have an increased median survival. The diagnostic test based on this mutation has a sensitivity of 85%, but involves a rather lengthy procedure. The prognostic value of a *BRCA1* mutation is perceived in different ways for breast cancer depending on the subgroup studied. In our study, the comet test applied to lymphocytes of new patients with sporadic breast cancer has a sensitivity of 78% in basal conditions and 83% following *in vitro* irradiation at 5 Gy. Therefore, taking into account the relatively simple implementation of this test, it would be interesting to study its prognostic value in the framework of a standardised study, especially in women without axillary nodal invasion.

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