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Chromosomal radiosensitivity in G_2-phase lymphocytes identifies breast cancer patients with distinctive tumour characteristics

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Summary A substantial proportion of women with breast cancer exhibit an abnormally high radiosensitivity as measured by the frequency of chromatid breaks induced in G_2-phase, PHA stimulated lymphocytes. Chromatid break frequencies were compared for a cohort of previously untreated sporadic breast cancer patients and hospital outpatient controls. In the breast cancer group 46% showed high radiosensitivity compared to 14% of controls ($P < 0.001$). Comparison of those breast cancer patients with a high G_2 radiosensitivity (G_2RS) versus those with a low G_2RS showed no difference in menopausal status or age but the high G_2RS group had on average a lower score on the Nottingham Prognostic Index. Predicted survival in the high G_2RS group at 15 years was 55% compared to 36% for the low G_2RS group. Furthermore, 81% of tumours from the high G_2RS were oestrogen receptor positive compared to 45% from the low G_2RS group. Thus high G_2RS identifies a sub-population of patients with distinctive tumour characteristics and with a predicted improved prognosis as compared with those in the low G_2RS group. Our findings imply that besides influencing risk of breast cancer the genetic factors determining G_2 radiosensitivity also influence the tumour characteristics and prognosis in these patients. © 2001 Cancer Research Campaign http://www.bjcancer.com

Keywords: breast cancer; chromosomal radiosensitivity; G_2-phase

The association between chromosomal radiosensitivity and cancer predisposition has been clearly demonstrated in a number of heritable conditions (Sanford et al, 1989). This was first established for patients with the recessively-inherited multi-system disorder ataxia-telangiectasia (A-T) (Taylor, 1983) and further demonstrated in 20 other inherited cancer-prone conditions (Scott et al, 1996, 1999).

Scott et al (1994) reported that, using peripheral blood lymphocytes stimulated with phytohaemagglutinin (PHA) in vitro, they could discriminate between A-T heterozygotes and normal controls according to the number of chromatid aberrations induced by low doses of radiation (the G_2 assay). They demonstrated that 42% of untreated breast cancer patients but only 9% of controls exhibit a G_2 sensitivity similar to that of A-T heterozygotes (Scott et al, 1994, 1998). Three other groups have confirmed these findings in breast cancer patients (Parshad et al, 1996; Patel et al, 1997; Terzoudi et al, 2000).

Increased chromosomal radiosensitivity among blood relatives of breast cancer patients with high G_2 scores have also been demonstrated. Clear evidence of Mendelian heritability of G_2 chromosomal radiosensitivity was established by Roberts et al (1999). A single major gene could account for 82% of the variance between family members. This strongly supports the view that enhanced radiosensitivity of peripheral blood lymphocytes is a marker for breast cancer-predisposing genes of limited penetrance.

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It is well established that breast cancers arising in carriers of the breast cancer susceptibility genes, *BRCA1* and *BRCA2* have different histological characteristics from each other and from breast cancers arising in patients unselected for family history (Lakhani et al, 1997; 1998). A substantial proportion of families with multiple breast cancers, however, are not attributable to these two genes *BRCA1* and *BRCA2* (Peto et al, 1999). The pathological characteristics of tumours arising in non-*BRCA1* or non-*BRCA2* families are different from *BRCA1* and *BRCA2* tumours (Lakhani et al, 2000). There is also evidence that the pathological characteristics of non *BRCA1*/*BRCA2* familial breast cancers differ from sporadic (nonfamilial) breast cancers (Lakhani et al, 2000).

In breast cancer, a reliable indicator of prognosis has been developed, the Nottingham Prognostic Index (Galea et al, 1992), using information on tumour size, histological grade and nodal status (see Table 2 for formula).

No studies have compared the characteristics of breast cancers in groups identified by their G_2RS. We have therefore undertaken such a study.

MATERIALS AND METHODS

Patients blood samples and clinical data

Peripheral venous blood was collected from 65 unselected hospital outpatients with primary breast cancer at the time of attendance for diagnosis and from 66 control surgical outpatients without malignancy. The blood samples (10 ml) were collected into lithium heparin tubes and transported at room temperature by car to the St Andrews laboratory (a distance of 20 miles). Age, menstrual

status, tumour size, histological nodal status, histopathological classification of tumour, histological grade and oestrogen receptor status were documented retrospectively for the breast cancer patients. Pathological assessment of tumours and oestrogen receptor status, recorded by standard protocols by a specialist breast pathologist, were undertaken independently of the G_2 assay. Results were collated after chromatid breaks were scored and were not known to those carrying out the G_2 assay.

All of the studies were undertaken with ethical approval from the Tayside and Fife Committees on Medical Research Ethics (218/97 and DE/JRW20069717).

G_2 assay

The blood samples were held in the laboratory overnight at room temperature and set up for culture the following day. Two samples were set up from each subject by adding 1 ml of blood to 9 ml prewarmed and gassed medium (RPMI 1640 containing 10% FCS, l-glutamine and antibiotics) to a 25 cm^3 flask. PHA (Murex HA15, 150 µl of the 5 ml stock) was added to each sample. The flasks were placed flat in a humidified and gassed incubator (5% CO_2/95% air) for 72 h.

At this time one flask was exposed to 0.4 Gy gamma irradiation from a caesium 137 gamma source (IBL437C). The other sample was mock irradiated. After 30 min incubation, colcemid (150 µl of 10 µg/ml stock) was added to each flask and returned to the incubator for a further 1 h. The cells and medium were then transferred to a 10 ml centrifuge tube and held on ice for 10 min. Cells were treated with ice-cold hypotonic solution (0.075M KCI) for 10 min, fixed and metaphase preparations made using standard protocols. Slides were coded for scoring blind and 50–100 metaphase spreads scored for aberrations under 63× oil immersion lens. The number of chromatid breaks per 100 metaphases was determined: the 'G_2 score'.

Statistical analysis

For comparisons between the G_2 scores in breast cancer patients and controls, the Mann-Whitney test was applied. The proportions of patients and controls in the high G_2RS group were estimated using the 90th percentile of the control group as the cut-off, as described by Scott et al (1999). Differences in the proportions in this high G_2RS cohort were compared for the breast cancer patients and controls using the two-tailed Fisher's exact test. Two groups of breast cancer patients were further selected on the basis of their G_2 lymphocyte scores; 21 patients with the highest and 20 with the lowest G_2 scores. Comparisons between the patients in the high G_2RS group and the low G_2RS group for oestrogen receptor positivity, nodal involvement and menopausal status were undertaken using a χ^2 test.

Comparisons of the median age and tumour diameter in these two groups used the Mann-Whitney test.

RESULTS

Reproducibility of the G_2 assay

Independent repeat assays on 7 donors allowed an estimate of the G_2 assay reproducibility (intra-individual variance) which gave a coefficient of variation (CV) of 8% compared with a CV of 19% for inter-individual differences between donors. Assays on blood collected at St Andrews and Dundee from the same donors on the same day enabled samples to be checked for possible differences introduced by the transport procedure. No differences were detected. The correlation coefficient was 0.76 ($P < 0.05$).

Comparison of the G_2 scores in breast cancer patients and hospital controls

Figure 1 illustrates the distribution of G_2 scores for patients and controls. The mean overall G_2 score for the breast cancer patients (84.8 ± 36.8) was greater than that for the control group (63.3 ± 18.5) (Table 1, $P < 0.0001$). The median values were significantly different ($P < 0.0001$). Using the 90th percentile of the control group as the cut-off point, the proportion of breast cancer patients with high G_2 sensitivity was 46.2% compared to 13.6% for the controls (since scores were in bins, 13.6% approximates the closest to the 90th percentile). These proportions were significantly different ($P < 0.001$) indicating that there was a substantial sub-group of breast cancer patients exhibiting increased lymphocyte radiation sensitivity (Table 1).

Comparison of the Nottingham Prognostic Index in breast cancer patients exhibiting high or low G_2 scores

For the breast cancer patients, those in the highest and lowest tertiles of G_2, scores were grouped using the 3 lowest and the 7

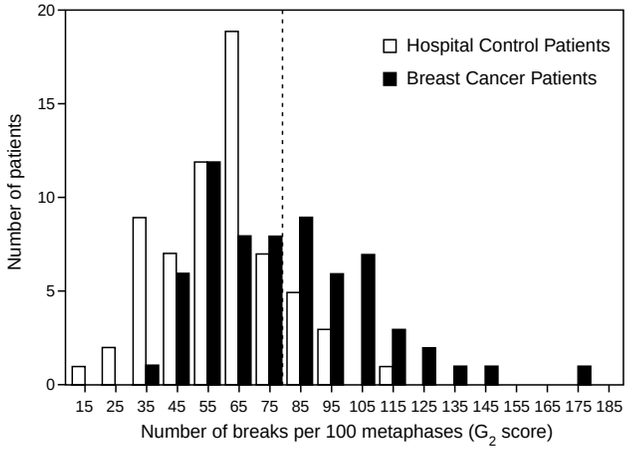

Figure 1 Comparison of the G_2 scores (number of chromatid breaks per 100 metaphases) measured in irradiated peripheral blood lymphocytes from breast cancer patients and hospital outpatient controls (.....90th percentile cut-off from the control population)

Table 1 Mean G_2 scores (number of chromatid aberrations per 100 metaphases scored in peripheral blood lymphocytes), population standard deviations, ranges and proportions of sensitive breast cancer patients and normals in the G_2 assay

	Normals	Breast cancer patients	
Mean G_2 score ± SD	63.6 ± 18.5	84.8 ± 26.8	$P < 0.0001$[a]
Range	24 – 118	42 – 181	
Sensitive sub-group	9/66 (13.6%)[c]	30/65 (46.2%)[c]	$P < 0.001$[b]
Number of subjects	66	65	
Median age	47	58	
Range	20 – 76	29 – 95	

[a] Mann-Whitney U test; [b] Two-tailed Fisher's exact test; [c] The 90th percentile was selected as the cut-off (as results were grouped in bins, more than 10% of patients had scores equal to, or greater than, this figure).

Table 2 Comparison of the age, G_2 score, tumour diameter, stage, grade and the Nottingham Prognostic Index for two cohorts of breast cancer patients with a high or a low G_2 score

	Age (years)	G_2 score	Tumour diameter (cm)	Stage	Grade	Nottingham Prognostic Index[a]
Low G_2 scores	53	47	2.2	2	3	5.44
	45	46	3.0	2	3	5.60
	58	52	1.5	1	1	2.30
	50	42	4.5	2	2	4.90
	55	50	2.5	2	3	5.50
	32	54	1.8	1	3	4.36
	29	58	2.0	1	3	4.40
	57	63	2.5	1	2	3.50
	41	62	1.3	2	2	4.26
	60	64	3.1	1	2	3.62
	50	64	2.0	1	3	4.40
	69	66	2.3	2	2	4.46
	77	64	2.7	3	3	6.54
	55	62	2.0	1	2	3.40
	64	60	1.2	1	2	3.24
	60	64	3.6	3	3	6.72
	69	62	3.5	3	3	6.70
	55	60	2.2	3	3	6.44
	67	66	2.6	1	2	3.52
	84	56	3.0	2	3	5.60
High G_2 scores	60	181	0.8	2	2	4.16
	66	115	1.5	2	3	5.30
	77	113	1.8	1	2	3.36
	62	120	1.4	1	2.5	3.76
	49	106	2.0	1	2	3.40
	58	114	1.5	1	3	4.30
	51	126	1.0	1	2	3.20
	60	107	2.0	3	2	5.40
	53	125	1.1	1	3	4.22
	70	105	1.5	1	3	4.30
	53	101	1.7	2	1	3.34
	37	116	2.0	2	3	5.40
	35	110	2.0	2	1	3.40
	60	96	2.1	2	2	4.42
	56	96	1.2	1	1.5	2.74
	82	92	1.5	1	3	4.30
	95	92	2.8	1	2	3.56
	83	92	1.4	1	2	3.28
	54	138	1.7	1	2	3.34
	73	104	1.6	3	2	5.32
	59	112	1.5	3	3	6.30

[a] The Nottingham Prognostic Index (Galea et al, 1992) = histological grade + stage + (tumour diameter cm × 0.2).

highest blocks of scores from the histogram (Figure 1). The Nottingham Prognostic Index (NPI) was calculated using data on the size of the primary tumour, axillary lymph node status and histological grade (Table 2). The 15-year survival is related to the NPI and can be used to define three sub-groups; a good prognosis group (GPG: NPI < 3.4) with an 80% 15-year survival, a medium prognosis group (MPG: NPI 3.4–5.4) with a 42% 15-year survival and a poor prognosis group (PPG: NPI > 5.4) with a 13% 15-year survival. In the Nottingham study (Galea et al, 1992), the proportions of patients presenting in each sub-group were GPG 29%, MPG 54% and PPG 17%. In our total unselected Tayside cohort, the proportions were similar; GPG 27%, MPG 51% and PPG 22% ($\chi^2 = 0.65$; $P \sim 0.5$) (Figure 2). However, when the breast cancer patients were separated into high and low G_2RS groups, the proportions changed markedly; GPG 15%, MPG 45% and PPG 40% in the low G_2RS group compared to GPG 38%, MPG 57% and PPG 5% in the high G_2RS group (Figure 2). In both cases these proportions were significantly different from those of the total unselected cohort (low G_2, $\chi^2 = 4.13$, $P < 0.05$; high G_2, $\chi^2 = 3.98$, $P < 0.05$).

Comparison of the tumours and patients in the high and low G_2RS groups

The tumours observed in the low G_2RS group were all invasive ductal carcinomas whereas in the high G_2RS group there were two lobular carcinomas and one tubulo-lobular type. There were no significant differences between the low and high G_2RS groups in the median ages of the patients ($P \sim 0.3$), the menopausal status ($\chi^2 = 1.2$, $P > 0.1$) and the histological nodal status ($\chi^2 = 0.22$, $P > 0.5$) (Table 3).

Both the median tumour diameter ($P < 0.001$) and the proportion of oestrogen receptor positive tumours ($P < 0.05$) were significantly different in the two groups. The low G_2RS group tended to have larger tumours which were oestrogen receptor negative

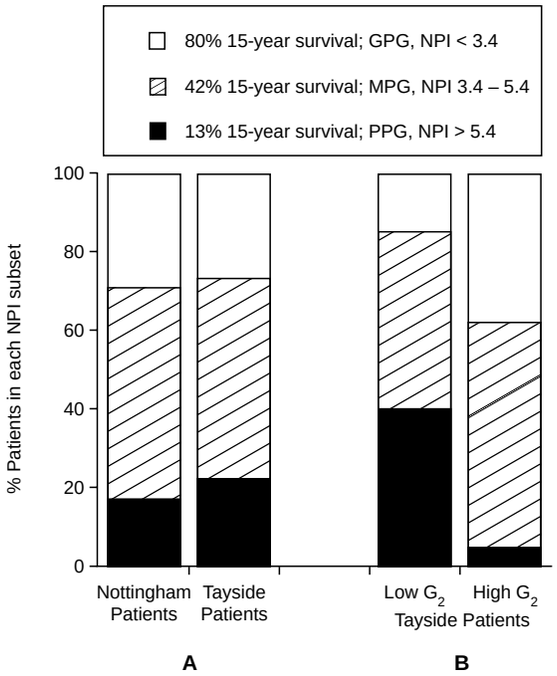

Figure 2 Comparison of the percentage of breast cancer patients in each category defined by the Nottingham Prognostic Index (Galea et al, 1992). (**A**) Comparison of unselected breast cancer patients from Nottingham and Tayside. (**B**) Breast cancer patients from the Tayside group selected on the basis of high and low G_2 score

whereas the high G_2RS group had tumours that were smaller and oestrogen receptor positive (Table 3).

A predicted 15-year survival can be calculated from the proportions of patients in each sub-group as indicated by the NPI. For the high G_2RS group this was 55% compared to 36% for the low G_2RS group. For the total unselected cohort, the predicted 15-year survival would be 46% (Table 3).

DISCUSSION

We have confirmed that a significant proportion of breast cancer patients exhibit an elevated G_2RS value relative to controls. The proportion of radiosensitive patients observed in the Tayside cohort (46.2%) was similar to that reported in the Manchester studies (42%, Scott et al, 1994; 39% Scott et al, 1999; χ^2 corr = 0.6, $P \sim 0.4$). These figures are consistent with data from epidemiological studies suggesting a substantial frequency of low penetrance genetic predisposition to breast cancer (Teare et al, 1994; Chen et al, 1995; Houlston and Peto, 1996). Increased chromosomal radiosensitivity has also been reported in breast cancer patients by Patel et al (1997), Terzoudi et al (2000) and Parshad et al (1996) although the numbers studied in these series do not permit close comparison of the proportions showing increased radiosensitivity.

Assay reproducibility between and within donor groups was similar to that previously reported (Scott et al, 1999). Problems with transport of blood samples were not encountered presumably because of the proximity of the centres in this study.

High lymphocyte G_2RS appears to be unrelated to sex, age or to environmental variables. There is now clear evidence that radiosensitivity, as measured by the G_2 assay, in breast cancer patients is inherited and likely to be a marker for a small number of low penetrance genes predisposing to breast cancer (Roberts et al, 1999). Thus from our data and those of Scott et al (1994;1999), women with a high G_2RS genotype are at approximately five-fold greater risk than those with a normal G_2RS: i.e. penetrance of this trait with respect to breast cancer is estimated at approximately 35%. Hence this heritable characteristic accounts for some 30% of all breast cancer cases, at least six times as many as all other currently recognized forms of heritable breast cancer combined. Patients in this study were not selected on the basis of family history and, given the estimated low penetrance of the cancer trait, detailed analysis of extended family trees would be required to demonstrate heritability.

The Nottingham Prognostic Index has proved useful in predicting survival in breast cancer patients and has been assessed prospectively (Galea et al, 1992; Brown et al, 1993; Balslev et al, 1994). The proportions of the three prognostic sub-groups in our cohort of unselected breast cancer patients were similar to those reported in the much larger study from Nottingham (Galea et al, 1992). However, when subdivided on the basis of the G_2 score, the results were quite different and patients in the low G_2RS group had a much poorer predicted prognosis than those in the high G_2RS group. This difference could not be explained on the basis of age or menopausal status. In fact rather more patients in the low G_2RS group were post-menopausal.

Several properties of the tumours in the low and high G_2RS groups showed distinct differences. In the high G_2 sensitive group, the tumours tended to be not only smaller at presentation but also oestrogen receptor positive. This would lead to a further improvement in prognosis for the high G_2RS group, compared to the low G_2RS group. Taking into account that the 15-year survival in age matched cancer-free controls is 83% then a predicted improvement from 36% to 55% 15-year survival in the low versus the high G_2RS group represents a dramatic difference.

Table 3 Characteristics of patients and tumours in the high G_2 sensitivity group and the low G_2 sensitivity group

	High G_2[a] sensitivity *n* = 21	Low G_2[a] sensitivity *n* = 20	
Median age (range)	60 (30–95)	56 (29–84)	$P \sim 0.3$[b]
Mean G_2 score (breaks per 100 metaphases)	112 ± 4	58 ± 2	
Oestrogen receptor[c] % +ve	81	45	$P < 0.05$[d]
Median tumour diameter (cm)	1.5	2.4	$P < 0.001$[b]
Histological nodal status (% +ve)	43	55	$P > 0.5$[c]
Menopausal status (% postmenopausal)	65	85	$P > 0.1$[c]
Predicted 15-year survival (%)	55	36	

[a]Highest and lowest tertile of the observed range; [b]Mann-Whitney U test; [c]Oestrogen receptor status defined using standard immunocytochemical methods using quick score analysis; [d]χ^2 test.

The above features of the tumours in the high G_2RS cohort are distinct from those associated with other hereditary forms of breast cancer notably those accompanying germline mutations in BRCA1 or BRCA2. However, the characteristics of the non BRCA1 / BRCA2 familial tumours reported recently are similar to those found by us in the high G_2RS group (Lakhani et al, 2000). The non *BRCA1* / *BRCA2* tumours were of lower grade indicating presumably a more favourable prognosis.

Only 3 tumours in the series were of 'special type' (2 lobular carcinomas and 1 tubulo-lobular carcinoma). These tend to carry a relatively good prognosis. All 3 were in the high G_2RS group. One previous report found no significant difference in G_2RS according to tumour grade (Scott et al, 1999). However there was also some evidence that patients with larger tumours (T2 compared to T1) had higher G_2 scores (Scott et al, 1999) which is contrary to our findings. That study was not designed specifically to examine the issue of tumour characteristics and detailed analysis of the findings was not reported.

The possible mechanisms underlying G_2RS and the identity of the low penetrance genes involved in cancer predisposition have not yet been established but it seems likely that chromatid breaks arise by a process involving genomic rearrangements (Bryant, 1998; Rogers-Bald et al, 2000). The cellular response to DNA damage is regulated by a network of proteins. Following the induction of double-stranded breaks in chromosomal DNA, a complex network is activated that regulates DNA repair and progression through the cell cycle (Wang, 2000). Genes in this network protect the integrity of the genome and mutational events in these genes can predispose individuals to cancer. According to one scheme, cancer susceptibility genes can be conveniently classified into two groups: gatekeepers and caretakers (Kinzler and Vogelstein, 1997). Gatekeeper genes are important in cell cycle control and apoptosis. Caretaker genes are involved in DNA repair and inactivation results in genetic instability. We hypothesise that inactivation of caretaker genes influences the processing of DNA damage and thus leads to increased chromatid damage (high G_2RS). The low penetrance gene/s involved in predisposition to breast cancer might thus be members of the caretaker gene family.

The differences that we have reported in this preliminary study of patients with breast cancers in low and high G_2RS groups, would warrant a more detailed study on a larger cohort of breast cancer patients to determine whether the high G_2RS group do indeed have an improved prognosis and comprise a distinct subset for whom specific screening and management policies may be appropriate. Our observations may also facilitate the identification of the low penetrance genes involved. It would not be possible to use the G_2 assay as a population screening method for breast cancer as it is technically demanding and time-consuming. However if these low penetrance genes can be identified, then it will be possible to determine whether specific polymorphisms serve as markers for increased risk of breast cancer.

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