British Journal of Cancer (1998) 78(9), 1214–1218

Homogeneously staining regions in 223 breast carcinomas: cytogenetic and clinicopathological correlations

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Summary A correlation analysis was performed on 223 breast carcinomas to assess the relationships between gene amplification, karyotypic and clinicopathological features. Homogeneously staining region (HSR) is the most frequent form of amplification found in breast cancer. HSR-containing tumours accounted for 60% of the cases. Although up to 40% of tumours with slightly altered karyotype contained HSRs, an excess of HSRs was found within the tumours whose karyotype showed the highest rates of rearranged chromosomes. HSRs were also found to be particularly frequent in small tumours of high histological grade and with a low expression of progesterone receptors. An excess of HSRs seems to be observed in younger patients, however, significant correlation could be demonstrated only for patients below 55 years and below 60 years, compared with older ones. With a 120-month follow-up for 152 patients, a significant association between the presence of HSRs and a shortened overall survival was observed. Altogether, the presence of HSRs appears to be a good indicator of poor prognosis. Further studies are needed to determine whether amplification of specific genes or cell ability to amplify is the most important parameter for tumour progression.

Keywords: amplification; breast cancer; histological grade; survival

Cytogenetic studies and clinicopathological data of breast cancer have established correlations between chromosome alterations and histological grading (Dutrillaux et al, 1991; Emerson et al, 1993; Pandis et al, 1996), proliferative activity (Remvikos et al, 1992) and steroid hormone receptor status (Magdelenat et al, 1992). Other studies have also proposed a possible relationship between prognosis and specific chromosome alterations, such as chromosome 1 rearrangements (Hainsworth et al, 1992) or the presence of homogeneously staining regions (HSRs) (Zafrani et al, 1992). However, most of these studies considered a limited number of cases, and their findings rarely reached statistical significance.

As proposed by Gerbault-Seureau et al (1987) and Saint-Ruf et al (1991), HSRs, as hallmarks of gene amplification, frequently occur in breast cancer cells. Their relationship with known proto-oncogene amplifications, such as *MYC*, *ERBB2* and *CCND1*, has been shown to be complex (Saint-Ruf et al, 1990), and studies using comparative genomic hybridization (CGH) have shown that multiple sites may be the targets of amplifications (Guan et al, 1994; Kallionemi et al, 1994; Muleris et al, 1994*a*, 1995; Trent et al, 1995). Thus, in addition to molecular studies (Bièche and Lidereau, 1995), it remains of interest to determine whether or not the presence of amplified DNA sequences forming HSRs is indicative of an adverse prognosis.

The results of a study of 223 primary breast cancers with karyotypic alterations are reported. They demonstrate a statistical correlation between the presence of HSRs and prognostic factors, confirming the tendencies observed in our preliminary analysis of 84 of these cases (Zafrani et al, 1992).

Received 27 November 1997
Revised 18 March 1998
Accepted 24 March 1998

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MATERIAL AND METHODS

Patients

Between 1984 and 1995, a cytogenetic study was successfully performed on 223 primary breast carcinomas. All patients were females, with ages ranging from 15 to 86 years, the mean age being 58 years. Fourteen patients had been treated with radiotherapy, chemotherapy or hormonotherapy before surgery. TNM staging of the tumours was performed according to the Union Internationale Contre le Cancer (UICC, 1988) (Table 1).

Pathological analysis

All but two tumours, histopathologically classified according to the World Health Organization recommendations, were infiltrating carcinomas. Ductal and lobular types contributed to 85% and 5% of cases respectively. The remaining cases were classified as tubular, medullary, mucinous, apocrine, papillary, pseudosarcomatous and undifferentiated carcinomas. Two cases were classified as ductal in situ carcinomas. Histological grading was performed using a modification of the criteria defined by Bloom and Richardson (Elston and Ellis, 1991). Axillary nodal involvement was recorded, as well as the histopathological size of the tumours.

Cytogenetic analysis

Cytogenetic analysis was performed on fresh tumours after surgery for most of the cases or on drill biopsies in some instances.

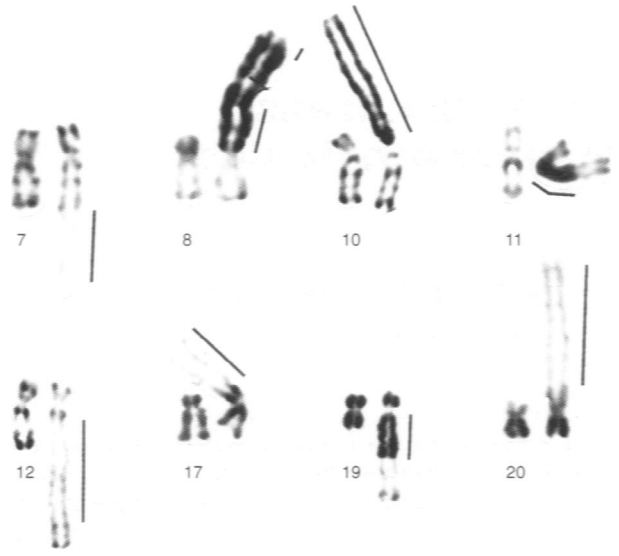

Figure 1 Examples of HSR carrier chromosomes nos. 7, 8, 10, 11, 12, 17, 19 and 20. The lines along the chromosomes indicate the extent and the localization of the HSRS. The corresponding normal chromosome is presented on the left side of the HSR carrier chromosome

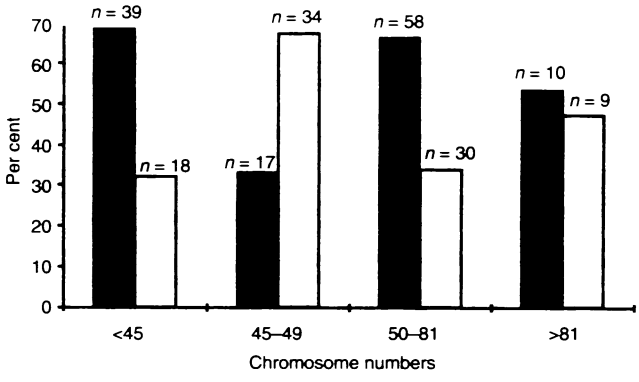

Figure 2 Histograms showing the percentages of the HSR+ (filled bars) and HSR– (empty bars) tumours as a function of chromosome number. *n*, number of cases

Short-term cultures, metaphase spreading and R-banding were performed as described previously (Gerbault-Seureau et al, 1987). Chromosomal counts and rate of rearranged chromosomes were established according to Dutrillaux et al (1991).

Steroid receptor assays

Oestrogen (ER) and progesterone (PR) receptors were measured as previously described (Magdelenat et al, 1992) using solid phase immunoenzymetric assays (ER-EIA and PR-EIA, Abott, USA) according to the manufacturer's recommendations. Briefly, tumour tissue samples were homogenized in a 10 mM Tris glycerol (10% v/v), 10 mM sodium molybdate, 1 mM dithiothreitol buffer containing 0.4 M potassium chloride. After centrifugation at 105 000 *g* (1 h, 4°C), the supernatant (cytosol) was used for ER and PR and total protein assays and the pellet for DNA assay: receptor status was stratified as follows (Magdelenat et al, 1992):

Table 1 Distribution of patients according to the UICC classification

	N0	N1a	N1b	N2	Nx	Total
T0	1	0	0	0	0	1
T1	29	2	7	0	0	38
T2	82	16	20	1	1	120
T3	12	6	6	0	0	24
T4	4	1	4	0	0	9
Total	128	25	37	1	1	192

ER–, PR– if < 500 fmol per g tissue (gt)
ER+, PR+ if ≥ 500 fmol per g tissue (gt)

The receptor status was available for 193 tumours.

Statistical analysis

Correlations between variables were tested using the χ^2 test. Survival curves were drawn using the Kaplan–Meier method (Kaplan and Meier, 1958) and compared with the log-rank test.

RESULTS

The presence of one HSR or more per metaphase was detected in 129 out of 223 (58%) tumours. As a rule, HSRs were present (HSR+) or absent (HSR–) in all metaphases from the same tumour. Among the 14 tumours treated by radio- or chemotherapy before surgery, eight had HSRs, a proportion (57%) similar to that of non-treated tumours. In HSR+ tumours, the number of HSRs per cell varied from one to five, but was fairly constant in a given tumour. DNA amplification was checked by CGH in 28 out of 129 cases of this study. Positive signals were observed in 27 tumours (Muleris et al, 1994*a*, 1995; Bernardino et al, 1998). In one of the two remaining cases, *ERBB2* gene amplification could be demonstrated by fluorescence in situ hybridization (FISH). Thus, the classical R-banding used in this study is assumed to be relevant for DNA amplification detection in HSRs. Examples of HSR carrier chromosomes are shown in Figure 1.

Representativeness of our series

The mean age of the patients in our study (58 years) was slightly higher than that of the large reference series of our institute (56 years).

TNM staging of the tumours was also compared with the reference series. In this series, 0.5%, 15%, 48%, 25% and 11.5% were classified as stage 0, I, IIa, IIb and III respectively, while in the reference series these percentages were of 5.5%, 29.5%, 30%, 23.5% and 11.5% respectively. These differences were due to the low availability of stage 0 or I tumours for cytogenetic studies because of small tumour sizes.

Correlations between the presence of HSRs and other cytogenetic features

As proposed, breast cancer cells that have undergone an endoreduplication can further evolve by chromosome losses and rearrangements, decreasing their chromosome numbers to about 50 (Dutrillaux et al, 1991). Taking this criterion to define the ploidy, 111 tumours were non-polyploid (near-diploid or hypodiploid) (< 50 chromosomes) and 112 were polyploid (≥ 50

Table 2 Relationship between clinicopathological parameters and presence of HSRs in the tumours using the χ^2 test, comparing subsamples a–s. For ER and PR, the observed values were compared with that of a theoretical distribution of HSR at random. *P*-values < 0.05 were considered significant

	No. of patients	HSR+ tumours no. (%)	*P*-value
Patient age (years)			
≤40	21	13 (62) a	a/b: NS
>40	202	116 (57.4) b	
≤50	79	49 (62) c	c/d: NS
>50	144	80 (55.5) d	
≤55	101	66 (66) e	e/f: 0.04
>55	122	63 (52) f	
≤60	123	79 (64) g	g/h: 0.03
>60	100	50 (50) h	
Tumour size (mm)*			
≤20	68	47 (69) i	i/j+k+l: 0.01
≤30	83	44 (53) j	i/j+k/l: 0.02
≤40	31	17 (55) k	i/j/k/l: 0.06
>40	33	14 (42.5) l	
Histological grade**			
1	23	6 (26) m	
2	101	53 (52.5) n	m/n/o: 0.0001
3	76	57 (75) o	
Hormonal receptors***			
ER–	73	47 (64.38) p	p/q: 0.066
ER+	120	61 (50.83) q	
PR–	85	57 (67.06) r	r/s: 0.006
PR+	108	51 (47.22) s	
ER+PR+	101	46 (45.5)	NS
ER+PR–	19	15 (79)	0.012
ER–PR+	7	5 (71.4)	NS
ER–PR–	66	42 (63.6)	0.027

*Data missing in eight cases. **Data concerning infiltrating carcinomas only. ***Data missing in 30 cases.

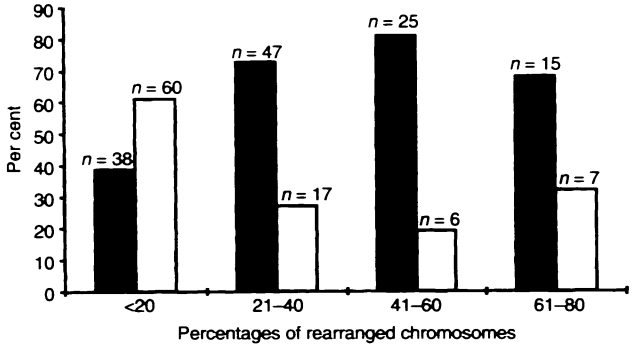

Figure 3 Histograms of the percentages of HSR+ (filled bars) and HSR– (empty bars) tumours in relation to the percentage of rearranged chromosomes. *n*, number of cases

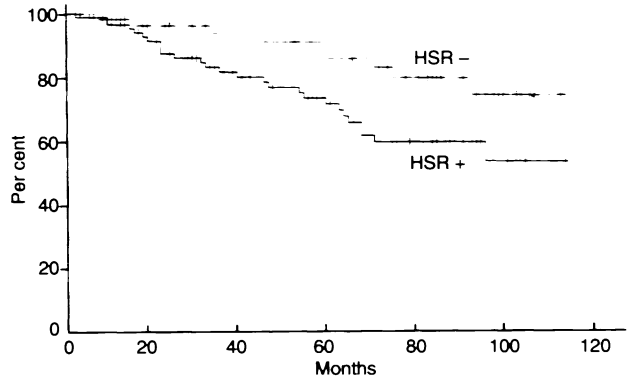

Figure 4 Percentages of overall survival in relation to the presence (HSR+) or absence (HSR–) of HSRs over a 117-month period (152 patients)

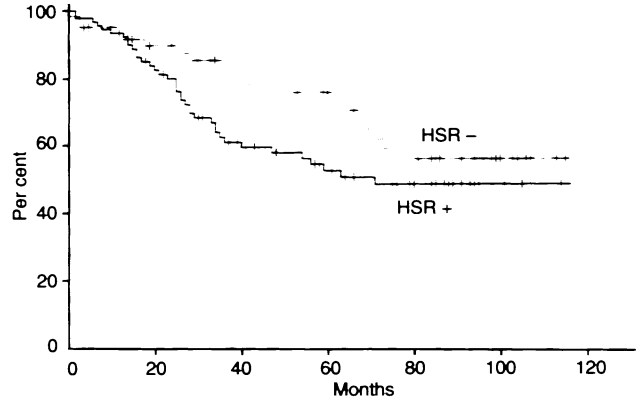

Figure 5 Percentages of disease-free intervals taking into account death, local relapse and metastasis occurrence in relation to the presence (HSR+) or absence (HSR–) of HSRs over a 117-month period (152 patients)

chromosomes). HSRs were observed in 55 (49.5%) and 74 (66%) of non-polyploid and polyploid tumours respectively. Thus, there are significantly more HSR+ polyploid than non-polyploid tumours (χ^2= 6.2; ν = 1; P = 0.0125). A more precise analysis with regard to HSR distribution in relation to ploidy showed that HSR– tumours were clustered at modal chromosome number around 46 (45–49). In contrast, HSR+ tumours had a wider distribution, but most of them were found among the most hypodiploid or hypotetraploid tumours (Figure 2). The excess of HSR+ tumours among hypodiploid (<45 chromosomes) and hypotetraploid (50–81 chromosomes) is very significant (χ^2= 17.7; ν = 3; P = 0.0005; data from Figure 2).

With regard to the correlation between the presence of HSRs and chromosome rearrangements, HSR– tumours had a lower rate of rearrangements than HSR+ tumours. When the tumours were grouped according to the percentage of rearranged chromosomes (<20%, 21–40%, 41–60%, >60%), the presence of HSRs appeared to be strongly correlated with this percentage (χ^2 = 28.6; ν = 3; P = 0.0001; data from Figure 3). It is noteworthy that even in tumours with few (<20%) rearranged chromosomes, the proportion of HSR+ tumours is not negligible (39%).

Correlations between presence of HSRs and clinicopathological data

Age of the patients

The proportion of HSR+ tumours was higher in younger than in older patients on average (Table 2). However, comparing younger and older patients, a significant excess of HSRs in younger patients was observed for 55 or 60 years cut-off only.

Tumour size

Surprisingly, there was an inverse relationship between tumour size and the presence of HSRs. For example, tumours below 20 mm were more frequently HSR+ than larger ones (Table 2).

Axillary node status

No correlations were found between the presence or absence of metastatic axillary nodes and HSRs.

Histological grade

There was a strong relationship between the presence of HSRs and high histological grade, the rate of HSR+ grade 3 tumours being about threefold that of grade 1 (Table 2).

Steroid hormone receptor status

There were more HSR+ tumours among those which had low rather than high hormone receptor expression, but the difference reached statistical significance only for progesterone receptors ($\chi^2 = 7.6$; $v = 1$; $P = 0.006$, Table 2).

Survival

The follow-up data were available for only 152 patients and ranged from 4 to 117 months. The overall survivals were 90% and 74% at 60 months for HSR– and HSR+ tumours respectively. At 110 months, the overall survivals were 74% and 54% respectively (Figure 4). The presence of HSRs was found to be significantly associated with a shortened overall survival ($\chi^2 = 4$; $v = 1$; $P = 0.04$).

Disease-free survival

As shown in Figure 5, the presence of HSR seems to be associated with a shortened disease-free survival, but the difference between HSR+ and HSR– tumours does not reach a statistical significance. Disease-free survivals were 76% and 52% at 60 months for HSR– and HSR+ tumours respectively. Although not statistically significant, the presence of HSRs seemed to be related to metastatic recurrence at 48 months, but not to local recurrence (data not shown).

DISCUSSION

Gene amplification is a common alteration of breast cancer cells. Molecular studies have shown that some genes, such as *ERBB2*, can be amplified and overexpressed in about 30% of the cases, and suggested that such amplifications could be related to prognosis (Slamon et al, 1987; Hynes, 1993).

DNA amplification can also be detected by cytogenetic analyses, which show that, in breast cancer, almost all amplifications are intrachromosomal and form HSRs (Gerbault-Seureau et al, 1987). This differs from other cancers such as gliomas, in which amplified genes are frequently extrachromosomal, forming double minutes (Muleris et al, 1994*b*). HSRs, in breast cancers, are frequently of a large size, accounting for more than 15% of the haploid genome per cell (Saint-Ruf et al, 1990). This suggested that amplifications either lead to very high numbers of copies of target DNA sequences, or that multiple sequences can be coamplified. Studies by CGH (Kallioniemi et al, 1994; Muleris et al, 1994*a*) provided a partial answer, showing that multiple chromosome bands could be involved in amplification in a given tumour with up to five different origins being detected in a single HSR (Guan et al, 1994; Muleris et al, 1995). This raises a number of questions about the meaning of these amplifications. Do they occur early during tumour genesis or are they a common event occurring during tumour progression? In the first eventuality, do they correspond to a special mechanism influencing tumour growth, metastatic potential and prognosis? This study was conducted to answer these questions.

Our study showed that a proportion of HSRs occur in tumours with minimal chromosome alterations: about 40% of tumours with less than 20% of rearranged chromosomes are HSR carriers. This suggests that HSRs can be formed during the early phase of tumour development. However, the strong correlation between the presence of HSRs and the rate of rearranged chromosomes per tumour shows that HSRs, which can occur early, are also formed during tumour progression. Thus, the amplification process occurs early and continues during tumour progression. The relationship between the presence of HSRs and age at tumour onset is of interest. Whatever the cut-off in relation to the age, HSR+ tumours are always more frequent in younger than in older patients, but the excess in younger patients reaches statistical significance at 55 and 60 years cut-off only. Among other interpretations, this could mean that breast cancers in post-menopausal patients have a lower tendency to form gene amplification. The effect of ageing on HSR incidence is, however, difficult to demonstrate because of the low number of cases in each age group.

Surprisingly, the probability of the presence of HSR was found to be inversely related to tumour size. Indeed, this may be also related to age, larger tumour sizes being observed in older patients, but it strongly suggests that tumour progression is quite different in young and old patients.

The strongest relationship with a pathological parameter was found between the presence of HSRs and high histological grade. The presence of HSRs was also found to be related to the loss of steroid hormone receptor expression and, in particular, to that of progesterone receptors, the highest rate of HSRs being observed in ER+/PR– and ER–/PR– tumours. The presence of HSRs appeared to be independent of axillary nodal status.

It remains to be determined whether the relationship with prognosis concerns the amplification process in general or is related to the involvement of specific genes. Unfortunately, there are no strong data to answer this question. Interpretation of the prognostic value of *ERBB2* amplification is controversial (Hynes and Stern, 1994). It is now admitted that it is associated with a poor prognosis in node-positive patients only (Noguchi et al, 1992; Marks et al, 1994). We do not know which of our HSR+ tumours had an *ERBB2* amplification, but we expect that a high proportion of them had it because the incidence of *ERBB2* amplification in non-selected cases is 20–30% (Brison, 1993).

Thus, a HSR+ tumour is typically of small size and occurs in younger patients with high histological grade disease and who are progesterone receptor negative. As a matter of fact, in our series, 86% of the tumours measuring 20 mm or less, PR– and of grade 3 were HSR+. This suggests that the presence of HSRs is related to adverse prognostic factors. Such a conclusion is strengthened by the data on 5-year survival, which show a lower survival for patients with HSR+ than HSR– tumours.

ACKNOWLEDGEMENTS

This research was supported by grants from La Ligue Nationale Contre le Cancer (Comité national et Paris) and by the Association pour la Recherche sur le Cancer (ARC). J Bernardino is a fellow from the ARC.

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