Analysis of the IGF-II receptor gene copy number in breast carcinoma

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Summary Insulin and the insulin-like growth factors (IGFs) may be important regulators of breast cancer growth. The IGF-II receptor is identical to the mannos 6-phosphate (Man-6-P) receptor, which is involved in lysosomal enzyme pathways. In order to determine whether the Man-6-P/IGF-II receptor gene copy number is altered in breast cancer we analysed specimens of invasive breast carcinoma from 51 patients by Southern blotting. No amplification of the receptor gene was observed whatever the clinical presentation of the tumour and irrespective of a concomitant amplification of c-erbB2 or int-2 genes in several tumours. As indicated by Northern blots, such amplification of the Man-6-P/IGF-II receptor gene was also detected in breast tumour tissues and non-tumour breast tissue. These results suggest that the receptor gene is stable in breast carcinoma and that, if anything, the receptor involvement in breast cancer progression may be the result of a disregulation of its expression at a post-transcriptional or post-translational level.

Materials and methods

Patients and pathological material

Tissue specimens were obtained during surgery, washed in saline and immediately frozen in liquid nitrogen. Specimens of invasive carcinoma were obtained from 51 patients treated for breast cancer at the University Hospital of Tours. The types of investigation, histopathological type classification and prognostic grades, stage of the disease, therapeutic steps, and follow-up have already been described (Bougnoux et al., 1991). The tumour was predominantly of the ductal type in 41 patients, lobular in three patients and of other types in the remaining patients. Two specimens were from local relapses, within breast tissue that had received radiation therapy (65 Gy). As reference, control tissues were also analysed: a fibroadenoma, a non-proliferative dysplasia, a portion of non-tumour tissue close to a carcinoma and two specimens of placenta from voluntary abortion products at 7.5 and 8 weeks of pregnancy.

DNA and RNA extraction

Genomic DNA was prepared from frozen powdered tissue by phenol extraction (Sambrook et al., 1989). After ethanol precipitation, the DNA samples were dissolved in 10 mM Tris - 1 mM EDTA (pH 7.4) and stored at -20°C. Total RNA was extracted as previously described (Chomczynski & Sacchi, 1987), redissolved in 10 mM Tris buffer, pH 7.5, and stored at -70°C.

Probe

The 7.9-kb XbaI/SalI fragment of Man-6-P/IGF-II receptor cDNA was excised from the pGEM-MPR8 kindly supplied by Dr W.S. Sly (St Louis University School of medicine, St Louis, MO, USA) and radiolabelled with [α-32P]dCTP (3000 Ci mmol⁻¹) by the random primer method of Feinberg and Vogelstein (1984). For Southern blot analysis of c-erbB2 the plasmid pSV2-erbB2 (Yamamoto et al., 1986) was digested to completion with HindIII to release the full-length 4.4-kb c-erbB2 cDNA, which was radiolabelled by the above-mentioned technique. The probe used to visualise the int-2 locus corresponded to a 0.9-kb SaeI fragment (designated S66) that spans the presumptive second exon of the human gene (Casey et al., 1986). We used actin cDNA (Minty et al.,

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DNA analysis

Ten micrograms of DNA was digested with EcoRI and XhoI and electrophoresed through a 0.8% agarose gel. The DNA was blotted onto Hybond-N membrane (Amersham, Buckinghamshire, UK) and cross-linked by alkaline treatment of the membrane in 0.4 M sodium hydroxide. Hybridisation was performed according to the method of Mahmoudi and Lin (1989): briefly, the membrane was hybridised at 68°C overnight with 2 x 10^6 c.p.m. ml^-1 radiolabelled probe in phosphate buffer 1 M, 20% SDS, 15% bovine serum albumin (BSA), 0.5 M EDTA and 10 mg ml^-1 denatured sonicated salmon sperm DNA. The blot was then washed to high stringency (0.2 SSC at 68°C) and autoradiographed for 1–3 days at ~70°C using Fuji medical X-ray film.

RNA analysis

Total RNA (50 μg per lane) was separated by electrophoresis in glyoxal gels (1.4% agarose), transferred to nylon membranes, hybridised with the α-32P-labelled mannose 6-phosphate/IGF-II receptor or actin cDNA probe, and washed according to the above-described method for DNA analysis.

Densitometric scanning of the gels

Quantification of the intensities of the autoradiographs was carried out using a Bioprolif densitometric scanning apparatus (Vilbert-Lourmat, France).

Results

Southern blot of breast cancer tissues DNA

DNA isolated from primary breast tumours was analysed with a Man-6-P/IGF-II receptor cDNA probe. The digestion pattern obtained from Southern blotting analysis of nine of the tumour DNA samples (lanes 1–9) and four non-tumour tissues, a fibroadenoma (lane 10), a non-proliferative dysplasia (lane 11), a non-tumour tissue close to a carcinoma (lane 12) and a 7.5 weeks’ gestation placenta (lane 13) is presented in Figure 1. Several restriction fragments of the Man-6-P/IGF-II receptor gene were visible. The pattern obtained was representative of all the samples examined, either tumour or reference non-tumour tissues. The differences in intensity between the fragments of different lanes reflected the differences in loading of tumour DNA as controlled by the densitometric scanning of the blot compared with the densitometric scanning after rehybridisation with the actin cDNA probe (data not shown) and with α-satellite DNA (Figure 2). No amplification of the receptor was detectable in the 51 tumour DNA samples.

In order to ensure that the tumours analysed were representative of breast cancer, c-erbB2 and int-2 genes, two genes known to display amplification in a number of breast tumours, were investigated in the breast tissues investigated for Man-6-P/IGF-II receptor gene copy number (Figures 3 and 4). Figure 3 shows the results obtained from Southern blotting analysis of 11 of the tumour DNA samples and of two non-tumour breast DNA samples. Moderate amplification of the 7-kb c-erbB2 fragment was seen in some tumours (lanes 3, 11 and 14). A high level of amplification was observed in lane 10. Figure 4 shows the results obtained from Southern blotting analysis of 14 tumour DNA samples: amplification of the 6-kb int-2 fragment was detected in some tumours (lanes 1–3, 13 and 14). The total number of tumour tissues investigated was 36 for c-erbB2 and 25 for int-2; eight and three tumours had a gene amplification. The number of

![Figure 1 Southern blot analysis comparing the gene copy number of Man-6-P/IGF-II receptor in breast tumour tissues, in breast non-tumour tissues and in placenta tissue. Tumour (lanes 1–9) and non-tumour (lanes 10–13) DNAs were digested with EcoRI and XhoI and hybridised to a 32P-labelled Man-6-P/IGF-II receptor cDNA probe (see text for details).](image1)

![Figure 2 Autoradiography of the Figure 1 blot hybridised with a 32P-labelled α-satellite DNA.](image2)

![Figure 3 Southern blot analysis comparing the gene copy number of c-erbB2 in breast tumour and in breast non-tumour tissues. Tumour (lanes 2–4 and 6–13) and non-tumour (lanes 1 and 5) DNAs were digested with EcoRI and XhoI and hybridised to a 32P-labelled c-erbB2 cDNA probe.](image3)
patients with gene amplification according to prognostic factors is presented in Table I.

**Northern blot**

We analysed total RNA of 15 of the mammary tumours analysed above and one non-tumour breast tissue. An apparently normal size 9-kb transcript (Oshima et al., 1988) was detected when total cellular RNA was subjected to Northern blot analysis. Figure 5 shows the result for two breast tumour tissues (Figure 5a, lanes 1 and 2) and non-tumour breast tissue (Figure 5a, lane 3) using XhoI/SalI fragment of Man-6-P/IGF-II receptor cDNA as a probe. In order to control for slight differences in loading of tumour RNA and for RNA degradation, all membranes were rehybridised with actin cDNA (Figure 5b, lanes 1–3). The same slight differences in RNA signals were observed in the two hybridisation experiments (Figure 5a and b).

**Table I** Comparative amplification of Man-6-P/IGF-II receptor gene with c-erbB2 and int-2 genes in invasive breast carcinoma

| Prognostic factor | Man-6-P/IGF-II | c-erbB2 | int-2 |
|-------------------|---------------|---------|-------|
|                    | Amplif. | Amplif. | Amplif. |
| Age (years)        | n²      | n²      | n²      |
| ≤45                | 12      | 10      | 8      |
| >45                | 39      | 26      | 17     |
| Stage              |         |         |       |
| I                  | 10      | 7       | 6      |
| II                 | 23      | 14      | 8      |
| III                | 17      | 14      | 10     |
| NA                 | 1       | 1       | 0      |
| Axillary lymph nodes |       |         |       |
| Negative           | 14      | 11      | 9      |
| Positive           | 19      | 13      | 10     |
| NA                 | 18      | 12      | 6      |
| Histological grade |         |         |       |
| I or II            | 30      | 17      | 11     |
| III                | 17      | 15      | 10     |
| NA                 | 4       | 4       | 4      |
| Vascular invasion  |         |         |       |
| Absent             | 33      | 24      | 17     |
| Present            | 12      | 10      | 6      |
| Unknown            | 6       | 2       | 2      |
| Oestrogen receptor |         |         |       |
| ≤10 fmol mg⁻¹      | 8       | 6       | 6      |
| >10 fmol mg⁻¹      | 43      | 30      | 19     |

*Number of tumours examined. †Number of tumours with gene amplification. ‡Pathological status. NA, not applicable.

**Discussion**

The data reported indicate that no amplification of the Man-6-P/IGF-II receptor gene took place in the tumour tissues of a set of 51 breast cancer patients. This observation was found independently of the clinical presentation of the tumour and irrespective of a concomitant amplification of c-erbB2 and int-2 genes in several tumours. Tumours are known to be heterogeneous. First, within the same tumour tissue sample some tumour cells can be found at different stages of tumour progression, therefore displaying different abnormalities. Second, tumour cells are annexed with stroma cells derived from the host, which in contrast are not expected to present such genomic abnormalities. Therefore, in these conditions, the simultaneous amplification of oncogenes such as c-erbB2 or int-2, along with the lack of amplification of the Man-6-P/IGF-II receptor gene, strongly argues against any underestimation of a potential amplification of the gene. In addition, the lack of amplification of the receptor gene is supported by the fact that the intensity of the tumour DNA signal is very similar to that of non-tumour tissues (Figure 1). It is improbable that the results presented represent biased selection of the tumour samples analysed for the following reasons. First, the distribution of patients according to age, stage and pathological type or prognostic grade is close to the usual presentation of breast cancer (Henderson et al., 1989). There was, however, some selection in favour of tumours of large size, because of the need for adequate material for analysis. Hence our series is enriched in larger tumours and contains a higher than usual proportion of patients with either positive axillary lymph nodes or of unknown lymph node status (since patients with tumours larger than 30 mm at presentation had a surgical biopsy of their tumour, prior to adjuvant chemotherapy, and no axillary dissection). This case selection would indicate that the tumours that we analysed were actually at an advanced rather than an early stage of tumour progression, and therefore perhaps more prone to possess genetic abnormalities than tumours obtained at earlier stages of the disease. Secondly, we found that 22% and 12% of the
tumour specimens examined had an amplification of c-erbB2 and int-2 respectively. This is in line with results already reported, with a range of 20–30% for the frequency of amplification for c-erbB2 (Slamon et al., 1987), and of less than 20% for int-2 (Lidereau et al., 1988). In addition, all tumours with int-2 gene amplification were oestrogen receptor positive, an observation previously reported (Borg et al., 1991). These findings suggest that the lack of amplification of Man-6-P/IGF-II receptor along with an amplification of other genes is likely to reflect a phenomenon specific to this gene.

The analysis of the receptor RNA level in some tumours and one non-tumour tissue indicates that the receptor gene is transcribed in all the tissues examined. We have not measured the level of the receptor protein product because of limited availability of tumour tissues. Therefore we cannot rule out the possibility that post-transcriptional or post-translational mechanisms could lead to a modification of expression of the receptor in breast cancer as has been observed in thyroid neoplasms (Yashiro et al., 1991).

It is widely accepted that multiple genetic alterations are essential for the development of malignant tumours, including human breast cancer. The gene alterations that have been found in human breast cancer are mostly amplifications of a small number of oncogenes. Other growth factor receptors not strictly defined as oncogenes may be good candidates for amplification in breast cancer. Indeed the IGF-I receptor, which has been shown to have prognostic value (Peyrat & Bonneteer, 1992), was reported to be sporadically amplified in breast cancer (Borns et al., 1992). Some human cell lines contain increased quantities of the insulin receptor protein although its gene is not amplified or overexpressed (Milazzo et al., 1992).

The Man-6-P/IGF-II receptor molecule in the trans Golgi network play an essential role in lysosomal enzyme trafficking, and this may explain the stability of its gene. Further experiments, such as immunolocalisation of the receptor protein, are required to clarify its role if any in breast cancer growth.

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