Association of INT2/HST1 coamplification in primary breast cancer with hormone-dependent phenotype and poor prognosis

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Summary The human proto-oncogene INT2 (homologous to the mouse INT2 gene, implicated in proviral induced mammary carcinoma) has been mapped to chromosome 11q13 and found to share band localisation with, among others, the HST1 proto-oncogene. Both genes are members of the fibroblast growth factor family. In the present study, coamplification (2–15 copies) of the INT2/HST1 genes was found in 27 (9%) of 311 invasive human breast carcinomas using slot blot and Southern blot analyses. Amplification was not correlated to tumour size, axillary lymph node status or stage of disease, neither to patient age nor menopausal status. However, 26 (96%) of the 27 amplified tumours were, often strongly, Oestrogen receptor positive compared to 65% of the unamplified cases (P = 0.001). These findings are in sharp contrast to the strong correlations of HER-2/new proto-oncogene amplification with advanced stage and steroid receptor negativity, previously observed in the same series of tumours. Patients with INT2/HST1 amplified breast cancer had a significantly worse disease-free survival than patients with unamplified genes (P = 0.015, median follow up 45 months). This correlation was confined to node-negative patients and persisted in multivariate analysis. No significant correlation to survival from breast cancer was found. It is concluded that amplification of the 11q13 region in breast cancer occurs in a particular subset of aggressive tumours, quite different from that identified by HER-2/new amplification. It remains to be shown that the selection for amplified genes at 11q13 is due to the activity of INT2, HST1 or yet another, still unidentified, neighbouring gene. However, the results are potentially of clinical value in separating a group of node-negative breast cancer for more intense treatment.

The putative proto-oncogene INT2 is known as one of several integration sites for mouse mammary tumour virus (MMTV), a retrovirus implicated in mammary tumorigenesis in certain strains of mice (Nusse, 1988a). INT2 encodes a predicted member of the fibroblast growth factor (FGF) family of potent mitogens or morphogens involved in angiogenesis, tissue induction and cell migration (Dickson & Peters, 1987; Thomas, 1988; Burgess, 1988). The human INT2 gene has been cloned, found to be 89% homologous to the mouse INT2, and mapped to chromosome 11q13 (Brookes et al., 1989; Case et al., 1986). Interestingly, another proto-oncogene, HST1 (HSTF1), was localised to the same chromosomal site and also found to be a FGF member (Adelaide et al., 1988, Yoshida et al., 1987, 1988a).

HST1 was initially detected as a transforming gene in DNA from human stomach cancer (Sakamoto et al., 1986), found virtually identical to the K5 oncogene from Kaposi sarcoma (Delli Bovi et al., 1987), and recently also identified as an alternative integration site for MMTV in mouse mammary tumours (Peters et al., 1989). INT2 and HST1 are closely linked in the mouse genome (Yoshida et al., 1988b) and only 35 kilobasepairs apart in the same transcriptional orientation in the human genome (Wada et al., 1988). This suggests that they originated through duplication of a common ancestral gene during evolution, and that this region may involve still other, yet unknown, related genes.

Amplification of the 11q13 region have been reported from various solid tumours including, besides breast cancer, squamous cell carcinomas (Zhou et al., 1988, Berenson et al., 1989), a stomach cancer and the vulvar carcinoma cell line A431 (Yoshida et al., 1988a), melanomas (Adelaide et al., 1988, Theillet et al., 1989), bladder and Oesophageal carcinomas (Tsutsumi et al., 1988; Tsuda et al., 1988, Theillet et al., 1989), and a hepatocellular carcinoma (Hatada et al., 1988). It usually entails the INT2 and HST1 genes and also the BCL1 locus, recognised as a chromosomal breakpoint in B-cell leukaemia (Tsujimoto et al., 1984), but not other genes located at the same or neighbouring bands (Ali et al., 1989). Multiple endocrine neoplasia type 1 (MEN-1), the pathogenesis of which seems to involve a putative FGF-membrane, have also been linked to a locus proximal to the INT2 gene at 11q12-q13 (Nakamura et al., 1989; Bale et al., 1989). Consequently, although no evidence yet exists, the plain fact that both selective amplifications and non-random translocation encompass the same chromosomal regions, points to its importance in the development of human cancer.

In breast cancer, amplification of the INT2 and HST1 genes have been found in 9–25% (Zhou et al., 1988, Liendeau et al., 1988; Varley et al., 1988; Tsuda et al., 1989, Theillet et al., 1989; Adnane et al., 1989; Fanti et al., 1989). When clinical follow-up was available, a correlation to poor survival was noted. In the present study we report on INT2/ HST1 coamplification in a particular subset of human breast cancer with hormone-dependent phenotype, and on the correlation to disease outcome among low-risk patient categories.

Materials and methods

Patients and tumour material

Patients were all from the southern Sweden health care region, diagnosed for breast cancer during the time interval of October 1982 and February 1985 and had a tumour sent for steroid receptor analysis. Tumours used for the present study were consecutive cases with a tissue amount allowing gene analysis, representing about 25% of all new cases of breast disease diagnosed during this time period. Cases ineligible for the study (e.g. benign disease, cancer in situ or samples from metatases) were excluded, as were tumours judged to be too cell-poor after cytopathological examination of tissue imprints. Patients presenting with bilateral cancer were not excluded if it was clear which primary tumour recurred.

Of the remaining 311 tumours, 27% were classified according to UICC as Stage I, 36% as Stage IIa, 24% as Stage IIb, 7% as Stage III, and 7% as Stage IV (distant spread at diagnosis or within 2 months after primary operation). Ten
patients not treated with axillary resection were unclassified. However, none of these had metastases at diagnosis. The range of patient age at operation was 31–92 years (median 63 years), 22% were premenopausal and 78% were postmenopausal. Adjuvant tamoxifen was given to 38% of the patients, adjuvant chemotherapy (cyclophosphamide) to 6%, whereas 45% received postoperative radiation (Sigurdsson et al., 1990). Recurrences were clinically confirmed and registered as loco-regional or distant. Deaths were distinguished as due to breast cancer or to intercurrent disease. Distant recurrences were found in 94 cases and loco-regional recurrences in 11 cases. Of 115 deaths, 81 were due to breast cancer. Median follow-up for all patients was 46 months, for those still living 53 months, and for those still living or dead in intercurrent disease 51 months. Only distant recurrences were considered in the calculation of distant disease-free survival, which also locoregional recurrences were included in disease-free survival. Death due to breast cancer was used as endpoint in breast cancer survival and death due to other causes were censored. Only patients with Stage I–III (M0) disease were included in the survival analyses.

Steriod receptor analysis

Measurements of Oestrogen (ER) and progesterone receptors (PgR) were performed within two weeks after surgery, at one laboratory and with radioligand binding techniques (isoelectric focusing and dextran-coated charcoal (DCC) with Scatchard analysis, respectively) as described previously (Norgren et al., 1982). The isoelectric focusing assay has previously been shown to be equivalent to the DCC assay for ER measurement (Fernö et al., 1983). Cut-off points of 10 fmol mg⁻¹ protein were used for classification of tumours as receptor positive or negative.

Gene analysis

DNA was extracted from pulversed tissue (Krieg et al., 1983) and checked for purity and high molecular weight integrity. According to fluorometric determination of DNA concentration, equal amounts (5 μg) of RNAase treated DNA were applied on Zetaprobe nylon membranes using a Bio-Dot SF blotting apparatus (BioRad laboratories, Richmond, CA). Ten μg of BamH1 or EcoR1 digested DNA were separated in 0.8% agarose gels and transferred to nylon membranes (Southern, 1975). Membrane hybridisation was carried out under stringent conditions according to the manufacturer's description with 10⁶ cpm ml⁻¹ multimple labelled (Amersham International plc, Buckinghamshire, England) DNA probes. For repeated hybridisation, probes were removed and membranes checked for absent signals. The DNA probes used for the study were; INT2 (0.9 kb SauI genomic DNA fragment, SS6) HST (0.59 kb AvrI cDNA fragment, ORF1) and progesterone receptor (2.6 kb BamH1-PsiI cDNA fragment, HPR-54). Degree of amplification was evaluated with densitometric analysis of short time exposed slot blot autoradiograms, in comparison with dilutional analysis of amplified samples and expressed as copies of the haploid genome. The PgR gene (11q22-q23) was used as internal control for the genes at 11q13.

Statistical analysis

The association of gene amplification with other categorised clinico-pathological variables was assessed by χ²-square analysis. Survival curves were calculated by the method of Kaplan and Meier (1958). Tests of differences between curves were made with the log-rank test for censored survival data (Mantel, 1966). Multivariate analyses were performed with Cox's partially nonparametric regression model (Breslow, 1975; Cox, 1972). The Biomedical Computer Program P series (Dixon, 1988) was used in all survival analyses.

Results

INT2/HST1 amplification

Hybridisation of the INT2 SS6 probe to BamH1 digested DNA (Figure 1) revealed the known polymorphism of this locus (two alleles; 8.4 and 5.6/2.8 kb fragments, respectively (Casey et al., 1986)) and the normal, approximately, 2:1 distribution of alleles among studied tumours. Both alleles were found affected by amplification but, in tumours heterozygous for the site, in no cases simultaneously. The HST pORF1 probe recognised four constant EcoR1 digested DNA fragments (Figure 2). The three shortest fragments (5.8, 2.8 and 0.8 kb) harbour the HST1 gene, while the largest fragment (8.0 kb) represents binding to the HST2 gene (Yoshida et al., 1988a). Amplification was found to exclusively affect the HST1 gene.

INT2 amplification was detected in 27 (9%) of 311 slot blot analysed tumours using the PgR gene as a single copy standard (Figure 3). The HST1 gene was found to be amplified in the same 27 tumours and to approximately the same degree, strongly suggesting that these two related genes are amplified as one amplilon unit. Degree of amplification

![Figure 1](image1)

**Figure 1** Hybridisation of the INT2 SS6 probe to BamH1 digested breast cancer DNA. A two allele polymorphism is seen; 8.4 and 5.6/2.8 kb, respectively. Amplification of the INT2 gene is seen in lane 3 (7 copies), lane 4 (3 copies), lane 11 (5 copies), lane 16 (8 copies) and lane 18 (15 copies). A partial rearrangement of the INT2 gene is seen in lane 16. Lane 1 shows HindIII digested lambda phage DNA.

![Figure 2](image2)

**Figure 2** Hybridisation of the HST pORF1 probe to EcoR1 digested breast cancer DNA. Bands at 5.8, 2.8 and 0.8 kb represent binding to the HST1 gene, while the band at 8.0 kb harbours the HST2 gene. Amplification (3–7 copies) of the HST1 gene is seen in lane 4–6.
ranged from 2–15 copies; 18 samples having 2–4 copies, 8 samples 5–10 copies, and 1 sample >10 (15) copies of the genes.

**INT2/HSTI** amplification in relation to other prognostic factors

**INT2/HSTI** amplification was not statistically correlated with axillary lymph node status. The tendency of gene amplification being more prevalent in node positive tumours was caused by the relatively high incidence of amplification in the node positive subgroup with few involved nodes. No association with tumour size or stage of disease was seen, neither to patient age or menopausal status (Table 1).

However, 26 (96%) of the 27 amplified tumours were ER positive, compared with 65% of the unamplified cases ($P = 0.001$). The ER concentration in amplified tumours was most often of high levels (>200 fmol/mg protein, Figure 4) and, furthermore, the single deviating sample was not totally devoid of ER, but just below the cut-off value used to classify tumours as ER positive. A similar trend, although not significant, was observed with PgR status. Also shown in Figure 4 is the quite different pattern of ER concentrations in **HER-2/neu** amplified tumours. **HER-2/neu** amplification was found in 52 (17%) of the 311 cases (Borg et al., 1990). Three of these were also amplified for the 11q13 region and, noticeably, also those with the lowest ER concentration among **INT2/HSTI** amplified cases.

As not all patients were given postoperative radiation and the same, or any, adjuvant therapy, a bias might be introduced in the calculation of survival differences. However, there was no distinction whatever between amplified and unamplified tumours in respect of therapy. Adjuvant tamoxifen, adjuvant chemotherapy and postoperative radiation were given to, respectively, 12 (44%), 2 (7.4%) and 12 (44%) of the 27 amplified tumours, and to, respectively, 106 (37%), 18 (6.3%) and 127 (45%) of the 284 unamplified tumours.

**INT2/HSTI** amplification in relation to survival

**INT2/HSTI** amplification was found to be a significant predictor of a shorter disease-free survival (DFS, $P = 0.015$) when analysing all M0 patients ($n = 291$, Figure 5a). There was a trend towards a worse prognosis of tumours with a
high copy number (≥5 copies) compared to those with a low degree of amplification (6 recurrences in 8 cases compared to 7 in 17 cases). Of a total of 13 recurrences in the 25 included amplified tumours, 3 were of loco-regional type while 10 were distant metastases. Thus, the calculation of amplification in relation to distant DFS resulted in a less prominent association (P = 0.12). Neither did the prediction of breast cancer survival (P = 0.19, Figure 5b) reach significance.

The presence of axillary lymph node metastases is widely considered as the most reliable risk factor in breast cancer, and it was natural to perform separate analyses of gene amplification in relation to survival in the node-negative and node-positive patient subgroups. As revealed in Figure 6, the significance of INT2/HST1 amplification as a predictor of DFS was totally confined to the node-negative group (P = 0.030, n = 160). No association was seen in node-positive patients (P = 0.73, n = 120). The corresponding correlations in node-negative patients to distant DFS (P = 0.092) or breast cancer survival (P = 0.21) were, again, not statistically significant.

As INT2/HST1 amplification affected mainly ER positive tumours, this category was analysed separately (Figure 7). A highly significant correlation (P = 0.002) was found to a shorter DFS in amplified ER positive tumours as compared with unamplified ER positive tumours. ER negative tumours had an intermediate DFS pattern (Figure 7). In the ER positive category, amplification was significantly or nearly significantly correlated also to distant DFS (P = 0.026) and breast cancer survival (P = 0.070).

To examine the significance of INT2/HST1 amplification as a predictor of DFS in combination with other prognostic factors, multivariate analyses (Table II) were performed on all M0 patients (actually 264, since lymph node status and PgR status were unavailable in 10 and 16 cases, respectively) as well as on node-negative patients separately (n = 148, PgR status not available in 12 cases). When analysing all M0 patients, lymph node status was the single most powerful predictor of DFS, followed by PgR status and tumour size. INT2/HST1 amplification was retained in the model as a nearly significant variable (P = 0.060) with a relative risk of 1.7 for amplified tumours. In node-negative patients, PgR status and INT2/HST1 amplification were the only and approximately equally significant independent variables (P = 0.011 and P = 0.013, respectively), with a relative risk of 4.0 (95% confidence interval 1.3–12) for amplified tumours. It should be pointed out that the number of tumours and events in some analyses are small and that the results of these must be cautiously interpreted.
The ability of cancer cells to increase their content of certain macromolecules by gene amplification in response to environmental stress or intratumoral competitive growth, is well established (Schimke, 1984). In cytogenetic studies of tumour cells grown for short terms to avoid in vitro artefacts, amplified DNA is seen mainly as extrachromosomal chromatin bodies in forms of double minutes or their precursors (Wahl, 1989). These genetic aberrations replicate autonomously, but lack centromers and are supposed to be randomly distributed during cell division and ultimately lost if not providing a selective growth advantage. The frequent finding of proto-oncogene amplification in human breast cancer point to its role in disease development. At least three different chromosomal regions are commonly affected: entailing the HER-2/neu and ERB Al genes at 17q11.2-1q2, the MYC gene at 8q24, and the INT2/HST1 genes at 11q13 (Callahan, 1989).

The present study suggests that amplification of the 11q13 region in breast cancer occurs in a quite different subset of tumours than affected by HER-2/neu amplification, a conclusion also drawn by Adnane et al. (1989). In contrast to HER-2/neu amplification, INT2/HST1 amplification was not associated with tumour size, an increased number of involved lymph nodes or distant spread. Furthermore, while HER-2/neu amplification was strongly connected with the absence of steroid receptors and probably with an autonomous growth, INT2/HST1 amplification occurred exclusively in ER positive tumours. This implies an importance of INT2/HST1 in earlier stages of certain breast cancers and that an interaction with or a dependence of Oestrogen stimulation may exist. Tumour progression to decreased hormone sensitivity would then be reflected in the loss of unstable genetic aberrations no longer involved in growth regulation, or in the overgrowth of other cell clones within the same tumour. Alternatively, one could consider ER positive and negative tumours as subsets of breast cancer rather than successive progression stages. INT2/HST1 and HER-2/neu amplification may then represent two pathways to reach this difference from a common precursor cell type, or be indicative of the presence of two different original cells.

The PgR gene, one of the major targets of Oestrogen action, was in an initial report (Law et al., 1987) mapped to the same chromosomal site as INT2. The location of the PgR gene was, however, later revised to a more distal site on the long arm (11q22-23, Rouot-Sau-Merck, 1987, Nat-Tei et al., 1988). The PgR gene was in the present study also found in no case to be coamplified with the genes at 11q13.

No evidence exists as yet for a specific physiological role of INT2 or HST1 in humans. An activity of the genes during mesoderm induction was, however, demonstrated in amphibian embryos (Paterno et al., 1989). Mouse HST1 was found expressed during a short interval in midstage embryos (Terada et al., 1989). The normal activity of INT2 in mice is also confined to the early embryonic development, where a stimulation of cell migration and tissue induction rather than of cell proliferation and angiogenesis was suggested (Wilkinson et al., 1989). Its reactivation in the adult mouse mammary gland by inserted proviral enhancers points to a causative role in the subsequent neoplastic formation. Interestingly, these tumours are initially hormone-dependent in that they arise only after several pregnancy cycles and regress also confined pregnancies. When tumours eventually progress to become autonomous, this seems to occur irrespective of further INT2 activity (Peters et al., 1984; Nusse, 1988b). A similar synergism between INT2 or HST1 and sex hormones in the earlier development of certain human breast cancers is conceivable, the former acting as inducers and the latter as promoters.

Arguing against this is the fact that INT2/HST1 amplification is found also in other malignancies in general considered not to be hormone-responsive. Also, it still remains to be confirmed that amplification of the genes actually coincides with a transcriptional activation, a controversial subject in human breast cancer: Liscia et al. (1989) used RNA:RNA interaction hybridisation and Northern blot analysis to show that some INT2/HST1 amplified tumours contained INT2, but not HST1, transcripts, implying that INT2 is the probable gene of significance in the amplicon. Several mRNA species of different sizes (2.4–4.6 kb) were observed (Liscia et al., 1989), none however equivalent to the single 1.7 kb INT2 transcript detected in teratocarcinoma cell lines and predicted from the physical map of the INT2 gene (Fantl et al., 1989). On the contrary, Theillet et al. (1989) saw both INT2 and HST1 transcripts with RNA:RNA in situ hybridisation, but found connection to gene amplification only in the case of HST1. Moreover, Fantl et al. (1989), using a sensitive RNAase protection assay to analyse both INT2 amplified and unamplified tumours, were unable to detect any expression of INT2 or HST1 and suggested that another gene in the vicinity of INT2 may be of importance. Terada et al. (1989), analysing a variety of cancerous and non-cancerous human cells and tissues for HST1 transcripts, also reported on negative findings except in some cases of testicular germ-cell tumours and a teratoma cell line. It must however be remembered that even a low level of expression of normally silent genes may be sufficient to induce aberrant growth. Neither can it be excluded that the findings of gene amplification in these clinical tumours is a reminiscence of an earlier activity.

Nevertheless, amplification of the 11q13 region has been
shown to be associated with a poor clinical outcome (Lider-
eau et al., 1988; Zhou et al., 1988; Tsuda et al., 1989). A
prognostic value of gene amplification in prediction of
disease-free survival was confirmed in the present study,
found to persist in multivariate analysis and to be confined
to node-negative patients. A subset of these latter patients, a
group in general considered as being of good prognosis, 
could not be found shown to have a disease-free survival
as bad as node-positive patients. At this median time of 46
months follow-up, the correlations had not yet translated
into survival differences. As the number of cases and relapse
events in the node-negative group are small, the results must
be critically interpreted. However, if shown to be valid in
future investigations, amplification of this chromosomal
region may become an important prognostic factor and use-
ful in selection of node-negative patients for adjuvant
therapy. Also, an increased knowledge of the genes at 11q13
will most certainly contribute to a deeper understanding of
human breast cancer etiology.

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