Loss of a c-H-ras-1 Allele and Aggressive Human Primary Breast Carcinomas

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ABSTRACT

The human H-ras protooncogene was shown to be expressed in 16 of 22 invasive ductal carcinomas of the breast. The K- and N-ras protooncogenes were either not expressed or expressed at low levels. No amplification or rearrangement of the three ras genes was detected among the 104 breast carcinoma DNAs tested. These results indicate that the overexpression of H-ras in human breast tumors is not correlated with alteration of the protooncogene. In addition, we did not find any point mutation at the codon 12 of the H-ras or K-ras protooncogenes in 32 and 64, respectively, tumor DNAs examined. However, in tumor DNAs from 14 of 51 patients, heterozygous for H-ras-1 related BamHI restriction fragments, one allele was lost. This allele loss did not alter ras M, 21,000 protein expression. Correlation with clinicopathological data showed, however, that the loss of one H-ras-1 allele in breast carcinoma DNAs is significantly linked to histological Grade III tumors, the lack of estrogen and progesterone receptors, and the subsequent occurrence of distal metastasis. Our results thus indicate that the loss of one H-ras-1 allele correlates with the most aggressive primary carcinomas of the breast.

INTRODUCTION

Activation of the ras protooncogenes family is thought to play a role in the development and/or the maintenance of certain human and animal malignancies. The ras genes acquire their transforming capacity, either by single point mutations in the codons 12 or 61 (1), leading to the expression of an aberrant gene product, or by the overexpression of the normal ras p21 (2). Recent immunohistochemical and immunological analyses of human primary breast carcinomas have shown that high levels of ras p21 are produced in 60% to 70% of the tumors tested (3, 4).

Mutated ras genes have been isolated and identified by transfection of DNA from a number of different human tumor cell lines (5–7) as well as hematopoietic (8) and solid tumors (9–11) onto NIH/3T3 fibroblasts. In addition, Kraus and coworkers (12) have isolated a transforming H-ras-1 gene (mutated at the codon 12) from a cell line derived from a carcinosarcoma in the breast. It has been estimated that approximately 10% of all human neoplasms carry a mutated ras gene (1). An association between tumor development and point mutation of H-ras has also been observed in chemically induced mammary tumors in the rat. It has been shown that, in 80% of the N-nitroso-N'-methylurea-induced rat mammary tumors, the tumor DNA scores positive in the NIH/3T3 transfection assay (13). The transforming gene isolated is always H-ras bearing a point mutation at codon 12 (13). In other studies, experimentally induced amplification of the normal human H-ras-1 protooncogene has been shown to have a transforming effect on the morphology of NIH/3T3 fibroblasts and to render the cells tumorigenic (14). These studies illustrate the potential importance of structural alterations of the ras genes in tumor development. Hence, we attempted to determine if the overexpression of ras p21 in human primary breast carcinomas could be linked to any structural alteration(s) of the ras genes, such as amplification(s), rearrangement(s), or point mutation(s).

The data presented here show that, of the three ras genes, only H-ras-1 is overexpressed in human primary breast carcinomas and that these high levels of expression cannot be linked to an amplification or a rearrangement of the gene. In addition, no point mutation could be detected in the H-ras-1 and K-ras-2 genes in any of the DNAs examined. However, in 27% of the tumor DNAs from heterozygous patients, one allelic restriction fragment containing H-ras-1 was lost in the tumor. This loss appears to be an important characteristic of human primary breast carcinomas, since it significantly correlates with the most aggressively growing tumors.

MATERIALS AND METHODS

Patients. Primary breast carcinomas from a total of 104 patients from the Centre Rene Huguenin (St. Cloud, France) have been tested. None of these patients had undergone radiation or chemotherapeutic treatment prior to survey. The tumors were classified following the WHO histological typing of breast tumors (15). Our study included 100 invasive ductal and 4 lobular carcinomas.

Follow-up information was only available for 80 patients surveyed a minimum of 6 mo after the end of therapy. In order to correlate the H-ras-1 allele loss to the patient prognosis, the statistical analyses were carried out on the 43 heterozygotes included in this group of 80 patients.

Tissues. Breast tumor samples (2 cm or greater) were frozen in liquid nitrogen immediately after surgical removal. The specimens were stored at −70°C until DNA or RNA was extracted. The histological type and grade of the tumors studied were established on paraffin-embedded sections at the Laboratory of Pathology from the Centre Rene Huguenin, St. Cloud, France. The criteria used for tumor grading included the evaluation of the degree of histological differentiation, nuclear atypia, and mitotic figures, according to the specifications of the WHO (15). Blood samples were obtained from 40 of the tumor-bearing patients, and peripheral lymphocytes were isolated on a Ficoll-Hypaque gradient (16). The MCF7 and T47D breast cancer, LS174 colon carcinoma, and T24 bladder carcinoma cell lines used as controls for RNA expression are routinely maintained in our laboratory.

Extraction of RNA and Selection of the Polyadenylated Fraction. Total cellular RNA was isolated from tissues using the guanidinium thiocyanate method (17). Polyadenylated RNA was prepared by binding to a polyribouridylic acid-Sephadex column (Bethesda Research Laboratories). Conditions used were those recommended by the supplier. Samples of polyadenylated RNA (4 μg) were denatured by incubation at 65°C for 15 min in 65% formamide-2.2 M formaldehyde-2× RNA running buffer [1× running buffer is 0.02 M 3-(N-morpholino)propanesulfonic acid (pH 7.0)-0.001 M EDTA-0.005 M sodium acetate]. The RNA samples were electrophoresed in a 1% agarose gel (containing 1× RNA running buffer and 2.2 M formaldehyde) and transferred to a nylon membrane (Genatran 45; Plasco, Inc.) as previously described (18).

Restriction Endonuclease Digestion and Agarose Gel Electrophoresis of High-Molecular-Weight DNA Samples. Cellular DNAs were extracted from tissues and lymphocytes as previously described (19). Conditions for restriction endonuclease digestions of DNA were those recommended by the suppliers (Bethesda Research Laboratories, New England Biolabs). Separation of the digested DNA samples by electroph-
phoresis on 0.8% agarose gels, ethidium bromide staining, and transfer to nylon membrane (Genatran 45; Plasco, Inc.) have been described (20).

Hybridization Conditions. After transfer of the DNA, nylon membranes were rinsed in 6× SSC [1× SSC is 0.15 M NaCl-0.015 M sodium citrate (pH 7.0)] and baked for 2 h at 80°C. Prehybridization and hybridization steps were performed in 50% formamide 5× Denhardt's solution (50× Denhardt's solution is 1% Ficoll-1% polyvinylpyrrolidone-1% BSA), 1% SDS, 3× SSPE (20× SSPE is 3 M NaCl-0.2 M NaH2PO4 (pH 6.5)), 2.5% dextran sulfate, and 0.001 M sodium phosphate (pH 7.5). The membranes were hybridized for 46 h at 37°C in heat-sealed plastic bags containing the hybridization solution mixed with 3× 106 cpm of 32P-labeled recombinant DNA per milligram of DNA, and then washed in the instructions of Biorad. The nitrocellulose was incubated in NTE-NP40 buffer (NTE-0.1% NP40) and 3% BSA overnight at 4°C. The nitrocellulose was then subjected to autoradiography to Kodak XAR films.

Probes. The human H-ras-1 2.9-kilobase pair recombinant clone (SsrI fragment) used in this study was kindly given by Dr. E. Chang (21). The N-ras probes consisted of 300-base pair HindIII fragment representing exon 1 and a 760-base pair HindIII fragment containing exon 2. These fragments were isolated from the plasmid pNR Sac (American Type Culture Collection No. 41031). The K-ras-2 probe is a 1.1-kilobase pair EcoRI fragment prepared from the plasmid pSW 11-1 (American Type Culture Collection No. 41027) (22). The β-actin probe is a generous gift of Dr. J. Fetherston (23). The recombinant DNAs used as probes were labeled with [32P]dCTP to a specific activity of 5× 109 to 1× 1010 cpm/μg of DNA by the nick-translation method of Rigby et al. (24).

Western Blotting. Tumor tissues were pulverized on dry ice. The powder was subsequently homogenized in a Potter homogenizer in 0.02 M Tris-HCl (pH 7.4)-0.1 M NaCl-0.005 M MgCl2-0.5% sodium deoxycholate-1% NP40-2 karyopyknotic index units of bovine aprotinin. Homogenates were briefly spun, and the supernatants were collected and stored frozen until use. One hundred μg of total protein extract were subjected to SDS-polyacrylamide gel electrophoresis on a 12% acrylamide gel. Proteins were transferred to nitrocellulose following the instructions of Biorad. The nitrocellulose was incubated in NTE-NP40 buffer (NTE-0.1% NP40) and 3% BSA overnight at 4°C. The nitrocellulose was then sequentially incubated in NTE-NP40 and 3% BSA solution containing: (a) for 3 h a 1/500 dilution of the p21 monoclonal antibody Y13-259 kindly provided by Dr. D. R. Lowy (25); (b) a dilution of Cappel rabbit anti-rat IgG for 2 h; and (c) 106 cpm of 32P-labeled Protein A for 1 h. All steps were done at 4°C and separated by two 10-min washes in cold NTE-NP40 buffer. The nitrocellulose was then subjected to autoradiography to Kodak XAR films.

Statistical Analysis. Some of the results obtained in this study were analyzed for statistical significance by the χ2 distribution (26). Differences between two populations were judged significant at a greater level than 95% of confidence (P < 0.05).

RESULTS

ras Expression in Human Primary Breast Carcinomas. The expression of the different c-ras genes has been examined in 22 primary breast carcinomas by the Northern blot technique. The T47D and MCF7 breast carcinoma, LS174 colon carcinoma, and T24 bladder carcinoma cell lines were used as positive controls for ras RNA expression. The MCF7 cell line has previously been shown to contain an amplified N-ras gene (27), and the T24 cell line has previously been shown to contain an amino acid 12-point mutation in the H-ras-1 gene (5). Representative results of this analysis are shown in Fig. 1. Nylon membranes were hybridized sequentially with the H-ras-1, K-ras-2, N-ras, and β-actin probes. This last probe was used to assess the quality and the quantity of the RNA transferred to the membrane. Sixteen of 22 tumors expressed the 1.4-kilobase H-ras RNA. In most of the primary breast carcinomas expressing H-ras, the level of RNA was higher than in T47D and MCF7 breast tumor cell lines (Fig. 1A, Lanes i and m). In addition, two of those tumors (Fig. 1A, Lanes b and c) showed elevated levels of H-ras RNA, comparable to that of the mutated T24 bladder carcinoma cell line (Fig. 1, Lane k). Six of the 22 primary tumors, on the other hand, expressed very little H-ras RNA as shown in Fig. 1A (Lanes i and j). This was not due to RNA degradation, since the 2.2-kilobase β-actin RNA species could be detected in each sample (Fig. 1, Lanes i and j). Similarly, hybridization with the N-ras probe revealed very low levels of expression of related RNA in these tumors (Fig. 1B, Lanes a and b). The 5.0- and 2.2-kilobase species of RNA could only be detected after 3 days of exposure, and their intensity remained lower than in MCF7 and T47D cell lines (Fig. 1B, Lanes c and d). No evidence for K-ras RNA could be detected in the tumor RNA even after 4 days of exposure (Fig. 1C, Lanes a and b), whereas the MCF7 breast and LS174 colon carcinoma cell lines, respectively, contain detectable amounts of this RNA species (Fig. 1C, Lanes c and d). Thus our analysis of RNA expression by the ras gene demonstrates that only H-ras is highly expressed in primary breast carcinomas.

Structure of the c-ras Genes in Human Primary Breast Carcinomas. We have screened 104 primary tumor DNAs for amplification and/or rearrangement of the H-ras-1, K-ras-2, and N-ras genes. In 40 cases we were also able to compare the restriction patterns in tumor and normal WBC DNAs from the same patient. No rearrangement nor amplification of any of the three ras genes could be detected (Table 1). In addition, none of the 64 tumor DNAs screened for point mutation at the codon 12 of the K-ras-2 gene showed the diagnostic 8.2- and 5.8-kilobase fragments after SsrI restriction enzyme digestion (Ref. 1; Table 1). Similarly, none of the 32 tumor DNAs examined contained the 412-base pair Mspl/HpaII restriction fragment which is diagnostic for an amino acid 12-point mutation in H-ras-1 (Ref. 28; Table 1).

H-ras-1 is polymorphic by BamHI restriction enzyme analysis (29). The basis for this polymorphism is the variable amplification (or deletion) of a 28-base pair tandem repetitive element located 3' to the H-ras-1 gene (30). The DNA from individuals who are homozygous for this locus contains a single H-ras-1 related BamHI restriction fragment, while DNA from heterozygous patients contains two such fragments. In some tumor DNAs from heterozygous patients, differences were observed in the intensity of the two constitutive H-ras-1 related BamHI restriction fragments (Fig. 2A). This difference of intensity between the two allelic restriction fragments was repeatedly detected in 14 of the 51 tumor DNAs from heterozygous patients. The hybridization pattern of four of these DNAs could be compared to that of the matching WBC DNAs (Fig. 2B). The results show that, in each case, the WBC DNA was heterozygous for H-ras-1 with both restriction fragments equally intense (Fig. 2B, Lanes b, d, f, and h), whereas in the tumor DNA, one fragment was either missing or reacted with the probe to a lesser extent (Fig. 2B, Lanes a, c, e, and g). The data are consistent with the observed loss of a H-ras-1 allele in 27% of the tumor examined. The variable intensity with which the lost allele reacts with the probe in tumor DNAs can most likely be accounted for by cellular heterogeneity in human breast carcinomas. Indeed, the tumor mass is composed of variable ratios of tumor cells to stroma or normal mammary epithelium and is in certain cases heavily invaded by plasma cells. It is...
Grade III tumors (9 of 12 tumors) and the number of tumors significantly altered by the loss of one c-H-ras allele. The breast carcinomas (Tumors 169, 239, 182, and 295) is not examined. Our data indicate that ras p21 expression in primary were determined by Western blot analysis. This procedure was (Lane a) and two tumors (Nos. 307 and 292) containing a estrogen receptor-negative tumors, 9 of 12 progesterone-negative tumors, 7 of 12 estrogen- and progesterone-negative tumors). Statistical analyses were performed, comparing clinicopathological parameters of the 12 patients lacking one H-ras allele in their breast tumor DNA to those of the 31 patients whose tumor DNA contained a normal heterozygous genotype. As shown in Table 3 the loss of one H-ras allele has a significant correlation with histopathological Grade III tumors (P < 0.02) as well as with the lack of one hormonal receptor (estrogen-negative tumors, P < 0.02; progesterone-negative tumors, P < 0.01) or both receptors (estrogen- and progesterone-negative tumors, P < 0.01). These parameters have previously been shown to be associated with the most aggressively growing and poorly differentiated breast tumors and are used, along with axillary lymph nodes metastasis, to determine the clinical prognosis of breast cancer patients. In the present study, no statistical correlation was observed between allele loss in the tumor and axillary lymph node metastasis (Table 3).

These patients have been monitored for the incidence of local recurrence of breast tumors and subsequent distal metastasis. At present, the majority of these patients are in the range of 1 to 5 yr postsurgery. Table 3 shows that the loss of one H-ras allele does not correlate with local recurrences, but it does correlate with the development of distal metastasis (P < 0.05). Thus, 42% of the patients with an allele loss in the primary tumor developed distal metastasis regardless of nodal status. In contrast, only 13% of the patients whose primary tumor contained a normal H-ras genotype later developed a distal metastasis.

DISCUSSION

The data presented here show that 70% of the primary breast carcinomas examined contained elevated levels of H-ras-1 mRNA, whereas K-ras and N-ras mRNAs were either not detectable or expressed at low levels. The proportion of carcinomas with high levels of H-ras-1 mRNA agrees with previously reported results showing enhanced expression of ras p21 in human breast carcinomas (3, 4). Therefore, the high levels of
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Fig. 2. Loss of one allelic H-ras-1 restriction fragment in human primary breast carcinomas. DNAs presented here correspond to the patients whose complete clinicopathological records were available and who are listed in Table 2. DNAs were cleaved by the restriction enzyme BamH1 and analyzed by the Southern blot technique. A, breast carcinoma DNAs 53 (a), 86 (b), 105 (c), 166 (d), 169 (e), 174 (f), 180 (g), 182 (h), 239 (i), 253 (j), and 315 (k). B, matched sets of breast carcinoma DNAs and WBC DNAs from the same patient. Tumor DNAs 166 (a), 253 (c), 295 (e), and 86 (g). Lymphocyte DNAs, Lanes b, d, f, and h; numbers are corresponding to the tumor DNAs. Black lines between Lanes a, b, c, d, e, f, g, h, i, j, and k in A indicate the 8.0-kilobase (kb) (above lines) and 6.5-kilobase pair (under lines) molecular weights.

Fig. 3. ras p21 expression in primary breast carcinomas presenting a H-ras-1 allele loss. One hundred μg of proteins from a cytosolic extract were loaded on a 12% polyacrylamide-SDS gel and analyzed by the Western blot technique. The samples are: Lane a, normal breast tissue; Lanes b and c, breast carcinomas 307 and 292 presenting a normal H-ras-1 genotype; Lanes d, e, f, and g, carcinomas 169, 239, 182, and 295, presenting a H-ras-1 allele loss; Lanes h, i, and j, respectively, 50 μg, 75 μg, and 100 μg of T24 bladder carcinoma cell line protein extract. The Me, 25,700 (25.7K) band represents the λ-chain of human IgG which reacts with the rabbit anti-rat IgG.

ras p21 found in human primary breast tumors are likely to be the result of H-ras-1 gene expression. However, Slamon and coworkers reported that, in addition to H-ras RNA, breast tumors contain detectable amounts of K-ras RNA (33). This difference with our data might be explained by cross-hybridization between K- and H-ras as a result of the probable use of low stringency hybridization conditions which are required for viral probes. Experimentally induced amplification of the normal H-ras-1 protooncogene in NIH/3T3 fibroblasts is accompanied by increased RNA and p21 expression (14). In addition, the cells become morphologically transformed and are tumorigenic (14). Recent immunohistochemical and radioimmunoassays of human mammary tissue demonstrated higher levels of ras p21 in invasive ductal carcinoma, with generally decreasing levels of expression in carcinoma in situ, hyperplastic and normal epithelial components of the same breast (34). Fibrocystic lesions without hyperplasia were almost negative for ras p21. These observations taken together support the hypothesis...
that enhanced expression of H-ras-1 could act as a contributing factor in breast tumor development. However, in the primary breast tumors examined here, H-ras RNA expression is not the result of a genetic rearrangement or amplification of H-ras-1. It is conceivable that the increased levels of H-ras RNA observed here are the result of mutations in cellular genes which regulate its expression or in regulatory elements near the H-ras locus. Point mutations in the ras genes have been reported to be a contributing factor in the development of certain types of human tumors and in carcinogen-induced rat breast tumors. In the present study no point mutations were observed at codon 12 of c-H-ras-1 or K-ras-2 using, respectively, the Mspl/Hpall and Ssrl restriction site polymorphisms. This does not exclude the possibility that point mutations have occurred at codon 12 which are not detected by these enzymes nor that codon 61 has been affected in any of the ras genes. However, the results of the present study are consistent with the low efficiency with which primary breast tumor DNAs morphologically transform NIH/3T3 in transfection assays (12) and suggest that point mutations in the ras genes occur at a low frequency in human breast tumors.

Southern blot analysis revealed that, in 27% of the tumor DNAs from patients heterozygous for the H-ras-1 locus, one allele was lost. At present, the extent of the deletion leading to the loss of one H-ras-1 allele in primary breast carcinomas is unknown. H-ras-1 is located on chromosome 11 (11:pl5) (35), and it is perhaps pertinent that a constitutive fragile site has been mapped in the vicinity, on Band p13 (36). However, the available cytogenetic analysis of primary breast tumors has revealed no consistent loss of the total or of major portions of chromosome 11 (37). Therefore, it is conceivable that the loss of a H-ras-1 allele corresponds to a small deletion. Alternatively, the apparent loss of a c-H-ras-1 allele may reflect the duplication of one of the chromosomes and the subsequent loss, by nondisjunction, of the other chromosome. Further analysis with probes corresponding to different regions of chromosome 11 should enable us to distinguish between these possibilities. Extensive studies have shown that a similar phenomenon occurs frequently on chromosome 13 in retinoblastomas (38) and on chromosome 11 in tumors of several tissues (30), including Wilms’ tumors (39). Indeed, several laboratories have reported that H-ras-1, as well as other loci located along the short arm of chromosome 11, is lost in Wilms’ tumors (39, 40). The concordance of events in primary breast carcinomas and Wilms’ tumors is intriguing enough to raise the question of the existence of a common mechanism. Hence, the deletion of a portion of the short arm of chromosome 11 could unmask a recessive oncogene or cause the loss of a dominant gene involved in the regulation of cellular proliferation.

Statistical analysis of the clinicopathological data from the patients whose tumor presented a H-ras-1 allele loss revealed that this phenomenon is significantly linked to parameters characteristic of tumor aggressiveness. Indeed, it correlates significantly with histopathological Grade III tumors (P < 0.02), the lack of estrogen and/or progesterone receptors (P < 0.01), and the occurrence of distal metastasis (P < 0.05). The biological significance of these correlations could be interpreted in at least two ways. (a) The loss of a portion of chromosome 11 contributes to the development of an undifferentiated tumor, or (b) the loss of a H-ras-1 allele and perhaps larger portions of chromosome 11 is not a contributing factor but rather is symptomatic of the chromosomal instability existing in tumor cells. Loss of heterozygosity has been reported to occur at several loci located on different chromosomes in melanomas (41). Irrespective of these considerations, the correlation between tumor aggressiveness and loss of one H-ras-1 allele suggests that evaluation of this gene in breast carcinomas could be used as an additional parameter in determining the prognosis of individual breast cancer patients.

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REFERENCES

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