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Differential Expression and Mutation of the *ras* Family Genes in Human Breast Cancer

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The expression of *ras* mRNA levels in 27 human sporadic breast cancer specimens was examined, and compared to the corresponding adjacent normal tissue using the RT-PCR technique. Eighteen out of the 27 specimens (67%) exhibited two- to four-fold increased expression of *ras* mRNA levels, compared to corresponding normal tissue. The rates of augmented mRNA expression were similar among the three *ras* genes. A statistically significant correlation of overexpression of *ras* genes in specimens classified as Stage I disease was observed, compared to tumors in a more advanced stage (II or III). The incidence of codon 12 point mutations of the K-*ras* gene in fresh tissue samples was also assessed in 61 human sporadic breast cancer cases. Point mutations were detected in four (6.5%) out of the 61 cases examined; no correlation was found with any clinicopathological parameter. This is the first report to our knowledge of the differential expression of the *ras* family genes in breast carcinoma. Our findings indicate that the aberrant expression of *ras* genes may be an initial event in breast cancer oncogenesis and that K-*ras* point mutations are rarely involved in the development of mammary neoplasias.

***Key Words:* *ras* gene; RT-PCR; breast cancer.**

Breast cancer is the most common type of cancer in women. Recent epidemiological data suggest that one in eight women will develop breast cancer over a lifetime (1). The development of an invasive breast tumor involves a multistep process that has been associated with the altered expression of several oncogenes and tumor-supressor genes (2). Breast carcinomas seem to derive from ductal or lobular epithelial hyperplasias, which acquire cumulative genetic changes leading to clonal outgrowths of progressively malignant cells (3). In chemically-induced breast adeno-carcinomas in rats 85% of the tumors carry transforming *ras* mutations (4). Moreover, several human breast cancer cell lines have been shown to contain mutational activation of *ras* oncogenes (5). *ras* mutations have been reported infrequently in human breast cancers; however, only a limited number of human breast carcinoma specimens have been analyzed for such alterations previous to our present study (6, 7).

Aberrant expression of the *ras* genes has been recognized in several human cancers and associated with the development of the disease (8). *In vitro* experiments have shown that overproduction of even the normal Ras protein is sufficient to confer a transforming potential on cultured cells (8, 9). Overexpression of the *ras* family genes has been detected in human mammary malignancies (10).

In the present study we examined 27 human sporadic breast cancer specimens analysing the expression levels of tumor *ras* mRNA, compared to the corresponding adjacent normal tissue, using the reverse transcription PCR (RT-PCR) technique. In addition, a sensitive PCR-RFLP assay was employed for the detection of codon 12 point mutations on K-*ras* gene, in 61 fresh tissue samples from respective sporadic human breast cancer patients (including the above 27 cases), as mutations of the *ras* family genes in breast cancer occur almost exclusively in this site (7, 11).

MATERIALS AND METHODS

Tissue specimens. Samples of macroscopically malignant tissue from 61 patients with breast cancer were surgically obtained and frozen at −70°C. In 27 cases adjacent normal breast tissue was also collected. All normal tissues examined contained normal ductal epithelium, and the malignant samples corresponded to primary breast tumors, the majority of which consisted of infiltrating ductal carcinomas. Tumors were staged according to the AJCC-TNM classification for breast cancer. Clinical data (lymph node metastasis, estrogen and progesterone receptors, age and family history) were available for all specimens examined.

RNA and DNA extraction. Total RNA was isolated from fresh tissues using Trizol (Life Technologies) following the manufacturer's instructions. RNA samples were digested with DNaseI (Gibco BRL) in order to discard genomic DNA. DNA from fresh tissues was extracted as previously described (12) and stored at 4°C until PCR amplification.

cDNA synthesis and PCR. For first strand cDNA synthesis, 1-5 μg of total RNA were reverse- transcribed in a 20 μl reaction volume

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TABLE 1

Primers, Amplification Conditions, PCR and RFLP Products for the Detection of Codon 12 Point Mutations and mRNA Expression of H-*ras*, K-*ras* and N-*ras* Genes

Gene	Primers	PCR profile	PCR product (bp)	RFLP products (bp)
H-*ras* (RNA)	5-GACGGAATAT AAGCTGGTGG-3 (S) 5-TAACTACCCC TCTGCACGGA-3 (A)	94° for 35s (denaturation) 60° for 40s (annealing) 72° for 40s (extension)	151	
K-*ras* (RNA)	5-ACTGAATATAAACTTG TGGTAGTTGGACCT-3 (S) 5-CAAATCACATTTATTT CCTACCAGGACCT-3 (A)	94° for 30s (denaturation) 58° for 40s (annealing) 72° for 45s (extension)	357	
N-*ras* (RNA)	5-AATCCAGCTAAT CCAGAACC-3 (S) 5-TGGTCTCTCATG GCACTGTA-3 (A)	94° for 30s (denaturation) 58° for 30s (annealing) 72° for 30s (extension)	150	
H-*ras* (DNA)	5-GAGACCCTGTAGG AGGACCC-3 (S) 5-GGGTGCTGAGACG AGGGACT-3 (A)	94° for 55s (denaturation) 62° for 50s (annealing) 72° for 50s (extension)	312	236 (wt) 291 (mut)
K-*ras* (DNA)	5-ACTGAATATAAACTT GTGGTAGTTGGACCT-3 (S) 5-TCAAAGAATGGTCCT GGACC-3 (A)	94° for 55s (denaturation) 58° for 45s (annealing) 72° for 45s (extension)	157	113 (wt) 142 (mut)
N-*ras* (DNA)	5-AACTGGTGGTGG TTGGACCA-3 (S) 5-ATATTCATCTACA AAGTGGTCCTGGA-3 (A)	94° for 55s (denaturation) 57° for 50s (annealing) 72° for 40s (extension)	83	41 (wt) 60 (mut)

Note. S = Sense primer; A = Antisense primer.

containing 2 μl of 10× PCR buffer, 50 ng of random hexamers, 50 mM $MgCl_2$, 200 ng dNTPs, 0.1M DTT and 200 U Reverse Transcriptase, (SuperScript II RT, Life Technologies) for 50 min at 42°C. PCR amplification of cDNA was performed in a 50 μl reaction volume containing 1 μg cDNA, 1 μM of each primer, 200 ng dNTPs, 5 μl of 10× buffer (670 mM Tris · HCl, pH 8.5; 166 mM ammonium sulphate; 67 mM magnesium chloride; 1.7 mg/ml BSA; 100 μM β-mercaptoethanol and 1% (w/v) Triton X-100) and 1 U of *Taq* DNA polymerase. The oligonucleotide primers, cDNA and DNA amplification conditions used and the subsequent PCR products have been previously described (13, 14) and are listed in Table I.

The PCR reactions were performed on a DNA thermal cycler (Perkin Elmer-Cetus Instruments, Norwalk, CT). The analysis of the expression of the three *ras* genes was performed as previously described (15). Preliminary experiments had revealed the conditions under which the amplification reaction remained in the exponential phase (data not shown) and thus the results could be used for quantification of the template. 10 μl of the PCR product was electrophoresed through a 10% polyacrylamide gel, silver stained and the intensity of the bands was analyzed by a UVP image analysis system.

The quantity and the quality of mRNA samples were normalized after amplification of β_2-microglobulin mRNA (15). The mRNA levels for each gene were expressed as the ratio of the intensity of the bands in tumor tissues versus the corresponding levels of normal tissues. We arbitrarily considered as overexpression, levels higher than 1.5-fold in malignant specimens compared to corresponding normal tissues.

Detection of ras mutations. Mutational analysis of codon 12 on the *ras* genes was performed as previously described (11) (Table I). Thirty μl of the PCR product were digested overnight with 20 U of the restriction endonucleases *MspI* (H-*ras*) and *BstNI* (K-*ras* and N-*ras*) in conditions recommended by the suppliers. 10 μl of the digestion product was electrophoresed through a 10% polyacrylamide gel and silver stained.

Statistical analyses. Statistical analyses were performed using the χ^2-test or Fisher's exact test. One-tailed P-values ≤ 0.05 were considered statistically significant.

RESULTS

The levels of expression of the H-, K-, and N-*ras* proto-oncogenes were assessed at the level of RNA in 27 of the above 61 specimens of human sporadic breast cancer. The levels of differential expression for the *ras* family genes were expressed as the ratio of the expression in each specimen versus the expression of its corresponding normal tissue. The results are summarized in Table II. Eighteen of the 27 (67%) tumors showed elevated expression of at least one of the *ras* family genes. N-*ras* exhibited overexpression in 10 (37%), K-*ras* in 9 (33%) and H-*ras* in 10 (37%) of the 27 tumor samples examined (Fig. 1). Nine cases (33%) did not exhibit overexpression of any member of the *ras* family genes. Moreover, six (22%) did not show mutational activation of *ras* genes. On the other hand, three samples (11%) exhibited transcriptional activation of all the three *ras* genes, while ten tumors (37%) overexpressed only one member of the *ras* family genes and five (18.5%) overexpressed two of the *ras* genes.

TABLE 2

Expression of the *ras* Family Genes in 27 Specimens from Patients with Human Sporadic Breast Cancer

Pt No.	Stage[a]	Expression[b] N-*ras*	K-*ras*	H-*ras*
1	II	N	N	N
2	I	1.9	1.8	1.5
3	I	2.4	2.5	1.6
4	I	1.9	N	N
5	II	1.8	N	1.6
6	III	N	2.8	N
7	II	N	N	N
8	II	N	2.4	N
9	I	N	N	1.5
10	III	N	2.9	N
11	III	N	N	N
12	II	2.1	2.5	1.6
13	III	N	N	N
14	II	N	N	N
15	II	2.3	N	1.5
16	I	N	2.3	1.7
17	II	N	N	N
18	I	N	2.2	N
19	II	N	N	N
20	I	2.8	N	N
21	II	N	N	N
22	II	N	N	1.6
23	II	2.7	N	1.5
24	I	1.8	3.0	N
25	I	1.7	N	N
26	II	N	N	N
27	II	N	N	1.8

[a] According to the TNM staging system for breast cancer.
[b] Levels of *ras* mRNA are expressed as the ratio of the levels in the tumor *vs* the normal tissue; N = Normal expression (ratio <1.5).

An association was found between overexpression of *ras* proto-oncogenes and the clinical stage of the disease: All nine specimens classified as Stage I disease exhibited overexpression of at least one of the *ras* family genes, regardeless of tumor histology, while ten samples from tumors in a more advanced stage (II or III) did not show expressional activation of *ras* genes (P = 0.019). This association primarily concerned postmenopausal women (P = 0.024), whose tumors expressed estrogen or progesterone receptors (P = 0.026). No relationship was found between the expression levels of the *ras* family genes and the other clinicopathological parameters evaluated. The incidence of codon 12 point mutations of the K-*ras* gene was assessed in 61 specimens of human sporadic breast cancer. K-*ras* mutations were found in four out of the 61 samples examined (6.5%) (Fig 2).

DISCUSSION

In the present study tumor and adjacent normal tissue specimens from 27 patients with breast cancer

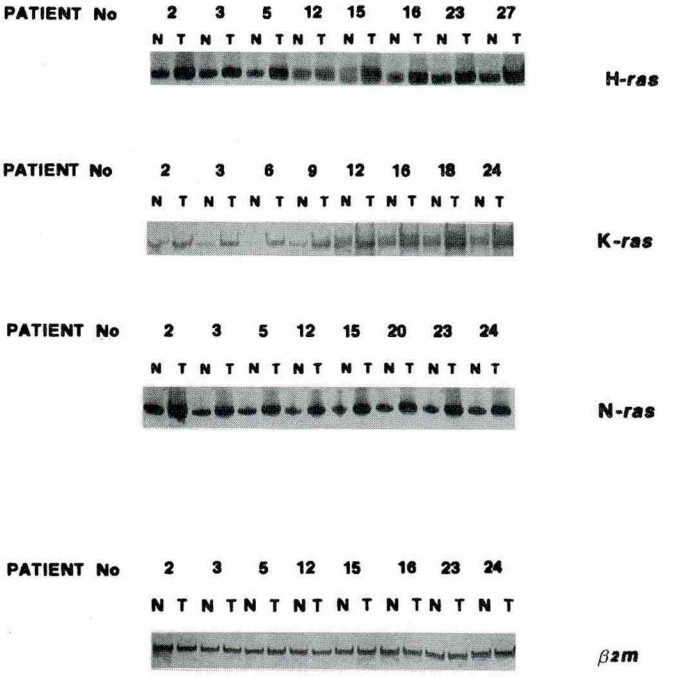

FIG. 1. Representative examples of H-, K- and N-*ras* mRNA overexpression in sporadic breast cancer. β2m = β_2-microglobulin mRNA. N: normal and T: tumor specimens.

were investigated for aberrant expression of H-, K-, and N-*ras* oncogenes. The pattern of expression of the *ras* genes was diverse. Overexpression of the N-*ras* was found in 10, K-*ras* in 9 and H-*ras* gene in 10 cases. It has been suggested that the participation of *ras* genes in breast carcinogenesis consists mainly of expressional activation, since *ras* mutations have been reported infrequently in breast cancer (7). Elevated levels of the p21 proteins—encoded by *ras* genes—compared to the corresponding normal tissues have been detected by immunohistochemical methods in 65-71% of cases (7, 10). To our knowledge, this is the first report on the differential expression of the *ras* family genes in breast carcinoma. Our data confirm the high incidence of *ras* overexpression reported previously for this type of malignancy, albeit overexpression rates for

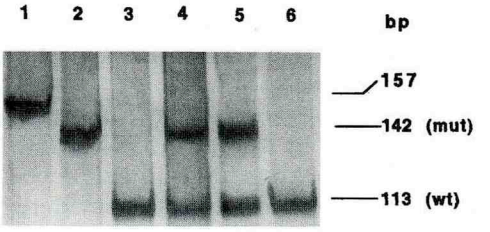

FIG. 2. Detection of K-*ras* codon 12 point mutations by a combined PCR-RFLP assay. Lanes 4 and 5 correspond to mutant specimens, while lanes 3 and 6 to normal specimens. Lane 2 corresponds to positive control for mutation (SW 480 cell line). Lane 1 corresponds to undigested PCR product.

breast cancer at the mRNA level are slightly lower than those reported for other tumor types (13, 14, 16). Hence, overproduction of $p21^{ras}$ is not due to the activation of only one member of the *ras* family, but all three *ras* genes are activated, in all combinations. This ascertainment is enhanced by the lack of correlation between the expression levels of any particular *ras* gene and the clinicopathological parameters of the patients.

Our finding, that *ras* mRNA overexpression is associated with tumors at an earlier stage, is in agreement with observations from related studies on other types of cancer (13, 17). Therefore, aberrant expression of *ras* genes may be an initial event in the development of breast cancer.

Lack of a statistical correlation between *ras* expression and hormone receptor status is in agreement with existing data (18). Immunohistochemically detected *ras* overexpression has not been found to be significantly associated with time to progression and overall survival (18). Nevertheless, it has been postulated that oncogene co-expression may serve as a prognostic correlate for recurrence and survival (19).

Mutations at codon 12 of the *ras* family genes concern almost exclusively the K-*ras* gene. Initially, using the PCR-RFLP method we did not detect point mutations at codon 12 of the H-*ras* and N-*ras* genes in a panel of 27 tumors for which adjacent normal tissue was available (data not shown), confirming previous reports (7, 11). However, four point mutations at codon 12 of the K-*ras* gene were found in 61 available breast cancer samples examined. Frequency of such mutations recorded in this study is consistent with those reported (6), confirming that mutational activation of K-ras rarely occurs in human breast cancer. Lack of correlation between point mutations and clinicopathological parameters is in agreement with published data (11, 20) and it is probably due to the limited number of these mutations in this type of cancer.

In contrast to the relatively low incidence of *ras* gene point mutations detected in human breast cancer, oncogene-induced animal models of mammary carcinoma bear a high frequency of such mutations (4), indicating etiological differences or alternative regulatory mechanisms. Likewise, capacity for infinite growth in culture usually occurs at a late stage of malignant progression and is frequently associated with activating *ras* mutations (7, 21). Thus, there is a discrepancy between the role that *ras* gene mutations are believed to play in human breast cancer progression and the *in vitro* experimental evidence (22, 23).

The present study provides further evidence that *ras* genes are probably involved in early stages of the mammary oncogenesis through augmented expression of the normal p21 protein. Recognition of the mechanisms resulting in aberrant expression of *ras,* as well as unveiling the influence of activation of the *ras* family genes in the Ras signaling pathway, should have a major impact in clarifying the oncogenetic process, possibly offering candidate therapy strategies.

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