Mutational activation of K-ras oncogene in human breast tumors

M. KOFFA1,2, V. MALAMOU-MITSI3, N.J. AGNANTIS3 and D.A. SPANDIDOS1,2

1Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, Athens;
2Laboratory of Virology, Medical School, University of Crete, Heraklion; 3Laboratory of Pathology, Medical School, University of Ioannina, Ioannina, Greece

Contributed by D.A. Spandidos, December 27, 1993

Abstract. ras genes are thought to play an important role in human cancer since they have been found to be activated frequently in several types of human tumors. From preliminary studies it has been found however, that ras mutations are extremely rare in breast tumors and therefore it was of interest to examine the frequency of such mutations. In this study we examined 65 cases of primary breast carcinomas from paraffin blocks, for the presence of point mutations in codons 12 of the K-ras and H-ras genes. The polymerase chain reaction (PCR) technique was used to amplify a codon 12 containing 157 bp and a 312 bp region of the K-ras and the H-ras genes respectively, followed by restriction fragment length polymorphism (RFLP) analysis to identify the point mutations. Eight out of the 65 tumors (12.3%) were found to carry a K-ras mutation in codon 12 but none was found to carry a H-ras mutation. It is suggested that the mutational activation of the K-ras gene may be involved in the development of a small percentage of breast tumors.

Introduction

It appears that ras gene mutations can be found in a variety of tumor types, although the incidence varies greatly. The highest incidence is found in adenocarcinomas of the pancreas (90%), the colon (50%) and in thyroid tumors (50%). For some tumor types a relationship may exist between the presence of a mutation and clinical or histopathological features of the tumor (1).

Breast cancer is of startlingly high incidence (approaching 1 in 9 women), but unfortunately current therapies for the disease are inadequate once it has metastasized. The disease is characterized by excessive morbidity and mortality. Normal as well as malignant growth is regulated by endocrine hormones and by local tissue factors, such as polypeptide growth factors. Breast carcinomas seem to progress as hyperplastic ductal or lobular epithelial growth, acquiring progressive genetic changes (including those of oncogenes and tumor suppressor genes) leading to clonal outgrowths of progressively malignant cells (2).

The chemical induction of breast adenocarcinomas in pubescent rats is a widely used model of carcinogenesis because the resulting tumors are histologically and behaviorally identical to human breast tumors. Notably 85% of these rat mammary carcinomas carry transforming ras mutations (3,4). Likewise, many investigators have reported that the mutational activation of ras oncogenes is likely involved in the etiology or progression of human breast cancer, particularly since the ras p21 protein has been shown to mediate autocrine production of growth factors in transformed cells and even bypass the estrogen dependency of human breast cancer cell lines (5,6). To date, however, few human breast cancer specimens have been analysed for the presence of transforming genes, although several human breast cancer cell lines have been shown to contain mutationaly activated ras oncogenes (7).

Rochlitz et al (8) have found the emergence of a K-ras mutation in codon 12 in the terminal stages of breast cancer progression, but other investigators were able to identify such mutations only in a primary tumor (9). Using oligonucleotide hybridization analysis, Spandidos has detected point mutations at amino acid position 12 (glycine to valine) of H-ras in 2/24 breast carcinomas tested (10).

Additionally many investigators have reported overexpression of the ras-encoded p21 proteins in malignant breast carcinomas (11,12), although the role of this overexpression in breast carcinogenesis has not been determined.

In this study we examined 65 cases of primary breast carcinomas from paraffin blocks, for the presence of point mutations in codon 12 of the K-ras and H-ras genes and found 8 cases (12.3%) carrying a K-ras mutation.

Materials and methods

Tumor specimens. Formalin fixed and paraffin-embedded tissue samples from 65 patients, with a diagnosis of primary breast carcinoma, were identified in the files of the Pathology Department, Medical School, University of Ioannina. Hematoxylin-eosin stained sections from all paraffin blocks...
were reviewed to reconfirm the tumor type and grade and representative blocks (one per case) were selected for further analysis.

**DNA extraction.** Four or five 10 μm thick sections from paraffin-embedded tissues were placed in 1.5 ml tubes and 300 μl digestion buffer, containing 100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS pH 8.0 and 0.1 mg/ml proteinase K was added. Samples were then incubated for 1 h at 60°C. Fresh proteinase K was added at the same concentration and the incubation was continued at 37°C O/N. The samples were then extracted once with phenol/chloroform and once with chloroform. The upper aqueous phase was transferred into fresh tubes and DNA was precipitated with the addition of 750 μl ethanol and 20 μl 5M NaCl. DNA was recovered with centrifugation for 15 min at 4°C, washed twice with cold 70% ethanol and resuspended in 20 μl double distilled water.

**Oligonucleotide primers and PCR amplification.** The oligonucleotides used for K-ras and H-ras codons 12 have been previously described (13,14). One μl of the extracted DNA of each sample was amplified in a volume of 50 μl under the following conditions:

**K-ras amplification:** The reaction solution contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 3.5 mM MgCl$_2$, 0.01% gelatin, 200 μM of each dNTP, 100 pmoles of each primer and 2.5 U Taq polymerase (Gibco). Samples were subjected to 40 cycles of amplification under the following conditions: 94°C for 50 sec; 59°C for 45 sec and 72°C for 50 sec.

**H-ras amplification:** The reaction solution contained 16.6 mM (NH$_4$)$_2$SO$_4$, 10 mM, β-mercaptoethanol, 6.7 mM MgCl$_2$, 67 mM Tris-HCl pH 8.8, 1.7 mg BSA, 6.7 μM EDTA, 200 μM of each dNTP, 75 pmoles of each primer and 2.5 U Taq polymerase (Gibco). Samples were subjected to 35 cycles of amplification under the following conditions: 94°C for 50 sec; 60°C for 40 sec and 72°C for 50 sec.

**RFLP analysis.** K-ras: 10-20 μl aliquots of the amplification products were digested for 3 h with 40 U BstNI. H-ras: 10-20 μl aliquots of the amplification products were digested O/N with 40 U MspI.

Enzymes were supplied by New England Biolabs and the conditions followed for digestion were those recommended by the supplier. Incubation temperatures were 60°C for BstNI and 37°C for MspI. Digestion products were electrophoresed through an 8% native polyacrylamide gel. Gels were stained with ethidium bromide and photographed on a UV light transilluminator. As positive control the cell lines SW480 and EJ were used.

**Results**

After reviewing the selected tissue sections, the histological classification (15) and grading (16,17) was as follows: There were 52 infiltrating ductal carcinomas Grade I-III (NOS type - 40 cases, Comedo type - 3 cases, Papillary type - 1 case, Mucinous type - 2 cases, Medullary type - 3 cases, Tubular type - 1 case and Cribriform type - 2 cases), 11 infiltrating lobular carcinomas Grade I-III and 2 ductal carcinomas *in situ*.

In this study we examined the presence of mutations in codon 12 of the K-ras and H-ras genes in these 65 cases of primary breast carcinomas. Using the known cell lines SW480 and EJ, with mutations in codon 12 of the K-ras and H-ras gene, respectively, experiments were performed to establish the PCR and RFLP techniques. Eight out of the 65 tumors (12.3%) were found to carry a point mutation in codon 12 of K-ras (for representative examples see Fig. 1), while there was no evidence for point mutations in codon 12 of H-ras (for representative examples see Fig. 2). Our study was limited to codon 12 of the K-ras gene, since mutations preferentially occur in this codon (18). The K-ras mutations were found in 1 out of 23 patients with infiltrating ductal NOS type Grade II, 4 out of 16 with infiltrating ductal NOS type Grade III, 2 out of 6 with infiltrating lobular type Grade II and 1 out of 4 with infiltrating lobular type Grade III. No K-ras or H-ras mutation was found in Grade I primary breast carcinomas. The results of PCR analysis of the 65 samples for K-ras mutations are summarized in Table I.

These data do not indicate a correlation between the presence of point mutations and the histological type of the tumor. However, it is notable that mutations were found only in tumors with Grade II and III, with a higher incidence in Grade III.
Table I. K-ras gene mutations in codon 12 in breast tumors.

<table>
<thead>
<tr>
<th>Histological type of tumor</th>
<th>Grade</th>
<th>Total No. of patients</th>
<th>No. of patients with K-ras mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infiltrating ductal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non otherwise specified type</td>
<td>I</td>
<td>1</td>
<td>none</td>
</tr>
<tr>
<td>Non otherwise specified type</td>
<td>II</td>
<td>23</td>
<td>1</td>
</tr>
<tr>
<td>Non otherwise specified type</td>
<td>III</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>Comedo type</td>
<td>II</td>
<td>3</td>
<td>none</td>
</tr>
<tr>
<td>Papillary type</td>
<td>I</td>
<td>1</td>
<td>none</td>
</tr>
<tr>
<td>Mucinous type</td>
<td>I</td>
<td>1</td>
<td>none</td>
</tr>
<tr>
<td>Mucinous type</td>
<td>II</td>
<td>1</td>
<td>none</td>
</tr>
<tr>
<td>Medullary type</td>
<td>III</td>
<td>3</td>
<td>none</td>
</tr>
<tr>
<td>Tubular type</td>
<td>I</td>
<td>1</td>
<td>none</td>
</tr>
<tr>
<td>Cribriform type</td>
<td>I</td>
<td>1</td>
<td>none</td>
</tr>
<tr>
<td>Cribriform type</td>
<td>II</td>
<td>1</td>
<td>none</td>
</tr>
<tr>
<td>Infiltrating lobular</td>
<td>I</td>
<td>1</td>
<td>none</td>
</tr>
<tr>
<td>Infiltrating lobular</td>
<td>II</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Infiltrating lobular</td>
<td>III</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Ductal carcinomas in situ</td>
<td></td>
<td>2</td>
<td>none</td>
</tr>
<tr>
<td>Total number</td>
<td></td>
<td>65</td>
<td>8 (12.3%)</td>
</tr>
</tbody>
</table>

Discussion

At the molecular level, breast cancer may be the result of a complex, dynamic, and stochastic process where there is more than one way to accomplish each of the steps necessary for malignant growth. The well documented biological heterogeneity of breast tumors may arise from the many possible molecular changes that can accomplish a given step with variable efficiency. Alternatively, this heterogeneity may also reflect various possible orders of acquiring the sum of steps necessary for malignant growth. As a result it seems likely that multiple genetic alterations act in concert to produce an invasive breast carcinoma with the ability to metastasize to distant organ sites. It is also suggested that the loss of specific chromosomal regions collaborates during progression of primary breast cancer (19), even by triggering activation of an oncogene by gene amplification due to the instability in the nucleus of malignant cells. Amplification of the erbB-2 oncogene in addition to loss of heterozygosity on chromosome 17 might promote breast cancer by affecting the proliferation of tumor cells (20).

Previous observations (8,9) are substantiated by the present study showing that involvement of K-ras gene mutations can occur in breast carcinoma, albeit at a low frequency. Considering the low frequency of activated K-ras, little can be concluded about the malignant potential of a tumor containing an activated ras gene. However, this low frequency of ras mutations provides evidence for an etiological difference between spontaneous human breast tumors and carcinogen-induced animal models of breast cancer which bear a high incidence of ras mutations (3,4). This molecular analysis also illustrates the biological and probable etiological differences that exist between breast adenocarcinomas and others histologically similar adenocarcinomas which have a high incidence of ras mutations (1). In addition the presence of point mutations (with an incidence of 12.3%) only in primary carcinomas with higher Grade (II and III) indicates that the mutational activation of the K-ras oncogene may be a late event and could play a role in the metastatic progression of human breast cancer. The study described here contributes to our understanding of tumor development and progression in breast cancer and to the future application of DNA diagnosis for clinical purposes; e.g., to estimate the prognosis of patients. It is suggested that the mutational activation of K-ras may alter the tumorigenic behaviour of a cell that already has malignant potential, thus being involved in the process of breast carcinogenesis.

References