Immunohistochemical Study of the ras Oncogene Expression in Human Breast Lesions*

N.J. AGNANTIS¹, C. PETRAKI¹, P. MARKOULATOS², and D.A. SPANDIDOS²,³

¹Department of Pathology, Hellenic Anticancer Institute, Athens, ²Hellenic Institute Pasteur, Athens, Greece, ³The Beatson Institute for Cancer Research, Garscube Estate, Bearsden, Glasgow G61 1BD, Scotland

Abstract. An immunohistochemical study of ras oncogene expression in human breast lesions was carried out using a monoclonal antibody, Y13 259, to the ras encoded p21 protein. A total of 75 cases of breast disease examined included: 33 simple and complex cystic disease; 22 simple and hyperplastic fibroadenomas; 18 ductal, lobular and mixed carcinomas and 2 in situ carcinomas. Most of the complex cystic disease, hyperplastic fibroadenomas and all types of carcinomas showed high p21 expression as indicated by staining intensity. These results suggest that elevated ras expression may play an important role in the development of some premalignant and malignant breast lesions.

In the last decade the great value of monoclonal antibodies in diagnostic histopathology and especially in solving problematic cases has been accepted. Monoclonal antibodies have played an important role in the immunohistochemical detection of several tumour antigens (1).

Oncogenes are genes which are involved in the conversion of normal cells to cancer cells. Several oncogenes have been identified as transforming genes in cell transformation assays by virtue of their homology to sequences present in acutely transforming oncopigenic retroviruses (2). Abnormal oncogene expression at either a qualitative or quantitative level has been demonstrated in a variety of human tumors (3).

Mammalian cells contain at least three functional ras genes: Ha-ras1, Ki-ras2 and N-ras (4). Ras genes code for a 21,000 dalton protein, ras p21, which is located on the inner part of the cytoplasmic membrane (5), binds GTP (6) and possesses GTPase activity (7-9). Ras gene mutations which alter amino acids 12 and 61 in the p21 protein have been found to be critical in cell transformation experiments (4). It is estimated that about 15% of human tumors carry structural mutations in the ras genes (10). Moreover, it has been found that many benign and malignant human tumors express elevated levels of ras gene transcripts (11-14) or p21 protein (15-20) as compared to noraml tissue. Amplification of ras proto-oncogenes may also lead to tumorigenesis (21, 22).

The availability of monoclonal antibodies to oncogene products allows the involvement of these oncogenes in various stages of human cancer to be determined (15-20, 23). Previous results from our laboratories using molecular hybridization analysis have shown that the Harvey ras proto-oncogene is activated in breast cancer (11). In the present study we have used an immunohistochemical method which employs monoclonal antibodies to the ras oncogene encoded p21 protein to determine the p21 expression in different human breast lesions.

Materials and Methods

The rat monoclonal antibody Y13 259 was raised to Harvey-ras p21 protein. The antibody recognized both Harvey and Kirsten-ras gene products. It was prepared from the hybridoma cell line as previously described (24). As controls for immunostaining, two Chinese hamster cell lines were used. The transformed line PH06T1 contains the mutant T24 human Ha-ras oncogene inserted within a high expression vector. CHL is the parental untransformed Chinese hamster lung fibroblast strain (25). Frozen sections of human breast lesions and cytocentrifuged preparations of cultured cells were prepared as previously described (17).

For immunostaining, sections were washed in PBS buffer pH 7.4. Monoclonal antibody Y13 259 diluted 1 to 100 in PBS was applied for 90 min at 37°C. After being washed 4 times in PBS, the secondary antibody anti-rat IgG biotin conjugate (from Sigma) diluted 1 to 100 in PBS was applied for 1 hour at 37°C. The sections were then washed 4 more times in PBS and were further incubated for 1 hour with the complex of streptavidin-peroxidase (from Sigma) diluted 1 to 100 in PBS. After the final washing, the reaction was developed with DAB solution (3-3 Diamino Benzidine Tetrahydrochloride from Sigma) at 0.5 mg/ml in PBS pH 7.4 which had been activated by the addition of 30% H₂O₂ to 0.2% immediately prior to use.

* This paper was originally presented at the First International Conference of Anticancer Research, 26-30 October 1985, Loutraki, Greece.

Correspondence to: Dr. D.A. Spandidos, The Beatson Institute for Cancer Research Garscube Estate, Bearsden, Glasgow, G61 1BD, Scotland, UK.

Key Words: ras oncogene, breast cancer, immunohistochemistry.
Table I. Staining intensity of breast tissues for Y13 259.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Number of cases-staining intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/−</td>
</tr>
<tr>
<td>Cystic disease</td>
<td></td>
</tr>
<tr>
<td>Simple</td>
<td>16</td>
</tr>
<tr>
<td>Complex</td>
<td>3</td>
</tr>
<tr>
<td>Fibroadenomas</td>
<td></td>
</tr>
<tr>
<td>Simple</td>
<td>13</td>
</tr>
<tr>
<td>Hyperplastic</td>
<td>4</td>
</tr>
<tr>
<td>Carcinomas</td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>3</td>
</tr>
<tr>
<td>Lobular</td>
<td>0</td>
</tr>
<tr>
<td>Mixed</td>
<td>0</td>
</tr>
<tr>
<td>In situ carcinomas</td>
<td>0</td>
</tr>
<tr>
<td>Adjacent normal</td>
<td>58</td>
</tr>
</tbody>
</table>

Results

Sections of breast tissues were analyzed by the immunohistochemical method using the ras p21 specific monoclonal antibody Y13 259. The intensity of staining in the sections was assessed independently by two investigators and was graded as equivocal or negative (+/−), moderate (+) or intense (++) positive. In the carcinoma group, 15 out of 18 were (+) or (++) positive and only 3 cases were (+/−). Finally, the 2 in situ carcinomas were (+) positive.

The ability of the Y13 259 monoclonal antibody to detect enhanced levels of ras p21 was confirmed with the following control cells as previously described (17). The negative control consisted of early passage Chinese hamster lung (CHL) cells which express low levels of the endogenous p21, while the positive control consisted of the FH067T1-1 cells overexpressing the human T24 bladder carcinoma Ha-ras1 oncogene (see Materials and Methods).

Discussion

Our results summarised in Table I indicate that expression of the ras gene p21 protein is elevated in breast cancer in epithelial hyperplastic lesions, particularly when the latter are associated with cellular atypia. A similar study by Williams et al (17) using the same monoclonal antibody, showed elevated expression of ras p21 protein in premalignant neoplastic polyps as compared to the normal colonic mucosa from the same patient. These findings, taken together with the results of molecular hybridisation analyses in studies concerning oncogene expression in human tumours (11-14), indicate that oncogenes are activated in early and late stages of tumorigenesis.

In recent studies, a monoclonal antibody (RAP-5) raised against a synthetic peptide corresponding to amino acid positions 10-17 of the ras p21 protein has been used to examine the levels of p21 in a variety of tumors (15, 19-22). Increased levels of ras p21 expression were found in benign and malignant lesions (15, 19-22). However, it is now apparent that the protein detected by RAP-5 is not ras p21 but a normal component of several cell types (26, 27). Hence it is unlikely to be a useful reagent for detection of ras proteins in human tissues (26, 27). By contrast, the monoclonal antibody Y13 259 raised against the viral Ha-ras p21 protein is specific for ras p21 (17, 24), and is thus potentially useful for detecting ras oncogene expression in human tissues. It should, however, be noted that Y13 259 cannot distinguish between the mutant and the normal p21 proteins.

The results in Table I do not support a correlation between ras p21 expression and tumor invasion. Such a correlation was claimed to occur in mammary and colon tumors using the RAP-5 monoclonal antibody (15, 20). The results in Table I are consistent with our previous findings that there is no correlation between tumor stage in breast (28, 29), colon (30) and head and neck (31) tumors and ras oncogene expression. The lack of correlation between ras expression and invasiveness in colorectal tumors has also been reported by others (16).

Oncogene expression studies in human tumors have shown that more than one oncogene may be activated in the same tumor (13, 14). These results support the model of carci-
nogenesis as a multi-stage process where many oncogenes could be activated before the development of the malignant cell (3).

Although the precise role of the ras p21 protein in the carcinogenesis process in not known, the idea of its being a signal transducer is very attractive (3). In experimental model systems, normal cells transformed by the mutant ras give rise to tumorigenic cells with metastatic properties (25, 32), and overexpression of the normal Ha-ras gene can cause immortalization of primary cells (25) and tumorigenic conversion of immortalized non-tumorigenic cells (25, 33). Ras expression may play a similar role in vivo. However, it is important to note that ras genes are expressed at high levels in certain normal tissues (34) without causing neoplasia. Also ras expression has been shown to induce differentiation (35). Thus elevated expression of ras genes is not necessarily abnormal.

Breast cancer is basically a disease of the mammary epithelium which is an actively dividing cell population that gives an opportunity for genetic mutations to produce cells capable of abundant neoplastic proliferation (36). Epithelial hyperplasia, either ductal or lobular or both, is a very frequent finding in biopsies done for cystic disease and is not necessarily followed by atypical proliferative alterations or cancer development. It may also be stabilised, or even regress and disappear (36).

Most pathologists agree that the Terminal-Ductal-Lobular-Unit (T.D.L.U.) of Wellings is the area where preneoplastic lesions arise, and this fact has been proved experimentally (36-40). An estimate of the time necessary for progression from normal to preneoplastic hyperplasia and finally to true neoplasia is 10 to 20 years (40). During this time, expression of a particular oncogene above a threshold level in an otherwise normal or premalignant cell might be enough to trigger the subsequent events leading to the malignant conversion. An important step in the diagnosis of early breast cancer is identification of a reliable marker which is expressed in those hyperplastic lesions that have preneccancous potential. It is hoped that the study of ras gene expression in a larger number of histological biopsies will contribute towards this aim.

Acknowledgements

We thank Athena Michaelides and Clare Maravella for technical assistance and Peggy Anderson for critical reading of the manuscript. The Beatson Institute is supported by the Cancer Research Campaign of Great Britain and the Hellenic Institute Pasteur by the Greek Ministry of Research and Technology.

References


Received April 15, 1986
Accepted August 20, 1986