ANTICANCER RESEARCH 6: 1157-1160 (1986)

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

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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 oncogenic 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-*ras*1, Ki-*ras*2 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 FHO6T1 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% H_2O_2 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.

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Key Words: ras oncogene, breast cancer, immunohistochemistry.

Table I. *Staining intensity of breast tissues for Y13 259.*

Tissue	Number of cases-staining intensity		
	+/−	+	++
Cystic disease			
Simple	16	1	0
Complex	3	11	2
Fibroadenomas			
Simple	13	1	0
Hyperplastic	4	3	1
Carcinomas			
Ductal	3	6	4
Lobular	0	1	2
Mixed	0	2	0
In situ carcinomas	0	2	0
Adjacent normal	58	0	0

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 (++). The results obtained with the different breast tissues are shown in Table I. A total of 75 cases of breast disease was examined: 33 involved cystic disease and these cases were divided into two groups, *viz.* simple and complex; 22 were fibroadenomas which were also divided into two groups, *viz.* simple and hyperplastic; 18 were carcinomas, mostly of ductal origin; finally, 2 cases of *in situ* carcinoma were also studied.

In the 22 fibroadenomas we did not examine any adjacent noraml tissue, because, since these were circumscribed and encapsulated benign tumours, the specimen consisted only of excisional biopsies. In all other 58 cases the adjacent normal tissue was examined separately, and in all cases it was found to be negative to very low levels (+/−).

In 16 cases of simple cystic disease (C.D.), the p21 protein was expressed at very low levels (+/−) and in only one case a slightly positive (+) expression was observed. In contrast, in the group of complex, C.D. the majority of the cases exhibited a (+) positivity and two cases were considered as (++) positive. It is of interest that complex C.D. is always associated with hyperplastic lesions which frequently show cellular atypia according to the Papanikolaou criteria of malignancy. These lesions are considered as preneoplastic. Moreover, it is internationally accepted that women with histologically verified complex C.D., have a higher risk (2 to 5 times) of developing cancer when compared with the general population.

In the group of fibroadenomas, similar results were found: 13 simple fibroadenomas expressed p21 at very low levels (+/−) and one was (+) positive. In contrast, half of the hyperplastic fibroadenomas were (+) or (++) 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 FH06T1-1 cells overexpressing the human T24 bladder carcinoma Ha-*ras*1 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 precancerous 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 Maravellia 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

1 Erlandson RA: Diagnostic immunohistochemistry of human tumors. An interim evaluation. Am J Surg Path *8:* 615-614, 1984.
2 Varmus HE: The molecular genetics of cellular oncogenes. Ann Rev Genet *18:* 553-612, 1984.
3 Spandidos DA: Mechanism of carcinogenesis. The role of oncogenes, transcriptional enhancers and growth factors. Anticancer Res *5:* 485-498, 1985.
4 Marshall CJ: Human Oncogenes. *In:* RNA Tumor Viruses. Molecular Biology of Tumor Viruses 2nd ed (Weiss RA, Teich NM, Varmus HE and Cofin J eds). Cold Spring Harbor Lab. New York, USA. Vol. 2, 1985, pp 486-558.
5 Willingham M, Pastan I, Shih T and Scolnick E: Localization of the src gene product of Harvey strain of MSV to plasma membrane of transformed cells by electron microscopic immunocytochemistry. Cell *19:* 1005-1014, 1980.
6 Shih T Y, Papageorge A G, Stokes P E, Weeks M O and Scolnick E M: Guanine nucleotide-binding and autophosphorylation activities associated with the p21 src protein of Harvey murine sarcoma virus. Nature *287:* 686-691, 1980.
7 Gibbs JB, Sigal IS, Poe M and Scolnick EM: Intrinsic GTPase activity distinguishes normal and oncogenic *ras* p21 molecules. Proc Natl Acad Sci USA *81:* 2674-2678, 1984.
8 McGrath JP, Capon DJ, Gueddel DV and Levinson AD: Comparative biochemical properties of normal and activated human *ras* p21 protein, Natue *310:* 644-649, 1984.
9 Sweet RW, Yokoyama S, Kamata T, Feramisco JR, Rosenberg M and Gross M: The product of *ras* is a GTPase and the T24 oncogene mutant is deficient in this activity. Nature *311:* 273-275, 1984.
10 Santos E, Sukumar S, Martin-Zanca D, Zarbl H and Barbacid M: Transforming *ras* genes. *In* Viruses and Cancer (Rigby PWJ and Wilkie NM eds). Cambridge University Press, 1985, pp.291-313.
11 Spandidos DA and Agnantis NJ: Human malignant tumours of the breast as compared to their respective normal tissue have elevated expression of the Harvey *ras* oncogene. Anticancer Res *4:* 269-272, 1984.
12 Spandidos DA and Kerr IB: Elevated expression of the human *ras* oncogene family in premalignant and malignant tumours of the colorectum. Br J Cancer *49:* 681-500, 1984.
13 Slamon DJ, Dekernion JB, Verma IM and Cline MJ: Expression of cellular oncogenes in human malignancies. Science *224:* 256-262, 1984.
14 Spandidos DA, Lamothe A. and Field JK: Multiple transcriptional activation of cellular oncogenes in human head and neck solid tumours. Anticancer Res *5:* 221-224, 1984.
15 Thor A, HoranHand P, Wunderlich D, Caruso A, Muraro R and Schlom J: Monoclonal antibodies define differential *ras* expression in malignant and benign colonic diseases. Nature *331:* 562-565, 1984.
16 Gallick GE, Kurzrock R, Kloetzer WS, Arlinghaus RB and Gutterman JV: Expression of p21 *ras* in fresh primary and metastatic human colorectal tumours. Proc Natl Acad Sci USA *82:* 1792-1799, 1984.
17 Williams ARW, Piris J, Spandidos DA and Wyllie AH: Immunohistochemical detection of the *ras* oncogene p21 product in an experimental tumour and in human colorectal neoplasms. Br J Cancer *52:* 687-693, 1985.
18 Kerr IB, Lee FD, Quintanilla M and Balmain A: Immunocytochemical demonstration of p21 *ras* family oncogene product in normal mucosa and in premalignant and malignant tumours of the colorectum Br J Cancer *52:* 695-700, 1985.
19 Viola MV, Fromowitz F, Oravez S, Deb S and Schlom J: *Ras* oncogene p21 expression is increased in premalignant lesions and high grade bladder carcinoma. J Exp Med *161:* 1213-1218, 1985.
20 Horan Hand P, Thor A, Wunderlich D, Muraro R, Caruso A and Schlom J: Monoclonal antibodies of predefined specificity detect activated *ras:* gene expression in human mammary and colon carcinomas. Proc Natl Acad Sci USA *81:* 5227-5231, 1984.
21 Pulciani S, Santos E, Lauver AV, Long LK, Aaronson SA and Barbacid M: Oncogenes in solid human tumors. Nature *300:* 539-542, 1982.
22 Pulciani S, Santos E, Long LK, Sorrentino V. and Barbacid M: *Ras* gene amplification and malignant transformation. Mol Cell Biol *5:* 2836-2841, 1985.
23 Sikora K, Evan G, Stewart J and Watson JV: Detection of the c-myc oncogene product in testicular cancer. Br J Cancer *52:* 171-176, 1985.
24 Furth ME, Davis LJ, Fleurdelys B and Scolnick EM: Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus of the cellular *ras* gene family. J Virol *43:* 294-304, 1982.
25 Spandidos DA and Wilkie NM: Malignant transformation of early passage rodent cells by a single mutated human oncogene. nature *310:* 469-475, 1984.
26 Ghosh AK, Moore M and Harris M: Immunohistochemical detection of *ras* oncogene p21 product in benign and malignant mammary tissue in man. J Clin Pathol *39:* 428-434, 1986.

27 Robinson A, Williams ARW, Piris J, Spandidos DA and Wyllie AH: Evaluation of a monoclonal antibody to *ras* peptide, claimed to bind preferentially to cells of infiltrating carcinomas. Br J Cancer In Press.
28 Agnantis NJ, Parissi P, Anagnostakis D and Spandidos DA: Comparative study of Harvey *ras* oncogene expression with conventional clinicopathologic parameters of breast cancer. Oncology *43:* 36-39. 1986.
29 Agnantis NJ and Spandidos DA: Expression of the human Harvey *ras* oncogene in breast cancer. *In :* Breast Cancer: Origins, Detection and Treatment. (Rich MA, Hager JC and Taylor - Papadimitriou J eds). Martinus Nijhoff Publishing, Boston, USA. 1986, pp 190-194.
30 Kerr I B, Spandidos DA, Finlay IG, Lee FE and McArdle CS: The relation of *ras* family oncogene expression to conventional staging criteria and clinical outcome in colorectal carcinoma. Br J Cancer *53:* 231-235, 1986.
31 Field JK, Lamothe A. and Spandidos DA: Clinical relevance of oncogene expression in head and neck tumors. Anticancer Res *6:* 595-600, 1986.
32 Muschel RJ, Williams JE, Lowy DR and Liotta LA: Harvey *ras* induction of metastatic potential depends upon oncogene activation and the type of recipient cell. Am J path *121:* 1-8, 1985.
33 Chang EH, Furth ME, Scolnick EM and Lowy DR: Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. Nature *297:* 479-483, 1982.
34 Spandidos DA and Dimitrov T: High expression levels of *ras* p21 protein in normal mouse heart tissues. Bioscience Rep *5:* 1035-1039, 1985.
35 Noda M, Ko M, Ogura A, Liu D-G, Amano T and Ikawa Y: Sarcoma viruses carrying *ras* oncogenes induce differentiation-associated properties in a neuronal cell line. Nature *318:* 73-75, 1985.
36 Gallager HS: The developmental pathology of breast cancer. Cancer *46:* 905-907, 1980.
37 Azzopardi JG: Problems in breast pathology. Vol. II in the series: Major problems in Pathology. W.B. Saunders Co., Philadelphia-London-Toronto, 1979, p. 104.
38 Cardiff RD, Wellings SR and Faulkin LJ: Biology of breast preneoplasia. Cancer *39:* 2734-2746, 1977.
39 Wellings SR, Jensen HM and Marcum RG: An atlas of subgross pahtology of the human breast with special reference to possible precancerous lesions. J Natl Cancer Institute *55:* 231-275, 1975.
40 Wellings SR: Development of human breast cancer. Adv Cancer Res *31:* 287-314, 1980.

Received April 15, 1986
Accepted August 20, 1986