

Ras protein expression as a marker for breast cancer

GLORIA M. CALAF^{1,2} and JORGE ABARCA-QUINONES³

¹Institute for Advanced Research, Tarapacá University, Arica 1001236, Chile;

²Center for Radiological Research, Columbia University Medical Center, New York, NY 10032, USA;

³School of Medicine, Saint-Luc Hospital, IMAG Unit (IREC), University of Louvain, Brussels 1200, Belgium

Received March 1, 2016; Accepted April 15, 2016

DOI: 10.3892/ol.2016.4461

Abstract. Breast cancer, the most common neoplasm in women of all ages, is the leading cause of cancer-related mortality in women worldwide. Markers to help to predict the risk of progression and ultimately provide non-surgical treatment options would be of great benefit. At present, there are no available molecular markers to predict the risk of carcinoma *in situ* progression to invasive cancer; therefore, all women diagnosed with this type of malignancy must undergo surgery. Breast cancer is a heterogeneous complex disease, and different patients respond differently to different treatments. In breast cancer, analysis using immunohistochemical markers remains an essential component of routine pathological examinations, and plays an important role in the management of the disease by providing diagnostic and prognostic strategies. The aim of the present study was to identify a marker that can be used as a prognostic tool for breast cancer. For this purpose, we firstly used an established breast cancer model. MCF-10F, a spontaneously immortalized breast epithelial cell line was transformed by exposure to estrogen and radiation. MCF-10F cells were exposed to low doses of high linear energy transfer (LET) α particles (150 keV/ μ m) of radiation, and subsequently cultured in the presence of 17 β -estradiol. Three cell lines were used: i) MCF-10F cells as a control; ii) Alpha5 cells, a malignant and tumorigenic cell line; and iii) Tumor2 cells derived from Alpha5 cells injected into nude mice. Secondly, we also used normal, benign and malignant breast specimens obtained from biopsies. The results revealed that the MCF-10F cells were negative for c-Ha-Ras protein expression; however, the Alpha5 and Tumor2 cell lines were positive for c-Ha-Ras protein expression. The malignant breast samples were also strongly positive for c-Ha-Ras expression. The findings of our study indicate that c-Ha-Ras protein expression may be used as a marker to predict the progression of breast cancer; this marker may also ultimately provide non-surgical treatment options for patients who are at a lower risk.

Introduction

Breast cancer is a progressive disease and the most common neoplasm affecting women of all ages. Similar to all progressive diseases, early and reliable diagnosis is the key to successful treatment. Benign epithelial breast disease represents a growing percentage of the pathological characteristics of this disease, which include numerous benign entities such as cysts, fibrosis, adenosis and duct ectasia, which require neither surgery nor follow-up (1,2). Lesions such as papillomas, sclerosing adenosis, lobular intraepithelial neoplasia, flat epithelial atypia, radial scars and atypical hyperplasias, which are all considered pre-malignant lesions, are signs of an increased risk of breast cancer (3-7).

It has been demonstrated that human malignant tumors of the breast have an elevated expression of the Harvey Ras oncogene when compared to their respective normal tissue samples (7). In a previous study, the authors performed a comparative analysis of Harvey Ras oncogene expression with conventional clinicopathological parameters of breast cancer (8). In another study, an immunohistochemical analysis of Ras oncogene expression in human breast lesions was performed (9). A high expression of p21 Ras oncogene in breast cancer patients is considered to have important clinical significance (10).

In breast cancer, the increased expression and/or activation of Ras are often associated with tumor aggressiveness (10,11). Available and emerging immunohistochemical and molecular studies have improved the classification of breast cancer, as well as the prognostic and predictive information regarding breast cancer pathology; thus, a new tumor classification has been proposed (12-17). In this new classification, the basal-like and the triple-negative types of breast cancer have a likelihood of distant recurrence and mortality compared with other types, and have a tend to affect younger women. For both subtypes, a need for effective biological markers has been reported in certain studies; thus, Ras expression may prove to be an effective prognostic marker (13,17).

The complexity of breast cancer pathology remains a challenge for the scientific community; therefore, protein biomarkers are needed as indicators of pathological, physiological, or pharmacological processes. Among these, estrogen receptor (ER) and Her2/neu are biomarkers that have been approved for the prognosis, diagnosis and treatment of this disease. Since breast cancer is a heterogeneous disease, some breast cancer cells lose their ability to express ER α among other proteins, resulting in

Correspondence to: Dr Gloria M. Calaf, Institute for Advanced Research, Tarapacá University, Calle Antofagasta 1520, Arica 1001236, Chile

E-mail: gmc24@columbia.edu

Key words: c-Ha-Ras, breast cancer risk

a disease which is therapy-resistant. To identify human breast cancer biomarkers between ER α -positive and -negative breast cancer, tissues need to be micro-dissected and protein expression can then be identified to be compared with either normal ductal epithelium or ductal epithelium containing ductal carcinoma *in situ* lesions. Ras and Her2/neu protein expression have been previously considered as biomarkers, since they are highly expressed in human breast cancer (11).

Breast cancer is a heterogeneous complex disease, a spectrum of many subtypes with distinct biological features, and this leads to differences in response patterns to various treatment modalities and clinical outcomes. In this context, the analysis of breast cancer using immunohistochemical markers remains an essential component of routine pathological examinations, and plays an important role in the management of the disease as regards diagnostic, prognostic and therapeutic strategies (18-22). Ras family members (H-Ras, K-Ras, N-Ras and M-Ras) are small GTPases which are activated indirectly by external stimuli. Since the cloning of HRas, the first human oncogene, the Ras/MPK pathway has been a preferential subject of cancer research (23,24), and it is known as an important pathway in the initiation and progression of cancer. Therefore, the aim of the present study was to evaluate Ras expression by immunohistochemical analysis in breast cell lines, as well as in normal, benign and malignant breast sample biopsies, in order to identify a marker that may be used as a prognostic tool for breast cancer patients.

Materials and methods

Breast cancer cell lines. An *in vitro* experimental breast cancer model (Alpha model), previously developed by our group by exposing the immortalized human breast epithelial cell line, MCF-10F, to low doses of high linear energy transfer (LET) α particles radiation (150 keV/ μ m) and subsequent growth in the presence or absence of 17 β -estradiol (25), was used in this study. This model consisted of human breast epithelial cells in different stages of transformation: i) a control cell line, MCF-10F; ii) a malignant and tumorigenic cell line termed Alpha5 (60 cGy plus estrogen/60 cGy plus estrogen); and iii) Tumor2 cells derived from cells originating from a tumor following the injection of the Alpha5 cell line into nude mice. The cells were grown in DMEM/F-12 (1:1) medium supplemented with antibiotics [100 U/ml penicillin, 100 μ g/ml streptomycin, 2.5 μ g/ml amphotericin B (all from Life Technologies, Grand Island, NY, USA)], 10 μ g/ml 5% equine serum (Biofluids, Rockville, MD, USA), 0.5 μ g/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA) and 0.02 μ g/ml epidermal growth factor (Collaborative Research Inc., Bedford, MA, USA).

Protein expression by immunoperoxidase staining. Exponentially growing cell line cells were plated on a glass chamber slide (Nunc Inc., Naperville, IL, USA), at a density of 1×10^4 cells/ml of medium and allowed to grow for 2-3 days until 70% confluent. The cells were fixed with buffered paraformaldehyde at room temperature, incubated with 1% H₂O₂ in methanol to block endogenous peroxidase and again washed twice with buffer solution. Subsequently, the cell cultures were then covered with normal horse serum for 30 min at room temperature and incubated with either anti-mouse or

anti-goat monoclonal or polyclonal antibodies: ER α (mouse, sc-8002), Neu (rabbit, sc-284) and H-Ras (mouse, sc-29) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 1:500 dilution overnight at 4°C and then incubated for 45 min with diluted biotinylated secondary antibody solution (Vector Laboratories Inc., Burlingame, CA, USA) and Vectastin Elite ABC reagent (Vector Laboratories Inc.) was used. The experiments were repeated twice in cells with identical passages *in vitro*.

Breast samples. All samples were obtained from archives entrusted by Professor P. Maldague and Professor A. Trouet from the School of Medicine, Saint-Luc Hospital, IMAG Unit (IREC), University of Louvain, Brussels, Belgium. This study was approved by the Ethics Committee of the University of Louvain and was conducted in accordance with institutional guidelines. The study consisted of the analysis of a total of 40 samples from the archives (all patients had provided with written informed consent prior to obtaining the samples). Normal tissues were obtained from 10 patients admitted for reduction mammoplasty with a family history for cancer; 15 samples had benign breast lesions and 15 specimens had breast cancer. All samples were diagnosed and classified according to the World Health Organization classification (26).

Immunohistochemistry. Processing of the breast samples was performed following a routine pathological examination. All samples investigated were tested for mouse H-Ras monoclonal antibody (Santa Cruz Biotechnology, Inc.). Immunolocalization was performed using a streptavidin-biotin immunoperoxidase method according to the protocol of the laboratory. Briefly, 5 μ m-thick sections obtained after formalin fixation and paraffin-embedding were deparaffinized in xylene and rehydrated with Tris-buffered saline (TBS). The sections were then treated with citrate buffer (pH 6.0) and hydrogen peroxide was used to quench endogenous peroxidase activity. The slides were then subjected to the primary antibody solution and placed in a moist chamber overnight at 4°C. After washing in TBS, a biotinylated link antibody was applied, followed by washing and the addition of streptavidin peroxidase. The localization of the antibody was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counterstaining with Mayer's hematoxylin (Sigma-Aldrich).

Results

The established breast cancer model (25) was shown to exhibit several characteristics of breast carcinogenesis. The normal cell line, MCF-10F, did not exhibit any of the features that characterize malignant cells, such as anchorage-independent growth in soft agar, invasion and tumor growth in nude mice. The Alpha5 cell line induced the development of mammary gland tumors in animals after injection and gave rise to the Tumor2 cell line (25). Fig. 1 shows the results of immunoperoxidase staining obtained to detect ER α , ErbB2 and c-Ha-Ras protein expression in the MCF-10F, Alpha5 and Tumor2 cell lines. The results indicated a positive expression in the Alpha5 and Tumor2 cell lines for all 3 markers. However, a negative expression was observed in the MCF-10F cell line. The Tumor2 malignant cells derived from the mice were positive for ER α ,

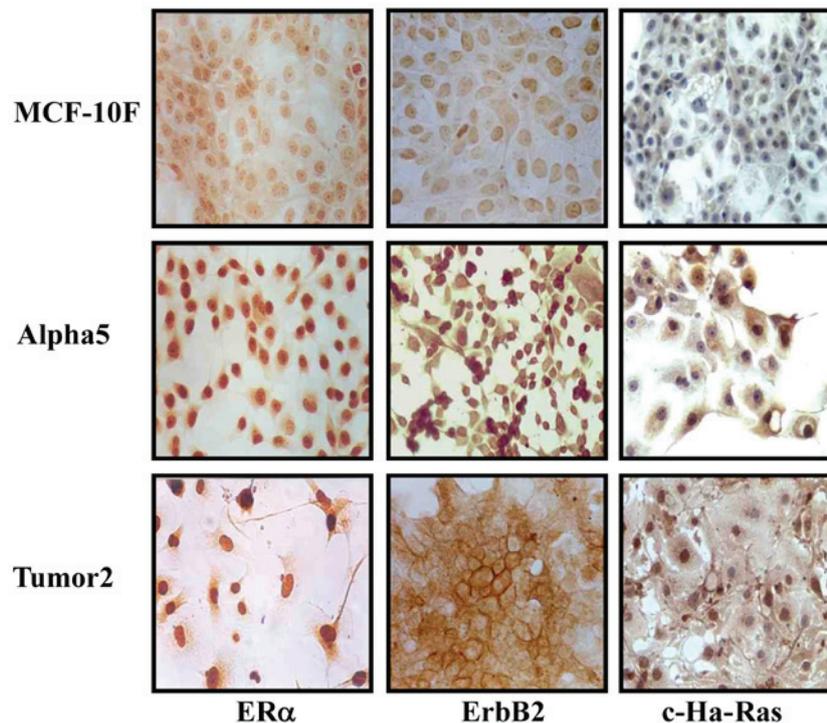


Figure 1. Representative images of estrogen receptor α (ER α), Neu and c-H-Ras protein expression determined by peroxidase staining in the MCF-10F, Alpha5 and Tumor2 cell lines. Primary antibodies used were mouse monoclonal antibodies to ER α , Neu and H-Ras.

ErbB2 and c-Ha-Ras protein expression, as with the original cell line, Alpha5.

An analysis of breast specimens obtained from the tissue archives of patients with a family history of breast cancer was also carried out. A routine pathological examination revealed marked fibrosis, a variety of cysts with or without apocrine metaplasia, some microcalcifications, some low duct hyperplasias and sporadic epithelial flat lesions in both groups. Our findings support the concept of the heterogeneity of expression in epithelial lesions and the value of immunohistochemical analysis in histopathological diagnosis. Representative images are shown in Figs. 2 and 3. The results indicated that case 1 (without a family history) was a low ductal hyperplasia. Proliferative ducts originating from small structures were observed (see inset). Ducts and ductules were positive for ER α and ErbB2; however, a negative c-Ha-Ras expression was observed in ducts and enlarged ductules. Case 2 (without a family history) exhibited lobules that were negative for ER α , ErbB2 and c-Ha-Ras. Case 3 (with a family history) exhibited ducts and ductules positive for ER α and ErbB2; however, the ductules were negative for c-Ha-Ras. Case 4 (with a family history) exhibited lobules negative for ER α , but positive for ErbB2 and c-Ha-Ras. Case 5 (with a family history) also exhibited lobules negative for ER α and c-Ha-Ras, but positive for ErbB2. Representative images of benign lesions are illustrated in Fig. 2. Insets (when available for each image) show other structures also present in that case, as ducts and ductules.

As shown in Fig. 3, the results indicated that case 6 (with a family history) was a lobular hyperplasia where the lobules were negative for ER α , but positive for ErbB2 and c-Ha-Ras. Case 7 (with a family history) also had lobules negative for ER α , but positive for ErbB2 and c-Ha-Ras. Case 8 (with a family

history) corresponded to ductal carcinoma where the cells were positive for ER α , ErbB2 and c-Ha-Ras. Case 9 (with a family history) exhibited structures positive for ER α , ErbB2 and c-Ha-Ras. Case 10 (with a family history) exhibited hyperplastic foci with structures negative for ER α , ErbB2 and c-Ha-Ras. Representative images of breast lesions are shown in Fig. 3. The results revealed normal or benign structures, apart from case 3; however, the report stated that those patients had malignant lesions. On the other hand, there was no association with the markers used clinically, as ER and ErbB2 protein expression were positive in benign, as well as in malignant breast lesions with or without hereditary predisposition. The present study revealed that the percentage of cells positive for ER α was low in normal mammary glands and non-proliferative benign breast disease or displasias, but increased in certain malignant lesions.

Representative images of H-Ras protein expression in normal (Fig. 4A) benign (Fig. 4B) and malignant (Fig. 4C) breast lesions are shown. The expression of H-Ras was negative in the normal breast samples, but weakly positive and heterogeneous in ductules and ducts. In the benign breast samples, the cyst epithelium was usually positive, with some differential patterns. In most cases, it was distributed in the cytoplasm and occasionally with membrane localization. The apocrine metaplasia foci exhibited a stronger expression, with different patterns between the samples analyzed. A significant increase in immunostaining was observed in the atypical ductal hyperplastic lesions, associated with epithelial ductal proliferation. The cancer specimens revealed a more variable staining pattern. The expression of H-Ras was almost invariably stronger in the cancer specimens than in the benign epithelium from the same patients. Some heterogeneity of expression was observed, with weakly positive or negative cancer cells adjacent to strongly

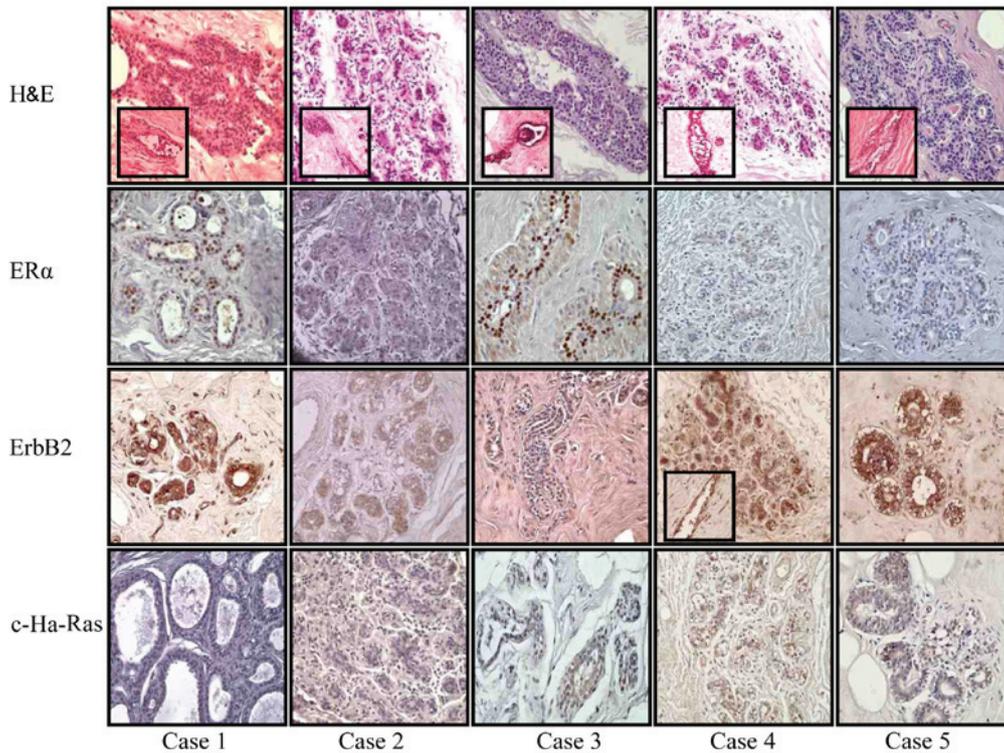


Figure 2. Representative images of estrogen receptor α (ER α), Neu and H-Ras protein expression in breast cancer specimens (cases 1-5) determined by peroxidase staining. Primary antibodies used were mouse monoclonal antibodies to ER α , Neu and H-Ras.

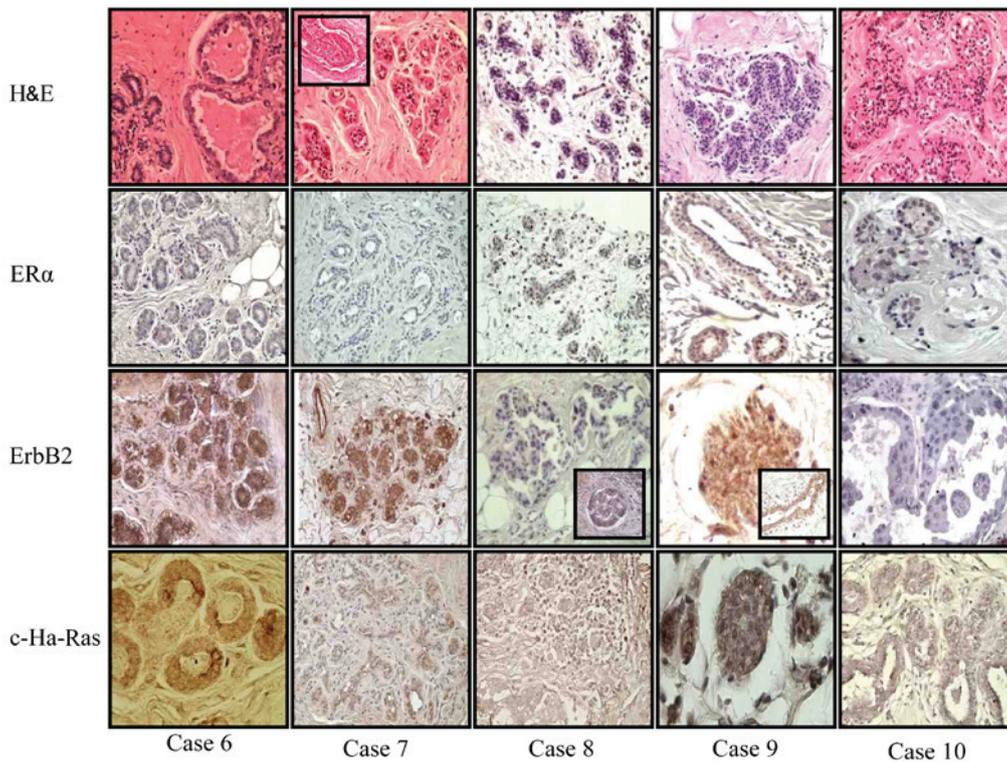


Figure 3. Representative images of estrogen receptor α (ER α), Neu and H-ras protein expression in breast cancer specimens (cases 6-10) determined by peroxidase staining. The primary antibodies used were mouse monoclonal antibodies to ER α , Neu and H-ras.

positive cells. Metastatic cancer cells within the stroma (clusters and isolated cells) exhibited a variable H-Ras expression. Only the tumor budding (clumps) directly associated with

carcinoma foci were more homogeneous, with similar patterns of tumor foci. The expression of H-Ras was similar in lobular and ductal carcinomas.

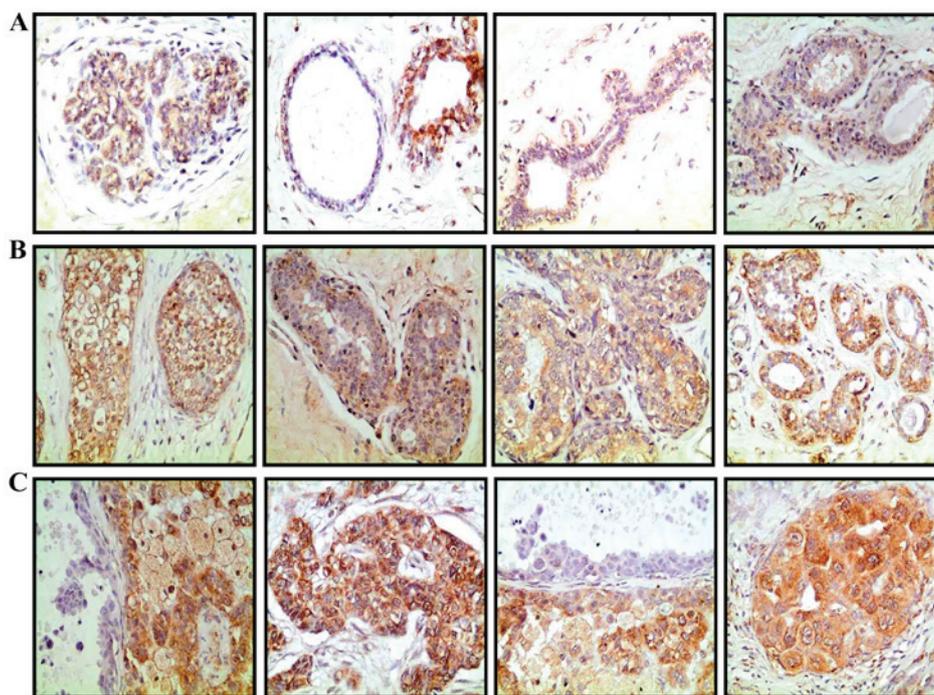


Figure 4. Representative images of c-Ha-Ras protein expression in (A) normal, (B) benign and (C) malignant breast cancer specimens determined by peroxidase staining. The primary antibody used was mouse monoclonal antibody to H-Ras.

Discussion

As with all progressive diseases, the early and reliable diagnosis is key to successful treatment. In our analyses, a previously established breast cancer model (25) was used. The normal cell line, MCF-10F, exhibited a negative protein expression of ER, ErbB2 and c-Ha-Ras, whereas the malignant Alpha5 and Tumor2 cell line exhibited a positive expression ER, ErbB2 and c-Ha-Ras. This model of progression indicated that malignancy can influence the positivity of these markers, as was also observed in our biopsy specimens. Immunohistochemistry plays an important diagnostic adjunct to morphological examination in the diagnosis of benign and malignant lesions (2,7,17,20). Markers to help predict the risk of progression, and to ultimately provide non-surgical treatment options to those at lower risk would be of great benefit. At present, there are no applied molecular markers available to predict the risk of carcinoma *in situ* progression to invasive cancer, and therefore, all women diagnosed with such a malignancy must undergo surgery.

Our results revealed positive c-Ha-Ras protein expression only in the malignant lesions in comparison to the benign lesions. The high c-Ha-Ras expression in intraductal proliferative lesions is very relevant. These epithelial lesions have a risk of progression to invasive breast cancer and related issues, particularly atypical ductal hyperplasia (4). However, the use of c-Ha-Ras expression as a diagnostic and prognostic marker should be selective and should preferably be used in conjunction with other markers. According to published data, the major value of c-Ha-Ras expression is in its clinical correlation with an improved prognosis of relapse (11,24).

In a previous study, the expression of transformation phenotypes was examined in the human breast epithelial cell line,

MCF-10F, with the chemical carcinogens, 7,12-dimethylbenz[a]anthracene, N-methyl-N-nitrosourea, N-methyl-N-nitro-N'-nitrosoguanidine and benzo[a]pyrene (27). This was done to clarify whether chemically induced neoplastic transformation correlates with alterations in the Ras gene. MCF-10F cells have two c-Ha-Ras alleles, identified by 1.0- and 1.2-kb restriction fragments. Treatment with carcinogens resulted in the loss of one of the alleles (1.0 kb). Polymerase chain reaction-amplified DNA from all carcinogen-treated cells was analyzed for point mutations in c-Ha-Ras at codons 12 and 61. All of the carcinogens induced a mutation of the remaining allele at the first position of codon 12 (GGC→AGC). Another frequent mutation occurred at the first position of codon 61 (CAG→GAG). The changes in c-Ha-Ras were associated with the emergence of colony formation in agar-methocel, but no specific changes in this gene correlated with the emergence of invasiveness or tumorigenesis, indicating that other genes may be involved in the process.

It has been concluded that c-Ha-Ras induces an additive effect on the expression of tumorigenesis in the human breast epithelial cell line, MCF-10F, treated with chemical carcinogens. This has provided a model for analyzing the role of c-Ha-Ras in human breast cancer (28). Data have also highlighted the importance of chromosome 11 in the radiation-induced malignant transformation of human breast epithelial cells and suggest the usefulness of the model in uncovering specific derangements during breast cancer progression (29). Among the loci of chromosome 11, the locus 11p15.5, where the c-Ha-Ras oncogene is located, had an incidence of allelic imbalance between 25 and 40%. Furthermore, direct sequencing analysis of codons 12 and 61 of the c-Ha-Ras oncogene identified various point mutations (29). On the other hand, studies have shown that the c-Ha-Ras oncogene is upregulated by the

effect of malathion alone and by the combination of estrogen with either malathion or parathion (30). This suggests that pesticides and estrogens affect human breast cells, inducing molecular changes indicative of transformation (30).

In this study, Ras protein expression was examined in samples of normal and hyperplasias, as well as malignant breast cancer lesions with hereditary predisposition in conjunction with other markers regularly used in clinical practice. Since Ras is often mutated in human cancers, much effort has been devoted to devising means of controlling the activity of Ras (23). Breast tumors have been shown to have an elevated expression of the Harvey Ras oncogene when compared to their respective normal tissue sections (7). The Harvey Ras oncogene has also been shown to have a significant correlation with the clinicopathological characteristics of breast cancer (8). Immunohistochemical analyses of Ras oncogene expression in human breast lesions have been carried out (9), as well as of the p21 Ras oncogene (10,21,22), with a high expression in breast cancer patients indicating clinical significance. It can thus be concluded that the oncogene, c-Ha-Ras, is a good marker to be used in breast cancer in patients and to predict response to therapy in such patients. The evaluation of Ras expression by immunohistochemistry in cell lines in an experimental breast cancer model of transformed cells by environmental substances and estrogen, as well as in normal, benign and malignant breast biopsies from patients, provided evidence that it can be used as good a prognostic tool for breast cancer patients.

Acknowledgements

The technical support of Guiliana Rojas, Georgina Vargas and secretarial assistance and suggestions of Leodán A. Crispin and Richard Ponce-Cusi are greatly appreciated. This study was supported by Grant support FONDECYT no. 1120006 (GMC).

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