# Clinical significance of elevated ras p21 oncogene expression in breast cancer patients.

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**ABSTRACT - In the present study we have examined the relationship of *ras* oncogene protein product p21 expression to the various clinicopathological parameters of the 40 patients studied. Previous observation by us (15a) and others (16, 17) are substantiated by the present study which showes that involvement of lymph nodes is correlated with high p21 levels ; in contrast all other parameters including tumor size and stage, as well as tumor grade, menopausal status and distant metastases, are independent of p21 levels. Hormone receptor status may be related to p21 levels as far as progesterone receptor level is concerned, however the results remain inconclusive. These data indicate that *ras* p21 oncoprotein may play a role in the biologic behaviour and/or pathogenesis of human breast cancer.**

## INTRODUCTION

THE P21 PROTEIN PRODUCT of the *ras* gene family is thought to be an important component in pathways regulating normal cell proliferation and differentiation (for reviews see ref. 1 and 2). This protein acquires transforming properties as a result of activating lesions that convert *ras* protoncogenes to oncogenes in a wide spectrum of malignancies (1,2,3,4). Transforming oncogenes of the *ras* family are capable of converting fibroblast cell lines to fully metastatic tumors (5,6,7). Also, tranfection of activated *ras* into poorly metastatic murine adenocarcinoma cells enhances metastatic potential (8,9).

Moreover, several human benign tumors and carcinomas contain high levels of *ras* transcripts (10,11) as well as of the *ras* p21 gene product as determined by immunohistochemical methods (11). Analysis of expression of the *ras* oncogenes in human malignant breast tumors and in their respective normal tissues has revealed a significant elevation of *ras* transcripts in malignant as compared to normal tissues. That observation suggests that the Harvey-*ras* oncogene may be specifically activated in the development of human breast cancer (12). We (11,13) and others (14,15) have shown previously that *ras* p21 may be specifically activated in the development of human malignant breast and colon tumours. Among twenty-four patients with breast cancer, we observed previously that higher H-*ras* expression may be related to the presence of lymph node metastases (15a). Similar findings have been presented by others (16,17), however the number of cases studied has been small and the above observation has to be substantiated further.

The purpose of the present study is to evaluate additional immunohistochemical data for *ras* p21 from 40 newly diagnosed breast cancer female patients and to compare them with different clinicopathologic parameters.

## MATERIALS AND METHODS

*Tumors* - Forty female patients were used in the present work. These consisted of 19 surgically treatable and 21 inoperable tumors. All patients were staged clinically for the presence of distant metastatic disease. Tissues were obtained during surgery from tumors of the breast. The presence of malignancy was confirmed histologically with the technique of frozen sections. Tumor grading was assessed on permanent sections by one and occasionally two experienced pathologists. Involvement of regional axillary lymph nodes was determined histologically, at the time of primary diagnosis, on nodes obtained by axillary sampling either from specimens of modified radical mastectomies or from biopsy material (selective nodal excision) in the case of lumpectomy.

*Immunoperoxidase studies* - The streptavidin-biotin complex immunoperoxidase assay was performed as previously described (13) on 5 μm sections from formalin fixed- paraffin embedded tissues of primary invasive breast cancer (28 of ductal with 7 intraductal associated, 5 of lobular with 3 *in situ* and 7 of mixed type). The primary antibody employed was a rat-derived monoclonal anti-*ras* p21 which was generated by Furth *et al.* in 1982 (18). This monoclonal antibody recognises both the mutated and the unaltered forms of all *ras* p21 species (18). Both 1 :100 and 1 :200 dilutions of a stock 1 mg/ml solution of purified antibody were used in all assays. The p21 antigen is localized in the inner side of the cytoplasmic epithelial membrane. The negative control consists of normal Chinese hamster lung (CHL) cells and the positive control has the same CHL cells transformed with the mutant T24 H-*ras* 1 oncogene derived from a human bladder carcinoma (13).

The staining intensity of the positive control was used as a marker for the higher expression of the p21 antigen and corresponded with ++. Consequently the mode rate p21 expression was marked with +. Finally, the negative p21 staining intensity agreed with the negative control and was labeled with -/+.

All slides were reviewed and scored jointly by two pathologists. According to the percentage of positive cancer cells present in each slide, the pathologist scaled the p21 expression as negative (< 25 %), moderate (>25 % to <75 %) or high (>75 %). This grading was done using 10 high power fields (HPF) per slide. No negative cancer case was found in the reviewed material. In very few cases that normal mammary glands or lesions with mild cystic disease were present in the same slide with cancer, the p21 expression was always judged as negative (see figures 1, 2 and 3).

Fig. 1. Infiltrating duct adenocarcinoma NOS type. Moderate (+) p21 staining intensity. Immunohistochemical stain X400.

| Estrogen-Progesterone receptor statuts | Total population N = 38 | Population with + p21 N = 12 | Population with ++ p21 N = 26 | P Values |
|---|---|---|---|---|
| ER-<br>ER+ | 9<br>29 | 3<br>9 | 6<br>20 | 1.<br>not significant (p > 0.1) |
| PR-<br>PR+ | 10<br>28 | 5<br>7 | 5<br>21 | 0.2875<br>not significant (p > 0.1) |
| ER-PR-<br>ER+PR+<br>ER+PR-<br>ER-PR+ | 5<br>24<br>5<br>4 | 3<br>7<br>2<br>0 | 2<br>17<br>3<br>4 | 0.2676<br>not significant (p > 0.1) |

Table IV. Correlation between p21 levels and hormone receptor status. P= Chi-square test with Yate's correction. ER= Estrogen receptors. PR= Progesterone receptors.

that enhancement of c-*ras* protein expression may have a clinical significance in breast cancer (14, 15, 16, 17).

We classified our patients according to lymph node status and hormone receptors, which are the dominant prognostic indicators in human breast cancer (21, 22). The expression of p21 in the primary tumor was higher when axillary lymph nodes were positive for metastases upon diagnosis and the difference reached statistic significance ($p < 0.05$). Estrogen receptor status and menopausal status were unrelated to p21 expression. These data are in agreement with other similar publications (16, 17). When we assessed the relationship between p21 expression and PR presence, we found it increased in PR positive tumors. However the difference did not reach a statistic significance ($p > 0.1$).

In contrast to other studies (16, 17) which showed that a correlation of tumor size or clinical stage of the disease and p21 expression may occur, and although in the present study T4 tumors more often had increased p21 levels, the difference was not statistically significant ($p > 0.1$).

No statistical correlation was observed between high p21 levels and tumor grade : this was in accordance with previous data (15a, 17).

When a distant metastasis was detected, we compared p21 levels of the primary tumor with this parameter and no correlation was observed ($p > 0.1$). Fromowitz *et al* (23) suggested that enhanced p21 expression is not obligatory for continued growth of distant metastases. The same authors (23) indicated that markedly enhanced p21 expression is associated with the earlier stages of aggressive breast cancers. However their primary antibody was the RAP-5, the specificity of which is controversial in the literature (14, 15, 16, 18, 24).

In contrast with all the above data the results of Candlish *et al* (25) suggest that the presence of *ras* p21 is not a useful marker of malignancy or of proliferating breast epithelium but represents a feature of normal cellular metabolism. Although they used the same MAb, Y13 259, we think that their findings are influenced by the use of acetone-fixed cryostat sections.

We believe that the recent paper of Going *et al* (26), in which improved fixation schedules for the optimal preservation of *ras* p21 are described, will allow us to evaluate our future data better, especially in the field of borderline breast and colon lesions which frequently cause diagnostic problems.

In conclusion, we have presented in this paper data which further support our previous observations in reference to Ha-*ras* oncogene expression and the conventional clinic pathologic status of breast cancer (15a).

The mechanism by which enhanced p21 expression of normal p21 protein may play a role in mammary cancer growth it is not yet known. Emerging evidence suggests that there are multiple steps in cancer development and oncogene activation may play an important role in the prognosis of breast cancer (16, 17, 27). Suppression of the activity of such cancer genes, if possible *in vivo*, may inhibit the growth and metastatic potential of breast cancer and have clinical applications in the future. ■

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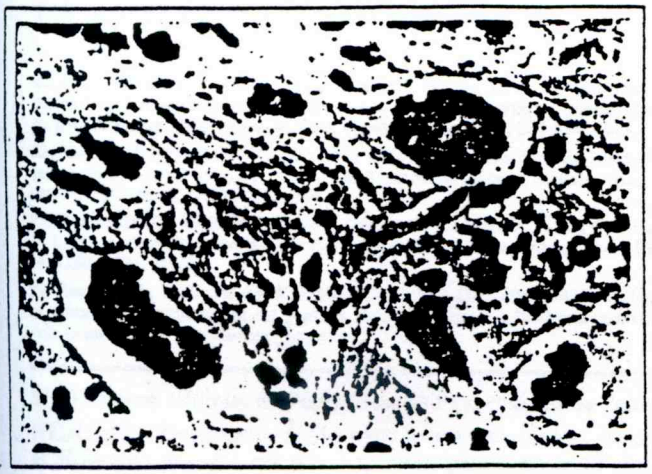

Fig. 2. Infiltrating duct adenocarcinoma NOS type. High (++) p21 staining intensity. Immunohistochemical staining X300.

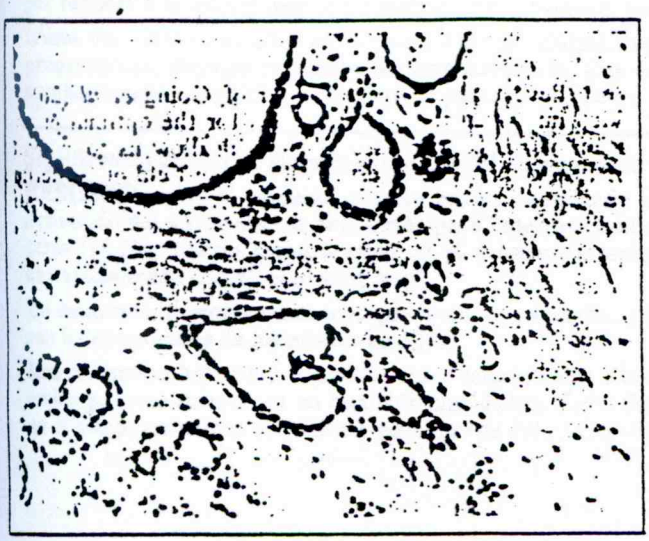

Fig. 3. Mild degree of cystic disease adjacent to a mammary adenocarcinoma. Negative (-/+) p21 staining intensity. Immunohistochemical stain X 120.

*Statistical analysis* - The relationship between p21 antigen staining and clinicopathologic variables was determined by the $\chi^2$ test distribution. Differences between two populations were judged significant at a level greater than 95 % confidence ($p<0.05$). Yate's correction was used in all correlations except in the comparison between p21 levels and axillary lymph node metastases because the expected frequency was almost 5 (i.e. 4.9). For this reason we did not use it (for further explanation see ref. 19).

## RESULTS

*ras p21 antigen expression in primary carcinomas in relation to the grading of the tumors and menopausal status-* The relationship between intensity of staining and tumor grading is shown in Table I. Correlation coefficient relating to p21 antigen with grade was $p>0.1$ and also $p>0.1$ for the menopausal status, both statistically nonsignificant (see Table I).

*Relationship between p21 levels to tumor size and stage of disease-* Statistical analyses were performed comparing the patients who showed high (++) p21 levels with those who expressed moderate (+) p21 expression. No significant correlation with the progression of tumor size was observed ($p>0.1$). When different stages of the disease were assessed, we also found no significant difference among the various stages and p21 activation ($p>0.1$). Table II summarizes these results.

*Correlation between p21 levels and axillary lymph node metastases as well as distant metastases-* As shown in Table III the high elevation of p21 oncoprotein was significantly correlated with axillary lymph node metastase ($p>0.05$), while the presence of distant metastases was not correlated ($p>0.1$).

| | Total population N = 40 | Population with + p21 N = 13 | Population with ++ p21 N = 27 | P values |
|---|---|---|---|---|
| **Tumor Grade** | | | | |
| I | 1 | 0 | 1 | |
| II | 25 | 9 | 16 | 0.6978 |
| III | 14 | 4 | 10 | not significant ($p>0.1$) |
| **Menopausal status** | | | | |
| Premenopausal women | 13 | 5 | 8 | |
| Postmenopausal women | 27 | 8 | 19 | 0.8429 not significant ($p>0.1$) |

Table I. Correlation between p21 levels and tumor grade as well as menopausal status. p= Chi-square test with Yate's correction.

| | Total population N = 40 | Population with + p21 N = 13 | Population with ++ p21 N = 27 | P values |
|---|---|---|---|---|
| **Tumor size** | | | | |
| T1 | 6 | 2 | 4 | |
| T2 | 14 | 6 | 8 | 0.6891 |
| T3 | 6 | 2 | 4* | not significant ($p>0.1$) |
| T4 | 14 | 3 | 11** | |
| **Stage of disease** | | | | |
| I | 5 | 2 | 3 | |
| II | 15 | 6 | 9 | 0.7950 |
| III | 4 | 1 | 3' | not significant ($p>0.1$) |
| IV | 16 | 4 | 12'' | |

Table II. Correlation between p21 levels and tumor size (T) as well as stage of disease (S). p= Chi-square test with Yate's correction. * T3 vs T1+T2= 1 ** T4 vs T1+T2+T3 = 0.4574' SIII vs SI+SII = 1'' SIV vs SI+SII+SIII = 0.6296

| | Total population N = 40 | Population with + p21 N = 13 | Population with ++ p21 N = 27 | P values |
|---|---|---|---|---|
| **Axillary Lymph Node Metastases** | | | | |
| Negative | 15 | 8 | 7 | |
| Positive | 25 | 5 | 20 | 0.0293* significant ($p<0.05$) |
| **Distant Metastases** | | | | |
| Negative | 33 | 12 | 21 | |
| Positive | 7 | 1 | 6 | 0.4911 not significant ($p>0.1$) |

Table III. Correlation between p21 levels and axillary lymph nodes metastases (L.N.M.) as well as distant metastases (DM). P= Chi-square test without Yate's correction* and with Yate's correction.

*ras p21 levels and hormone receptors-* We examined whether the p21 oncoprotein enhancement in breast cancer correlates with the presence of ER and PR, known biochemical markers for hormone dependence (20). It can be seen in Table IV that ER-positive tumors expressed a moderate positivity (+) of p21 in 9/29 and a high elevation (++) of p21 in 20/29. A similar pattern was observed in PR- positive tumors. Nevertheless both receptors, alone or in combination, were found to be statistically nonsignificant ($p>0.1$) and a larger number of patients is required in order to resolve the issue.

## DISCUSSION

In this study we examined *ras* p21 protein (the product of c-*ras* gene family) in primary tumors of breast cancer patients, in order to clarify our previous observations (13, 15a) as well as observations by others, which indicated

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*Received : February 6, 1989 ; accepted : June 2, 1989.*

**Signification clinique de l'élévation de l'expression de l'oncogène *ras* p21 chez les malades porteuses d'un cancer du sein.**

**RESUME - Dans cette étude nous avons examiné la relation entre l'expression de l'oncogène *ras*, la production de protéine p21 et différents paramètres clinicopathologiques chez 40 malades. De précédentes observations par nous-même (15a) ou par d'autres (16,17) furent confirmées par le présent travail qui montre que l'envahissement ganglionnaire est corrélé avec des taux élevés de p21 ; ceci contraste avec d'autres paramètres y compris la taille de la tumeur, le stade, le grade histologique, la relation par rapport à la ménopause et les métastases à distance, ces facteurs sont tous indépendants des taux de p21.**

**L'état des récepteurs d'hormones peut être en relation avec les taux de p21 au moins pour ce qui concerne le récepteur de la progestérone, mais ce résultat n'est pas concluant. Ces données suggèrent que l'oncoprotéine p21 peut jouer un rôle dans le comportement biologique et/ou dans la pathogénèse du cancer du sein chez la femme.**

**Significancia clínica de elevada expresión del oncogene *ras* p21 en pacientes con cáncer de seno.**

**RESUMEN - En el presente estudio hemos examinado la relación de la expresión de la proteína p21 como producto de la activación del oncogene *ras* con varios pará ?metros clinicopatológicos de 40 pacientes. Previa observación por nosotros (15a) y otros (16,17), fueron comprobados en el presente estudio, los cuales presentaron una implicación de los nodulos linfáticos, correlacionados con altos niveles de p21.**

**En contraste, a otos parametros que incluyen : tamaño, progresión de la tumor, asi como el estado menospausico y metástasis son independientes de los niveles de p21.**

**Los receptores hormonales, pueden estor relacionados a los niveles de p21, estando implicado el receptor de la progesterona. Sin embargo estos resultados no han sido elucidados. Estos datos, indican que la oncoproteína *ras* p21, juega un papel importante, en el comportamiento biológico y patogenésis del cáncer del seno.**