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Heterogeneous immunoreactivity of frozen human benign and malignant breast lesions to C-MYC and C-Ha-ras cellular oncogenes

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Summary. *C-myc* and *c-Ha-ras* oncoprotein expression was studied by immunohistochemistry and gene detection by *in situ* hybridization on serial frozen sections of 32 breast lesions (19 benign biopsies and 13 infiltrating carcinomas). *C-myc* protein was expressed in 15/19 benign and 12/13 malignant lesions; *c-myc* gene was detected in 17/19 benign and 13/13 malignant lesions. Although a higher proportion of benign biopsies (8/9) showed more than 50% of protein-positive cells than malignant specimens, this cannot predict the outcome of a lesion. Conversely, p21 *ras* protein was expressed only in 2/19 benign lesions and in most cases of grade I to III carcinomas. The *c-Ha-ras* gene was always detected in a small percentage of cells, in both benign and malignant lesions. The results obtained with atypical hyperplasia, a doubtful proliferating lesion, suggests that p21 *c-Ha-ras* protein expression is not restricted to breast carcinomas.

Although Southern blot is commonly considered as a very sensitive technique for oncogene analysis, no amplification of *c-myc* and *c-Ha-ras* gene has been demonstrated either in benign or malignant lesions. The detection, on serial frozen sections, of proteins and DNA of *c-myc* and *c-Ha-ras*, showed a possible amplification of the *c-myc* and *c-Ha-ras* genes in various benign and malignant lesions.

Key words: *c-myc* oncoprotein, *C-Ha-ras* oncoprotein, Breast lesions, Immunohistochemistry, *In situ* hybridization

Introduction

Many factors may influence the development of breast carcinomas. Cellular oncogenes have frequently been reported to be involved in the conversion of normal cells to cancer cells (Weinberg, 1989). Studies on *c-myc* and *c-Ha-ras* oncogene expression suggest that these genes may play an important role in breast malignant transformation (Field and Spandidos, 1990). The human *c-myc* gene encodes for a protein of 62 kD called *c-myc* p62 which has been detected in a variety of human tumours (Alitalo et al., 1987). The *c-myc* protein is a nuclear protein which is involved in both DNA synthesis and replication (Studzinski et al., 1986). The human *ras* genes encode for proteins of 21 kD called *ras* p21, homologous to G proteins; they possess GTPase activity and are located in the internal part of the cytoplasmic membrane; these proteins are thought to function as a signal transducer (Barbacid, 1990). *Ras* p21 has been found overexpressed in a number of tumours when compared to adjacent normal tissues.

This study was aimed at the detection of *c-myc* and *c-Ha-ras* oncogenes on serial frozen sections in human breast lesions, with the use of immunohistochemistry for oncoproteins and *in situ* hybridization with biotinylated probes or Southern blot for genes. The *in situ* hybridization with non-isotopic probes was chosen for its convenience on tissue sections. In this respect, thirty two biopsies were tested for the presence of *c-myc* and *c-Ha-ras* proteins and DNA.

Materials and methods

Tissue samples

Thirty two specimens of breast lesions (19 benign and 13 malignant) were surgically removed from patients aged from 19 to 76 years (Tables 1, 2). One part of each specimen was snap frozen and stored in liquid nitrogen; the other part was fixed in Bouin's fluid and paraffin-embedded. Eight µm serial frozen sections were prepared on gelatin-coated slides; they were fixed in cold acetone for 10 min for immunohistochemical analysis or in 4% paraformaldehyde for 10 min for *in situ* hybridization.

Lymphocytes obtained from one blood sample were

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used for DNA extraction; the lymphocyte DNA served as DNA amplification control.

Control cell lines

Different epithelial and fibroblastic cultured cells were used as control to detect *c-myc* DNA oncogene amplification: HeLa cells, derived from a human carcinoma of the uterine cervix, containing 1-5 copies of *c-myc* oncogene (Lazo et al., 1989); CaSki cells, derived from a human carcinoma of the uterine cervix without *c-myc* gene amplification; MCF7 and Colo 320 HSR cells, derived from human mammary and colon carcinomas with 5-10 copies and 30 copies of *c-myc* oncogene per cell respectively (Alitalo et al., 1983; Escot et al., 1986); and MRC5, fibroblastic cells issued from human embryo lung without amplification of *c-myc* gene.

Antibodies

C-myc protein was tested with the mouse monoclonal antibody OM-11-905 (Cambridge Research Biochemicals), directed against a conjugated synthetic peptide. For *c-Ha-ras* protein detection, a mouse monoclonal antibody, RAP-5, was used (Ohuchi et al., 1986); it is directed against a peptide reflecting aminoacid position 10-17 of *c-Ha-ras* gene product.

Probes

DNA probes were: the recombinant plasmid pRyc 7.4 containing a fragment of human *c-myc* gene including a portion of exons 2 and 3; and the plasmid pGEM 3 containing 0.6 kb Sma I fragment including the fourth exon of human *c-Ha-ras* 1 gene. These plasmids (kindly provided by C. Theillet, Montpellier) were amplified; the DNAs were purified though serial precipitations and they were biotinylated by nick-translation using 11-dUTP (Sigma) and a nick-translation kit (BRL, Gaithersburg, Md, USA), for *in situ* hybridization. The probes were labelled by nick translation using a ^{32}P-dCTP (Amersham, Cardiff, UK, SA: 3000 Ci/mol), for Southern blot analysis.

Oncoprotein detection

The presence of the oncoprotein was assessed on serial frozen sections with immunoperoxidase technique using avidin-biotin-peroxidase complex (Hsu et al., 1981). Briefly, after rehydration, endogenous peroxidase activity was blocked by incubation with 0.5% hydrogen peroxide in phosphate-buffered saline (PBS) for 15 min at room temperature. Non-specific background staining was reduced by incubation with 10% normal serum in PBS-0.1% bovine serum albumin (BSA) for 20 min at room temperature. The sections were successively incubated with primary antibodies at room temperature for 2h, sheep biotinylated anti-mouse IgG diluted 1:50 in PBS-0.1% BSA for 30 min at room temperature; streptavidin-biotinylated horseradish peroxidase complex at 1:100 in PBS-0.1% BSA for 30 min at room temperature. After washing, the slides were incubated in the dark with 3-amino-9-ethyl carbazole (Biolyon, Lyon, France), washed in distilled water for 5 min, counterstained with haematoxylin, and washed and mounted in polyvinyl alcohol medium. The specificity of the reaction was assessed by the absence of staining in sections incubated with PBS instead of primary antibody or with the conjugate alone.

In situ hybridization technique

The slides were successively incubated with 0.2N HCl for 20 min at room temperature and in 2xSSC (0.3M NaCl, 0.03M Na citrate) for 30 min at 50 °C; they were further exposed to 0.5 µg/ml proteinase K in 2xSSC for 15 min at room temperature; the proteinase K (Merck, Darmstadt, Germany) was prepared at 2 mg/ml and preincubated for 1h at 37 °C before use. After rapid washing in 2 mg/ml glycine in PBS, the slides were post-fixed in 4% paraformaldehyde and washed in PBS.

The *in situ* hybridization conditions were those described by Guérin-Reverchon et al. (1989) with high stringency (TM-17 °C); the DNA-DNA hybrids were detected with a three-step procedure and streptavidin-alkaline phosphatase complex. The slides were counterstained with 0.1% fast-green solution (Sigma, St-Louis, Mo, USA) and mounted in a glycerin-gelatin solution. Positive nuclei showed a purple precipitate under a light microscope.

To determine whether the signals observed after *in situ* hybridization were due to DNA or RNA, other adjacent sections were digested with RNase B (Boehringer, Mannheim, Germany) at 200 µg/ml diluted in 2xSSC and RNase T1 (Boehringer, Mannheim, Germany) at 100 U/ml in 2xSSC or DNase I (Boehringer, Mannheim, Germany) at 200 µg/ml in 2xSSC before hybridization (Burns et al., 1987).

Quantification of positive cells

The results of histochemistry and *in situ* hybridization were analyzed semi-quantitatively on each section by counting the number of positive cells with a magnification of x 200.

Southern blot analysis

DNA extraction of four available biopsies (2 benign and 2 malignant lesions) and one sample of human reference lymphocytes, was performed according to Maniatis et al. (1982). The tissues were minced in 1M phosphate buffer pH 7.4, without glycerol. The crude nuclear fraction was separated from the cytoplasmic fraction by centrifugation at 1000 g for 5 min. The nuclei were lysed overnight by 2% SDS (sodium dodecyl sulphate) in 1M phosphate buffer at pH 8.0, and digested with proteinase K at 50 µg/ml. The DNA was

obtained after two phenol (Appligene, Strasbourg, France) and chloroform treatments. DNA aliquots (10 µg) were digested for 12h at 37 °C in appropriate buffers from the supplier, either with a mixture of EcoR1 and Sma 1 restriction enzymes (Boehringer, Mannheim, Germany) for *c-myc* detection, or with Bam H1 (Boehringer, Mannheim, Germany) for *c-Ha-ras* detection. They were then subjected to horizontal electrophoresis in a 0.7% agarose gel. The DNA fragments were transferred onto nylon filters (Hybond N, Amersham, Cardiff, UK) and heated at 80 °C. The filters were prehybridized in 21 ml of buffer containing 5 x Denhardt's, 1% SDS, 12% SSPE (0.3M NaCl, 0.17M NaH_2PO_4, H_2O, 0.02M EDTA), 0.4M NaCl, 10% dextran sulphate, 10mM Na_2HPO_4, 1 mg/ml sonicated salmon sperm DNA and 50% of deionized formamide, for 12h at 42 °C. They were hybridized 48h at 42 °C with 3-5 x 10^8 cpm of ^{32}P labelled probes (SA: 10^8-10^9 cpm/µg DNA) per filter; they were successively washed four times for 15 min in 2xSSC-0.1% SDS at room temperature, one h in 1xSSc-0.1% SDS at 50 °C and twice for 30 min in 0.1xSSC - 0.1% SDS at 65 °C. The filters were exposed for autoradiography to Hyperfilm (Amersham, Cardiff, UK) at -80 °C for two weeks.

Results

Histological examination of biopsies

Each tissue section was examined after hemalun-phloxin safran staining to classify benign and malignant lesions according to their histological features (Tables 1, 2). The breast microscopic classification used was that of Dupont and Page (1985) and Page et al. (1985) dividing benign lesions into two groups as non-proliferative and proliferative lesions. Infiltrating carcinomas were characterized according to the international TNM classification of breast tumours (1988), by their tumor size (T), the number of invaded lymph nodes (N) versus the total number of axillary nodes surgically removed and remote metastasis (M); their histoprognosis grade is given according to the Bloom and Richardson classification (1957); oestrogen and progesterone receptors were detected by biochemical techniques (Table 2).

Myc and p21 ras oncoprotein detection

In preliminary experiments, we compared the reactivity of monoclonal antibodies against *myc* (OM-

Table 1. *C-myc* and *c-Ha-ras* detection in benign breast lesion.

PATIENTS	AGE	HISTOLOGY	*c-myc* PROTEIN	*c-myc* DNA	*c-Ha-ras* PROTEIN	*c-Ha-ras* DNA
Non proliferative lesions						
1	38	FA	-	+	-	++
2	30	FA	+++	++	-	++
3	44	FA	++++	+	-	+
4	44	FA	++++	-	-	+
5	19	FA	++	++	-	+
6	53	FA	++++	++	-	++
7	56	FAP grade 1	++	+	-	++
8	ND	CD	++	+	-	-
9	34	CD	+++	+	-	+
10	37	CD	-	+	-	++
11	37	CD	+++	-	-	-
12	41	CD	++	++	-	++
13	58	CD	+	++	-	++
14	43	E	++++	++	+	++
15	50	E	ND	++	-	+
16	58	E	-	++	-	+
17	51	E	+	++	-	++
Proliferative lesions						
18	51	AH	++	+	-	++
19	51	AH	+++	++	+++	++
Normal tissues			++	++	-	-

-: no positive cells; +: <5% positive cells; ++: 6-20% positive cells; +++: 21-50% positive cells; ++++: >50% positive cells; ND: not determined; FA: fibroadenoma; FAP: fibroadenoma phyllode grade I; CD: cystic disease; E: epitheliosis; AH: atypical hyperplasia.

Table 2. *C-myc* and *c-Ha-Ras* detection in breast carcinomas.

PATIENTS	AGE	SBR GRADE	SIZE (mm)	AXILLARY NODE*	ER	PGR	*c-myc* PROTEIN	*c-myc* DNA	*c-Ha-Ras* PROTEIN	*c-Ha-Ras* DNA
1	33	I	35	1/16	35	255	+++	++	+	++
2	44	I	17	0/25	204	659	++	+	-	+
3	67	I		1/10	0	0	+	+	+	+
4	53	I	25	8/20	294	225	+	+	+	+
5	48	II		0/18	0	0	+++	+	+	+
6	49	II	35	5/16	2	0	++	+	+	+
7	48	II	48	3/17	ND	ND	++	++	++	+
8	54	II	19	0/15	236	575	ND	+	++	+
9	42	II	50	0/8	19	103	-	+	-	+
10	37	III	40	0/17	ND	ND	+++	++	-	+
11	49	III	13	ND	13	0	++	+	+++	++
12	76	III	20	0/16	0	0	++	++	+++	+
13	64	III	46	3/8	44	958	++	++	+++	+

SBR grade: Bloom and Richardson grade (1957); *: invaded nodes/total number of axillary nodes removed; ER: estrogen receptors (fmoles/mg protein); PGR: progesterone receptor (fmoles/mg protein); -: no positive cells; +: <5% positive cells; ++: 6-20% positive cells; +++: 21-50% positive cells; ++++: >50% positive cells; ND: not determined.

11-905) and p21 *ras* (RAP 5) oncoproteins on frozen and paraffin-embedded sections. The staining of epithelial cells was uniform on paraffin-embedded sections with the two antibodies (data not shown). On frozen sections, *myc* protein was detected in the nuclei of epithelial cells; the intensity of the signal varied with the lesions. P21 oncoprotein was located at the plasma membrane when the antibody was diluted at 1:100 or more. The signal was more diffuse in the cytoplasm at lower antibody dilutions. Therefore, according to these results, our investigation was conducted on frozen sections.

Detection on serial frozen tissue sections of c-myc protein in benign and malignant breast lesions

Of 18 benign breast lesions, 13 non-proliferative and 2 proliferative lesions were positively stained for *c-myc* protein (Table 1), using OM-11-905 monoclonal antibody. An intense labelling with a granular aspect was seen in nuclei of ductal epithelial cells mainly in fibroadenomas; the staining was either moderate or weak in the other lesions.

The number of positive ductal epithelial cells differed with the benign lesions. More than 50% of cells were positive in 8/15 specimens: 7 non proliferative and one proliferative lesion. In 7 other biopsies, less than 20% of cells were weakly or moderately stained: 6 non proliferative and one proliferative lesion.

The last three non proliferative lesions did not show any detectable signal. No staining was seen in fibroblastic cells of positive biopsies. Normal tissues adjacent to the lesions did not react with the monoclonal antibody.

In infiltrating carcinomas (Table 2), the nuclei of malignant cells were intensely stained for *c-myc* protein (Fig. 1) in 11/12 cases. Three lesions contained more than 50% of positive cells (Patients N° 1, N° 5, N° 10) and 8 showed less than 20% of positive cells (3 patients of grade I, 2 of grade II and 3 of grade III).

Detection on serial frozen tissue sections of c-myc DNA in benign and malignant lesions

With *in situ* hybridization and *c-myc* DNA biotinylated probe, a weak or an intense nuclear labelling of ductal epithelial cells was observed in 17/19 benign breast lesions (Table 1); only 3 non-proliferative fibroadenomas (patients N° 3, N° 4, N°6) exhibited an intense labelling which was limited to foci of a few positive cells. In ten of 17 positive samples, 5 to 20% of ductal epithelial cells were stained without any significant difference in the degree of malignancy: 9 non proliferative and one proliferative lesion. Less than 5% of ductal cells showed a signal in 7 cases: 6 non proliferative and one proliferative lesion. Normal tissues adjacent to the lesions did not react with the *c-myc* probe.

In 13/13 carcinomas (Table 2), the infiltrating cells showed an intense or a moderate reaction, limited to foci of a few positive cells (Figs. 2A, 2B). The percentage of positive cells was not clearly related to the tumour grade. Five lesions contained 5-20% of positive cells: grade I (one patient), grade II (one patient) and grade III (3 patients). Eight other lesions showed less than 5% of positive cells: grade I (3 patients), grade II, (4 patients), grade III (one patient). There was no correlation of immunoreactivity regarding the lymph nodes, metastasis or primary tumor size, level of estrogen or progesterone receptors.

Codetection of c-myc and c-myc DNA in benign and malignant lesions

C-myc protein and *c-myc* gene were codeteced in 13/18 benign breast lesions and 11/12 breast carcinomas. The results were discordant in 5 benign non-proliferative lesions and one malignant lesion (grade II). In benign biopsies, only the protein was detectable in two non-proliferative lesions; *c-myc* gene was found in the 3 other cases. In the carcinoma from patient N° 9, only the *c-myc* gene was detected. There was no significant relation between the *c-myc*-positive carcinomas and the other prognostic factors (Table 2).

Detection on frozen tissue sections of p21-ras protein in benign and malignant breast lesions

In benign lesions (Table 1) *c-H-ras* protein was detected in only 2/19 biopsies with the monoclonal antibody RAP-5 diluted at 1:100, in less than 5% of ductal epithelial cells; one epitheliosis and one atypical hyperplasia. In these lesions, the positive staining was located in the plasma membrane of the epithelial cells sometimes being very weak and diffuse in the cytoplasm. No staining was observed in fibroblastic cells. Normal tissues from adjacent zones of the lesions were consistently negative. Conversely, a *c-Ha-ras* protein labelling of malignant cells was observed in 10/13 carcinomas (Table 2). The signal was mainly located in the membrane of epithelial cells (Fig. 3) and the intensity of the reaction varied with cell differentiation. More than 50% of cells were positive in 3/4 grade III tumours and about 20% in 2/5 grade II tumours. Less than 5% of positive cells were found in 3/4 grade I tumours and 2/5 grade II tumors.

Incidentally, a very strong *c-ras* protein signal was observed in the biopsy N° 13 (Table 2), which had a poor prognosis; it was a grade III tumour, without detectable estrogen receptors and showed high levels of progesterone receptors. The reaction was moderate in 2/4 grade II tumours. A weak signal was seen in grade I (3 patients) or in grade II (2 patients) biopsies. Three samples were negative. Among these, two cases had a favorable prognosis: biopsy N° 2 which was T1N0 carcinoma, grade I, showed high levels of estrogen (204 fmoles/mg protein) and progesterone receptors (659 fmoles/mg protein); biopsy N° 9 was T2bN0 carcinoma grade II, had low levels of estrogen (19 fmoles/mg

protein) and progesterone (103 fmoles/mg protein) receptors. For biopsy N° 10, the prognosis could not be confirmed in the absence of hormonal receptor status.

Detection on serial sections of frozen tissue sections of p21-ras gene in benign and malignant breast lesions

With *in situ* hybridization and *c-Ha-ras* biotinylated probe, 17/19 benign biopsies (Table 1) showed a positive signal located in the nuclei of epithelial cells: in 6 of them, 5% of cells were weakly stained; and in 11 other cases, 5 to 20% of cells were positive. A moderate labelling was seen in proliferating lesions and fibroadenomas. Two samples of cystic disease were negative (N° 8 and N° 12). Normal tissues adjacent to the lesions did not react with the *c-Ha-ras* probe.

Infiltrating carcinomas (Table 2) consistently showed a signal of *c-Ha-ras* DNA. About 5% of undifferentiated cells were positive (Fig. 4) in 11/13 samples; the other two biopsies exhibited about 20% of labelled cells (grade I, one patient, grade III, one

Fig. 1. Immunoperoxidase staining of *c-myc* protein in a carcinoma grade I (patient Nº 4, Table 2), using monoclonal antibody OM-11-905 and avidin-biotin peroxidase complex. Sections were counterstained with haematoxylin. *C-myc* reactivity was localised in the nuclei of ductal cells. x 425

Fig. 2. *In situ* hybridization with a *c-myc* DNA probe: **a.** Foci of few cells are positive in a carcinoma grade II from patient Nº 9, Table 2. x 290. **b.** Only one or two cells are positive in grade II carcinoma from patient Nº 6, Table 2. x 460

Fig. 3. Immunoperoxidase staining of *c-Ha-ras* protein in a grade II carcinoma (patient Nº 7, Table 2), using monoclonal antibody RAP-5 and avidin-biotin peroxidase complex. Sections were counterstained with haematoxylin. *C-Ha-ras* reactivity was localised on the membrane of carcinoma cells. x 500

Fig. 4. *In situ* hybridization with a *C-Ha-ras* DNA probe. Few cells are positive in a grade II carcinoma from patient Nº 5, Table 2. x 290

patient).

Codetection on serial sections of frozen tissue sections of p21-ras protein and c-Ha-ras gene in benign and malignant breast lesions

p21 *ras* protein and gene were codetected in 2/19 benign (Table 1) breast lesions (one epitheliosis and one atypical hyperplasia); 2/19 samples of cystic disease did not exhibit any positive signal; the other 15 cases displayed a positive reaction only for DNA.

p21 *ras* protein and gene were codetected in 10/13 malignant lesions whatever the tumour grade; the other 3 specimens (one of grade I, one of grade II, and one of grade III), exhibited the DNA in a few cells but not the protein.

Controls on cultured cells and tissue sections

The cultured cells HeLa, MCF7, Colo 320 HSR, CaSki and MRC5 were tested for the detection of oncoprotein and DNA oncogenes. Colo 320 HSR (Fig. 5A) and MCF7 cells (Fig. 5B) were positive for *c-myc* protein and DNA, HeLa cells were only slightly *c-myc* DNA-positive and CaSki and MRC5 cells were negative. For *c-Ha-ras* protein detection, a positive lesion was introduced in each reaction.

The nuclear *c-myc* or *c-Ha-ras* DNA signal was abolished by a DNase digestion in most cells of adjacent positive tissue sections from benign and malignant lesions and not by treatment with the RNase. This indicates that the signals obtained with *in situ* hybridization on tissue sections were mainly due to DNA-DNA hybrids.

Fig. 5. *In situ* hybridization with a *c-myc* DNA probe on control cells. **a.** Colo 327 cells derived from colonic cancer, containing 30 copies of *c-myc* gene. x 460. **b.** MCF7 cells derived from a breast cancer, containing 5 copies of *c-myc* gene. x 460. Note that all Colo cells are positive whereas only a few MCF7 cells are positive (arrow).

Southern blot analysis

Total DNA extracted from four samples, two of benign (Table 1, patients N° 15, N°19) and two of malignant (Table 2, patients N° 1, N° 11) breast lesions, was analysed for the eventual modifications of *c-Ha-ras* and *c-myc* protooncogene genomes with the Southern blot. One peripheral lymphocyte DNA was used as reference of one copy of each gene. Bands of high molecular weight (> 7.3) and 7.3, 4.2, and 1.4 kb corresponding to *c-myc* and *c-Ha-ras* oncogene respectively were obtained with DNAs from breast lesions, after restriction of enzyme digestion. Although the internal control of GAPDH gene showed that when similar amounts of total DNA were transferred to the nitrocellulose membranes, similar signals were obtained; no oncogene amplification was observed in any breast lesions (Fig. 6).

Discussion

Using serial sections of frozen breast lesions and immunohistochemical analysis, we have shown that the oncoprotein *c-myc* was expressed in most benign and malignant samples whereas p21 *ras* oncoprotein was found in most malignant lesions and was absent in benign biopsies. On the contrary, with *in situ* hybridization, the *c-myc* and *c-Ha-ras* gene were detected in most cases.

The ability of different monoclonal antibodies to detect enhanced levels of *c-myc* p62 and *ras* p21, respectively, has been described in breast cancers (Theillet et al., 1986; Spandidos, 1987; Papamichalis et al., 1988). However, the results of immunohistochemical study detection of oncogene products are not always consistent and different patterns of oncogene expression have been obtained.

C-myc protein is widely distributed in different normal tissues and marked differences are apparent in its intracellular distribution, depending mainly on the method of tissue fixation (Loke et al., 1988; Mizukami et al., 1991).

With frozen sections and the monoclonal antibody OM 11 905 (Evan et al., 1985), we found elevated *c-myc* protein expression in a variety of benign and malignant

lesions, but the extent of positive cells and the intensity of signal varied with the lesions; adjacent normal breast tissues were consistently negative. These results are in agreement with those previously reported on frozen sections (Tauchi et al., 1989) or on formalin-fixed tissue sections (Evan and Hancock, 1985; Spandidos, 1987; Papamichalis et al., 1988; Locker et al., 1989). Similar findings were obtained with an ELISA test (Spandidos et al., 1989a) for evaluation of *c-myc* protein expression. The monoclonal antibody which was defined by immunohistochemical methods, Western blot and immunoprecipitation, recognizes a nuclear p62 *c-myc* oncoprotein. The amplification of *c-myc* gene observed by immunohistochemistry in breast lesions has been confirmed with molecular biology techniques by several authors. Studies on DNA have shown that *c-myc* is amplified in 18 to 58% of breast cancers (Escot et al., 1986; Biunno et al., 1988; Garcia et al., 1989). An amplification of 2- to 15-fold was found in malignant lesions but was reduced in benign lesions (Field and Spandidos, 1990; Donovan-Peluso et al., 1991). In our results, all malignant lesions and 90% of benign biopsies were *c-myc* DNA-positive. However, only a few cells (5-20%) were positive in the 32 samples. Previous *in situ* hybridization experiments have shown that *c-myc* amplification could be detected in cell lines containing more than 5 copies of *c-myc* gene; this was confirmed by Southern blot analysis (Pélisson et al., 1992). Our results obtained on breast tumors favoured the amplification of *c-myc* in most of the lesions, but a small number of cells were positive. The interest of *in situ* hybridization is to establish a correlation between gene expression and histological data.

Besides gene amplification, gene rearrangements have also been demonstrated in some samples (Varley et al., 1987; Bonilla et al., 1988; Garcia et al., 1989). DNA amplification and/or genetic rearrangements are the results of transcriptional dysregulation. In such cases, RNA was overexpressed (Escot et al., 1986; Whittaker et al., 1986); this was confirmed in short term

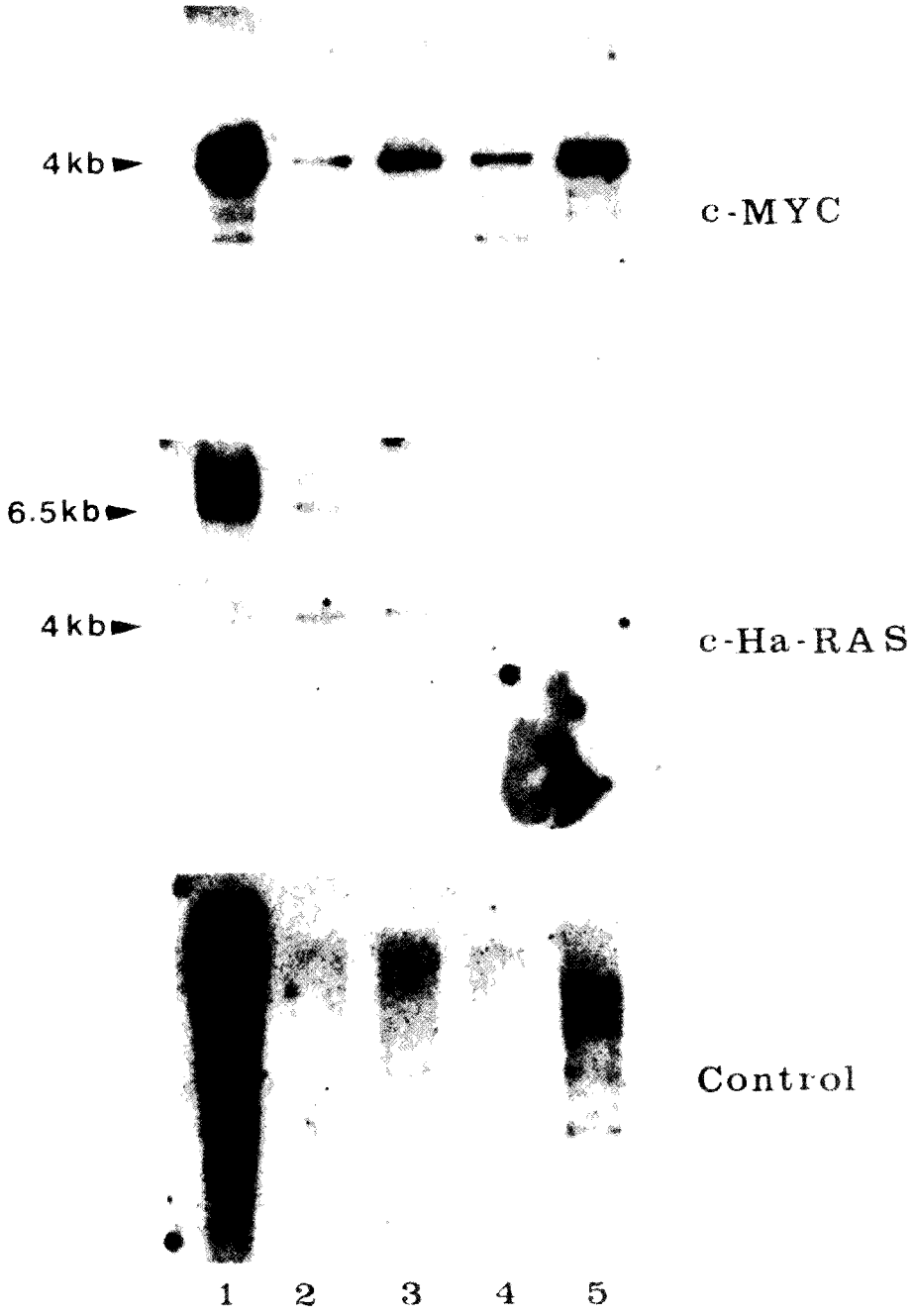

Fig. 6. DNA analysis with Southern blot. For *c-myc* probe, 10 µg of each DNA were digested with a mixture of Eco-R1 and Sma1 restriction enzymes and electrophoresed. For *c-Ha-ras* probe and control gel. 10 µg of each DNA were digested with Bam H1 and electrophoresed. Lane 1: Grade I adeno-carcinoma (patient N° 1 from Table 2); lane 2: Cystic disease (patient N° 15 from Table 1); Lane 3: Grade II adenocarcinoma (patient N° 11 from Table 2); Lane 4: Atypical hyperplasia (patient N° 19 from Table 1); Lane 5: Control of lymphocyte DNA. Control gel: The quantity of DNA on each gel was controlled after hybridization with GAPDH probe. M: Molecular weight markers in 1% agarose gel from Raoul DNA (Appligène) giving 9 bands ranging from 7378 to 1416 bp.

cultures derived from the breast tumours (Benz et al., 1989) and in breast carcinoma cell lines (Kozbor and Croce, 1984; Lavialle et al., 1989).

The amplification of *c-myc* gene was found to be correlated in some cases with tumour cell aneuploidy and with inflammatory breast carcinomas, an aggressive form of breast cancer (Escot et al., 1986; Clair et al., 1987; Spandidos, 1987; Lavialle et al., 1989). For other researchers, there was no correlation with histological grade, estrogen, progesterone receptors or lymph node metastases (Garcia et al., 1989; Tauchi et al., 1989).

In our study, there was no significant difference in immunohistochemical staining with any of the prognostic factors; however, the signal was more intense in positive cell foci of malignant lesions than those of benign lesions. No amplification of the gene *myc* was detected with the Southern blot. It has previously been shown that extraction of RNA and DNA from samples containing different numbers of tumour cells can cause underestimation of RNA expression or gene amplification due to the dilution of sequences from tumour cells with those deriving from inflammatory or stromal cells. From experiments of *in situ* hybridization using detection of RNA-RNA hybrids, applied to breast tumours for which the status of *c-myc* locus was known, it could be concluded that *c-myc* oncogene is consistently expressed in human ductal carcinomas, including cases which may result from negative Northern blot analysis (Mariani-Constantini et al., 1988).

The variable levels of amplification of *c-myc* gene in breast tumours suggest that activation of *c-myc* might be an important factor in the development of some human breast carcinomas.

Ras oncogenes have been implicated in a variety of tumours; they have been identified in 10-15% of human cancers, this percentage being lower in breast lesions (Barbacid, 1990). The expression of *ras* is often increased in tumours relative to normal tissues (Slamon et al., 1984; Spandidos and Agnantis, 1984; Mariani-Constantini et al., 1988). It has mainly been studied by immunohistochemical techniques with monoclonal antibodies including Y13 259 (Furth et al., 1982) and RAP5 (Horan-Hand et al., 1984); similar findings were obtained with an ELISA test (Spandidos et al., 1989a). These techniques did not discriminate between the different p21 proteins or between normal and activated forms. Some authors have identified increased levels of immunoreactivity in malignant cells of mammary carcinomas (Horan-Hand et al., 1984) as compared to those of corresponding benign tumours, dysplastic lesions or normal epithelium; similar findings have been obtained with immunoblot analysis in a variety of primary carcinomas (De Bortoli et al., 1985; Tanaka et al., 1986; Clair et al., 1987) or with radioimmunoassay (De Biasi et al., 1989). Other authors, however, have not been able to detect any significant difference between normal and malignant tissues (Ghosh et al., 1986). In our immunohistochemical study using RAP monoclonal antibody, we found that *ras* p21 gene expression was enhanced in all malignant breast lesions as compared to most benign lesions. These findings are in agreement with those previously reported (Horan-Hand et al., 1984; Ohuchi et al., 1986; Czerniak et al., 1989). Controversial results were found with RAP monoclonal antibody which also recognizes a cytoplasmic cellular compartment different from p21 protein (Ghosh et al., 1986; Robinson et al., 1986). One explanation is that many of these studies were done with formalin-fixed biopsies whereas in our case, fresh frozen biopsies were used. Furthermore, RAP 5 has been used diluted at 1:100 and none of the normal epithelial cells reacted positively either in normal tissues or in tissue adjacent to the lesions. Unexpectedly, in two non-proliferative lesions, one epitheliosis and one atypical hyperplasia, we found that less than 5% of cells were positive. Similarly, this has been detected in self-regressing human tumours such as skin keratoacanthomas which very seldom progress into squamous cell carcinomas; 34% of these tumours appear to contain *c-Ha-ras* oncogene activated by point mutation in codons 12 and 61 (Theillet et al., 1986; Leon et al., 1988).

DNA analysis of breast carcinomas has shown an overexpression of *Ha-ras* gene which is not correlated with any alteration (Theillet et al., 1986) an enhanced level of an RNA has also been demonstrated (Spandidos and Agnantis, 1984). In our study, the absence of gene amplification with Southern blot could be explained by the presence of mutations and/or rearrangements or by the dilution of a rare amplified gene in the total cellular DNA since only few cells were positive with *in situ* hybridization.

These findings illustrate that in human tumours *ras* oncogene activation is not sufficient in itself to induce full neoplastic phenotypes (Leon et al., 1988). *Ras* oncogenes are likely to participate in early stages of tumour development, including the onset of neoplasia in some cases.

In our experiment, the detection on serial frozen tissue sections of proteins and DNA of *myc* and *ras* oncogenes was rare in benign lesions and frequent in malignant breast biopsies. Among 19 benign samples only two of them exhibited *c-myc* protein, *c-myc* DNA, p21 *c-Ha-ras* protein and *c-Ha-ras* DNA, one epitheliosis and one atypical hyperplasia. The other 17 did not express p21 *ras* protein.

On the contrary, 9/12 breast carcinomas showed positive reactions for both oncoproteins and oncogenes. The three others gave different results.

We observed that *c-myc* protein was similarly distributed in benign lesions and carcinomas. Only 4 out of 32 samples did not express *c-myc* protein: one malignant and 3 benign lesions. Among the 3 benign lesions (Table 1), there were two biopsies classified as «no increased risk» according to Page et al. (1985); i.e. no risk of subsequent carcinoma development (one cystic disease, patient N° 10, one epitheliosis, patient N° 16); the other was a fibroadenoma (patient N° 1). The *c-myc* protein negative carcinoma from patient N° 9

(Table 2) was a grade II, T2bN0 with low levels of estrogen and progesterone receptors. According to clinical observations and histological features, the prognosis was not very good; no *c-myc* and *c-Ha-ras* proteins were detectable whereas few cells were DNA positive. In such a case, it is possible that the prognosis was better than in a similar case with high expression of *c-myc* and *c-Ha-ras* oncoprotein; unfortunately, the follow-up of these patients was not available.

One lesion, atypical hyperplasia, from patient N° 19 (Table 2), classified as «moderately increased risk to develop a cancer» by Page et al. (1985), showed elevated levels of *c-Ha-ras* and *c-myc* proteins in a high number of cells (20-50%) but only 6-20% of cells were DNA positive. Five years after removal, there was no recurrence. As the risk is increased by 4 to 5 times in women with such lesions as compared with the general population, these patients should be followed for longer periods.

In one case of epitheliosis (patient N° 14, Table 1) or mild epithelia hyperplasia of usual type for Page et al. (1985), listed as «no proliferative disease», a high number of positive cells for *c-myc* protein (more than 50% of cells) and a low number of positive cells for *c-Ha-ras* (less than 5%) were observed. We do not know the outcome of this patient to compare with the other epitheliosis tested which did not recur within 10 years.

Several studies have been done on both *myc* and *ras* genes, in breast carcinomas but not on benign lesions; they showed obvious enhanced levels of these genes and in most cases an active transcription (Slamon et al., 1984; Whittaker et al., 1986; Biunno et al., 1988; Garcia et al., 1989; Benz et al., 1989; Spandidos et al., 1989b). Human breast cancer is a heterogeneous and complex neoplastic disease where many factors may influence biological behaviour.

In conclusion, our findings are in agreement with those of others who reported that immunohistochemical detection of *c-myc* and *c-Ha-ras* oncogene products in breast cancer was usually related to a poor prognosis. Although the number of biopsies is small for each group, the detection of proteins and DNA in serial frozen sections indicates that *c-myc* and *c-Ha-ras* genes could be amplified in a variety of benign and malignant breast lesions. However, other cellular oncogenes could be involved in the development of breast carcinomas.

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