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Identification of micrometastasis at the time of diagnosis of the primary cancer has clinical utility. The presence of epithelial cells in the bone marrow has been found to be a good predictor of disease and relapse free survival. The number of cancer cells in the marrow is insufficient for the pathologists to analyze them morphologically. It is clear that the significance of these cells in breast cancer etiology and progression can be facilitated by culturing these micrometastatic cells and determining their genetic mutations. Therefore, the aims of this proposal was to

A. Define the culture conditions conducive for the selective growth of cancer cells. The culture conditions was standardized using established human breast cancer cells MCF-7, T47D and MDA-MB-231.

B. Determine the validity of molecular markers, mutant p53 and hsp27, two cellular proteins whose expression reflects increase in metabolic stress as a result of malignant transformation and correlate the expression of these proteins to the propensity of metastatic dissemination.

We discovered that epithelial cells can be selectively grown on a microporous hydrophobic polymer, polyvinylidene difluoride (PVDF) using a vertical culture condition that is permeable to oxygen diffusion. Our studies also show that the expression of mutant p53 is correlated with the propensity of metastatic dissemination In conclusion, we have developed a novel culture method that can be utilized to selectively grow epithelial cancer cells and have preliminary evidence to suggest that expression of mutant p53 is a molecular marker that determines the potential for metastatic dissemination.

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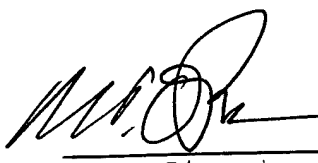

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INTRODUCTION

Primary breast cancer, if detected early, is curative by conventional methods of treatment that include surgery followed by radiation and /or chemotherapy. Metastatic dissemination is difficult to control and does not lend itself to appreciable curative rates. While efforts to curtail metastatic disease is being made with newer and improved chemo-therapeutic regimens, early detection of metastatic disease will impact on mortality and severity of disease in patients of all ages.

To this end we have been developing methods to identify and characterize micrometastatic disease. Our studies indicate that the presence of micrometastatic cells is associated with poor prognosis and early relapse (1). We believe that this group of patients represent possible candidates for adjuvant therapy. It is possible to eradicate these micrometastatic cells in the bone marrow prior to the lodging of these cells in the target organ and thus prevent overt metastasis. The micrometastatic cells were detected in the bone marrow using a panel of antibodies (AE1, C26, T16) directed against cytokeratins. The reactivity of these antibodies have been established earlier and have been found to be epithelial-specific. We have been trying to establish the correlation of the presence of epithelial cells with clinical outcome. Our results are consistent with the finding that presence of epithelial cells in the bone marrow is an independent prognosticator of breast cancer relapse and survival outcome (five year follow-up). The presence of bone marrow positivity, presumably, enhances the propensity to metastatic dissemination and thus decreases long term disease free survival.

Detection of micrometastatic cells in the bone marrow by epithelial specific markers limits the clinical utility of these cells. For the elucidation of the pathological and biological characteristics of these cells, it is imperative that these cells have to be isolated and cultured. To this end specific aims of this proposal was to

I. Define the culture conditions and the attachment matrices that would be conducive for the selective proliferation of epithelial cells. The standardization of the culture conditions was done using human breast cancer cells both estrogen receptor positive, MCF-7 and T47D and estrogen receptor negative, MDA-MB-231. The non-tumorigenic cell line MCF-10 was used as a control.

II. Determine the validity of molecular markers, mutant p53 and HSP27, two cellular proteins whose expression reflects increase in metabolic stress as a result of malignant transformation and correlate the expression of these proteins to the propensity of metastatic dissemination. The expression of these proteins was examined by

immunohistochemistry and reverse-transcriptase polymerase chain reaction (RT-PCR).

For the purpose of this report results of the two specific aims will be presented separately.

SPECIFIC AIM I :

DEVELOP AN IN VITRO EXPERIMENTAL CULTURE SYSTEM CONDUCIVE FOR THE PROLIFERATION OF EPITHELIAL CELLS THAT CAN BE ADAPTED FOR THE GROWTH OF MICROMETASTATIC EPITHELIAL CELLS.

METHOD OF APPROACH :

One of the principal approaches was to use polymeric microporous support membranes as containers for the cell culture. The choice of such an approach was based on the fact that breast cancer cells exhibit an adherence preference for microporous surfaces and that involves culturing human breast cancer cells in a vertical tube configuration. Under these conditions, it was experimentally possible to regulate the access of oxygen to the cells and to the attachment site (2). The hydrophobic polymer used in this work, polyvinylidene difluoride (PVDF), is chemically and biologically inert. The tubes are charged with media and the cells are seeded into the tubes and permitted to sediment at the bottom. Cell cultures are examined microscopically by cutting the polymer tubes into sections and fixing and staining the attached cells on one or more sections. Cell colonies can be 'passed' for further culturing by inserting into fresh polymer tubes cut segments of tubes with attached cells. This novel experimental approach provided an opportunity to examine the adherence, attachment and migration characteristics of breast cancer cells. This information is critical for designing an experimental system to grow micrometastatic epithelial cells.

An *in vitro* culture approach such as this provides an opportunity to culture these cells in the absence of serum, thus making it possible to test the effect of serum in a dose dependent manner on the growth and adherence of human breast cancer cells. The standardization of the culture conditions was done using human breast cancer cells, both estrogen receptor positive, MCF-7 and T47D and estrogen receptor negative, MDA-MB-231. The non-tumorigenic cell line MCF-10 was used as a control.

RESULTS OBTAINED :

GROWTH OF EPITHELIAL CELLS ON NOVEL MATRICES

The novel cell culture approach encountered an unusual pattern of cell proliferation. For example MCF-7 cells in addition to the usual compact colonies form extended sparsely populated or even empty appearing extracellular matrix (ECM)

which in due course of time gets progressively filled with cells by proliferation or migration into the domain of these matrix structures and because of their appearance have termed it gauze matrices.

This gauze matrix formation is a novel pattern of cell growth which is partly dependent on the attachment membrane and has been instrumental in inducing novel pattern of cell growth and migration. Gauze matrix formation is principally on the vertical walls of the polymer tube. The migrating cells leave behind a trail of translucent material attached firmly to the surface of the microporous membrane. Accumulation of these trails forms a matrix and the formation of the gauze matrices is an ongoing process that is presumably modulated by secretory materials from the epitheliai cells as the pattern, architecture and morphology may be cell specific.

The human breast cancer cell line, MCF-7, forms gauze like matrices. Compact colonies develop within forty eight hours and begin to die off within a week. Cells within gauze matrices are maintained in the same tube in a viable state for at least twenty eight days in the absence of fetal bovine serum in the culture media.

Peripheral blood lymphocytes from human blood samples start dying within twenty four hours in the absence of serum addition in the culture media. Peripheral blood lymphocytes (PBL) mixed into a MCF-7 cell culture, begin to die within twenty four hours in the absence of FBS while the MCF-7 cells form gauze matrices and remain fully viable within them. The formation of gauze matrices offers novel approaches to selective cell culturing and purification of specific cell types from a mixture of cells of varied origin.

EFFECT OF SERUM AND RELATED FACTORS ON THE FORMATION OF GAUZE MATRICES

Fetal bovine serum (FBS), an important growth regulatory mix has profound effects on the growth and adhesion of various breast epithelial cell lines, the estrogen receptor positive MCF-7, T47D cell lines, and the estrogen receptor negative cell line, MDA-MB-231, and the non-tumorigenic cell line, MCF-10. In the absence of FBS in the media, the colonies formed by the four cell lines tested are characterized by a filamentous gauze-like extracellular matrix sparsely populated by round shaped cells. No cell-cell adhesion was observed. The gauze matrix colony is suggestive of phases of the metastatic process. Here the cells are separated and round out and reside only within the bounds of the matrix of their own creation.

The formation of compact colonies is stimulated by FBS in a dose dependent manner. In the absence of FBS, only gauze matrix colonies are formed. At 2 % FBS, both types of colonies, gauze matrix and compact co-exist. At FBS concentrations greater than 4 % only compact colonies are formed. This observation may have biological significance as well and serum factors may also circulate in the blood of

cancer patients and their concentration may influence the metastatic process.

The commonly used growth factors, insulin, epidermal growth factor, keratinocyte growth factor, bovine pituitary extract when added singly or in combination to the serum factor do not inhibit the formation of gauze matrix colonies nor do they stimulate the formation of compact colonies. The addition of serum to serum free cultures (SFM) after forty eight hours of incubation destroys the gauze matrix colonies. There is no appearance of compact colonies. Therefore, we postulate that the gauze matrix colony and the compact colony evolve to distinct entities that are different not only morphologically but may be different at the biochemical and signal transduction level.

FIGURE LEGENDS - SPECIFIC AIM #1

Figure 1 : Design of the cell culture system

Figure 2: MCF-7 cell culture; compact colony

Figure 3: MCF-7 cell culture; gauze matrix colony

Figure 4: MCF-7 cell culture; gauze matrix genesis (A)

Figure 5: MCF-7 cell culture; gauze matrix genesis (B)

Figure 6: Bone marrow cell culture

Figure 7: Bone marrow cell culture: gauze matrix colony

Figure 8: MCF-7 compact colony in 6% FBS

Figure 9: Gauze matrix colony of MCF-7 in SFM

Figure 10: Gauze matrix colony of MCF-7 in SFM

Figure 11: Co-existence of compact and gauze matrix colonies of MCF-7 in 2 % FBS

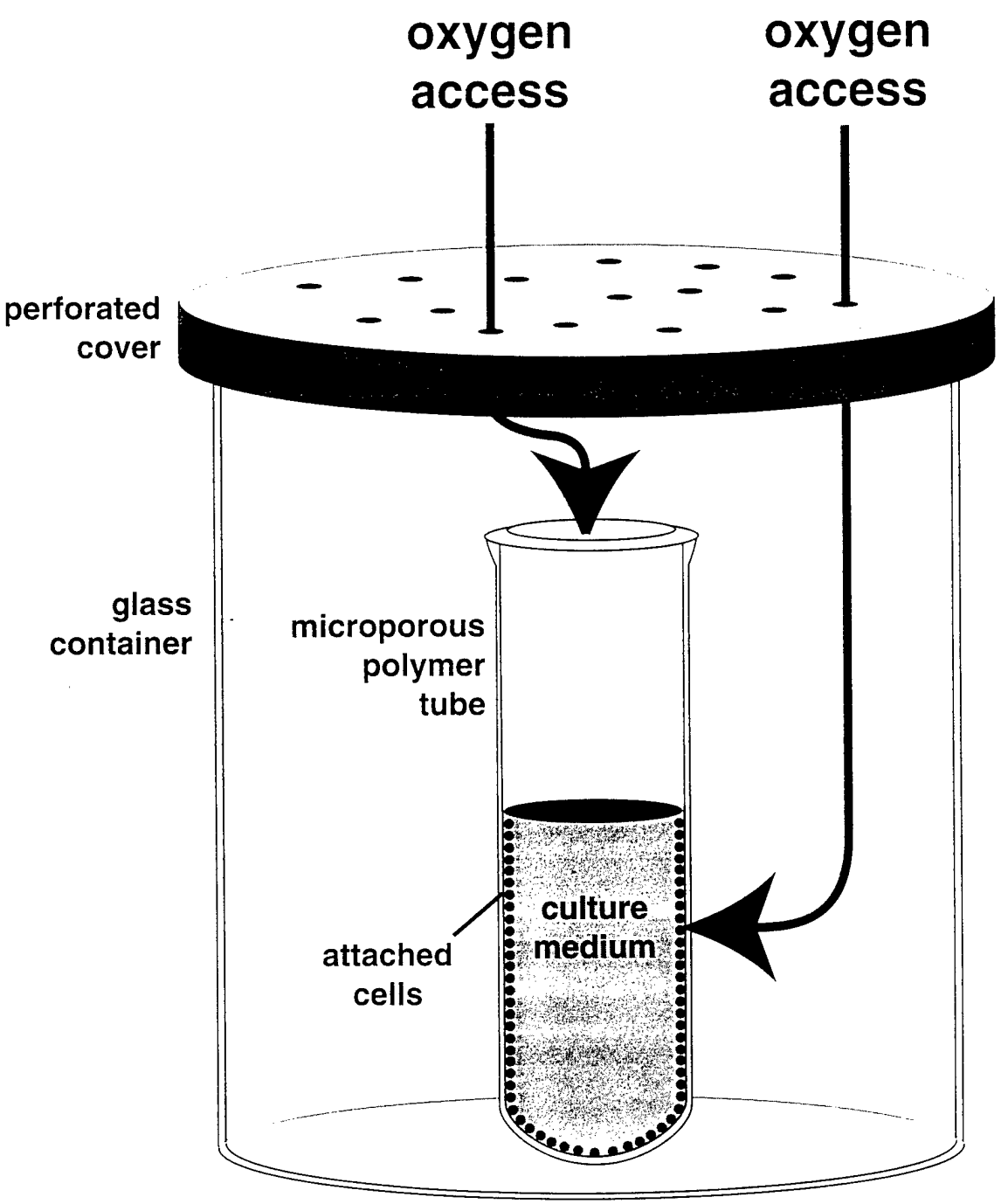


Figure 1 : Design of the cell culture system

Figure 2: MCF-7 cell culture; compact colony

Note absence of cells outside the confines of the gauze matrix. Note the separation of individual cells and the three-dimentional appearance of the matrix.

Figure 3: MCF-7 cell culture; gauze matrix colony

Figure 4: MCF-7 cell culture; gauze matrix genesis (A)

Figure 5: MCF-7 cell culture; gauze matrix genesis (B)

Figure 6: Bone marrow cell culture

Figure 7: Bone marrow cell culture: gauze matrix colony

Figure 8: MCF-7 compact colony in 6% FBS

Figure 9: Gauze matrix colony of MCF-7 in SFM

Figure 10: Gauze matrix colony of MCF-7 in SFM

Figure 11: Co-existence of compact and gauze matrix colonies of MCF-7 in 2 % FBS

SPECIFIC AIM II:

SIGNIFICANCE OF THE EXPRESSION OF MOLECULAR MARKERS p53 AND HSP27 IN PRIMARY HUMAN BREAST CANCER AND ITS ASSOCIATION WITH METASTATIC DISSEMINATION

No single marker fully characterizes human breast cancer. Several oncogenes p53, HER-2/neu, ras and myc have been implicated in human breast cancer but only serve to define subsets of tumor cell (3). We attempted to investigate the role of mutant p53 in primary human breast cancer and its association with metastatic dissemination. It is assumed that malignant transformation results from cumulative mutations either random or in a definite order which produce metabolic stress and adaptive proliferative advantage over non-transformed cells, markers of stress may be deregulated. Two markers of this metabolic stress are mutant p53 and the heat shock protein, HSP27. We examined the expression of mutant p53 and HSP27 in primary breast cancers by immunohistochemical analysis and RT-PCR. The expression of mutant p53 protein correlated well with transcription of the gene. Correlative studies were performed with prevalence of bone marrow micrometastasis (BMM) and expression of the markers of metabolic stress.

METHOD OF APPROACH:

Mutant p53 has been widely reported in human breast cancer and the mutation spans exons five through eight. There have been reports of conservation of the mutations and such studies have been extremely useful in identifying the lineage and origin of cells in the metastatic lesions. In order to establish the association of the expression of p53 and HSP27 in primary cancer, we analyzed the expression of these two markers by immunohistochemistry in forty seven primary human breast tumors frozen in OCT. The expression of these two markers was correlated to the metastatic dissemination which was determined by the prevalence of bone marrow micrometastases or positive lymph node involvement.

Immunohistochemical staining procedures were standard and have been previously described (3). Briefly the fixed sections are brought to room temperature and blocked with non-specific proteins. The primary antibody for p53 detection was Ab-6 that was obtained from Oncogene Science and used at a dilution of 1: 100. The HSP27 antibody, clone G3.1 was obtained from Neomarkers (Fremont, California) and

used at a dilution of 1: 250. After extensive washing of the primary antibody secondary antibody is applied followed by avidin-biotin immunoperoxidase staining with diaminobenzidine as the chromogen. The slides are counterstained by hematoxylin.

For RNA and PCR amplification, total RNA is isolated by RNAzol (Biotecx, Texas). The slides are overlayered with five hundred microliters of RNAzol and incubated on ice for fifteen minutes. The solution is carefully collected and transferred into an eppendorf tube and the slide further washed with an additional five hundred microliters of RNAzol to ensure complete transfer. 0.2 ml of chloroform is added, incubated for fifteen minutes at 4^0 C, vortexed, and the mixture centrifuged at 12000 X g for fifteen minutes. The RNA collected from the aqueous phase is precipitated by an equal volume of isopropanol. The RNA is dissolved in nucleic acid free media, incubated with RQ DNase (0.1 U/microliter) and RNAsin (2U/ microliter) and then purified by phenol/chloroform extraction and reprecipitated by two volumes of ethanol. The purified RNA is used for cDNA synthesis. First strand cDNA synthesis and subsequent amplification is done using a kit from Perkin-Elmer. The advantage of this kit is that cDNA synthesis and amplification of several genes is done in a single tube. PCR amplification is done at 95^0 C for five minutes at step 1, Step 2, 95^0 C for one minute, Step 3, 55^0 C for forty five seconds, Step 4, 72^0 C for one minute, with a cycling of Step 4 to Step 2 for forty five cycles and then further elongation carried out for ten minutes and finally soaked at 4^0 C. The amplified products were analyzed by horizontal agarose gel electrophoresis. The primers used to amplify exons four through ten for p53 were

p53 sense: 5' GGGACAGCCAAGTCTGTGACT 3',

p53 antisense: 5' CCTGGGCATCCTTGAGTT 3'

RESULTS OBTAINED

Mutant p53 was expressed in seven of the forty seven primary tumors analyzed and the expression was found to be both intraductal and infiltrating. Expression was verified by both techniques that employed immunohistochemistry and RT-PCR. All the tumors that showed high level of expression by immunohistochemistry showed amplification for mutant p53. There was complete concordance between the two techniques. p53 mutation was characterized and confirmed by sequence analysis. Expression of mutant p53 was correlated with clinical parameters that related to prevalence of bone marrow micrometastasis and lymph node metastasis

Four of the seven p53 positive tumors (57%) were also positive for bone marrow micrometastasis and six of the forty p53 negative tumors were positive for bone marrow micrometastasis (15 %). This difference is statistically significant at $p = 0.02$. Three of the seven p53 positive tumors had positive lymph node metastases. Expression of HSP27 was localized in intraductal and infiltrating tissues at a frequency of thirty five percent. Expression of HSP27 was not correlated with either prevalence

of bone marrow micrometastases, expression of mutant p53 or lymph node metastases (4). Representative expression of p53 and HSP27 is presented in figures 12-17.

These results suggest that expression of mutant p53 is associated with the propensity of metastasis since the tumors that expressed p53 was associated with a greater frequency with either lymph node metastasis or prevalence of bone marrow micrometastasis. Such an association was not observed with the heat shock protein HSP27. Thus, in future characterization of the micrometastatic cells present in the bone marrow, expression of mutant p53 can be used as a marker that could presumably define an aggressive subset of breast cancer cells.

We chose two stress related markers, one p53 that is related to genetic and metabolic stress and is essential guardian of the cell cycle and the other HSP27 generally induced as a result of temperature stress. Within the limitations of our study expression of HSP27 was not found to have a correlative significance with metastasis .

FIGURE LEGENDS - SPECIFIC AIM #2

Figure 12: Expression of p53, infiltrating

Figure 13: Expression of p53, intraductal

Figure 14: Expression of p53, infiltrating

Figure 15 : Expression of HSP2, infiltrating

Figure 16: Expression of HSP27, intraductal

Figure 17: RT-PCR analysis of p53 positive and negative tumors

Figure 18: RT-PCR analysis of p53 negative tumors for actin expression

Figure 19: Representative sequence analysis for p53 mutation in positive tumor

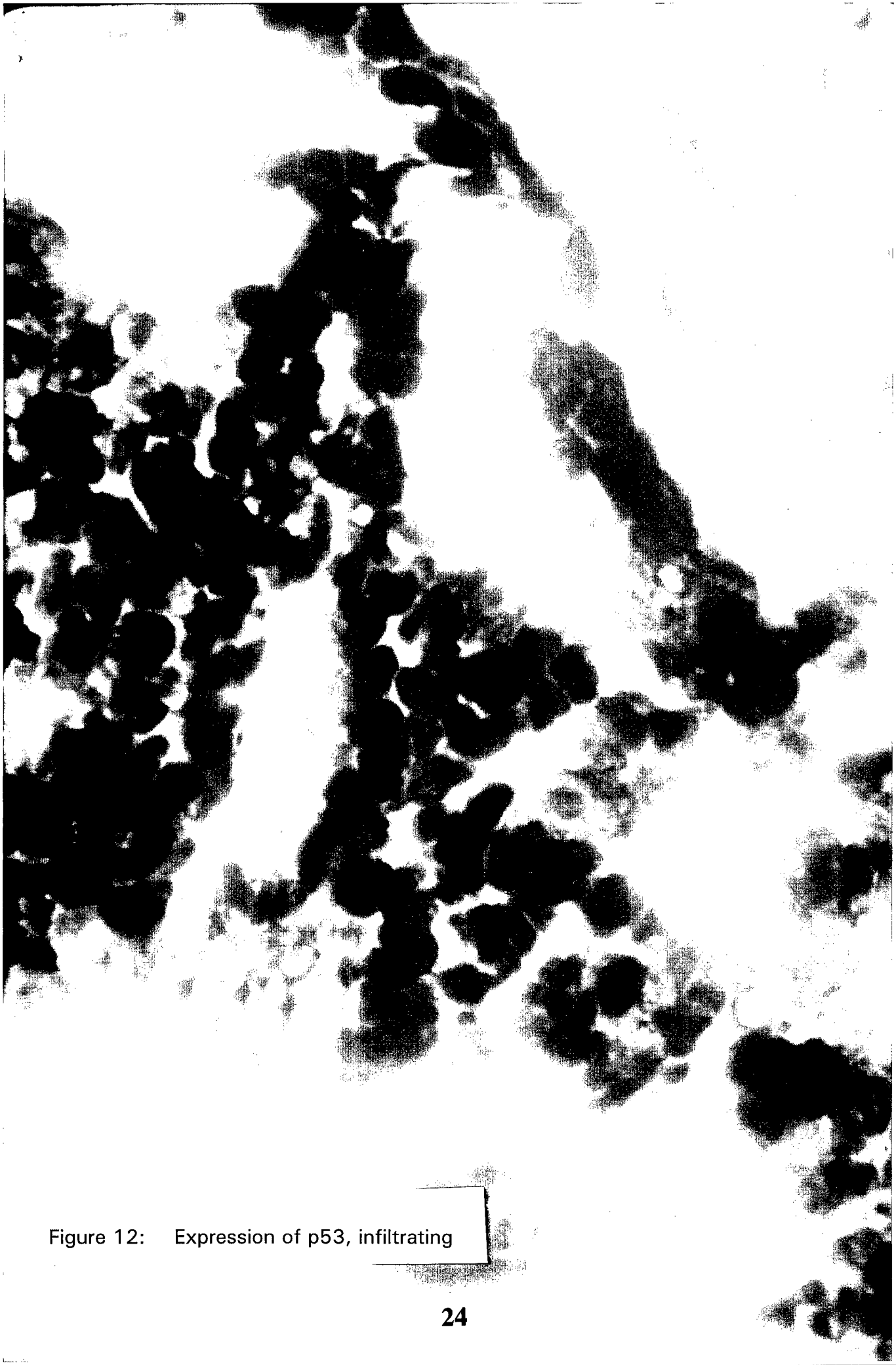


Figure 12: Expression of p53, infiltrating

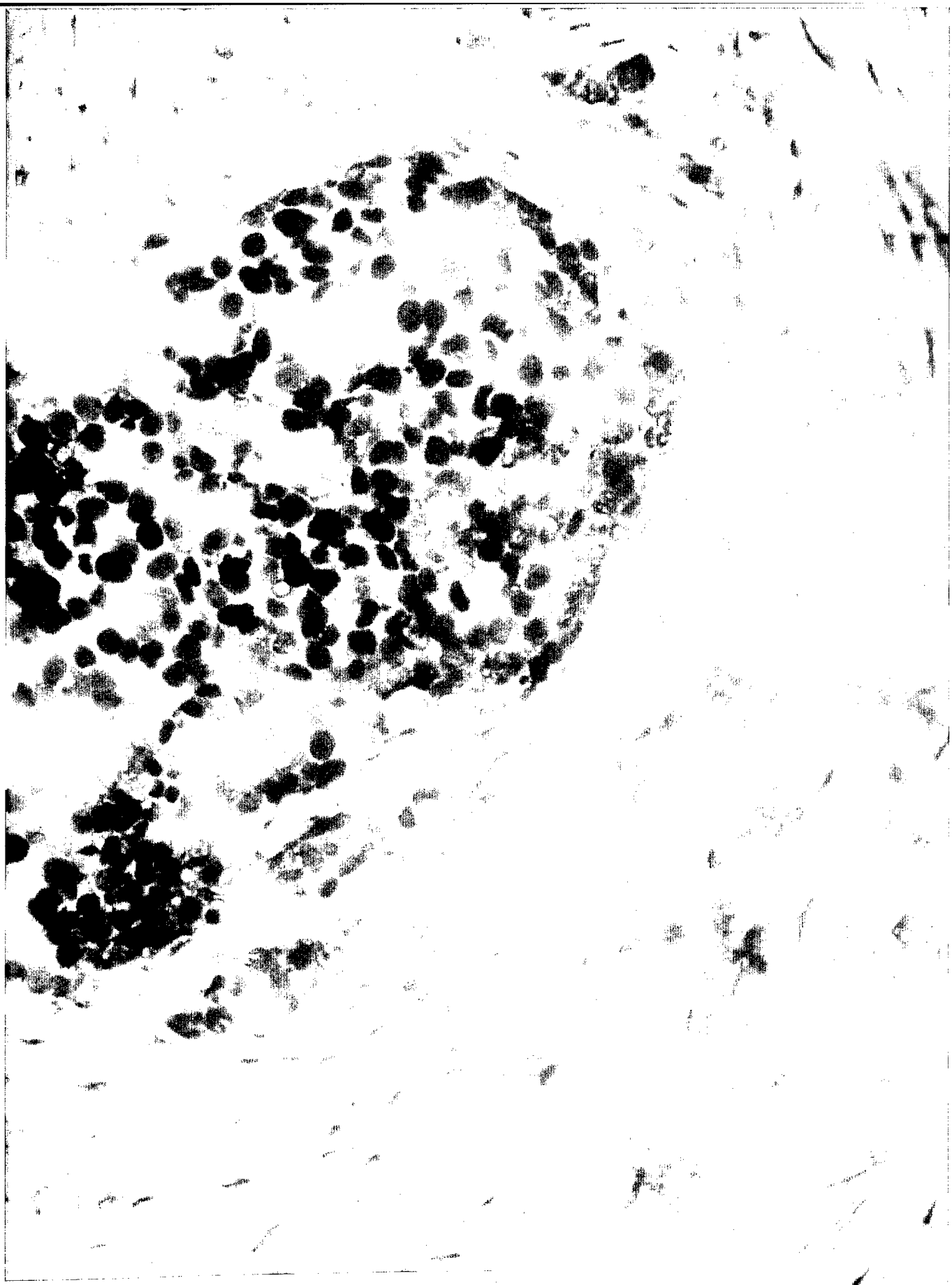


Figure 13: Expression of p53, intraductal

Figure 14: Expression of p53, infiltrating

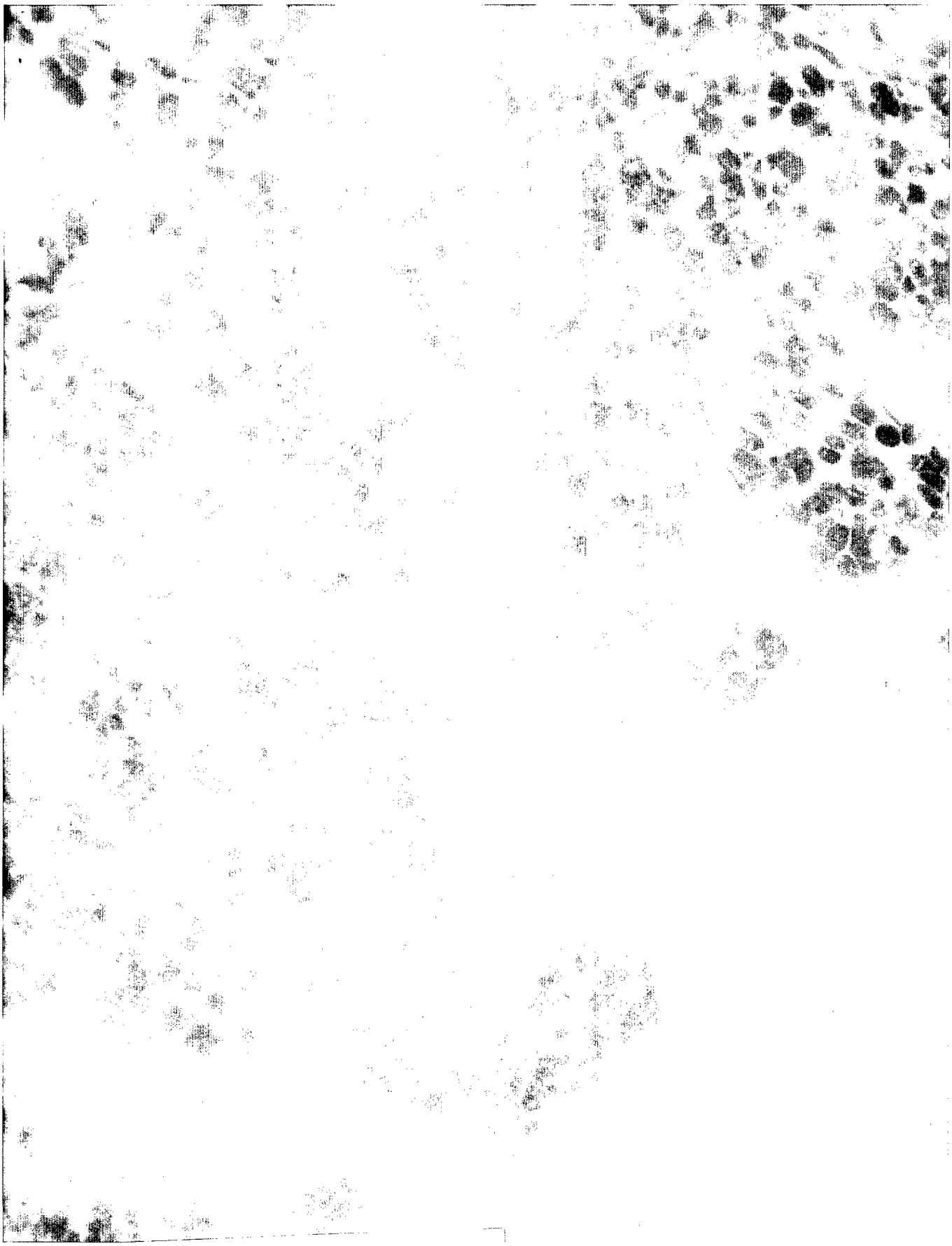


Figure 15 : Expression of HSP2, infiltrating

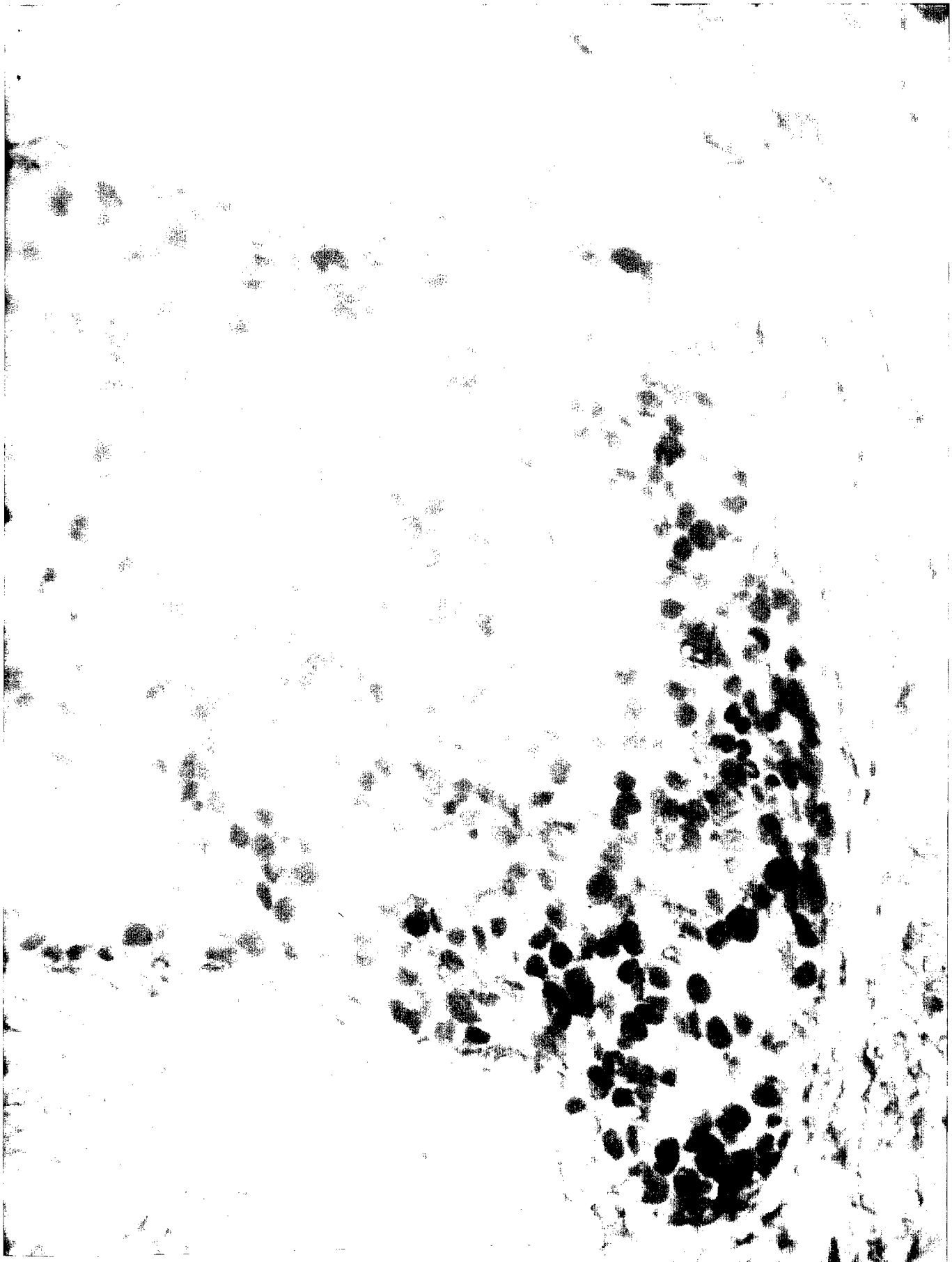
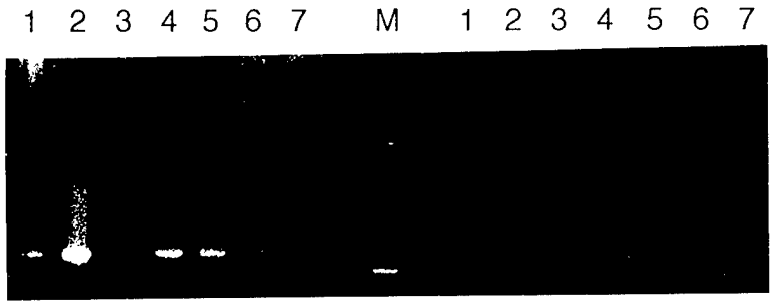


Figure 16: Expression of HSP27, intraductal

AMPLIFICATION OF MUTANT p53 by RT-PCR



p53 +ve tumors p53 -ve tumors

Figure 17: RT-PCR analysis of p53 positive and negative tumors

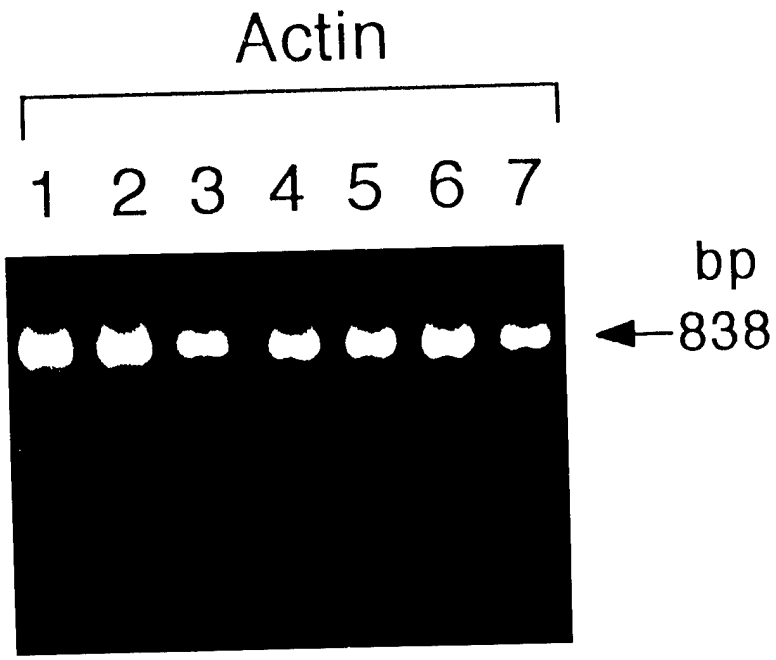
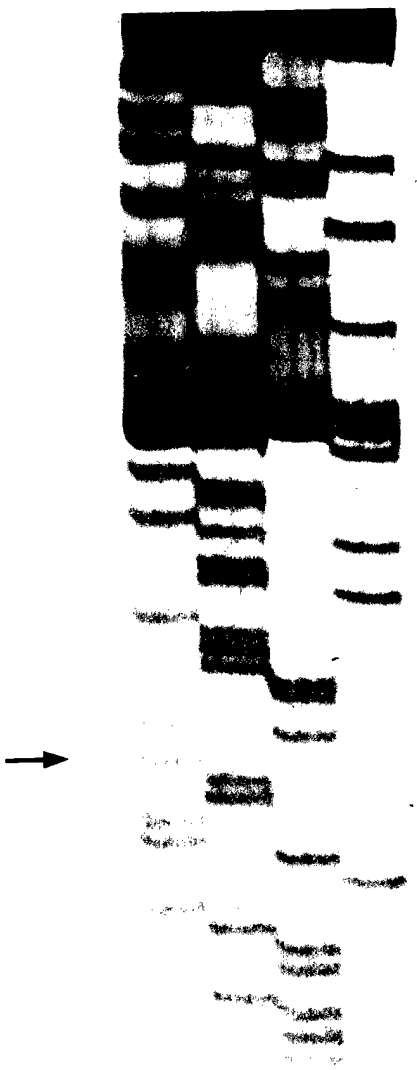


Figure 18: RT-PCR analysis of p53 negative tumors for actin expression



A C G T

Point mutation in exon 7 of p53 tumor DNA

CGG → CAG codon 248

(Arg) (Gln)

Figure 19: Representative sequence analysis for p53 mutation in positive tumor

CONCLUSIONS DERIVED FROM THE STUDY

1. We have succeeded in developing a novel cell culture method utilizing microporous PVDF membrane that can be used to selectively facilitate the growth of epithelial cells. The utility of this technique in isolating and culturing epithelial cells from the bone marrow needs to be examined in future experiments

2. Expression of mutant p53 can be used as a molecular marker to characterize micrometastatic epithelial cells since tumors that expressed mutant p53 were associated with a greater frequency with either lymph node metastasis or bone marrow micrometastasis .

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