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Expression of Heat Shock Protein 70 and c-myc Protein in Human  
Breast Cancer : An Immunohistochemical Study

(ヒト乳癌における熱ショック蛋白質70と c-myc 蛋白質の発現に  
関する研究)

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EXPRESSION OF HEAT SHOCK PROTEIN 70 AND c-myc PROTEIN IN HUMAN  
BREAST CANCER: AN IMMUNOHISTOCHEMICAL STUDY

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## ABSTRACT

The major heat shock protein, HSP70, protects cells from a variety of stressful stimuli, while c-myc protein allegedly stimulates expression of HSP70 by transacting on the HSP70 promoter. This study was aimed at correlating expression of HSP70 with that of c-myc protein in benign and malignant breast lesions. For this purpose, the indirect immunoperoxidase and immunoblotting techniques using monoclonal antibodies were employed. Fresh frozen sections were prepared from five fibroadenomas and 59 breast carcinomas. Immunohistochemically, both proteins were localized in the nuclei and/or cytoplasm of neoplastic and nonneoplastic epithelial cells. Expression of HSP70 and c-myc protein was comparable in malignant cells of 37 (63%) carcinomas. In 17 (29%) carcinomas, c-myc protein expression predominated over HSP70 while in 5 (8%) carcinomas HSP70 was predominant. All five fibroadenomas and most nonneoplastic epithelial cells adjacent to cancer showed strong reactivities of both proteins. Immunoblot analysis for HSP70 revealed a clear single band in the extract of tumors with strong HSP70 staining, but no or faint bands were seen in the extract of immunohistochemically HSP70-negative carcinomas. The current study, for the first time, demonstrated the expression of HSP70 in human cancer cells in vivo. The discrepancy in expression of both proteins in a certain percentage of breast carcinomas suggests the presence of mechanisms of HSP70 production not involving the c-myc protein-triggered promoter pathway.

## INTRODUCTION

Exposure of cells to environmental stress induces a series of heat shock proteins (HSPs). These stresses include nonphysiologic temperature<sup>1-10)</sup>, exposure to drugs<sup>1)</sup>, induction by mitotic reagents<sup>10-12)</sup>, and starvation of glucose<sup>9)</sup> and serum<sup>13)</sup>. HSPs are known to be encoded by members of a multigene family<sup>14)</sup>. The predominant and highly conserved form of HSPs is HSP70 of approximately 70 kDa in molecular size<sup>15)</sup>. In most mammalian cells, there are two major forms of HSP70. A constitutive form is 73 kDa while 72 kDa protein is heat-inducible and is also produced by exposure to nonphysiologic stresses<sup>5)</sup>. These two proteins are encoded by different genes<sup>1)</sup>, whereas the 73 and 72 kDa proteins are chemically and immunologically similar<sup>2,3,5,8)</sup>. HSP70s are known to be distributed in both the cytoplasm and nuclei of cultured cells, and are allegedly concentrated in nuclei, particularly in nucleoli by heat shock<sup>3,4,6)</sup>. Following return to heat shock-treated cells to normal growth temperature, both the synthesis of 72 kDa stress protein and its nucleolar staining diminish<sup>3)</sup>. The association of HSP70 with the damaged cytoskeletal structure in the cytoplasm has also been documented<sup>16-18)</sup>. HSP70s protect cells from stressful environmental stimuli although the exact mechanisms of protection are not clear at present<sup>15)</sup>. HSP-70-like proteins are also known to be crucial for normal cell growth<sup>7)</sup>.

The c-myc oncogene and oncoprotein show a high degree of evolutionary conservation<sup>19)</sup>. The oncogene product is expressed in nuclei in certain phases of cell cycle and cell

differentiation<sup>20-23</sup>). Recently, Ariga *et al.*<sup>24</sup>) showed that c-myc protein is a sequence-specific factor involved both in initiation of DNA replication and in regulation of RNA transcription. The product of a rearranged mouse c-myc gene is capable of stimulating expression of chimeric genes containing a *Drosophila* HSP70 promoter region and its 5'-flanking sequence<sup>25</sup>). The c-myc protein also stimulates to elevate the level of appropriately initiated protein expression from the human HSP70 promoter<sup>26</sup>), and heat shock also increases the rate of c-myc protein synthesis<sup>27</sup>). So far, amplification and enhanced expression of c-myc oncogene have been demonstrated in human breast carcinoma tissues and cell lines<sup>28-32</sup>).

Our current study was aimed at elucidating the expression of HSP70 and the correlation of HSP70 localization with c-myc protein localization in benign and malignant human breast tumors. Analyses were performed by immunohistochemical and immunoblotting techniques using monoclonal antibodies.

## MATERIALS AND METHODS

Sixty-four surgically resected breast tumors (59 carcinomas and 5 fibroadenomas) were immediately frozen after embedding in O.C.T. compound (Miles Inc., Elkhart, IN, USA). Their histologic types were determined on the basis of the criteria proposed by the Japan Mammary Cancer Society<sup>33)</sup>. The carcinomas were further classified into three groups by nuclear pleomorphism after Elston et al<sup>34)</sup>, as shown in the footnote of Table 2. Fresh frozen sections, 6  $\mu$ m thick, were cut and fixed in a 1:1 mixture of ethanol and acetone at 4<sup>0</sup>C for 10 minutes and in 10% formalin at room temperature for 30 minutes for c-myc protein.

Immunostaining was performed using the indirect immunoperoxidase technique. A mouse monoclonal HSP70 antibody, RPN1197, from Amersham International (Amersham Bucks, U.K.), specifically recognizes 72 kDa-HSP70 without reactivity to any other kinds of HSPs and related proteins<sup>8)</sup>. A mouse c-myc monoclonal antibody, OM-11-906, from Cambridge Research Biochemicals (Cambridge, U.K.), reacts with c-myc proteins from human (p62<sup>c-myc</sup>) and mouse (p64<sup>c-myc</sup>) and (p66<sup>c-myc</sup>), but not with chicken (p110<sup>c-myc</sup>) and N-myc protein<sup>35)</sup>. Horseradish peroxidase-labeled antimouse Ig G were from Amersham International. Peroxidase activity was located by dipping sections in 0.05 M Tris-HCl solution, pH 7.6, containing 25 mg% diaminobenzidine and 0.003% hydrogen peroxide for 5 minutes. Sections were counterstained with 5% methyl green in 0.1 M Veronal acetate buffer, pH 4.0. Normal mouse serum at dilution of 1:100 was used as a negative staining control. Antigen localization patterns were evaluated as either nuclear predominance, cytoplasmic predominance or both nuclear and

cytoplasmic localization. The degree of antigen expression was scored from (-) to (++), according to the number of cells that were stained positively in the nuclei and/or cytoplasm, namely, -: negative or equivocal, +: less than 50% of cells positive, and ++: more than 50% of cells positive.

Portions of the fresh nonnecrotic cancer tissue were stored at  $-70^{\circ}\text{C}$  for immunoblot analysis. Representative tissue samples (0.1 g) were homogenized with 0.9 ml of 20 mM Tris-HCl buffer, pH 7.6, containing 1 mM ethylenediamine tetraacetic acid (EDTA) and 50 mM phenylmethyl sulfonylfluoride. The homogenates were mixed with the same volume of 120 mM Tris-HCl, pH 6.8, containing 4% sodium dodecyl sulphate (SDS), 40% glycerol, 10 mM EDTA and 5% 2-mercaptoethanol in Eppendorf's microcentrifuge tubes and then placed in boiling water for 5 minutes. After centrifugation at 14,000 rpm for 10 minutes, 5  $\mu\text{l}$  of the supernatant were applied to SDS-polyacrylamide gel electrophoresis according to the method of Laemmli<sup>36)</sup>. Proteins were blotted to a nitrocellulose filter (Schleicher & Schuell, Dassel, W. Germany) by the method of Towbin et al.<sup>37)</sup> and immunostained by the indirect immunoperoxidase method.

## RESULTS

HSP70 and *c-myc* protein were localized in the nuclei and/or cytoplasm (Figs. 1-5). Control sections incubated with normal mouse serum were totally unstained. All 5 fibroadenomas, as well as most nonneoplastic mammary epithelial cells adjacent to cancer, showed strong positivity (++) for both substances, mainly in the nuclei. HSP70 immunoreactivity was demonstrated in 44 (75%) carcinomas; (++) in 32 (54%) tumors and (+) in 12 (20%) (Table 1). Fifteen (25%) carcinomas were negative or equivocal for HSP70. In 7 (12%) carcinomas the cytoplasm was the main site of HSP70 localization (Table 2). Thirty-one (53%) tumors expressed HSP70 in both the nuclei and cytoplasm. Nuclear HSP70 staining was predominant in 6 (10%). Heterogeneous (mosaic) staining patterns of HSP70, in which positivity varied from cell to cell, were demonstrated in 11 (19%) carcinomas (Fig. 5). When the tumors were graded according to the degree of nuclear atypia, grade 3 atypia was seen in 5 carcinomas, grade 2 atypia in 20, and grade 1 in 34 (Table 2). Characteristically, tumors with HSP70 localized mainly in the nuclei tend to accompany less nuclear atypia (Fig. 1) and four of five tumors with grade 3 atypia showed negative staining for HSP70 (Fig. 3). A similar tendency was observed when the pattern of *c-myc* protein expression was correlated with nuclear atypia in cancer cells (Table 3). The carcinomas were histologically classified into 52 invasive ductal carcinomas (7 papillotubular carcinomas, 13 solid-tubular carcinomas and 32 scirrhous carcinomas), 3 mucinous carcinomas, 2 medullary carcinomas and 2 invasive lobular carcinomas. No significant correlation was seen between the



expression pattern of HSP70 or c-myc protein and the histologic typing.

c-myc protein immunoreactivity was (++) in 40 (68%) carcinomas, (+) in 12 (20%), and (-) in 7 (12%). HSP70 was exclusively expressed in epithelial cells while c-myc protein was demonstrated in both epithelial and stromal cells. Staining intensity of HSP70 in cancer cells was often weaker than that in nonneoplastic epithelial cells adjacent to the cancer tissue (Fig. 1). In 37 (63%) carcinomas, expression of HSP70 and c-myc protein was comparable, as shown in Figure 2 and Table 1. In 17 (29%) carcinomas, c-myc protein expression predominated over HSP70 (Figs. 1 and 3) while in 5 (8%) carcinomas HSP70 was predominant (Fig. 4).

Results of the immunoblot analysis using cancer extracts corresponded well with those of immunostaining. HSP70 immunoreactivity was visualized as a clear single band around the molecular weight of 70,000 dalton on the membrane filter (Fig. 6). The bands were not visible or faint in the extract of carcinomas immunohistochemically negative for HSP70 (Lanes 1 and 2). The extract of breast carcinomas strongly immunostained for HSP70 showed a dense single band of HSP70 (Lanes 3 and 4).

## DISCUSSION

HSPs consist of a protein family highly conserved during the phylogenetic development<sup>15)</sup>. HSP70, the main member of this family, is known to be crucially important for self-protection against a variety of stresses<sup>1-13)</sup> and for normal growth<sup>7)</sup>, and its expression is allegedly controlled by the c-myc protein transacting on the HSP70 promotor<sup>25,26)</sup>. By using a monoclonal antibody to human HSP70, we demonstrated immunoreactive HSP70 in frozen sections of human breast tissues, and the expression of HSP70 was compared with that of c-myc protein. Both HSP70 and c-myc protein were localized in the nuclei and/or cytoplasm of benign and malignant epithelial cells. Specificity of immunostaining was confirmed by negative staining with normal mouse serum and by the immunoblotting technique for HSP70. The discordant expression of both substances were noted in a considerable percentage of cancer tissues, with c-myc protein expression being predominant in 29% of carcinomas and HSP70 predominant in 8% of carcinomas. These data suggest the presence of mechanisms of HSP70 expression not involving the c-myc protein-triggered promotor pathway in human breast carcinomas. A heterogenous staining pattern of HSP70 observed in 19% of tumors suggested varied levels of expression of this protective substance in individual cancer cells within a single tumor tissue.

No studies have so far been focused on the expression of HSP70 in human cancer cells in vivo. To our knowledge, only two reports have described localization of immunoreactive HSP70 in nonneoplastic human tissues; one in atherosclerotic lesions<sup>38)</sup>

and another in alcoholic liver disease<sup>18)</sup>. Since nuclear and nucleolar localization of HSP70 has been observed under the stressful (heat-shocked) conditions in cultured cells<sup>3,4,6)</sup>, HSP70 might function as an important cell protector within the nucleus. In fact, HSP70 has a capacity to bind to nucleotides<sup>3,4)</sup>. The physiologic roles of HSP70 in the cytoplasm have also been presented<sup>16-18)</sup>.

In the current study, nuclear localization of HSP70 was often seen in benign mammary epithelial cells and in breast carcinomas with low nuclear atypia. In contrast, breast carcinomas with high nuclear atypia frequently lacked HSP70 expression. These findings suggest that the pattern of HSP70 localization might reflect the heat shock-resistant status of cancer tissues. In this sense, the susceptibility to thermotherapy<sup>39,40)</sup> is a good target of study in relation to the HSP70 expression and its intracellular localization pattern in breast cancer. Although it has been shown that thermotherapy is effective irrespective of the histologic types of cancer<sup>41)</sup>, no detailed examination has been performed in view of the relationship between the HSP70 status and thermoresistance of cancer. It is supposed that breast cancer with high atypia is susceptible to thermotherapy because of its frequent lack of HSP70 expression. This hypothesis is now being our target of clinical investigation.

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Table 1. Immunohistochemical reactivity of HSP70 and c-myc protein in 59 breast carcinomas

		HSP70			Total
		-	+	++	
c-myc protein	-	5	0	2	7
	+	4	5	3	12
	++	6	7	27	40
Total		15	12	32	59

-: negative or equivocal

+: less than 50% of cancer cells positive

++: more than 50% of cancer cells positive

cf. All five fibroadenomas showed intensive reactivity (++) of both substances.

Table 2. Patterns of HSP70 immunostaining in breast carcinomas in relation to nuclear atypia

Pattern of HSP70 staining	Nuclear atypia <sup>a)</sup>			Total
	grade 1	grade 2	grade 3	
Nuclear predominance	5	1	0	6
Cytoplasmic predominance	3	4	0	7
Both nuclear & cytoplasmic	22	8	1	31
Negative	4	7	4	15
Total	34	20	5	59

<sup>a)</sup> Grade of nuclear atypia was determined according to the following criteria proposed by Elston et al.<sup>34)</sup>

grade 1: Little variation seen in nuclear size

grade 2: A moderate degree of variation seen in nuclear size

grade 3: Marked variation seen in nuclear size and shape

Table 3. Patterns of c-myc protein immunostaining in breast carcinomas in relation to nuclear atypia

Pattern of c-myc protein staining	Nuclear atypia			Total
	grade 1	grade 2	grade 3	
Nuclear predominance	19	2	1	22
Cytoplasmic predominance	2	1	1	4
Both nuclear & cytoplasmic	11	14	1	26
Negative	2	3	2	7
Total	34	20	5	59

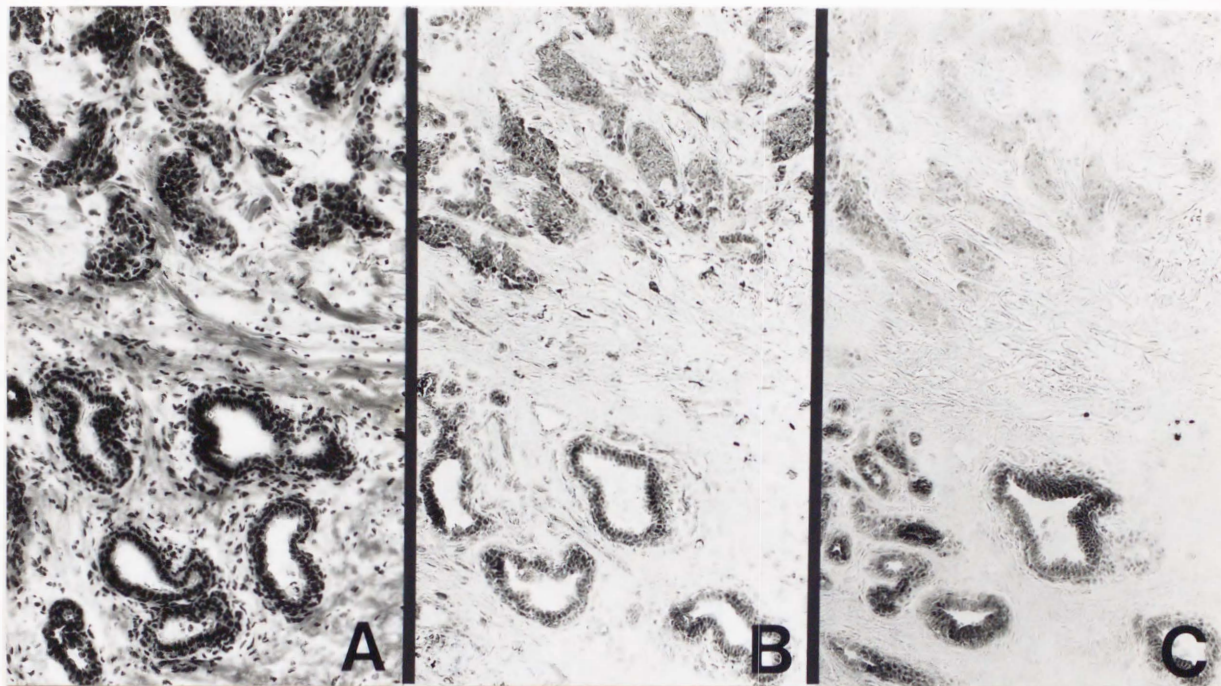


Figure 1. Comparative immunostaining of *c-myc* protein (B:++) and HSP70 (C:+) in a grade 1 scirrhous carcinoma and adjacent noncancer breast tissue (x100, A:H&E). Both substances are localized mainly in nuclei. Benign ductal cells show strong nuclear immunoreactivity for both substances. The cancer cells show weaker staining of HSP70 when compared with the normal mammary lobule. *c-myc* protein is positive also in nuclei of mesenchymal cells.

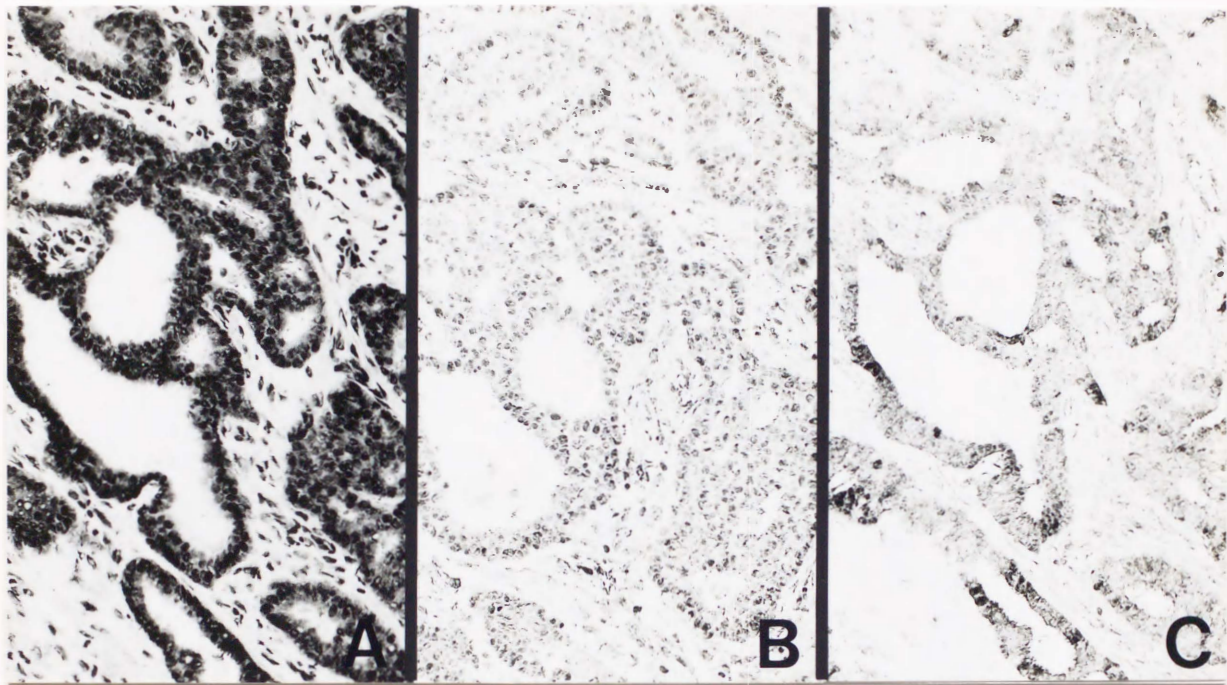


Figure 2. Comparative immunostaining of *c-myc* protein (B:++) and HSP70 (C:++) in a grade 1 scirrhous carcinoma (x150, A:H&E). Note nuclear and mesenchymal staining of *c-myc* protein and cytoplasmic staining of HSP70.

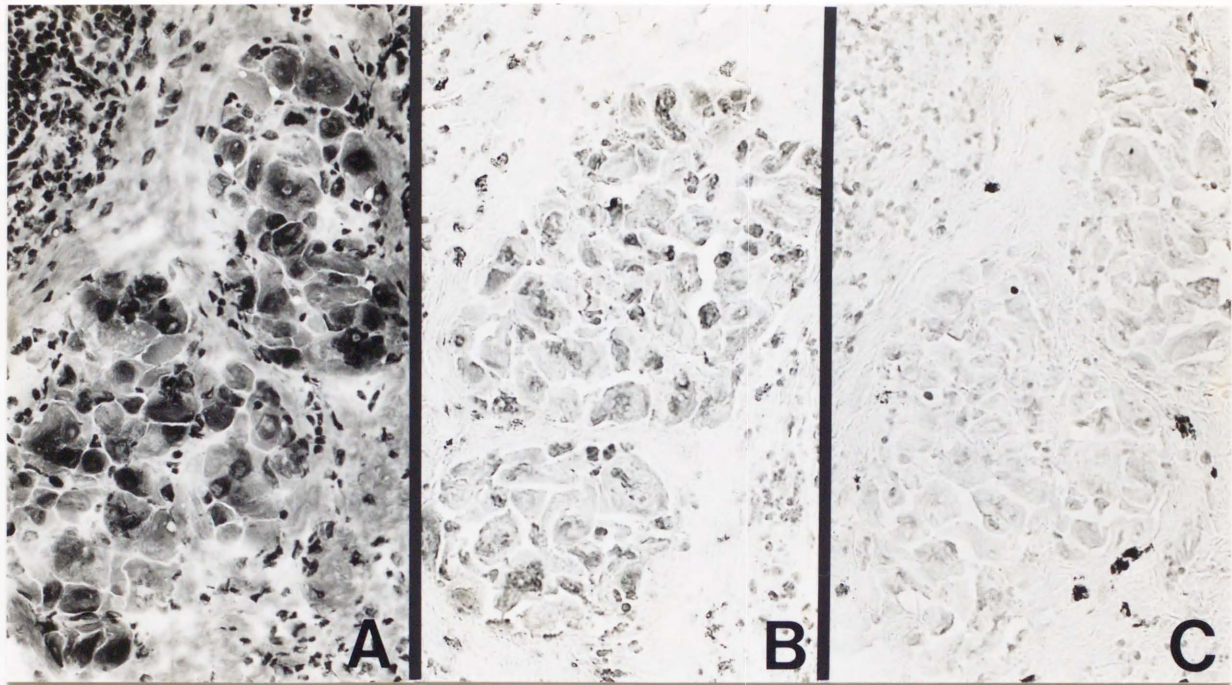


Figure 3. Comparative immunostaining of *c-myc* protein (B:++) and HSP70 (C:-) in a grade 3 papillotubular carcinoma (x200, A:H&E). HSP70 staining is negligible in contrast to active nuclear expression of *c-myc* protein. Endogenous peroxidase activity is seen in granulocytes infiltration around the tumor nests.

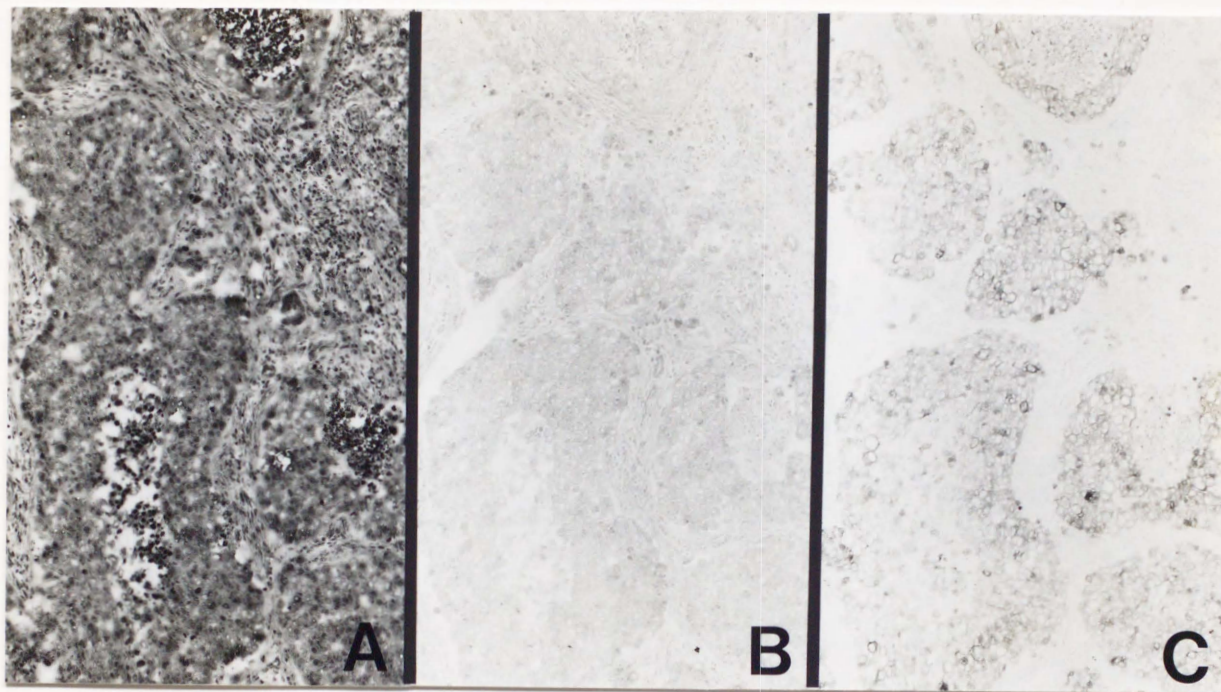


Figure 4. Comparative immunostaining of c-myc protein (B:-) and HSP70 (C:++) in a grade 2 scirrhous carcinoma (x75, A:H&E). In this cancer, HSP70 is predominant over equivocally stained c-myc protein. HSP70 is observed in the cytoplasm.



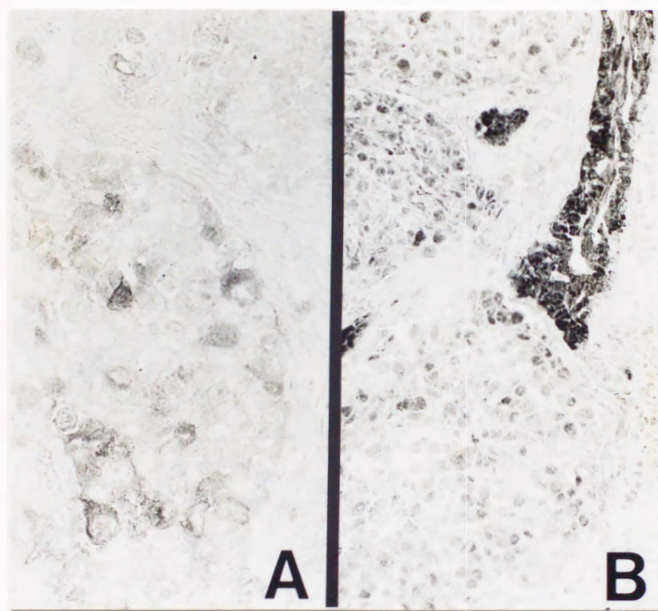


Figure 5. Heterogenous (mosaic) expression of HSP70 in two grade 2 scirrhous carcinoma. A proportion of cells are positive for HSP70 in the cytoplasm (A, x300) or in nuclei (B, x150). Noncancerous ducts are strongly immunoreactive for HSP70 (B).

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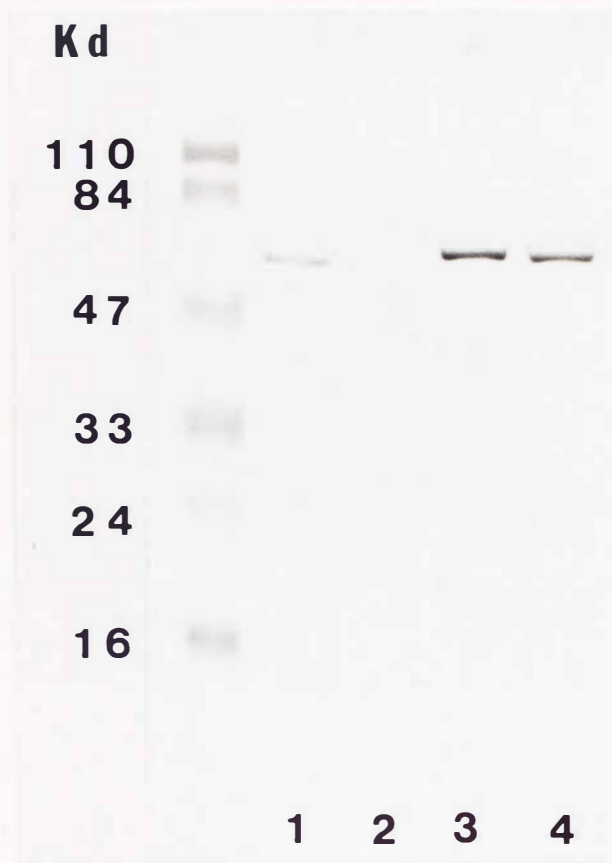


Figure 6. Immunoblot analysis of breast carcinoma extracts for HSP70. Lanes 1 & 2: carcinomas immunohistochemically negative or equivocal for both HSP70 and *c-myc* protein. Lanes 3 & 4: carcinomas strongly positive by immunostaining for both HSP70 and *c-myc* protein. Results of immunoblotting well correspond to those of immunohistochemical staining. The weak band seen in lane 1 might reflect the admixture of noncancerous ducts in the cancer tissue as shown in Figure 5B.



inches 1 2 3 4 5 6 7 8  
cm 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

# Kodak Color Control Patches

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# Kodak Gray Scale



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**A** 1 2 3 4 5 6 **M** 8 9 10 11 12 13 14 15 **B** 17 18 19

