Jpn. J. Cancer Res. 88, 273–280, March 1997

Immunoreaction at 43 kDa with Anti-ubiquitin Antibody in Breast Neoplasms

Keiichi Iwaya,[1,2] Hideki Nishibori,[1] Takuya Osada,[1] Yoshihiro Matsuno,[1] Hitoshi Tsuda,[1] Seiji Sato,[3] Hiroaki Kono,[3] Takashi Fukutomi,[4] Minoru Suzuki,[2] Chikao Torikata,[2] Akihiro Iwamatsu[5] and Setsuo Hirohashi[1,6]

[1]Pathology Division, National Cancer Center Research Institute, [4]Department of Surgery, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104, [2]Department of Pathology, National Defense Medical College, 3-2 Namiki, Tokorozawa 359, [3]Kyowa Hakko Kogyo Co., Ltd., 600-1 Minamiissiki, Nagaizumi-cho, Suntoh-gun, Shizuoka 411 and [5]Kirin Brewery Co., Ltd., Central Laboratories for Key Technology, 1-13-5 Fukuura, Kanazawa-ku, Yokohama 236

Protein ubiquitination has been implicated in ATP-dependent protein turnover and normal cell proliferation. To investigate whether the ubiquitin-mediated system is functionally involved in the cancerous state, we examined changes in protein ubiquitination in 52 surgically resected primary breast tumors. Immunohistochemically, ubiquitin (Ub) was identified in the cytoplasm of cancer cells, which were stained more strongly than adjacent normal ductal epithelium. Corresponding immunoblot analysis of normal and neoplastic regions of human breast showed that the immunoreaction for Ub at about 43 kDa was increased in all of the tumors (100%), regardless of the clinical stage or histologic grade. This protein, which gave a single spot on two-dimensional gel electrophoresis, had partial amino acid sequences which were identical to those of actin family members. Our results suggest that ubiquitination of this 43-kDa protein may be involved in the carcinogenesis or biological characteristics of human breast neoplasms.

Key words: Ubiquitin — Actin — Breast neoplasm — Two-dimensional electrophoresis — Amino acid sequence

Cellular proteins are in a state of constant turnover in normal cells. The process is extensive and highly selective; specific proteins are degraded within cells at greatly different rates. Several distinct mechanisms are responsible for intracellular protein degradation, such as the lysosomal processes and the soluble ATP-dependent proteolytic pathways. Prominent among these is the ubiquitin-mediated proteasome-dependent proteolytic system.[1–3]

Ub is present in all eukaryotic cells and is one of the most highly conserved proteins known.[4] It exists in cells either freely or joined covalently to a variety of cytoplasmic, nuclear, and integral membrane proteins.[5,6] Recent biochemical and genetic evidence indicates that conjugation of Ub to intracellular proteins is essential for their selective degradation.[7,8] A non-proteolytic role has also been suggested for Ub, in which its reversible joining to an acceptor protein modulates the protein function. This may be the case for the ubiquitinated histones H2A and H2B present in chromatin.[9,10]

The Ub proteolytic system may also be involved in cell cycle progression, since it selectively degrades nuclear oncoproteins such as p53, N-*myc*, c-*myc*, c-*fos*, and E1A *in vitro*.[11] For example, normal p53 is a short-lived intracellular protein which appears to function as a tumor suppressor; however, stabilization of the protein has been associated with point mutation of the gene, which results in neoplastic transformation.[12] Changes in the regulation of degradation and an impaired turnover of nuclear oncoproteins *in vivo* would result in changes of the protein levels.

Recent immunohistochemical studies have demonstrated a strong immunoreaction for Ub in the majority of malignant tumors, and changes in the formation of the Ub complex with cellular proteins have been described in renal cell carcinoma.[13,14] However, the functional role of Ub attachment is not clear, and the target proteins have not yet been identified.

In order to identify the types of proteins targeted for ubiquitination, we examined differences in cellular proteins conjugated with Ub between regions of tumor tissue and normal breast tissue, using immunochemical and amino acid sequencing analyses.

MATERIALS AND METHODS

Patients We investigated a total of 52 primary breast tumors which had been surgically resected at the National Cancer Center Hospital, Tokyo, between 1991 and 1922. The tumors and their associated non-neoplastic

[6] To whom correspondence should be addressed.

Abbreviations: Ub, ubiquitin, 1-D, one-dimensional; 2-D, two-dimensional; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; DTT, dithiothreitol; PVDF, polyvinylidene difluoride membranes.

Table I. Clinicopathological Factors in 52 Breast Patients

Histologic type	
non invasive carcinoma	2
invasive ductal carcinoma	46
invasive lobular carcinoma	3
papilloma	1
Histologic grade	
grade 1	6
grade 2	18
grade 3	28
Clinical stage	
I	17
II	21
III	14
Total	52

tissues were divided into two parts: (1) formalin-fixed paraffin-embedded tissue for routine histopathological diagnosis and for immunostaining with anti-Ub polyclonal antibody, (2) fresh frozen tissue for 1-D and 2-D PAGE followed by immunoblotting or amino acid sequencing. The clinical stage of the cancer at the time of surgery was defined according to the TNM system. Histologic grading of the primary tumor was performed using a modification of the WHO classification system.[15] The clinicopathological data of the 52 patients are listed in Table I.

The 52 patients in the present study ranged in age from 31 to 79 years (mean, 54.4 years). All but one had undergone radical or modified radical mastectomy; the exception involved an excisional biopsy. Tumor sizes as measured from the largest cut section ranged from 1.3 to 10 cm.

Immunohistochemistry We used a polyclonal antibody from rabbits which had been given injections of bovine red cell Ub coupled with bovine serum albumin.[16] This antibody is known to react with human Ub. To determine whether the polyclonal antibody would react with both free and protein-conjugated Ub, Ub-lysozyme conjugates were prepared by incubating lysozyme and ATP with a Ub-protein ligation system consisting of E1, E2, and E3.[17] Immunoblot analysis showed that the anti-Ub antibody reacted with lysozyme-Ub conjugates (data not shown).

Immunohistochemical staining was performed according to the conventional method. Sections were deparaffinized in xylene, and rehydrated in a descending ethanol series, ending in water. They were then incubated for 30 min in 0.3% hydrogen peroxidase in methanol, and preincubated in 2% normal swine serum in PBS in order to abolish endogenous peroxidase activity and to diminish any non-specific antibody binding. The sections were incubated overnight at 4°C with the polyclonal antibody at a dilution of 1 : 1000, and then incubated for 30 min with biotinylated goat anti-rabbit immunoglobulin as a secondary antibody (Vector Laboratories Inc., Burlingame, CA) diluted 1 : 200. Subsequently, they were incubated for 30 min with avidin-biotinylated peroxidase complex using a Vectastain ABC Kit (Vector Labs.) diluted 1 : 100 in PBS. The peroxidase reaction was performed using 3,3′-diaminobenzidine tetrahydrochloridehydrogen peroxidase as a chromogen in Tris buffer at pH 7.6 for 5–10 min. The sections were counterstained with hematoxylin, dehydrated and mounted. Between each step, the slides were washed 3 times (5 min each) with PBS.

Immunoblot analysis Breast tumor and non-tumorous tissues were minced on ice using plastic surgical scissors. Approximately 200 mg of tissue was suspended in 1 ml of lysis buffer containing 1% (v/v) Triton X, 50 m*M* Tris buffer at pH 7.5, 1 m*M* phenylmethylsulfonyl fluoride, and 0.1 μg/ml aprotinin. The suspension was homogenized in a Polytron homogenizer, incubated for 30 min on ice, and then centrifuged for 30 min at 10,000*g* at 4°C. For 1-D PAGE, 25 μl of the resulting soluble fraction was mixed with the same amount of Laemmli solution (2% SDS, 10% glycerol, 100 m*M* DTT, 60 m*M* Tris buffer at pH 6.8, and 0.001% bromophenol blue), denatured for 5 min at 100°C, and then applied to 10% polyacrylamide gel. For 2-D PAGE, the soluble fraction was saturated with solid urea, mixed with the same volume of O'Farrell buffer (9.5 *M* urea, 2% (w/v) Nonidet P-40, 1.6% Ampholine pH 5–7, 0.4% Ampholine pH 3–10, and 5% β-mercaptoethanol) at room temperature, and then loaded on a Mini-PROTEAN II (Bio-Rad, Richmond, CA) electrophoresis apparatus. We used pH 5–7 immobilized gradient strips for the first dimension, and 10% polyacrylamide gel for the second dimension. After the 1-D or 2-D SDS-PAGE, the fractionated proteins were transferred onto PVDF (Immobilon-P, Millipore, Bedford, MA) in a transfer buffer consisting of 25 m*M* Tris buffer, 192 m*M* glycine, 20% methanol, and 0.1% SDS. The membranes were reacted with two antibodies against Ub: the above-mentioned polyclonal antibody, and a commercially available rabbit polyclonal antibody (Dako, Glostrup, Denmark). The process employed was as follows: (1) incubation for 3 h with a blocking buffer consisting of 5% skim milk (Difco, Detroit, MI) and 1% bovine serum albumin dissolved in PBS; (2) incubation overnight at 4°C with primary antibody; (3) washing 3 times with 0.1% Tween-20 in PBS; (4) incubation for 30 min with peroxidase-conjugated anti-rabbit IgG (DAKO) diluted 1 : 200 with PBS; (5) washing with 0.1% Tween-20 PBS 3 times; and (6) incubation with enhanced chemiluminescence reagent (ECL, Amersham, Buckinghamshire, U. K.) for color development and photography with Hyperfilm-ECL (Amersham).

Purification of the protein from 2-D gels Proteins were fractionated by 2-D PAGE using a PROTEAN II (Bio-Red, 180×165×10 mm) apparatus with the same sample preparation and composition as described above for the first and second gels.

After electrophoresis, the gel was stained with 0.3 *M* $CuCl_2$ for 5 min and washed with water 2 times (5 min each), and target protein spots were excised from several stained gels with a minimum of polyacrylamide. The proteins in the excised gel slices were electroeluted at 100 V for 4 h and then concentrated by a Centrilutor 100 (Amicon, Danvers, MA). After 10% SDS-PAGE, the protein was blotted on PVDF membrane (Immobilon-psq, Millipore) with Tris-ε-aminocaproic acid-methanol buffer. It was visualized as a single band by staining with 0.1% Ponceau S in 1% acetic acid for 5 min, and then the membrane was washed 3 times with water.

***In situ* reduction, and peptide mapping by reverse-phase HPLC** The PVDF-immobilized protein was reduced and *S*-carboxymethylated *in situ* prior to digestion.[18] *Achromobacter* protease I (0.5 pmol) digestion of the 43-kDa protein was performed in 90 m*M* Tris-HCl buffer (pH 9.0) containing 8% acetonitrile at 30°C for 16 h. Digested peptides were subjected to reverse-phase HPLC on a Wakosil-II 5C18 AR column (2.0×150 mm 300 Å, Wako Pure Chemicals, Osaka). The digestion solution was diluted with water to decrease the acetonitrile concentration before injection. Elution was carried out with a linear gradient of 2–50% Solvent B in 30 min at a flow rate of 0.25 ml/min using the following solutions: Solvent A, 0.05% trifluoroacetic acid in water; solvent B, 0.02% trifluoroacetic acid in 2-propanol: acetonitrile, 7 : 3 (v/v). The fractionated peptides were collected manually by monitoring the absorbance at 214 nm using a Hitachi L4000 detector.

Amino acid sequencing and composition analysis Amino acid sequencing was performed using a Shimadzu PSQ-2 gas-phase sequencer. The resulting PTH-amino acids were identified by isocratic HPLC as described previously.[19]

RESULTS

The Ub immunoreaction was found in all 52 breast tumor specimens. The expression of Ub in the tumor cell cytoplasm was constant, regardless of clinical stage or histologic features, including 1 papilloma, 15 early clinical cases, and 3 invasive lobular carcinomas. Six of the 52 (12%) breast tumors were found to express Ub in the nuclei. While the Ub immunoreaction in cancer cells was intense (Fig. 1), it was detected focally and weakly in myoepithelial cells, mammary glandular epithelial cells, fibroblasts, and endothelial cells.

Immunoblot analysis, using the same antibody that reacted with both free Ub and protein-conjugated Ub, was carried out to compare the Ub immunoreaction in normal tissues with that in breast tumor tissues. As ahown in Fig. 2, the profiles of the ubiquitinated proteins in breast tumors and normal tissues were similar. Comparisons of these profiles showed that the ubiquitinated bands were increased in tumor tissues, in accord with the distinct staining for Ub observed in the tumor cell cytoplasm. The largest difference in immunoreaction which distinguished tumor from normal tissue was observed at about 43 kDa. This immunoreaction was detected in all 52 tumor tissues, regardless of histologic type, clinical stage, or histologic grade. It was also detected by the DAKO polyclonal antibody (data not shown). While the immunoreaction was seen at about 43 kDa in specimens of normal tissue like those in cases 1, 5 and 6 in Fig. 2, it was weak and migrated slightly differently from that of tumor tissue. The 43-kDa protein was mainly fractionated in the Triton X-soluble fraction, while in the Triton X-insoluble fraction the immunoreaction for Ub at 43 kDa was very weak.

In tumor and normal breast tissue samples from cases 4 and 5, which showed strong immunoreaction at 43 kDa in the tumor samples, 2-D electrophoresis and immunoblot analysis were performed using the Mini-PROTEAN II electrophoresis apparatus. Two-D electrophoretic separation identified tumor-associated immunoreaction at a common single spot showing a consistent isoelectric point on the Mini-PROTEAN II gel (Fig. 3). In normal breast tissue the profiles of immunoreactive spots differed between the two cases.

With the use of PROTEIN II gel, the immunoreaction at around 43 kDa was examined in detail to identify the target of this immunoreaction. In case 5 there were six immunoreactive spots corresponding to the immunoreaction at 43 kDa on the Mini-PROTEAN II gel, with similar isoelectric points (Fig. 4). Since the polyclonal antibody used in this study reacted not only with Ub itself, but also with all the fragments generated by *Achromobacter* protease I, the six spots were examined to determine whether they contained Ub or Ub fragments. The individual spots were collected from over fifty gels for each tumor, and they were digested by *Achromobacter* protease I for analysis of the peptide maps. Among the peptide maps from the six spots, four were distinct; two showed the same patterns as others. The partial amino acid sequences suggested that three maps were derived from members of the keratin family and the other from a member of the actin family. However, Ub or its fragments were not found in them.

Subsequently, in ten cases the immunoreactivity of the six spots was examined. The immunoreaction indicated by the arrow in Fig. 4 was detected in all cases, but the other five spots (1 to 5) were not detected in six cases. The constant protein spot from several copper-stained 2-

D gels was cut out and electroeluted in four cases. The protein was again separated on 10% acrylamide gel and blotted onto PVDF membrane. The purified protein on the membrane appeared as a single band using Ponceau S staining, and its immunoreaction with anti-Ub antibody was confirmed.

Fig. 5 shows a peptide map obtaind from the *Achromobacter* protease I digestion of the constant spot. A total of

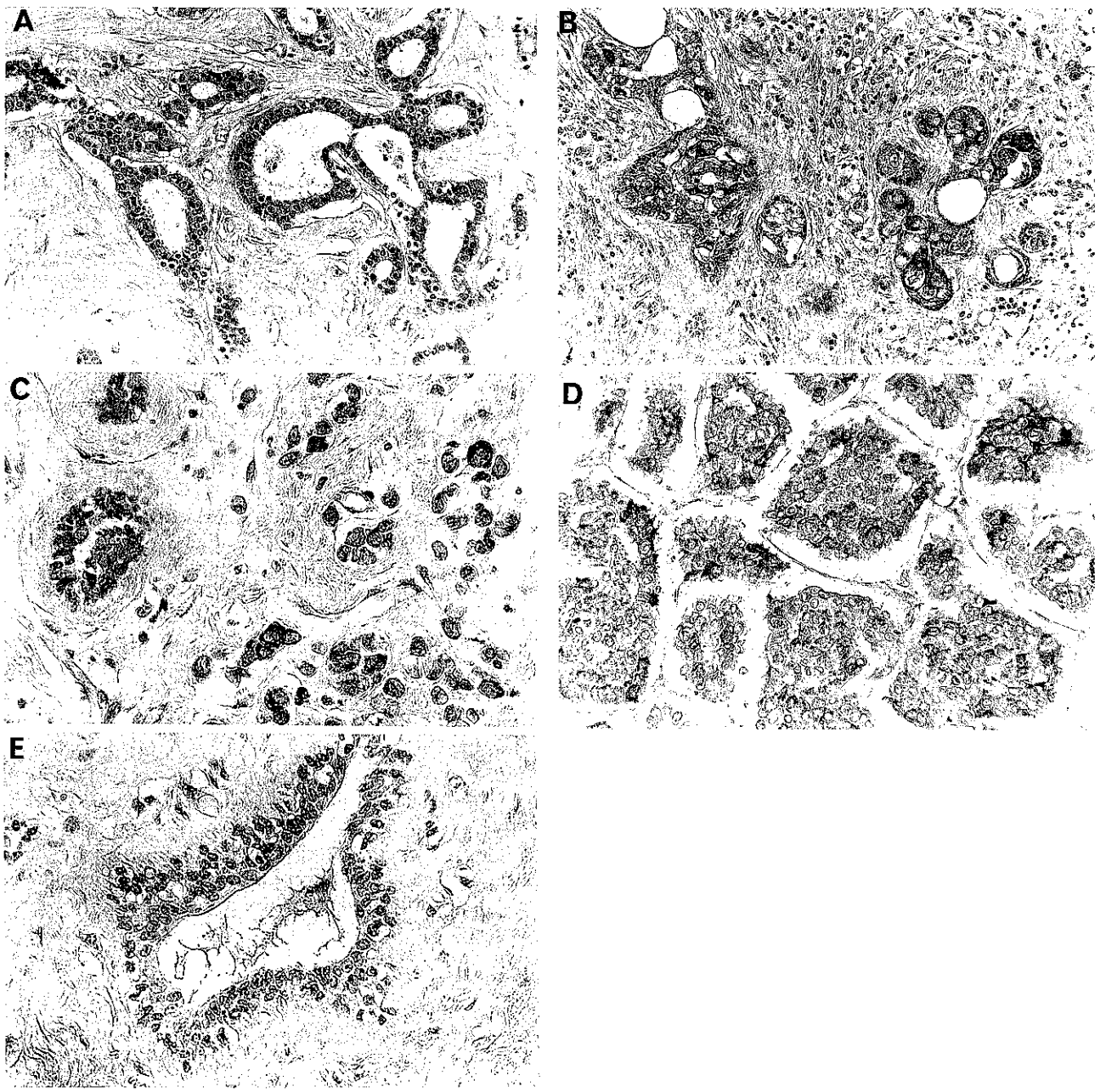

Fig. 1. Immunohistochemical staining for ubiquitinated protein in breast tumor. A, Invasive ductal carcinoma cells forming ductules show intense cytoplasmic staining with anti-Ub antibody. Focal calcification is seen. B, Invasive ductal carcinoma cells forming irregular lobules show cytoplasmic staining. C, Scattered carcinoma cells invade the fibrous stroma. Cytoplasmic staining in the carcinoma cells is strong and consistent, whereas adjacent normal duct cells stain faintly. Fibroblasts and endothelial cells are stained focally. D, Invasive ductal carcinoma forming papillary structure shows cytoplasmic staining. E, Same slide as in Fig. 1D. Normal duct cells are negatively stained for Ub.

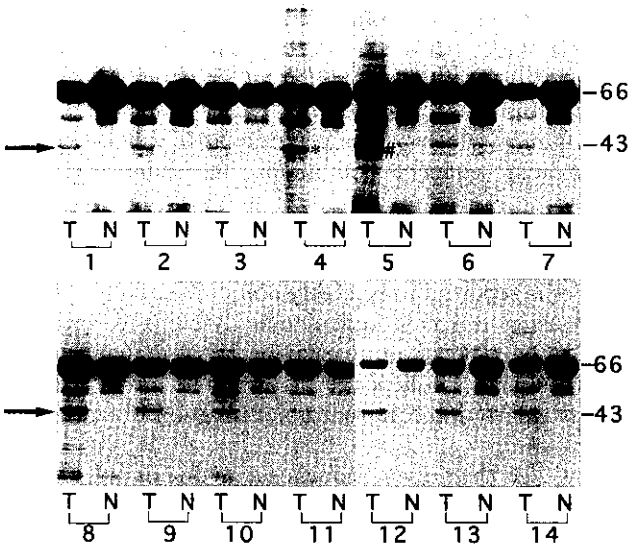

Fig. 2. Comparison of immunoreaction for Ub between breast tumor tissue and normal tissue — immunoblot analysis of 1-D separation. The case number is shown at the bottom of each lane. The Triton X-soluble fraction of breast tumor tissue (T) and the corresponding non-neoplastic tissue (N) were loaded on 10% polyacrylamide gel. The anti-Ub antibody shows immunoreaction at 43 kDa in each tumor tissue (arrow).

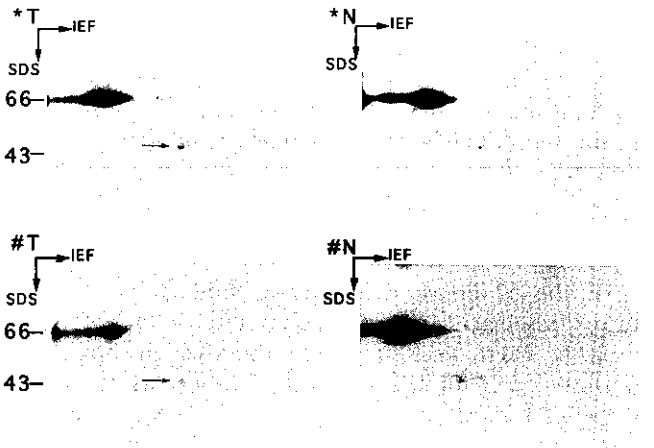

Fig. 3. Comparison of immunoreaction for Ub between breast tumor tissue and normal tissue — immunoblot analysis of 2-D separation. 2-D electrophoresis was performed in cases corresponding to cases 4 (*) and 5 (#) in Fig. 2 above. In both cases, the 43-kDa immunoreaction was seen as a single spot with the same isoelectric point in tumor tissue (arrow). The 66-kDa large spot that reacted in all membranes was determined to be human serum albumin by amino acid analysis.

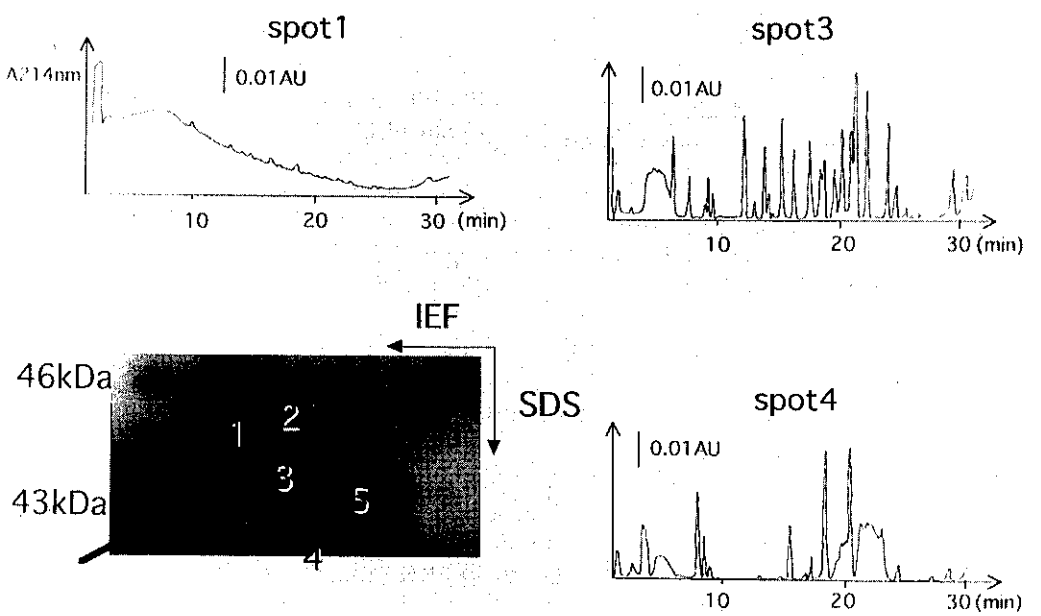

Fig. 4. Large-gel 2-D separation of 43-kDa immunoreactive material. 2-D electrophoresis was performed with a large gel (185 $\times$165$\times$10 mm) in case 5 from Fig. 2 above. The 43-kDa immunoreactive material was divided into six spots. A large spot indicated by the arrow was consistently identified in the ten cases examined. The other five numbered spots were not detected in all cases. All these spots were subjected to *in situ* reduction, and peptide mapping by reverse-phase HPLC. Spots 1 and 2 gave the same peptide map and spots 3 and 5 also showed the same pattern. Three peptide maps corresponded to those of different members of the keratin family.

196 residues were sequenced, from 11 peptides (Table II), and these were found to be identical with conserved residues of actin family members. In four cases, the peptide maps and sequence data were found to correspond reproducibly, and no sequence contamination was noted.

DISCUSSION

In the 52 breast cancer cases studied, we found a tumor-associated immunoreaction for Ub with a 43-kDa protein. There were six protein spots which showed immunoreaction for Ub in 10 breast cancer cases. Peptide mapping by *Achromobacter* protease I digestion and subsequent partial amino acid sequencing did not reveal Ub or Ub fragments among them. However, a constant spot commonly detected in every case was found to be a member of the actin family by amino acid sequence analysis. Although the complete amino acid sequence of this protein was not obtained, it is possible that the protein was not actin itself but rather a member of the actin family, which has an evolutionarily well conserved amino acid sequence and almost the same isoelectric point.

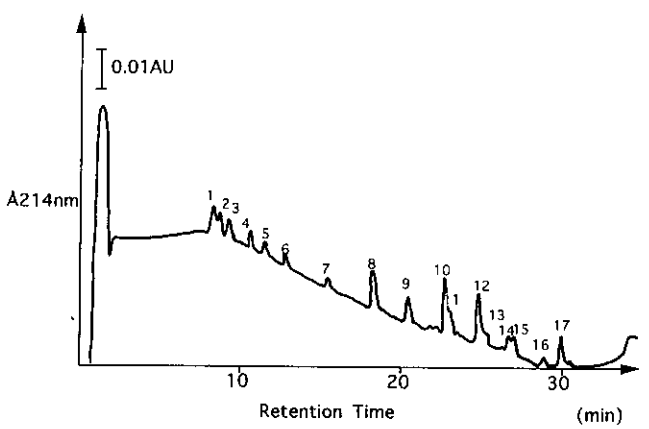

Fig. 5. HPLC map of peptides released after *in situ* digestion with *Achromobacter* protease I. Numbers indicate peptides for which sequence analysis was carried out.

Some forms of actin are ubiquitinated. In some insects, a significant portion of the actin found in the direct flight muscles is reported to be stably ubiquitinated.[20] Another study using a whole reticulocyte lysate and antibody against the Ub-activating enzyme showed that actin is degraded in a Ub-dependent manner *in vitro*.[21] These reports clearly indicate that ubiquitination of actin does occur.

However, the present sequence data do not indicate the presence of ubiquitin fragments. Since the N-terminal fragment of actin, in which the first lysine residue is suspected to be the binding residue for Ub,[2] was not included in the fragments examined here, the N-terminal sequence was examined directly. Although the Ub sequence could not be determined, we did find that the first lysine residue was not cleaved by *Achromobacter* protease I (data not shown). The 43-kDa protein was completely digested at all the other lysine residues. This incomplete digestion suggests that the 43-kDa protein may form a complexed structure around the first lysine N-terminal residue that could mask the residue and interfere with digestion. Ub itself is known to be hardly digested by *Achromobacter* protease I. These findings suggest that Ub and the N-terminal fragment of the 43-kDa protein may remain on the PVDF membrane as a complexed large fragment and not appear as a digested peak.

Our immunochemical data, which indicate that the 43-kDa protein is conjugated with Ub, suggest that this protein in breast tumor cells changes its conformation due to conjugation with Ub and undergoes alteration of its spatial distribution and relative stability. From the

Table II. Partial Peptide Sequences of 43-kDa Protein

No.	Fragment	Sequence
1, 2	51–61	(k) DSYVGDEAQSK (R)
3	374–375	(k) CF
4	285–291	(k) CDVDIRK (D)
5	329–336	(k) IIAPPERK (Y)
6	360–373	(k) QEYDESGPSIVHRK (C)
7, 8	316–326	(k) EITALAPSTMK (I)
9	62–68	(k) RGILTLK (Y)
10	69–84	(k) YPIEHGIVTNWDDMEK (I)
11, 12	292–315	(k) DLYANTVLSGGTTMYPGIADRMQK (E)
15	192–213	(k) ILTERGYSFTTTAEREIVRDIK (E)
17	216–238	(k) LCYVALDFEQEMATAASSSSLEK

partial identity between the sequence of this protein and that of actins, at least part of the function of the 43-kDa protein is suggested to be similar to that of actin, and the protein may be involved in the mechanism of tumor invasion or malignant transformation. For example, the edges of tumors are rich in actin structures, suggesting that the invasiveness of tumor cells may be related to these structures.[22] Indeed, in hepatoma cells, Ub has been found by electron microscopy to be present within microvilli, within which actin is organized.[23] Another study has suggested that variant actin, which migrates very closely to β- and γ-actins on 2-D gels, is correlated with oncogenic transformation.[24] The 43-kDa protein may be a candidate for a new variant actin. While further definition of the role of the 43-kDa protein in breast tumor tissue awaits both structural and genetic analyses, the present study has at least identified a common denominator in breast cancer.

Finally, the present findings may lead to the development of a clinical marker for breast cancer. We found immunoreaction at 43 kDa with anti-Ub antibody in 100% of the breast tumors tested, including 15 cases at the early clinical stage. It is possible that changes in the immunoreactivity of the 43-kDa protein represent an early event in breast carcinogenesis.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Keiji Tanaka for providing the Ub-protein ligation system, and to the staff of the photo center for photography. This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare of Japan for the Comprehensive 10-Year Strategy for Cancer Control, and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

(Received October 8, 1996/Accepted December 9, 1996)

REFERENCES

1) Hershko, A. and Ciechanover, A. The ubiquitin pathway for the degradation of intracellular proteins. *Prog. Nucleic Acid Res. Mol. Biol.*, **33**, 19–56 (1986).
2) Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K. and Varshavsky, A. A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science*, **243**, 1576–1583 (1989).
3) Glotzer, M., Murray, A. W. and Kirschner, M. W. Cyclin is degraded by the ubiquitin pathway. *Nature*, **349**, 132–138 (1991).
4) Ozkaynak, E., Finely, D. and Varshavsky, A. The yeast ubiquitin gene: head-to-tail repeats encoding a polyubiquitin precursor protein. *Nature*, **312**, 663–666 (1984).
5) Jentsch, S., McGrath, J. P. and Varshavsky, A. The yeast DNA repair gene RAD6 encodes a ubiquitin-conjugating enzyme. *Nature*, **329**, 131–134 (1987).
6) Leung, D. W., Spencer, S. A., Cachianes, G., Hammonds, R. G., Collins, C., Henzel, W. J., Barnard, R., Waters, M. J. and Wood, W. I. Growth hormone receptor and serum binding protein: purification, cloning and expression. *Nature*, **330**, 537–543 (1987).
7) Hershko, A., Leshinsky, E., Ganoth, D. and Heller, H. ATP-dependent degradation of ubiquitin-protein conjugates. *Proc. Natl. Acad. Sci. USA*, **81**, 1619–1623 (1984).
8) Bachmair, A. and Varshavsky, A. The degradation signal in a short-lived protein. *Cell*, **56**, 1019–1032 (1989).
9) Thorne, A. W., Sautiere, P., Briand, G. and Crane-Robinson, C. The structure of ubiquitinated histone H2B. *EMBO J.*, **6**, 1005–1010 (1987).
10) Nickel, B. E., Allis, C. D. and Davie, J. R. Ubiquitinated histone H2B is preferentially located in transcriptionally active chromatin. *Biochemistry*, **28**, 958–963 (1989).
11) Ciechanover, A., DiGiuseppe, J. A., Bercovich, B., Orian, A., Richter, J. D., Schwartz, A. L. and Brodeur, G. M. Degradation of nuclear oncoproteins by the ubiquitin system *in vitro*. *Proc. Natl. Acad. Sci. USA*, **88**, 139–143 (1991).
12) Finlay, C. A., Hinds, P. W. and Levine, A. J. The *p53* proto-oncogene can act as a suppressor of transformation. *Cell*, **57**, 1083–1093 (1989).
13) Ishibashi, Y., Takada, K., Joh, K., Ookawa, K., Aoki, T. and Matsuda, M. Ubiquitin immunoreactivity in human malignant tumors. *Br. J. Cancer*, **63**, 320–322 (1991).
14) Kanayama, H., Tanaka, K., Aki, M., Kagawa, S., Miyaji, H., Satoh, M., Okada, F., Sato, S., Shimbara, N. and Ichihara, A. Changes in expression of proteasome and ubiquitin genes in human renal cancer cells. *Cancer Res.*, **51**, 6677–6685 (1991).
15) Tsuda, H., Hirohashi, S., Shimosato, Y., Hirota, Y., Tsugane, T., Watanabe, S., Terada, M. and Yamamoto, H. Correlation between histologic grade of malignancy and copy number of c-*erb*B-2 gene in breast carcinoma. *Cancer*, **65**, 1794–1800 (1990).
16) Nishibori, H., Matsuno, Y., Iwaya, K., Osada, T., Kubomura, N., Iwamatsu, A., Kohno, H., Sato, S., Kitajima, M. and Hirohashi, S. Human colorectal carcinomas specifically accumulate Mr-42,000 ubiquitin-conjugated cytokeratin 8 fragments. *Cancer Res.*, **56**, 2752–2757 (1996).
17) Tamura, T., Tanaka, K., Tanahashi, N. and Ichihara, A. Improved method for preparation of ubiquitin-ligated lysozyme as substrate of ATP-dependent proteolysis. *FEBS Lett.*, **292**, 154–158 (1991).
18) Iwamatsu, A. *S*-Carboxymethylation of proteins transferred onto polyvinylidene difluoride membranes followed by *in situ* protease digestion and amino acid microsequencing. *Electrophoresis*, **13**, 142–147 (1992).
19) Aoyama, H., Iwamatsu, A., Dibo, G., Tsunasawa, S. and

Sakiyama, F. An improved isocratic HPLC separation of PTH-amino acids at the subpicomole level and its application to protein sequence analysis. *J. Protein Chem.*, **7**, 191 (1988).
20) Ball, E., Karlin, C. C., Beall, C. J., Saville, D. L., Sparrow, J. C., Bullard, B. and Fyrberg, E. A. Arthrin, a myofibrillar protein of insect flight muscle, is an actin-ubiquitin conjugate. *Cell*, **51**, 221–228 (1987).
21) Mayer, A., Siegel, N. R., Schwartz, A. L. and Ciechanover, A. Degradation of proteins with acetylated amino termini by the ubiquitin system. *Science*, **244**, 1480–1483 (1989).
22) Byers, H. R., Etoh, T., Doherty, J. R., Sober, A. J. and Mihm Jr., M. C. Cell migration and actin organization in cultured human primary, recurrent cutaneous and metastatic melanoma. *Am. J. Pathol.*, **139**, 423–435 (1991).
23) Schwartz, A. L., Ciechanover, A., Brandt, R. A. and Geuze, H. J. Immunoelectron microscopic localization of ubiquitin in hepatoma cells. *EMBO J.*, **7**, 2961–2966 (1988).
24) Hamada, H., Leavitt, J. and Kakunaga, T. Mutated β-actin gene: coexpression with an unmutated allele in a chemically transformed human fibroblast cell line. *Proc. Natl. Acad. Sci. USA*, **78**, 3634–3638 (1981).