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Advances in Brief

A Matrix Attachment Region (MAR)-binding Activity Due to a p114 Kilodalton Protein Is Found Only in Human Breast Carcinomas and Not in Normal and Benign Breast Disease Tissues[1]

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Abstract

A M_r 114,000 protein (p114) that specifically binds to nuclear matrix attachment DNA (matrix attachment region, MAR) from a breast carcinoma cell line SK-BR-3 was purified to near homogeneity. p114 strongly binds to a wild-type A+T-rich MAR probe with high unwinding propensity with a dissociation constant (K_d) of 10^{-9}, while it exhibits substantially reduced binding to a mutated A+T-rich non-MAR probe, which lacks unwinding propensity. This binding specificity and affinity is similar to the previously cloned thymocyte-associated MAR-binding protein SATB1. By Southwestern blot analysis, the MAR-binding activity of p114 is detectable in human breast carcinomas but is undetectable in normal breast tissues, benign breast diseases, and immortalized epithelial MCF-10A cells. Thus, the MAR-binding activity of p114 is not merely reflecting cell proliferation, but it strongly associates with breast carcinomas. The p114 MAR-binding activity was found in all 43 human breast carcinoma specimens tested, without exception. Much stronger p114 MAR-binding activity was detected in poorly differentiated than well-differentiated carcinomas. p114 may be a reliable diagnostic and possibly prognostic marker for breast cancer.

Introduction

Human breast cancer is characterized by a multiplicity of genetic alterations affecting both proto-oncogenes and tumor suppressor genes (1). The oncogene c-*erb*B-*2*/*neu* is, for example, one of the genes frequently overexpressed early in breast tumor promotion (2). Approximately 20% of breast carcinomas show evidence of c-*erb*B-2 activation, which correlates with a poor prognosis, primarily in patients with metastasis to axillary lymph nodes. In addition, mutations of the *p53* tumor suppressor gene and the associated accumulation of the p53 protein are frequently found in breast tumors (3), and altered p53 expression is considered to have prognostic value (4). Although these proteins can be useful markers for some breast carcinomas, there is a significant proportion of tumors with a rapid proliferation rate that do not overexpress c-erbB-2 or p53 proteins, and the prognosis of patients with highly proliferative axillary node-negative cancer is poor, regardless of their protein expression status (5). Therefore, other proteins are likely to be involved in the uncontrolled growth of breast cancer cells.

In the eukaryotic nucleus, DNA is organized into a three-dimensional structure consisting of topologically constrained loop domains. Maintenance of this structure is mediated by the attachment of specific segments of the DNA to an insoluble skeletal framework referred to as the nuclear matrix (6). These DNA segments, which are located at the base of the loop domains, are designated MARs[3] (7, 8). Attachment of MARs to the nuclear matrix presumably occurs through interaction of the MARs with MAR-binding proteins present in the nuclear matrix. Because this interaction influences the loop organization of DNA, MARs and their binding proteins may have an important functional role in multiple cell processes, particularly transcription and replication of DNA. Many MARs are found at the boundaries of transcription units, where they may delimit the ends of an active chromatin domain, and MARs are often located near regulatory elements, including enhancers (8). Recent evidence shows the biological significance of MARs in tissue-specific gene expression: (*a*) MARs surrounding the immunoglobulin heavy chain (IgH) gene enhancer are essential for transcription of a rearranged μ gene in transgenic B lymphocytes (9); and (*b*) a cell type-specific MAR-binding protein, SATB1, which is predominantly expressed in thymocytes, has been cloned, indicating that MARs can be specific targets for tissue-specific factors (10).

Nuclear matrix proteins vary in a cell type-specific manner, suggesting that the nuclear matrix may play an important role in the tissue-specific three dimensional organization of DNA (11). Additionally, the protein composition of the human breast tumor cell nuclear matrix is different from that of normal breast cells (12), and some of these nuclear matrix proteins unique to breast tumor cells may correspond to MAR-binding proteins. We investigated whether there is a breast tumor protein that has a high affinity for MARs and is absent in normal breast cell counterparts. In this study, we report a new MAR-binding protein that possesses MAR-binding activity that is detectable in human breast tumor specimens at varying levels, depending on the differentiation status of the tumors. The MAR-binding activity of this protein is undetectable in normal and benign lesions of breast tissues, as well as in immortalized human epithelial cells. This protein may, therefore, play an important role in breast tumorigenisis and may have a high potential as an effective diagnostic and prognostic marker.

Materials and Methods

Tissue Specimens, Cell Lines, and Extracts. Surgical specimens of human primary breast carcinomas, benign breast lesions, and normal breast tissues were procured for this research by the Breast Tumor Surgical Team, Tochigi Cancer Center, Japan; Human Tissue Bank of University of California, San Diego, CA; and Cooperative Human Tissue Network, which is funded by the National Cancer Institute (USA). After surgical removal of the breast lesions, the specimens were immediately subjected to pathological analysis according to the standard diagnosis criteria (13). Staging followed International Union Against Cancer criteria. The specimens were dissected, trimmed,

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[3] The abbreviations used are: MAR, matrix attachment region; DCIS, ductal carcinoma *in situ*; PCNA, proliferating cell nuclear antigen.

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flash frozen in liquid nitrogen, and stored at −80°C. Forty-three breast carcinoma specimens were used in this study, including 33 cases of infiltrating ductal carcinomas, 6 cases of DCIS with microinvasion, 2 cases of DCIS, and 1 case each of infiltrating lobular carcinoma and mucinous carcinoma. For benign lesions, 17 specimens were studied including fibroadenoma, proliferating fibrocystic change, atypical ductal and atypical lobular hyperplasia, and fibrocystic change. Immortalized breast epithelial cell lines, MCF-10A, breast carcinoma cell lines, ZR-75-1, SK-BR-3, and MDA-MB-231, were obtained from American Type Culture Collection. These cell lines were maintained as exactly recommended by American Type Culture Collection. Tissue and cell extracts were prepared as described (14). Briefly, the cell pellet and minced tissues were resuspended in approximately 3× volume of extraction buffer [0.4 M KCl, 10 mM sodium phosphate buffer (pH 7.4), 10% glycerol, 0.5 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin and aprotinin]. Cells and tissues were lysed in a Dounce homogenizer (50 strokes with pestle A). The extract was centrifuged for 1 h in a T865.1 rotor in a Sorval centrifuge at 38,000 rpm, and the supernatant was either assayed directly for binding activity using the Southwestern analysis or used for affinity purification of MAR-binding proteins.

Western Blotting. Proteins separated by SDS-PAGE were electrophoretically transferred to Immobilon P membranes (Millipore) in 20 mM sodium phosphate buffer (pH 6.8). Biotinylated high-molecular-weight protein markers (Bio-Rad) were included for internal molecular mass standards. The filters were blocked in 5% BSA in TST [20 mM Tris-HCl (pH 7.4), 0.5 M NaCl, and 0.05% Tween 20], washed in TST buffer minus BSA, and incubated at 4°C for 1.5 h with either monoclonal antibody to α-tubulin (Sigma Immuno Chemicals) or monoclonal antibody PC10 to PCNA (Santa Cruz Biochemicals) at a 1:1000 dilution. Other antibodies used were rabbit anti-SATB1 antibody (10), rabbit anti-SAF-A antibody (15), and mouse anti-nucleolin antibody (14). After being washed in TST, the filters were incubated simultaneously with a 1:15,000 dilution of either goat anti-rabbit or mouse horseradish peroxidase conjugate and streptavidin-labeled horseradish peroxidase to detect the biotinylated protein markers (Bio-Rad). Following extensive washing in TST containing 0.15% Tween 20, the blots were incubated with ECL (enhanced chemiluminescence) reagent solutions (Amersham Biochemicals) and exposed to XAR film for visualization of protein bands.

DNA Binding Studies with Protein Blots (Southwestern Blotting). Proteins were dissolved in the SDS-PAGE loading buffer containing 2% SDS and 5% (v/v) 2-mercaptoethanol by incubation at 68°C for 1 min before being applied to a 7.5% polyacrylamide mini gel layered with 3.75% stacking gel. After electrophoresis, separated proteins were transferred electrophoretically at 50 V/cm at 1 mA for 1 h onto Immobilon P membrane (Millipore). The membrane was treated with 4% BSA in the TST buffer at 25°C for 1 h. The membrane was then washed in binding buffer [20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM DTT, and 0.25% BSA] for 1 h. To 20 ml of binding solution, heat-denatured salmon sperm DNA to a final concentration of 2 μg/ml and poly(deoxyinosinic-deoxycytidylic acid) to a final concentration of 5 μg/ml was added, and the membrane was preincubated at room temperature for 30 min. ^{32}P-labeled wild-type $(25)_7$ probe (approximately 10^8 cpm/μg DNA; Refs. 10, 16 and 17) was added and further incubated for 30 min at room temperature. The membrane was washed four times each for 10 min in binding buffer. It was then exposed to XAR film. To visualize the biotinylated protein markers (Bio-Rad), the same membrane was washed for 1 h at room temperature in three changes of binding buffer containing 2 M NaCl and 0.5% Tween 20, and Western Blotting was performed as described above.

Gel Mobility Shift Assay. The experiment was done basically as described previously (10) with some modifications. Wild-type $(25)_2$ and mutated $(24)_2$ were used as DNA probes (0.5 ng) in the present study. In a 20-μl binding reaction mixture, 25 ng of double-stranded Bluescript *XmnI-ScaI* fragment was used as a nonspecific competitor DNA instead of 2 μg of poly(deoxyinosinic-deoxycytidylic acid), and 100 mM KCl was used instead of 50 mM KCl.

DNA Affinity Purification of MAR-binding Proteins. The MAR affinity column was prepared exactly as described (14). Cell extract was prepared from approximately 10^9 cells, incubated with nonspecific DNA competitors, centrifuged, and diluted with buffer Z [25 mM HEPES (K^+) (pH 7.8), 12.5 mM $MgCl_2$, 1 mM DTT, 20% (v/v) glycerol, and 0.1% (v/v) NP40] as described previously (14). Cell extract was re-adjusted to 0.3 M KCl in buffer Z and was loaded on a mutated non-MAR DNA affinity column to first remove nonspecific A+T-rich DNA-binding proteins. The proteins in the flow-through fraction were then loaded on the MAR affinity column pre-equilibrated with buffer Z containing 0.3 M KCl and washed with the same buffer; then bound proteins were eluted with buffer Z containing 0.4, 0.6, 0.8, and 1.0 M KCl. The MAR-binding protein was eluted at 0.6–0.8 M KCl.

Results and Discussion

Detection and Purification of a MAR-binding Protein (p114) from a Human Breast Carcinoma Cell Line, SK-BR-3. To identify a new, nonubiquitous MAR-binding protein(s) in breast carcinomas, we searched for a protein that confers binding specificity similar to that of SATB1. SATB1 binds double-stranded, A+T-rich DNA sequences that readily unwind under negative superhelical strain, but it does not bind to mutated sequences that are A+T-rich yet resistant to unwinding (10). MARs typically contain a specific region(s) that exhibits such high unwinding capability (16), and this unusual structural property is important for MARs to confer high affinity to the nuclear matrix (17). The cDNA encoding the SATB1 protein was previously cloned by taking advantage of the high affinity of the protein to the MAR sequences with high unwinding capability (10). SATB1 was found to be a sequence context-specific binding protein that recognizes DNA sequences in which one strand exclusively consists of A, T, and C nucleotides that are well mixed (ATC sequences). MAR regions containing ATC sequence clusters exhibit high unwinding capability (16, 17).[4]

In an attempt to detect and purify a new MAR-binding protein from breast carcinomas, we used two similar A+T-rich DNA sequences with contrasting DNA structural properties (17): (*a*) a 25-bp sequence, 5′-TCTTTAATTTCTAATATATTTAGAA-3′ [wild-type (25)], originally derived from a MAR 3′ of the IgH enhancer and which contains the core unwinding element (underlined) of this MAR; and (*b*) a 24-bp sequence 5′-TCTTTAATTTCTACTGCTTTA-GAA-3′ [mutated (24)], where three adenines are mutated and one thymine is deleted within the core unwinding element of the 25-bp sequence. Upon multimerization, a heptamer of the 25-bp sequence, wild-type $(25)_7$, strongly binds to SATB1, unwinds under superhelical strain, exhibits a strong affinity for the nuclear matrix, and augments SV40 promoter activity in stable transformants. In contrast, similarly multimerized mutated $(24)_8$, an octamer of the mutated sequence, does not bind SATB1, resists unwinding, shows a very weak affinity for the nuclear matrix, and does not enhance promoter activity (17). Two DNA affinity columns were prepared: one containing double-stranded concatemers of wild-type $(25)_n$ (n = various number of repeating units), which represent strong MAR sequences, and the other concatemers of mutated $(24)_n$, which represent non-MAR, A+T-rich sequences. The strategy for purifying a MAR-binding protein from breast carcinoma cell extracts is to first remove nonspecific A+T-rich DNA-binding proteins from the extracts by passing the proteins through the mutated $(24)_n$ affinity column. Although nonspecific A+T-rich DNA-binding proteins are expected to bind to this column, MAR-binding protein(s) with similar binding specificity as SATB1 should pass through this column. The flow-through fraction from the mutated $(24)_n$ column was then applied onto the wild-type $(25)_n$ DNA column to isolate protein(s) that specifically bind to MARs. Following this protocol, a MAR-binding protein was purified to near homogeneity using cell extracts prepared from a breast adenocarcinoma cell line SK-BR-3 cells. Silver staining analysis showed that a single protein (p114) of approximately M_r 114,000 was purified from the second wild-type $(25)_n$ column by initial washing with the buffer containing 0.3 M KCl and 0.4 M KCl, and subsequently eluting the bound protein with 0.6 and 0.8 M KCl (Fig. 1*A*, *Lane 2*). Southwestern blot analysis of the crude extracts and the purified protein

[4] Unpublished results.

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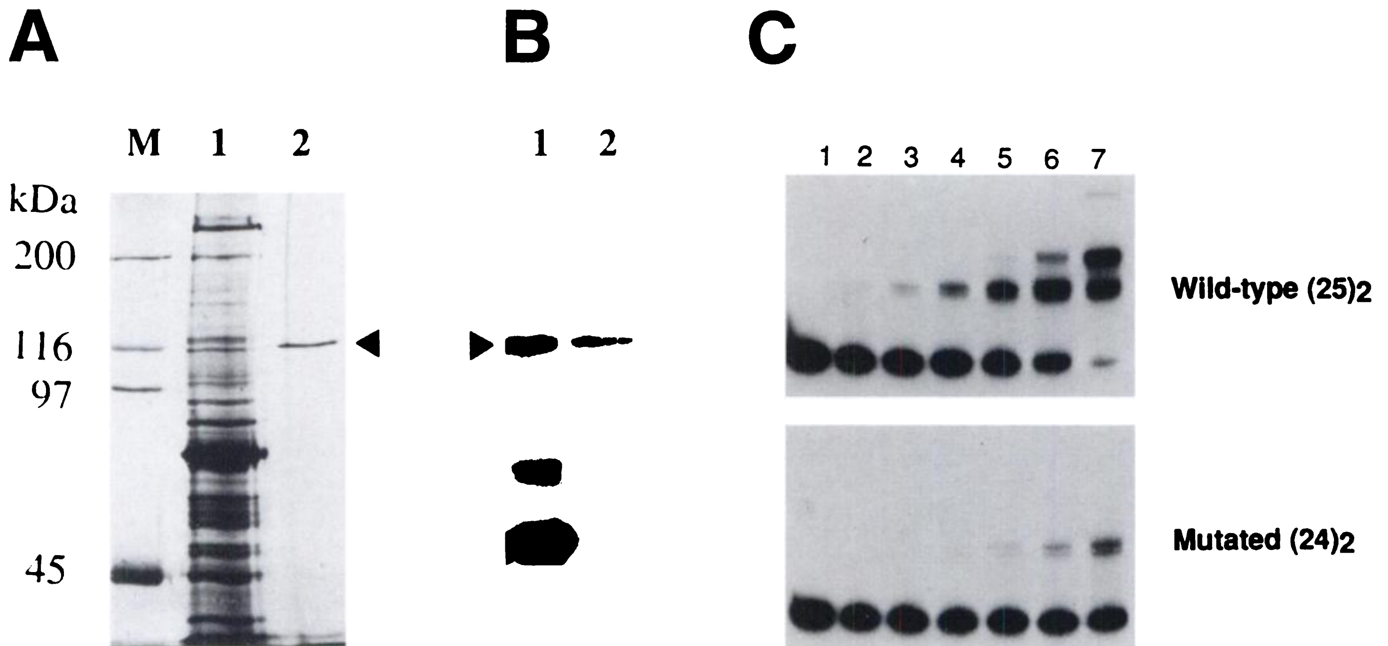

Fig. 1. Affinity purification of a MAR-binding protein from a breast carcinoma cell line, SK-BR-3. *A,* silver staining analysis was performed for 1 μg protein of a crude cell extract prepared from the SK-BR-3 cells (*Lane 1*) and column-purified p114 (10 ng) from the SK-BR-3 extract (*Lane 2*). *M,* molecular size marker. *Arrowhead,* p114. *B,* Southwestern blot analysis was performed with 40 μg of cell extracts from SK-BR-3 cells (*Lane 1*) and 40 ng of column-purified p114 (*Lane 2*). The proteins were loaded on a gel, separated by electrophoresis, blotted onto a membrane, and hybridized with radiolabeled wild-type $(25)_7$ probe. *C,* gel-mobility shift assay was performed for purified p114 from SK-BR-3 cells with either a radiolabeled wild-type $(25)_2$ (*top panel*) or mutated $(24)_2$ probe (*bottom panel*). The DNA probes were incubated with varying amounts of protein: *Lane 1,* 0 ng; *Lane 2,* 0.037 ng; *Lane 3,* 0.11 ng; *Lane 4,* 0.33 ng; *Lane 5,* 1 ng; *Lane 6,* 3 ng; and *Lane 7,* 9 ng in 20 μl of binding reaction mixture and electrophoresed on a 12% native polyacrylamide gel.

using the radiolabeled wild-type $(25)_7$ showed that, among multiple proteins that bind wild-type $(25)_7$ in the crude extracts, only p114 was selected and purified through these two columns (Fig. 1*B, Lanes 1* and *2*). The remaining proteins in the crude extracts that showed positive signals on the Southwestern blot are apparently nonspecific, A+T-rich binding proteins that bind both wild-type $(25)_n$ and mutated $(24)_n$ (Fig. 1*B, Lane 1*). p114 was also purified to near homogeneity from several human tissue specimens of infiltrating ductal carcinoma (data not shown).

Western blot analysis for p114 indicated that p114 is not recognized by either the anti-SATB1 or anti-nucleolin antibodies (data not shown). Nucleolin has been shown recently to selectively bind MARs (14). Furthermore, anti-SAF-A antibody (kindly provided by Dr. Arnt Richter, University of Konstanz, Germany) did not recognize p114 (data not shown). SAF-A is apparently a ubiquitous MAR-binding protein strongly attached to the nuclear matrix and recently identified as a hnRNP U protein (15, 18). Another ubiquitous MAR-binding protein, ARBP, has been described as a M_r 95,000 protein (19) and, therefore, believed to be distinct from p114. Therefore, p114 is likely to be a novel MAR-binding protein.

The Purified p114 Strongly and Selectively Binds to an A+T-rich DNA with High Unwinding Propensity. The p114 fraction shown in Fig. 1*A, Lane 2,* was examined by gel mobility shift assay using the dimers of wild-type 25-bp and mutated 24-bp sequences, wild-type $(25)_2$ and mutated $(24)_2$. This fraction showed a very strong affinity to wild-type $(25)_2$, with an estimated dissociation constant

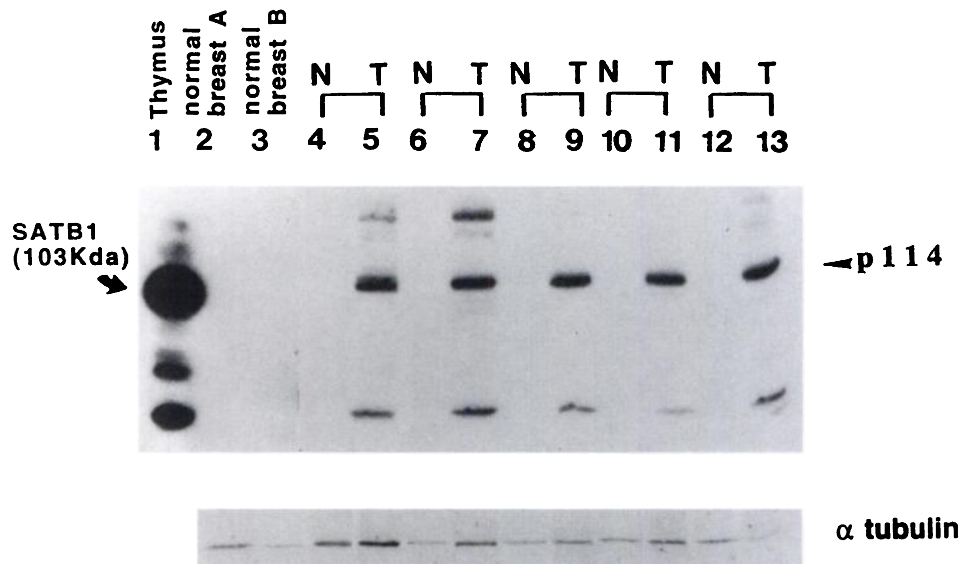

Fig. 2. The p114 MAR-binding activity is present in breast carcinoma specimens but not in normal breast tissues. Southwestern blot analysis was performed. Forty μg of proteins in tissue extracts prepared from five cases of infiltrating ductal carcinoma (*Lanes 5, 7, 9, 11,* and *13*), their adjacent normal tissues (*Lanes 4, 6, 8, 10,* and *12*), and two normal breast tissues derived from reduction mammoplasties of healthy individuals (*Lanes 2* and *3*) were loaded on a SDS-polyacrylamide gel. After electrophoresis, the proteins were renatured; then the gel was blotted and hybridized with the radiolabeled wild-type $(25)_7$ probe. Proteins from breast tumor specimens (*T*) and their corresponding normal adjacent tissues (*N*) are indicated by *brackets.* Extracts from thymocytes shown in *Lane 1* contains 4 μg of protein. The positions of SATB1 and p114 are indicated. The same amount of proteins used in the Southwestern blot was subjected to Western blot analysis using anti-α-tubulin antibody.

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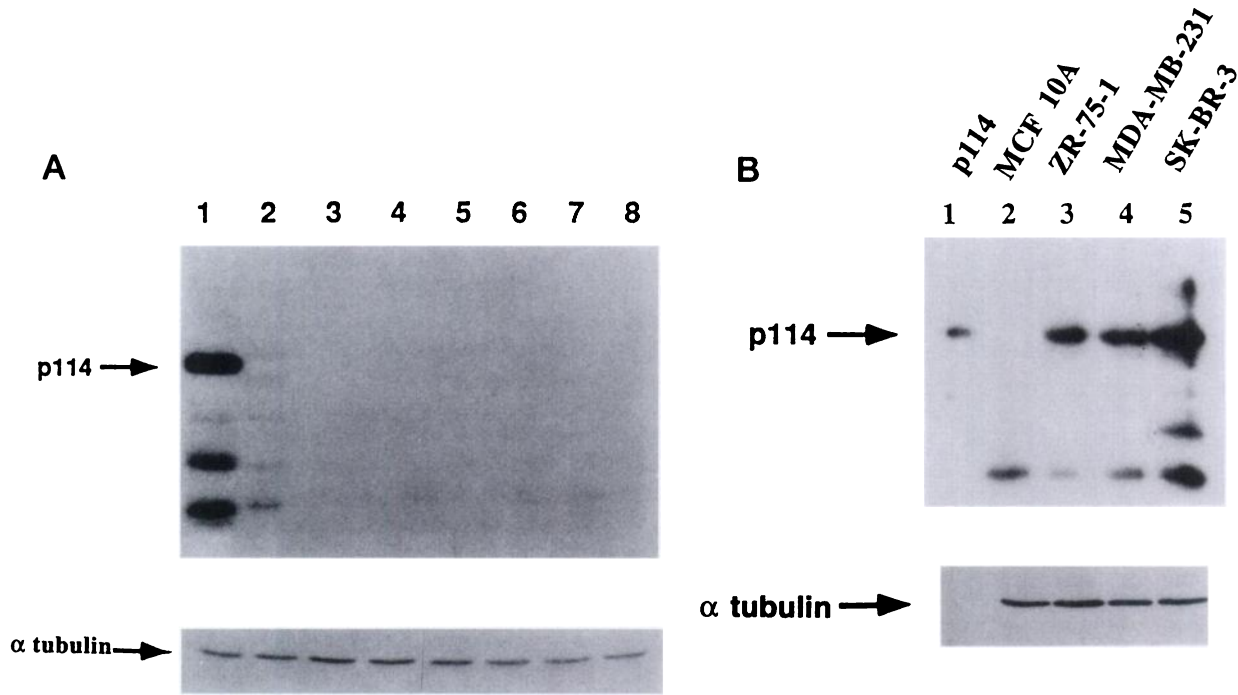

Fig. 3. The p114 MAR-binding activity is present in breast carcinomas but not in benign breast diseases or nonmalignant immortalized epithelial cells. Southwestern blot analysis using radiolabeled wild-type $(25)_7$ as the probe was performed with *A*, 40 μg of proteins extracted from various breast diseases: *Lane 1*, infiltrating ductal carcinoma; *Lane 2*, fibroadenoma; *Lane 3*, fibrocystic changes; *Lane 4*, proliferative fibrocystic change; *Lane 5*, atypical ductal hyperplasia; *Lanes 6–8*, atypical lobular hyperplasia; *B*, *Lane 1*, purified p114 (40 ng); 20 μg of proteins in the cell extracts from MCF-10A (*Lane 2*), ZR-75-1 (*Lane 3*), MDA-MB-231 (*Lane 4*), and SK-BR-3 (*Lane 5*). The same amount of proteins used for Southwestern blot in *A* and *B* was subjected to Western blot analysis using anti-α-tubulin antibody.

No.	TNM Staging		Lymph Node Status	Histologic Grading	p114 Activity
1	$T_2N_3M_1$	III_B	7/33	III	52.9
2	$T_4N_3M_1$	IV	n.a.	III	43.8
3	$T_2N_2M_0$	III_A	10/38	III	29.3
4	$T_2N_3M_0$	III_B	4/28	III	23.5
5	$T_{4b}N_3M_1$	IV	34/67	III	23.4
6	$T_2N_1M_0$	II_B	3/23	III	22.4
7	$T_2N_1M_1$	IV	1/15	III	21.4
8	$T_2N_2M_0$	III_A	18/51	III	17.0
9	$T_{4b}N_2M_1$	IV	12/37	III	16.0
10	$T_2N_1M_0$	II_B	3/21	III	15.5
11	$T_2N_0M_0$	II_A	0/13	III	14.7
12	$T_1N_2M_0$	III_A	3/34	III	14.6
13	$T_2N_1M_0$	II_B	3/40	III	13.3
14	$T_2N_1M_0$	II_A	2/29	III	13.3
15	$T_4N_1M_0$	III_A	12/15	(a)	10.7
16	$T_2N_0M_0$	II_A	0/18	III	10.3
17	$T_{4b}N_2M_0$	III_B	24/32	II	24.0
18	$T_1N_1M_1$	IV	1/15	II	17.6
19	$T_2N_0M_0$	II_A	0/24	II	16.2
20	$T_2N_1M_0$	II_B	2/21	II	15.9
21	$T_2N_2M_0$	III_A	5/15	II	15.0
22	$T_{4b}N_0M_1$	IV	0/31	II	13.0
23	$T_2N_0M_0$	II_A	0/32	II	12.4
24	$T_2N_0M_0$	II_A	0/39	II	12.3
25	$T_1N_1M_0$	II_A	1/22	II	11.8
26	$T_1N_0M_0$	I	0/26	II	10.2
27	$T_1N_2M_0$	III_A	2/29	II	8.4
28	$T_2N_0M_0$	II_A	0/12	II	7.3
29	$T_1N_1M_0$	II_A	1/29	II	5.5
30	$T_2N_1M_0$	II_B	1/24	II	5.0
31	$T_1N_0M_0$	I	0/33	II	3.9
32	$T_2N_1M_0$	II_B	2/17	I	5.0
33	$T_2N_1M_0$	II_B	2/11	I	5.0
34	$T_1N_1M_0$	II_A	5/14	(b)	4.8
35	$T_1N_1M_0$	II_A	9/52	I	1.0
36	DCIS		0/38		6.5
37	DCIS/microinvasion		0/39		5.5
38	DCIS/microinvasion		1/8		5.2
39	DCIS/microinvasion		0/13		4.6
40	DCIS/microinvasion		0/29		3.5
41	DCIS/microinvasion		0/34		1.5
42	DCIS/microinvasion		0/15		1.0
43	DCIS		n.a.		1.0

(a) infiltrating lobular carcinoma
(b) mucinous carcinoma

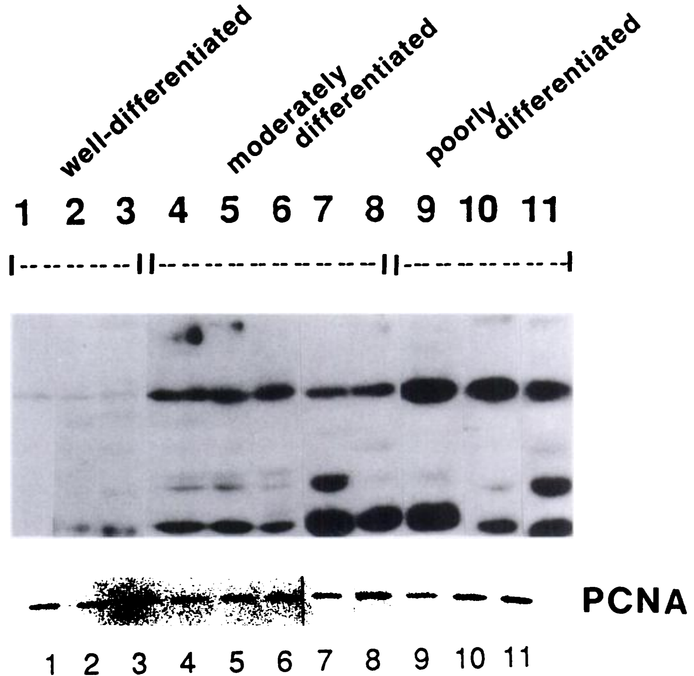

Fig. 4. Southwestern blot analysis of p114 MAR-binding activity in breast ductal carcinomas of various histological gradings and clinical stages. Forty μg of proteins extracted from breast carcinoma tissues (infiltrating ductal carcinomas) were subjected to Southwestern blot analysis using the radiolabeled wild-type $(25)_7$ probe. *Left panel*, summary of data for 43 breast carcinoma specimens, including clinicopathological status. The level of p114 MAR-binding activity was estimated by laser densitometric analysis of the p114 band on Southwestern blots. Lymph node status information was not available (*n.a.*) for certain carcinoma specimens. Histological grades are shown for infiltrating ductal carcinomas. Other types of carcinomas tested are indicated: *a*, infiltrating lobular carcinoma; *b*, mucinous carcinoma. *No.*, tumor identification number. *Right panel*, Southwestern blot analysis of selected carcinoma specimens. Well differentiated (*Lanes 1–3*), moderately differentiated (*Lanes 4–8*), and poorly differentiated tumors (*Lanes 9–11*). *Lanes 1–3*, tumor number 41–43; *Lane 4*, tumor number 17; *Lane 5*, tumor number 20; *Lane 6*, tumor number 18; *Lane 7*, tumor number 24; *Lane 8*, tumor number 19; *Lane 9*, tumor number 1; *Lane 10*, tumor number 2; *Lane 11*, tumor number 3. The same amount of protein used for Southwestern blot was subjected to Western blot analysis using anti-PCNA antibody.

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(K_d) in the range of 10^{-9} M (Fig. 1*C, upper panel*), as determined under conditions of protein excess (14). However, the protein exhibited much reduced binding, approximately 30-fold less, to the mutated $(24)_2$ probe (Fig. 1*C, bottom panel*). This indicates that p114 is not a nonspecific, A+T-rich DNA binding protein. Thus, p114 has a binding specificity and a strong affinity for MARs similar to SATB1.

A MAR-binding Protein Exists in Human Breast Carcinomas but not in Benign Breast Disease and Normal Tissues. Having identified p114 as a MAR-binding protein from SK-BR-3 cells and infiltrating ductal carcinomas, we next examined whether p114 is specifically expressed in breast carcinomas or if it is also expressed in normal breast tissues. Southwestern blot analysis was performed for five cases of infiltrating ductal carcinomas and their adjacent normal tissues and for two normal breast tissues from healthy individuals A and B. The MAR-binding activity due to p114, indicated by an arrowhead, was detected in all five carcinomas (Fig. 2, *Lanes 5, 7, 9, 11,* and *13*) and was undetected in normal breast tissues (Fig. 2, *Lanes 4, 6, 8, 10,* and *12*). To confirm that a similar amount of protein was loaded in each lane, the same amount of protein used in the Southwestern blot was subjected to Western blot analysis using anti-α-tubulin antibody (Fig. 2). We also performed a gel-mobility shift assay, which is more sensitive than Southwestern methods, using crude whole-cell extracts prepared from breast carcinomas and their adjacent normal breast tissues. Proteins extracted from normal breast tissues showed no MAR-binding activity at all, while proteins extracted from carcinoma specimens exhibited a strong MAR-binding activity (data not shown).

To further investigate whether the p114 MAR-binding activity is specifically present in carcinoma cells, we examined 17 cases of benign breast lesions including fibroadenoma, proliferative fibrocystic change, fibrocystic change, atypical ductal hyperplasia, and atypical lobular hyperplasia (20) by the Southwestern blot method. Atypical ductal and lobular hyperplasias are proliferative epithelial lesions with premalignant potential. None of the benign lesions (including atypical ductal and lobular hyperplasias) showed a p114 band on a Southwestern blot. Data of representative specimens are shown in Fig. 3*A, Lanes 2–8*.

We also examined three breast carcinoma cell lines and a spontaneously immortalized human epithelial MCF-10A (21) by the Southwestern blot analysis. Although p114 MAR-binding activity was detected in all breast carcinoma cell lines tested, it was undetected in MCF-10A cells. In contrast, an A+T-rich, DNA-binding protein of lower molecular weight was commonly present in crude extracts from all four cell lines tested, including MCF-10A (Fig. 3*B*). This particular protein is apparently a nonspecific, A+T-rich DNA-binding protein, because it was removed by the DNA affinity column containing mutated $(24)_n$. Again, the quantity of proteins loaded in each lane was comparable as confirmed by Western blot using anti-α-tubulin antibody. The data show that the p114 MAR-binding activity represents a true difference between malignant cells and nonmalignant proliferating cells. Thus, p114 has excellent potential to become a tumor marker for malignancy and would provide a unique diagnostic tool for breast lesions. For example, it may have a potential usage in differentiating between atypical ductal hyperplasia from DCIS, which is not always straightforward (22).

Stronger p114 MAR-binding Activity Is Associated with Poorly Differentiated Breast Carcinomas. It is important to determine whether p114 MAR-binding activity is detectable in every breast carcinoma or only in a subpopulation of breast carcinomas. For this purpose, a total of 43 breast carcinoma specimens of different histological gradings and clinical stages were examined for MAR-binding activity by the Southwestern method with the wild-type $(25)_7$ probe. All 43 specimens showed positive signals for p114 without exception. Weak but clear signals were detected, even for DCIS, with or without microinvasion. In addition to infiltrating ductal carcinomas, strong positive signals were detected for infiltrating lobular carcinoma and mucinous carcinoma. This is significant because all other known markers are found only in a subset of breast tumors; for example, overexpression of c-erbB-2 or p53 proteins is found only in about one-half of the tumors with a rapid proliferation rate (5). The data for all 43 specimens are summarized in Fig. 4, *left panel,* and results from Southwestern blot analysis of selected specimens are shown in Fig. 4, *right panel.*

The intensity of the p114 band varied among tissue specimens for the same amount of protein. The relative intensity was determined by laser densitometric analysis. The p114 band intensity was generally found to be greater for poorly differentiated infiltrating ductal carcinomas than for moderately differentiated carcinomas. Well-differentiated predominantly intraductal carcinomas with histological grade I displayed substantially weaker signals for the p114 band. The maximum difference in the p114 band intensity between well-differentiated carcinomas and poorly differentiated carcinomas is approximately 50-fold (Fig. 4, *right panel, Lanes 1–3 versus Lanes 9* and *10*). We also performed Western analysis for these specimens using monoclonal antibody against PCNA, which is a cell proliferation marker (23). By Western blotting, Keyomarsi *et al.* (24) showed that breast carcinomas have a substantially higher amount of PCNA in comparison to that of normal breast tissues. All extracts from these breast cancer tissues (Fig. 4, *right panel*) exhibited comparable amounts of PCNA on a Western blot. Although it is possible that some carcinoma specimens dissected may be more heavily contaminated by normal cells than others, the Western blot data for PCNA strongly suggest that this set of tumor specimens well represent the tumor portions of the dissected breast cancer tissue. Unlike PCNA, which is present in normal proliferating cells, p114 MAR-binding activity is restricted to carcinoma cells.

Furthermore, despite similar PCNA amounts detected in each carcinoma specimen (Fig. 4, *right panel*), the p114 MAR-binding activity progressively increased as the differentiation status became poorer. In addition, the p114 signal is undetectable in proliferating MCF-10A cells (Fig. 3B), at least by the experimental procedure described; these results, taken together, indicate that p114 MAR-binding activity is not a mere reflection of cell proliferation.

The results presented in this study strongly suggest that the MAR-binding activity of p114 has a high potential as a reliable marker for distinguishing malignant from nonmalignant breast lesions. The protein not only provides a valuable tool for the early detection of breast carcinomas, but it will also open a new avenue toward identifying the critical difference in the regulatory mechanisms between carcinomas and normal cells at a molecular level. To assess the diagnostic potential of p114 at a single-cell level, as well as to study its role in breast tumorigenesis, our laboratory is focusing on cDNA cloning of and generating immunological reagents for this protein.

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References

1. Devilee, P., and Cornelisse, C. J. Genetics of human breast cancer. Cancer Surv., *9:* 605–630, 1990.
2. Ravdin, P. M., and Chamness, G. C. The c-*erb*B-2 proto-oncogene as a prognostic and predictive marker in breast cancer: a paradigm for the development of other macromolecular markers–a review. Gene (Amst.), *159:* 19–27, 1995.
3. Callahan, R. p53 mutations, another breast cancer prognostic factor. J. Natl. Cancer Inst., *84:* 826–827, 1992.
4. Davidoff, A. M., Humphrey, P. A., Iglehart, J. D., and Marks, J. R. Genetic basis for p53 overexpression in human breast cancer. Proc. Natl. Acad. Sci. USA, *88:* 5006–5010, 1991.

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5. Isola, J., Visakorpi, T., Holli, K., and Kallioniemi, O-P. Association of overexpression of tumor suppressor protein p53 with rapid cell proliferation and poor prognosis in node-negative breast cancer patients. J. Natl. Cancer Inst., *84:* 1109–1114, 1992.
6. Nelson, W. G., Pienta, K. J., Barrack, E. R., and Coffey, D. S. The role of the nuclear matrix in the organization and function of DNA. Annu. Rev. Biophys. Chem., *15:* 457–475, 1986.
7. Cockerill, P. N., and Garrard, W. T. Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. Cell, *44:* 273–282, 1986.
8. Gasser, S. M., and Laemmli, U. K. A glimpse at chromosomal order. Trends Genet., *3:* 16–22, 1987.
9. Forrester, W. C., van Genderen, C., Jenuwein, T., and Grosschedl, R. Dependence of enhancer-mediated transcription of the immunoglobulin μ gene on nuclear matrix attachment regions. Science (Washington DC), *265:* 1221–1225, 1994.
10. Dickinson, L. A., Joh, T., Kohwi, Y., and Kohwi-Shigematsu, T. A tissue-specific MAR/SAR DNA binding protein with unusual binding site recognition. Cell, *70:* 631–645, 1992.
11. Fey, E. G., and Penman, S. Nuclear matrix proteins reflect cell type of origin in cultured human cells. Proc. Natl. Acad. Sci. USA, *85:* 121–125, 1988.
12. Khanuja, P. S., Lehr, J. E., Soule, H. D., Gehani, S. K., Noto, A. C., Choudhury, S., Chen, R., and Pienta, K. J. Nuclear matrix proteins in normal and breast cancer cells. Cancer Res., *53:* 3394–3398, 1993.
13. Millis, R. R., Hanby, A. M., and Girling, A. C. The Breast. *In:* S. S. Sternberg, D. A. Antonioli, D. Carter, S. E. Mills, and H. A. Oberman (eds.), Diagnostic Surgical Pathology, Ed. 2, pp. 323–408. New York: Raven Press, 1994.
14. Dickinson, L. A., and Kohwi-Shigematsu, T. Nucleolin is a matrix attachment region DNA-binding protein that specifically recognizes a region with high base-unpairing potential. Mol. Cell. Biol., *15:* 456–465, 1995.
15. Fackelmayer, F. O., Dahm, K., Renz, A., Ramsperger, U., Richter, A. Nucleic acid-binding properties of hnRNP-U/SAF-A, a nuclear-matrix protein which binds DNA and RNA *in vivo* and *in vitro*. Eur. J. Biochem., *221:* 749–757, 1994.
16. Kohwi-Shigematsu, T. and Kohwi, Y. Torsional stress stabilizes extended base unpairing in suppressor sites flanking immunoglobulin heavy chain enhancer. Biochemistry, *29:* 9551–9560, 1990.
17. Bode, J., Kohwi, Y., Dickinson, L., Joh, T., Klehr, D., Mielke, C., and Kohwi-Shigematsu, T. Biological significance of unwinding capability of nuclear matrix-associating DNAs. Science, *255:* 195–197, 1992.
18. von Kries, J. P., Buck, F., and Stratling, W. H. Chicken MAR binding protein p120 is identical to human heterogeneous nuclear ribonucleoprotein (HnRNP) U. Nucleic Acids Res., *22:* 1215–1220, 1994.
19. von Kries, J. P., Buhrmester, H., and Stratling, W. H. A matrix/scaffold attachment region binding protein: identification, purification, and mode of binding. Cell, *64:* 123–135, 1991.
20. Shnitt, S. J., and Connolly, J. L. Pathology of benign breast disorders. *In:* J. R. Harris, S. Hellman, I. C. Henderson, and D. W. Kinne (eds.), Breast Diseases, Ed. 2, pp. 15–30. Philadelphia: J. B. Lippincott Co., 1991.
21. Soule, H. D., Maloney, T. M., Wolman, S. R., Peterson, W. D., Brenz, R., McGrath, C. M., Russo, J., Pauley, R. J., Jones, R. F., and Brooks, S. C. Isolation and characterization of a spontaneously immortalized human breast epithelial cell lines, MCF-10. Cancer Res., *50:* 6075–6086, 1990.
22. Parham, D. M., and Jankowski, J. Transforming growth factor alpha in epithelial proliferative diseases of the breast. J. Clin. Pathol., *45:* 513–516, 1992.
23. Bravo, R., Frank. R., Bundell, P., *et al.* Cyclin/PCNA is the auxiliary protein of DNA polymerase delta. Nature (Lond.), *326:* 515–517, 1987.
24. Keyomarsi, K., O'Leary, N., Molnar, G., Lees, E., Fingert, H. J., and Pardee, A. B. Cyclin E, a potential prognostic marker for breast cancer. Cancer Res., *54:* 380–385, 1994.

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A Matrix Attachment Region (MAR)-binding Activity Due to a p114 Kilodalton Protein Is Found Only in Human Breast Carcinomas and Not in Normal and Benign Breast Disease Tissues

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