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S100A9 expression in invasive ductal carcinoma of the breast: S100A9 expression in adenocarcinoma is closely associated with poor tumour differentiation

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Abstract

S100A9 is associated with myelomonocytic cell differentiation and is also expressed in some epithelia. However, there have been few studies on S100A9 in adenocarcinoma (AC) because the expression in normal epithelia is limited to squamous epithelia. Our previous studies on pulmonary AC and liver carcinomas suggested that S100A9 expression in carcinomas of glandular cell origin is related to poor tumour differentiation. In this study, we examined S100A9 expression in invasive breast carcinoma and evaluated the relation of the expression to the tumour differentiation in 70 cases of invasive ductal carcinoma (IDC) of the breast. S100A9 gene and protein expression was detected in MCF-7 breast carcinoma cells. The rate of S100A9 immunopositivity in IDC showed a higher correlation with poor tumour differentiation, especially in nuclear pleomorphism ($P=0.0002$) and mitotic activity ($P=0.0001$). Furthermore, transcriptional expression of S100A9 in sections of IDC could be detected in cases with a high S100A9 immunopositivity. No significant differences in the number of myelomonocytic cells expressing S100A9 were found among cases. There was no correlation between S100A9 immunopositivity and lymph node metastasis ($P=0.32$). S100A9 immunopositivity in non-invasive ductal carcinoma was also associated with poor tumour differentiation. No immunopositive reaction was observed in invasive lobular carcinomas with a classic cytological appearance and non-neoplastic duct cells. We conclude that S100A9 in glandular epithelial cells is newly expressed under cancerous conditions and is over-expressed in poorly differentiated AC.
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1. Introduction

The group of human S100 calcium-binding proteins is known to comprise 20 members [1] and to be widely distributed in various tissues [1–3]. Although the biological functions of this protein family have not been clearly elucidated, it is considered to influence diverse cellular processes including cell-cycle progression, cell differentiation and malignant transformation [1–5]. Recently, the S100 protein family has become of particular interest because of its differential expression in normal or neoplastic tissues [1–6]. The correlation between carcinoma differentiation, invasion and metastasis and the production of S100 protein has also been extensively studied [7,8].

S100A9 was originally discovered in human granulocytes and macrophages and has been shown to be associated with myelomonocytic cell differentiation [1,9–11]. This protein is also expressed in certain types of epithelial and carcinoma cells [5,12–14]. S100A9 expression is ubiquitously observed in the squamous epithelia under normal and cancerous conditions [12–14]. However, there have been few studies on S100A9 in specific types of carcinomas, except for squamous cell carcinoma (SCC), because its expression is not demonstrated in the epithelia of non-squamous-typed cells under normal

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conditions [12,13]. Recently, S100A9 gene expression has been detected in cultured human adenocarcinoma (AC) cells derived from various organs [15,16]. Furthermore, our recent study [16] showed that S100A9 is also expressed in human pulmonary AC, suggesting that its expression is closely associated with tumour differentiation and shows a higher correlation with poor differentiation. S100A9 may be associated not only with myelomonocytic cell differentiation, but also with the differentiation of carcinomas of glandular cell origin [16,17].

In this study, we examined the gene and protein expression of S100A9 in primary invasive breast carcinoma (IBC) and evaluated the relation of S100A9 expression to the differentiation of invasive ductal carcinoma (IDC) of the breast.

2. Materials and methods

2.1. Cell and culture

An established human IBC cell line, MCF-7, was maintained in Dulbecco modified Eagle medium (DMEM) (Nissui, Tokyo, Japan), supplemented with 10%(v/v) heat-inactivated fetal bovine serum (Sigma Japan, Tokyo) and 100 μg/ml each of sodium ampicillin and kanamycin sulphate at 37 °C under humidified 5% (v/v) CO_2/95% (v/v) air. As a control, neutrophils were prepared from peripheral blood as previously described [10].

2.2. Reverse transcription-polymerase chain reaction (RT-PCR) in MCF-7 cells

Total RNA was isolated from MCF-7 cells using an RNeasy mini kit (QIAGEN, Tokyo). First-strand cDNA was synthesised as described previously [3,16]. The S100A9 cDNA was amplified by PCR using sense (5′-GTCGCAGCTGGAACGCAACA-3′) and antisense (5′-CCTGGCCTCCTGATTAGTGG-3′) primers [3]. The specificity of these primers has been confirmed by sequencing of the PCR product [3]. β-actin cDNA was also amplified [3,16]. As a control, CD68 cDNA (GenBank NM-001251) was also amplified by PCR using sense (5′-GGACTACCAAGAGCCACAA-3′) and antisense (5′-ATCCAAAGCTGAGGTGTCC-3′) primers from the human leukocyte cDNA library (Wako, Osaka, Japan). The annealing temperature was 56 °C, and the transcripts were analysed on 2%(w/v) agarose gel as stated [3,16]. The intensity of ethidium bromide fluorescence was measured by Scion Image.

2.3. Antibody

The establishment and characterization of the monoclonal antibody, mAb60B8, against S100A9 have been described elsewhere [10,11]. As the S100A9 antigen is resistant to formaldehyde, its presence can also be demonstrated in routine pathological materials by immunohistochemistry [11,12,16,17].

Recombinant S100 proteins were prepared and purified as described previously [6].

2.4. Western immunoblotting on MCF-7 cells

Sonic extracts were prepared from the MCF-7 cells and neutrophils as described previously [10]. Western immunoblotting was performed with mAb60B8 staining [18].

2.5. Immunohistochemistry

2.5.1. Tissue specimens

The histological subtype of breast carcinoma was classified according to the World Health Organisation criteria [19]. Seventy primary IDCs were included in this study. All of the tumours (2.1 cm mean dia.; ranging from 0.8 to 3.5 cm) were fixed in 10%(v/v) formalin, and paraffin-embedded tissue sections were routinely stained with haematoxylin-eosin. IDCs were histologically graded according to the criteria of the Nottingham method [20]. We interpreted nuclear atypia as scoring 1 if nuclei were uniform in size and shape with evenly distributed chromatin and inconspicuous nucleoli, and nuclear atypia scored 3 when nuclei showed pleomorphism and hyperchromatism with large nucleoli [21]. Furthermore, mitotic counts were assessed as the number of mitoses per 10 consecutive high-power fields at ×400 magnification using an Olympus BHS 523 5W (objective ×40, field dia. 0.50 mm, field area 0.196 mm^2) in areas with the highest density of mitotic figures, avoiding fields containing less than 50% tumour cells.

The breast tissues used as controls consisted of the following: invasive lobular carcinoma (ILC; $n=20$, mean dia. 3.8 cm mean dia.; ranging from 1.0 to 8.0 cm), non-invasive ductal carcinoma (NIDC; $n=25$, 4.1 cm mean dia.; ranging from 1.0 to 10.2 cm), lobular carcinoma in situ ($n=2$, 1.8 cm and 2.1 cm dia.each), duct hyperplasia ($n=5$), blunt duct adenosis ($n=5$) and normal breast tissues ($n=10$). We dealt with ILC as a control material and did not evaluate tumour differentiation because the histological grading is controversial [22,23]. However, ILCs were also classified by their growth pattern and cell morphology, including nuclear atypia [23,24]. The histological grading of NIDCs was by Holland's classification [25]. The latter three kinds of control tissues were derived from non-cancerous areas of IDC cases.

The histological subtype and degree of tumour differentiation of the breast carcinomas used in this study are listed in Table 1. Special types of IBC [19], such as medullary carcinoma, mucinous carcinoma, and Paget's disease of the nipple, were excluded from this study.

Table 1
Histological classification of the breast carcinomas used in this study

Subtype	Number of cases ($n=117$)
Invasive ductal carcinoma	70
Grade I	29
Grade II	24
Grade III	17
Invasive lobular carcinoma	20
Classic	16
Alveolar	1
Large cell	3
Non-invasive ductal carcinoma	25
Well	12
Mod	8
Poor	5
Lobular carcinoma in situ	2

Classic: classic form, Alveolar: alveolar variant form, Large cell: large cell variant form, Well; Well-differentiated carcinoma, Mod: Moderately differentiated carcinoma, Poor: Poorly differentiated carcinoma.

2.5.2. Staining of tissue sections

The protocol employed for immunohistochemistry is described elsewhere [11,16,17]. In brief, the deparaffinised, representative tissue sections were trypsinised and immunostained with mAb60B8, followed by the streptavidin-biotin technique using a Histofine SAB-PO (Multi) kit (Nichirei Co., Tokyo). As a negative control, the antibody preincubated with an excess of recombinant S100A9 for 18 h at 4 °C, or 0.01 M phosphate-buffered saline, were used instead of primary antibody [11].

No apparent immunoreactivity was detected in these control sections.

2.5.3. Evaluation of S100A9 immunostaining in tumour cells

The rate of immunopositivity observed in the tumour cells was scored as follows (16): score 0 (non-reactive), no immunoreactive tumour-cells were seen; score 1, less than 10% positive cells; score 2, greater than 10% but less than 50% positive cells; score 3, greater than 50% positive cells. The control materials were evaluated in the same way.

Immunostaining intensity was not evaluated in this study because we knew from our previous studies that staining intensity shows a positive correlation with the percentage of positively stained cells [16,17].

2.5.4. Quantification of myelomonocytic cells expressing S100A9 in IDC

In the tissue sections of IDC, mAb60B8-labelled myelomonocytic cells were counted in 30 microscopic fields (15 fields each in the tumour centre and invasion front) at ×400 magnification and expressed as the number of positive cells per 1 mm^2.

2.6. Detection of S100A9 transcripts in the tissue sections of IDC

To investigate the relation between the degree of tumour cell immunopositivity and the amount of S100A9 mRNA expression in IDC cases, we determined mRNA by RT-PCR, and compared S100A9 mRNA at each score of S100A9 immunopositivity. Tissue sections from 10 cases each with S100A9 immunopositivity scores 0, 1, 2, and 3 were examined. Each total RNA was isolated from a slice of 4 μm paraffin-embedded tissue sections from these 40 cases using a Paraffin Block RNA Isolation Kit (Ambion Inc, USA). First-strand cDNA was synthesised in a volume of 20 μl, containing 500 ng total RNA, 0.5 μl oligo $(dT)^{16}$ primer, 10 pmol dNTPs, 5 U *RAV2-RTase* and 1st Strand Synthesis buffer (TaKaRa, Kyoto, Japan). Synthesis was performed at 36 °C for 10 min, 42 °C for 50 min, 52 °C for 10 min and 99 °C for 5 min. PCR amplification was performed as previously described [3,16] using 1 μl first-strand cDNA as a template, and primers for S100A9 and 54 cycles. The annealing temperature was 56 °C, and the transcripts were analysed on 2%(w/v) agarose gel as stated [16]. For the semiquantitative analysis, the intensity of ethidium bromide fluorescence was measured by Scion Image.

2.7. Statistical analysis

Results were analysed for statistical significance by the comparison of two proportions (z-test), χ^2 test, Student t-test or Mann–Whitney U-test where appropriate, and $P<0.01$ was considered significant.

3. Results

3.1. Expression of the S100A9 gene in MCF-7 cells

Expression of S100A9 mRNA in the human IBC cell line, MCF-7, was detected by RT-PCR (Fig. 1a). The CD68 gene was not expressed in MCF-7 cells while CD68 cDNA was amplified by 36-cycle PCR from the human leukocyte cDNA library. S100A9 and β-actin cDNAs in MCF-7 cells were amplified PCR cycle dependently (Fig. 1b).

3.2. Expression of the S100A9 protein in MCF-7 cells

The mAb60B8 reacted with recombinant S100A9 and showed no cross-reactivity other S100 proteins except for a faint reaction with S100A8 (Fig. 2a,b). S100A9 protein expression in MCF-7 cells was immunodetected with mAb60B8 (Fig. 2c). The antibody stained a single, discrete protein band of 14 kDa.

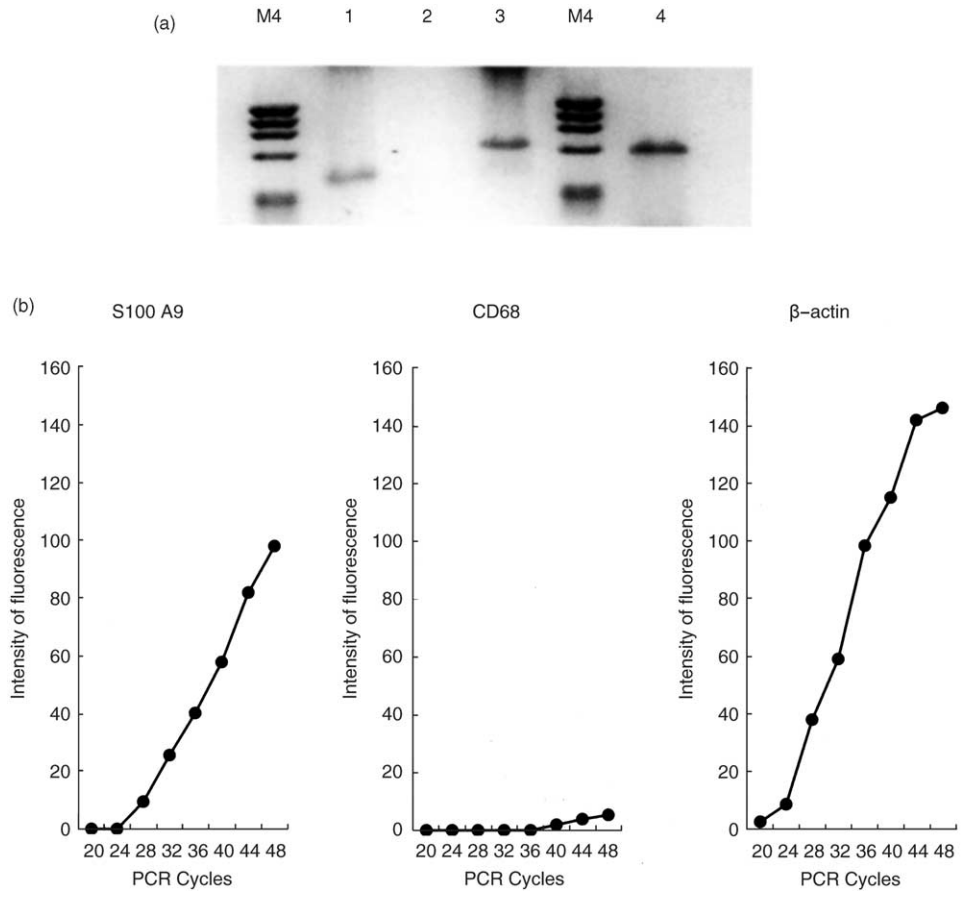
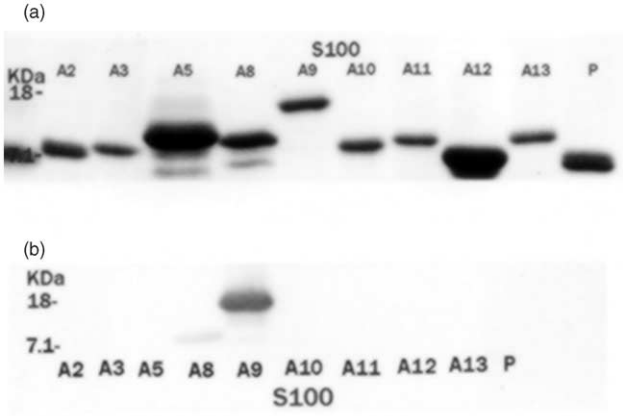


Fig. 1. Expression of S100A9 mRNA in MCF-7 cells. (a) Transcripts of S100A9 (lane 1) and β-actin (lane 3) in MCF-7 cells are amplified by 36 cycle reverse transcription-polymerase chain reaction (RT-PCR). CD68 (lane 2) is not expressed in MCF-7 cells while CD68 cDNA is amplified by 36-cycle PCR from the human leukocyte cDNA library (lane 4). M4: DNA size marker (Wako; 1353, 1078, 872, 603 and 310 bp from the top). (b): The S100A9 and β-actin cDNAs in MCF-7 cells are amplified PCR cycle dependently.

3.3. Immunohistochemical evaluation of S100A9 expression in IDC

S100A9 protein expression was immunohistochemically detected in the tumour cells of 35 of the 70 IDC cases. Immunoreactivity for S100A9 was heterogeneous among the tumour cells, and the staining intensity of the individual carcinoma cells varied from absent to intense. The S100A9 immunopositivity rate in each histological grade of tumour differentiation was 4/29 (13.8%) in grade I IDCs, 14/24 (58.3%) in grade II IDCs and 17/17 (100%) in grade III IDCs (Table 2; Fig. 3). Especially in grade III IDC, the rate of cases in which the percentage of positive cells scored 3 was significantly higher than that of grade II IDC ($P=0.0050$, z-test; Table 2).

Usually, the histological grading of a carcinoma is accomplished by a general evaluation of both the cellular and structural atypism, and nuclear pleomorphism is a basic index of cellular atypism. The relation between S100A9 immunoreactivity and nuclear pleomorphism or tubule formation in the 70 IDCs is shown in Tables 3 and 4, respectively. In the IDCs with a nuclear pleomorphism score of 3, S100A9 immunopositivity was higher than in IDCs in which the nuclear pleomorphism score was 1 or 2, and the rate of the cases with positivity scores of 3 was also significantly higher than that of cases in which the nuclear pleomorphism score was 2 ($P=0.0002$, z-test; Table 3). In contrast, for the rate of the cases with immunopositivity scores of 3, there was no significant difference between the cases with tubule formation scores of 3 and 2 ($P=0.15$, z-test; Table 4). On the other hand, the S100A9 immunopositivity rate for each score of mitotic counts was 8/32 (25%) in IDCs that scored 1, 13/22 (59.1%) in IDCs that scored 2 and 14/16 (87.5%) in IDCs that scored 3, and mitotic counts showed a significant correlation with S100A9 immunoreactivity ($P=0.0001$, χ^2-test). Lymph node metastasis was observed histologically in 26 of the 70 IDC cases. The S100A9 immunopositivity rate in these 26 cases was 15/26 (57.7%), showing no significant correlation between S100A9 immunopositivity and node metastasis ($P=0.32$, χ^2-test).



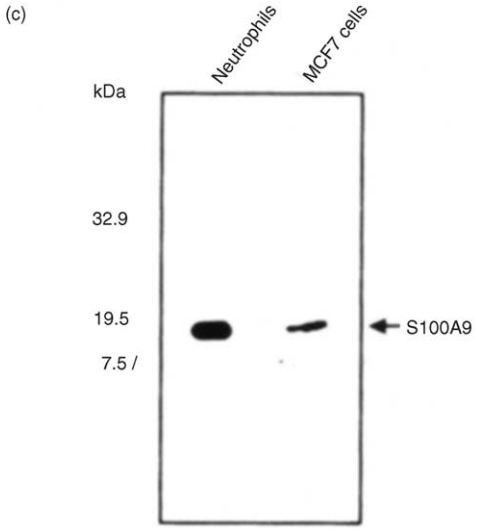


Fig. 2. Characterisation of mAb60B8 and Western immunoblot of S100A9 in MCF-7 cells. (a) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of recombinant S100 proteins; 2–4 μg S100 protein were applied in each lane followed by Coomassie brilliant blue staining. (b) Cross-reactivity of mAb60B8. S100 proteins were immunostained with the antibody after Western blotting. The intensity of each image was estimated as S100A8 = 138 and S100A9 = 1648 by *Densito graph 2.02* (ATTO). (c) Expression of S100A9 protein in MCF-7 cells. A strongly positive band is seen for the extract taken from neutrophils as a control. The lysate (160 μg protein) of MCF-7 cells and neutrophils (20 μg protein) was applied in each lane.

In the carcinoma cases used as controls, immunopositivity for S100A9 was detected in tumour cells of the ILC and NIDC, but not the lobular carcinoma in situ. No immunopositivity was found in duct hyperplasia, blunt duct adenosis or normal duct cells of the breast. In ILC cases, S100A9 immunopositivity was detected only in the large-cell variant, and the positivity in this form scored 2 or greater. The positively stained ILC cells were identified by both morphology and immunohistochemistry for epithelial markers on the serial sections. The tumour cells in this variant had abundant cytoplasm and nuclear pleomorphism, scoring 2 or 3

Table 2
Relationship between S100A9 immunoreactivity and the histological grade of the tumour differentiation in 70 invasive ductal carcinomas

S100A9 immunoreactivity	Histological grade		
	Grade I ($n=29$)	Grade II ($n=24$)	Grade III ($n=17$)
Non-reactive (Score 0. $n=35$)	25	10	–
Reactive ($n=35$)	4	14	17
Score 1	4	6	2
Score 2	–	6	4
Score 3	–	2	11

n; number of cases.

with the criteria of the Nottingham method [20]. In contrast, the classic and alveolar variant forms, i.e. the forms in which no significant positive reaction was found, consisted of small or medium-sized cells with scanty cytoplasm and relatively uniform round nuclei. In contrast, among the NIDC, S100A9 immunopositivity in each histological grade of tumour differentiation was 0/12 in well-differentiated NIDCs, 3/8 in moderately differentiated NIDCs and 5/5 in poorly differentiated NIDCs. Furthermore, the finding of greater than 50% positive tumour cells was limited to 5 cases of poorly differentiated NIDC.

3.4. Density of myelomonocytic cells expressing S100A9 in IDC

Myelomonocytic cells showed strong positivity, regardless of the histological features of the breast. The density of mAb60B8-labelled myelomonocytic cells within IDC tissue sections ranged from 5 cells/mm^2 to 35 cells/mm^2 (mean ± S.D.: 16.90 ± 9.48 cells/mm^2). The density of the myelomonocytic cells for each score of S100A9 immunopositivity is shown in Table 5. No significant differences in the number of myelomonocytic cells expressing S100A9 were found for each score of S100A9 immunopositivity ($P > 0.05$, Mann–Whitney U-test; Table 5).

3.5. S100A9 mRNA expression in the tissue sections of IDC

Expression of S100A9 mRNA could only be detected easily in sections of the 10 cases in which S100A9 immunopositivity scored 3, and S100A9 cDNA fragments were faintly amplified from all tissue sections of the 10 cases that had positivity scores of 2 (Fig. 4). In contrast, transcriptional expression of S100A9 was not detected in any of the 20 cases in which S100A9 immunopositivity scored 0 or 1 at 54 cycles of RT-PCR (Fig. 4). The levels of S100A9 mRNA expression in 10 cases each with S100A9 immunopositivity scores of 0, 1,

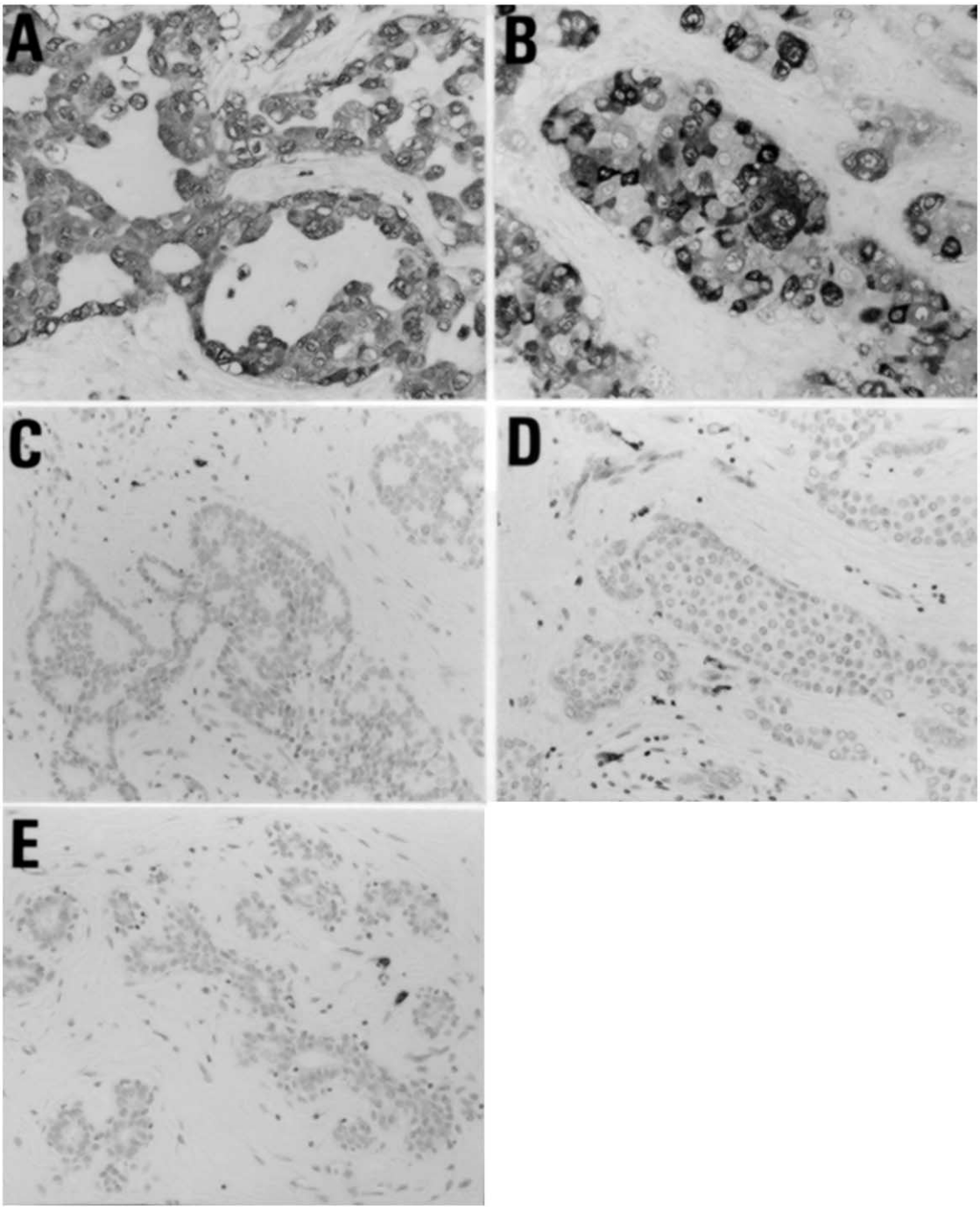


Fig. 3. Immunohistochemical staining for S100A9 in invasive ductal carcinomas (IDCs) (×165). (A, B) A positive reaction is seen in numerous tumour cells of the IDCs showing a tubular or solid growth pattern with apparent nuclear and cytoplasmic polymorphism. (C, D) No staining of tumour cells for S100A9 is visible in IDCs with little nuclear and cytoplasmic polymorphism, regardless of the presence or absence of tubular formation. However, positive staining is observed in small numbers of infiltrating myelomonocytic cells like normal breast tissue (E). (A) grade II IDC (3, 2, 1); (B) grade III IDC (3, 3, 2); (C) grade I IDC (1, 1, 1); (D) grade I IDC (1, 3, 1). The parenthesised numbers are scores of nuclear pleomorphism, tubular formation, and mitotic activity respectively.

2, and 3 are shown in Fig. 5. The level in the cases in which S100A9 immunopositivity scored 3 was significantly higher (97.98 ± 8.44) than that in those which scored 2 (35.22 ± 6.91)($P < 0.0001$, Student t-test). S100A9 mRNA levels in the cases in which the positivity score was 0 or 1 measured 0 at 54 cycles of RT-PCR.

4. Discussion

In this study, gene and protein expression of S100A9 was detected in MCF-7, a widely used IBC cell line. Recently, we also confirmed S100A9 expression in human pulmonary AC cell lines [16]. It is clear that

Table 3
Relationship between S100A9 immunoreactivity and the nuclear pleomorphism in 70 invasive ductal carcinomas

S100A9 immunoreactivity	Score of the nuclear pleomorphism		
	1 ($n=18$)	2 ($n=31$)	3 ($n=21$)
Non-reactive (Score 0. $n=35$)	16	19	–
Reactive ($n=35$)	2	12	21
Score 1	2	6	6
Score 2	–	4	4
Score 3	–	2	11

Table 4
Relationship between S100A9 immunoreactivity and the tubular formation in 70 invasive ductal carcinomas

S100A9 immunoreactivity	Score of the tubular formation		
	1 ($n=19$)	2 ($n=22$)	3 ($n=29$)
Non-reactive (Score 0. $n=35$)	15	10	10
Reactive ($n=35$)	4	12	19
Score 1	4	4	4
Score 2	–	4	6
Score 3	–	4	9

S100A9 is expressed not only in SCC, but also in certain types of AC [5,15,16]. We could detect S100A9 immunopositivity in breast carcinoma cells, but not in non-neoplastic epithelia of the breast. The immunopositivity rate in IDC showed a higher correlation with poor tumour differentiation, especially with cellular atypism and mitotic activity. However, there was no significant correlation between S100A9 immunopositivity and node metastasis.

Since S100A9 is a secretory protein [1,2], we examined whether S100A9 immunopositivity in IDC cells is derived from protein over-expression or the accumulation of protein accompanied by the dedifferentiation of carcinoma cells. The transcriptional expression of S100A9 could only be detected clearly in the tissue sections of cases in which a high S100A9 immunopositivity was observed. For the number of myelomonocytic cells expressing S100A9, there were no significant differences between each score of S100A9 immunopositivity. Therefore, we consider that S100A9 derived from myelomonocytic cells scarcely affects case comparisons of tissue sections of IDC and that a high S100A9 immunopositivity indicates the over-expression of S100A9 protein in carcinoma cells.

S100A9 immunopositivity rate in NIDC was also associated with poor tumour differentiation. In ILC cases, we observed S100A9 immunopositivity only in the large-cell variant form. This variant shows the classic growth pattern, but the tumour cell morphology is outside that of classic ILC [23,24]. Accordingly, we think that S100A9 expression in ILC is also related to the degree of cellular atypism of the tumour cells.

From the above results, we suggest that: (1) S100A9 is newly expressed in mammary gland epithelia under cancerous conditions; (2) The level of S100A9 expression in breast carcinoma is closely associated with the histological differentiation and cytonuclear features of the carcinoma; (3) S100A9 expression in ductal carcinoma is not directly correlated with the invasiveness and node metastasis of the carcinoma. The expression of S100A4, S100A7, S100A1 and S100B in IBC has been examined previously [8,26–28]. Of these four S100 proteins, S100A7, S100A1 and S100B have no correlation with the histological grade of IBC [26,27]. The relation between S100A4 expression and the histological grade of IBC is controversial [8,28]. Of the above three suggestions, (1) and (2) were in accord with our previous results regarding pulmonary AC and liver carcinomas [16,17]. As mentioned, we conclude that S100A9 in glandular epithelial cells is newly expressed under cancerous conditions and is over-expressed in poorly differentiated AC, being related to tumour differentiation.

Recently, it has been reported that S100A9 is also over-expressed in gastric AC [5]. However, the functional role of S100A9 in the carcinogenesis of AC has never been elucidated. In the lung [16], liver [17] and breast, we could find no S100A9 over-expression, not only in the normal epithelia, but also in adenomatous hyperplasia and well-differentiated AC (including grade I hepatocellular carcinoma of the Edmondson–Steiner classification). In contrast, S100A9 over-expression was easily observed in ACs with poorly differentiated cytonuclear features such as cellular atypism and mitotic activity. Therefore, S100A9 expression in AC is closely

Table 5
The density of myelomonocytic-cells expressing S100A9 in each score of S100A9 immunoreactivity in 70 invasive ductal carcinomas

Tumour cell immunoreactivity	Score 0	Score 1	Score 2	Score 3
Myelomonocytic cell density[a]	17.18±10.42	19.60±8.26	14.50±7.05	15.90±9.54

[a] All values are expressed as means (positive cells/mm^2) with standard deviation. Mann–Whitney U-test: Score 0 versus Score 1, U = 246 (n1 = 35, n2 = 12), $P=0.379$; Score 0 versus Score 2, U = 150 (n1 = 35, n2 = 10), $P=0.496$; Score 0 versus Score 3, U = 191 (n1 = 35, n2 = 13), $P=0.395$; Score 1 versus Score 2, U = 32, $P>0.05$; Score 1 versus Score 3, U = 54, $P>0.05$; Score 2 versus Score 3, U = 70, $P>0.05$.

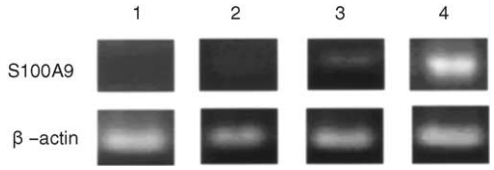


Fig. 4. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of S100A9 expression in the tissue sections of invasive ductal carcinomas. RT-PCR product was obviously detected in cases with S100A9 immunopositivity score 3 (lane 4), but not in the cases (with positivity score 0 (lane 1) or 1 (lane 2), after 54 cycles of RT-PCR. S100A9 cDNA fragments were faintly amplified in the cases that had positivity scores of 2 (lane 3). Lane 1, S100A9 immunopositivity score 0; lane 2, immunopositivity score 1; lane 3, immunopositivity score 2; lane 4, immunopositivity score 3.

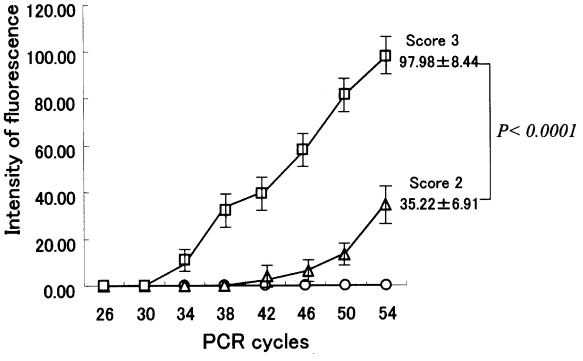


Fig. 5. Semiquantitative analysis of reverse transcription-polymerase chain reaction expressions in each score of S100A9 immunopositivity in invasive ductal carcinomas. S100A9 mRNA level in the cases ($n=10$) with positivity scores of 3 (□), is significantly higher than that in those ($n=10$) that had positivity scores of 2 (△) ($P<0.0001$). ○, S100A9 immunopositivity scores 0 and 1; △, immunopositivity score 2; □, immunopositivity score 3.

associated with cytomorphological changes and cell proliferation, accompanied by the dedifferentiation of AC cells, rather than with the early carcinogenic event.

It is known that some members of the S100 protein family regulate the reorganisation of cytoskeletal filaments [1,2]. S100A9 is also thought to be involved in the Ca^{2+}-dependent regulation of the assembly or disassembly of cytokeratin (CK) in epithelial cells [14]. Generally, cell shape and cell differentiation are influenced by the organisation of cytoskeletal filaments [29]. If S100A9 expression is simply correlated with the cellular atypism of carcinoma cells, undifferentiated carcinomas should show a high expression rate. However, S100A9 expression is hardly observed in undifferentiated carcinoma [16,17], so we suggest that epithelial differentiation in carcinoma cells is necessary for S100A9 expression. It is known that the CK expression patterns in epithelial cells undergo various modulations and alterations during cancerisation and subsequent dedifferentiation [29,30], and it is thought that CK expression diminishes and disappears in undifferentiated carcinoma [30]. Although the molecular biological mechanisms of the alterations in cellular CK protein composition are still unclear, such modifications of intermediate filament expression often occur irregularly, resulting in phenotypical heterogeneity [30] or, in the case of neoplasms, tumour cell heterogeneity. In poorly differentiated IDC, the coexpression of glandular epithelial CKs and one or more of the squamous epithelial CKs is described [31].

On the basis of the above considerations, we deduce that S100A9 expression in AC is associated with changes in the CK protein composition accompanied by the dedifferentiation of the carcinoma, and that the immunohistochemical heterogeneity of S100A9 protein expression is derived from the characteristics of CK protein composition in individual carcinoma cells. The relation between S100A9 expression in carcinoma cells and CK expression patterns appears to be an important area for further investigation.

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