

S100A8 and S100A9 Expression Is a Crucial Factor for Dedifferentiation in Thyroid Carcinoma

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Abstract. *Background:* S100A8 plays a role in various functions of myeloid cells by forming a heterocomplex with S100A9. S100A8 and S100A9 are also known to be overexpressed in certain species of carcinomas. *Materials and Methods:* In this study, the protein expression of S100A8 as well as that of S100A9 was investigated in thyroid tumors. *Results:* All of the undifferentiated carcinomas were immunopositive for S100A8 and S100A9 and overlap between staining patterns of both proteins was observed. In poorly differentiated carcinomas, all the cases were negative for S100A8, while slight immunopositivity of S100A9 was seen in 2 cases. Papillary carcinoma, follicular carcinoma, follicular adenoma and medullary carcinoma and normal follicles were negative for both proteins. *Conclusion:* S100A8 plays an important role in dedifferentiation of thyroid carcinoma possibly by forming a complex with S100A9.

Thyroid carcinoma is one of the most common malignancies originating from the endocrine organs. There are two histological types, papillary and follicular carcinomas, arising from the follicular cells. These two histological types have different clinical characteristics, that is, papillary carcinoma frequently metastasizes to the regional lymph node and follicular carcinoma predominantly metastasizes to the distant organs. These carcinomas generally have mild biological characteristics but when they dedifferentiate and become

undifferentiated carcinoma, the lesions display extremely rapid growth with a dire prognosis (1). Poorly differentiated carcinoma is an entity proposed by Sakamoto *et al.* (2) and Carcangiu *et al.* (3). In 2004, poorly differentiated carcinoma was adopted as an independent histological type showing an intermediate prognosis between that of well and undifferentiated carcinomas in the World Health Organization (WHO) classification (4); a tumor is diagnosed as poorly differentiated carcinoma when any of the three histological patterns, insular, trabecular or solid, is recognized in the majority of the tumor together with an infiltrative pattern of growth, necrosis and obvious vascular invasion. Unlike these carcinomas, medullary carcinoma, another histological type of thyroid carcinoma, originates from the calcitonin-producing cells (C-cells). Approximately, 25% of them express a hereditary autosomal-dominant trait based on germline rearrangement during transfection (*RET*) gene mutation (5).

S100 proteins belong to the superfamily of EF-hand calcium-binding proteins, and there are more than 20 components (6-8) in this family. Among these, S100A8 and S100A9 are known to form a stable heterocomplex in myeloid cells and their expression levels are elevated during many inflammatory diseases (6-10). They play a role in leukocyte adhesion and transendothelial migration. Previous studies have proven that these proteins are also expressed in normal, inflammatory and neoplastic squamous epithelia (11, 12). Furthermore, we have demonstrated that S100A9 protein is overexpressed in various carcinomas of glandular cell origin, being associated with poor tumor differentiation (13-15). In thyroid carcinoma, S100A9 expression is observed exclusively in undifferentiated carcinoma with few exceptions, indicating that S100A9 plays an important role in carcinoma dedifferentiation (16). In the current study, the protein expressions of S100A8 as well as S100A9 was

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examined immunohistochemically in thyroid tumors in order to investigate the relationship between the expression levels of these two proteins.

Materials and Methods

Tissue specimens. Tissue specimens were obtained from 160 patients, who underwent surgery in the Department of Surgery, Kuma Hospital. The lesions consisted of 40 undifferentiated (anaplastic) carcinomas, 40 poorly differentiated carcinomas, 20 papillary carcinomas, 20 follicular carcinomas, 20 follicular adenomas and 20 medullary carcinomas. Poorly differentiated carcinoma was diagnosed by the criteria in the WHO classification (4). For immunohistochemical study, the tissues were fixed with 10% formalin and paraffin embedded.

Antibody. The establishment and characterization of anti-S100A8 and anti-S100A9 mouse monoclonal antibodies have been described elsewhere (15, 17-20).

Immunohistochemistry. The immunohistochemical study was performed on serial sections prepared from the above formalin-fixed, paraffin-embedded tissues. The sections from each representative tissue-block were deparaffinized and dehydrated. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 15 min. For S100A8 staining, the sections were subjected to heat antigen retrieval in 0.03 mol/L citrate buffer (pH6.0) for 40 min at 95°C. In S100A9 staining, the sections were treated with 0.4 mg/ml proteinase K (Dako, Copenhagen, Denmark) for 6 min at room temperature. After rinsing in phosphate-buffered saline (PBS), 10% bovine serum (Wako, Osaka, Japan) was applied for 20 min to block nonspecific reactions. The sections were incubated with each primary antibody (at a concentration of 1:250 for S100A8 and 1:1000 for S100A9) for 1 hour at room temperature. After rinsing in PBS, these labeled antigens were detected with peroxidase-labeled anti-mouse and anti-rabbit immunoglobulins (Nichirei, Tokyo, Japan). The peroxidase reaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride. Finally, the sections were counterstained with hematoxylin. Infiltrating macrophages and neutrophils showed intense staining for S100A8 and S100A9, and were regarded as internal positive controls. Sections for the negative control were prepared using mouse immunoglobulins instead of the primary antibody.

Immunohistochemical evaluation. Signals for S100A8 and S100A9 were detected predominantly in the cytoplasm, but nuclear staining was also seen. All slides were evaluated by two independent observers (Y.I. and K.A.) without knowledge of the clinical data of each patient and both observers re-examined specimens with discrepant scores to arrive at a consensus score. We examined one or more fields of carcinoma in one representative tissue section (at least 500 cells) and calculated labeling indices of S100A8 and S100A9. The cases were evaluated as immunopositive for S100A8 or S100A9 when cytoplasmic and/or nuclear staining for each protein was observed in more than 1% of the tumor cells. Quantification of the S100A8- and S100A9-positive cases was performed by classifying them into four grades of immunopositivity: score 1, 1 to 5%; score 2, 6 to 25%; score 3, 26-50%, and score 4, more than 50% of the tumor cells were immunoreactive. Negative cases were scored as 0.

Statistical analyses. Fisher's exact probability test was adopted to examine the relationship between variables. A *p*-value less than 0.05 was considered significant.

Results

Normal follicular cells in the non-neoplastic parts were negative for S100A8. Moreover, none of the poorly differentiated carcinoma, papillary carcinoma, follicular carcinoma, follicular adenoma or medullary carcinoma were evaluated as positive for S100A8. (Figure 1a, b). Conversely, all of the undifferentiated carcinomas were immunopositive for S100A8 and 14 out of the 40 cases (35%) were scored as 4, *i.e.* more than 50% of the tumor cells were immunoreactive (Figure 1c). Table I summarizes the S100A8 protein expression in the thyroid tumors.

The S100A9 protein expression in the thyroid tumors in this study is summarized in Table II. The undifferentiated carcinomas were also immunopositive for S100A9 in all cases. In addition, overlapping between S100A8 and S100A9 staining patterns in corresponding tissue areas was seen in all the undifferentiated carcinomas, but positivity of S100A9 was also observed in S100A8-negative tumor cells (Figure 1c, d). S100A9 was negative in the normal follicles, papillary, follicular and medullary carcinomas and follicular adenoma (data not shown). However, out of the 40 poorly differentiated carcinomas, 2 cases (5%) were scored as 1.

Table III indicates the relationship between the immunoreactivity for each S100 protein in this study. The two expressions were correlated with each other ($p < 0.0001$), and the immunopositivity score of S100A8 was equal to or lower than that of S100A9 in each case (Table III).

Discussion

Previously, few studies have been conducted on the expression of S100A8 protein in a large series of non-squamous-type carcinoma. However, we recently demonstrated that S100A8 and S100A9 overexpression in invasive ductal carcinoma of the breast correlates with various clinicopathological parameters reflecting aggressive behavior such as poor histological grade, mitotic activity, vessel invasion, node metastasis, pT category, pStage and so on (20). In thyroid carcinoma, S100A8 expression was expressed in all the undifferentiated carcinomas in this study, but it was entirely negative or showed less than 1% immunoreactivity in the other types of tumors. These findings suggested that S100A8 plays an important role in dedifferentiation of thyroid carcinoma.

In poorly differentiated carcinomas, slight S100A9 immunopositivity was found in 2 cases. Poorly differentiated carcinoma of the thyroid is thought to be an intermediate type between well-differentiated and undifferentiated

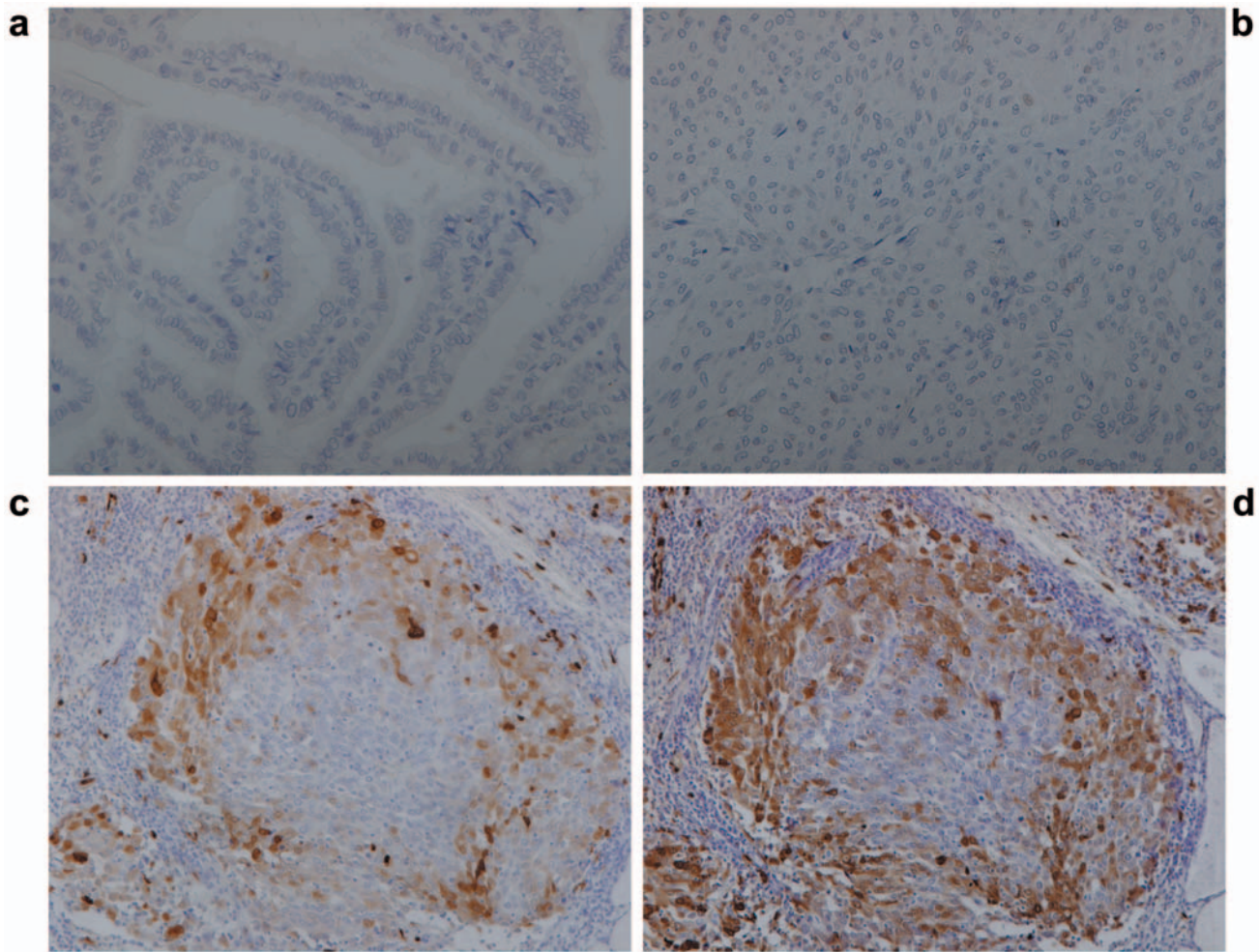


Figure 1. Immunohistochemical expression of S100A8 and S100A9 proteins. a, Lack of S100A8 expression in papillary carcinoma. b, Lack of S100A8 expression in medullary carcinoma. c, Expression of S100A8 in undifferentiated carcinoma. d, Expression of S100A9 in undifferentiated carcinoma.

carcinoma, and the diagnosis is predominantly based on cellular architecture such as insular, trabecular or solid growth pattern rather than on cellular atypism (4). In contrast, in undifferentiated carcinoma, cellular atypia is one of the representative characteristics. It is therefore suggested that S100A8 protein expression in thyroid carcinoma is closely related to the increased cellular atypism caused by dedifferentiation of the carcinoma.

The expression pattern of S100A8 protein was basically similar to that of S100A9 protein, and both expressions were found only in the undifferentiated carcinoma. Moreover, in the undifferentiated carcinoma, overlap between staining patterns of the two proteins was also found. These findings have also been observed in prostatic adenocarcinoma and breast carcinoma and suggest an S100A8/S100A9 heterocomplex formation in undifferentiated thyroid carcinoma (20-22). Although each protein may have

individual functions (23, 24), it is considered that a S100A8/A9 heterocomplex is the most functionally relevant form for these proteins and that the stability of S100A8 depends on S100A9 and *vice versa* (11, 24). Recently, Vogl *et al.* reported that S100A8 is an active component of the heterocomplex (25). It is therefore suggested that S100A8 contributes to the dedifferentiation of thyroid carcinoma under stable and functional conditions by forming a complex with S100A9. It is supposed that S100A8 plays a more crucial role in dedifferentiation of thyroid carcinoma than S100A9.

It has been proven that S100A8 and S100A9 promote the migration and metastasis of lung carcinoma cells in mice (26). Furthermore, S100A8 and S100A9 are known to contribute to invasion of breast carcinoma cells and proliferation of colon carcinoma cells (27, 28). However, the physiological role of both proteins in thyroid carcinoma

Table I. *S100A8 expression in thyroid tumors.*

Histology	S100A8 expression scores					Total
	0	1	2	3	4	
Undifferentiated carcinoma	0	8	13	10	9	40
Poorly differentiated carcinoma	40	0	0	0	0	40
Follicular carcinoma	20	0	0	0	0	20
Follicular adenoma	20	0	0	0	0	20
Papillary carcinoma	20	0	0	0	0	20
Medullary carcinoma	20	0	0	0	0	20

$p < 0.0001$ (undifferentiated carcinoma vs. others).

Table II. *S100A9 expression in thyroid tumors.*

Histology	S100A9 expression scores					Total
	0	1	2	3	4	
Undifferentiated carcinoma	0	3	8	15	14	40
Poorly differentiated carcinoma	38	2	0	0	0	40
Follicular carcinoma	20	0	0	0	0	20
Follicular adenoma	20	0	0	0	0	20
Papillary carcinoma	20	0	0	0	0	20
Medullary carcinoma	20	0	0	0	0	20

$p < 0.0001$ (undifferentiated carcinoma vs. others).

remains an open question. Previous *in vitro* studies have suggested that in prostate carcinoma cells, S100A8 and S100A9 expression is enhanced together with their receptor, receptor for advanced glycation end products (RAGE) and that the S100A8/A9 complex induces the phosphorylation of p38 mitogen activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) by activating nuclear factor- κ B (NF- κ B) (21, 22), which is composed of two subunits (p50 and p65) and a key regulator of genes modulating cellular proliferation and apoptosis (29). Indeed, antisense inhibition of the p65 subunit prevented undifferentiated carcinoma cell lines from forming colonies in soft agar (30, 31). One possibility is that the S100A8/A9

Table III. *Relationship between S100A8 and S100A9 expression in thyroid tumors.*

	S100A9 expression score					
	0	1	2	3	4	Total
	0	118	2	0	0	120
S100A8 expression score	1	0	3	5	0	8
	2	0	0	3	10	13
	3	0	0	0	5	10
	4	0	0	0	9	9
Total	118	5	8	15	14	160

$p < 0.0001$.

complex enhances the development of undifferentiated carcinoma through the NF- κ B signaling pathway.

In conclusion, we demonstrated that S100A8 expression level is elevated almost exclusively in undifferentiated thyroid carcinoma together with S100A9 expression. Further studies are required to elucidate the physiological mechanism, which would contribute to the establishment of a therapeutic strategy for undifferentiated thyroid carcinoma.

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