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The Ca^{2+}-binding S100A2 protein is differentially expressed in epithelial tissue of glandular or squamous origin

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Summary. It has been previously shown that S100A2 is downregulated in tumor cells. The level of immunohistochemical S100A2 expression was therefore characterized in 424 normal and tumoral (benign and malignant) tissues of various origins, but mostly epithelial (with either glandular, squamous, respiratory or urothelial differentiation). We also investigated whether S100A2 could be co-localized with cytokeratin K14, an intermediate filament protein expressed in basal proliferative keratinocytes. Our data show that S100A2 has a low level of expression in non-epithelial tissue. In epithelial tissue S100A2 expression decreases remarkably in the tumors when compared to the normal specimens, and was correlated with the level of keratin K14. This decrease in S100A2 staining from normal to cancer cases is more pronounced in glandular than in squamous epithelial tissue. In addition, the patterns of S100A2 staining also differ between glandular and squamous tissue. These data suggest distinct functional roles for S100A2 in epithelial tissue of squamous or glandular origins.

Key words: S100A2, Tumor suppressor gene, Immunohistochemistry, Epithelium, Carcinoma

Introduction

S100 proteins represent the largest subfamilies of Ca^{2+}-binding proteins characterized by the EF-hand structural motif (Shäfer and Heizmann, 1996; Donato, 1999). While other Ca^{2+}-binding proteins act mainly as buffers, the physiological and structural properties of S100 proteins suggest that they are trigger or activator proteins. They are involved in biological functions such as cell proliferation, apoptosis, motility, exocytosis or cytoskeletal organization (Shäfer and Heizmann, 1996; Gimona et al., 1997; Heizmann and Cox, 1998; Donato, 1999). To date, some 19 different proteins have been assigned to the S100 protein family (Donato, 1999), and the genes encoding thirteen S100 proteins are located in a cluster on human chromosome 1q21 (Shäfer and Heizmann, 1996).

S100A2 (previously labeled S100L or CaN19) has already been shown to be ubiquitously expressed in a subset of cells in kidney, lung, and breast epithelia, and moderately so in the liver and in cardiac and skeletal muscles; there is little or no S100A2 expression in the adrenal gland, the intestine and the brain (Glenney et al., 1989; Ilg et al., 1996; Wicki et al., 1997; Camby et al., 1999). Confocal laser microscopy has shown that S100A2 is located primarily in cell nuclei (Mandinova et al., 1998; Mueller et al., 1999), a subcellular location unique among all other S100 proteins, which are either cytosolic or extracellular (Ilg et al., 1996).

S100A2 is of particular interest because its cDNA coding was identified in a search for novel tumor-suppressor genes in human mammary epithelial cells (Lee et al., 1991). Furthermore, a down-regulation of S100A2 was reported in tumors of various origins such as in breast carcinomas (Lee et al., 1992; Pedrocchi et al., 1994), melanomas (Maelandsmo et al., 1997) and other neoplasms (Ilg et al., 1996). This down-regulation suggests that the protein may play a role in inhibiting tumor progression or suppressing tumor cell growth (Wicki et al., 1997).

The aim of the present study has been to deal with the characterization of the level of S100A2 immunohistochemical expression in a large set of 424 normal and tumoral (benign and malignant) tissues of various histological origins, but epithelial in the great majority of cases (with either glandular, squamous, respiratory or urothelial differentiation). As S100A2

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expression appears to be linked to squamous-cell differentiation (Shrestha et al., 1998), we also investigated whether S100A2 could be co-located with cytokeratin K14, an intermediate filament protein characteristic of epithelial cells and expressed in basal proliferative keratinocytes (Southgate et al., 1999).

Materials and methods

Specimens

A first series of 318 cases was analyzed for S100A2 immunoreactivity. This series included 140 normal cases, and 70 benign and 108 malignant tumors. As indicated in Table 1, the 140 normal cases included 61 epithelial tissues with malpighian (n=23), glandular (n=22), respiratory (n=10) and urothelial (n=6) differentiation, and 79 adipous tissues. The other structures listed in Table 1 came from these normal cases or normal tissues found around the tumors. The histopathological diagnoses of the 178 tumors are given in Table 2.

In a second series of experiments we analyzed 48 normal and 58 cancer tissues from head and neck squamous cell epithelias. These two series of patients were age- and sex-matched. The 48 normal samples included 9 larynx, 7 hypopharynx and 32 oral cavity samples (8 from the tongue, 10 from the tonsils, 6 from between the tongue and the tonsils, and 8 from the buccal mucosa). All these specimens were obtained either from fresh autopsy material or from surgery performed for non-tumor pathologies, as detailed previously (Choufani et al., 1999). The 58 squamous cell head and neck carcinomas (HNSCCs) were primary tumors (not metastases or recurrences) and included 18 larynx, 9 hypopharynx and 31 oral cavity cancers (7 tumors of the tongue, 7 of the tonsils, 7 between the tongue and the tonsils, and 10 of the buccal mucosa). All the patients (aged between 43 and 83) had undergone partial or radical surgery. The diagnoses were established on the basis of the histological criteria described by Hyams et al. (1988).

Immunohistochemical staining

The tissue specimens were fixed in 4% buffered formaldehyde and embedded in paraffin. Two sections of 5 µm each were taken from each sample and subjected to processing with the antibodies against human S100A2 protein and cytokeratin K14 and kit reagents, as previously described (Camby et al., 1999). Briefly, incubation with antibodies was carried out at 25±1 °C for 60 minutes. The dilution used was 1:500 for the S100A2 antibody and 1:500 for the anti-K14. Immunoreaction was developed by applying the avidin-biotin-peroxidase complex (ABC) kit reagents (Vector Labs, Burlingame, CA), with diaminobenzidine/H_2O_2 as the chromogenic substrates. The control tissues were incubated with the corresponding pre-immune sera with the second antibody alone, or with polyclonal anti-S100A2 antibody preabsorbed with human recombinant S100A2. Counterstaining was performed with hematoxylin. The antibody against human recombinant S100A2 was raised in rabbits. Its specificities have been described earlier (Huang et al., 1996; Ilg et al., 1996). The mouse monoclonal antibody against K14 (NCL-LL02) was purchased from Novocastra Laboratories (distributed by Prosan, Merelbeke, Belgium).

The immunohistochemical patterns of the clinical samples obtained through surgery and from autopsy material were similar (data not shown).

Evaluation of immunohistochemical staining

The immunohistochemical staining of the clinical specimens was carried out in accordance with a methodology adapted from Gamallo et al. (1993) and described elsewhere (Bronckart et al., 2001). Briefly, the relative abundance of immunoreactive cells was characterized by the LI variable by counting the percentage of positive cells in at least 20 fields (x400) per specimen. The LI scores were 0 for an absence of staining, 1 for 1-10% of reactive cells or tissue area, and 2 for more than 10% of reactive cells or tissue area. The staining intensity was characterized by the I variable (which was assessed in the histological fields used for the assessment of the LI variable). The I score was graded 0 (no staining, or staining equivalent to the background staining in the negative control), 1 (weak staining), and 2 (intense staining). The final LI/I scores for a tissue specimen corresponded to the most frequent of the 20 LI/I scores assessed for each specimen.

The scores were examined independently by two investigators. In cases of disagreement, a third investigator also examined the scores.

Statistical analyses

The Chi-square tests were used to evaluate the possible associations between the different histopathological- and immunohistochemical-related variables. All the statistical analyses were carried out using Statistica (Statsoft, Tulsa, OK, USA).

Results

S100A2 expression in normal, benign and malignant specimens of various origins

S100A2 immunohistochemistry was first performed on a series of 318 cases including a wide diversity of normal (n=140), benign (n=70) and malignant (n=108) histological types. The results are detailed in Tables 1 (normal cases, including different structures) and 2 (tumoral [benign and malignant] cases) and morphologically illustrated in Fig. 1. Most of the tissues of nonepithelial origins, including adipous, nervous or mesenchymatous specimens, were S100A2-negative (LI

= I = 0, i.e. absence of any immunoreaction). This was observed in both the normal (Table 1) and the tumoral (Table 2) tissue. In sharp contrast, all the different epithelial tissues analyzed displayed an intense S100A2 immunoreaction with either a nuclear or a cytoplasmic location. As detailed below, we observed that the S100A2 staining pattern differed according to the glandular or squamous origin of the epithelial tissues. By combining all the data relating to the epithelial cases (Tables 1, 2 and 4, including the series of 106 squamous tissues described below), we observed a statistically significant ($P<0.01$; Chi^2 test) decrease in the number of S100A2 (nuclear- or cytoplasmic-) immunopositive cases between the normal or benign tumoral epithelial tissues and the malignant ones. In fact, S100A2 was expressed in 117/148 (79%) of the normal or benign tumoral epithelial tissues, and in only 69/132 (52%) of the malignant ones.

We investigated whether S100A2 was co-expressed with cytokeratin K14 in epithelial tissue because the decrease in nuclear S100A2 immunopositivity was most marked in the squamous epithelial tissue (see below). A very significant level of association was obtained between the cytoplasmic immunohistochemical expression of S100A2 and the immunohistochemical expression of K14 ($P<0.000001$; Chi^2 test). In particular, negative S100A2 cytoplasmic expression was strictly associated with negative K14 expression.

Difference in S100A2 expression between glandular and squamous epithelial tissues

Table 3 illustrates the different (cytoplasmic or nuclear) S100A2 staining patterns (diffuse, granular and

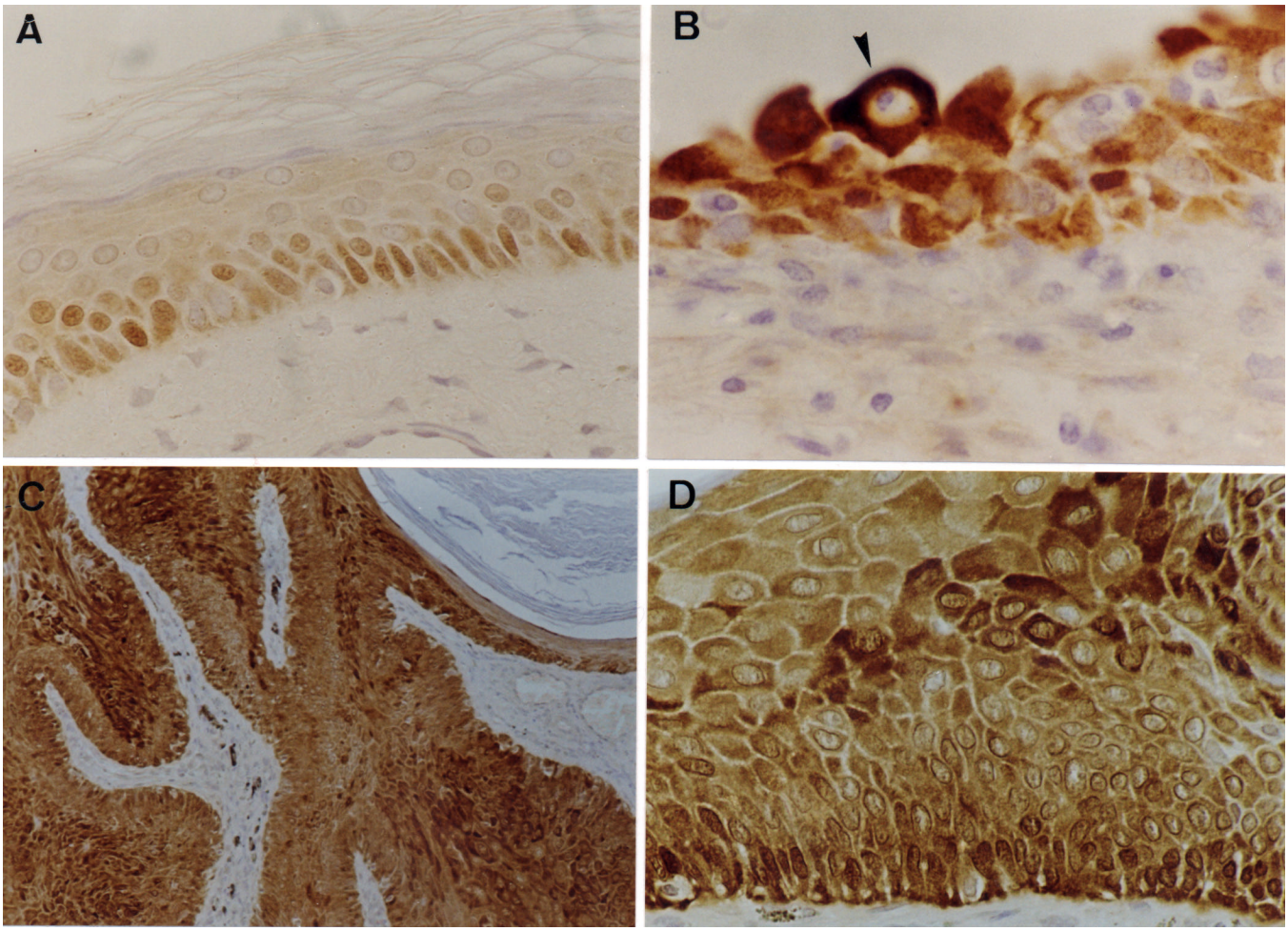

Fig. 1. Immunohistochemical illustrations of the level and pattern of expression of S100A2 in normal skin **(A)**, in a benign prostatic hyperplasia **(B)**, and in a molluscum pendulum analyzed at low **(C)** and high **(D)** magnification. In normal skin, the S100A2 positive cells are located in the basal layer and the subcellular location relates essentially to the nuclei **(A)**. In the benign prostatic hyperplasia, some cells exhibit dramatic S100A2 positivity in their cytoplasms (arrow), while S100A2 is not present in the nuclei **(B)**. In the molluscum pendulum, the epithelial cells are strongly S100A2 immunopositive, while the connective tissue remains negative **(C)**. Whereas at a higher magnification the nuclei of basal cells are positive, they become negative in the more superficial epithelial cell layers. The cytoplasm remains positive in the superficial layers **(D)**. A, x 200; B, D, x 400; C, x 100

focal, or negative) observed in the glandular as opposed to the squamous epithelial tissues. Significant differences were observed between the cytoplasmic and the nuclear S100A2 staining patterns. Indeed, most of the squamous tissues exhibited diffuse as well as focal and granular S100A2 nuclear staining. In contrast, the majority (43/74) of the glandular cases were negative. The few positive cases exhibited essentially a granular and focal S100A2 nuclear immunoreaction (Table 3). With the exception of the cytoplasmic staining in the benign cases, the differences observed between the glandular and the squamous cases (independently of whether they were normal, benign or malignant) remained statistically significant when each diagnostic group was considered individually, i.e. normal cases, benign or malignant tumors (Table 3).

S100A2 expression in an additional series of squamous (normal and cancer) tissue samples from the head and neck

We performed a second series of analyses in an additional series of 106 squamous tissue samples from the head and neck, including 48 normal and 58 cancer cases. The results are summarized in Table 4. We observed that the cases were either actually positive, exhibiting more than 50% of positive cells, or almost negative. The few cases which were weakly positive exhibited less than 10% of S100A2-positive cells. A cut-off value of 10% at the S100A2 nuclear immunopositivity level was thus chosen to separate the negative or weakly S100A2-positive cases from the clearly positive ones. A statistically significant decrease in S100A2-immunopositive cases was observed in the cancer group (Table 4). In contrast, such a difference could not be evidenced in the cytoplasm. In sharp contrast, the S100A2 staining intensity changed strikingly regardless of whether the nucleus or the cytoplasm was taken into consideration in the normal versus the cancer cases. Indeed, in the normal cases, S100A2 immunopositivity was mainly found in the nucleus while in the cancer cases it was mainly seen in the cytoplasm (Table 4).

The significant decrease in S100A2 nuclear immunoreactivity observed in the cancerous as compared to the normal squamous cell tissue was also associated with a prognostic value. Indeed, clinical stage III and IV cancers (n=22) exhibited a significantly ($P<0.01$) lower proportion of S100A2-positive cells than clinical stage I and II cancers (n=29; data not shown).

Discussion

Deregulated expression of S100 proteins has been associated with a wide range of different diseases, particularly with cancers. This is due to their differential expression in neoplastic tissues, their involvement in metastatic processes, and the clustered organization of at least thirteen S100 genes on human chromosome 1q21, a region frequently rearranged in several types of tumor (Schäfer and Heizmann, 1996; Donato, 1999). In this context, a tumor-suppressor role for S100A2 is proposed. Different reasons support this possible role, such as the repressed expression in a large number of tumors, regulation by immediate early genes which are important regulators of normal growth control, and selective hypermethylation within the promoter region (a inactivation mechanism of human tumor suppressor genes) (Wicki et al., 1997). In a recent study (Nagy et al., 2001), we investigated whether S100A2 could play a tumor suppressor role in certain epithelial tissues by acting at cell migration level. In accordance with this hypothesis, our results showed that S100A2 had a clear inhibitory influence on cell motility in the case of human head and neck squamous cell carcinoma lines. The present study confirms a specific role for S100A2 in

Table 1. S100A2 expression in normal tissue.

HISTOLOGICAL TYPE	No. OF CASES ANALYZED	No. OF POSITIVE CASES	NUCLEAR STAINING	CYTOPLASMIC STAINING
Epithelium				
malpighian	23	16	granular	granular
glandular	22	8	granular	granular
respiratory	10	4	granular and focal	granular and focal
urothelial	6	6	granular and focal	granular and focal
Adipous tissues	79	0	absent	absent
Muscles				
smooth	14	1	absent	diffuse
striated	9	9	absent	diffuse
Vessels				
capillary	169	0	absent	absent
veinous	80	0	absent	absent
arterial	87	27	absent	diffuse
Nerves	11	0	absent	absent
White blood cells	187	0	absent	absent
Lymphoid tissues	5	0	absent	absent

epithelial tissue. Indeed, while normal non-epithelial tissue (including adipous, nervous and mesenchymatous types) is S100A2-negative, our data show a decrease in S100A2 staining in epithelial tissue from normal to cancer cases

The present data showing differential expression of S100A2 in tissue of various origins are fully corroborated by previously published material on the brain (Camby et al., 1999), the skin (Boni et al., 1997; Maelandsmo et al., 1997; Shrestha et al., 1998), the salivary glands (Huang et al., 1996), the chondro-osseous tissues (Muramatsu et al., 1997), the breast (Lee et al., 1992; Wicki et al., 1997) and the colon (Bronckart et al., 2001). Our data also illustrate that the decrease in S100A2 staining from normal to cancer cases is more pronounced in glandular than in squamous epithelial tissue. In addition, the patterns of nuclear S100A2 staining also differ between tissues of glandular and squamous origins (see Table 3). These data suggest distinct functional roles for S100A2 in epithelial tissue of squamous or glandular origins. Our present data show clearly that squamous cell carcinomas retain a

Table 2. S100A2 expression in tumoral (benign versus malignant) tissue.

HISTOLOGICAL TYPE	BENIGN CASES		MALIGNANT CASES	
	No. of cases analyzed	No. of positive cases	No. of cases analyzed	No. of positive cases
NON-EPITHELIAL ORIGIN				
Nervous tumors				
Meningiomas	5	0	2	0
Schwanomas	5	0	2	0
Medulloblastomas			5	0
PNETs			5	0
Gliomas			5	0
Adipous tumors				
Lipomas	5	0		
Liposarcomas			5	0
Mesenchymal tumors				
Hemangiomas	5	0		
Mesotheliomas			5	0
Leiomyomas	5	0		
Leiomyosarcomas			5	0
EPITHELIAL ORIGIN				
Squamous: Skin- or epidermis-related tumors				
Moluscum pendulum	5	5		
Cholesteatomas	5	5		
Spinocellular carcinomas			5	5
Basocellular carcinomas			5	5
Glandular: Uro-genital or digestive system tumors				
Prostatic hyperplasia	5	5		
Prostatic adenocarcinomas			10	0
Renal adenocarcinomas			10	0
Mammary fibroadenomas	5	5		
Mammary adenocarcinomas			7	2
Colon dysplasia	5	2		
Colon adenocarcinomas			6	6
Cholangiocarcinomas			5	4
Pancreas adenocarcinomas			5	1
Other: Uro-genital or digestive system tumors				
Urothelial carcinomas			5	5
Liver cirrhosis	5	5		
Hepatocarcinomas			6	2
Other: Thyroid tumors				
Multinodular goiters	5	4		
Microfollicular adenomas	5	5		
Macrofollicular adenomas	5	5		
Papillary carcinomas			5	4
Anaplastic carcinomas			5	3

The tumors are listed in function of their origin: non-epithelial or epithelial (squamous, glandular or other). The great majority of the cases (>95%) exhibited focal and/or granular staining in both the nuclei and the cytoplasms.

Table 3. Subcellular location of S100A2 in epithelial tissues according to their glandular or squamous origins.

1. S100A2 immunohistochemical staining in the nuclei*.

EPITHELIAL TISSUE ORIGIN	LABELING PATTERN		
	Positive		Negative
	diffuse	granular and focal	
squamous (n=32)	16	15	1
glandular (n=74)	3	28	43

Pearson-Chi^2 → P<0.000001 for all cases (n=106);
→ P=0.0006 for normal cases only (n=15);
→ P=0.01 for benign tumors only (n=31);
→ P<0.000001 for malignant tumors only (n=60).

2. Labeling index in the cytoplasm**

EPITHELIAL TISSUE ORIGIN	LABELING PATTERN		
	Positive		Negative
	diffuse	granular and focal	
squamous (n=32)	23	8	1
glandular (n=74)	21	27	26

Pearson-Chi^2 → P=0.00004 for all cases (n=106).
→ P=0.004 for normal cases only (n=15);
→ P=n.s. (0.07) for benign tumors only (n=31);
→ P=0.01 for malignant tumors only (n=60).

*: the S100A2 nuclear staining was labeled as diffuse (the entire cell nucleus was stained), focal and granular (only a part of the nucleus contained positive S100A2 granules), or negative; **: the S100A2 cytoplasmic staining was labeled as for the nuclear staining. All these cases come from Tables 1, 2 and 4.

Table 4. S100A2 expression in normal and cancerous squamous head and neck tissue.

1. Determination of the labeling index in the nuclei*:

DIAGNOSTIC	LABELING INDEX			
	In the nuclei		In the cytoplasm	
	< 10%	> 10%	< 10%	> 10%
normal (n=48)	1	47	4	44
cancer (n=58)	29	29	1	57
Chi^2 test	P<0.0003		n.s. (P>0.05)	

2. Determination of the staining intensity**.

DIAGNOSTIC	STAINING INTENSITY			
	In the nucleus		In the cytoplasm	
	absent to weak	actually positive	absent to weak	actually positive
normal (n=48)	1	47	40	8
cancer (n=58)	29	29	25	58
Chi^2 test	P<0.000001		P=0.00002	

*: for each case analyzed we record the number of either positive nuclei or positive cytoplasms in at least 20 distinct fields, with each field including between 300 and 800 cells specific to each of the histological types analyzed (i.e. 300 to 800 normal cells for a normal case, 300 to 800 cancer cells for a cancer case, etc.); **: the way in which we determined whether the staining intensity was negative, weakly positive or distinctly positive is detailed under Materials and Methods.

significant level of S100A2 positivity, but that the subcellular S100A2 location is modified from the normal to the cancer state. This translocation may play a role in the down regulation of the tumor suppressor effect.

Gimona et al. (1997) report a differentiation-related function for S100A2 in LLC-PK1 cells. In the case of laryngeal squamous cell carcinomas Lauriola et al. (2000) show that S100A2 expression can be very heterogeneous in function of tumor grade and the degree of squamous differentiation. More particularly, S100A2 expression has been positively associated with squamous-cell differentiation, and negatively with tumor grading (Shrestha et al., 1998; Lauriola et al., 2000). In accordance with our observations, Lauriola et al. (2000) exhibit a positive association between S100A2 and K14 expression - a fact which also argues in favor of the expression of S100A2 in direct relation to a given state of differentiation in epithelial cells. Indeed, basal and proliferative keratinocytes express K14 and, when they terminally differentiate, keratinocytes switch off K14 and start K10 expression whereas K16 replaces K10 in response to hyperproliferative stimuli (Paramio et al., 1999). The K10-induced inhibition of keratinocyte proliferation is reversed by the coexpression of K16, but not by that of K14, and these results agree with the expression pattern of these proteins in the epidermis. In fact, Paramio et al. (1999) report that the characteristics of this process indicate that K10 and K16 act on the retinoblastoma Rb protein signal transduction pathway, while K14 does not. Concerning the involvement of S100A2 in the process of cell differentiation, it should be noted that the genes for the S100 proteins on human chromosome 1q21 (including S100A2) are colocalized with genes playing important functions in terminal differentiation of the human epidermis, suggesting a close functional cooperation among these genes (Mischke et al., 1996). Furthermore, in line with the suggestion that S100A2 is a tumor-suppressor candidate, it has been suggested that S100A2 may protect normal keratinocytes against carcinogens (Deshpande et al., 2000).

In conclusion, the data from the present study show that in epithelial tissue S100A2 is co-expressed with keratin K14 and its level of expression decreases dramatically in tumoral when compared to normal epithelial tissue. From being essentially nuclear in normal cells, the subcellular location becomes both cytoplasmic and nuclear in tumoral cells. In addition, S100A2 expression differs between glandular and squamous tissue, suggesting distinct functional roles for S100A2 in epithelial tissue of squamous or glandular origins.

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