PERGAMON

European Journal of Cancer 36 (2000) 1050–1059

European Journal of Cancer

www.ejconline.com

Differential expression of the Ca^{2+} binding S100A6 protein in normal, preneoplastic and neoplastic colon mucosa

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Received 17 September 1999; received in revised form 4 January 2000; accepted 13 January 2000

Abstract

The expression of calcium-binding protein S100A6 was investigated in normal colon tissue, in colon adenomas and in colorectal carcinomas. Using an immunoblotting approach we detected four S100A6 variants with Mwt of 10 kDa and p*I* of 5.05 (isoform I), 5.15 (isoform II), 5.23 (isoform III) and 5.32 (isoform IV) that were differentially expressed in the analysed samples. The quantitative examination of S100A6 variant expression in 25 pairs of colorectal carcinoma and matched control mucosa proved a statistically significant increased abundance of S100A6 isoforms I ($P=0.004$) and III ($P=0.025$) in malignant tissue, and conversely, an increased level of S100A6 isoform IV in healthy tissue ($P=0.022$). The expression of isoforms I and III and the loss of isoform IV were also observed in colon cancer cell lines. In addition, the immunohistochemical study of 16 primary colorectal carcinomas revealed both in the non-paired Student *t*-test and in the Mann–Whitney test the statistically significant accumulation of S100A6 protein ($P<0.001$) in the invasive margin of the tumour. The immunohistochemical analysis of S100A6 protein in polyps differing in clinical severity gave a strong staining that was maximal in dysplastic lesions. Thus, our results indicate a possible, statistically significant correlation (non-paired Student *t*-test $P=0.036$) between S100A6 expression and colon carcinoma progression.

Keywords: S100 calcium-binding proteins; Colon; Cancer; Immunoblotting

1. Introduction

Colorectal tumorigenesis is generally assumed to be a multistep process in which the role of calcium ions in the control of the proliferation and differentiation of epithelial cells is increasingly appreciated [1]. The members of the S100 family of small acidic Ca^{2+}-binding proteins that are expressed in a cell-type specific fashion seem to be excellent candidates to function as cell-type specific mediators of Ca^{2+} signalling. Furthermore, the participation of S100 proteins in the control of cell growth is also supported by the disregulation of their levels in various neoplastic diseases [2,3].

The S100A6 protein, formerly designated as calcyclin, was the first S-100 protein specifically identified as being related to the state of cellular proliferation. The deregulation of *S100A6* gene expression during malignant transformation has, so far, been described for human malignant melanoma [4], squamous cell carcinoma of the oral mucosa [5] and human breast cancer tissues [6]. Additionally, mRNA encoding the S100A6 protein and mRNAs for S100A10 (calpactin I light chain) and S100A4 (calvasculin) were found to be expressed in most human epithelial tumour cells and their levels increased in parallel with increases in the S phase population of cells [7]. The expression of the S100A6 protein can be, at least in some cases, under the positive control of oncogenes since increased expression of S100A6 protein was observed in metastatic ras-transformed cells [8].

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0959-8049/00/$ - see front matter
PII: S0959-8049(00)00043-5

Knowledge of the expression of calcium-binding proteins in the course of colon carcinogenesis is rapidly expanding. Studies carried out using human colon cancer cell lines revealed the overexpression of S100A11 and S100A10 mRNAs, as well as the presence of an alternatively spliced mRNA for calretinin [9–11]. Recently, the expression of calretinin by a large proportion of undifferentiated colorectal carcinomas was described. Additionally, the degree of its expression coincided with an increase in the number of metastases in the regional lymph nodes and in other organs [12]. Similarly, an association between an increased level of S100A4 mRNA in colon adenocarcinomas and the invasion and metastasis of tumour cells has been reported by Japanese authors [13].

We recently reported the upregulation of S100A9 and S100A8 proteins in neoplastic colon mucosa, using two-dimensional gel electrophoresis (2-DE) for the separation of proteins directly extracted from surgically resected colorectal carcinomas [14].

In the present study, we used a new polyclonal anti-human recombinant S100A6 antibody [15] to explore S100A6 protein expression in normal, preneoplastic and neoplastic colon mucosa.

2. Materials and methods

2.1. Tissue specimens

Matched sets of 25 colorectal carcinomas and normal colon mucosa used for 2-DE analysis were obtained within 30 min after surgical resection. Non-necrotic tumour tissues and control mucosa samples that were taken from each patient at 5–10 cm from the tumour mass were immediately homogenised in double the volume of 9.0 M urea lysis buffer (9.0 M urea, 2.0% Triton X-100, 3.0% CHAPS, 70 mM DTT and 2% carrier ampholytes, pH 8.5–10). Then cell lysates were centrifuged at 15 000*g* for 5 min, 4°C and subsequently stored at −80°C. The protein concentration was determined by the modified bicinchoninic acid (BCA) protein assay [16].

For immunohistological staining, 16 biopsies of colorectal carcinoma with sufficiently wide normal perilesional mucosa were examined. The samples of colorectal carcinomas for both electrophoretic as well as immunohistochemical analyses were collected from various locations in the colon (right, transverse, and left colon) and were staged according to Dukes' classification. None of the patients in this group was receiving any drug therapy.

Four adenomatous polyps (two malignant, one tubular and one tubulovillous) obtained after colonoscopic excision were prepared for 2-DE study in the same way as the surgically resected tissues. For the immunohistochemical study, a group of 24 paraffin-embedded biopsies consisting of 6 tubular, 6 tubulovillous, 6 villous and 6 malignant polyps (either severe dysplasia or carcinoma *in situ*) was selected.

2.2. Cell lines

DLD and Caco colon tumour cell lines were commercially obtained from the American Type Culture Collection and maintained at 370°C in 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine serum. When cells were 80–90% confluent, they were quickly washed in ice-cold buffer, scraped and lysed by vortex-mixing in 300 μl of 9.0 M urea lysis buffer. Insoluble debris was removed by centrifugation (15 000*g* for 5 min, 4°C) and the protein concentration was determined by the modified BCA protein assay.

2.3. Two-dimensional gel electrophoresis

The 2-DE was performed by standard procedure as described in [14]. Commercial strips with a non-linear immobilised pH 3–10 gradient (Pharmacia-Biotech, Prague, Czech Republic) were used for isoelectric focusing. These strips were wetted by rehydration buffer containing 2 M thiourea, 7 M urea, 4% CHAPS, 0.5% Triton×100, 10 mM DTT and 0.4% Pharmalytes pH 3–10) overnight [17]. Isoelectric focusing was performed in Multiphore II apparatus (Pharmacia-Biotech) with a 6000 V Power supply from Serva. For both analytical 2-DE and protein electrotransfer 200 μg of protein of tissue as well as cell lysates was loaded. In the second dimension, the 16.5% T, 3% C Tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for the resolution of proteins between 5 and 20 kDa was used [18]. Six slab gels were run using Protean II xi 2-D Multi Cell Unit (Bio-Rad, Prague, Czech Republic) with a Model 3000 xi power supply (Bio-Rad) at a constant current of 300 mA for 20 h. After electrophoresis the separated proteins were either silver-stained [19] or submitted to electrotransfer. The isoelectric points and molecular weights of individual proteins were approximated using polypeptide SDS–PAGE standards (Bio-Rad).

2.4. Western blot analysis

Proteins were first separated by 2-DE, then blotted using the Transblot Cell (Bio-Rad) at 0.1 mA, overnight, on to 0.2 μm PVDF membrane (Boehringer Mannheim, Prague, Czech Republic). The membrane was blocked for 1 h in 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated with polyclonal goat anti-human recombinant S100A6 antibodies [15] diluted 1:800 in blocking solution overnight. This step was followed by incubation with horseradish peroxidase-coupled rabbit anti-goat

antibody diluted 1:2000, purchased from Dako. Between each step, the membranes were washed extensively in washing solution (TBST). The membranes were developed against X-ray film following the use of the Boehringer Mannheim (BM) chemiluminescence blotting substrate (Boehringer Mannheim). Control incubations were done with solution containing only diluted secondary antibody. We did not get any positive reaction. The exposed films were scanned using a laser densitometer (4000×5000 pixels, 12 bits/pixel; Molecular Dynamics, Palo Alto, CA, USA) and quantitation of individual S100A6 variants was performed by ImageQuant software (Molecular Dynamics). The reproducibility of electrotransfer was verified by colloidal silver staining of blots after immunodetection [20].

2.5. Immunohistochemistry and evaluation of staining

Tissue sections of 4 μm were cut from formalin-fixed, paraffin-embedded blocks and examined for S100A6 expression using a standard peroxidase technique. After blocking for 20 min, tissue sections were incubated with S100A6 antibodies for 24 h at 4°C, and washed three times in phosphate-buffered saline pH 7.2 (PBS). The slides were then incubated with horseradish peroxidase-coupled anti-goat antibody (Sigma, Prague, Czech Republic) for 45 min, at 37°C. Excess antibody was washed off with PBS. Finally, 0.05% 3,3 diaminobenzidine tetrahydrochloride chromogen solution (Sigma) in PBS containing 0.02% hydrogen peroxide was added for 10 min at room temperature to visualise the antigen–antibody complex formed. The S100A6 staining intensity was scored using computerised image analysis (Lucia M software, version 3.00, Laboratory Imaging, Prague, Czech Republic). The grabbed images have been captured and converted to the 256 gray scale. The percentage of the whole area, total integral optical density (area×density of objects) and density extrapolated to 10 000 pixels were counted. Regarding the tumour tissue, six fields from four different locations (original magnification×500) including the central part of the tumour, the margin of the tumour, tumour-adjacent normal mucosa and connective tissue were analysed for each tumour. In the case of polyps, six fields (original magnification×400) from both polypous and non-polypous mucosa were examined by the same approach as the tumour lesions.

The acquired values were used for statistical analysis. Negative staining controls were obtained by incubating with normal goat serum purchased from Dako.

2.6. Statistical analysis

Comparison between two characteristics was assessed using the non-parametric Mann–Whitney test and the non-paired Student *t*-test. Relationships were considered statistically significant when $P < 0.05$.

3. Results

The aim of our study was to investigate S100A6 protein expression in normal, preneoplastic and neoplastic colon mucosa. We applied two-dimensional gel electrophoresis followed by immunoblotting as a superior technique for the detection of possible post-translational protein processing [21]. Fig. 1 shows the S100A6 immunostaining in normal and malignant colon mucosa collected from patient No. 1 suffering from colon adenocarcinoma, Dukes' B. Three variants of S100A6 protein (I, II and IV), with II the most dominant, were present in macroscopically healthy tissue (Fig. 1a). All these isoforms exhibited only charge heterogeneity. In the tumour (Fig. 1b), the expression of the dominant variant (II) was preserved, however, the most acidic isoform (I) was more abundant compared with normal tissue, the most basic isoform (IV) was hardly detectable and, finally, a new more basic isoform (III) appeared. To verify the existence of four different S100A6 isoforms, we mixed the proteins extracted from normal and malignant tissue and performed the immunostaining once more (Fig. 1c). This new immunoblot confirmed the previous results since four charge variants of S100A6 protein were detected. We calculated their

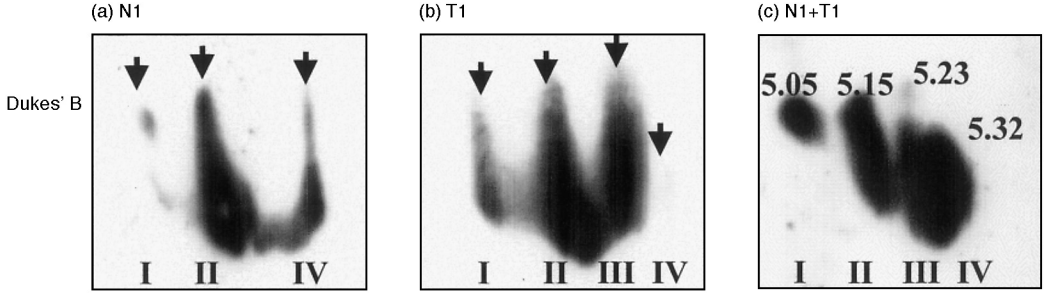

Fig. 1. Two-dimensional gel electrophoresis immunoblotting of S100A6 protein. (a) N1, normal colon mucosa collected from patient No. 1; (b) T1, colorectal carcinoma; (c) N1 + T1 mixed sample extracted from normal tissue and colorectal carcinoma. The positions of individual S100A6 isoforms are designated by Roman numbers I, II, III and IV.

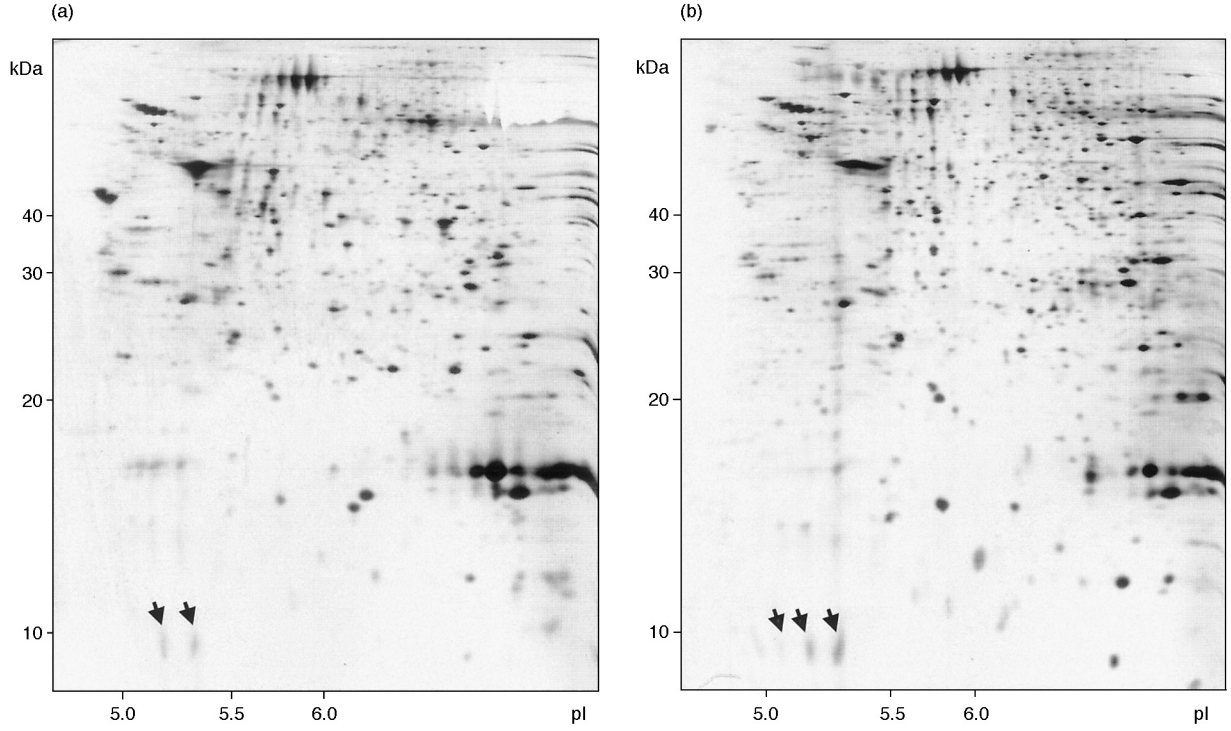

Fig. 2. Silver-stained two-dimensional protein pattern of samples of normal (a) and malignant colon mucosa (b) collected from patient No. 2. The positions of S100A6 isoforms are indicated (arrowed). The corresponding 2-DE immunoblot of patient No. 2 is shown on Fig. 3a.

approximate p*I* values and found that the p*I* value of the most basic isoform 5.32 is identical to the theoretical p*I* value of human S100A6 protein computed from the nucleotide sequence located in SwissProt database. We tried to translate the results from immunodetection to the corresponding silver-stained spots on the gel, however, S100A6 isoforms were poorly stainable by ammoniacal silver. Hence, in only a few cases could we match the immunostained spots with a corresponding silver-stained gel (Fig. 2a,b). Then we examined the remaining 24 matched cancer/normal pairs. The S100A6 was expressed in all individuals tested, although not all S100A6 variants occurred in all patients. Nevertheless, like in patient No. 1, differential quantities of S100A6 isoforms I, III and IV comparing normal with malignant tissue were revealed. Globally, we found that from a group of 25 patients the S100A6 isoforms I and III were upregulated in tumour tissue collected from 18 (72%) and 12 patients (48%), respectively. Conversely, the level of S100A6 isoform IV expression was augmented in normal colon mucosa of 14 patients (56%). The representative 2-DE immunoblots and distribution of S100A6 isoforms I, III and IV in all examined patients are depicted in Fig. 3(a–d). Utilising the Mann–Whitney test, the S100A6 isoform I, III and IV abundance alterations were found to be significant ($P < 0.05$). The results of statistical analysis including mean values of isoform intensities in normal and malignant colon mucosa are summarised in Table 1.

To verify the epithelial origin of S100A6 protein we carried out a 2-DE immunoblotting analysis of the S100A6 expression in two tumour cell lines derived from colon adenocarcinomas (Fig. 4). In both cases, the presence of isoform III, the fairly faint detection of isoform I and the loss of isoform IV were observed. These two cell lines clearly differ in the expression of the S100A6 isoform I that was only weakly immunostainable in Caco cells. It is interesting to note that whilst Caco cells retain many differentiated functions of normal epithelial cells, the DLD cell line is derived from

Table 1
Distribution of four S100A6 isoforms in normal and malignant colon mucosa

Isoform	pI/MW	Normal mucosa (densitometry)		Malignant mucosa (densitometry)		Mann–Whitney test
		Mean	± S.D.	Mean	± S.D.	$P < 0.05$ (is significant)
S100A6 (I)	5.05/10 kDa	267	540	3389	7026	0.004
S100A6 (II)	5.15/10 kDa	22 960	15 870	28 260	20 720	0.567
S100A6 (III)	5.23/10 kDa	1437	3875	7961	105 60	0.025
S100A6 (IV)	5.32/10 kDa	14 960	15 140	4640	7742	0.022

metastasing adenocarcinoma, Dukes' C and *in vitro* cultivation of this cell line results in the spontaneous occurrence of invasive cell variants [22].

We also examined the expression of S100A6 variants in four polypoid lesions differing in severity (Fig. 5). As in Fig. 1, we obtained a strong positive reaction for isoform II in all polypoid lesions. Additionally, the level of isoforms I and III expression as in colon neoplasms closely corresponded with the malignant potential of analysed polyps.

Next we performed immunohistochemical staining of S100A6 protein in 16 primary colon tumours and in their perilesional normal mucosa. These areas were previously defined by a pathologist after a consecutive section had been stained with haematoxylin–eosin. The S100A6 staining in normal mucosa was heterogeneous (Fig. 6a) and was maximal in the upper areas of the crypt and in the surface epithelium. In contrast, tumour tissue showed strong positive staining of the complete tumour mass (Fig. 6b). The maximal staining intensity of S100A6 protein in the tumour was found at its invasive margin, just at the border with the adjacent normal colon mucosa (Fig. 6c). Additional strong staining was

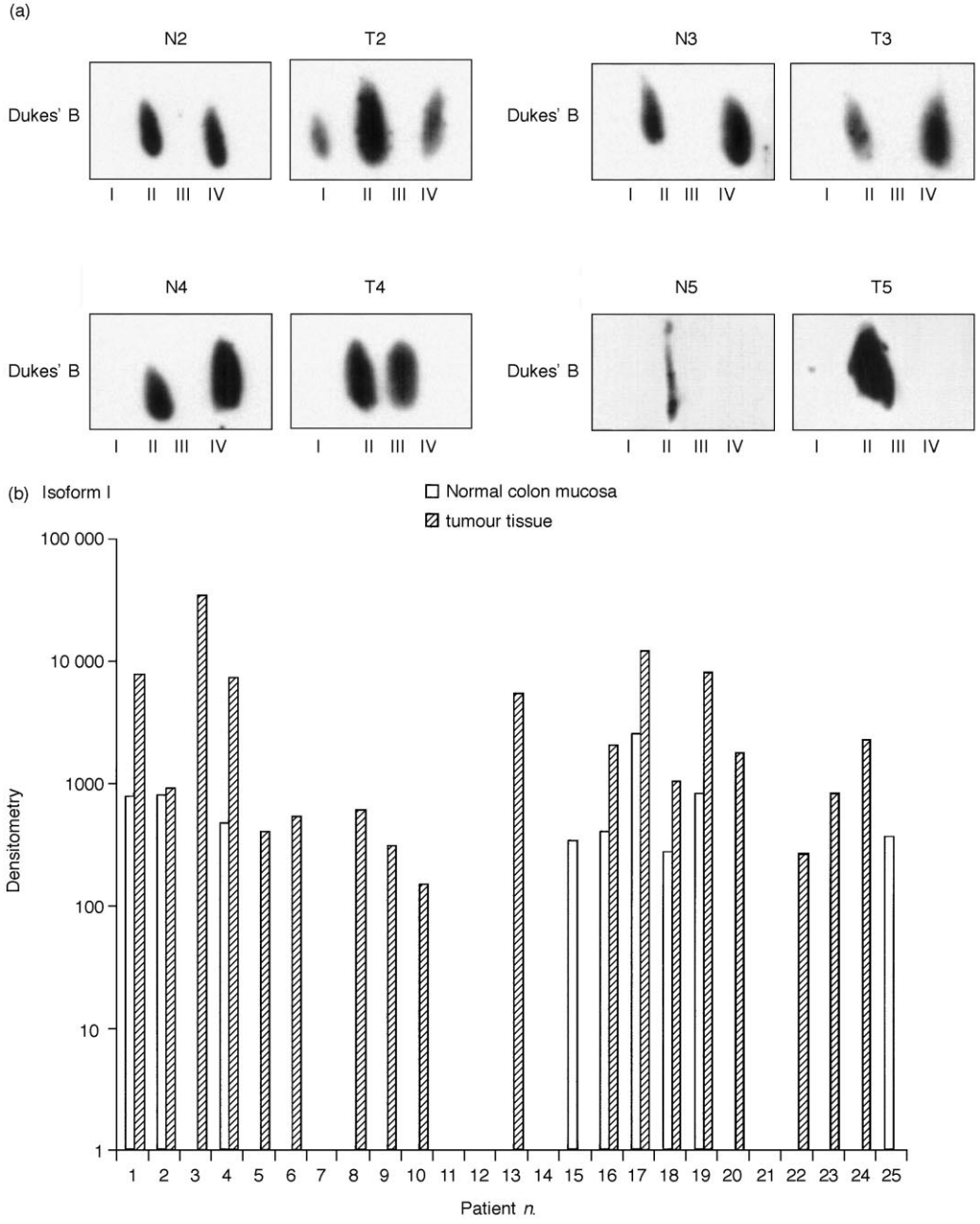

Fig. 3. Two-dimensional gel electrophoresis immunoblotting of S100A6 protein of a representative four matched sets of normal mucosa and colorectal carcinoma (a) and box-plot comparisons of the levels of S100A6 isoform I (b); S100A6 isoform III (c); and S100A6 isoform IV (d); in individual normal/cancer pairs.

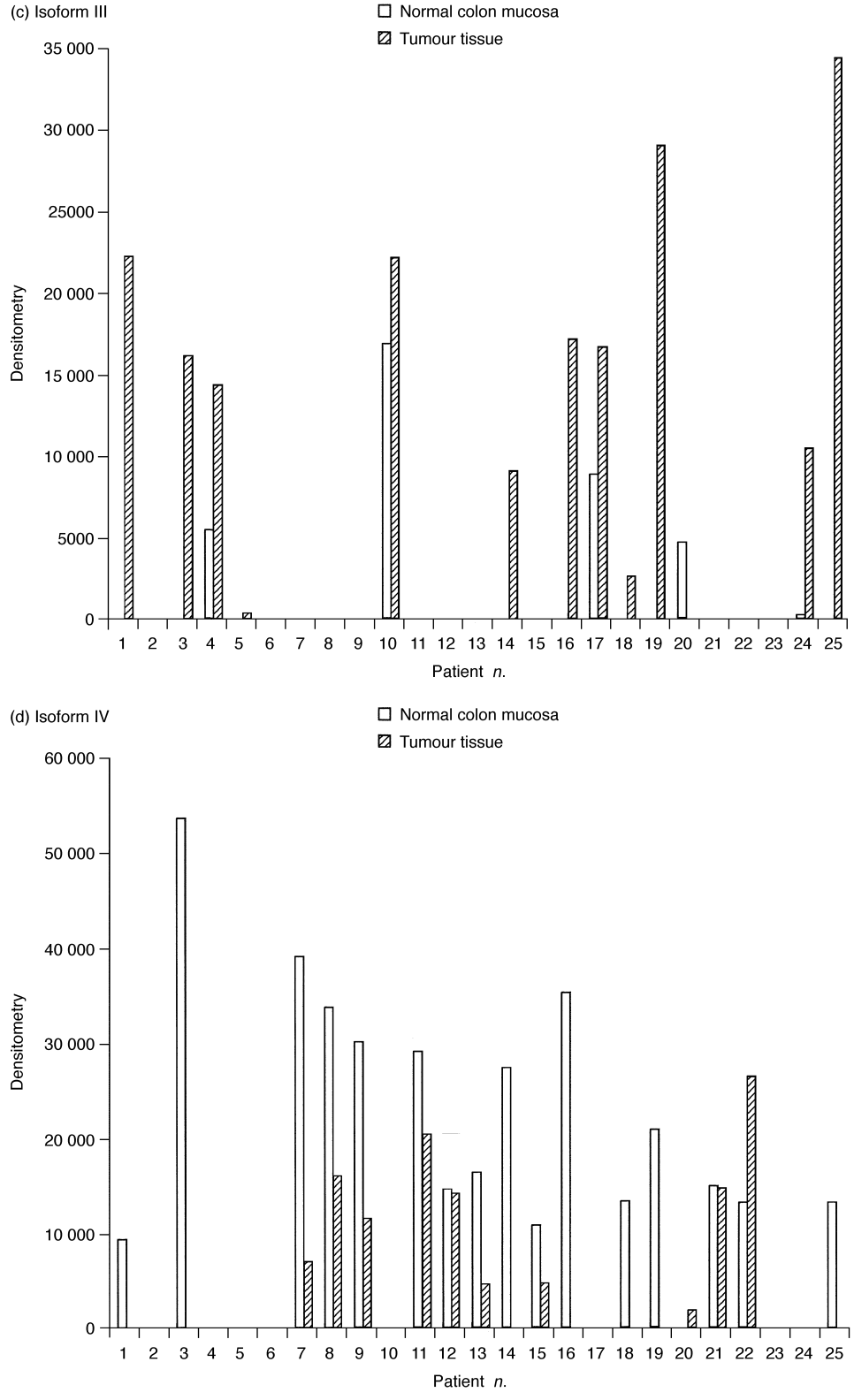

Fig. 3. continued.

found in a micro-metastasis of colorectal cancer in connective tissue (Fig. 6d). Statistical analysis showed a significant difference in the expression of S100A6 between connective tissue on the one hand and normal mucosa or tumour mass on the other ($P < 0.001$), thus, again, confirming the epithelial origin of S100A6. Comparing the middle part or the margin of the tumour with perilesional normal mucosa, a statistical significance was

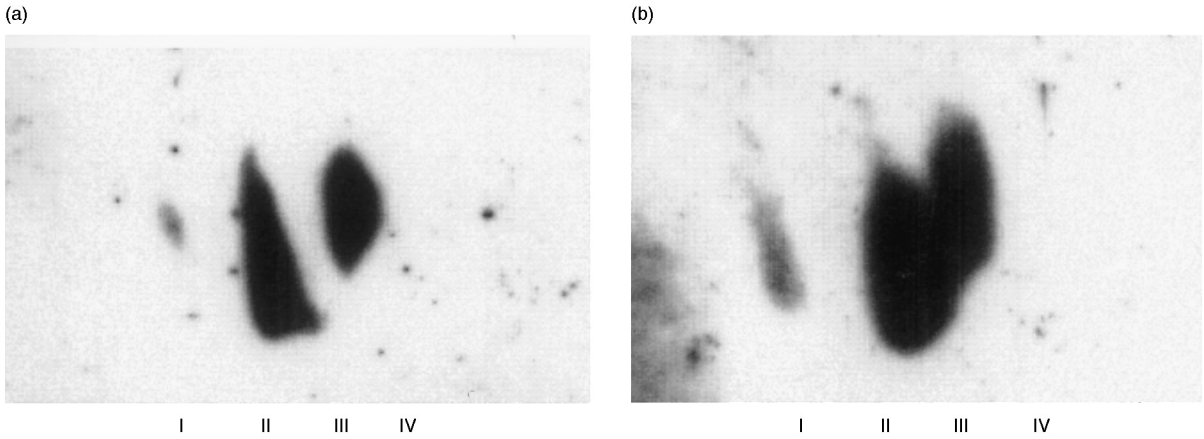

Fig. 4. Two-dimensional gel electrophoresis immunoblotting of S100A6 protein in Caco cell line (a) and DLD cell line (b).

found by the Mann–Whitney test and the non-paired Student *t*-test for the edge of the tumour only ($P < 0.001$).

Regarding polypoid tissue, we commonly detected strong homogeneous staining of S100A6 protein that was further augmented in dysplastic areas (Fig. 6e, f). Furthermore, to find a possible correlation of S100A6 protein expression with the transition of benign preneoplastic polypoid lesions to neoplasia, the S100A6 staining intensity in the groups of villous and malignant polyps was selected for statistical analysis. In this case, the application of the non-paired Student *t*-test showed a significant difference ($P = 0.036$), whilst the Mann–Whitney test showed a relationship close to significance ($P = 0.068$).

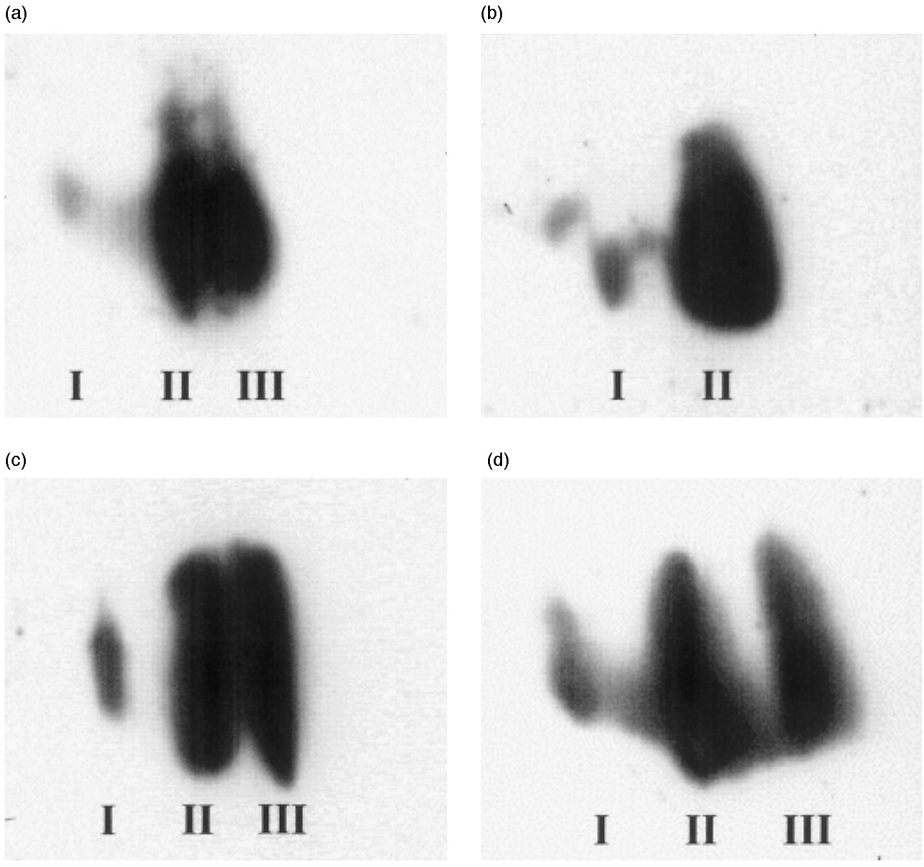

Fig. 5. Two-dimensional gel electrophoresis immunoblotting of S100A6 protein in polypoid lesions. (a) tubular polyp; (b) tubulovillous polyp with marked proliferation activity; (c) malignant polyp (carcinoma *in situ*); (d) malignant polyp (severe dysplasia) surgically resected together with carcinoma from patient No. 1.

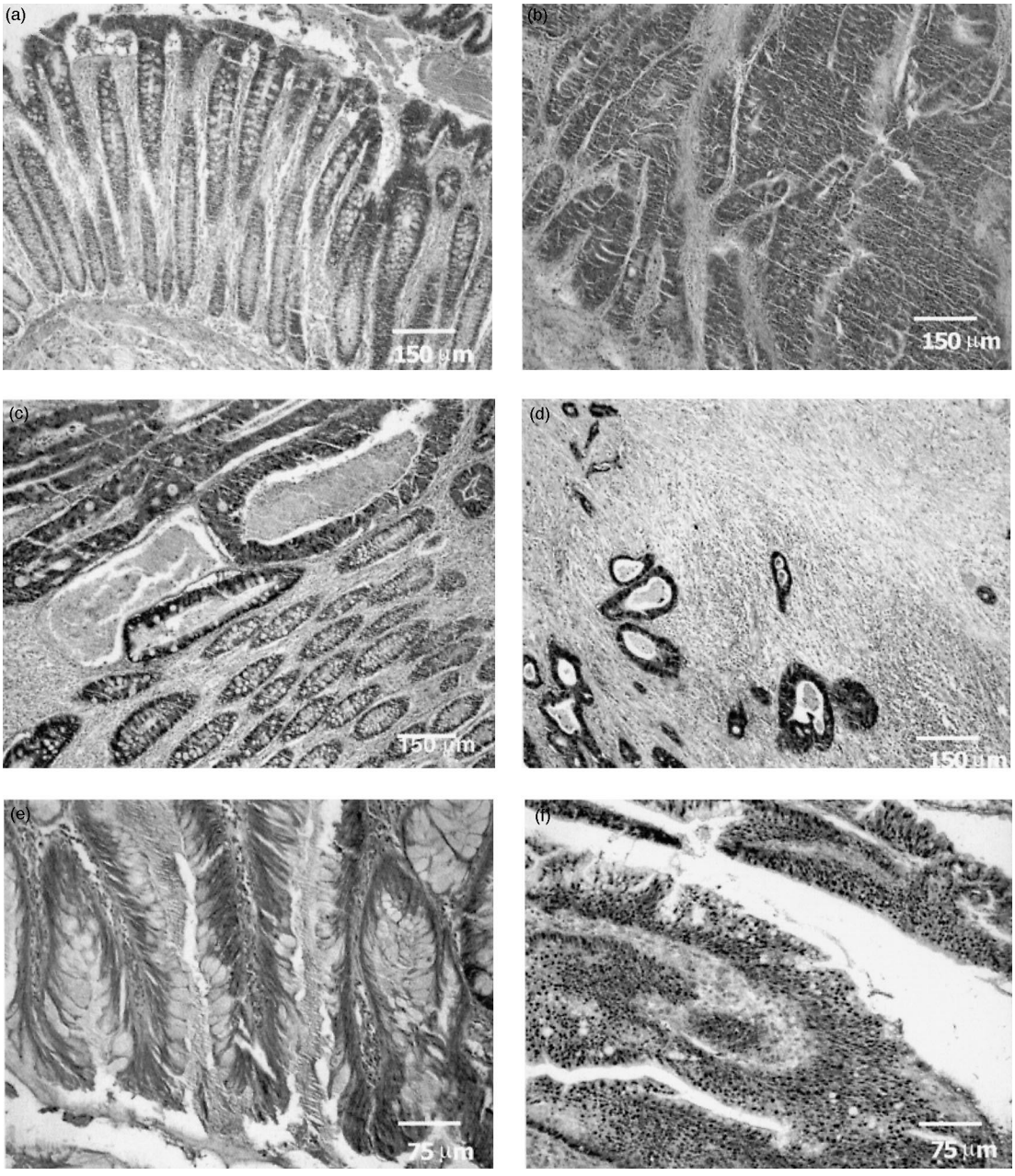

Fig. 6. Immunohistochemical staining of S100A6 protein. (a) adjacent non-malignant colonic mucosa; (b) the central part of colorectal cancer, Dukes' C; (c) transitional part of colorectal carcinoma, Dukes' C with adjacent normal colonic mucosa; (d) micro-metastasis in connective tissue; (e) villous polyp; (f) malignant polyp, dysplastic area. As indicated, the scale bar corresponds to 150 microns (μm) (a–d) or 75 microns (e, f).

4. Discussion

Ca^{2+} ions are suggested to play an important role in the development of colorectal cancer. In addition to reducing the amount of free secondary bile acids and ionised fatty acids in the lumen of the colon, they directly control proliferation and terminal differentiation in normal colon crypts probably via surface Ca^{2+} receptors [1]. The cellular actions of Ca^{2+} ions are mediated by site-specific binding to specialised proteins. From this point of view, monitoring the expression of selected calcium-binding proteins that can mediate calcium signalling in colon mucosa is worthwhile. There exist three main families of Ca^{2+} binding proteins [23]. The first group involves ER Ca^{2+} binding proteins like calreticulin, Grp78, endoplasmin and protein disulphide isomerase. Calreticulin was found to be up-regulated in malignant breast lesions and, additionally, its N-terminal domain significantly reduced growth of human colon carcinoma in athymic mice because of suppression of tumour angiogenesis [24,25]. Annexins belong to the second group of Ca^{2+} binding proteins. The function of annexins is still not clearly defined, but several members of this family have been implicated in the regulation of

membrane transport and sorting along the endocytic pathway. Annexins exhibit characteristic tissue and subcellular distribution, indicating their participation in differentiation and other physiological functions specific to these tissues. Annexin IV that was found to bind to the nuclear envelope in a Ca^{2+}-dependent manner is almost exclusively found in epithelial cells [26,27]. In our search for proteins whose expression is associated with colorectal cancer development we found significant overexpression of annexin IV and annexin III in neoplastic tissue (data not shown). The third class of Ca^{2+} binding proteins is represented by proteins which possess one or more E–F hand helix–loop–helix structural motifs. This group encompasses a large array of proteins amongst which particularly members of the S100 protein family attract attention. There are evident links between the expression of some members of the S100 protein family, especially S100A2, S100A4, S100B and S100A6, and neoplastic disorders [2]. The S100A6 mRNA is highly abundant in several normal human tissues, including colon and mammary gland [4]. For the latter, a further increase in S100A6 mRNA level was observed in the course of its malignant transformation [6]. In this study, we searched for expression of S100A6 protein in the course of malignant transformation of colon mucosa. We used the 2-DE technique because the changes in the dynamics of post-translational modifications of proteins can be fundamental for their association with pathogenesis [28]. Regarding the S100A6 protein, the synthesis of three charge variants in human keratinocytes was described [29]. Comparing 25 matched sets of normal and neoplastic colon mucosa we provide evidence for four charge S100A6 isoforms that exhibit differential expression under normal and pathological conditions. Whilst the most basic isoform IV (p*I* 5.32), which seems to be without any modification, preferentially accumulates in healthy tissue, isoforms I (p*I* 5.05) and III (p*I* 5.23) predominate in transformed mucosa. A possible association of isoforms I and III with the early phase of colon tumorigenesis is corroborated by their expression in malignant polypoid lesions and the Caco cell line.

The basis of the charge heterogeneity of S100A6 isoforms is, so far, not known. Ca^{2+}-induced structural changes of S100A6 protein seem not to be involved since they are very modest in comparison with other Ca^{2+} sensors [30]. In contrast, the different types of post-translational modifications such as phosphorylation, acetylation, formylation or S-sulphation, which were described for some members of S100 protein family, could also be possible for S100A6 subunits [3]. Preliminary results obtained from electrospray mass spectrometry indicate acetylation and formylation of S100A6 molecules which probably influence their cell relocation (data not shown). Recently, two isoforms of chicken S100A6 protein differing with respect to the presence or absence of a C-terminal lysine were identified [31] and the native protein was shown to exist as non-covalently associated homodimers (AA and BB) or heterodimers (AB).

In addition, the possible role of S100A6 variants in colon carcinogenesis remains questionable. Generally, the S100A6 protein has been implicated in the regulation of cell growth and proliferation because its mRNA is preferentially expressed in the G_1 phase of the cell cycle [32]. The effects of S100A6 on cell cycling are probably mediated via interactions with various ligands including Ca^{2+}/phospholipid-binding proteins of the annexin family, glyceraldehyde-3-phosphate dehydrogenase, and a 30 kDa protein present in Ehrlich ascites tumour cells [33]. It is possible that the post-translational modification of S100A6 will induce the conformational changes which direct the binding of S100A6 variants to a specific target, and, thereby elicit a distinct cascade of cellular events. Furthermore, it is known that some combinations of S100 isoforms can influence the phosphorylation status of their ligands and, hence, their activities [34].

In accordance with Ilg and colleagues [15] immunohistochemical analysis demonstrated an increased expression of S100A6 protein in transformed colon mucosa in comparison with normal colon mucosa. However, comparing the central part or the transition area of tumour tissue with normal perilesional mucosa, a statistically significant difference in staining intensity was found only for the margin of the tumour mass. The increased S100A6 production in preneoplastic adenomas, which is even further augmented with the progress of premalignant lesion toward neoplasm, is of great clinical interest. This immunohistochemical finding coincides with the appearance of isoforms I and III found in malignant polyps by 2-DE immunoblotting study. An association of increased levels of S100A6 production with premalignant tissue was also observed for preneoplastic lesions in human lung [35] and might be of prognostic significance in these tumour types.

Acknowledgements

The authors thank Jana Michaličková, Alena Firychová, Jitka Žáková and Šárka Průchová for their excellent technical assistance. This work was supported by grants from the Ministry of Health, Czech Republic (project no. 4075-3), from the Grant Agency of Czech Republic (project no. 310/98/P238) and by the Swiss National Science Foundation (project no. 31-50510.97).

References

1. Whitfield JF, Bird RP, Chakravarthy BR, Isaacs RJ, *et al.* Calcium — cell cycle regulator, differentiator, killer, chemo-

preventor, and maybe, tumor promoter. *J Cell Biochem* 1995, **22**, 74–91.
2. Schäfer BW, Heizmann CW. The S100 family of EF-hand calcium-binding proteins: functions and pathology. *Trends Biol Sci* 1996, **21**, 134–140.
3. Heizmann CW, Cox JA. New perspectives on S100 proteins: a multi-functional Ca^{2+}-, Zn^{2+}- and Cu^{2+}-binding protein family. *BioMetals* 1998, **11**, 383–397.
4. Maelandsmo GM, Florenes VA, Mellingsaeter TE, *et al*. Differential expression patterns of S100A2, S100A4 and S100A6 during progression of human malignant melanoma. *Int J Cancer* 1997, **34**, 464–469.
5. Berta GN, Ghezzo F, D'Avolio A, *et al*. Enhancement of calcyclin gene RNA expression in squamous cell carcinoma of the oral mucosa, but not in benign lesions. *J Oral Pathol Med* 1997, **26**, 206–210.
6. Pedrocchi M, Schäfer BW, Mueller H, *et al*. Expression of Ca^{2+}-binding proteins of the S100 family in malignant human breast-cancer cell lines and biopsy samples. *Int J Cancer* 1994, **57**, 684–690.
7. Matsumoto T, Murao S, Kito K, *et al*. Modulation of S-100 genes response to growth conditions in human epithelial tumor cells. *Pathol Int* 1997, **47**, 339–346.
8. Chambers AF, Tuck AB. Ras-responsive genes and tumor metastasis. *Crit Rev Oncogenesis* 1993, **4**, 95–114.
9. Tanaka M, Adzuma K, Iwami M, *et al*. Human calgizzarin; one colorectal cancer-related gene selected by a large scale random cDNA sequencing and Northern blot analysis. *Cancer Lett* 1995, **89**, 195–200.
10. Wildrick DM, Priebe-Malven M, Boman BM. Expression of the gene for the light chain of calpactin 1 (annexin II) in cell lines DiFi, HT-29, and WI-38. *Anticancer Res* 1992, **12**, 1721–1724.
11. Gander JC, Bustos-Castillo M, Stüber D, *et al*. The calcium-binding protein calretinin-22k, an alternative splicing product of the calretinin gene is expressed in several colon adenocarcinoma cell lines. *Cell Calcium* 1996, **20**, 63–72.
12. Gotzos V, Wintergerst ES, Musy JP, *et al*. Selective distribution of calretinin in adenocarcinomas of the human colon and adjacent tissues. *Am J Surg Pathol* 1999, **23**, 701–711.
13. Takenaga K, Nakanishi H, Wada K, *et al*. Increased expression of S100A4, a metastasis-associated gene, in human colorectal adenocarcinomas. *Clin Cancer Res* 1997, **3**, 2309–2316.
14. Stulik J, Österreicher J, Koupilova K, *et al*. The analysis of S100A9 and S100A8 expression in matched sets of macroscopically normal colon mucosa and colorectal carcinoma: the S100A9 and S100A8 positive cells underlie and invade tumor mass. *Electrophoresis* 1999, **20**, 1047–1054.
15. Ilg E, Schäfer BW, Heizmann CW. Expression pattern of S100 calcium-binding proteins in human tumors. *Int J Cancer* 1996, **68**, 325–332.
16. Hill HD, Straka JG. Protein determination using bicinchoninic acid in the presence of sulfhydryl reagents. *Analyt Biochem* 1988, **170**, 203–208.
17. Rabilloud T, Adessi C, Giraudel A, Lunardi J. Improvement of the solubilization of proteins in two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 1997, **18**, 307–316.
18. Schägger H, Von Jagow G. Tricine-sodium polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analyt Biochem* 1987, **166**, 368–379.
19. Hochstrasser DF, Merril CR. Catalysts for polyacrylamide gel polymerization and detection of proteins by silver staining. *Appl Theor Electrophoresis* 1988, **1**, 35–40.
20. Van Oostveen I, Ducret A, Aebersold R. Colloidal silver staining of electroblotted proteins for high sensitivity peptide mapping by liquid chromatography–electrospray ionization tandem mass spectrometry. *Analyt Biochem* 1997, **247**, 310–318.
21. Anderson NG, Anderson NL. Twenty years of two-dimensional electrophoresis: past, present and future. *Electrophoresis* 1996, **17**, 443–453.
22. Vermeulen SJ, Nollet F, Teugel E, *et al*. The αE-catenin gene (CTNNA1) acts as an invasion-suppressor gene in human colon cancer cells. *Oncogene* 1999, **18**, 905–915.
23. Gilchrist JSC, Czubryt MP, Pierce GN. Calcium and calcium-binding proteins in the nucleus. *Mol Cell Biochem* 1994, **135**, 79–88.
24. Franzen B, Linder S, Alaiya AA, *et al*. Analysis of polypeptide expression in benign and malignant human breast lesions: down-regulation of cytokeratins. *Br J Cancer* 1996, **73**, 1632–1638.
25. Pike SE, Jones KD, Cherney B, *et al*. Vasostatin, a calreticulin fragment, inhibits angiogenesis and suppresses tumor growth. *J Exp Med* 1998, **21**, 2349–2356.
26. Dreier R, Schmid KW, Gerke V, Riehemann K. Differential expression of annexins I, II and IV in human tissues: an immunohistochemical study. *Histochem Cell Biol* 1998, **110**, 137–148.
27. Raynal P, Kuijpers G, Rojas E, Pollard HB. A rise in nuclear calcium translocates annexin IV and V to the nuclear envelope. *FEBS Lett* 1996, **392**, 263–268.
28. Parekh RB, Rohleff C. Post-translational modification of proteins and the discovery of new medicine. *Curr Opin Biotechnol* 1997, **8**, 718–723.
29. Celis JE, Rasmussen HH, Gromov P, *et al*. The human keratinocyte two-dimensional gel protein database (update 1995): mapping components of signal transduction pathways. *Electrophoresis* 1995, **16**, 2177–2240.
30. Sastry M, Ketchem RR, Crescenzi O, *et al*. The three dimensional structure of Ca^{2+}-bound calcyclin: implications for Ca^{2+}-signal transduction by S100 proteins. *Curr Biol* 1998, **6**, 223–231.
31. Allen BG, Andrea JE, Sutherland C, *et al*. Molecular cloning of chicken calcyclin (S100A6) and identification of putative isoforms. *Biochem Cell Biol* 1997, **75**, 733–738.
32. Calabretta B, Kaczmarek L, Mars W, *et al*. Cell-cycle-specific genes differentially expressed in human leukemias. *Proc Natl Acad Sci USA* 1985, **82**, 4463–4467.
33. Zeng FY, Gerke V, Gabius HJ. Identification of annexin II, annexin IV and glyceraldehyde-3-phosphate dehydrogenase as calcyclin-binding proteins in bovine heart. *Inter J Biochem* 1998, **25**, 1019–1025.
34. Wilder PT, Rustandi RR, Drohat AC, Weber DJ. S100B (ββ) inhibits the protein kinase C-dependent phosphorylation of a peptide derived from p53 in a Ca^{2+}-dependent manner. *Protein Sci* 1998, **7**, 794–798.
35. Kayser K, Andre S, Bovin NV, *et al*. Preneoplasia-associated expression of calcyclin and of binding sites for synthetic blood group A/H trisaccharide — exposing neoglycoconjugates in human lung. *Cancer Biochem Biophys* 1997, **15**, 235–243.