Journal of BUON **13:** 101-107, 2008
© 2008 *Zerbinis Medical Publications. Printed in Greece*

ORIGINAL ARTICLE

Two-dimensional electrophoresis and immunohistochemical study of calreticulin in colorectal adenocarcinoma and mirror biopsies

K. Vougas[1], E. Gaitanarou[1], E. Marinos[2], C. Kittas[2], I.E. Voloudakis-Baltatzis[1]

[1]Department of Cell Biology, Molecular Biology and Electron Microscopy, "G. Papanicolaou" Research Center of Oncology and Experimental Surgery, "Saint Savas" Anticancer Hospital of Athens, Athens; [2]Department of Histology and Embryology, University of Athens, Medical School, Athens, Greece

Summary

Purpose: *The purpose of this study was to analyse the polypeptide patterns of colorectal adenocarcinomas and mirror biopsies and to investigate the expression of calreticulin and the relationship of this chaperon to colon cancer.*

Materials and methods: *The investigation was carried out on 21 adenocarcinomas and 21 mirror biopsies using high-resolution two-dimensional (2D) electrophoresis and immunohistochemical PAP method.*

Results: *2D electrophoresis revealed several polypeptide patterns that were shown to be upregulated in colorectal adenocarcinomas compared to their mirror biopsies. One polypeptide spot being upregulated in colorectal adenocarcinoma, turned out to be calreticulin. The overexpression of calreticulin was confirmed by further examination in immunohistochemical level.*

Conclusion: *Calreticulin was found overexpressed in colon cancer tissues as compared to the corresponding mirror biopsy tissues. The overexpression was particularly intense to high-malignancy tissues and particularly in the poorly differentiated regions of the tissue. Calreticulin showed a direct relationship to the disease stage, a fact strongly indicating that the functional role of calreticulin is directly associated with tumor growth and metastasis.*

Key words: calreticulin, colon adenocarcinoma, mirror biopsies

Introduction

Colon cancer is a main cause of death in Western Europe. The means of dealing with the disease through surgical therapy and chemotherapy often proves inadequate to offer a satisfactory disease control.

Researchers using proteomics technologies attempt to identify markers for early diagnosis, tumor specific antigens to act as targets for tumor-directed therapies and discover differences in protein expression profiles between tumor and mirror biopsy cells in order to elucidate the control mechanisms of carcinogenesis, tumor growth and metastasis.

Calreticulin is a ubiquitous protein found in a wide range of species and in all nucleated cell types. It is a Ca+2 binding chaperone, which is resident in the lumen of the endoplasmic reticulum (ER). Calreticulin binds (buffers) Ca+2 in the ER lumen with high capacity and also participates in the folding of newly synthesised proteins and glycoproteins [1-4]. Together with calnexin (a type I integral membrane protein of the ER which exhibits a high degree of amino acid sequence and structure similarity to calreticulin), they are perhaps the most important chaperones involved in ER quality control and secretory pathways. They directly affect the folding and post-translational modification of virtually all glycosylated, secreted or integral membrane proteins that pass through the ER. This means that their effects are expansive because the majority of cell surface receptors, channels and transports are glycosylated as are many proteins that reside in intracellular organelles including the ER itself. The action of calreticulin is vital

Correspondence to: Dr. Irene Voloudakis-Baltatzis. Department of Cell Biology, Molecular Biology and Electron Microscopy, "G. Papanicolaou" Research Center of Oncology and Experimental Surgery, "Saint Savas" Anticancer Hospital, 171, Alexandras Ave, 115 22 Athens, Greece.
Tel: +30 210 6409234, Fax: +30 210 6409522, E-mail: Ibalt@ath.forthnet.gr

Received 03-08-2007; Accepted 19-09-2007

in a diverse range of cellular functions including cell-cell communication and recognition, immune responses, ion, solute and metabolite fluxes. Glycosylation of newly synthesized proteins acts as a signal for enhanced folding and quality control and for the degradation of misfolded proteins. Furthermore, it has been shown that calreticulin affects transcriptional control of selected genes involved in cell adhesion [5,6]. Calreticulin also seems to participate in a not yet clear pathway, which results in decrease of tyrosine phosphorylation [6,7] also having effect on cell adhesion.

In our lab we produced 2D SDS-PAGE protein maps of mirror biopsy and malignant tissues derived from patients with colon adenocarcinoma. Through thorough study of these maps several proteins have been shown to be either overexpressed or underexpressed in both types of the above tissues. One of them, being upregulated in cancer, turned out to be calreticulin. This protein of interest was further examined by immunohistochemistry in order to confirm the observed overexpression and to obtain further information on the topology of this overexpression.

Materials and methods

In this study 21 adenocarcinomas and 21 mirror biopsy tissues (morphologically healthy tissue close to the tumor site) were examined, which were obtained surgically from the first Propaedeutic Surgical Clinic, School of Medicine, University of Athens, Hippocration Hospital. Samples were immediately placed in ice-cold saline, in the operating room. In the laboratory half of the tissue was washed free of blood with ice- cold saline frozen in liquid nitrogen and kept at –70° C until further use. Ten of the above tissues (5 adenocarcinomas and 5 mirror biopsies) were fixed in 25% glutaraldehyde for immunohistochemical study.

Homogenisation

All the procedures were carried out on ice. The tissue was cleaned of excess fat. It was then washed with isotonic sucrose homogenisation buffer pH 7.4 (0.25 M sucrose, 10 mM Tris-HCl, 1.5 mM EDTA, 0.5 mM DTT, 25 Mm KCl, 5 Mm $MgCl_2$ and 0.2 Mm PMSF as a protease inhibitor) for blood present to be removed. Following the wash the tissue was cut to small pieces using a pair of scalpels. Blood was washed from the cut tissue using again the homogenisation buffer. The tissue was homogenized in a Potter Elvjhem homogenizer in a total of 8 ml homogenisation buffer. The homogenate was filtered through nylon gauze 40 threads/cm to remove tissue debris. Following that, the filtered homogenate was centrifuged in a refrigerated benchtop centrifuge at 1000 g for 15 min at 4° C. The supernatant containing the cytoplasm and various membrane elements of the cell, such as Golgi complex, ER lysosomes and mitochondria, was further centrifuged at 100000 g for 1 h at 4° C.

The pellet containing the membrane formations mentioned earlier was collected and, after a short lyophilisation to remove the excess liquid, was incubated O/N with redyPrep sequential extraction kit reagent III (Bio-Rad Laboratory; Urea-Thiourea Solution) to solubilise all the membrane-bound insoluble proteins. The solution was then centrifuged at 25000 g for 1 h at room temperature. The pellet was discarded and the total protein content of the supernatant containing all the solubilised proteins was determined using the Bradford method [8]. The supernatant was stored at –80° C.

The supernatant, containing the soluble protein content of the cells was collected and buffer-exchanged against Tris buffer pH 7.4 for 4 h. The buffer exchanged protein solution was then lyophilised O/N. The lyophilised proteins were solubilised in Sample Buffer (9 M Urea, 4% w/v CHAPS, 1% DTT, 0.2 mM PMSF) urea and the total protein concentration was determined with the Bradford method [8]. The protein solution was stored at –80° C.

2D gel electrophoresis

2D-PAGE analysis of cytosolic polypeptides from 16 colorectal carcinoma and 16 mirror biopsies tissues were performed as described by O' Farrel [9] and modified by Hochstrasser et al. [10] using the Bio-Rad 2D-PAGE system. The lyophilised protein samples were dissolved in sample solution (9M urea, 4% w/v CHAPS, 1% DTT) and the protein content was determined using the Ramagli and Rodriguez modification of the Bradford protein assay [8]. First dimensional isoelectric focusing (IEF) was carried out in 180×1.5 mm rod gels. 75 μg of protein were always loaded onto the basic end of each IEF gel with carrier ampholytes, pH range 4-8 (concentration 4%) and pH 3.5-10 (concentration 1%) [10]. Samples were electrophoresed at 700 V for 19 h. Each sample was electrofocused in duplicate, with and without the creatine kinase (cK) charge. After extrusion, gels were stored in extended form in parafilm strips at –80° C until used. IEF gels were allowed to soak in equilibration buffer [10] for 5 min before being loaded onto the second dimension gels and overlayed with a small amount of agarose (0.025 M Tris base, 0.192 mM glycine, 0.1% SDS, 0.5% agarose). Second dimensional SDS-PAGE was performed at constant current (35 mA/gel) at + 8° C using 1.5 mm thick 12% polyacrylamide gels.

Silver staining densitometry and computer analysis of 2D-gels

Proteins were visualised on 2D gel separations by silver staining with the Sigma silver stain kit. Gels were stained in large Pyrex glass dishes. For increased sensitivity the staining procedure was repeated. Cleanliness of glassware, uniformity of incubation times and efficiency of shaking were essential for reproducible high staining. Gels were photographed and scanned immediately after staining and stored in plastic bags with distilled H_2O. 2D gels were digitised using the 620 CCD Densitometer of Bio-Rad with a resolution of 132 μm and optical density range 0.0-3.0. The analysis was carried out by Bio-Rad 2D-Analyst II software running on a AST Premium 386/33 computer.

Resin samples

Five adenocarcinoma tissues and the respective 5 mirror biopsies were fixed in a 2.5% solution of glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.2 for 1 h and then in a 1% osmium tetroxide (OSO_4) solution in 0.1 M sodium phosphate buffer pH 7.2 for 1 h.

The specimens were then dehydrated in ethanol and embedded in Araldite resin. Semithin sections (1 μm) were cut for light immunohistochemical study.

Immunohistochemistry (PAP method)

The semithin sections from adenocarcinomas and mirror biopsy tissues derived from patients were examined. The disease stage according to the Dukes classification based on the histopathology reports is shown in Table 1. These sections were treated in ethoxide for 5-10 min and dehydrated via graded alcohol. They subsequently were treated in 0.3% H_2O_2 for 8-10 min. Following incubation for 30 min in a blocker solution containing 1/30 normal goat serum in phosphate-buffered saline (PBS), sections were immunostained with antiserum to calreticulin (anti-calreticulin, N.B 600-101 A5 rabbit antiserum, Abcam Ltd, Cambridge). Afterwards, PAP was applied, diluted in bovine serum albumin (BSA) and PBS 1/400 for 30 min. Finally the sections were incubated for 5 min in a solution containing diaminobenzidine (DAB), H_2O_2 and PBS.

Following that, the slides were rinsed twice with tap water and counterstained in hematoxylene for 2 min. They were then visualised using an optical microscope and were photographed using a Nikon Coolpix 3100.

Western blots

SDS-PAGE samples were transferred to PVDF membranes (Bio-Rad). The membranes were blocked for

Table 1. Histopathological features of colon specimens

No.	*Gender*	*Age (years)*	*Tumor type*	*Dukes stage*	*Tissue differentiation*	*Lymph nodes*
Patients whose mirror biopsy and cancer tissue samples were used for 2D-SDS PAGE analysis						
1	Male	76	Adenocarcinoma	A	High	–
2	Female	61	Adenocarcinoma	A	High	–
3	Male	80	Adenocarcinoma	A	Moderate	–
4	Male	52	Adenocarcinoma	A	Moderate	–
5	Male	40	Adenocarcinoma	B	High	
6	Female	N/A	Adenocarcinoma	B	Moderate	–
7	Female	N/A	Adenocarcinoma	B	Moderate	–
8	Male	73	Adenocarcinoma	B	Moderate	–
9	Female	N/A	Adenocarcinoma	B	Moderate	–
10	Female	63	Adenocarcinoma	B	Moderate	–
11	Female	76	Adenocarcinoma	B	Moderate	–
12	Male	67	Adenocarcinoma	B	Moderate	–
13	Female	85	Adenocarcinoma	C	Moderate	1/2
14	Female	63	Adenocarcinoma	C	Moderate	2/11
15	Female	76	Adenocarcinoma	C	Moderate	15/18
16	Female	85	Adenocarcinoma	C	Low	4/11
Patients whose mirror biopsy and cancer tissue samples were used for immunohistochemical study of calreticulin expression						
1	Female	56	Adenocarcinoma	A	Moderate	–
2	Male	63	Adenocarcinoma	A	Moderate	–
3	Male	66	Adenocarcinoma	B	Moderate	–
4	Male	72	Adenocarcinoma	C	Low	12/12
5	Male	84	Adenocarcinoma	C	Low	2/17

N/A: not available; Lymph nodes: 1/2 means 1 positive out of 2 lymph nodes

1 h in 3% Blocker (Bio-Rad) in TTBS buffer (tris-HCl, NaCl, tween-20, pH 7.5). Following that, the membranes were washed in PBST twice and incubated with the primary antibody (Rabbit Anti-Calreticulin Polyclonal antibody from Abcam), diluted 1:1000 in 1% BSA TTBS buffer, for 2 h. The membrane was then washed with TTBS for 5 min and incubated for 2 h with the secondary antibody conjugated with HRP (Goat Anti-Rabbit IgG-HRP from Abcam), diluted 1:3000 in 1% BSA TTBS buffer. The membrane was then washed with TTBS buffer and incubated for 30 min with Opti-4CN solution from Bio-Rad, for colorimetric detection. The membrane was then washed in ddH20 for 15 min and was allowed to dry. It was then documented and stored.

Results

2D SDS-PAGE

The majority of the tumors were moderately differentiated and derived from different stages of colon adenocarcinoma (Table 1). Duplicate patterns obtained for each colon sample revealed a high degree of consistency in pattern, complexity and intensity of silver stain.

Calreticulin expression levels in the cancer samples 2D gels were increased when compared with the expression levels of the protein in the mirror biopsy samples and this was observed in all 3 disease stages. The spot with coordinates pI=4.39, MW=59197 Da was identified as calreticulin using the Western blot technique.

The volume measurements of all samples are tabulated in Table 2, where they are categorised by disease stage and tissue type. The average volume for each disease stage and each tissue type was calculated and the results are shown in Table 3.

Dukes stage A: Calreticulin's expression in cancer tissues was increased by an average 18.34 volume units compared to the calreticulin's levels in the mirror biopsy tissue (Table 3).

Dukes stage B: Calreticulin's expression in cancer tissues was increased by an average of 31.10 volume units compared to the calreticulin's levels in the mirror biopsy tissues (Table 3). Examining each case separately reveals that 1 out of 7 patients presented the same calreticulin's expression levels in both cancer and mirror biopsy tissues.

Dukes stage C: Calreticulin's expression in cancer tissues was increased by an average of 86.39 volume units compared to the calreticulin's levels in the mirror biopsy tissues (Table 3). The levels of calreticulin in the mirror biopsy tissues of this stage seem to be greatly elevated compared with the mirror biopsy tissues of the other stages.

Table 2. Calreticulin expression studies on 2D SDS-PAGE gels

Dukes A	
Cancer tissue	*Mirror biopsy tissue*
59.38	43.52
65.60	37.80
65.02	51.80
66.86	50.41
Dukes B	
Cancer tissue	*Mirror biopsy tissue*
126.28	97.34
99.90	39.69
45.41	28.97
23.69	21.32
35.16	35.06
49.09	27.84
88.35	Not detected
Dukes C	
Cancer tissue	*Mirror biopsy tissue*
139.93	45.90
237.19	52.09
327.10	316.98
89.25	32.93

The calreticulin spot volumes indicating calreticulin expression have been tabulated for every case (mirror biopsy, cancer tissue) of all 3 disease stages

2D gel sections from all 3 disease stages indicating calreticulin are illustrated in Figure 1 (A-D).

Immunohistochemistry

The results revealed that calreticulin immunoreacted in both specimens in mirror biopsies and colorectal adenocarcinomas, in all Dukes stages (A, B, C), but with different density (Table 4).

In stage A, colorectal adenocarcinomas showed normal immunoreactivity (Figure 2a) and weak in mirror biopsies (Figure 2b). In stage B adenocarcinomas, calreticulin immunoreacted stronger than in stage A (Figure 3a). Also, the mirror biopsies immunoreacted moderately (Figure 3b). The last group of stage C immunoreacted stronger in colorectal adenocarcinomas and in mirror biopsies (Figure 4a and b).

Table 3. Average calreticulin expression in the 3 disease stages

Disease stage (Dukes)	*Cancer tissue (average volume)*	*Mirror biopsy tissue (average volume)*
A	64.22	45.88
B	66.84	35.74
C	198.37	111.98

The calreticulin spot volumes indicating calreticulin expression on Table 2 have been averaged for each disease stage and tissue type

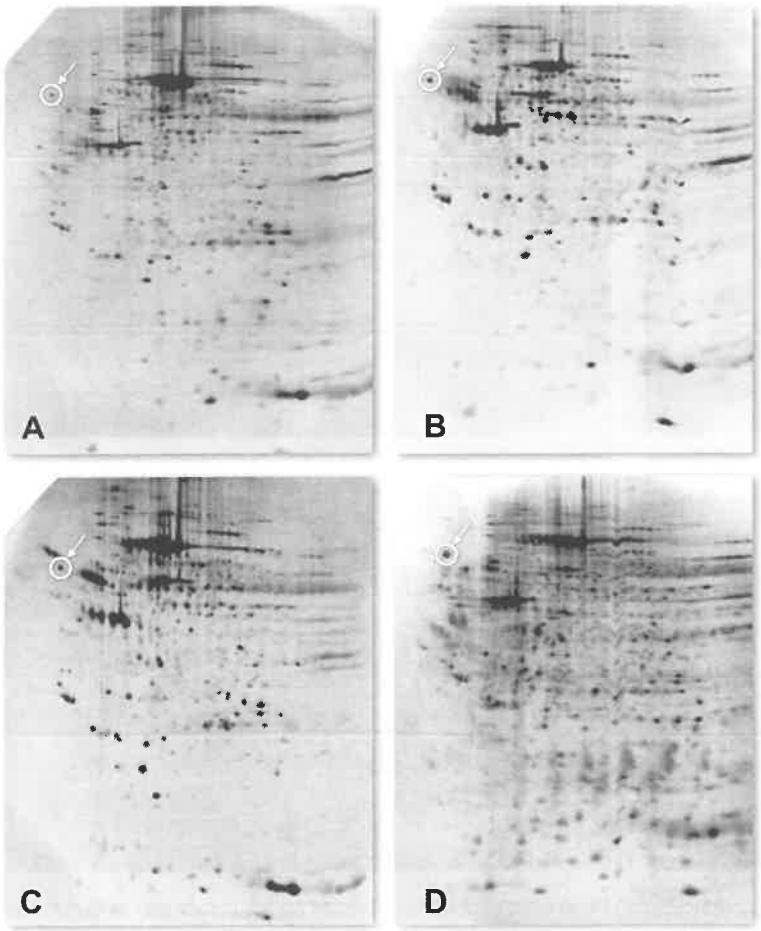


Figure 1. Calreticulin expression in various disease stages (A: mirror biopsy; B: Dukes A; C: Dukes B; D: Dukes C). The calreticulin spot is circled and indicated with arrow.

Discussion

The densitometry performed on the 2D gels for calreticulin reveals a proportional relationship of calreticulin expression levels and disease stage.

As the disease progresses to stage B the difference between cancer and mirror biopsy state increases in terms of calreticulin expression (Table 2) as indicated by densitometry of the 2D gels. It must be noted that in 1 out of 7 Dukes B cases analysed with 2D gels calreticulin expression levels in the cancer tissues were equal to the ones of the mirror biopsy tissue. This probably means that the mirror biopsy tissue is not actually healthy tissue. This implies that the early biochemical events of tumor growth cause no morphological differentiation on the affected tissues. Surprisingly, in stage C calreticulin levels were elevated in both cancer and mirror biopsy tissues as compared to the corresponding levels of the first 2 stages by approximately 3 orders of magnitude. This fact is vividly depicted on Table 3, where the average volumes were calculated for all 3 disease stages. This finding implies that the presently considered mirror biopsy tissue close to the tumor has biochemically evaded the mirror biopsy state (as mentioned earlier). Furthermore, there is a strong indication that the currently unknown reason for calreticulin expression in the cancer process is much more intense in stage C as compared to the first 2 stages. Even though calreticulin expression levels in Dukes C mirror biopsy tissue are elevated, the difference between calreticulin

Table 4. Results of immunohistochemical calreticulin expression studies

Patient no.	*Tissue type*	*Dukes stage*	*Calreticulin expression*
1	Adenocarcinoma	A	±
	Mirror biopsy	–	±
2	Adenocarcinoma	A	+
	Mirror biopsy	–	+
3	Adenocarcinoma	B	++
	Mirror biopsy	–	+
4	Adenocarcinoma	C	+++
	Mirror biopsy	–	++
5	Adenocarcinoma	C	+++
	Mirror biopsy	–	+

Calreticulin expression is empirically determined
± indicates very faint expression and +++ indicates very strong expression

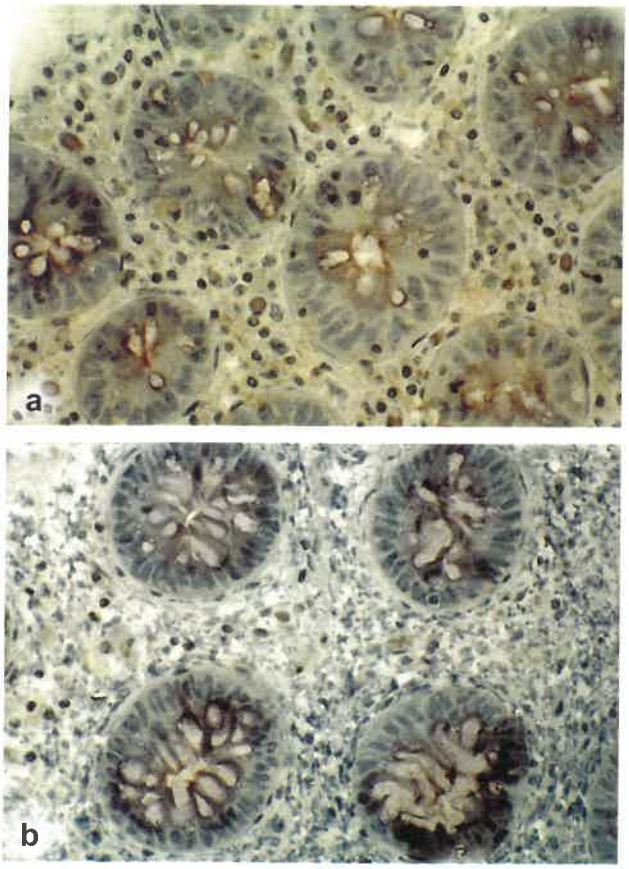


Figure 2. Sections showing the immunoreactivity of calreticulin in colorectal adenocarcinoma (Dukes A) from (a) adenocarcinoma and (b) mirror biopsy (× 40).

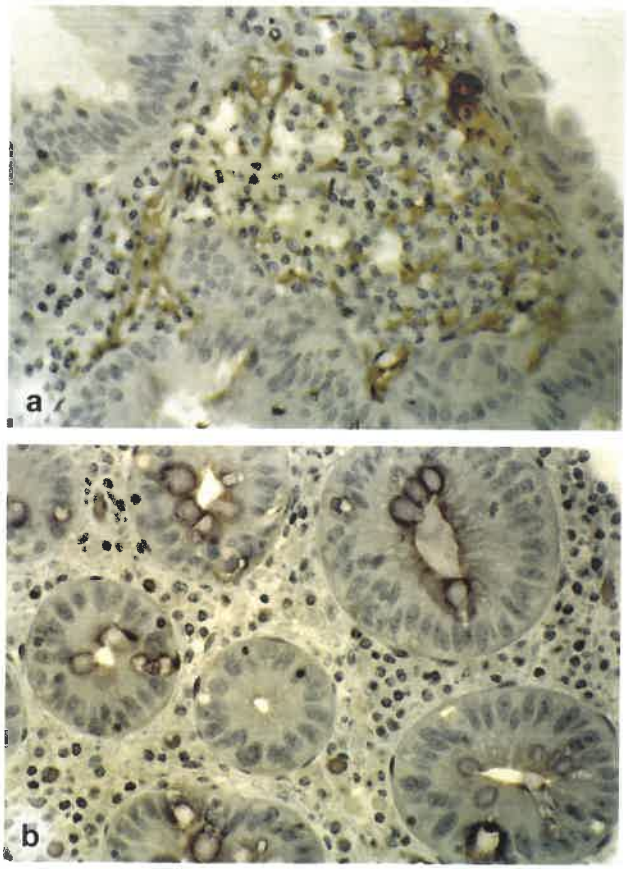


Figure 3. Sections showing the immunoreactivity of calreticulin in colorectal adenocarcinoma (Dukes B) from (a) adenocarcinoma and (b) mirror biopsy (× 40).

levels in mirror biopsy and cancer tissues of that stage is the greatest noted (Table 2). These findings are in complete agreement with the immunohistological studies that are presented in this paper.

An additional observation is that calreticulin is mainly overexpressed in the poorly differentiated regions of the tissues under study. This suggests that the overexpression characteristic is closely associated with tumor aggressiveness, since poorly differentiated tumors seem to be more aggressive than the ones with moderate or high differentiation.

Several years ago it was found that calreticulin could bind to KxGFFKR motif within the a-subunits of intergrins and regulate intergrin-mediated adhesion [11]. It was demonstrated that calreticulin-null murine ES cells exhibited severely impaired intergrin function, both intergrin-mediated adhesion to extracellular matrix proteins and intergrin-triggered calcium influx from extracellular sources [11]. It was also demonstrated that cells overexpressing calreticulin present increased cell adhesion to the extracellular matrix [7].

Furthermore, it was demonstrated that overexpression of calreticulin in mouse L fibroblasts correlated with increased cell adhesiveness. Specifically, when overexpressing calreticulin fibroblasts aggregate effectively at a shear stress, which prevents aggregation in other cell lines and when released from contact inhibition, they display a pattern of mobility resembling that of epithelia. It was shown that cells overexpressing calreticulin have significantly decreased tyrosine phosphorylation of cellular proteins, including b-catenin, a component of cadherin-based junctional complexes [7]. In particular, when dephosphorylated on tyrosine, b-catenin is stabilized within junctional complexes where it may participate in linking junctional components to the cytoskeleton [12-14]. In contrast, when phosphorylated on tyrosine, b-catenin displaces to the cytoplasm [15,16].

Additionally, recent evidence indicates that calreticulin may affect gene expression [5,6,17,18], and cells overexpressing calreticulin have elevated expression of N-cadherin and vinculin, which are both adhesion-related proteins [5,6].

Summarizing, 2D SDS-PAGE electrophoresis and immunohistochemistry were used to analyse calreticulin expression. Calreticulin was found overex-

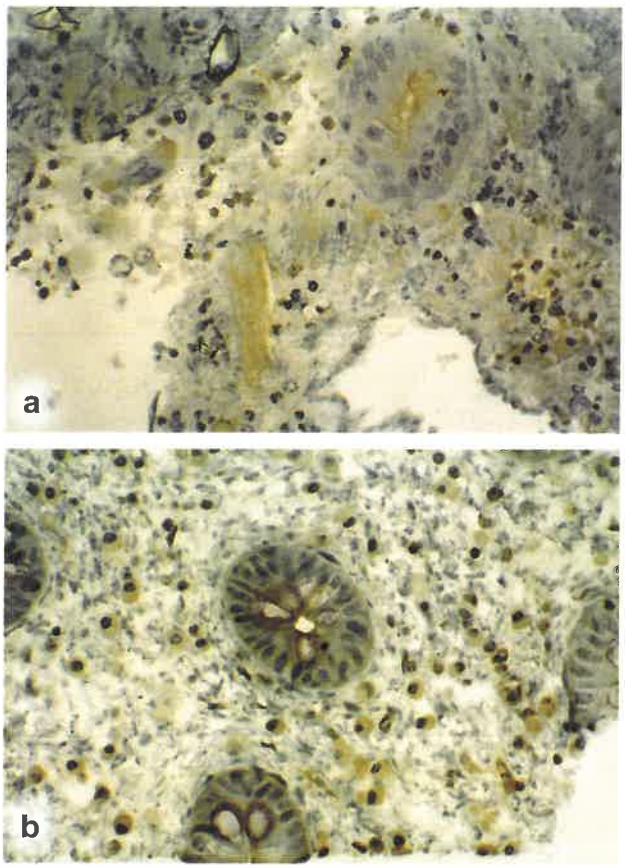


Figure 4. Sections showing the immunoreactivity of calreticulin in colorectal adenocarcinoma (Dukes C) from (a) adenocarcinoma and (b) mirror biopsy (× 40).

pressed in colon cancer tissues as compared to the corresponding mirror biopsy tissues. The overexpression was particularly intense to Dukes C tissues and particularly in the poorly differentiated regions of the tissue. Furthermore, the stage C mirror biopsy tissues displayed increased calreticulin levels as compared to the mirror biopsies of the other stages. Bearing in mind that cell adhesion mechanisms are an element of extreme importance for tumor growth and metastasis, the fact that overexpression of calreticulin reveals a direct relationship analogous to the disease stage strongly indicates that the functional role of calreticulin is directly associated with tumor growth and metastasis.

Acknowledgements

The authors wish to thank H. Georga and H. Tsiamanta for their excellent technical assistance.

References

1. Jacob CA, Chevet E, Thomas DY, Bergeron JJ. Lectins of the ER quality control machinery. Results Probl Cell Differ 2001; 33: 1-17.
2. Michalak M, Corbett EF, Mesaeli N, Nakamura K, Opas M. Calreticulin: one protein, one gene, many functions. Biochem J 1999; 344: 281-292.
3. Ellgaard L, Molinari M, Helenius A. Setting the standards: quality control in the secretory pathway. Science 1999; 286: 1882-1888.
4. Yoshida Y, Chiba T, Tokunaga F et al. E3 Ubiquitin ligase that recognizes sugar chains. Nature 2002; 418: 438-442.
5. Opas M, Szewczenko-Pawlikowski M, Jass GK, Mesaeli N, Michalak M. Calreticulin modulates cell adhesiveness via regulation of vinculin expression. J Cell Biol 1996; 135(6 Pt 2): 1913-1923.
6. Fadel MP, Dziak EL, Ferrier J, Mesaeli N, Michalak M, Opas M. Calreticulin affects focal contact-dependent but not close contact-dependent cell-substratum adhesion. J Biol Chem 1999; 274: 15085-15094.
7. Fadel MP, Szewczenko-Pawlikowski M, Leclerc P et al. Calreticulin affects beta-catenin-associated pathways. J Biol Chem 2001; 276: 27083-27089.
8. Ramagli LS, Rodriguez LV. Quantitation of microgram amounts of proteins in two-dimensional polyacrylamide gel electrophoresis sample buffer. Electrophoresis 1985; 6: 559-563.
9. O' Farrell PH. High resolution two-dimensional electrophoresis of proteins. J Biol Chem 1985; 250: 4007-4021.
10. Hochstrasser DF, Harrington MG, Hochstrasser AC, Miller MJ, Merril CR. Methods for increasing the resolution of two-dimensional protein electrophoresis. Ann Biochem 1988; 173: 424-435.
11. Coppolino MG, Dedhar S. Calreticulin. Int J Biochem Cell Biol 1998; 30: 553-558.
12. Hazan RB, Kang L, Roe S, Borgen PI, Rimm DL. Vinculin is associated with the E-cadherin adhesion complex. J Biol Chem 1997; 272: 32448-32453.
13. Hazan RB, Norton L. The epidermal growth factor receptor modulates the interaction of E-cadherin with the actin cytoskeleton. J Biol Chem 1998; 273: 9078-9084.
14. Barth AI, Nathke IS, Nelson WJ. Cadherins, catenins and APC protein: interplay between cytoskeletal complexes and signaling pathways. Curr Opin Cell Biol 1997; 9: 683-690.
15. Eastman Q, Grosschedl R. Regulation of LEF-1/TCF transcription factors by Wnt and other signals. Curr Opin Cell Biol 1999; 11: 233-240.
16. Waser M, Mesaeli N, Spencer C, Michalak M. Regulation of calreticulin gene expression by calcium. J Cell Biol 1997; 138: 547-557.
17. Liewellyn DH, Kendall JM, Sheikh FN, Campbell AK. Induction of calreticulin expression in HeLa cells by depletion of the endoplasmic reticulum Ca2+ store and inhibition of N-linked glycosylation. Biochem J 1996; 318 (Pt 2): 555-560.
18. Nguyen TO, Capra JD, Sontheimer RD. Calreticulin is transcriptionally upregulated by heat shock, calcium and heavy metals. Mol Immunol 1996; 33: 379-386.