Different expression of calgizzarin (S100A11) in normal colonic epithelium, adenoma and colorectal carcinoma

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Abstract. The aim of the study was to detect proteomic markers usable to distinguish colorectal carcinoma from colon adenoma for a better understanding of the molecular mechanisms in the process of tumourigenesis. Therefore, we microdissected colon carcinoma tissue, epithelial colon adenoma tissue as well as normal adjacent colon epithelium and determined protein profiles by SELDI-TOF MS. A multitude of significantly different signals was detected. For their identification colon biopsis were lysed and subjected to a two-dimensional gel electrophoresis for separation. Subsequently, we identified nearly 100 proteins by tryptic digestion, peptide fingerprint mapping and database search. Calgizzarin (S100A11; S100C) identified by peptide fingerprint mapping correlated very well with a significantly differentially expressed signal found in prior protein profiling. Using an immunodepletion assay we confirmed the identity of this signal as calgizzarin. To localise calgizzarin in tissues we performed immunohistochemistry. For further confirmation of the identity of calgizzarin we re-analysed IHC-positive as well as IHC-negative tissue sections on ProteinChip arrays. This work demonstrates that biomarkers in colorectal cancer can be detected, identified and assessed by a proteomic approach comprising tissue-microdissection, protein profiling and immunological techniques.

Introduction

Colorectal cancer is ranked the third most common form of cancer worldwide in terms of incidence (estimated to result in 945000 new cases, 9.4% of the world total population) and mortality (492000 deaths, 7.9% of the total) in 2000 (1). The majority of colorectal cancers are non-hereditary and sporadic, which makes early detection even more important. Tumourigenesis in sporadic colorectal cancer (CRC) has been extensively studied and can be seen as a multistep process, with each step representing specific gene mutations or epigenetic changes (2). The progression from adenoma to sporadic CRC results from the accumulation of genetic and epigenetic alterations involving activation of oncogenes and inactivation of tumour suppressor genes (3). Several other molecular mechanisms were also shown to be involved in tumour development, but tend to occur infrequently. Despite the dominant role of the adenoma-carcinoma sequence these changes are thought to be responsible for the different biological nature and clinical behaviour of CRC. In addition epidemiological studies revealed a strong impact of environmental and dietary factors.

Regardless of the enormous efforts and introduction of new parallel genomic and proteomic techniques in recent years, relevant markers that can be used for early diagnosis or improved therapy in cancer have only been established in a few tumour diseases (4). One point might be that the *in situ* situation in tumours is neglected, because results from starting material like serum and non-microdissected tissue cannot be traced back to the biological properties or the heterogeneity of the tumour itself. Hence, microdissection, proteomic techniques and immunohistochemistry have been combined in a technical triade (5).

The proteomic technique, SELDI-MS (surface enhanced laser desorption/ionization-mass spectrometry), uses chromatographic surfaces able to retain proteins depending on their physico-chemical properties followed by direct analysis via time of flight mass spectrometry (TOF-MS) (6). This technique does not require large amounts of samples making it ideal for small biopsies or microdissected tissue which are required to produce the homogeneous tissue samples indispensable in cancer research (7-9). Microdissected tissue material, free of contaminating and unwanted tissue components, is extremely important for producing clean data for biomarker identification in cancer diagnostics and in elucidating clonal heterogeneity of tumours. In the present study, the epithelial tumour cells respectively adenoma cells had to be separated from all surrounding tissue constituents. This separation was done with an extremely precise technique such as laser based microdissection. This technique has previously been combined

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with ProteinChip technology to identify protein markers in other cancers (10-12).

We analysed pure microdissected populations of cells from colorectal carcinoma tissue, colon adenoma epithelium as well as normal colon epithelium using SELDI-MS ProteinChip technology.

The protein patterns derived by this analysis showed a multitude of significant differences. To identify this differentially expressed proteins crude extracts of biopsis were separated by 2D gel electrophoresis followed by tryptic digestion and peptide fingerprint mapping. Among nearly 100 identified proteins calgizzarin (S100A11; S100C) correlated very well to a significantly different marker which was overexpressed in CRC found in prior protein profiling. The identity of this protein was also confirmed as calgizzarin by immunological techniques.

Materials and methods

Laser microdissection of tissue sections. All 13 CRC samples and matched normal mucosa (n=19) as well as 30 adenoma tissue samples were obtained after surgical resection at the Department of General and Visceral Surgery of the Friedrich-Schiller-University Jena; these were collected fresh, snap-frozen in liquid nitrogen, and stored at -80˚C. Tumour specimens were categorized according to their WHO classification. Most of the tumours were classified as pT2 and pT3.

Laser microdissection was performed with a laser microdissection and pressure catapulting microscope (LMPC; Palm, Bernried, Germany) as described elsewhere (10). Briefly, we microdissected on native air-dried cryostat tissue sections ~3000-5000 cells each in a maximum of 20-30 min. Proteins were extracted by 10 μl lysis buffer (100 mM Na-phosphate (pH 7.5), 5 mM EDTA, 2 mM $MgCl_2$, 3 mM 2-ß-mercaptoethanol, 0.1% CHAPS, 500 μM leupeptin, and 0.1 mM PMSF) for 30 min on ice. After centrifugation (15 min; 15000 rpm) the supernatant was immediately analyzed or frozen in liquid nitrogen for a maximum of one day.

Profiling of microdissected normal colon epithelium, adenoma tissue and epithelial carcinoma tissue. The protein lysates from microdissected tissues (carcinoma, adenoma and normal) were analysed on strong anion exchange arrays (Q10; Ciphergen Biosystems Inc., Fremont, CA) as described elsewhere (10). In brief, array spots were preincubated by a washing/loading buffer containing 100 mM Tris-buffer, pH 8.5 with 0.02% Triton X-100 followed by application of 2 μl of sample extract on ProteinChip arrays, which were incubated at room temperature for 90 min in a humidity chamber. After washing three times with the same buffers and two final washing steps with water, 2x0.5 μl sinapinic acid (saturated solution in 0.5% TFA/50% acetonitrile) was applied. Mass analysis was performed in a ProteinChip Reader (Series 4000, Ciphergen Biosystems Inc.) according to an automated data collection protocol. Spectra were normalised with total ion current and cluster analysis of the detected signals and the determination of respective P-values for normal, adenoma and carcinoma tissue was carried out with the CiphergenExpress Program (Version 3.0; Ciphergen Biosystems Inc.). For P-value calculation, normalised spectra with signals in the range between 2.5 and 200 kDa exhibiting a signal-to-noise ratio (S/N) of at least 10 were selected and analysed with the Mann-Whitney U test for non-parametric data sets.

Two-dimensional gel electrophoresis. Samples for two-dimensional gel electrophoresis (2-DE) were prepared directly from surgical material of colon tumour and corresponding normal colon epithelium tissue assessed by a pathologist. Proteins were isolated and 2-DE was performed as described elsewhere (10). In brief, isoelectric focusing (IEF) was carried out on a Multiphor II (Amersham) using 7 cm IPG strips. Vertical SDS-PAGE was performed in a cooled Protean II xi Cell (Bio-Rad) using 4-12% Bis-Tris Zoom™ gel (Invitrogen). The gels were stained with Simply Blue Safe Stain (Enhanced Coomassie, Invitrogen).

In-gel digestion. Protein patterns of the 2-DE gels from normal colon epithelium and tumour tissue were comparatively analysed and protein spots with a molecular mass of nearly 10-50 kDa were excised. In-gel digestion of proteins was performed as described elsewhere (10). In brief, excised gel pieces were destained and dried. After rehydration and digestion with 10 μl of a trypsin solution (0.02 μg/μl; Roche) at 37˚C for 7 h supernatants were applied directly on a NP20 Protein Chip array (Ciphergen Biosystems Inc.). An empty gel piece underwent the same treatment as a control. After addition of the matrix (CHCA; Ciphergen Biosystems Inc.), peptide fragment masses were analysed using the ProteinChip Reader. The spectra for the peptide mapping experiments were externally calibrated using five standard proteins including Arg8-vasopressin (1082.2 Da), somatostatin (1637.9 Da), dynorphin (2147.5 Da), ACTH (2933.5 Da) and insulin ß-chain (3495.94 Da). Proteins were identified using the fragment masses generated through trypsin digestion by searching in a publicly available database (http://129.85.19.192/profound_bin/WebProFound.exe).

Immunodepletion assay. Anti-calgizzarin serum (2 μl) (gift of Dr Jean-Christophe Deloulme) were incubated with 10 μl protein A-agarose (Sigma) for 15 min on ice. A pellet was generated by centrifugation and the supernatant was discarded. The pellet was washed two times with a buffer containing 20 mM Hepes (pH 7.8), 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA and 0.05% NP-40. Afterwards, 5 μl of a lysate from laser-dissected CRC were incubated with this pellet for 45 min on ice. As a negative control 5 μl of the lysate were incubated with protein A-agarose without the specific antibody for 45 min on ice. After incubation samples were cleared by centrifugation and 3 μl of each supernatant were analysed by ProteinChip arrays.

Immunohistochemistry. Cryostat sections (8-μm) of colon cancer tissue and colon adenoma tissue were placed on slides, air dried for approximately 60 min at 20˚C and fixed in paraformaldehyde as described (10). After fixation, slides were treated in the microwave at 80 watts (3x3 min) in 10 mM citric acid pH 6.0 to inhibit endogenous peroxidatic activity. Subsequently, they were rinsed twice with TBS pH 7.4, and incubated overnight at 4˚C in humidity chamber with the corresponding primary anti-calgizzarin serum. Slides were

Table I. Significantly different signals which separate CRC, adenoma and normal tissue samples detected on Q10 ProteinChip arrays. The signal representing the subsequently identified calgizzarin is displayed bold.

MW (kD)	P-value
4.968	$1.04x10^{-2}$
5.087	$3.83x10^{-5}$
8.212	$1.24x10^{-2}$
8.406	$2.91x10^{-3}$
9.757	$3.24x10^{-2}$
10.179	$2.42x10^{-5}$
10.357	$9.38x10^{-5}$
10.396	$9.83x10^{-6}$
10.532	$7.77x10^{-6}$
12.001	$9.64x10^{-3}$
12.189	$6.18x10^{-3}$
16.810	$6.01x10^{-3}$
16.937	$8.93x10^{-3}$
18.480	$5.5x10^{-3}$
23.826	$2.61x10^{-4}$
24.785	$2.08x10^{-3}$
27.973	$3.18x10^{-4}$
46.581	$4.09x10^{-4}$

rinsed 3x10 min in TBS and the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and the Jenchrom pxbl-kit (MoBiTec, Göttingen, Germany) were used according to the manufacturer's instructions to visualize the localization of the antibody. Negative controls were incubated with the labeled secondary antibody only. Sections cut in parallel to the IHC-treated sections were stained by HE for better identification of different tissue areas. IHC staining was evaluated by a pathologist.

Results

Protein profiling of microdissected CRC, colon adenoma and normal colonic epithelium. Areas corresponding to about 3000-5000 cells per tissue probe were excised, and 62 tissue sections in total (13 carcinoma, 30 adenoma and 19 normal colonic epithelium tissues) were successfully dissected by a pathologist. All protein lysates from the microdissected tissues were applied to Q10 arrays and analysed on a ProteinChip Reader Series 4000 instrument. In the range between 2.5-200 kDa, up to 158 peaks were detected with normalised intensities. After evaluation with the CiphergenExpress Program, a multitude of significantly different signals were detectable for CRC, adenoma and normal tissue samples (Table I).

Identification of a differentially expressed signal. For identification of signals histologically checked pieces of different colon tissues were lysed and subjected to 2-DE for separation. Numerous protein spots in the range of about 10-50 kDa were excised from the gels and peptide fingerprints of the tryptic digested spots were determined using SELDI-TOF MS. In this way we were able to identify nearly 100 proteins by database search (profound; http://129.85.19.192/profound_bin/WebProFound.exe). One of these proteins, calgizzarin, correlated very well with a significantly different signal found in prior protein profiling. This signal of ~12 kDa showed an increased expression in samples derived from colorectal carcinoma and discriminated significantly between CRC and adenoma tissue and normal colon tissue ($P=9.64x10^{-3}$) as well as between CRC and adenoma or normal colon epithelium, respectively ($P=2.35x10^{-3}$ or $3.01x10^{-3}$, respectively). The distribution of the intensities for the different tissues is given in Fig. 1. Representative examples of SELDI-MS spectra from CRC, colon adenoma and normal colon epithelium are shown in Fig. 2 in the range from 10.5 to 14 kDa. The identification of further significant signals is in progress.

The reassurance that calgizzarin matches to the differentially expressed peak at 12 kDa found by ProteinChip analysis was done with an immunodepletion assay using

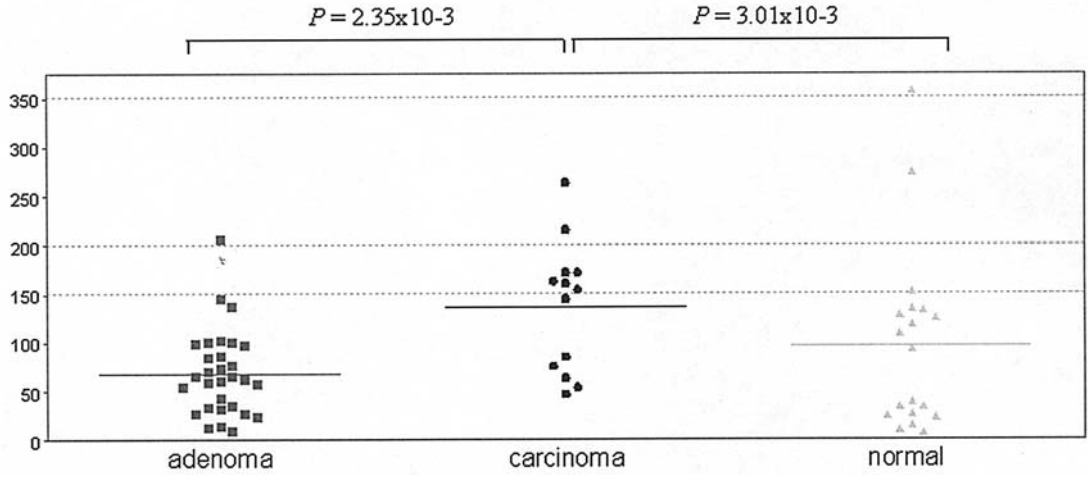

Figure 1. The distribution of the intensities of 12 kDa peak in colon adenoma samples (adenoma), CRC samples (carcinoma) and normal colon epithelium samples (TZ LCM). The spectra were obtained using Q10 arrays. X-axis indicate the sample groups, Y-axis the intensity (μA).

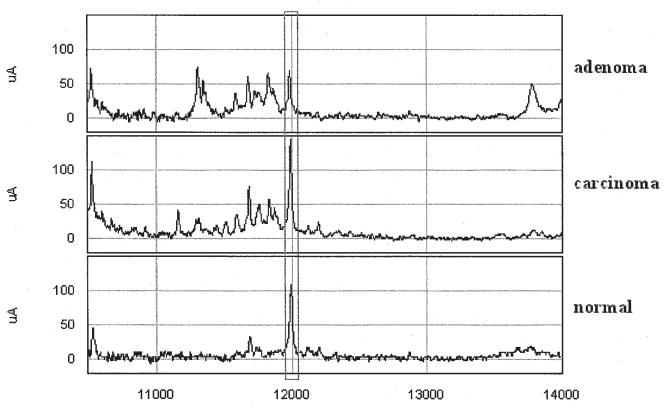

Figure 2. Representative examples of SELDI-TOF MS spectra of colon adenoma, CRC and normal colon epithelium. Data are obtained using Q10 array. The peak of interest at 12 kDa is marked with a frame.

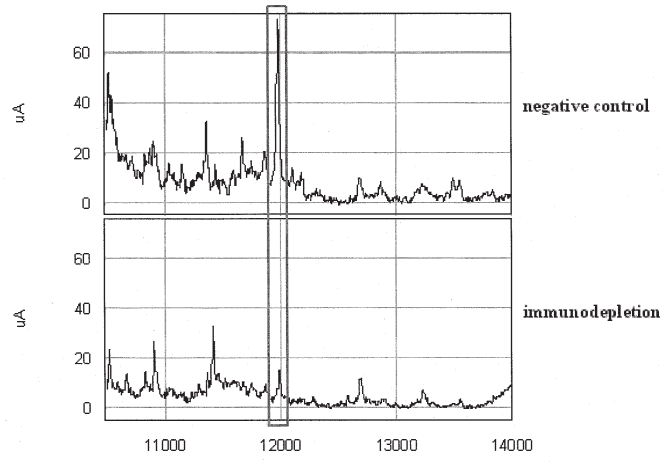

Figure 3. Immunodepletion assay of CRC. A peak (marked with a frame) representing calgizzarin was detectable in the negative control and clearly decreased in the corresponding depleted probe.

microdissected colorectal cancer tissue as starting material. Analysis of the supernatant of the immunodepletion assay by ProteinChip arrays showed that the peak corresponding to calgizzarin was significantly reduced. In the negative control without the specific antibody the peak at 12 kDa was clearly detectable (Fig. 3).

Characterisation of calgizzarin by immunohistochemistry. To characterise the identified marker and to localise calgizzarin in tissue sections, we examined their expression in several colon epithelium tissue samples by immunohistochemistry using a specific anti-calgizzarin serum. Negative controls without the primary antibody or without antibody at all demonstrated negative results. Adenoma cells, normal colon epithelial cells as well as CRC cells demonstrated cytoplasmic signals for calgizzarin in all tissue samples examined. The CRC cells showed an unequivocally increased signal compared to the signal detected in normal epithelial cells or epithelial adenoma cells, respectively (Fig. 4). Quantitative differences between the expression of calgizzarin in normal epithelial colon cells, adenoma cells and colorectal carcinoma cells are as clearly noticeable as in ProteinChip array results.

To further confirm that the localised calgizzarin is identical to the peak found by ProteinChip areas of similar size from carcinoma and adenoma tissue that were prior positively analysed in IHC were obtained by tissue laser microdissection. In protein lysate from carcinoma tissue a signal identical in mass to the peak obtained with the initial SELDI-MS analysis was detected on a Q10 array. In the protein lysate derived from colon adenoma this peak was significantly reduced (Fig. 5).

Discussion

Genomic and proteomic techniques are used for profiling studies of colon tumours (13-15). Besides these studies, protein profiling experiments were carried out using the proteomic technique surface-enhanced laser desorption/

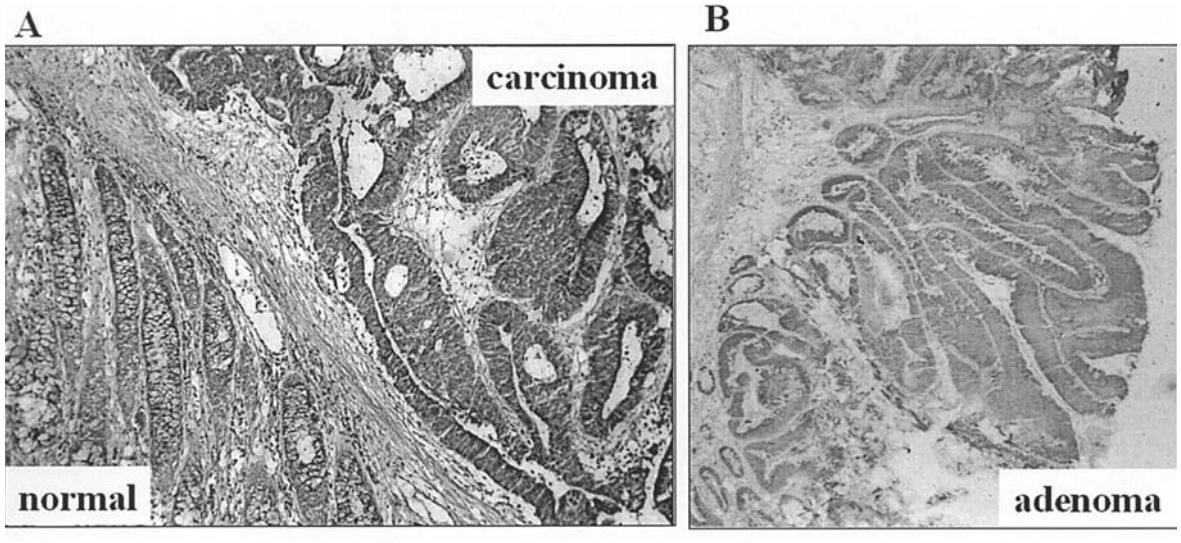

Figure 4. Immunohistochemistry of calgizzarin. (A) CRC (carcinoma) and normal colon epithelium (normal) and (B) colon adenoma (adenoma) with a magnification of x100.

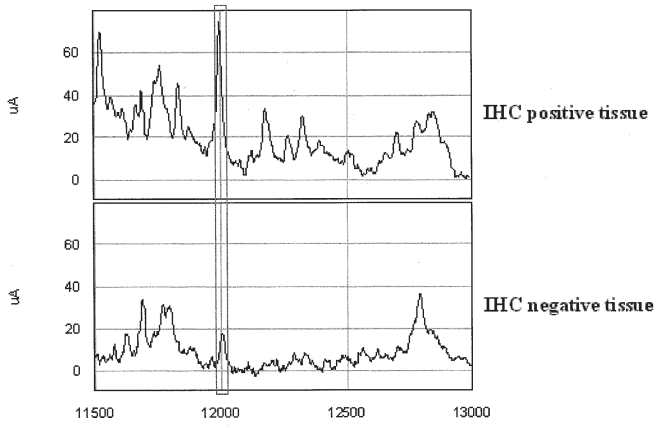

Figure 5. IHC-positive areas of similar size of tumorous and adenoma tissue were microdissected and analysed on ProteinChip arrays. A signal (marked with a frame) with a molecular mass of 12 kDa representing calgizzarin was detectable in protein lysate derived from CRC. In the protein lysate from the colon adenoma fraction this signal is significantly decreased.

ionisation time-of-flight mass spectrometry (SELDI-TOF MS) (ProteinChip technology) (16-18). In the present study, we analysed 62 microdissected samples all derived from CRC, colon adenoma and normal colon epithelium by ProteinChip technology to find significantly differences between CRC and colon adenoma for better understanding of the molecular mechanisms behind the process of tumourigenesis. Among a multitude of various signals which significantly differ between CRC and adenoma and normal epithelium one signal corresponded very well to the molecular mass of calgizzarin (also named S100A11 or S100C, respectively). This signal was up-regulated in colorectal cancer and distinguished significantly between colorectal carcinoma and colon adenoma. We were able to confirm the identity of this signal as calgizzarin by an immunodepletion assay. An elevated calgizzarin expression in CRC compared to normal colon epithelium is described on cDNA level as well as on protein level (19,20). In the present study we confirmed this up-regulation in CRC compared to normal colon epithelium by ProteinChip technology as shown recently by 2D gel electrophoresis (21). Beside this we determined also the protein pattern of samples derived from epithelial colon adenoma tissue and compared, to our knowledge for the first time, the expression ratio between CRC and adenoma on protein level. In a recent study, the transcriptional profiles of intestinal tumours in mice were analysed by cDNA microarrays and here as well as in human colorectal cancer cell lines an up-regulation of the *calgizzarin* gene derived from adenocarcinomas compared to adenoma was described (22). Calgizzarin belongs to the group of S100 proteins involved in the Ca^{2+} signaling network, and regulates intracellular activities such as cell growth and motility, cell cycle progression, transcription, and cell differentiation (23,24). It is a nuclear phosphoprotein linked to suppression of DNA synthesis in normal confluent human fibroblasts, although in neoplastic cells such as HeLa and Saos-2 cells loss of nuclear calgizzarin localisation has been observed (25). In a study using also mass spectrometry a specific induction of calgizzarin protein expression in response to the treatment with transforming growth factor-ß (1) was measured in fibroblasts (26).

IHC demonstrates the heterogeneous distribution of calgizzarin in all tissue samples examined underlining the importance of tissue microdissection prior to all analyses. Microdissection enabled exact separation of the epithelial and mesenchymal tissue components and the separation of benign and malignant cell complexes. Only in this way it is possible to detect and to quantify the different levels of expression of calgizzarin in biological different colon tissues. The clonal heterogeneity of the tumour itself concerning the transcriptome and the proteome is morphologically hard to recognise and therefore cannot be completely solved by microdissection, unless repeated cycles of microdissection, protein profiling and immunohistochemical analysis with specific antibodies is employed.

In this case the differential expression of calgizzarin could be visualised so clearly by IHC as it could be done with ProteinChip analysis. Re-analysis of IHC positive areas of CRC, colon adenoma and normal colon epithelium by microdissection and ProteinChip analysis display again a clear difference in expression level.

In conclusion, through protein profiling, we were able to detect, identify and characterise calgizzarin as a marker differentially expressed in CRC and colon adenoma. The combination of tissue microdissection with SELDI-MS and IHC in a proteomic triade opens up the possibility for a more detailed insight and understanding of tumour progression by reducing the complexity of the proteome using a defined cell population.

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