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Multivariate methods aid in pinpointing promising tumor marker candidates from colorectal biopsies

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ABSTRACT

The application of proteomic techniques to the search for disease markers is widely reported nowadays. However, the data rendered by these methods is highly complex and requires mining through statistical methods. Since univariate tests are prone to false positives and require post-test correction, multivariate methods seem more suitable for the task. Here we show an example of their utility, applying both principal component analysis (PCA) and linear discriminant analysis (LDA) to the hydrophobic subproteome of the colorectal mucosa. In order to find proteins specifically altered by colorectal cancer, we compared both the tumor and the adjacent healthy mucosa. PCA followed by variable selection, and corroboration by LDA, pointed out the proteins vimentin and prohibitin as promising candidates for the diagnosis of colorectal tumors.

Keywords: Principal component analysis; discriminant analysis; multivariate statistics, colorectal cancer; hydrophobic proteins.

Abbreviations

2-DE: two-dimensional electrophoresis; **FDR:** false discovery rate; **SGoF:** sequential goodness of fit; **PCA:** principal component analysis; **PC:** principal component; **LDA:** linear discriminant analysis; **CRC:** colorectal cancer; **MS:** mass spectrometry.

1. Introduction

Proteomics has become a very popular field in the aid of biomarker discovery, mostly because it allows the detection of many marker candidates at a time. This is an important advantage, as until present no single marker has been demonstrated to differentially diagnose one disease. Most of the markers employed nowadays lack either sensitivity or specificity, and thus several markers or other complementary methods (imaging techniques, exploration, etc.) have to be used for the final diagnosis [1].

The way proteomic results have been analyzed, especially those derived from two-dimensional electrophoresis (2-DE) or related methods, has led to an ever-increasing number of potential biomarkers, many of which eventually fail upon validation. Most of the literature available reports these candidates after independent analyses of each variable (for example, after comparison of the mean level of a spot in two study groups by the Student's *t* test). The major hindrance on these comparisons is that they usually consist on a high number of variables (e.g. spots in the case of a 2-DE experiment) and hence require multiple testing. However, the methods commonly employed, called univariate as they test one variable at a time (as the Student's *t* test mentioned), lack power when applied for multiple testing. Thus, there is a need for more efficient methods to analyze these types of datasets. Several alternatives have been proposed, being the simplest to implement some type of post-test correction after

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multiple testing of the independent variables, as the traditional Bonferroni method, the false discovery rate (FDR)-based alternatives [2], or other more recent and powerful methods as the sequential goodness of fit (SGoF) [3]. However, all the variables can be considered at once when using multivariate methods. These tests obviate the need for a post -test correction, thus there is an agreement they are an interesting path for further exploration in proteomic data analysis. To date, many groups have applied these methods with just a profiling or descriptive purpose, trying to find out if the 2-DE maps as a whole contain enough information to distinguish groups of samples [4-7]. Nonetheless these methods can be used to pinpoint one or several specific spots differentiating the sample groups. In this light, there are different and opposing proposals as whether to try to minimize or maximize the number of protein candidates eventually selected.

Recently Marengo *et al*. [8] developed a modification of the principal component analysis (PCA), which they called "Ranking PCA", coupling PCA to a variable selection algorithm that incorporates in each cycle the variable that gives more differential information between the groups. This is based on the "principle of exhaustiveness", aiming to find all the molecules with relevance on the disease studied, even if the information they provide is redundant. On the opposite side, we had published before an application of the PCA [9], which was also based in variable selection, but aiming at finding the minimum number of non-redundant potential markers to differentiate our groups. PCA is a multivariate method for dimension reduction, i.e., it reduces a high number of starting variables (spots in our case) to a new reference system containing a smaller set of variables called principal components (PCs). All the initial variables have to be quantitative and independent. The method calculates the *eigenvalues* and *eigenvectors* of a correlation matrix derived from the original matrix of data. Each PC is the linear combination of the original variables; they are built orthogonal to each other, and they hierarchically explain the maximum possible variance contained in the starting dataset. The final aim of the PCA is to find the smallest group of PCs able to explain the maximum variance from the original data. In that study we also explored the utility of the linear discriminant analysis (LDA) to further investigate the potential of the multivariate methods in highlighting relevant discriminant proteins. The LDA is defined as a classification method, as it allows “classification” or “discrimination” of the sample groups. It also defines which variables are necessary to reach the best classification possible. Thus from the original data it produces a function ("diagnostic function") formed by the linear combination of the variables providing the best (more accurate) classification of the samples into their original groups. These "discriminant variables" or the diagnostic function can then be used to classify new unknown samples. In our group, we use the LDA as a tool to assess the correct classification of samples given by the variables selected by PCA.

In our first description of this approach [9], we applied PCA and variable selection methods to a set of 2-DE maps were *N*-glycoproteins from serum had been resolved. To prove the utility of the method on maps obtained from other types of samples, we now applied the PCA-variable selection strategy to a 2-DE dataset obtained from the comparison of hydrophobic proteins from normal tissues and tumors from colorectal cancer (CRC) patients [10]. From all the potential tissue biomarkers highlighted by the 2-DE comparative strategy, multivariate methods allowed us to narrow down the candidates to two proteins, which were further validated with promising preliminary results.

2. Material and Methods

2.1. *Comparison of 2-DE maps from healthy and tumoral colorectal mucosa*

The procedure to obtain 2-DE maps from tumoral colorectal mucosa and its neighboring healthy counterpart is described in [10]. Briefly, pairs of samples from 5 patients with CRC were collected. Hydrophobic fractions were extracted by temperature-dependent phase partitioning using the ReadyPrep Protein Extraction Kit (Membrane I) (Bio-Rad), based on the use of Triton X-114. The procedure was repeated to ensure purity of the hydrophobic proteins. These were then separated by 2D-PAGE in 17-cm polyacrylamide gels, along a 4-7 pH gradient. Protein maps were visualized by a silver staining protocol compatible with mass spectrometry (MS) analysis [11]. The reproducibility of the 2D-PAGE for the same sample run in different days was calculated as 85% [10]. Map images were acquired with a GS-800 (Bio-Rad) calibrated densitometer, and compared with the PDQuest 7.1.1 software (Bio-Rad). Protein spots were detected, background was subtracted and images were filtered. The intensity level of a spot in a gel was normalized to the total protein intensity detected for the entire gel. Therefore the spots were relatively quantified and the protein amount was expressed as a relative volume (relative intensity x area of the spot).

The study was approved by the Galician Ethical Committee for Clinical Research (code 2006/326), and complied with the tenets of the Helsinki Declaration, the Oviedo Agreement, the Organic Law for Data Protection 15/1999 and the Royal Decree 1720/2007. Informed consent was obtained from patients or guardians, and anonymity was warranted through the use of clinical history numbers.

2.2. *Multivariate analyses*

Levels (relative volumes) of the spots compared between healthy and tumoral mucosa were analyzed by PCA and LDA as previously described [9]. Notice the use of paired samples minimizes the chances of detecting protein differences due to normal individual variability. Furthermore, only those spots present in all the 10 maps obtained from the 5 patients were tested, avoiding the inclusion of null values

and the application of replacement (inference) methods.

For dimension reduction, PCA was applied, and the PCs with *eigenvalues* higher than 1 were selected and considered statistically significant if $p < 0.05$ by the Mann-Whitney *U* test. Next, we examined the correlation matrix (component matrix), which contains the correlation between the original variables and each of the PCs extracted in the analysis. For the significant PCs, we selected the variables (spots) with a correlation higher than 0.8 (that is, where more than 80% of the "spot information" was contributing only to that PC).

In order to evaluate the discriminant function by LDA, we employed a chi-square transformed from Wilks' lambda, so that the classification function was significant (hence "useful") when $p < 0.05$. Results from the LDA were corroborated by leave-one-out cross-validation, removing in turn each one of the 10 cases and classifying them on the basis of the nine remaining ones.

2.3. *Identification by mass spectrometry*

Proteins were identified following the MS protocols described before [10]. First, protein digests were submitted to MALDI-TOF/TOF. Those not successfully identified were further processed through Cap-LC-nESI-Q-TOF. All the parameters for database search were kept as described in reference [10].

2.4. *Detection of caveolin-1, prohibitin and vimentin*

Total colorectal tissue and both the hydrophobic and the soluble fractions derived from it were resolved in 10% SDS-PAGE gels. Then they were transferred to PVDF membranes by Western blot. The primary antibodies employed were goat anti-human caveolin-1 (1 µg/mL; AbD Serotec); mouse anti-human vimentin Ab-2 (2 µg/mL; clone V9; Neomarkers); and mouse anti-human prohibitin (1 µg/mL; clone II-14-10; Neomarkers). Secondary antibodies were rabbit anti-goat or goat anti-mouse IgG (H+L), conjugated with alkaline phosphatase (1/2,000; Bio-Rad). Gel images were acquired with a GS-800 calibrated densitometer (Bio-Rad) and analyzed with Quantity One (v. 4.4.1, Bio-Rad).

3. Results and Discussion

3.1. *Origin of the dataset*

In a previous work [10], we extracted hydrophobic proteins from healthy and tumoral colorectal mucosa samples from 5 CRC patients. We then separated these proteins by 2-DE, obtaining 10 maps that were matched and analyzed by the univariate Student's *t*-test for paired samples. The 41 proteins that showed significantly altered levels were submitted to MALDI-TOF/TOF and Cap-LC-nESI-Q-TOF, and 23 of them were identified. From them we chose the protein vimentin for validation, on the basis of its levels, the available literature, and relevance for CRC, showing it was indeed altered in this pathology [10].

3.2. *Detection of caveolin-1 corroborates the enrichment of the sample*

To assess the enrichment in hydrophobic proteins in the samples used for the present study, we specifically detected caveolin-1, a plasma membrane protein. Results from the starting tissue were compared against the hydrophobic and hydrophilic fractions (figure 1). Caveolin-1 was detected as a band of approximately 21 kDa, almost 5 times more abundant in the hydrophobic fraction than in the starting tissue homogenate. In the hydrophilic sample only residual contamination was observed.

3.3. *Application of PCA and LDA*

The dataset with the relative volume of the 41 spots significantly differing in healthy and tumoral mucosa was analyzed first by PCA. This method allowed a reduction of the original data to 9 PCs, explaining 100% of the total variability (table 1). Applying the Mann-Whitney *U* test to these components, we found PC1 was significant with more than 99% confidence ($p < 0.01$). The plot in figure 2A shows how the PC1 effectively separates the 10 samples in their true original group (healthy mucosa or tumor). Comparison of this result with our previous application of PCA to a proteomic dataset [9] shows that with a complex starting sample (as the serum in that study) the information given by the first principal component is not so relevant (i.e. not statistically significant). However, in the present study the highly-enriched and

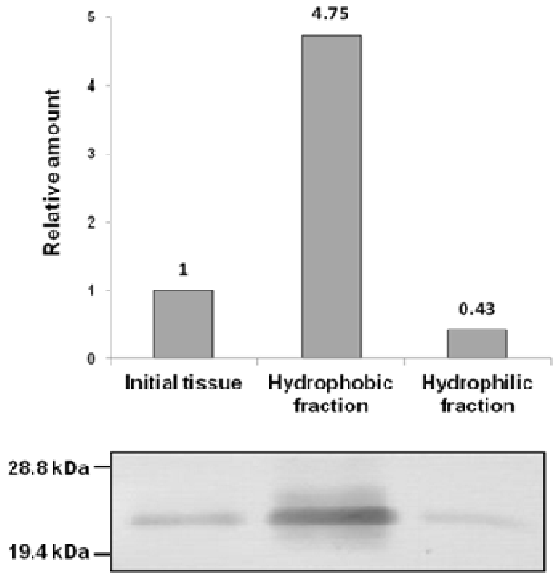

Figure 1. Detection of caveolin-1 in the initial tissue, and in the hydrophobic and hydrophilic fractions derived from it. Bars show the relative amount of caveolin-1, considering the starting tissue has a value of 1.

Table 1. Principal components (PCs) calculated from the 41 spots altered in colorectal tumors.

Component	*Eigenvalues*		
	Total	% Variance	Cumulative %
PC1	17.184	41.913	41.913
PC2	6.601	16.101	58.014
PC3	4.899	11.948	69.963
PC4	3.977	9.701	79.663
PC5	2.926	7.135	86.799
PC6	2.056	5.015	91.814
PC7	1.454	3.545	95.359
PC8	1.116	2.721	98.080
PC9	0.787	1.920	100.00

less-complex initial sample (hydrophobic proteins, estimated to be 10% of the total starting tissue [10]) surely contributed to our ability to detect the spots providing significant "differential information" through the first principal component. Even samples that have been enriched but are still complex (as in [12]) fail usually to provide such a neat separation through the first principal component.

As shown in table 1 and figure 2A, the separation of the groups given by PC1 is 100% effective. However, the individual contribution of each PC (percentage of variance explained, table 1) is small. Therefore we selected the variables with higher correlation with PC1 (the most informative and significant). A subsequent PCA using only the values of the spots with more than 80% correlation to PC1 (8 variables) resulted in a new set of PCs detailed in table 2. The new PC1, named as PC1', was again significantly different between healthy mucosa and tumor tissue ($p < 0.01$), and explained 72.6% of the variance, reaching with the first three components a 90% cumulative variance. As shown in figure 2B, it also allowed graphical separation of the two groups of tissues. More interestingly, the tumor tissues showed a closer distribution than the normal tissues, indicating a higher homogeneity. This effect has been noticed before by us [9, 12] and other authors [4, 13] in different types of samples and cancers, and highlights the good performance of this set of 8 spots for the diagnosis of the tumor tissue.

From this set of 8 spots selected by the new PCA we could identify the proteins vimentin, alpha-1B-glycoprotein, and prohibitin (table 3). The other 5 spots included in the set could not be identified. Since the alpha-1B-glycoprotein is involved in acute phase and inflammatory processes [14], which are not specific for CRC but appear also in benign pathologies of the colon and rectum, we discarded this protein and repeated the PCA with only the other two identified proteins (vimentin and prohibitin). We obtained again a similar explanation of variability (73% versus the previous 72.6%) by the first PC (named as PC1'' in this case). Again, we found a neat graphical separation of the cases by group (figure 2C). Therefore vimentin and prohibitin by themselves could be as informative as the 8 spots together.

To corroborate this, we applied LDA to the values obtained for vimentin and prohibitin in all our healthy and

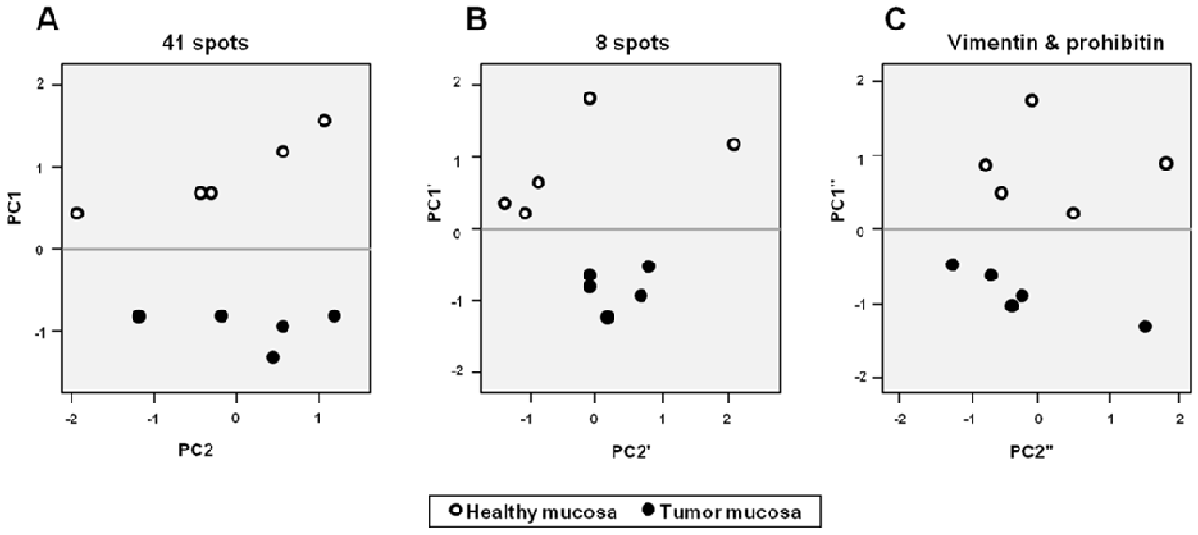

Figure 2. Separation of the healthy (open circles) and tumor (filled circles) mucosa samples on the basis of the first (and significant) principal component of each analysis. A) PCA on the 41 spots with different levels in healthy and tumor colonic mucosa; B) PCA on the 8 spots with higher contribution to the PC1 in the previous analysis; C) PCA performed just on the 2-DE spot values for vimentin and prohibitin. In all the analyses, healthy samples obtained positive values for the first (significant) principal component, whereas tumor samples showed negative values.

Table 2. Principal components (PCs) calculated from the 8 spots with higher correlation (≥ 80%) with PC1.

Component	*Eigenvalues*		
	Total	% Variance	Cumulative %
PC1'	5.805	72.568	72.568
PC2'	0.860	10.745	83.313
PC3'	0.540	6.750	90.063
PC4'	0.426	5.328	95.391
PC5'	0.227	2.843	98.234
PC6'	0.117	1.459	99.693
PC7'	0.022	0.279	99.972
PC8'	0.002	0.028	100.00

tumor samples. We found thus a discriminant function explaining 100% of the tissue variability, i.e. the difference between healthy and tumoral mucosa. This function classified each sample in its correct group with a high confidence level (99.8%; $p = 0.002$), even higher than the one obtained for the initial set of 41 proteins (96.6%; $p = 0.034$). In both cases, 100% of the samples were correctly classified after leave-one-out cross-validation.

3.4. Validation of vimentin and prohibitin

Since the multivariate analyses pointed out at vimentin and prohibitin as potential tissue biomarkers to distinguish the healthy colorectal mucosa from its tumoral counterpart, we aimed next to corroborate these results and verify the changes in these two proteins by specific immunodetection.

Vimentin is a type-III intermediate filament ubiquitously expressed by cells of mesenchymal origin, as fibroblasts, chondrocytes, macrophages, and endothelial cells [15]. It seems to act as a scaffolding protein to stabilize connective tissues and cells, or in signal transduction [16-17]. We quantified the relative levels of the spot identified as vimentin in all the maps from healthy and tumor tissues obtained from the 5 CRC patients. As shown in figure 3 (panels A through C), either the vimentin levels were decreased in tumors, or it was altogether absent. Representation of the levels of vimentin in each group of samples allowed us to find a cut-off point, which can distinguish the healthy tissues from the tumors (figure 3B). As mentioned above, the protein vimentin had been studied before by our group [10]. In that occasion, slot and Western blot analyses showed there was a decrease of vimentin in tumor tissues, with at least 3 isoforms of the protein showing different variations in amount. These specific changes were again corroborated in other patients (figure 3D). In certain carcinomas, such as breast cancer or melanoma, vimentin is up-regulated during epithelial-mesenchymal transition [18]. However, this phenomenon has not been observed in CRC; in fact, a reduced expression as the one found by us was observed. These results can be explained by the fact that the vimentin gene has been found methylated and epigenetically silenced in colorectal tumors and adenomas [19-20].

Table 3. Characteristics of the 8 spots with high correlation (≥ 80%) with the significant PC1.

Spot no. (as in [10])	Fold change in tumor tissue	Protein	Correlation with PC1 (%)
3	-7.6	Vimentin	80.5%
19	-5.8	not identified	82.8%
20	-2.5	Alfa-1B-glycoprotein	86.6%
21	-2.4	not identified	89.9%
27	+3.0	Prohibitin	80.2%
31	-10.7	not identified	84.2%
35	+4.9	not identified	80.7%
39	-8.7	not identified	81.2%

Prohibitin is a highly conserved and widely expressed protein. At the subcellular level it has been localized to the cell membrane, mitochondrial inner membrane and cytoplasm, as well as to the nucleus, depending on the cell type and situation. Its subcellular localization influences its multiple roles within the cell. Although the best characterized function of prohibitin is as a chaperone involved in the stabilization of mitochondrial proteins, it has also been implicated in the regulation of proliferation, apoptosis, transcription, and as a cell-surface receptor. Recent data indicated a role of prohibitin in pathogenesis, including its potential involvement in cancer (reviewed in [21]). When we examined the levels of prohibitin in our samples, we found an increment in the amount of protein in 4 of the 5 patients, while the remaining one showed a slight decrease (figure 4). That made it impossible to establish an effective experimental cut-off for the relative levels of the protein in the tumor tissue regarding its healthy counterpart (as shown in figure 4B). Our finding that prohibitin is up-regulated in most of the colorectal tumors is in agreement with previous reports describing an up-regulation in CRC [4, 22]. Recently, Chen *et al.* [23] confirmed the increased expression of prohibitin in the adenoma-carcinoma sequence, only in those adenomas further developed into CRC and not in the non-malignant ones. These results are in line with our observations.

Prohibitin is a good example of the superiority of the multivariate methods over the univariate ones. As mentioned above, it does not vary in the same direction in all the patients analyzed. However, the multivariate approach highlights it not as a marker by itself, but as part of a group (in this case together with vimentin) that provides the best dis-

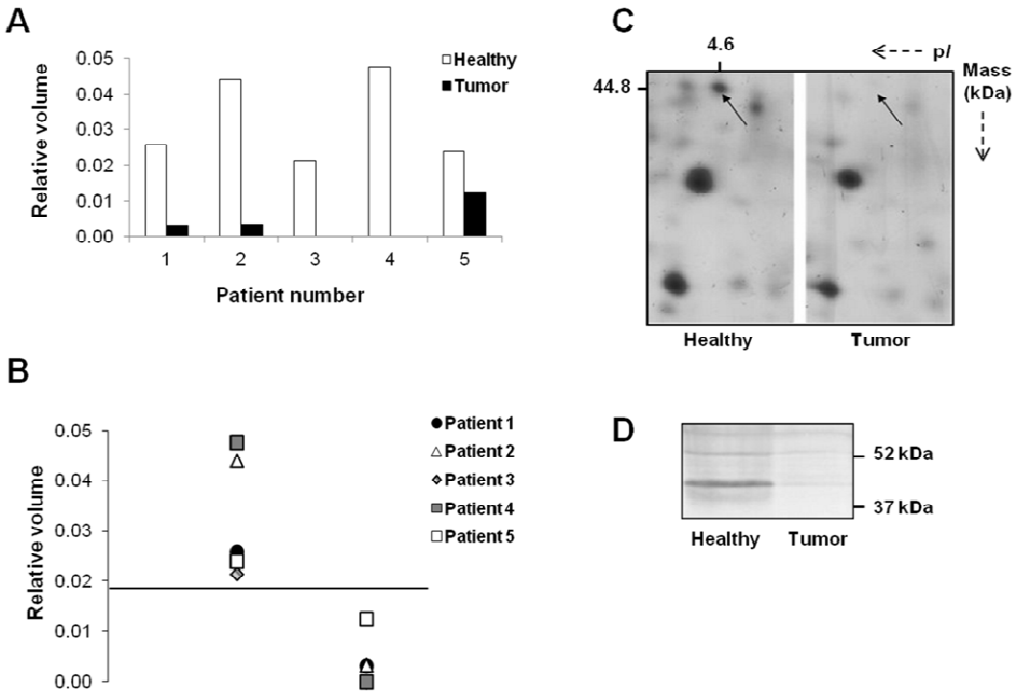

Figure 3. Validation of the differential expression of vimentin. A) Vimentin levels in the healthy and tumor tissues corresponding to each CRC patient. B) The difference between the vimentin levels in the healthy and the tumor mucosas can be visualized by a cut-off. C) Areas of 2 -DE maps from a healthy tissue and a tumor where the vimentin spot (pointed by arrows) is located (p*I*: isoelectric point). A decrease in the relative levels is clearly seen in this example. D) Immunodetection of vimentin isoforms in a healthy colorectal tissue and a tumor, corroborating the decrease of the protein levels in the latter.

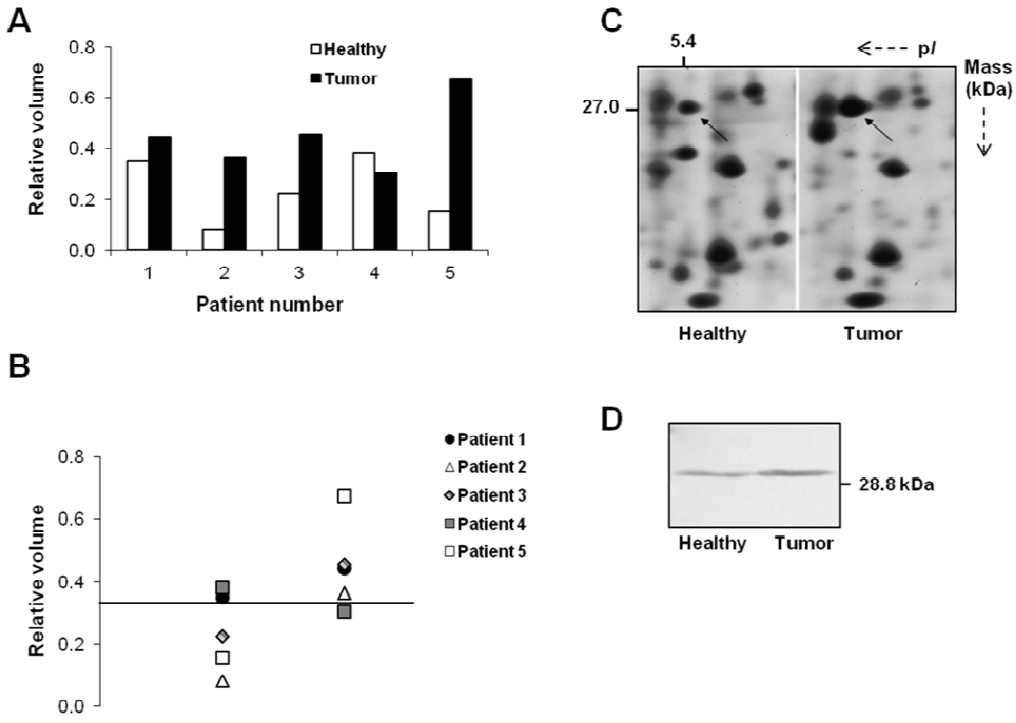

Figure 4. Analysis of the prohibitin levels. A) The relative amount of prohibitin increased in 4 of the 5 patients studied. B) A plot of the individual sample values showed it was not possible to establish an experimental cut-off to specifically distinguish healthy tissues from tumors. C) Prohibitin spot (arrow) shown in representative 2-DE maps from a healthy tissue and a colorectal tumor (p*I*: isoelectric point). An increase in the spot levels can be observed in the tumor. D) Specific immunodetection of prohibitin in a healthy colorectal tissue and a tumor.

crimination between the two types of samples studied (healthy *vs* tumoral). If we had done a simple univariate test, we would have found prohibitin was differentially expressed between the two tissues (for instance, $p < 0.05$ by a paired *t*-test), but we would have probably discarded it since it does show the opposite variation in 1 out of 5 cases. Vimentin would have stood successfully the univariate testing ($p < 0.05$); however a detailed examination of figure 3A reveals that patient 5 does not show such a high fold-variation (< 2 fold) as the other sample pairs, though the difference between the tissues is clearly seen in the prohibitin level (figure 4A). This example intuitively shows the major advantage of the multivariate approach, by selecting more than one protein to construct a panel that is expected to be more sensitive than a biomarker alone.

4. Concluding Remarks

This study has shown the utility of multivariate methods, especially PCA, for the evaluation of a 2-DE dataset, as well as for the selection of a reduced set of potential markers for the disease. In this case, the proteins vimentin and prohibitin were selected through this approach and showed significant changes in their levels between healthy and tumoral colorectal mucosa. Vimentin was able to correctly classify all the samples studied, whereas prohibitin could not do this alone. However, PCA and LDA showed that both proteins together were able to discriminate correctly 100% of the samples. Further studies will clarify if it is vimentin alone or the combination of both proteins that gives the best results for diagnosis of colorectal tissues.

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