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Application of Relative Warp Analysis to the Evaluation of Two-Dimensional Gels in Proteomics: Studying Isoelectric Point and Relative Molecular Mass Variation

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We propose a geometric-morphometrics method (relative warp analysis) to be used in proteomic comparisons. This approach was applied to a dataset from a comparison between 5 controls and 5 patients with colorectal cancer disease published elsewhere. The spots in the 2-D maps were used as landmarks in a morphometric study, and the differences in shape (spot distribution) among them were obtained. The shape variables were used to compare controls and patients. These components mostly ignore random or experimental effects affecting all the proteins in any of the two dimensions studied. Furthermore, the method allows the researcher to find those proteins which contributed the most to the local shape component detected. Applying this approach, we detected variations in the position (isoelectric point and/or relative molecular mass) of some spots that may reflect differences in the amino acidic sequence or post-translational modifications.

Keywords: two-dimensional gel electrophoresis • geometric-morphometrics • relative warps • spot position • isoelectric point • relative molecular mass

Introduction

The study of the protein content, particularly in human serum, has become fundamental for identifying biomarkers of different cellular or organism states such as development, stress, metabolic or disease processes.[1−6] Furthermore, it is well-known that different biological states prime distinct types of post-translational modifications (PTMs), i.e., phosphorylation, glycosylation, or acetylation, that are related to signal transduction processes among others, and that can be altered due to pathological conditions and even in normal situations such as development or differentiation.[7−15] These and other modifications shift protein physical properties such as isoelectric point (p*I*) or relative molecular mass (M_r).[16]

In recent years, a rapidly emerging set of key proteomic technologies has facilitated the identification of a large number of proteins in complex samples or mixtures.[17,18] Two-dimensional gel electrophoresis (2D-PAGE) is the most widely used technique in proteomics for separating complex biological mixtures containing a large number of proteins. This comprehensive technology uses two sequential electrophoretic runs to separate the proteins in a particular sample, first regarding their p*I* (through an immobilized pH gradient) and then depending on their M_r (through a porosity gradient). A snapshot (hereinafter regarded as map) of the protein content in the investigated sample is then obtained. A typical analysis consists of the comparison of the spot relative volumes (quantities) among maps of control and case samples.[19] Recently, some studies have also used multivariate methods to improve the detection of differences in protein quantity among distinct 2-D maps.[20] Unfortunately, the analysis of the sole protein amount systematically ignores most protein changes, being unable to detect variations due to protein phosphorylation, glycosylation, acetylation or even partial degradation.

Recently, a three-way Principal Component Analysis (PCA) has been proposed as a tool to incorporate data on the p*I* and M_r when dealing with 2-D maps.[19,21] This approach uses information from spot *X* and *Y* coordinates in the map and reduces it by PCA. However, it presents two major drawbacks: first, it may confound true differences in p*I* or M_r with technical problems due to random or uncontrolled effects; and second, with that method it is extremely difficult to detect individual proteins contributing to the overall effect.

The geometric-morphometrics methods are the best available alternative to obtain size and shape components from any biological form or image.[22−25] Traditionally, morphometrics and geometric-morphometrics methods have been widely used for issues such as species classification and species boundaries studies,[22,26−28] but they have been proven useful in other important topics, such as epidemiological[29] and diagnostic studies.[30] Here, we propose a geometric-morphometrics method called Relative Warp (RW) analysis[22,23] to investigate changes in protein distribution (regarding protein modifications) among control and case samples. With this method, the user needs to define a series of points (which may be some specific spots or

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a whole set) called landmarks, that represent a form of the biological image. Applied to 2-D maps, the method can detect the mobility of some proteins related to others (the local component of variation), and thus the results are just slightly impaired by experimental pitfalls during 2D-PAGE affecting to all proteins simultaneously.

We have re-analyzed 2-D gels of serum *N*-glycoproteins (obtained after separation through a specific lectin chromatography) from 5 colorectal cancer (CRC) patients and 5 healthy donors presented elsewhere[31] by the above-mentioned morphometric method, to investigate its potential utility in proteomics. Using this methodology, we were able to study local variation in 'the shape' of the spot distribution, and hence measure the variability of spot positions in 2-D maps. Eventually, we found spots showing differences in their p*I* or M_r, in relation with the disease condition studied.

Materials and Methods

1. Sample Collection and Preparation. In the present study, we have used serum samples from 5 healthy donors and 5 CRC patients, which were previously analysed for differences in protein expression by means of 2D-PAGE.[31] All procedures involving human samples were performed according to the clinical ethical practices of the Spanish Government. Informed consent was obtained from each subject's guardian.

Blood samples were obtained by venipuncture, clotted, and centrifuged to get the sera that were stored at −85 °C until used. One mL of filtered serum was applied to a Concanavalin A-Sepharose (Sigma-Aldrich Chimie) column. A fraction containing mainly *N*-glycoproteins was selectively eluted with 0.5 M methyl-α-D-mannopyranoside.

2. Two-Dimensional Gel Electrophoresis and Protein Identification. The detailed procedure has been described before.[31] Briefly, 150 μg of lyophilized *N*-glycoproteins were first separated by isoelectric focusing in 17-cm, pH 4−7, linear ReadyStrip IPG Strips (Bio-Rad). After equilibration, the IPG strips were transferred onto 9−16% gradient polyacrylamide gels and SDS-PAGE performed in a Protean II xi Cell (Bio-Rad). Finally, gels were stained with ammoniacal silver. Protein identification was accomplished either by MALDI-TOF mass spectrometry (MS) or by nanoHPLC−ESI−MS/MS.

3. Computer Analysis of Two-Dimensional Patterns. Silver-stained gels were scanned using a GS-800 calibrated densitometer (Bio-Rad) and protein patterns were analysed with the PDQuest 7.1.1 software package (Bio-Rad). The same gel set had been previously interrogated for differences in intensity.[31] Briefly, the spots were detected by the software based on the spot parameters chosen by the user (biggest, smallest, and least intense spot), background was subtracted and the resulting filtered images were edited to correct inaccuracies. Spots within the images corresponding to the control and the case samples were matched independently. A representative standard gel containing the data from all the images matched was obtained from each comparison. Then, these analyses within groups (control; patient) were compared between groups (control vs patient).

The final number of spots chosen for the morphometric analysis described here was 45. They were automatically selected on the basis of the PDQuest software ability to calculate both their *X* and *Y* coordinates in all the gels, without a priori knowledge about the spots. This selection procedure had to be applied since the geometric-morphometrics method employed does not admit null values in the dataset.

4. Relative Warp Analysis of Two-Dimensional Maps. Changes in spot position between control and patient 2-D maps were studied by a geometric-morphometrics approach. First, we obtained detailed information of the 45 protein locations (coordinates on the gel) using the 'export matchset' option in the PDQuest program. These coordinates defining different spots were considered equivalent to the landmarks (coordinate points describing the geometry of a bidimensional image) used in typical morphometric studies.[25,32]

Under a geometric-morphometrics framework, the form is disentangled in size and shape components, the latter including uniform (changes affecting all landmarks simultaneously) and local (affecting some landmarks) shape components.[22,23] This is convenient in the present case because both the size and the uniform component of 2-D maps describe changes in the overall migration pattern regarding the *X* (p*I*), the *Y* (M_r), or both axes simultaneously. Such kind of effects can be typically caused by random variation in the experiment. Here, size and uniform components were not considered because they are not informative, a priori, about true changes in protein charge state or mass. In contrast, any change in the relative position of some landmarks in relation to others, named the local component (captured as the RWs; see Bookstein[22]), mostly ignores scale artifacts in the *X* and *Y* directions and could be explained only by a true protein shift in our case.[23,25,27]

We will not attempt here a detailed description of the RWs which can be obtained elsewhere,[22,23] rather we will give a brief explanation about their meaning and how they were obtained. The estimation of shape components in 2-D maps was accomplished aligning the raw coordinates of the individuals using the Procrustes generalized orthogonal method,[33] which determines a *reference configuration* by minimizing the sum of squared distances between homologous landmarks from different individuals. The RW analysis finds a function fitting all those homologous landmarks to the reference configuration. As a result, the orthogonal axes (principal warps) describing shape deformations of the reference configuration at different spatial scales, were obtained. The specimen deviations from the reference configuration are called the partial-warp scores. These partial warps describe the shape differences among individuals. The RWs are obtained by a PCA onto the partial warp values.[22,23] RWs were computed scaling the centroid size to 1 after the alignment, excluding the uniform components from calculations, and with the scaling option $\alpha = -1$, that weights preferentially the closest landmarks.[23,27] All RW calculations and the representation of their deviations were done by a simple and user-friendly software, the tpsRelw program developed by Rohlf[34] (http://life.bio.sunysb.edu/morph/morphmet/tpsrelww32.exe). Thus, the differences in spot position between controls and patients are detected via the RWs.

The individual contribution of each protein can be expressed showing the vectors of the deformation caused by positive and negative displacements along the RW. This was accomplished using the RWs visualization options from the tpsRelw program.

5. Reproducibility of the Method. The reproducibility of the 2D-PAGE analysis for the protein intensities has been described elsewhere.[31] The performance of the matching process with the PDQuest software has been already estimated by Garrels[35] reporting a number of inconsistencies when matching corresponding proteins about 2.4% of the total spots (reproducibility of 97.6%). This should be our maximum error. In fact, the error

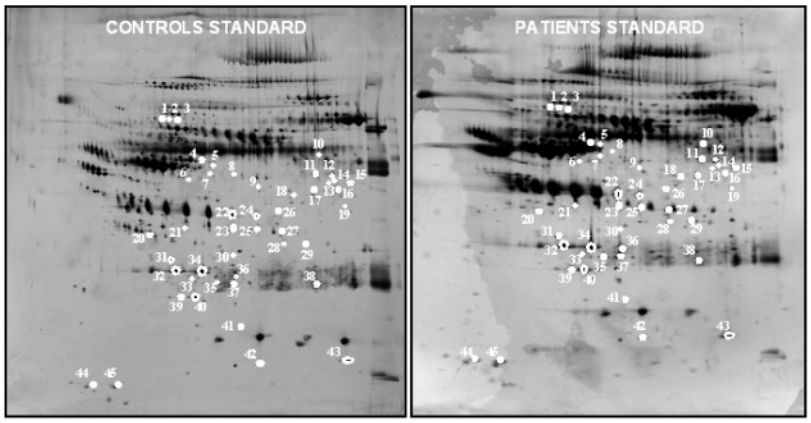

Figure 1. Representative 2-D standard gels for the comparison between the 5 control and 5 case samples analyzed. The subset of 45 spots used for the geometric-morphometrics analysis is highlighted.

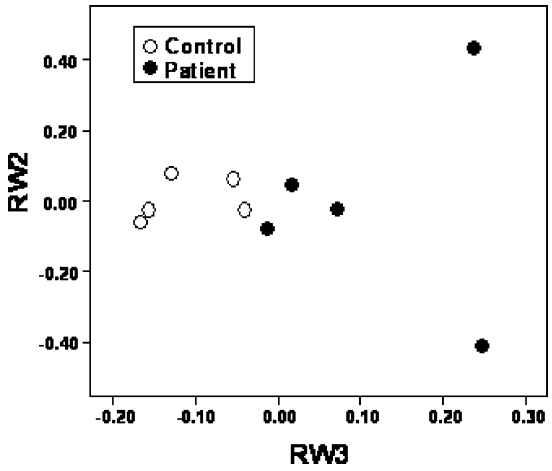

Figure 2. Plot of relative warp 2 (RW2) and relative warp 3 (RW3) values for the 5 controls and 5 patients.

of the RW analysis (including matching error and data analysis error) was calculated by comparing the relative variance between the same sample run at two different days and the variance of the whole group of samples. On average, the reproducibility was 99.4% for the three main RWs (range between 99.5% for RW3 and 99.4% for RW2). The reproducibility of the remaining RWs was lower (data not shown), but they were not informative in our particular case (see Results).

Results

Samples from 5 healthy donors and 5 CRC patients were processed and submitted to 2D-PAGE as stated in 'Materials and Methods'. In Figure 1 we show the standard gels for donors (controls) and patients and highlight the 45 spots chosen for the morphometric analysis.

We looked for changes in the p*I* and/or the M_r of the selected proteins among maps from the 5 controls and 5 patients by studying the RWs of the spot positions. The analysis revealed that the main nine RWs explained a 99% of the local variability in protein position (RW1 = 53%; RW2 = 18%; RW3 = 9%; RW4 = 8%; RW5 = 4%; RW6 = 3%; RW7 = 2%; RW8 =1%; RW9 = 1%). However, only the RW3 detected significant differences among controls and patients ($U = 0$; $Z = -2.61$; $P = 0.009$). This result is graphically represented in Figure 2, where the values of all samples for RW2 and RW3 are plotted. It is obvious from the figure that patients showed positive (or closest to 0) RW3 values, while controls presented the most negative ones. This result reveals a significant difference in the protein set regarding p*I* and/or M_r among controls and patients.

This finding requires a method to visualize which landmarks (spots) contributed the most to the variation in RW3. One suitable approach is displaying the vectors showing the net movement of the landmarks, that describe the protein contribution to the shape change in RW3. In Figure 3 we show the contribution of each spot to this particular RW.

Discussion

A correct diagnosis, prognosis, and monitoring of cancer and other diseases needs of as many identified markers as possible. One of the most exciting areas of proteomic studies is the search for such kind of markers using the 2D-PAGE technology, which is based on a blind search with no preconceived ideas about the existence or identity of the biomarkers, thus increasing the number of potential candidates discovered.[36] Classical analysis of 2-D maps has dealt with differences in intensities of isolated proteins or a lineal combination of all proteins (principal components − PCs) between the contrasting groups of individuals.[21]

Sera from 5 healthy donors and 5 CRC patients were processed and submitted to 2D-PAGE as explained in 'Materials and Methods'. We have already shown[31] 27 proteins which differed in spot intensity (relative abundance) among the controls and the cancer patients studied here. In that work, however, we did not attempt to study changes in the p*I* and M_r of the proteins displayed in the maps, although it is well-known that the cancer process investigated affects not only the amount of proteins but also their structure and p*I* and M_r properties. In this work, we apply the RW method to find proteins varying their position in relation with the control or CRC patient condition.

The RW analysis presented here is very reproducible. It considers spots that were first automatically matched by the PDQuest software, therefore, we could think that the error of the process is that committed by the matching algorithms. This error was estimated for the QUEST system by Garrels[35] in 2.4% of the total spots (reproducibility of 97.6%). Moreover, Rosengren and co-workers[37] observed that PDQuest outperformed other softwares in the matching process, with less false positive matchings. After the RW analysis we achieved a reproducibility of 99.4% for the three main RWs (range between 99.5% for RW3 and 99.4% for RW2).

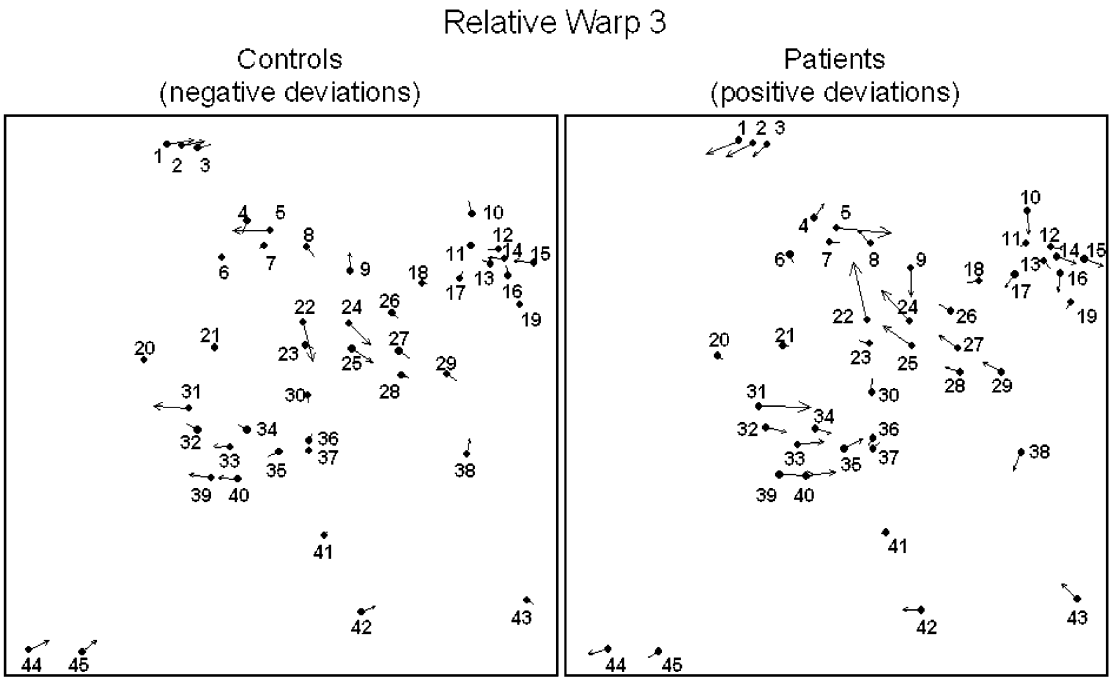

Figure 3. Plot of the relative warp 3 values as vectors (rescaled 10x to visualize the differences) showing the net movement of the landmarks (spots).

This novel application of geometric-morphometrics allows us the detection of significant local variation in p*I* and M_r (described by variation in RW3, Figure 2), clearly separating the control and patient populations. This could be a future application of the proposed method for diagnosis: the spot position patterns of large control and patient populations would be recorded, and then it could be statistically assessed if an unknown sample, with a particular protein pattern, corresponds to the phenotype of a healthy or an affected person.

Furthermore, the method allows the graphical identification of those proteins which contributed the most to the changes detected between the groups, theoretically related to the disease condition studied. There are several reasons that could account for the shift: PTMs as glycosylation, phosphorylation, acetylation, etc., and also changes in the amino acidic sequence of the protein (amino acid polymorphisms), the existence of truncated forms, etc. Vectors depicted in Figure 3 show the dimension of the change in protein position. We have identified some proteins (either by MS or MS/MS) showing major deviations, along with proteins displaying minor changes and proteins not showing any move.

One of the spots that presented the biggest vectors of deviation was spot 5. It was identified as angiotensin precursor (primary accession number P01019 in Swiss-Prot protein knowledgebase, http://www.expasy.org/sprot/), a protein that contents angiotensin I, II, and III. Angiotensinogen is expressed by the liver and secreted in plasma. Its circulating levels are dynamically regulated as an important determinant of blood pressure and electrolyte homeostasis, and it is up-regulated during acute-phase response.[38] Interestingly, at least 10 polymorphic variations of this protein have been described (see information on the mentioned database), four of them related with a disease condition (hypertension), and even one of them (Y281C) altering the structure, glycosylation and secretion of the protein.[39]

Another outstanding change affects spot 31. This corresponds to alpha-1-microglobulin (AMBP or protein HC, P02760). It is produced by the liver as a glycoprotein of 27 kDa and secreted, thus appearing in body fluids as plasma, urine, and cerebrospinal fluid. It belongs to the lipocalin superfamily of hydrophobic ligand binding proteins, that have been used as biochemical markers of disease because of their relation with inflammatory processes, cancer, lipid disorders, liver, and kidney function.[40]

Spots 23 and 24 were identified as haptoglobin (HPT, P00738) and they constitute an interesting example of the results achieved after RW analysis. Several authors have stated that HPT is increased in CRC,[41−43] and it had been connected to this cancer as a serum tumor marker, even together with carcinoembryonic antigen.[41,42] As shown in Figure 3, spot 24 presented a large shift. In relation to that, a 40-kDa isoform of HPT has been recently described as a cancer-associated glycoform of the protein, suggesting that a change in the structure (that would be reflected in the p*I* and M_r of the protein, and therefore in its position), rather than in the amount of HPT, occurs in colon cancer patients.[44] Furthermore, there have been reports of alterations in HPT carbohydrates in serum from ovarian cancer, including increased fucosylation[45] and a shift from biantennary to triantennary oligosaccharides.[46]

On the contrary, the application of RW analysis finds that another HPT isoform (i.e., spot 23) has no relevant shift. Interestingly, there are several recent reports of changes in the PTMs affecting only a certain isoform of a protein in relation with cancer processes. For instance, Charrier and co-workers[47] showed that free prostate specific antigen (PSA) from benign prostate hyperplasia patients presented more acidic isoforms with regard to prostate cancer patients. Edberg et al.[48] described different PTM patterns of the isoforms HMGA1a and HMGA1b (encoded by the oncogene *HMGA1*) isolated from nonmetastatic and metastatic cells, suggesting that the isoforms likely exhibit differences in their biological function.

Spot 40 also shows a shift in position, although not so noticeable as in previous examples. It corresponds to apolipoprotein A-I (Apo-AI, P02647). This protein is synthesized in the liver and small intestine and secreted to plasma, being the major protein of plasma HDL and appearing also in chylomicrons. The expression of Apo-AI is associated with colonic adenocarcinoma progression, and thus it is a potential marker of the aggression.[49] In relation to PTMs, it has been reported that phosphorylation of apoA-I resulted in an increase of two negative charges, consequently causing a shift to a more acidic p*I*.[50]

The application of the RW analysis also allowed us to detect spots that were not shifting their position in relation to the disease condition. For instance, spot 18 was identified as complement factor H-related protein 1 (Q03591), whose changes in abundance have been reported in urinary bladder cancer,[51] although to date there is no report regarding PTMs related to disease. Another protein that did not show a shift was spot 20, identified as clusterin (P10909). At least 17 isoforms of the protein have been identified in plasma, and changes in its expression levels have been related to CRC both in tumors[52,53] and serum.[31] In the particular case of spot 20 we have done glycosylation analysis by nanoHPLC−MS/MS, finding a minor change in the glycan moiety of the patient isoform (Rodriguez−Piñeiro, A. M.; Thomsson, K. A., Goteborg University, unpublished results). This minor change is not expected to affect the position of the isoform, consistent with the result of the RW analysis.

Finally, spot 41 corresponds to concanavalin A (gij1421224 at NCBI protein database, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein), the lectin used in the chromatographic separation of the proteins analyzed. Although it is not human, it appears in 2-D gels as it can be washed out of the column during the elution. Interestingly enough, this spot does not show even a minor movement between control and patient groups. We consider that this is a good negative control of the RW analysis.

A limitation for the application of the RW method to 2D-PAGE is that, as the classical 2-D analysis (of spot intensity/quantity/relative volume), it requires the presence of a given spot in the majority of the gels.[54] Moreover, a specific limitation of the application of RW to 2D-PAGE is that the spots need to have their coordinates calculated in all the gels.[25] In a previous study using the same images,[31] 363 spots were considered as the dataset for quantitative comparison, since all the spots were present in all maps. However, in the present study the 363 spots had to be reduced to 45 spots, due to the lack of some coordinate (*X* or *Y*) in one of the 10 individuals. The reason the PDQuest software lost some coordinates could vary from case to case. For instance, if the software did not automatically recognize one spot in the detection step, being manually added later on (a typical procedure in proteomics[54]), the spot could not be fitted to a Gaussian function and the center (therefore the position coordinates) was not calculated.

Nevertheless, the morphometric technique employed is not limited by the number of spots used, rather, the larger the number of landmarks (spots), the better.[25] Moreover, the differences found in a small dataset would be still present in a larger dataset. Some of the authors (A. C.-R. and E. R.-A.) have done a morphometric work on gastropods shells using 12 landmarks, finding qualitatively similar results when using exclusively 4 representative landmarks.[28]

One possible solution to increase the number of spots used, would be to extrapolate their positions taking into consideration the vectors from a spot to the neighboring ones in other gels belonging to the same group. This would be the same strategy used in some statistical tests that do not support missing values and replace them with a proper function. However, we considered that this approach should not be used for a first report of the utility, since it could introduce more variability in a still nonestablished method. Another possibility would be to scan the images of the 2-D maps as in other morphometric studies,[28] and then record the coordinates of the spots manually. Obviously, the main problem of this approach is the introduction of operator-related errors.

Recently, Marengo and co-workers[21] described a strategy similar to the one proposed here (using spot position) to study p*I* and M_r properties in 2-D maps. They accomplished a three-way PCA to differentiate groups in two sets of control-case samples. However, as in any morphometric analysis of PCs, the general effects affecting all landmarks simultaneously are confounded with the local variation affecting some landmarks in relation to others.[22] On the contrary, the RW method separates the local variance from global effects.[22,23,25] Therefore, applied to 2-D maps, it corrects for bias affecting all spots simultaneously at any of the two dimensions of the separation. This has special importance in the case of 2D-PAGE, because technical drawbacks and random variation would probably have an effect on the location of all the spots resolved (at least in one of the two dimensions). The RW analysis has also the advantage of being able to describe the particular contribution of each protein to the difference observed between controls and patients (as depicted in Figure 3), being more useful to detect putative markers of the disease. Thus, the advantages of our method regarding the three-way PCA are that it corrects for technical errors affecting one or both dimensions, and detects the individual protein contribution.

Although it is not the aim of the article, it is obvious that trying to find which modification is affecting the proteins, causing their change in position in 2-D gels, would be of great interest. A new tool called Protein Modification Screening Tool (ProMoST), recently implemented,[16] could hypothetically complement our method. Researchers could use our approach to statistically assess changes in spot position in 2D-PAGE among different groups of samples and, afterward, using the ProMoST software, they would theoretically identify the PTM that could be responsible for the observed change.

Conclusions

The application of a geometric-morphometrics method (relative warp analysis) to the comparison of controls and CRC patients 2-D serum maps allowed us to identify proteins that vary in their p*I* and M_r in relation to the absence or presence of the disease. A potential utility of the method could be as a diagnostic tool: resembling the development of the microarray technology, it would be feasible in the future to use the approach to distinguish healthy individuals from those affected by a certain disease.

We encourage other researchers to try the method and report their results, considering that a corroboration with larger datasets is needed to check its general feasibility and utility in proteomics.

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