Spectrophotometric assessment of nuclear proteins: a preliminary study

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Qualitative evaluation of protein content in formalin fixed, paraffin-embedded tissues is usually performed by means of cytofluorimetric analysis. On the other hand, several studies underline the opportunity to measure the concentration of nuclear proteins, which is often accomplished by using complex techniques and instrumentation. In the present work, we suggest a new application for the spectrophotometric evaluation of protein content on extracted and isolated nuclei, based on EDTA treatment of specimens and chemical extraction of proteins, followed by direct spectrophotometric measurement at UV wavelengths. We also demonstrate how this parameter correlates with other diagnostic factors, such as the proliferation index (MIB-1) and the DNA content (ploidy) of cells. This method is simple and effective, yet less expensive than other protein quantitation protocols.

Key words: isolated nuclei, nuclear protein, spectrophotometry, flow cytometry, linear regression.

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For many years, qualitative evaluation of cellular protein content has been performed on fresh, frozen or formalin fixed, paraffin-embedded samples by means of a cytofluorimetric technique [Pollack et al., 1984]. This method is almost always coupled with the cytofluorimetric determination of DNA content (DNA index); proteins and DNA are labeled with two different fluorochromes. Data obtained with this method outline cell clones with abnormal protein (or DNA) content, compared to normal (diploid) controls. Otherwise, these data do not give any quantitative measure of nuclear proteins.

In some early studies, proteins were linked to the histone component in pathological tissues [Rowekamp et al., 1974]. More recently, other studies stressed the importance of protein quantitation in cell cultures treated with toxic compounds, such as ethanol [Mahadev et al., 1998]. In order to achieve the best output, sophisticated techniques, e.g. high-pressure liquid chromatography (HPLC) [Jackson et al., 1985], microspectrofluorimetry associated to digital imaging procedures [Bottiroli et al., 2004], or assembled instruments for the multiparametric evaluation of tumors [Caspersson et al., 1983], were used. These methods are effective, but often expensive and time-consuming.

In the diagnostic evaluation of mammary and endometrial neoplasms, the comparison between protein content quantified by direct spectrophotometry and other important prognostic or diagnostic parameters (DNA content, proliferation rate) is considered highly useful [Moberger et al., 1989]. In recent studies, mass spectrometry has been used as a non-invasive quantitative method for the evaluation of microsatellite instability for protein-based biomarker profiling of patients [Petricoin and Liotta, 2003]. On the other hand, these measures are often performed on culture cells or small fragments of whole tissue, thus including the extracellular stroma.

In this study, we propose a simple, fast and cost-effective method to evaluate the protein content of extracted nuclei, isolated from fresh or frozen tissue. We present preliminary results on a limited number of cases, mostly breast tumors, performing a tentative statistical correlation with other important diagnostic parameters.

Materials and Methods

Biological samples

Twenty-seven samples from formalin-fixed, paraffin-embedded surgical specimens were examined. Nine of them were normal controls from non-pathological kidney, spleen or breast, and 18 were breast tumors with known DNA content (DNA index, as measured by monoparametric flow cytometry) and proliferative rate (MIB-1 immunohistochemical determination).

Nuclei isolation and count

Sections were deparaffinized in xylene and rehydrated in a descending ethanol series. They were then digested in a pepsin solution (Roche, Germany). After centrifugation at 2,000 RPM for 10 minutes, nuclear suspensions were filtered through a 70-µ nylon mesh to remove unfractionated tissues or other large debris and resuspended in the following solution: 0.04 g ethylenediamine tetraacetic acid (EDTA), 0.097 g Tris base, 0.661 g Tris-HCl, 0.29 g NaCl, and 0.5 ml Nonidet P40, in a final volume of 100 mL. This solution eliminates most of the residual cytoplasm and allows a better cytofluorimetric output. In order to evaluate the efficacy of EDTA treatment, cytological hematoxylin-stained slides were prepared. A small amount of nuclear suspension was stained with Trypan Blue and counted in a Bürker chamber for the standardization of protein content and cellular concentration (see below).

Flow cytometry

DNA was stained with 4,6 diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Germany) and nuclei were analyzed using a Partec CA II flow cytometer (Partec, Germany) equipped with an HBO 100 Mercury arc lamp. Cytometry was standardized with normal human spleen. DNA histograms were obtained from 10,000 cells and were considered non diploid when at least two separate G0-G1 peaks were present.

The DNA index was calculated from each histrogram as the ratio of the G0-G1 peak of the non diploid population to G0-G1 peak of normal cells present in the tumor [Hiddeman et al., 1984; Wersto et al., 1991].

Immunohistochemistry

Five µm-thick sections were treated with normal goat serum for 10 minutes, primary monoclonal anti-MIB-1 antibody (clone M7240, DAKO, Italy) for 45 minutes, and thoroughly washed in 0.1 M Tris-HCl buffer. Hybridization with secondary biotinylated antibody (DAKO) for 30 minutes was followed by Streptavidin-Biotin treatment for 30 minutes and diaminobenzidine (DAB) chromogenic reaction for 8 minutes. Nuclei were counterstained with hematoxylin and slides were mounted in Eukitt medium after progressive dehydration.

Protein extraction

Suspended nuclei were centrifuged at 3,000 RPM for 10 minutes, then treated with a lysis buffer (50 mM KCl, 10 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$, 0.45% Nonidet P40, 0.45% Tween-20) and 19 µg/mL proteinase K. The volume of lysis buffer was directly proportional to the nuclei concentration. The samples were left overnight at 58°C, then proteinase K was inactivated at 95°C for 30 minutes. After cooling to room temperature, the samples were centrifuged at high speed (13,000 RPM) for 10 minutes in order to precipitate membranes and other heavy material, leaving DNA and proteins in the above aqueous solution. Nuclei extracted from filtered and EDTA-treated cells now contained only proteins of the nucleolemma and nucleoplasm.

Spectrophotometric assessment of nuclear proteins

Nuclear proteins were completely denatured at this point, most of them being cleaved to peptones (short aminoacidic chains) or single aminoacids. Spectrophotometry can directly measure the protein content of a given solution, calculating from absorbance at 280 nm wavelength. This is directly proportional to the concentration of aromatic aminoacids (tyrosine, tryptophane), as in the Lambert-Beer function: $A = \varepsilon \bullet b \bullet c$ (A, absorbance; ε, molar absorption coefficient; b, optical path; c, concentration). Measures were performed on an Eppendorf Biophotometer (Eppendorf, Germany). Instead of protein standards

Table 1. Examined samples. Means and standard deviations for protein concentration in every group (normal controls, diploid tumors, aneuploid tumors) are reported.

Group	*Tissue*	*Ploidy*	*DNA index*	*MIB-1 (%)*	*Prot. (mg/mL)*	*Mean*	*SD*
Controls	Kidney	diploid	1.00	N/A	4.4	4.9	2.02
	Breast	diploid	1.00	N/A	2.7		
	Breast	diploid	1.00	N/A	3.7		
	Kidney	diploid	1.00	N/A	4.4		
	LN	diploid	1.00	N/A	7.2		
	Spleen	diploid	1.00	N/A	6.9		
	Kidney	diploid	1.00	N/A	5.4		
	Breast	diploid	1.00	N/A	2.0		
	LN	diploid	1.00	N/A	7.7		
DT	Breast	diploid	1.00	23	10.3	6.6	2.50
	Breast	diploid	1.00	19	7.3		
	Breast	diploid	1.00	15	4.0		
	Breast	diploid	1.00	1.5	6.7		
	Breast	diploid	1.00	24	4.5		
AT	Breast	aneuploid	1.16	27	13.3	13.0	4.73
	Breast	aneuploid	1.99	2	16.1		
	Breast	aneuploid	1.56	40	17.8		
	Breast	aneuploid	1.90	10	20.7		
	Breast	aneuploid	2.76	40	11.3		
	Breast	aneuploid	1.67	32	15.0		
	Breast	aneuploid	1.56	40	19.7		
	Breast	aneuploid	2.30	37	9.9		
	Breast	aneuploid	2.50	50	13.4		
	Breast	aneuploid	1.80	8.4	7.8		
	Breast	aneuploid	2.06	40	6.4		
	Breast	aneuploid	3.00	88	10.9		
	Breast	aneuploid	2.08	10	6.6		

SD: standard deviation. LN: lymph node. DT: diploid tumors. AT: aneuploid tumors.

(solutions at known concentration), we applied a simple conversion factor between absorbance and concentration (1 unit of absorbance = 1.33 mg/mL), as reported in the spectrophotometer data sheet *(http://www.eppendorf. com/en/cat_stat/pdf_2003/20345_kap_07.pdf).*

Protein and DNA absorbance spectra are partially overlapped. For this reason, our calculations were corrected as reported both in early and more recent literature [Layne, 1957; Stoscheck, 1990], following the formula: protein concentration (mg/mL) = (1.33 x A280) – (0.76 x A260).

Before any measure, the instrument was set to zero using the lysis buffer as a blank. To avoid out-of-scale concentrations, each sample was diluted 1:5 with the same buffer. Three independent measures on each sample were performed.

Statistical analysis

Data were elaborated in a Microsoft Excel spreadsheet, with automatic calculation of means and standard deviations. In the same spreadsheet, the following statistical analyses were performed: mean and standard deviation for normal samples, diploid tumors, aneuploid tumors; 2-tails, Student's t-test for the evaluation of statistical differences between the protein content of the three groups; linear regression analysis between protein content, DNA index (1 for diploid, >1 for aneuploid) and MIB-1; calculation of regression line, regression function and statistical significance, expressed by the R^2 value (good correlation between dependent and independent variables if R^2>0.5 or 50%).

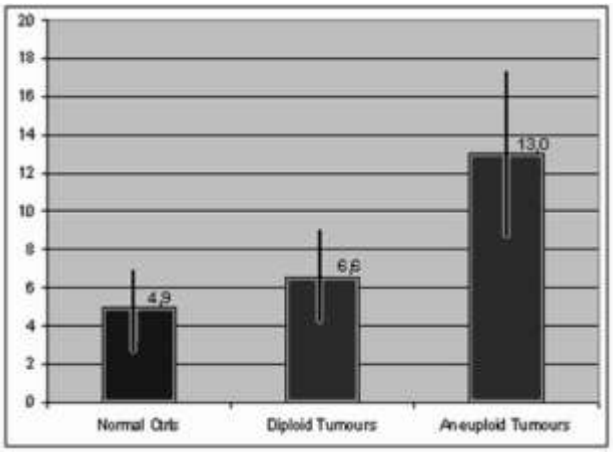

Figure 1. Statistics on nuclear protein content (mg/ml) assessed by direct spectrophotometry. The difference between diploid (normal or tumor) and aneuploid cells is evident. Student's T Test results are: Controls to Diploid Tumors 0.20955 (not significant); Controls to Aneuploid Tumors 0.00011 (significant); Diploid to Aneuploid Tumors 0.01151 (significant).

Results

The results are summarized in Table 1 showing DNA index and MIB-1 percentage for each sample.

Table 1 reports the average protein content of nuclei in normal controls, diploid and aneuploid tumors, along with their standard deviations. The histogram in Figure 1 outlines the sharp difference in protein content between diploid and aneuploid samples, as confirmed by the Student's t-test.

There was no correlation between the DNA index and MIB-1 percentage as determined by linear regression analysis (although the R^2 of about 35% was relatively high), as shown in Figure 2.

Similarly, considering all of the 18 neoplastic cases, the protein content was related neither to the

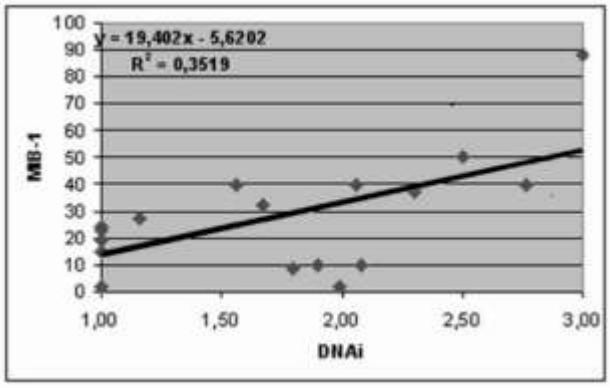

Figure 2. Linear regression analysis between DNA index and MIB-1 (percentage), performed on all the 18 tumor samples. R^2 value is not significant.

DNA index (R^2=5%) or to the MIB-1 (R^2=2.5%). These data are reported in Figure 3.

Quantitation of nuclear proteins did not seem to be dependent on the other two cited parameters. On the other hand, interesting results emerged when we considered only the tumors with a high proliferation rate (MIB-1>10%). In this group, the regression analysis between the MIB-1 and the DNA index showed a high degree of correlation (R^2=69%). Furthermore, the plots DNA index/protein concentration and MIB-1/protein concentration revealed an "atypical" subset of 5 cases with high DNA index and high MIB-1, but relatively low protein content. All these cases had a DNA index >2 (hypertetraploid), as shown in Figure 4.

When excluding also this subset from the analysis, a good linear regression was seen both between protein and the DNA index (R^2=75%) and between the protein concentration and MIB-1 (R^2=86%, Figure 5).

We can summarize our results as follows:

1. The content of nuclear proteins in aneuploid cells is significantly higher than in both normal (control) and tumor diploid cells. There is no statistical difference between normal and pathological diploid samples.
2. Considering all the tumor cases, protein content, DNA index and MIB-1 are not related to each other.
3. In tumors with a high proliferation rate (MIB-1>10%), this variable is directly proportional to the DNA index (ploidy).
4. Five highly proliferating tumors are nonetheless "atypical", because their nuclear protein content is relatively low, but the MIB-1 and the DNA index are particularly high. All these cases are hypertetraploid (DNA index >2).

Discussion

In this study, proteins from isolated, homogenized nuclei of frozen tissue were precisely quantified. The measure was performed directly, reading the spectrophotometric absorbance of aromatic aminoacids at the appropriate wavelength. Protein concentration was then related to other parameters considered to be crucial for the identification of cancer clones, such as DNA index and percentage of MIB-1 positivity [Sun et al, 2003; Munoz et al, 2003].

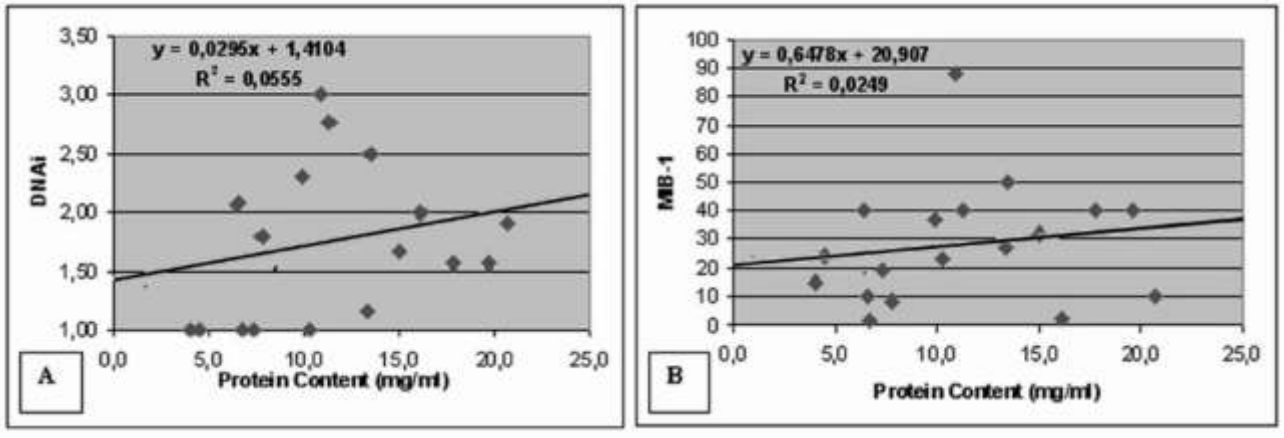

Figure 3. A, linear regression analysis between protein concentration and DNA index. B, linear regression analysis between protein concentration and MIB-1 (percentage). Both analyses were performed on all 18 of the tumor samples, and the resulting R^2 values are not significant.**

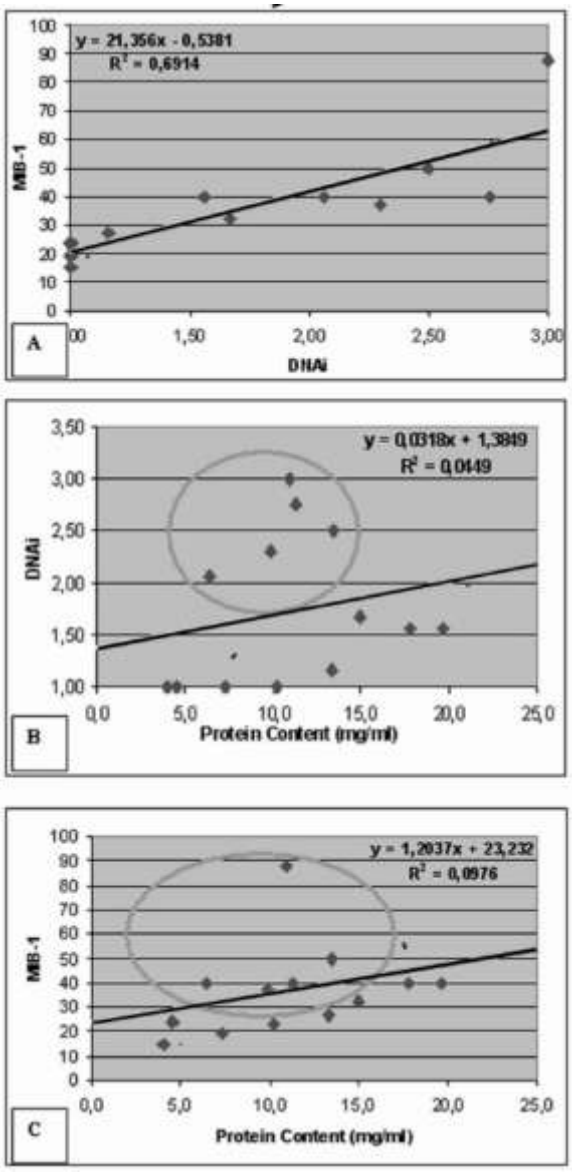

Figure 4 (left column). A, linear regression between DNA index and MIB-1 percentage in the 13 tumor samples with high proliferation rate, i.e. MIB-1>10% (R^2=69%). B and C, the regression analysis of protein content vs. DNA index and MIB-1 outlines a specific subset of 5 hypertetraploid cases with low protein concentration but high MIB-1 and DNA index (outlined with green circles). In B and C, R^2 values are not significant.

Our statistical findings lead to two conclusions.

Firstly, both normal (control) and tumor diploid cells contain a low amount of nuclear proteins, compared to aneuploid tumor cells. This phenomenon has two possible explanations: aneuploid cells necessarily need a lot more structural chromatin-associated proteins (histones), and/or their metabolic rate is much higher, leading to an improved production of metabolic enzymes and transcription factors [Tripodi et al., 2000]

Secondly, the protein content is directly proportional both to MIB-1 and DNA index, but only in highly proliferating (MIB-1>10%) non-hypertetraploid (DNA index <2) cases. This conclusion is subtle, but even more interesting. It means that a particular subset of hypertetraploid samples, which behaves differently from other aneuploid tumors, expresses a relatively high MIB-1, but has low protein content. A possible explanation of this behavior is that hypertetraploid cells, having accumulated an abnormal quantity of DNA beyond a critical threshold, undergo severe metabolic damage, it thus being impossible for them to follow the normal cell cycle. This eventually results in increased apoptotic rates, as previously described in literature [Attallah et al.,

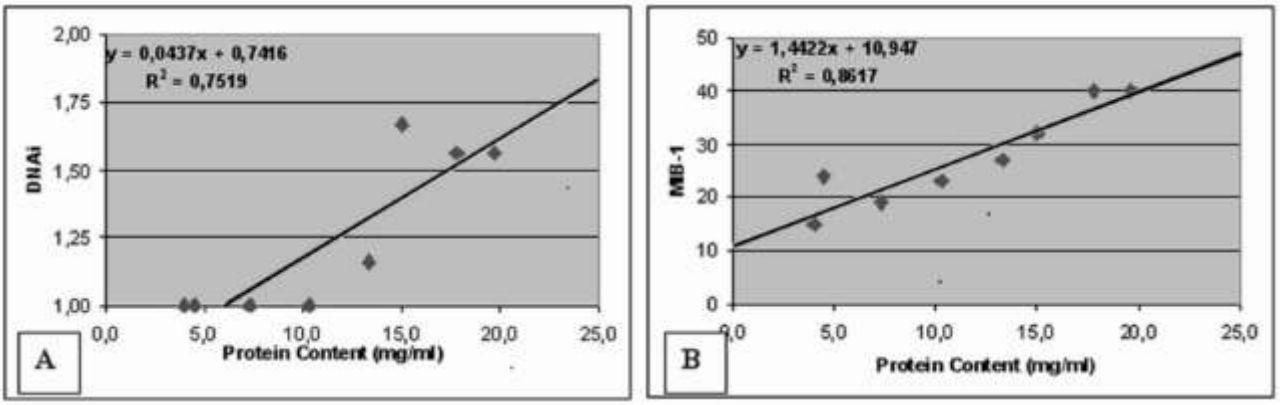

Figure 5. Both DNA index (A) and MIB-1 (B) are strictly related to nuclear protein concentration in the 8 highly proliferating, non-hypertetraploid tumors (R^2, respectively 75% and 86%).***

1996; Fukasawa et al., 1997; Verdoodt et al., 1999]. In hypertetraploid cells, a randomly regulated and uncontrolled replication of DNA, and the inability to divide every S-phase, lead to abnormal genetic content, two- or threefold the diploid set (up to DNA index =3). This process is scarcely coupled with an appropriate synthesis of structural or functional proteins, thus possibly explaining our findings.

It must be stressed that we performed our observations in a progressively limited set of samples (low statistical significance), and, therefore, our conclusions should be only considered indicative.

Direct and precise quantification of nuclear protein content in normal and pathological cells allows the collection of valuable data on the biological features of tumors, and to relate them to other prognostic parameters. Verifying the correlation between nuclear proteins, DNA content and proliferative rate, we found a particular subset of hypertetraploid tumors. Probably, their protein synthesis rate is abnormally low, because of the macroscopical genetic aberrations accumulated in their DNA.

Our study provides only preliminary data, but we are now considering more cases, with the aim to validate our findings. Collecting samples from neoplastic tissues other than breast will evaluate the correlation of parameters regardless of the tumor site. A careful study of hypertetraploid tumors will provide further characterization of the biological features and diagnostic/prognostic significance of these particularly abnormal cells.

Quantification of nuclear proteins by means of spectrophotometric assessment on isolated nuclei appears to be an effective, fast, low-cost and precise tool for the measure of this important parameter, strongly related to other diagnostic features.

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