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Review

Defining Prognostic Factors in Malignancies Through Image Analysis

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INTRODUCTION

THE INCREASING amount of literature on objective evaluation and quantitation in pathology has involved almost all fields of diagnostic practice and scientific investigation. As a research tool, quantitative methods are considered to play a relevant role in the correct interpretation of scientific results, improving their reproducibility both within and between different laboratories, and image analysis is one of the most important.

The attention of pathologists is now turned towards the standardisation of protocols, statistical guidelines and large conclusive studies in quantitative pathology, aimed at providing the necessary objectivity in diagnostic work. Moreover, image analysis systems have to be frequently upgraded to extend the spectrum of potential applications. The benefits of quantitative methods are particularly tangible in areas where conventional diagnosis may be difficult. The quantitative assessment of cell and tissue properties may well widen the pathologist's overall medical expertise, since it generally represents a more accurate approach to the knowledge of morphobiological features.

In tumour pathology, prognostic information is almost always requested by oncologists, as a complementary insight to the diagnosis.

Accordingly, providing factors that can predict the biological behaviour of tumours and orientate the clinical decision-making processes may be the most challenging applications of quantitative image analysis in pathology. Even though no new prognostic factors can be ascertained, it would be interesting to define the classic parameters of prognosis, more in detail, by quantitation. Overall these topics form the subject of the present review.

TECHNICAL PRINCIPLES AND METHODOLOGIES

Equipment

Basically, an image analysis system includes a microscope, a videocamera, a computer and a display screen. The original image seen under the microscope is captured by the videocamera, displayed as a digitised image on the display screen and processed by a software program.

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Analysis is performed on grey or true colour red–green–blue (RGB) images depending upon the acquisition system (videocamera, filters) and the objects or parameters to be measured. Measurements can be obtained through automatic or semi-automatic interactive procedures. A motor control processor for autofocus, a stage automation on the light microscope, as well as an automatic computer control of the microscope lamp power supply are important accessory features for many applications.

Image analysis systems are available from commercial suppliers or can be compiled personally using separate components [1].

Almost all commercial systems allow a series of programmed analyses to be conducted based on morphometric, stereologic and tissue architecture measurements, including 3-dimensional reconstruction and syntactic structure analysis (SSA) such as 'minimum spanning tree SSA' and 'Voronoy diagrams'. Appropriate software has been used for quantitation of oncogene products, ploidy analysis, quantitation of proliferative indices, neovascularisation and immunohistochemical reactions. Some systems offer interesting options including image archiving, cytogenetic karyotyping, automated cytology screening, continuing educational teaching, autoradiographic image evaluation, prognostic algorithm definition and on-line statistical elaboration.

The 'know-how' in pathology quantitation is based on computer science concepts of hypermedia, image and data storage, laboratory databases, expert systems, decision analysis and artificial intelligence. A computer-assisted workstation is becoming the pathologist's natural environment in which information acquisition, image processing and analysis, data storage and elaboration may be conveniently organised.

Morphometric measurements

One of the simplest applications of morphometry deals with linear measurements, such as the Breslow index assessment in primary melanomas, or the infiltration depth evaluation in microinvasive squamous cell carcinomas of the uterine cervix [1].

All image analysis software programs are also able to define planimetric cell nuclei characteristics, including perimeter,

area, maximum diameter, indentation, convolution, cleaving, ellipsing, orientation, crowding, textural features, such as chromatin distribution and optical density, as well as nucleolar features. Counting elements such as the number of cells, mitoses, nucleoli and piknotic nuclei is another feasible application of quantitative image analysis. Other more specific options are possible such as numbering Alcian-blue-stained metaplastic cells in the stomach, or silver-stained nucleolar organiser regions in many tumour cell types.

The study of tissue architecture

Several advanced quantitative methods such as stereology, syntactic structure analysis and 3-dimensional reconstruction will only be mentioned in this review. They usually provide quantitative information on tissue architecture as they are low-resolution techniques and may have potential diagnostic and prognostic implications in pathology [2, 3].

Quantitative immunohistochemistry

The need for objective evaluation and quantitation has become increasingly important in immunohistochemistry, along with the improvement in sensitivity and the wide use of immunohistochemical analyses. Examples comprise hormone receptor assays, oncogene expression such as c-erb-B2 and proliferative antigen expression such as PCNA and Ki-67 [1]. In this investigative field, quantitative determinations consist of percentage assessment of immunopositive cells, optical intensity values of immunostaining patterns and measurement of immunostained areas. Consequently, quantitative immunohistochemistry has been shown to have a great impact on the intra- and interlaboratory quality assurance of immunohistochemical procedures [4].

DNA-image cytometry

Measurements of cellular DNA are performed by image analysis on Feulgen stained tissue sections, reacting stoichiometrically with DNA, through a standardised procedure. However, it should be pointed out that the true value of cell DNA content cannot be obtained by cytometric measurements that actually quantify the DNA staining intensity. This parameter may strongly depend on the overall cellular proliferative activity and, possibly, on gross cytogenetic anomalies [5]. Moreover, a number of physical and staining limitations critically affect determination of the absolute amount of DNA actually present in a nucleus. To some extent, the same is true for flow cytometry, which is usually compared to 'static' image cytometry, as a complementary means of DNA measurement. Similarly it has been stated that the concept of 'DNA ploidy' does not coincide with that of cellular ploidy, because several conditions, depending on physiological cell cycle, may change the number of chromatids and, consequently, the DNA content, but not the number of chromosomes, i.e. the ploidy, each chromosome being composed of 1 or 2 chromatids [5]. Therefore, in image cytometry, DNA indices are assessed by quantitative evaluation of nuclear optical density at point-by-point image resolution. Following a segmentation process which discriminates meaningful objects, the grey digital values corresponding to the nucleus are elaborated to obtain the measurement of integrated optical density (IOD) which depends on the amount of DNA-specific stain in the nucleus. Hence, the true cellular DNA content is found when a coefficient of normalisation K is introduced, as a multiplicative term, to adjust the IOD value [5].

The software packages include visualisation of the ploidy histogram usually displaying the following statistics regarding the whole cell population: number of cells, DNA index, 2C deviation index, 5C exceeding rate and malignancy index. DNA assessment is currently considered more accurate in image cytometry than in flow cytometry, because DNA analysis is applied to '*in situ*' pathological material allowing the pathologist to combine information from both morphological and molecular observations. This is especially useful when a diploid cell population is ascertained by flow cytometry, to confirm that the diploid status may actually be imputed to tumour cells [5].

Standardisation

In 1990 the Committee for Diagnostic Quantitative Pathology Working Group of the European Society of Pathology stressed the need for standardisation [6], partially concerning the processing of specimens to be analysed, such as fixation and dehydration, section thickness, staining density, size of measured field, intensity of light source and instrumentation calibration. A detailed description of each step-by-step procedure is beyond the scope of this review. However, standardised processes and measurements precede any evaluation of quality assurance.

Examples of data elaboration

Two statistical multivariate techniques are currently used to elaborate quantitative variables: discriminant function analysis and cluster analysis. In the first approach, data are obtained from the analysis of two or more samples, the diagnoses of which are known. Thus, such an analysis provides an allocation rule for diagnosing any new case.

Cluster analysis is adopted when no predefinite diagnostic groups are established. Each quantitative parameter contributes to defining similarities and differences between any pair of cases. Diagnostic group aggregation is based on either agglomerating or dividing cases. Comprehensive accounts on discriminant function analysis and cluster analysis have been reported elsewhere [7–10].

From a pragmatic point of view, the rationale for morphometric application, as detailed by True [11], is:

(1) It limits interobserver variability in the quantitation of cell and tissue features;
(2) It provides a reproducible gradation of quantitative features;
(3) It improves sensitivity in detecting minimal changes;
(4) It allows the evaluation of the effect of changes in methods of tissue processing;
(5) It allows the assessment of quality control;
(6) It permits the development of teaching programmes;
(7) It improves availability of research tools;
(8) It requires investment of relatively limited human and economic resources.

APPLICATION OF IMAGE ANALYSIS TO TUMOUR PATHOLOGY

This section focuses on quantitatively determined prognostic factors in tumour pathology, as potentially recognised by the applications of morphometry, DNA cytometry, quantitative AgNOR assessment, and where relevant, other

advanced quantitative techniques. The pertinent literature on breast, ovary, renal, urinary bladder, larynx, lung and thyroid cancer, as well as non-Hodgkin's lymphomas are reviewed.

Breast cancer

In invasive breast cancer (IBC), tumour size provides relevant prognostic information. This needs to be confirmed in very low ranges of tumour dimensions, where concepts such as minimal invasive cancer and microinvasion could be defined more precisely through image analysis. In the pT1 tumour group, some tumours have an aggressive clinical behaviour while others do not. Therefore, morphometric parameters including the analytical evaluation of tumour sizes may identify patients with a more favourable outcome.

In breast cancer, the interobservers' tumour grading variability has been assessed by the κ statistic ranging from 0 (no agreement) to 1 (total agreement): κ values of 0.5 and 0.4 have been reported in counting mitoses and in grading nuclear pleomorphism, respectively, giving an overall poor concordance [12]. Nevertheless, the prognostic value of the tumour grade as determined by conventional examination has been confirmed by morphometric application. Taking into account chromatin texture patterns, mean nuclear area, number of mitoses and mean nuclear shape factors, a reliable case separation in the Bloom–Richardson score scale from 4 to 8, i.e. grade I to III, has been consistently demonstrated [13, 14].

Moreover, histological architectural tumour characteristics such as the percentage of glandular formation and the amount of *in situ* component in IBC have been found to be inversely correlated with high recurrence rates [15].

Other quantitative parameters, affecting patients' survival, have been identified as the tumour cell area/stroma ratio (the greater this ratio, the more prolonged the patient's survival) [16] and the nuclear or nucleolar pleomorphism (the more pleomorphic nuclei or nucleoli, the shorter the overall survival, as evidenced by greater standard deviation (S.D.) of corresponding morphometric measurements) [17].

Interestingly, in a morphometric study on IBC, the tumour cell mean nuclear area is important to establish the presence of lymph node metastases in metastatic and non-metastatic tumour cases [18].

To estimate the predictive value of quantitative variables, a morphometric prognostic index (MPI) for IBC, based on tumour size, histological grade and nodal status, has been proposed [19] as follows:

$\mathrm{MPI} = (0.3341 \times \mathrm{MAI}^{1/2}) + 0.2342 \times \text{tumour size in cm}) - (0.7654 \times \text{lymph node status})$, where MAI = mitotic activity index (number of mitoses $\times$ 10 high-power field) represents the tumour grade; and lymph node status is 1 if positive for metastases or 2 if not.

In breast pathology, one of the most vexing problems is the differential diagnosis between borderline lesions such as atypical hyperplasia and well-differentiated *in situ* carcinoma. Unfortunately, no pertinent diagnostic criteria have been defined by the assessment of currently quantifiable parameters.

Prognostic information by AgNOR counts are of little value. However, it is worth noting that AgNORs increase in number and size when cell activity increases, possibly implying DNA abnormalities [20]. In a recent report, the mean area of AgNOR per nucleus has been proposed as the sole AgNOR-related variable of prognostic relevance [21].

More interestingly, in a study on 178 IBC, tumour angiogenesis, quantitatively evaluated as total endothelial area, was found to be prognostically significant in univariate and multivariate analyses of disease-free survival and overall survival [22]. When the tumour subset of lymph node negative invasive breast carcinomas was considered, the increasing total endothelial area was an independent unfavourable prognostic indicator for overall survival [22]. Conversely, in another study, no correlation was found between the percentage of factor VIII-related antigen-stained area and the prognosis [23].

DNA image cytometry has been shown to be a reliable prognostic indicator in breast cancer. In fact, abnormal DNA ploidy is associated, with increasing histological grade [24], tumour size [25], lymph node metastases [26], oestrogen receptor status [27], c-erb-B2 oncoprotein, p53 tumour suppressor gene product overexpression and increased cell proliferation as assessed by the Ki-67 labelling index.

Even though the presence of DNA abnormalities is considered as a prognostic independent variable, oestrogen receptor status seems to modify the impact of DNA ploidy on survival [28], which is reduced in hormone receptor negative cases.

Ovarian cancer

The prognosis in ovarian cancer strongly depends on the stage of disease as assessed by the International Federation of Gynaecology and Obstetrics (FIGO) staging system. Nevertheless, a number of other factors have been investigated as possible prognostic independent variables or in conjunction with staging. Baak and associates have demonstrated that a combination of morphometric parameters were associated with a poor prognostic outcome. They are the mitotic activity index above 30 (mitotic figures assessed in 25 fields at $\times$400 magnification—objective 40$\times$, numerical aperture 0.75–); the volume percentage epithelium above 60% (the epithelial/stromal tissue ratio in the atypical areas as measured by a point counting technique using a point grid placed on a projection microscope at 200$\times$ magnification) and the shortest nuclear axis of 25 randomly selected epithelial cell nuclei per case [29].

Moreover, a significant strong correlation has been found between the aforementioned combined variables and tumour histological grade as conventionally ascertained by pathologists [29].

Accurate prognostication becomes outstandingly important in true stage I ovarian cancers, for which the question is still whether adjuvant therapy should be currently advised. In this setting, the value of morphometry, used in conjunction with staging and DNA cytometry, has been emphasised in order to identify patients' prognostic groups [29]. Stage I ovarian cancer has been further prognostically defined in a study where the volume-corrected mitotic index (M/V index), which is the number of mitoses per square millimetre of neoplastic tissue in the microscopic fields, has been shown to correlate with the clinical outcome [30]. Interestingly, several quantitative studies have been addressed to ovarian 'borderline' tumours, because prognostic information concerning such cumbersome tumours has been claimed to provide consistent therapeutic guidelines. In a single case, a morphometry-assisted diagnosis was used to correctly allocate a tumour in the borderline rather than malignant category [31]. In another study, based on 20 borderline tumours, the

morphometric measurements (especially mitotic activity index) correlated with the prognosis, but were inaccurate in predicting the extra-ovarian spread of these tumours [32]. The predictive value of DNA-static cytometry in ovarian carcinoma, especially of the serous type, is considered higher than the value linked to qualitatively assessed morphologic tumoral characteristics [33], even though it has yielded controversial results in predicting the biological behaviour of borderline tumours [34, 35]. This may indicate that further quantitative investigations dealing with this debated field of pathology should be planned. Currently, it has been clearly demonstrated that similar DNA ploidy results have been obtained through flow cytometry and static computer-assisted cytometry techniques, by using cell suspensions from borderline serous and mucinous tumours [36].

Renal cancer

Despite the increasing knowledge of alternative forms of therapy of renal cell carcinoma (RCC), surgical ablation remains the only effective modality in localised disease. RCC is usually considered a tumour with an unpredictable course: it has a very slow progression rate in some patients, regardless of the presence of metastatic disease at diagnosis and, conversely, pursues an aggressive behaviour, in other cases, with a rapidly fatal outcome. Hence, only accurate definition of prognostic factors would provide reliable information on patients' survival. Various pathologic parameters have been considered in the assessment of RCC prognosis. Tumour size and histocytological grade form the basis of numerous pathological staging systems which have proved to be effective in predicting the clinical outcome in individual cases. Other pathological findings such as renal vein invasion, perirenal adipose tissue infiltration, cell type and histological pattern have been reported as potential predictors, but with discordant results in the literature [37].

The prognostic importance of morphometric measurements in renal cell carcinoma has been stated by Murphy and associates [38] and Gutierrez and associates [39]. The former developed a quantitative analysis system which allowed the correct prediction of outcome in 19 out of 20 patients. Using this system, 150 nuclei per case, selected randomly from neoplastic fields, were measured by 25 nuclear shape descriptors for each nucleus (i.e. roundness factor, ellipticity, concavity and convexity of nuclear boundary, etc.) [38]. Gutierrez and associates, in a series of 95 RCC, found that a mean nuclear area of less than 35 μm^2 was associated with a 97% 5-year survival rate, compared with 17.2% 5-year survival of cases with a mean nuclear area greater than 35 μm^2 [39].

One of the characteristics of RCC is the intratumoral heterogeneity, consisting of phenotypical differences between the cell clones within the same tumour. A measure of this cellular variability, as assessed by karyometric analysis, may be not only an indicator of the tumoral genetic instability, but may offer additional prognostic information as well [40]. Among the karyometric features, DNA static measurements may consistently quantify the degree of this intratumoral variability. This is usually impossible to obtain by flow cytometry because of the bias intrinsic to random sampling.

Several studies have used a morphometric approach in the prognostic assessment of stage I RCC. Considering a mean nuclear area of 32 μm^2 as the decision threshold, Tosi and associates in a series of 41 stage I RCC, were able to discriminate those associated with a worse prognosis having, in 100% of cases, a mean nuclear area greater than 32 μm^2 [41].

Other instances have demonstrated that the nuclear morphometric measurements have the capacity to differentiate renal oncocytomas from RCCs [42]. Furthermore, the stereological estimation of volume-weighted nuclear volume, in a series of 36 variable sized besophilic renal tumours, does not support the assumption of using a 3 cm tumour diameter as the dividing line between renal adenomas and carcinomas [43]. As far as DNA-static cytometry is concerned, the results of a recent study by Nenning and associates on 110 RCC showed a significant correlation between the size of the tumour, lymph node metastases and distant metastases on the one hand and the computed DNA parameters on the other [44]. Accordingly, a positive association has been reported between aneuploidy and development of metastases in a series of 48 RCC, but, in a multivariate analysis, DNA assessment appeared to be a dependent prognostic variable, with the expected exception of stage IV RCC [45].

Urinary bladder cancer

Superficial bladder carcinomas encompass a wide spectrum of disease ranging from prognostically favourable pTaG1 tumours to more aggressive *Cis* or invasive poorly differentiated pT1 G3, requiring careful evaluation and close monitoring.

Regardless of stage, grade 3 carcinomas account for 6–23% of all superficial urothelial bladder cancers. The variable incidence rate may reflect different criteria used by pathologists in histological grade assessment, and contrasts with the current opinion that good reproducibility of grading is warranted in low- and high-grade tumours. Interestingly, although G3 tumours are said to have marked nuclear polimorphism, many mitotic figures and conspicuous cellular atypia, it is difficult, at a practical level, to quantify these histological findings. Stage by stage, grade 3 tumours seem to be a heterogeneous category including tumours with variable prognosis. In our unpublished study on 26 patients with pT1G3 tumours treated with BCG-intravesical instillations, it was found that the mean nuclear area of the deepest part of the tumours seems to be a discriminant factor in separating the groups of patients with and without progression of disease.

The high inter- and intra-observer inconsistency among pathologists in grading urothelial bladder tumours has been repeatedly stressed. In several studies the tumour grade assessed by morphometric descriptors such as nuclear size and shape has been consistently evaluated to obtain the separation of the three WHO grades of lesion by discriminant analysis and reproducibility tests [46, 47]. Moreover, significant correlations have been found between increasing nuclear area and high tumour grade, depth of the invasion and survival [48]. By a multimodal quantitative approach, consisting of AgNOR count, PCNA and MIB-1 immunohistochemistry positive scores, nuclear morphometric measurements and DNA ploidy assessment by flow cytometry, 50 cases of transitional cell carcinoma of the urinary bladder, conventionally graded as G1, G2, and G3, were evaluated. Single or combined quantitative methods allowed correct discrimination of low- (G1 and G2) and high- (G3) grade tumours, thus supporting the assumption of the existence of only two prognostic subgroups in the urothelial tumour setting [49].

Laryngeal squamous cell carcinoma

Laryngeal squamous epithelial dysplastic lesions include a wide range of entities with variable potential for progression

towards invasive carcinomas. Therefore, correct management of these lesions implies prompt identification of prognostic factors both on morphological and biological grounds.

Morphometry has been claimed to provide a prognostic guide in the follow-up of squamous epithelial dysplasia in laryngeal biopsies, but this has not been confirmed by large conclusive studies. In a limited series of 10 cases of dysplastic lesions, progression of disease in invasive cancer was correctly predicted in 6 of these cases by nuclear morphometry [50].

Invasive squamous carcinoma has been investigated in conjunction with other similar malignancies of the head and neck in a study by Helliwell and associates [51]. A number of morphological tumour characteristics such as grading, keratinisation, nuclear pleomorphism, nucleolar prominence, mitotic rate, lymphocytic infiltration, pushing or infiltrating borders, percentage of necrosis and stromal desmoplasia were semiquantitatively evaluated. Moreover, the area and diameter of the two largest nuclear profiles in each section, as well as the surface area to volume (s/v) ratio of tumour islands were assessed by morphometric analysis. The response to cisplatin chemotherapy of different tumour groups was assumed as the end-point of the study. Among other figures, the combination of low s/v ratio and pushing borders, as well as the differentiated histology were associated with good response, while a lack of inflammatory reaction or desmoplasia correlated with poor response [51].

Lung cancer

Cytological differential diagnosis between bronchial epithelial dysplasia and carcinoma has been attempted by a quantitative evaluation of cell and nuclear features in smears. Differences have been found to permit diagnostic group separation [52].

In histological material, well-differentiated adenocarcinoma and alveolar hyperplasia, as well as adenocarcinomas, squamous and small cell carcinomas have been reliably distinguished by measurements of nuclear area with its S.D. [53], or through discriminant analysis of nuclear parameters [54]. In squamous cell carcinoma, a morphometric index, based on measurements of tumour, stroma, necrosis, mitosis/necrobiosis ratio, reciprocal cell count and number of apoptotic bodies, has proved to be of prognostic relevance: tumours in patients with short-term survival exhibited dismal features as greater epithelial volume, necrosis and high mitotic rate [55].

The wide spectrum of neuroendocrine tumours has been analysed with diagnostic and prognostic purposes, either by quantitating the nuclear differences between oat and intermediate-cell varieties in small cell carcinoma category [56], or by assessing the nucleus/cytoplasm ratio as strong predictor of biological behaviour [57].

Moreover, a constant increasing trend in DNA content has been demonstrated from typical carcinoid, atypical carcinoid and small cell carcinoma, with evident prognostic implications [58]. DNA analysis is also useful in recognising squamous dysplasia as a premalignant condition [59].

Thyroid cancer

In thyroid follicular tumours, it is often difficult to differentiate between malignant and benign entities. It is currently recommended that a diagnosis of carcinoma should be rendered when unequivocal capsular or vascular neoplastic invasion may be demonstrated. This is true in histological sections, but in fine-needle aspiration biopsy the differential diagnosis is based on the cell morphology and aggregation. Hence, several studies have been conducted on cytological material obtained from follicular adenomas, follicular carcinomas and non-toxic goitres. Nuclear parameters currently investigated include nuclear perimeter, nuclear area and nuclear cytoplasmic ratio. Based on the latter two, an allocation diagnostic rule allowed correct diagnosis for 11 out of 13 adenomas and for 18 out of 20 carcinomas [60]. In another study, a threshold value for the mean nuclear area of 90 μm^2 correctly discriminated 100% of the adenomas and 76% of the carcinomas [61].

Combined nucleolar and nuclear features have found to provide further insights into follicular adenoma–carcinoma differential diagnosis [62]. Nevertheless, even histological diagnosis is hampered by important limitations, when only cytological features are considered. Based on a stereology and flow-cytometry investigation on follicular adenomas and well-differentiated carcinomas, it has been postulated that DNA aneuploid follicular lesions have greater nuclear area, perimeter and volume, regardless of their histological diagnosis [63]. Expanding these concepts, it has been proposed that some adenomas may actually be pre-invasive carcinomas [63, 64].

In a study on 28 patients with papillary carcinoma, recurrence, occurring in 6 patients, was found to correlate with tumour size, standard deviation of the estimated nuclear area (value > 17 μm^2) and cellularity mean index corresponding to the percentage of tumour volume composed of tumour cells (value > 40%) [65].

Interestingly, several studies have shown that DNA aneuploidy is strongly associated with the malignant phenotypes [66], while lack of correlation has been demonstrated between DNA ploidy patterns and tumour progression and survival [67].

In medullary carcinomas, unstable DNA histograms coupled with tumoral high stages allow identification of patients with shorter disease-free interval [68]. Finally, morphometric parameters are of little value in assessment of the malignant potential of Hurthle cell tumours [69].

Non-Hodgkin's lymphomas

The majority of morphometric studies on non-Hodgkin's lymphoma (NHL) have been performed in the past decade. The initial analytical investigations were aimed at defining morphometric subtypes of NHL in comparison with currently adopted histological classifications. Notably, the reference in the main classification systems (Lukes and Collins, Kiel, Working Formulation and R.E.A.L.) to morphological cell features has been founded on the qualitative and, to a great extent, subjective estimation of nuclear and cellular sizes, shapes and components (nucleoli, chromocentres, chromatin texture). Therefore, an objective evaluation in these settings, by quantitation of morphological features, would complement the descriptive analysis. Nevertheless, in the novel R.E.A.L. classification, no mention has been made of the possible application of image analysis to NHL pathology, in spite of apparent difficulties in subclassifying some entities, such as large cell lymphomas, as ascertained by reproducibility tests [70].

Similar qualitative approaches have inspired the concepts of 'tumour grade' and 'prognostic groups': the first referred to histological parameters such as cell and nuclear size,

density of chromatin and proliferation fraction; the second, to the biological behaviour of tumours.

One of the most elementary tasks in the quantitation of NHLs consists of quantitatively evaluating features of lymphoma cell nuclei as compared to nuclei of normal or reactive lymphoid cells. In follicular lymphoma, the neoplastic nuclei may be identical in size to that of unstimulated mantle cells, but are quite different in nuclear profiles, being more irregular than their normal counterparts [71]. Interestingly, the presence of nuclear clefts is usually underestimated by visual examination which is able to find only 5% of irregular nuclei in a cell population actually containing 25–30% of them. Moreover, other comparative studies have demonstrated that the nuclear area is an effective parameter in distinguishing cell types in the normal range of benign lymphoid cells as well as in the spectrum of NHL cells [72, 73]. This would mean that morphometry could be a reliable tool in classifying NHLs. Based on discriminant analysis, the diagnoses of centroblastic and immunoblastic lymphomas have been confirmed with an 88% accuracy, taking into account the following parameters: nucleolar area, cytoplasm/nucleus ratio, number of nucleoli per cross-section, relative nucleolar eccentricity and percentage of immunoblasts [74]. The incomplete separation of diagnostic groups denotes that a minimal but not negligible fraction of cases fall within a transitional category. In the setting of T-cell NHLs, it has been observed that the occurrence of large cell types is usually associated with increasing nuclear pleomorphism. When the nuclear area is more than 25 μm^2, the neoplastic nuclear irregularities strongly contrast with the relative uniformity of large paracortical reactive lymphocytes [75].

Moreover, nuclear area and its coefficient of variation are known to be important clues in recognising the subclasses of T-cell NHL [76]. One of these, the early cutaneous T-cell lymphoma has been investigated by morphometric analysis of nuclear contour irregularities. The nuclear features of lymphoid cells may be accurately quantitated by examining the specimen with a 1000× oil immersion lens after appropriate technical procedures of tissue processing. Considering perimeter and nuclear area, the nuclear contour index (NCI) has been defined for diagnostic purposes [77].

Through low-resolution techniques, the possibility of quantitatively examining the nodal architecture has been applied in distinguishing follicular lymphoma from florid reactive hyperplasia, based on the differences in size and shape of reactive and neoplastic follicles.

In lymphoid malignancies, relatively few studies pertain to DNA ploidy and AgNOR count: there are only a few on the prognostic significance of DNA analysis [78, 79], and the association of high AgNOR numbers with increased proliferative activity and poor clinical outcome in high-grade lesions [80].

A very interesting application of image analysis in lymphoid malignancies is quantitative immunohistochemistry. This issue is in continuous evolution because there are theoretically no limits in quantitating the numberless features of immunohistochemical analyses. For example, the count index of immunopositive cells has been applied to the immunoassay of nuclear proliferation antigen Ki-67, finding it of prognostic significance in different subsets of NHLs [81].

Quantitation of immunostaining patterns by optical intensity values and stained area measurements are other feasible options. In addition, image analysis allows the discrimination of elements in a mixed cell population which score positively for a specific marker. Morphometric analysis of positive cells permits comparative studies on cell morphology and marker expression. Image analysis of immunocytochemistry and *in situ* hybridisation in cytospin preparations of lymphoid cell populations (Figure 1) is, at present, a relevant part of our field of investigation.

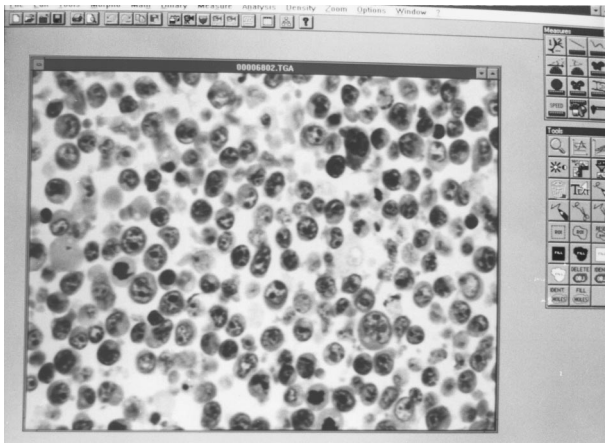

Figure 1. An example of 'acquired' image, showing a neoplastic lymphoid cell population derived from cytospin preparations. The digitised image is suitable for analysis.

CONCLUSIONS

Image analysis appears to predict survival outcome of many, if not all, tumour entities, with a relatively high degree of accuracy and less subjectivity than visual examination. Computer-aided analysis has the evident advantage of selecting the objects to be measured and may pursue at least two lines of development: (1) standardised applications in quantifying the simple morphologic characteristics of the objects; and (2) quantitative evaluations of histochemical, immunohistochemical and molecular tests. However, misunderstandings must be avoided: quantitative pathology does not necessarily mean automatic diagnosis; similarly the pathologist's responsibilities cannot be assigned to computer applications.

We conclude this review referring to a quotation from Williams Thompson (Lord Kelvin), 1883: ''I often say that when you can measure what you are speaking about, and express it in numbers, you know something about it; but when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind''.

This citation has also been reported in the book by Hamilton and Allen [82], which may be considered the 'state of the art' in this discipline.

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