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Morphometric index of adult renal cell carcinoma

Comparison with the Fuhrman grading system

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Abstract Various grading systems have been proposed for renal cell carcinoma (RCC), using nuclear, cytoplasmic, and architectural features. The available evidence suggests that nuclear grading is a better prognostic indicator than other types of grading schemes. Nuclear morphometry may still improve the correlation of the nuclear grading with survival, however, because observer consistency is lacking in the subjective grading of RCC. The aim of this study was to investigate morphometrically whether RCC cases show a continuous spectrum of nuclear changes or whether there are discrete groups of cancer that correspond to the four Fuhrman grades. Karyometry was performed on 5- µm-thick, haematoxylin- and eosin-stained sections from 60 cases of conventional (clear cell) RCC. The analysis also included the evaluation of normal renal tissue (proximal tubules) adjacent to cancer. In each case the difference between the value of the cancer and the corresponding normal epithelium was calculated to represent, quantitatively, the degree of similarity between the tumour tissue and the internal normal control. When the differences were sorted into ascending order, a steady increase in values was observed for both the nuclear and the nucleolar features. A monotonic trend was evident for the differences in the mean maximum nuclear diameter and mean nucleolar area. When the differences between the values in the cancer and in the corresponding normal epithelium of these two features were summed up, the method resulted in a continuous variable, or nuclear morphometric index, related to the degree of deviation of each individual RCC from its internal normal control. The lowest index values were observed in of Fuhrman grade I cases, whereas values ranging from 2.679 to 5.422 were associated with cases graded II. Values equal to or higher than 5.951 were seen in the cases assigned to either grade III or grade IV. Partial overlap was present between the index values in grades III and IV. The RCC cases can be represented by a continuous index that corresponds to the morphological grading based on the Fuhrman scheme. This study shows that the index may be useful in supplementing the pathologist's grading. This issue can be further addressed with follow-up studies.

Key words Renal carcinoma · Karyometry · Quantitative analysis · Morphometry · Diagnostic distance

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Introduction

It is now widely accepted that renal cell carcinomas (RCCs) are not a single tumour type but consists of a variety of tumours that can be classified according to morphology and genotype [7, 26, 53]. There is evidence to indicate that tumour stage is an important prognostic factor for RCC irrespective of tumour type [28, 49]. Tumour grade appears to provide useful survival information [21].

Various grading systems have been proposed for RCC, using nuclear, cytoplasmic, and architectural features [11, 21]. Nuclear grading systems are the most widely used. The available evidence in the literature suggests that nuclear grading is a better prognostic indicator than other grading methods [11]. Nuclear grading has prognostic value for conventional (clear cell) and papillary RCC. The data supporting the validity of nuclear grading for chromophobe carcinoma are not well established, but it seems reasonable to grade these tumours for ongoing

clinicopathological studies [31]. Of the several proposed nuclear grading systems available, that of Fuhrman et al. is the most widely used in Europe [20, 33].

There are two major problems with the proposed grading schemes [11]. The first is that the degree of correlation of the various systems with survival is not satisfactory. This is due, at least in part, to the inadequate description of grading criteria published in some classifications. The other problem is the somewhat poor observer consistency in grading of RCC [29] when composite grading systems that require assessment of several morphological features are used. In recent studies nuclear morphometry and stereology have been applied in an attempt to improve the prognostic information usually derived from grading schemes [2, 4, 8, 15, 19, 22, 23, 32, 42, 44, 45, 48, 56].

The aim of this study was to investigate morphometrically whether RCC cases show a continuous spectrum of nuclear changes or whether discrete groups of cancer (as suggested by the Fuhrman grading system) exist.

Material and methods

Patient population

The present series included 60 kidney tumours diagnosed as conventional (clear cell) renal carcinoma, Stage I (e.g., pT1 N0 M0) [47] at the Institute of Pathological Anatomy, University of Ancona School of Medicine, Italy. This study did not include types of RCC other than the clear cell type. All the patients, 39 men and 21 women, underwent simple or radical nephrectomy between 1986 and 1999 without any preoperative adjuvant chemotherapy or irradiation. The patients' age range was 34–88 years, with a mean of 65 years. The size of the tumours ranged from 3 to 5 cm (mean 3.5 cm).

The nephrectomy specimens were handled routinely, with submission of at least three sections of tumour and also a section from the adjacent part of the renal parenchyma with a normal appearance and all surgical margins. Regional lymph nodes were also examined for the presence of metastasis. Formalin-fixed, paraffin-embedded, haematoxylin- and eosin-stained sections were revised and tumours were graded according to the criteria of Fuhrman et al (Table 1) [20]. There were 14 cases of grade I, 15 of grade II, 19 of grade III and 12 of grade IV.

Quantitative analysis

Quantitative analysis was performed by one of us (R.P.) on 5-µm-thick haematoxylin- and eosin-stained sections of each tumour and its accompanying normal renal tissue (proximal tubules). A Zeiss-Kontron IBAS-AT Image Analyser (Munich, Germany) combined with a light Zeiss microscope equipped with a 100× oil immersion objective was used.

Table 1 The Fuhrman grading system [20]

Grade	Characteristics
I	Nuclei round, uniform, approximately 10 µm; nucleoli inconspicuous or absent
II	Nuclei slightly irregular, approximately 15 µm; nucleoli evident
III	Nuclei very irregular, approximately 20 µm; nucleoli large and prominent
IV	Nuclei bizarre and multilobated, 20 µm or greater; nucleoli prominent; chromatin clumped

The analysis of each sample began with the selection of the measurement area. Concerning RCC, the most cellular area was chosen for analysis. This was in general located at the periphery of the tumour nodule, near the capsule. Areas of necrosis and haemorrhage were avoided. As normal renal tissue, the epithelial lining of proximal tubules located at least 1 cm from the carcinoma was evaluated. All distinguishable nuclei in a microscopic measurement field were systematically selected, starting from the upper left corner of the measurement field. Altogether, adjacent fields were analysed until a total of 50 nuclei were measured. The selected nuclei had clearly visible boundaries. Disintegrated nuclei and disintegrated cells were not measured. The boundaries of the nuclei on the screen were accurately outlined with the help of a magnetic tablet with a cursor.

In each case the mean and standard deviation of the following features were calculated: maximum nuclear diameter, nuclear area, roundness factor and nucleolar area. The maximum nuclear diameter is the largest of the Feret diameters measured in 32 different directions (i.e., at an angular resolution of 5.7°). The nuclear roundness factor is a size-independent indicator of the regularity of a profile. It was calculated according to the following formula: 1 divided by (4×π×nuclear area divided by the squared nuclear perimeter). Its value is 1.0 for a circle and greater than 1.0 for irregular structures.

The values of the maximum nuclear diameter were highly correlated with those of the nuclear area (correlation coefficient: 0.99). Therefore, with the Fuhrman grading system in mind, the maximum nuclear diameter was preferred to the nuclear area. Correlation coefficients lower than 0.90 were obtained between the features retained in this presentation, e.g. maximum nuclear diameter, roundness factor, nucleolar area and nucleolar frequency.

To find the number of nuclei to be measured, the running mean procedure was applied. For the features investigated, the measurement of 30 nuclei was sufficient to have a cumulative average within the 95% limits. To be sure of adequate sampling, 50 nuclei were measured at random in each case. In general, 10–13 fields were sufficient to reach this number.

In each case the number of nucleoli was also counted in 500 nuclei both in the normal tubules and in the areas of carcinoma where karyometry was performed. The result of the evaluation was expressed as a percentage of nucleolated nuclei or nucleolar frequency.

Results

The nuclear size and roundness analysed in the proximal tubular epithelia of the 60 cases showed a narrow range of values. In fact, the mean of the maximum nuclear diameter of the individual specimens spanned from 6.17 to 7.58 µm, whereas the mean nuclear roundness factor ranged from 1.003 to 1.032. In a similar way to the nuclear features, the nucleoli showed very little variation in size and frequency. The values of the mean nucleolar area ranged from 1.31 to 2.37 µm^2, whereas the percentage of nucleolated nuclei ranged from 0.2% to 7.6%. There was no relationship between the small variability in the feature values and the grade of the accompanying cancer.

The nuclear size and roundness measured in the 60 renal carcinomas showed a wide range of values. The mean of the maximum nuclear diameter was between 6.69 and 15.03 µm, whereas the mean nuclear roundness factor ranged from 1.015 to 1.209. The values of the former feature, ranked in ascending order, are reported in Fig. 1, which also shows the values in the normal epithelium for comparison. The nucleoli also showed wide

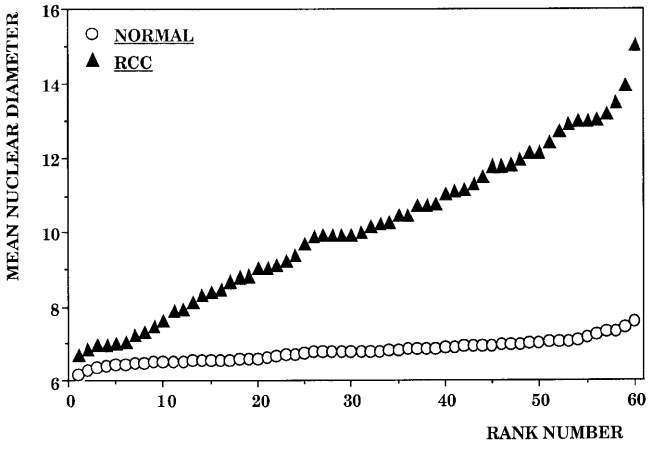

Fig. 1 Mean of the maximum nuclear diameter of the 60 cases of renal cell carcinoma (*RCC*) and of the corresponding normal tubular epithelium (*normal*)

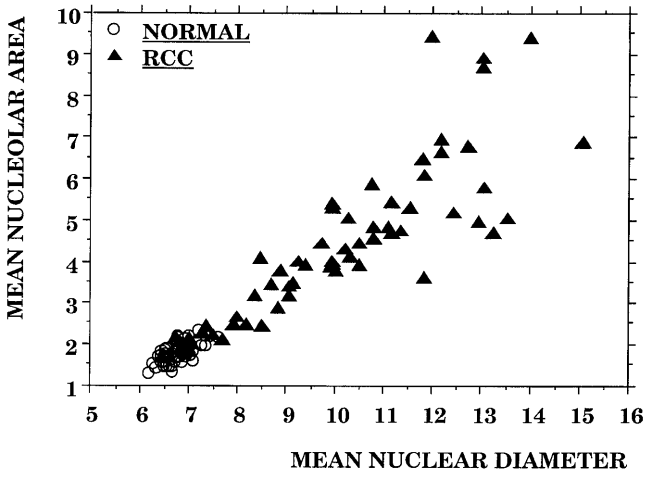

Fig. 2 Scattergram obtained with the mean maximum nuclear diameter and mean nucleolar area. Both the normal samples and cancers are represented

variation in size and frequency. The mean nucleolar area ranged from 1.82 to 9.44 μm^2, whereas the nucleolar frequency ranged from 0.2% to 85.2%.

To investigate the presence of field-to-field variations within one section, representative slides from 5 cases were selected. In each section, the same observer (R.P.) measured all nuclei in 5 random fields, and the mean and standard deviation were calculated for each field. Analysis of variance showed no significant field-to-field differences. The intra- and inter-observer variation was assessed in 5 cases. Two observers (R.P. and A.S.) performed the measurements. Correlation coefficients (Spearman-Rank test) greater than 0.95 were obtained by repeated measurements of the maximum nuclear diameter and nucleolar area. The nuclear roundness factor and the nucleolar frequency were less reproducible, the correlation coefficients of the repeated evaluations being lower than 0.90.

Figure 2 shows the scattergram obtained with the following two features: mean maximum nuclear diameter

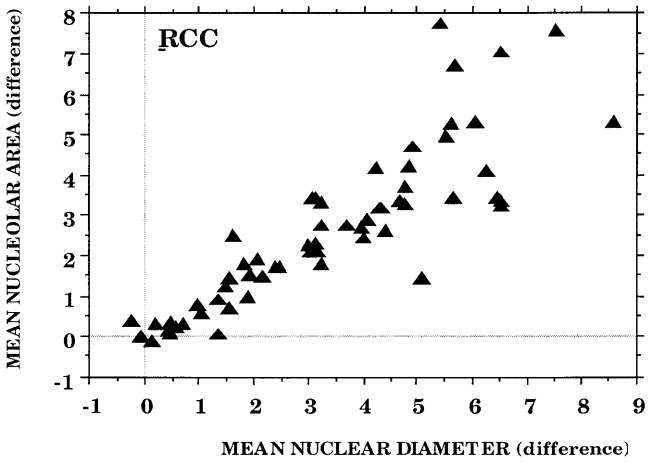

Fig. 3 Scattergram based on the differences of the mean maximum nuclear diameter, and mean nucleolar area in RCC, showing that both features are relevant in the grading and also making a full spectrum of feature values in carcinomas apparent

and mean nucleolar area. Both the normal samples and cancers are represented. The values derived from the proximal tubular epithelium are located in the lower left corner of the bivariate graph, where they form a homogeneous and well-demarcated cluster. The carcinomas occupy a wide area of this scatterplot, which extends from the lower left corner, where they are overlapped with the controls, to the upper right corner. Figure 2 gives a clear graphical representation of the value distribution of RCC in comparison with normal.

The difference calculated between the value of the individual cancer and the corresponding normal epithelium gives a quantitative representation of the degree of similarity between the tumour areas and the internal normal control. Based on the differences of the mean maximum nuclear diameter and mean nucleolar area, as shown in the scattergram of Fig. 3, a full spectrum of the distribution of the carcinomas is apparent. In particular, the scattergram shows that the cases form a continuum of changes. The higher the difference the lower is the similarity to normal.

When the differences were sorted in ascending order, a steady increase in values was observed for the four features in the analysis, a monotonic trend being more evident for the differences for the mean maximum nuclear diameter and the mean nucleolar area. The differences for these two features were summed up to derive a diagnostic distance of each tumour from its normal (this distance measure derived from the sum of the absolute difference in values for any feature is called the Manhattan distance). This is an index that expresses the degree of deviation of each individual cancer from its internal normal control. Figure 4 shows the individual index values ranked in ascending order. The graph shows a distribution of the cases along an almost straight line, the values ranging from –0.070 to +15.069.

Morphologically, the cases with lower index values showed more similarity to the normal proximal epithelium than the cases with higher values, whose deviation

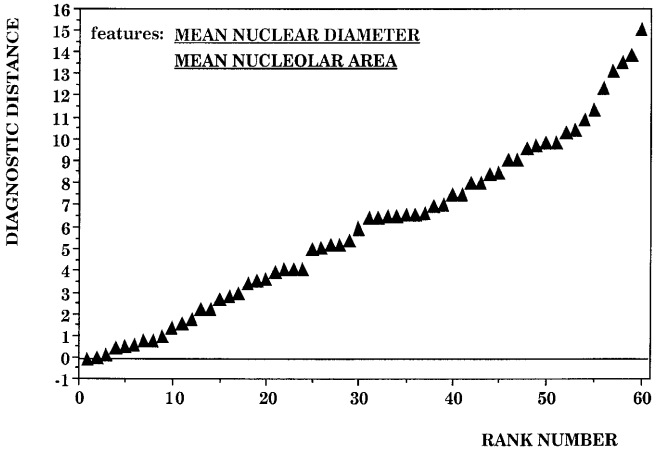

Fig. 4 Diagnostic distance of each tumour case is an index that expresses the degree of deviation of each individual cancer from its internal normal control. The values for the cancer cases studied are shown. The scale of values for features is continuous and no naturally distinguishable grades are revealed

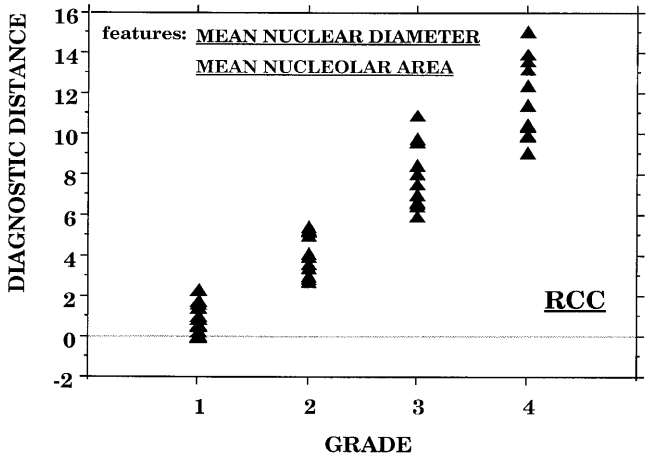

Fig. 5 Comparison between the index values and the nuclear grades, based on the Fuhrman scheme

from normal was more evident and easily identifiable. Therefore, the cases can be represented by a continuous index that corresponds to the morphological appearance of the tumours. An attempt was made to sort the cases in ascending order of severity of their morphological changes. The kind of ordering obtained with the histological examination corresponded only to some extent to the sequence obtained with the index calculation.

Figure 5 shows the degree of correspondence between the index values and the nuclear grades based on the Fuhrman scheme. The lowest index values (equal to or lower than 2.25) were observed in cases of grade I, whereas values ranging from 2.679 to 5.422 were associated with grade II. Values equal to or higher than 5.951 were seen in cases of either grade III or IV. There was no overlap between the index values in cases assigned to grade I and II or between those for grades II and III. Partial overlap was present between the index values in grades III and IV. This involved 9 cases, 3 of grade III and 6 of grade IV. The feature values of normal controls for these 9 cases were very similar. One of our group (R.Ma.) reviewed these cases blindly to see whether some differences could be seen, especially on the basis of the degree of chromatin clumping. The presence of heavy clumps of chromatin is considered to be a feature of the Fuhrman grade IV [20]. Subjectively, it was not possible to identify any morphological feature that could distinguish between any two subgroups in these 9 cases.

Attempts were made to see whether the partial overlap between the cases graded III and IV could be resolved with a different number of features in calculation of the distance from normal. When the difference related to the nuclear roundness factor was added, the index values did not show a substantial change in comparison with the previous index values based on the differences of two features. This is because the form factor has very low absolute values compared with those of the nuclear diameter and nucleolar area. Neither the inclusion of the difference of other features nor the removal of one of the two features solved the problem of the overlap. Moreover, these attempts produced a poorer correspondence between the index values and the grade of the other cases. The construction of an index based not on the differences from normal but on the original feature value also did not improve the situation.

The results presented above are related to the means of the features evaluated in each case. This was derived from the values of the individual nuclei. In each case the standard deviation was also calculated. When this was utilised in the analysis, the correspondence between the resulting distance measure and the grade was not better than that presented above (data not shown).

Discussion

Quantitative cell analyses have been applied to renal cancer pathology for diagnostic and prognostic purposes [1, 4, 8, 10, 15–18, 22, 23, 32, 39, 40, 42–46, 50, 54–56, 59, 61]. Nuclear morphometry has been used in the differential diagnosis between benign and malignant lesions as well as for an accurate distinction between different types of RCC [9, 22, 30, 51, 58, 60]. Quantitative analysis has also been adopted to predict survival in RCC. In addition, karyometry has been used to improve the type information usually derived in staging and grading RCC [11, 27]. The reasons for applying nuclear morphometry to the latter are that the degree of correlation of the various systems with survival is not satisfactory and that there is lack of observer consistency in assigning grading to RCC [11].

In most reported studies, nuclear cross-sectional area was the only karyometric feature evaluated against patient survival [13, 35, 37]. Tosi et al. [56] were among the first to investigate nuclear morphometry as a prognostic factor in RCC. They found that, using a mean nuclear area of 32 μm^2 as the decision threshold, none of

Table 2 Comparison between the nuclear diameter values in the different Fuhrman grades with those of the present morphometric study

Grade	Fuhrman Nuclear diameter	Morphometry Mean maximum nuclear diameter (μm)	
		Range	Mean
I	Approximately 10 μm	6.6–8.4	7.4
II	Approximately 15 μm	8.3–10.1	9.2
III	Approximately 20 μm	9.7–12.9	10.9
III	20 μm or larger	11.7–15.0	12.9

the short-term survivors were below that threshold and only 17% of the long-term survivors exceeded that value. Ruiz et al. [44], Eskelinen et al. [15] and Gutierrez Bannos et al. [23] have confirmed these findings. Ruiz et al. [44] found that the values of the karyometric features, except for shape factor, tend to increase in proportion to stage and that nuclear area was a significant predictor of outcome in T1-2N0M0 patients. Eskelinen et al. [15] observed that the mean nuclear area of the 10 largest nuclei was among the most important predictors of recurrence-free survival.

Other measures of nuclear size gave conflicting results, with nuclear area being shown to be either a dependent or an independent variable, when all the features were tested by multivariate analysis [11]. The maximum nuclear diameter has been found to be of independent significance. Gonzàlez-Càmpora et al. [22] demonstrated that there was a relationship between an adverse outcome and the maximum nuclear diameter and nuclear elongation. Also Ruiz-Cerda et al. [45] found that nuclear diameter was an important factor to the prediction of RCC behaviour. Monge et al. [32] sought to determine the predictive value of selective nuclear morphometry for patient outcome in RCC. They found that, in multivariate analysis carried out by the Cox method, the feature with the most predictable value related to survival was the tumour stage. The morphometric feature with the highest impact in the test was maximum nuclear diameter. In our study we found that the values of the nuclear area were highly correlated with those of the maximum nuclear diameter; the latter was preferred to the former because it corresponds to one of the diagnostic characteristics used in the Fuhrman grading system, even though there was poor correspondence of the values of the nuclear diameter in the different Fuhrman grades with those of the present study (Table 2).

Descriptors of nuclear shape have provided variable results as predictors of outcome, with estimation of elongation being correlated with survival and various outcome groups, whereas estimations of nuclear roundness and regularity proved to be of less clear prognostic importance. For instance, Pound et al. [42] and Carducci et al. [8] found that a variety of nuclear shape descriptors allowed accurate identification of those patients with an adverse outcome. Other authors have demonstrated that the measurement of nuclear shape factors is not as accurate as that of nuclear area and diameter [4, 44, 56]. For instance, Ruiz-Cerda et al. [44] found that the nuclear shape factor did not guarantee high consistency of the results. We came to the same conclusion in the present study when observer variation was investigated. The nuclear roundness factor was less reproducible than other nuclear measures. Also, consistency with the frequency of nucleolated nucleoli was not as high as with nucleolar area. The explanations for the problems with roundness factor and nucleolar frequency are that the accuracy of the former is dependent on the hand drawing of the nuclear contour with the cursor, whereas the accuracy of the latter is affected by the subjective analysis of the cells under the microscope. Our data on the nucleolar frequency are similar to those obtained by Helpap et al. in a study dealing with the nucleolar status in RCC [25]. They also found that frequency of nucleoli is correlated to the tumour grade. However, the degree of observer dependency of their analysis was not reported.

In our study it was seen that the isolated assessment of a quantitative feature does not suffice to describe the nuclear abnormalities and that the best representation of the degree of change is the combination of at least two features. This is in agreement with a conclusion reached by Barth et al. [5] in an investigation in which they showed that the correct morphometric tumour grading depends on the combination of features. Others also pointed out the need of combining more than one feature. For instance, Nativ et al. [38] performed a study on the value of nuclear morphometry for differentiating localised from metastatic RCC. They found that the mean nuclear area and nuclear regularity factor enabled accurate prediction of metastatic potential in 85% of cases. Donhuijsen et al. [14] observed that particularly favourable and unfavourable cases could be separated from average ones if the nuclear area and perimeter were evaluated simultaneously. They also observed that there is a broad distribution of values for individual cases so that no exact demarcation of prognostically different groups can be identified.

Our work has shown that the feature values represent a continuum of increasing abnormalities, and that this corresponds to the nuclear morphological changes of RCC. Part of what we do in pathology is to compare the features that we see in a particular sample with some standard or baseline in our memory. The baseline may be “normality” but is likely to differ depending on the feature we are assessing. Given a suitable baseline, we then determine how far removed the features of a given case are from that point, allowing us to determine the degree of morphological abnormality and make a diagnostic decision. However, different pathologists use different baselines in making an observation, or often their perceptions of “normality” or “abnormality” are different, leading to disagreement in feature assessment and diagnosis. In contrast, when we characterise a case by measuring a single histological feature or a number of features, we have the ability to give that case a unique posi-

tion either along a univariate axis or in multidimensional feature space. Given that we know the numerical position of some baseline within this feature space, it is convenient to express cases by their distance from this baseline. The full spectrum of potential morphological change can be represented in this fashion, and objective thresholds can be set at what are considered to be clinically relevant points along the distance scale [24].

In the current investigation the normal proximal epithelium was used as the reference point in the determination of the exact position of each individual case along the continuous spectrum of nuclear changes, the degree of abnormality being expressed by the Manhattan distance. It was found that only those cases with a low distance value are identical to the normal epithelium. Very few studies have used the comparison between RCC and the normal renal tissue for an accurate determination of the RCC status. Barth et al. [5] demonstrated that in tissue sections normal tubular epithelia and RCC grade 1 show no differences in the mean and standard deviation for the nuclear area, differences being present between the normal and the other tumour grades. Urger et al. [57] performed morphometric analysis of neoplastic renal aspirates and benign renal tissue. They used the Euclidean distance calculation (square root of the sum of the squared differences in values for each feature) to show the existence of two subgroups of RCC differing in their degree of similarity to normal. Murphy et al. [34] used the Manhattan distance measure to calculate the degree of separation between groups of RCC patients with different survival times.

In the present investigation we found that the diagnostic distance measure forms a continuous index whose values increase in proportion to the Fuhrman grade. The lowest index values were observed in the cases assigned to grade I, whereas values ranging from 2.679 to 5.422 were associated with grade II. Values equal to or higher than 5.951 were seen in the cases assigned to either grade III or grade IV. This might be interpreted as possible evidence that a three-grade system is more representative of the nuclear changes in RCC than a four-grade system. A few other papers demonstrated that the values for the estimated features show a strong tendency to increase in proportion to histological grade [6, 23, 36, 41]. In particular, Delahunt et al. [12] showed that all the nuclear quantitative features examined in their study were correlated with a three-division nuclear grading classification. Our observations are in agreement with the investigation by Gutierrez Bannos et al. [23], who combined Fuhrman grades III and IV in a study on nuclear area versus nuclear grade in the prognosis of RCC. Stoerkel and associates have proposed reducing the number of nuclear grades to three to improve the discriminatory power of grade [52].

Others adopted an approach similar to ours in an investigation dealing with image morphometric nuclear grading of intraepithelial neoplastic lesions of the breast and cervix [3]. The quantitative method resulted in a continuously scaled variable, or nuclear grading scale, expressed in standard deviation units from measured normal nuclei from breast or cervix. For a given histological preinvasive neoplastic lesion the mean nuclear grade of measured nuclei was shown to be analogous to the histopathological nuclear grade of the same lesion assigned subjectively by the pathologist.

In conclusion, the RCC cases can be represented by continuous index that corresponds to the morphological appearance of the tumours and is basically correlated with the Fuhrman scheme. This study shows that it may be very useful in supplementing the pathologist's histopathological grading by providing objective, quantitative and reproducible measures of nuclear morphometry. Quantitative studies of the nuclear chromatin texture are under way in our institute to investigate further whether the grade-III and -IV cases, especially those for which the current study showed value overlap, have distinctive patterns that are not be detected subjectively.

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