Value of quantitative nucleolar features in the preoperative cytological diagnosis of follicular neoplasias of the thyroid

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Abstract
Nucleolar prevalence, size, and outline were investigated on cytological material from cold thyroid nodules obtained by fine needle aspiration. The percentage of nucleolated nuclei in follicular adenoma (32 cases) was less than in follicular carcinoma (26 cases). In adenoma most nuclei contained one nucleolus, and nuclei with two or more nucleoli were less common than in carcinoma where most cases showed the highest nucleolar diameter values. There was some overlap between adenomas and carcinomas, however, when the mean of the 10 largest values of the major nucleolar diameter was considered. In follicular carcinoma the percentage of margined nucleoli—that is, those touching the nuclear membrane—was, in general, greater than 20%; in adenoma the values were equal to or lower than 16%.

The overlap index showed that the percentage of margined nucleoli and nucleolated nuclei are the two best discriminatory features between adenoma and carcinoma.

The role of cytopathology, and in particular fine needle aspiration biopsy, in the diagnosis of follicular lesions of the thyroid is highly controversial. Some say that it is impossible to differentiate between follicular adenoma and carcinoma; others believe that refinement of the cytological diagnosis gives the cytopathologist more confidence in identifying follicular carcinoma.1-12 For instance, Suen proposed the semiquantitative analysis of several cytological features for distinguishing groups of follicular lesions according to their probability of malignancy.13 She pointed out that the evaluation of some cell features or components, such as nucleoli, have more discriminatory value than others, such as the nuclear size and outline. Other authors also included the evaluation of nucleoli among the cytopathological features for the identification of benign and malignant follicular lesions.14-19 Because a cytological feature in one group overlaps with that in another group and because the evaluation is routinely performed on a purely subjective basis, however, the validity of the identification of follicular adenoma and follicular carcinoma has not proved to be as good as that of other thyroid carcinomas, such as the papillary, medullary, and anaplastic variants.15-14

During the past few years the quantitative analysis of cytological features has been tested to improve the accuracy of the discrimination between benign and malignant lesions.16-20 Even though there are some differences between these two broad categories, a disturbing overlap has been observed, thus reducing the potential routine application of quantitative methods to the cytological diagnosis of cold thyroid nodules, unless a discriminant analysis is applied.26

The aims of this study were (i) to quantify features related to the nucleolar prevalence, size, and margination in follicular adenoma and carcinoma using a routine microscope with an eyepiece with an incorporated horizontal micrometer; and (ii) to evaluate the importance of the nucleolar features in the differentiation of benign (adenoma) and malignant (carcinoma) lesions.

Methods
The study was carried out on cytological material from 58 cases of follicular neoplasia of the thyroid (cold nodules) obtained by fine needle aspiration biopsy. The specimens were included when at least two slides per case with a minimum of eight to 10 tissue fragments or clusters of cells obtained with multiple aspirations were available. By using a double-headed microscope, two of our team (RM and MS) reviewed the histological slides of the resected specimen and reached a common diagnosis in all the cases. In this way we were able to use the histological reports as a reference diagnosis for the 58 cases of follicular lesions: 32 follicular adenomas and 26 follicular carcinomas. Histologically the follicular carcinomas were all angioinvasive; tumours with an oxyphil appearance were not included. All smears had been wet-fixed in 95% ethyl alcohol and stained according to a hypochromic Papanicolaou staining procedure, which visualises nucleoli and outlines the distribution of chromatin. Nucleoli appear more round than chromatin particles and have a regular contour. Of the two slides available in each case, the most cellular was selected for the quantitative study.

The nucleolar prevalence was evaluated blind using a Leitz Dialux 20 EB microscope at the final microscopic magnification of 1000 times (100 times objective magnification; 10 times ocular magnification; oil immersion) by one of us (AB). The evaluation was performed on 100 nuclei adopting the vertical stratified
nucleoli touching and the nucleolar diameter; the prevalence of the bletons of follicular adenoma adopted successively by measured not stained, Poorly fixed, in method and farther the slide; from this point, parallel lines were scanned in the y direction, with each successive line farther towards the end of the slide. Poorly stained, poorly fixed, and clotted nuclei were not evaluated. To visualise all the nucleoli present in each nucleus the microscope was focused more than once so as to scan the whole nucleus in depth. The nucleolar size and outline were measured successively by the same observer who adopted the same type of selection rules and used the same microscope. The evaluations of nucleoli were performed at 1000 times magnification and the following two raw variables were determined in 100 nucleoli in areas of the slides corresponding to those in which the prevalence was evaluated: (i) major nucleolar diameter; and (ii) number of nucleoli touching the nuclear membrane. The raw diameter data were expressed in arbitrary units (au), according to which 1 corresponds to the distance between two contiguous lines in the micrometer, 2 to the distance between three contiguous lines, and so on (one au corresponds to 0.581 μm). No nucleoli with a major diameter lower than 2 au—that is, about 1 μm—were observed. In each case the time required for all the countings and measurements was about 20 minutes. Reproducibility was tested by duplicate evaluations of the raw features in eight cases (four follicular adenomas and four follicular carcinomas), showing correlation coefficients of ≥0.95. As shown by others, 100 nucleoli and 100 nucleoli represent an acceptable sample size for the evaluation of nucleolar quantitative variables. The following features were derived: Nucleolar features related to prevalence: Percentage of nuclei with nucleoli (%NNu) Percentage of nuclei with one nucleolus (%NNu1), of nuclei with two or more nucleoli (%NNu2). %NNu1 and %NNu2 were calculated in relation to the total number of nuclei (100) counted in each case. Nucleolar features related to size: Mean major nucleolar diameter (NuD; μm) Mean of the 10 largest values of the major nucleolar diameters (10 NuD; μm) Features related to the shift of the nucleoli to the periphery of the nucleus (nucleolar margination) Percentage of marginated nucleoli (%MaNu)—that is, the percentage of nucleoli touching the nuclear membrane.
The overlap index ($O_i$) was calculated for all the variables to quantify the degree of overlap between the two diagnostic categories and to identify those which permit the best differentiation of follicular adenomas from follicular carcinomas. It is a non-parametric index originally developed by Hartz\(^1\) for comparing the utility of a laboratory test. $O_i$ is calculated as follows:

(a) Combine the $n_1$ observations from sample 1 and the $n_2$ observations from sample 2, and then rank all the observations from the smallest to the largest.

(b) For tied observations, first assign raw ranks to the tied values as if they were not tied. The corrected rank is the average of the raw ranks of the tied numbers.

(c) Add ranks for one of the samples, such as sample 1. Generally it is more convenient to add ranks for the smaller size sample. Call it the sum of the ranks $T_i$.

(d) Calculate $T_i = n_1 + (n_1 + n_2 + 1)/2$. $T_i = T_j$ when the medians of the two samples are the same—that is, when there is complete overlap.

(e) Calculate $O_i = 1 - \left| \frac{2(T_i - T_j)}{n_1 n_2} \right|$. The value of $O_i$ ranges from zero if there is no overlap to one if the observations from the two samples have the same medians.

The sum of the ranks was obtained using a Macintosh II computer whose statistical package contains a program for the Mann-Whitney U test.

Results

As for the nucleolar features related to prevalence, the follicular adenomas had mean category values lower than those of carcinoma. In follicular adenoma, out of 48-34\% (SD 17-39\%; median 44-0\%; 95\% confidence interval, 42-07 to 54-61\%) nucleolated nuclei, %NNu1 was 40-90\% (SD 13-45\%; median 39-0\%; 95\% CI 36-05 to 45-75\%) and %NNu2 was 7-15\% (SD 5-06\%; median 6-0\%; 95\% CI 5-48 to 9-39\%). In follicular carcinoma, out of 73-61\% (SD 15-71\%; median 77-0\%; 95\% CI 67-26 to 79-96\%) nucleolated nuclei, 59-61\% (SD 15-71\%; median 59-0\%; 95\% CI 54-18 to 65-11\%) and 12-76\% (SD 7-62\%; median 13-0\%; 95\% CI 10-51 to 17-41\%) of nuclei were mononucleolated and binucleolated or more, respectively (fig 1).

As for the mean major nucleolar diameter, the mean follicular adenoma category value was 1-824 μm (SD 0-174 μm; median 1-853 μm; 95\% CI 1.761 to 1.887 μm)—that is, lower than in follicular carcinoma (mean 2-140 μm; SD 0-299 μm; median 2-045 μm; 95\% CI 2-02 to 2-261 μm). Similarly, in follicular adenoma the mean of the 10 largest values of the major nucleolar diameter was 2-553 μm (SD 0-226 μm; median 2-5 μm; 95\% CI 2-472 to 2-635 μm)—that is, lower than in follicular carcinoma.

- Figure 2: Scattergram to show the mean of the major nucleolar diameter ($A$) and the mean of the 10 largest values of the major nucleolar diameter ($B$) for each case examined. $O_i$ stands for overlap index and indicates the degree of overlap between follicular adenomas and follicular carcinomas. The lowest $O_i$ value was obtained for the major nucleolar diameter. Horizontal bars indicate the median for each group.

- Figure 3: Scattergram to show the percentage of margined nucleoli for each case examined. $O_i$ stands for overlap index and indicates the degree of overlap between follicular adenomas and follicular carcinomas. The $O_i$ was the lowest of any of the features investigated. Horizontal bars indicate the median for each group.
Figure 4 Scatterplot of the two variables with the smallest O—that is, %MaNu and %NuN. The continuous line present on the plane of the graph was drawn so as to separate cases of follicular adenomas from follicular carcinomas as much as possible. One carcinoma (encircled on the scatterplot) is included among the follicular adenomas; three adenomas (arrowed) fall among the carcinomas.

(mean 2.927 μm; SD 0.371; median 2.92 μm; 95% CI 2.777 to 3.077 μm) (fig 2).

As for the percentage of marginated nucleoli, the mean category value obtained in follicular carcinoma was 23.23% (SD 3.97%; median 24.0%; 95% CI 21.62 to 24.83%)—that is, higher than in follicular adenoma, in which the mean was 15.95% (SD 4.33%; median 16.0%; 95% CI 14.03 to 17.15%) (fig 3).

Even though values related to prevalence, size, and margination were in general greater in follicular carcinoma than in follicular adenoma, there was no clear-cut separation between cases of follicular adenoma and follicular carcinoma on the basis of single feature analysis. Therefore, the overlap index was applied to identify the features with the smallest overlap. The test showed that the O value of the percentage of marginated nucleoli and of the percentage of nucleolated nuclei are the smallest—that is, 0.20 and 0.29, respectively. For the other features the O values were as follows: %NNu1 and NuD: 0.35; 10 HNuD: 0.39; %NNu2: 0.50. The scatterplot of the two variables with the smallest O—that is, %MaNu and %NuN (Spearman's rank correlation coefficient 0.38, p < 0.01) is shown in fig 4. The continuous line present on the plane of the graph was arbitrarily drawn to separate cases of follicular adenoma from follicular carcinoma as much as possible. One out of 26 carcinomas (encircled on the scatterplot) however, is included among the follicular adenomas, with three out of 32 adenomas (arrowed) among the carcinomas.

Discussion
Our quantitative study of nucleolar features on cytological preparations shows that prevalence, size, and outline are generally greater in follicular carcinoma than in follicular adenoma, and that good distinction between adenomas and carcinomas is achieved with the two variables having the smallest overlap index value—that is, the percentage of marginated nucleoli and the percentage of nucleolated nuclei.

As for the nucleolar prevalence, two subgroups of qualitative terms are used in cytopathology practice.7-11 The first, which corresponds to our feature “percentage of nucleolated nuclei,” includes terms such as “nucleoli absent, infrequent, inconsistent, always present.” The other, which corresponds to the percentages of nuclei with one, two, or more nucleoli, includes terms such as “single or multiple.” According to some authors,7-11 both subgroups are important in distinguishing follicular lesions of various types. In benign cases, in fact, nucleoli are in general infrequent and single; in carcinoma they are always present and multiple. In a previous paper we analysed quantitatively the nucleolar prevalence in benign and malignant thyroid neoplasias of several types and we found that nucleoli in the carcinoma groups are frequently nucleolated with percentages of two or more nuclei greater than in non-cancer groups.25 It was also observed that features associated with nucleolar prevalence have more discriminatory value than nuclear features, including the integrated optical density or DNA content. Van Diest et al investigated the prognostic value of quantitative nucleolar features in cytological material from breast cancers.30 Their results also suggested that nucleolar morphometric variables have a discrimination value exceeding that of nuclear variables and that the number of nucleoli per 100 nuclei was the best single variable.

As for nucleolar size, qualitative terms whose use in subjective cytological evaluation has been suggested,7-11 are “small, hypertrophic, prominent nucleoli, macronucleoli.” Small nucleoli are currently observed in benign nodules; hypertrophic, prominent nucleoli (macronucleoli) are a common feature of malignant cases. Our features related to the nucleolar diameter quantified this observation. In particular, the mean of the 10 largest values of the major nucleolar diameter, selected on the basis of the results of the study by Huntington et al32 in which the malignancy potential of 100 intraocular melanomas was assessed with a micrometer technique, can be considered the quantitative equivalent of prominence. Similarly, according to Kelemen et al's study to identify nucleolar prominence as a diagnostic variable in prostatic carcinoma,33 “nucleoli appeared to be prominent... when the largest
Nucleoli in thyroid follicular neoplasia

Nucleoli in the peripheral position included the not malignant cases. In particular, the production of RNA in the nucleoli and the cytoplasmic demand for RNA was regarded as an expression of "the level of production of RNA in the nucleolus and the cytoplastic demand for RNA." In our experience, the overlap between benign and malignant cases was larger than with the other "nucleolar" features. In a study on cells obtained from paraffin wax embedded material, the eccentricity—that is, a feature associated with location, was not helpful in cell classification. We do not have a definitive explanation for the discrepancy with our results, because the formula for the calculation of the eccentricity was not given and it was not specified whether the variable was measured in round as well as non-round nuclei. The percentage of margined nuclei, in our opinion, however, is a feature which is easy to evaluate in all nuclei without taking into consideration the prerequisite for nucleolar eccentricity—that is, that the nucleus has to be round. Our data on nucleolar location are similar to those obtained on cytological specimens of hyperplastic and neoplasic prostatic lesions by Helpap; the percentage of peripherally located nuclei in carcinoma was greater than in benign cases. We have not investigated the proportion of nucleoli in the central and intermediate positions. On a purely subjective basis, however, we observed that in benign thyroid lesions most of the nucleoli are not centrally located as in the prostate. This observation had yet to be confirmed quantitatively.

The overlap index adopted in our study helped us to identify the two nucleolar features which show the smallest overlap. Their combination in a scatterplot gives an idea of the spatial distribution of the cases and of the good differentiation of follicular adenomas from follicular carcinomas. On the division made by the continuous line drawn on the graph (fig 4), a case of carcinoma is included among the adenomas. The corresponding histological slides were examined again. It was observed that, from the morphological point of view, the case was not homogeneous, but showed areas with well-formed follicles, similar to those of thyroid adenomas, together with areas having a trabecular pattern. Three adenomas fell in the part of the graph plane occupied by the carcinomas. Their prevalent histological architecture was that of a microfollicular or trabecular (cellular) pattern, as generally observed in our cases of follicular carcinomas.

In conclusion, our results agree with the concept that nucleolar changes may be characteristic of cancer cells in general and with Baroggi et al's proposal that nucleolar abnormalities should be used in the identification of cancer cells. These changes, even though not specific for neoplasia, have been regarded as an expression of "the level of production of RNA in the nucleolus and the cytoplasmic demand for RNA". In particular, regarding nucleolar margination, Oberling and Bernhard stated that "one can assume that transportation of nucleolar products towards the cytoplasm is highly facilitated by this arrangement." From the practical point of view, values for all features were greater in carcinoma than in adenoma. Nevertheless, the overlap between the two groups, even when data are combined, seems to limit an immediate diagnostic application for the technique. Studies are needed to identify either further cytological quantitative features or other multivariate analyses to avoid the overlap.

12. Suen KC, Quenville NF. Fine needle aspiration biopsy c
13 Sun HC. How does one separate cellular follicular lesions of the thyroid by fine needle-aspiration biopsy? Diagn Cytopathol 1988;4:78-81.