

Research Article

Differentiation between Papillary Carcinoma and Adenomatous Nodule using Three-dimensional Nuclear Analysis is Useful for Morphologically Indeterminate Cases of Thyroid Tumor

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Abstract

Six nuclear parameters (number of focus layers, pixel count, three-dimensional luminance coefficient variation (3D-CV), roundness, intranuclear inclusion, and nuclear groove) were measured to examine the usefulness of discriminant analysis between papillary carcinoma of the thyroid (Papillary ca) or adenomatous nodule/goiter (A. nodule) using Mahalanobis distance.

Forty-two of 442 indeterminate specimens were histologically diagnosed with Papillary ca or A. nodule. Six nuclear parameters for each specimen were examined to conduct discriminant analysis between papillary ca and A. nodule using Mahalanobis distance.

The number of nuclear focus layers, pixel count, and intranuclear inclusion [×10⁻¹] of Papillary ca were higher than those of A. nodule (3.91 ± 0.44 , 888 ± 257 , and 2.2 ± 4.2 vs. 3.59 ± 0.54 , 785 ± 111 , and 1.3 ± 3.5). The 3D-CV [×10⁻³], roundness [×10⁻¹], and nuclear groove [×10⁻¹] of Papillary ca were lower than those of A. nodule (12.1 ± 5.7, 8.464 ± 0.144, and 2.2 ± 4.2 vs. 15.1 ± 5.2, 8.602 ± 0.078, and 3.3 ± 4.9). Positive discrimination rates of each specimen were 96.3% and 73.3% for Papillary ca and A. nodule, respectively.

The discriminant analysis for each specimen is reasonable and useful for providing high discrimination rates.

Keywords: Thyroid cytology; Indeterminate cases; Papillary carcinoma; Adenomatous nodule; Three-dimensional analysis; Discriminant analysis

Introduction

Papillary ca is the most common thyroid tumor, accounting for the majority (over 90%) of malignant thyroid tumors. Papillary ca shows characteristic findings, such as (1) ground-glass nuclei, (2) nuclear cytoplasmic inclusion, and (3) nuclear groove [1,2], although its cellular atypia is milder than those of cancers derived from other organs. Indeterminate tumors according to the cytological report of The Bethesda System for Reporting Thyroid Cytopathology (BSRTC) [3] include Papillary ca and adenomatous nodule/goiter (A. nodule), whose differential diagnosis is so difficult that it poses a risk of overdiagnosis. Papillary ca shows mild nuclear atypia and often appears through the formation of a flat sheet-like mass. Thus, the differential diagnosis of malignancy is difficult when characteristic findings, such as nuclear cytoplasmic inclusion and nuclear groove, are unclear. In cases of A. nodule, the follicular epithelium occasionally develops hyperplasia with enlarged nuclei, papillary arrangement, and a layered structure, precluding differentiation from Papillary ca. Thus, in patients whose differential diagnosis between Papillary ca and A. nodule is difficult, cells were three-dimensionally measured in order to develop a method for making an objective judgment. A discriminant analysis using Mahalanobis distance was conducted on the basis of histological diagnosis.

Materials and Methods

Samples

In 2011, 6735 patients underwent fine-needle aspiration biopsy of the thyroid at Ito hospital (Table 1). At most institutions in Japan, diagnoses are made according to the Thyroid Handling Criteria 6th Edition. The criteria are based on the BSRTC. According to our criteria, the number of inadequate cases was as low as 185 (2.7%), with 4,685 benign cases (69.6%), 442 indeterminate cases (6.6%), 164 malignancysuspected cases (2.4%), and 1,259 malignant cases (18.7%). Of the 442 indeterminate cases, patients diagnosed with Atypia of Undetermined Significance or Follicular Lesion of Undetermined Significance (AUS/ FLUS) was selected. Finally, 38 patients (42 specimens) histologically diagnosed with Papillary ca or A. nodule were included. Cell specimens with eosinophilic changes, nuclear overlapping, and artifacts (denaturation, drying, and strong staining) were excluded. At first, the

Cytology	Specimen	%
Inadequate	185	2.7
Benign	4,685	69.6
Indeterminate	442	6.6
Malignancy suspected	164	2.4
Malignant	1,259	18.7
Total	6735	

Table 1: Fine-needle aspiration biopsy of the thyroid (2011).

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Received October 18, 2013; Accepted November 06, 2013; Published November 08, 2013

Citation: Washiya K, Furutate S, Sakurai M, Sasaki E, Yoshioka H, et al. (2013) Differentiation between Papillary Carcinoma and Adenomatous Nodule using Three-dimensional Nuclear Analysis is Useful for Morphologically Indeterminate Cases of Thyroid Tumor. J Cytol Histol 4: 194. doi: 10.4172/2157-7099.1000194

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examiner photographed atypical cells in the cytological specimen while blinded to the histological results. From the images, four collaborators selected 705 cells (mean: 16-17 cells/specimen), determined to be appropriate for measurement according to the BSRTC criteria (Table 2). After their nuclei were measured and the discriminate analysis of malignancy was carried out, histological diagnosis was confirmed. They included 23 cases (27 specimens) of Papillary ca and 15 cases (15 specimens) of A. nodule. For fine-needle aspiration cytology, direct smear specimens were subjected to superimposition.

Imaging/Measurement/Analysis/Discrimination

This study was conducted as previously reported [4-6]. The measurement, analysis, and discrimination are summarized below.

A total of 41 three-dimensional images were photographed per nucleus using a virtual slide imager equipped with a 3CCD digital camera, with a 40x objective lens focused on the center of the nuclei in cytological specimens and 20 images automatically taken with movements up and down along the Z-axis by 0.25 μ m. Measurements were conducted using Photoshop[®] CS5. The following six nuclear parameters were analyzed:

- 1) Pixel count (nuclear area)
- 2) Number of focus layers (three-dimensional structures of nuclei): The number of images with nuclei microscopically and morphologically focused was determined from the 41 layer images per nucleus; a larger number of images indicated more three-dimensional nuclei.
- 3) Three-dimensional luminance coefficient variation (3D-CV): The nuclear luminance CV values of focus layer images were determined to calculate the standard deviation (SD) of CV differences between the layers. This was referred to as 3D-CV, an index that reflects the three-dimensional distribution of nuclear chromatin.
- 4) Roundness (irregular nuclear image): Roundness was determined as 4π (area/outer perimeter²), which indicated how close the measured nucleus was to a perfect circle; 1.0=a complete circle and values closer to 0.0= a more irregular circle (nucleus)
- 5) Intranuclear inclusion: Specimens with intranuclear inclusion were determined as 1, while those without intranuclear inclusion were determined as 0.

Histology	Specimen	Cell count
Papillary ca	27	354
A. nodule	15	351
Total	42	705

Table 2: Test (AUS/FLUS) specimens.

6) Nuclear groove: Specimens with a nuclear groove were determined as 1, while those without a nuclear groove were determined as 0.

Normality (Shapiro-Wilk test) and homoscedasticity (Levene test) were tested from these measurements. A significant difference test (Mann-Whitney's U or Welch test) was conducted. In addition, cells with nuclear atypia were differentiated into Papillary ca and A. nodule, as described below. The box M test of statistical analysis software SPSS® ver.16 demonstrated no homoscedasticity for the two groups (Papillary ca and A. nodule). Thus, to determine variables indicating differences in variance and covariance, Mahalanobis distance was employed to identify a group closer to the center of the group. Mahalanobis distances were determined for the nuclei to be analyzed, in order to discriminate groups closer to the center of the Papillary ca or A. nodule group. A discriminant analysis was conducted by: (1) determining Mahalanobis distances for 705 nuclei using four parameters (number of focus layers, pixel count, 3D-CV, and roundness) in order to discriminate groups closer to either of the groups, and (2) determining the mean values of six nuclear parameters (number of focus layers, pixel count, 3D-CV, roundness, intranuclear inclusion, and nuclear groove) for each specimen for discrimination. Examples of calculating Mahalanobis distances are shown below in Tables 3 and 4.

Results

Indeterminate cases should comprise less than 20% of appropriate cases according to the criteria. Of the 6,550 patients who underwent fine-needle aspiration cytology of the thyroid, 442 (6.74%) were indeterminate, maintaining a high diagnostic rate. The mean values \pm SD of Papillary ca and A. nodule, determined for each nucleus using the four parameters, are shown in Table 5. The number of nuclear focus layers was higher for Papillary ca (3.94 ± 0.91) than for A. nodule (3.62 ± 0.77). The nuclear pixel count was higher for Papillary ca (912 ± 352) than for A. nodule (810 ± 179). The nuclear roundness × 10^{-1} was lower for Papillary ca (8.502 ± 0.294) than for A. nodule (8.593 ± 0.276). These three parameters were significantly different between Papillary ca (12.8 ± 9.6) than for A. nodule (15.0 ± 14.7) but the difference was not significant.

Subsequently, the mean values \pm SD of Papillary ca and A. nodule, determined for each specimen using the six nuclear parameters, were compared (Table 6). The number of nuclear focus layers was higher for Papillary ca (3.91 \pm 0.44) than for A. nodule (3.59 \pm 0.54). The nuclear roundness \times 10⁻¹ was lower for Papillary ca (8.464 \pm 0.144) than for A. nodule (8.602 \pm 0.078). These two parameters were significantly different between Papillary ca and A nodule (p<0.05). The nuclear pixel count was higher for Papillary ca (888 \pm 257) than for A. nodule

(F-3.94 P-912 S-0.850 D-0.01278)	1.21369592	5.0812E-05	3.3512389	3.728176228	F-3.94
	5.0812E-05	9.3876E-06	0.10140288	0.020978229	P-912
	3.3512389	0.10140288	12248.9773	-425.4763702	S-0.850
	3.72817623	0.02097823	-425.47637	1256.386344	D-0.01278

Table 3: Mahalanobis distance to the center of the Papillary ca group (Papillary ca distance).

(F-3.61 P-810 S-0.859 D-0.01497)	1.75203718	-0.0001035	-13.64993	1.90911238	F-3.61
	-0.0001035	3.3641E-05	0.08338937	0.04126653	P-810
	-13.64993	0.08338937	4964.76779	19.233331	S-0.859
	1.90911238	0.04126653	19.233331	1369.63136	D-0.01497

*F: Number of focus layers, P: Pixel count, S: Roundness, D: 3D-CV.

Table 4: Mahalanobis distance to the center of the A. nodule group (A. nodule distance).

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	Number of focus layers ± SD	Pixel count ± SD	3D-CV×10-3 ± SD	Round	ness × 10 ⁻¹ ± SD
Papillary ca	3.94 ± 0.91 *1	912 ± 352 _*2	12.8 ± 9.6	8.502 ± 0.294]*3t
A. nodule	3.62 ± 0.77	810 ± 179	15.0±14.7	8.593 ± 0.276	

*1, 2, 3 Significantly different for the three parameters (p<0.05)

Table 5: Mean ± SD of four parameters for each nuclei of Papillary ca and A. nodule.

	Number of foc	us layers ± SD	Pixel count ± SD	3D-CV×10 ⁻³ ± SD	Roundness >	× 10⁻¹ ± SD	Intranuclear Inclusion × 10 ⁻¹ ± SD	Nuclear groove×10 ⁻¹ ± SD
Papillary ca	3 3.91 ± 0.44	<u>]</u> *1	888 ± 257	12.1 ± 5.7	8.464 ± 0.144	*2	2.2 ± 4.2	2.2 ± 4.2
A. nodule	3.59 ± 0.54		785 ± 111	15.1 ± 5.2	8.602 ± 0.078		1.3 ± 3.5	3.3 ± 4.9

*1, 2 Significantly different for the two parameters (p<0.05)

Table 6: Mean ± SD of the parameters for each specimens of Papillary ca and A. nodule.

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 (785 ± 111) . The intranuclear inclusion $\times 10^{-1}$ was higher for Papillary ca (2.2 ± 4.2; 6 of 27 specimens) than for A. nodule (1.3 ± 3.5; 2 of 15 specimens). The 3D-CV $\times 10^{-3}$ was lower for Papillary ca (12.1 ± 5.7) than for A. nodule (15.1 ± 5.2). The nuclear groove $\times 10^{-1}$ was lower for Papillary ca (2.2 ± 4.2; 6 of 27 specimens) than for A. nodule (3.3 ± 4.9; 5 of 15 specimens). But these differences were not significant.

The results of discriminant analysis are shown below:

(1) Results of discriminant analysis by each nucleus

The results of discriminant analyses of Papillary ca and A. nodule for 705 nuclei are shown in Tables 7 and 8, respectively. Discrimination rates were 64.4 and 54.4% for Papillary ca and A. nodule, respectively.

(2) The results of discriminant analyses for each specimen using the six parameters are shown in Tables 9 and 10. Discrimination rates were as high as 96.3% for Papillary ca and 73.3% for A. nodule. The diagnostic accuracy for A. nodule was also high considering that cytologically indistinguishable nuclei were measured.

Discussion

First, cell images by nucleus were analyzed using the four parameters. The mean pixel count \pm SD and number of layers \pm SD of Papillary ca were higher than those of A. nodule (Table 5), demonstrating an enlarged, irregular, three-dimensional nuclear structure for Papillary ca. These results are consistent with previous reports that DNA and the nuclear protein and size increase in malignant cells [7-10]. Also, cases of A. nodule may show enlarged nuclei, but have fewer focus layers and less three-dimensional structures. In addition, the nuclei are filled with larger amounts of euchromatin (fine chromatin condensation) as nuclear proliferation increases in malignant cells [11]. The nuclear 3D-CV of Papillary ca is lower than that of A. nodule (Table 5). The nuclear chromatin of Papillary ca shows a less variable three-dimensional structure and is filled with fine chromatin. The roundness of Papillary ca is lower than that of A. nodule, suggesting more circular nuclei in A. nodule.

The discriminant analysis of each nucleus using Mahalanobis distance yielded discrimination rates of 64.4% for Papillary ca and 54.4% for A. nodule. These are not high enough, suggesting that it was extremely difficult to morphologically determine the malignancy of each nucleus that appears in an indeterminate cytological specimen.

Thus, intranuclear inclusion and nuclear groove were examined for each specimen. The six nuclear parameters, including the above two, were employed to conduct an analysis of each specimen. Intranuclear inclusion was more frequently detected in Papillary ca, while a nuclear groove was more frequently detected in A. nodule (Table 6). The nuclear groove results may be explained by the fact that cytologically indeterminate cases were included as subjects in this study. The discrimination rate of Papillary ca using Mahalanobis distance was as high as 96.3% (Table 9). The discrimination rate of A. nodule was 73.3%, a higher value than that by nucleus (Table 10). A cytological diagnosis was not made for each cell, but was comprehensively made by observing whole cells for each specimen. Thus, the discriminant analyses for each specimen were reasonable and yielded high discrimination rates. The mean number of layers, pixel count, 3D-CV, and roundness for each specimen showed the same tendencies as presented in Tables 5 and 6.

The cell images were examined based on the above results. Figure 1 shows the cell images of specimens which were cytologically indeterminate, but were histologically diagnosed as Papillary ca. Cell aggregates diagnosed as Papillary ca by both histology and discriminant analysis (Figure 1a) were compared with those diagnosed as Papillary ca by histology and as A. nodule by discriminant analysis (Figure 1b), demonstrating that Figure 1a shows a larger mean number of nuclear layers, higher pixel count, and lower 3D-CV. This verifies that Papillary ca shows spherical nuclei and densely distributed fine chromatin particles (powder). Figure 1b shows a cell image (only one specimen) in which the morphological diagnosis of Papillary ca was difficult, resulting in misdiagnosis as A. nodule by discriminant analysis. Thus, a whole specimen, as well as partial images, should be examined when making a

	Nucleus	Discrimination rate
Papillary ca	228	64.4
A. nodule	126	35.6

Table 7: Discrimination of Papillary ca.

	Nucleus	Discrimination rate
pillary ca	160	45.6
nodule	191	54.4

Table 8: Discrimination of A. nodule.

	Specimen	Discrimination rate
Papillary ca	26	96.3
A. nodule	1	3.7

Table 9: Discrimination of Papillary ca.

	Specimen	Discrimination rate
Papillary ca	4	26.7
A. nodule	11	73.3

Table 10: Discrimination of A. nodule.



Figure 1: Histology: Indeterminate cell images of Papillary ca. a: Discriminant analysis: Papillary ca Spherical nuclei with densely distributed fine chromatin fine particles (powder). b: Discriminant analysis: A. nodule flat nuclei with weak staining, indistinguishable from Papillary ca. c: Discriminant analysis: Papillary ca intranuclear inclusion diagnosed as Papillary ca by nuclear parameter analysis. d: Discriminant analysis: Papillary ca by nuclear parameter analysis. d: Discriminant analysis: Papillary ca by nuclear parameter analysis. d: Discriminant analysis: Papillary ca Nuclear groove, Diagnosed as Papillary ca by nuclear parameter analysis.

diagnosis. The cell aggregates with the intranuclear inclusion (Figure 1c) and clear nuclear groove (Figure 1d), diagnosed as Papillary ca by histology, were also diagnosed as Papillary ca by discriminant analysis.

Figure 2 shows the cell images of specimens which are indeterminate by cytology, but were diagnosed as A. nodule by histology. They could be diagnosed as A. nodule in Figure 2a, c, and d, but not in Figure 2b, e, and f. The pixel counts and mean number of layers in Figure 2a, c, and d were lower than those of Papillary ca, while the 3D-CV and roundness were higher than those of Papillary ca, resulting in the correct diagnosis of A. nodule. Thus, the nuclei of A. nodule were smaller and less spherical than those of Papillary ca and showed no chromatin condensation. The intranuclear inclusions that appeared in A. nodule (Figure 2c) showed small and slightly unclear images. The intranuclear cytoplasmic inclusions suggesting Papillary ca should meet the following criteria: (1) clear boundary, (2) smoothness inside and chromatin condensation outside the boundary, (3) the same color as the cytoplasm, and (4) >10% occupancy of nuclear size [12]. The inclusions as shown in Figure 2c are considered to be pseudo-inclusions because they do not meet the above criteria.

Figure 2b shows a cell image with nuclear irregularity, light and shade, and nuclear groove, which preclude morphological diagnosis. Of the cases of A. nodule, four specimens showed intranuclear pseudoinclusions with partially unclear boundaries (Figure 2e), a nuclear groove (Figure 2f), and aggregates whose mean number of layers and 3D-CV were equivalent to those of Papillary ca. These were misdiagnosed as Papillary ca even by discriminant analysis. Specimens abundant in such cells should be carefully examined as indeterminate cases.

Changes in the nuclear chromatin structures of cancer cells are mainly caused by epigenetic changes [13,14]. DNA methylation is one

of the most stable epigenetic changes [15]. DNA methylation is involved in chromosome stabilization and chromatin structuring, as well as gene expression regulation, through modification by the covalent binding of a methyl group to cytosine in CpG island sequences in the genome [15]. Electron microscopic observations of the nuclear chromatin of cancer cells demonstrate that euchromatin increases along with their proliferative activities. The ground-glass nuclei of Papillary ca show heterochromatin eccentrically located near the nuclear membrane and a clear appearance with increased euchromatin on the whole. The threedimensional parameter analysis of nuclei using optical microscopic images in the present study demonstrated that the nuclei of Papillary ca were filled with fine chromatin with little variation in their threedimensional structures. DNA methylation occurs at an earlier stage of the multistage carcinogenesis than genetic abnormalities [15,16]. DNA methylation abnormalities that determine malignancy and the prognosis of cancer patients have already been accumulated [17,18]. DNA methylation is stable and less susceptible to denaturation than RNA and protein expression [19]. Thus, DNA methylation may be applicable for analyses of morphological changes.

Cytological specimens dehydrated and fixed with 95% alcohol maintain nucleic acids and proteins without change, providing more stable storage conditions than formalin-fixed pathological specimens [20]. Our method allows analyses with Papanicolaou-stained specimens prepared in routine clinical practice, providing the advantage of being able to use many previous specimens. The staining properties of cytological specimens vary with conditions. However, our method employs parameters unaffected by different staining properties [4], thus providing a useful tool for objective analyses of nuclear chromatin.

Only a few reports have been published on the objective analyses



Figure 2: Histology: Indeterminate cell images of A. nodule. a: Discriminant analysis: A. nodule small, nonspherical nuclei no chromatin condensation. b: Discriminant analysis: Papillary ca indistinguishable cell image with irregular nuclei, light and shade, and nuclear grove. c: Discriminant analysis: A. nodule nuclear pseudo-inclusion small, unclear intranuclear inclusion, not suggesting Papillary ca. d: Discriminant analysis: A. nodule nuclear grove flat nuclei with weak staining and mild atypia. e: Discriminant analysis: Papillary ca intranuclear inclusion with partially unclear boundary Indistinguishable aggregates whose three-dimensional nuclear structure and 3D-CV are similar to those of Papillary ca.

of histological or cytological images of thyroid diseases. Salmon et al. investigated Feulgen-stained tissue specimens of follicular tumors and hyperplastic nodules regarding the DNA contents and chromatin texture, reporting a significant decrease in chromatin condensation and heterogeneity from normal to neoplastic thyroid tissue was observed [21]. Tseleni-Balafouta et al. conducted a nuclear morphometry analysis using fine needle aspiration (FNA) smears of follicular tumors and A. nodules, reporting significant differences were found between carcinomas and hyperplastic nodules, as well as between carcinomas and adenomas for both nuclear morphometric variables. No significant differences were found between the nuclear variables in either hyperplastic nodules or adenomas [22]. Priya and Sundaram performed an evaluation using analysis of variance (ANOVA) [23], demonstrating the follicular carcinomas had a higher largest-to-smallest dimension ratio than patients with adenomatous goiters, but not the difference between Papillary ca and A. nodule. Murata et al. conducted digital image analysis using Image processor (PIP-4000, ADS), reporting that the nuclei of Papillary ca showed a larger size, more irregular shape, and higher contrast of the chromatin pattern than those of the benign group [24]. These results show the differences in the nuclei that appear in Papillary ca. Thus, it remains unclear whether the finding of a higher contrast of chromatin suggests the characteristics of groundglass nuclei. In addition, the above four reports differ in that the cases included those other than indeterminate ones and the nuclei were not three-dimensionally analyzed. Our method is effective in that it allows three-dimensional and objective analyses using nuclear parameters unaffected by the staining properties of specimens.

The clinical features, biological properties, and malignancy of thyroid

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tumors vary with tissue types. Thus, the histopathology of a thyroid tumor before an operation is indispensable for conducting appropriate treatment. For this purpose, a combination of ultrasonography and fine-needle aspiration cytology is the most effective [25]. In the future, morphologically indeterminate cases will be prospectively examined, and the differential diagnosis of follicular neoplasm will be further investigated.

Acknowledgment

This study was supported by a grant for Hirosaki UniversityInstitutional Research (2013).

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