Cancer Cytopathology

Biliary cytology: a diagnostic tree for adenocarcinoma based on a cohort of 135 patients with Endoscopic Retrograde Cholangio-Pancreatography for stenosis of the extrahepatic bile duct

Journal:	*Cancer Cytopathology*
Manuscript ID	CNCY-21-0329.R1
Wiley - Manuscript type:	Original Article
Date Submitted by the Author:	13-Jan-2022
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Keywords:	bile, cytology, cytomorphological criteria, diagnostic tree, atlas

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Short running title: Diagnostic tree and atlas of bile

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Funding statement

No outside funding was disclosed.

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Acknowledgements

We thank Catherine Crozet for her excellent technical support.

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Diagnostic tree for malignant biliary stenosis and a biliary cytology learning atlas of 24 general cytological criteria.

Abstract

Introduction: The diagnosis of well-differentiated adenocarcinoma on bile aspiration is a well-known challenge. Our study aimed to improve diagnostic performance and to provide a biliary cytology learning atlas.

Methods: In this context, our monocentric retrospective study included 135 cases of informative biliary samples collected between 2009 and 2018, classified as benign, atypical or malignant. A double assessment was carried out by a novice and an experienced cytopathologist in biliary cytology to establish the specificities, sensitivities and the inter- and intra-observer kappa indices agreements of 24 cytological criteria, illustrated in a learning atlas.

Results: A multivariate logistic regression was used to assess if the most specific and reproducible criteria were associated with malignancy. A scoring system was statistically determined: 6 points were attributed in the presence of 3 dimensional cluster, anisonucleosis and nuclear to cytoplasmic ratio > 0.5 whereas 4 points were given in the presence of enhanced nuclear membrane. A score higher than 10 points resulted in a malignancy diagnosis with 96% sensitivity and 97% specificity.

Conclusion: Our diagnostic tree of malignancy based on 4 criteria allowed, together with a multidisciplinary approach, the diagnosis of adenocarcinoma with a 100% specificity and a sensitivity of respectively 88% or 72% depending on the presence of “single malignant cell”, or on the presence of 3 combined criteria (“3D-cluster, anisonucleosis and a N/C ratio > 0.5”). It comes with a learning atlas useful for the training and accuracy of cytopathologists in this uncommon cytology.

Keywords: Bile, cytology, cytomorphological criteria, diagnostic tree, atlas

INTRODUCTION

Pancreatic ductal adenocarcinoma and cholangiocarcinoma of the biliary tract are the two most common causes of malignant bile duct strictures, whose incidence has increased in the last 30 years, [1] with a global incidence rate of 7.7 to 11 and 1.4 to 2.1 per 100,000 person-years respectively. [2]

A suspicion of malignant stenosis of the extrahepatic bile duct leads patients to undergo ERCP (Endoscopic Retrograde Cholangiopancreatography) for diagnostic purposes and, if possible, therapy by the placement of a biliary prosthesis. The specificity of the cytological examination of these biliary samples is remarkably high but reported sensitivities for the diagnosis of malignancy are variable. [3-6] As a result, cytological analysis of bile is very useful to assess the diagnosis of pancreatobiliary carcinoma, enabling early and appropriate management, without performing a new invasive procedure. However, the cytological examination of bile is a challenge for the cytopathologist, who must be aware of diagnostic pitfalls such as false positives (Primary Sclerosing Cholangitis (PSC), stent-related atypia) or false negatives (samples with low cell-counts or very well-differentiated adenocarcinomas).[7-9]

Numerous cytological criteria have been proposed on biliary brush samples[10,11] but, to our knowledge, only three studies have performed multivariate analyses of malignant cytological criteria on biliary aspiration samples, which are often less crushed and just as cellular as brush specimens.[12,15,16] These three studies, each performed by 3 experienced cytopathologists, showed variable specificities (between 65 and 86.4%) and variable sensitivities (between 76.9 and 98%) and a proportion of well-differentiated adenocarcinomas of 43% and 63% respectively for 2 out of the 3 studies.[12,15] Since cytological examination of biliary aspiration is not a common practice worldwide, our study aimed to define specific, sensitive and reproducible cytological criteria for malignancy on bile aspiration samples by both a novice and a 5 years experienced cytopathologist in biliary cytology and was designed for training

purposes. Our first goal was to propose a diagnostic tree to help in diagnosis of extra-hepatic bile duct adenocarcinomas, and focused on the specificity rather than the sensitivity in order to avoid false positives. Our second goal was to elaborate an atlas illustrating the main studied cytological criteria and to assess its use in training novice cytopathologists in biliary cytology, especially in well-differentiated adenocarcinomas.

MATERIALS AND METHODS

Patients and cytological samples

Seventy two malignant biliary cytological samples were retrospectively collected from the Grenoble Alpes University Hospital pathology department between January 1st, 2012 and December 31st, 2018. All the biliary samples were obtained under ERCP for biliary stenosis at the Grenoble Alpes University Hospital. From the 72 bile aspiration specimens available 3 were excluded are they were pauci-cellular and/or poorly preserved. The 69 malignant retained samples came from pancreatic and extra-hepatic biliary tract adenocarcinomas all proven by histology (8 pancreatoduodenectomy specimens, 45 pancreatic or biliary tract punctures or 16 pancreatic metastasis). They corresponded to 56 pancreatic ductal adenocarcinomas, 10 extra-hepatic cholangiocarcinomas, 1 adenosquamous carcinoma and 2 ampullary adenocarcinomas. Eighty one percent of them were well-differentiated, compared to respectively 43% and 65% of well-differentiated adenocarcinomas in the 2 studies previously mentioned [12,15]; 36 corresponded to men and 33 to women, ranging from 28 to 90 years old. This non-interventional, monocentric, retrospective study involving data and samples from human participants was carried out at the Grenoble University Hospital according to French current regulation and complies with Reference Methodology n°004 issued by French Authorities (CNIL). Subjects were all informed and did not oppose; written consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The raw data supporting the conclusions of this

article will be made available by the authors within respect of General Data Protection Regulation, without undue reservation.

Supplementary biliary samples classified as benign (N=58) and atypical (N=11), with (N=49) or without (N=20) a stent in place were retrospectively included for the second reading between January 1st, 2009 and December 31st, 2014. None of them showed evidence of malignancy during a follow-up of 5 years minimum (5-9 years). This prolonged follow-up demonstrates that the atypical cytologies corresponded to reactive atypia and these 11 atypical specimens were therefore considered as non-malignant for the statistical study. From these 69 selected non-malignant cases, 3 were excluded because of bad preservation or acellular slides. The 66 retained cases corresponded to secondary stenosis after surgery for lithiasic disease (N=58) or PSC (N=8).

Collection of bile samples

Interventional endoscopy technique

Patients with biliary stenosis underwent ERCP under general anesthesia on an X-ray table using a video side-viewing duodenoscope positioned in the second duodenum facing the papilla. A 0.035-inch-diameter guide-wire was introduced into the biliary tract and an injection of contrast medium (Telebrix; Guebert; France) was performed. The opacification objectified the presence of a biliary stenosis. After biliary sphincterotomy, a disposable cytology brush was slipped over the guide-wire through the stenosis (Cook®, France) with several passes in the stenosis. Three to 10 mL of bile were collected half before and half after brushing the stenosis. Brushing is performed before stenosis dilatation. Because the length of the guide-wire tract at the distal end of the device was 6 cm, the entrance hole of the guide wire had to be above the papilla to optimize bile aspiration. If required, the procedure was completed by the installation of a temporary prosthesis pending the etiological diagnosis of stenosis.

Cytological technique

Each bile aspiration specimen before and after brushing was cooled on ice and sent to the pathology department as soon as possible to avoid degenerative changes. Each specimen, one bile before brushing and one bile after brushing, was centrifuged (2,000 revolutions per minute for 10 minutes), and the deposit was smeared into 4 slides: 2 slides were stained with Papanicolaou after using a Merckofix fixing spray (Merck France, Calais, France) and 2 slides were air-dried and stained with May Grünwald Giemsa (MGG).

Cytological examination

For the first reading each of the 69 malignant samples was independently examined by a cytopathologist novice in biliary cytology (AB) and by a 5 year-experienced cytopathologist in biliary cytology (DG), both not blinded to the final diagnosis.

The presence or absence of 24 cytological criteria were studied, based on the *Papanicolaou Society of Cytopathology* fascicle [7] and on prior studies on biliary brushings.[10,11] These criteria were divided in 5 categories (Table 1) corresponding to (1) the background of the smears, (2) the presentation of the epithelial cells or clusters: “epithelial cell density” was considered adequate if at least 5 clusters of cells were observed in each of the 8 slides ; “nuclear overlap” was retained if moderate or marked ; “3D-cluster” was characterized by a disorganized overlapping atypical cells cluster ; lumen within clusters characterized the “acinar arrangement”; “single malignant cell” was defined as a single cell with intact cytoplasm, enlarged nucleus occupying at least 50% of the cell area, chromatin changes, irregular nuclear shape; “cytophagy” corresponded to an epithelial cell-in-cell arrangement, (3) the cytomorphological aspect of the nuclei: “anisonucleosis” was noted if a size variation of at least 4 times between the smallest and largest nuclei within a cell cluster was observed ; “irregular nuclear shape”; “enhanced nuclear membrane” was noted if visible at a x20 objective lens ; increased N/C ratio (“N/C ratio > 0.5”) was noted when the nucleus occupied

more than 50% of the cell area ; “chromatin changes” included a clear, coarse chromatin or hyperchromatism ; “prominent nucleolus” was noted when a nucleolus was visible at a x20 objective lens (4) the appearance of the cytoplasm : “vacuolated cytoplasm” ; “enhanced cytoplasmic membrane” when the limits of the cytoplasm were too clearly visible, (5) and two accompanying criteria such as “neutrophils phagocytosis” and the presence of “degenerative cell”. The criteria from the categories (2) and (3) were analyzed in comparison with the normal flaps of ductal cells arranged in a honeycomb pattern. At the end of the first reading, an atlas (Fig. 1) was elaborated to better illustrate the cytological criteria studied, especially those with lower inter-observer agreement.

The second reading was performed on the 69 malignant cases and on the 66 non-malignant (benign and atypical) cases in order to assess the presence of the 24 studied criteria and to determine the specificity and sensitivity (Table 2) of each criterion and the inter-observer agreements for the 24 cytological studied criteria.

For this second reading AB and DG were blinded to the final diagnosis, and a delay of three months was respected between the first and second reading.

A third reading was performed three months later by the experienced cytopathologist (DG) on 31 samples from the 135 cases, selected at random to evaluate the intra-observer agreement on the criteria retained for the diagnostic tree.

Statistical analysis

The Cohen’s kappa coefficient (k)[13] was computed to assess inter-observer and intra-observer agreement for the 24 cytological criteria. Generally, a k value less than 0.4 implies poor agreement. A k value between 0.4 and 0.75 corresponds to moderate to good agreement while a k value higher than 0.75 indicates excellent agreement.[14]

Sensitivity and specificity of the 24 cytological criteria were computed as well as their 95% confidence intervals (95% CIs) using the Wilson method.

We developed a score including the four non-100% sensitive and non-100% specific criteria harboring the highest specificities in order to improve their diagnostic performance by combining them. A logistic regression was used to assess if these criteria were associated with malignancy. A scoring system was then developed considering the values of the regression coefficients obtained from the logistic regression.

XLSAT and STATA 16.1 were used for the analyses.

RESULTS

Inter-observer agreements between novice and experienced cytopathologists in biliary cytology (Table 1).

From the 24 studied cytological criteria the first reading highlighted 4 criteria with an excellent kappa value: “pigment rich background, bloody background, anisonucleosis, irregular nuclear shape” and 6 criteria with a good kappa value: “inflammatory background, acinar arrangement, single malignant cell, N/C ratio > 0.5, clear chromatin and enhanced cytoplasmic membrane”.

The second reading, performed after the elaboration of the learning atlas highlighted 14 other criteria which became highly reproducible with an excellent k value corresponding to the following criteria: “clean background, inflammatory background, epithelial cell density, nuclear overlap, 3D-cluster, single malignant cell, enhanced nuclear membrane, N/C ratio > 0.5, clear chromatin, coarse chromatin, hyperchromatism, cytoplasmic vacuole, enhanced cytoplasmic membrane and neutrophils phagocytosis”. Moreover 4 criteria presented a good agreement: “necrotic background, mucinous background, acinar arrangement, and prominent nucleoli”. Kappa indices agreements of all the 24 criteria improved between the two readings and in the second reading 75% of them (18/24) were excellent.

These results show the interest of the atlas for our training purpose. Indeed, we demonstrated that this atlas can represent a learning support for cytopathologists novices in biliary cytology. It highly improves the agreement between cytopathologists by providing a clear definition and illustration of the cytological criteria.

Specificity and sensitivity of cytological criteria for the diagnosis of malignancy (Table 2)

To reduce the number of retained cytological criteria, three criteria (clear, coarse chromatin and hyperchromatism) were pooled into a single criterion entitled chromatin changes, as the inter-observer agreements were excellent (kappa $\geq$ 0.84).

The frequency and the percentage of all the criteria were reported in 3 diagnostic groups: malignant, atypical and benign cases. In addition, the sensitivity and specificity of each criterion were analyzed in the malignant and non-malignant cases.

The only one 100% specific criterion was represented by the presence of "single malignant cell".

Three 100% sensitive criteria (irregular nuclear shape, chromatin changes and the presence of cytoplasmic vacuole), were found in all malignant cases. This was not surprising as their presence is linked to the definition of malignant cells (isolated or clustered).

Four other criteria harbored high specificities (between 89-98%): "3D-cluster, anisonucleosis ($\geq$ 1:4), N/C ratio > 0.5 and enhanced nuclear membrane".

All the remaining studied criteria with low sensitivity (< 49%) and/or a specificity less than 89% were not retained for further analysis.

Scoring system (Tables 3A and 3B)

The four non-100% sensitive and non-100% specific criteria harboring the highest specificities (between 89-98%) were also very reproducible (k $\geq$ 0.88) and were chosen to develop a scoring system of biliary malignancy. Each 4 criterion was pondered in a logistic regression analysis and a score was attributed based on their regression coefficients (Table

3A). A final score resulted from the sum of each criterion's score. Table 3B reports the sensitivities and specificities associated with each value of the final score. A final score > 16 was associated with the best specificity for malignancy, and more specifically, the presence of 3 criteria, namely "3D-cluster, anisonucleosis and N/C ratio > 0.5", allowed to reach a specificity of 100% (95% CI: 94-100%) and a sensitivity of 72% (95% CI: 61-82%) for the diagnosis of adenocarcinoma. As shown in table 3A, the criterion "enhanced nuclear membrane" which showed the lowest score appeared less essential for the accurate diagnosis and wasn't retained for the diagnostic tree.

Malignant diagnostic tree (Fig. 2)

The main goal of this diagnostic tree was to avoid false positive results either by identifying a single malignant cell (the only 100% specific criterion) or by summing the following 3 criteria: "3D-cluster, anisonucleosis (≥ 1:4) and N/C ratio > 0.5", reaching a 100% specificity (final score > 16).

The intra-observer agreement of the 4 selected criteria included in this diagnostic tree was assessed by a third reading conducted by the experienced cytopathologist (DG) blinded to the final diagnosis on 32 biliary samples randomly selected among the 135 cases. All the 4 criteria were highly reproducible with excellent intra-observer kappa indices (Table 4).

The diagnostic tree was then applied to the second reading of both the experienced cytopathologist (DG) and the novice cytopathologist in biliary cytology (AB) in order to assess its internal validity on the 135 samples. No false positive or false negative results were obtained when the diagnostic tree was applied to the experienced pathologist's second reading. When applying it to the novice's second reading, both diagnostic specificity and sensitivity were 97%. Indeed, there were 3 false negatives (3 well-differentiated adenocarcinomas) and one false positive (PSC).

DISCUSSION

Our study is the first to propose an original diagnostic tree reaching a 100% specificity in a large population of well-differentiated adenocarcinomas regardless of the reader's experience in biliary cytology. We also produce a bile cytology learning atlas improving intra- and inter-observer agreements for the training of cytopathologists novices in biliary cytology.

The main goal of our study was to determine the most specific, sensitive and reproducible cytodiagnostic criteria for the diagnosis of adenocarcinomas, especially the well-differentiated ones which represent a diagnostic challenge.

We first studied a scoring system based on 4 specific, sensitive and reproducible criteria for malignancy ("3D-cluster, anisonucleosis, N/C ratio > 0.5 and enhanced nuclear membrane"). When bile specimens harbored two of them with a score higher than 10, the specificity reached 97% and the sensitivity of diagnosis of malignancy was 96%. As we focused on the specificity rather than the sensitivity in order to avoid false positives, we built our diagnostic tree with the more efficient (specific and reproducible) cytological criteria of malignancy. The decisive diagnostic tree encompasses the only 100% specific criterion (single malignant cell) and three out of the four scoring system's criteria ("3D-cluster, anisonucleosis and enhanced nuclear membrane"). Indeed the "enhanced nuclear membrane" criterion was not retained for the elaboration of the diagnostic tree as it was not seen by the MGG staining and as its individual score and sensitivity were the lowest of the 4 scoring system's criteria.

Our study highlighted three 100% sensitive criteria named "chromatin changes, vacuolated cytoplasm and irregular nuclear shape". In other words, they are sufficient to affirm the malignancy and represent strong criteria in our series including 81% of well-differentiated adenocarcinomas compared to 2 other previous studies reporting only 43% and 63% of well-differentiated adenocarcinomas respectively.[12,15]

To our knowledge only 3 multivariate studies performed on biliary cytology reported a scoring system encompassing 4 or 6 criteria for malignancy in the literature. [12,15,16] Unlike our study, their scoring systems were established by experienced cytopathologists. The more recent one (2019) conducted in Japan was based on 89 cholangiocarcinomas.[12] The presence of 3 out of 4 criteria (abnormal chromatin, irregularly arranged nuclei, irregularly overlapped nuclei and irregular cluster margins) reached a diagnostic specificity of 98% and a sensitivity of 87%. The second study by Jin YH *et al*. was performed on a cohort of 43 adenocarcinomas and showed that the presence of 3 out of the 4 following criteria (hyperchromatism, coarse chromatin, increased N/C ratio, loss of honeycomb arrangement) reached a diagnostic specificity of 90% and a sensitivity of 65%. Finally, another Japanese team reported in a small cohort of 22 adenocarcinomas a scoring system based on 6 criteria (loss of honeycomb arrangement, enlarged nuclei, loss of polarity, bloody background, flat nuclei and cell-in-cell arrangement). When more than 3 out of the 6 criteria were found, the specificity reached 76.9% and the overall sensitivity of diagnosis of malignancy was 86.4%.[15]

Two out of the 4 final retained criteria for establishing our proposed diagnostic tree were already reported in these three bile cytology studies even if their terminology of the criteria and/or their definition differs somewhat: "**N/C** ratio > 0.5" by Jin et al. (increased N/C ratio) and Nakajimet al. (named enlarged nuclei) and "3D-cluster" criterion combines some criteria of the 3 studies (loss of honeycomb arrangement, irregularly arranged nuclei, irregularly overlapped nuclei and loss of polarity). The criteria "single malignant cell" and "anisonucleosis" were not retained in their scoring system.

Moreover, our diagnostic tree performed well whatever the cytopathologist's experience in biliary cytology. Applying the diagnostic tree to the experienced cytopathologist's second reading, no false positive or false negative were noted. When applying it to the novice's second reading, both diagnostic specificity and sensitivity were 97%, better than the 3

multivariate studies performed by experienced cytopathologists. The only false positive was observed in a context of PSC, a classic pitfall. It should be noted that the 2nd reading was blind to any clinical information. That is why some authors have already pointed out the need of a multidisciplinary management not to ignore this diagnostic trap. [8,17-19] In the specific context of PSC or stents, Goyal et al. even recommend an anisonucleosis > 1:6 to avoid false positives. [17]

Three false negative results were also noted, all corresponding to well-differentiated adenocarcinomas where the "single malignant cell" criterion had not been found and less than three criteria had been identified. Furthermore it is interesting to note that each case included 8 bile cytology slides. It is commonly accepted that increasing the number of slides to be read reduces the cytopathologist's concentration and therefore increases the risk of missing single malignant cells.

Since bile cytology is an uncommon and not widely taught cytology, our second objective was to produce a learning atlas to increase the inter-observer agreements between cytopathologists and to train cytopathologists novices in biliary cytology. It can be noted that the novice's results are good from the first reading, which can be explained by the fact that the studied-criteria corresponded to "general" cytological criteria and that the novice had experience in cytology. Our study demonstrates that by redefining the less reproducible criteria and by elaborating an atlas illustrating each studied cytological criterion, all the inter-observer agreements improved between the first and the second readings and 75% of them became excellent. These results represent another strength of our study since none of the three multivariate analyses performed on bile specimens investigated the reproducibility of the selected criteria.[12,15,16]

MGG staining did not allow the assessment of three criteria, namely "enhanced nuclear membrane, prominent nucleoli and chromatin changes" pointing the superiority of Papanicolaou staining for biliary samples.

To conclude, our diagnostic tree of malignancy on bile aspiration specimens is based on 4 efficient criteria of well-differentiated adenocarcinoma which allow together with a multidisciplinary approach, a diagnosis of adenocarcinoma with a specificity of 100% and a high sensitivity. It comes with a learning atlas useful for the training and accuracy of cytopathologists. This atlas could also be the subject of data integration in artificial intelligence. Indeed, the precise definitions and illustrations of the cytological criteria defined in our atlas, could be used as a support for learning an artificial intelligence software dedicated to the screening of biliary adenocarcinomas, providing a real help to cytopathologists, using scanners able to analyze 3D images, especially 3D-clusters as part of our diagnostic tree.

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Table legends

Table 1. Inter-observer agreements between a novice (AB) and an experienced cytopathologist (DG) in biliary cytology for the 24 criteria in the 69 malignant cases: first and second readings.

Table 2. Each criterion used with its frequency and percentage (%) in malignant and non-malignant cases (atypical and benign), their sensitivity, specificity with their 95% confidence intervals (CIs 95%).

Table 3.A. Scoring system.

Table 3.B. Diagnostic performance of the combination of the retained criteria.

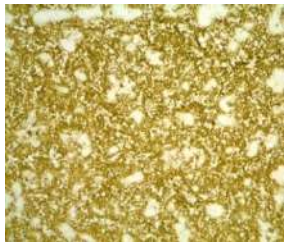
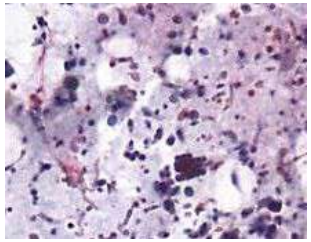
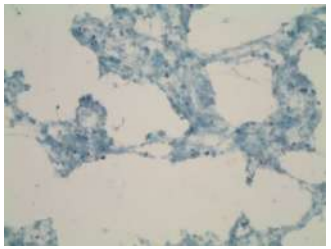
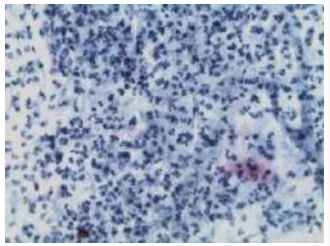
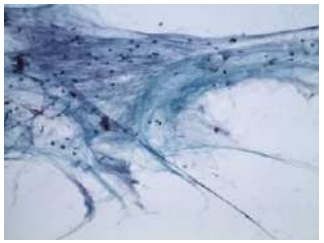
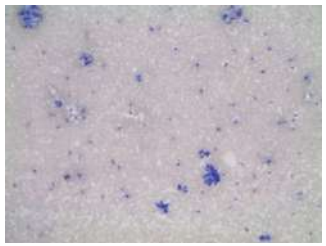
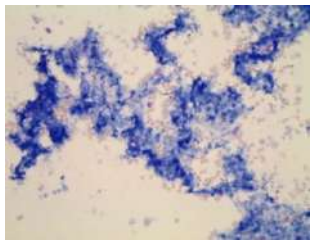
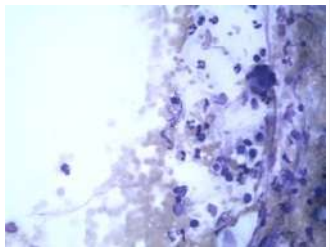
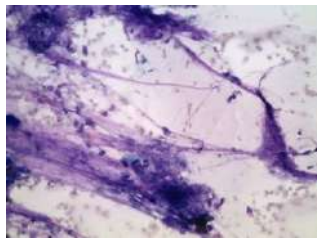
Table 4. Kappa intra-observer indices (DG) of the 4 criteria of the malignant diagnostic tree

Figure legends

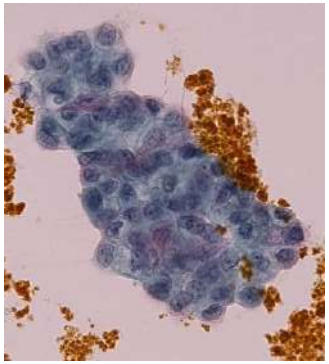
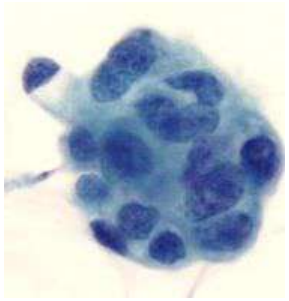
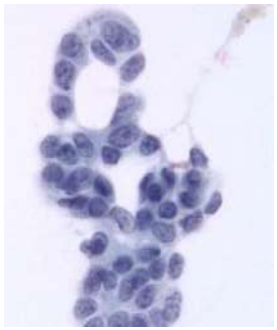
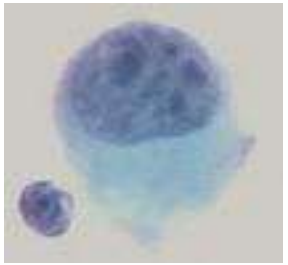
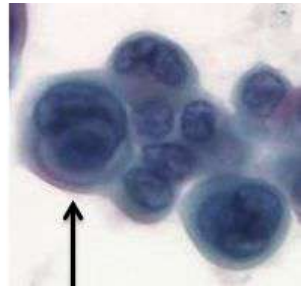
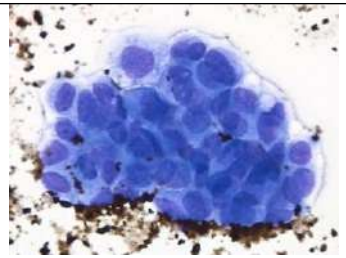
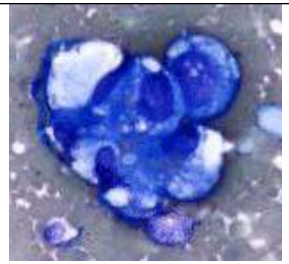
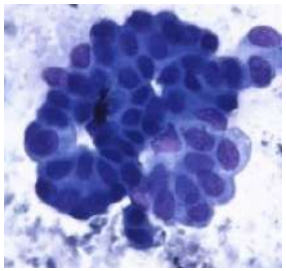
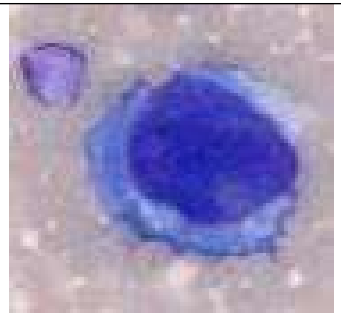
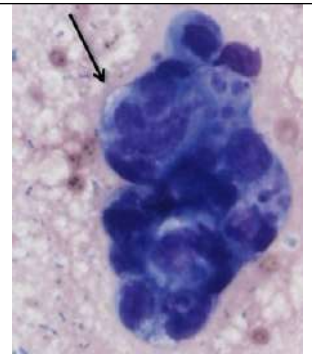
Figure 1. Bile cytology learning atlas illustrating the studied cytological criteria. PAPA: Papanicolaou stain, MGG: May Grünewald Giemsa stains.

Figure 2. Diagnostic tree to assert the diagnosis of adenocarcinoma on bile aspiration specimens. Papanicolaou staining x 40.

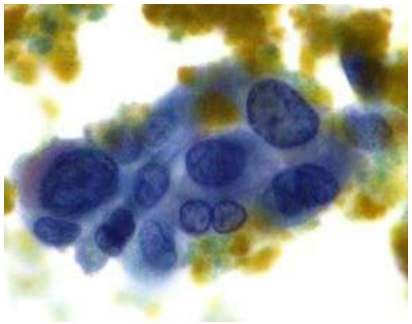
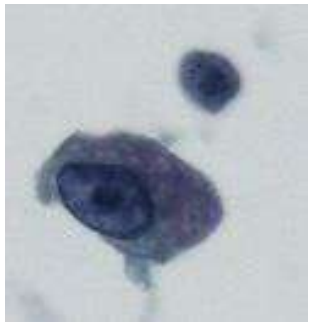
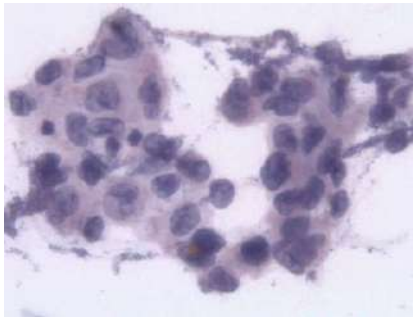
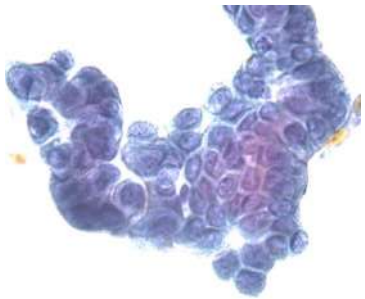
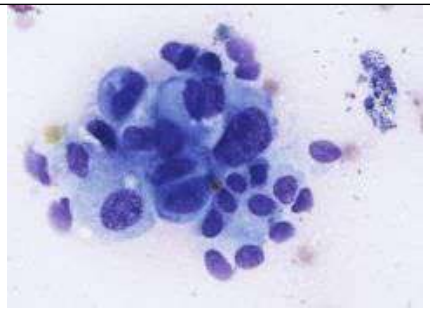
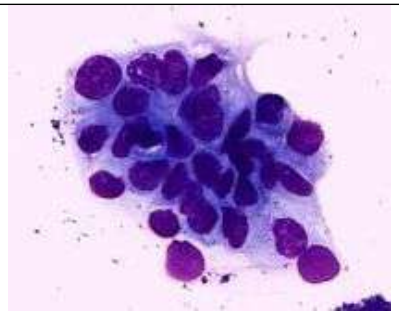
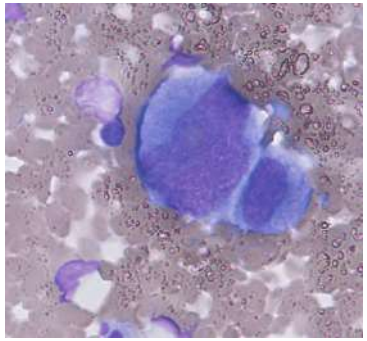
BACKGROUNG OF BILE CYTOLOGY

	Pigment-rich	Bloody	Necrotic	Inflammatory	Mucinous
PAPA	 x 5	 x 20	 x 20	 x 40	 x 40
MGG	 x 5	 x 5	 x 20	 x 40	 x 40

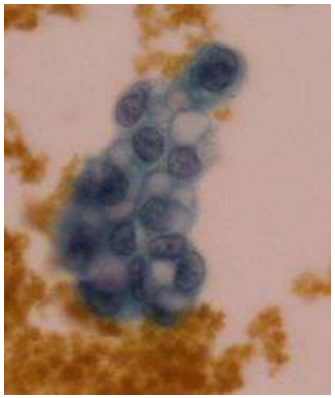
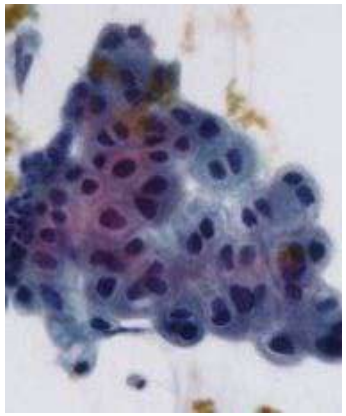
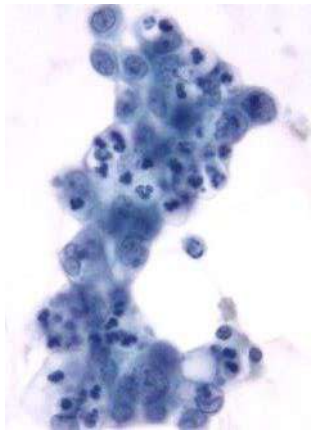
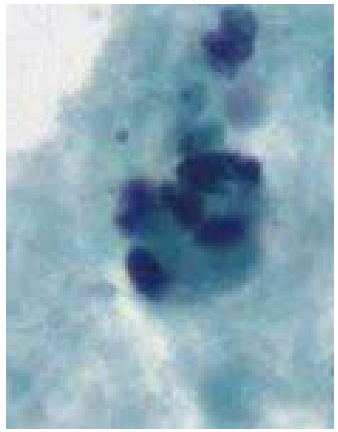
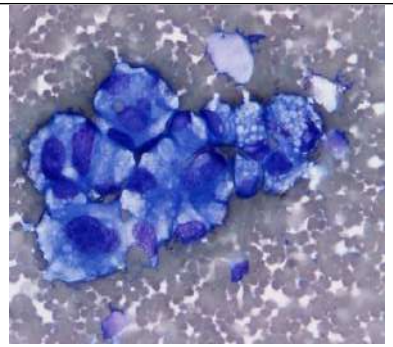
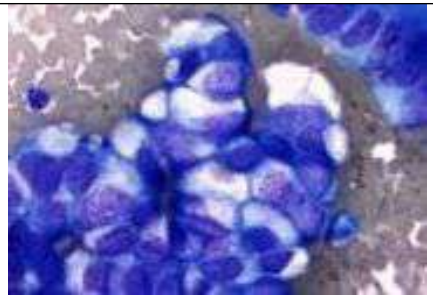
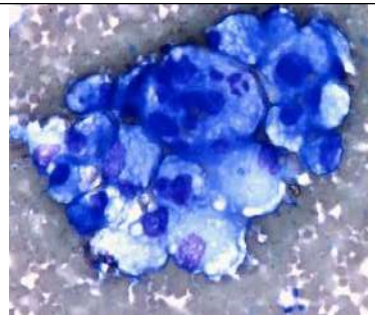
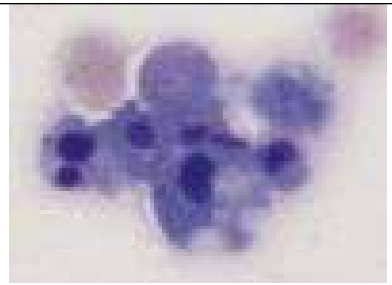
PRESENTATION OF EPITHELIAL CELLS OR CLUSTERS

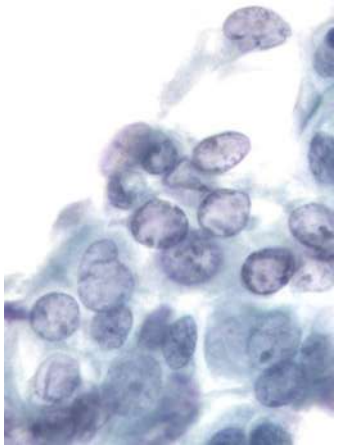
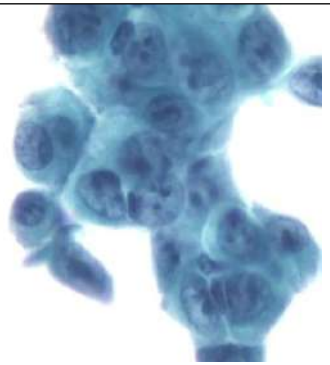
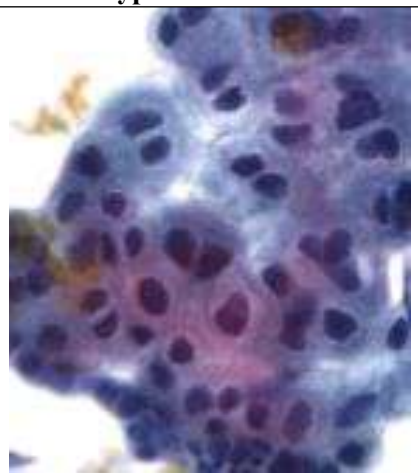
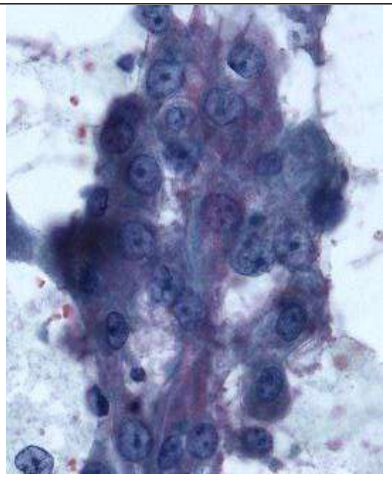
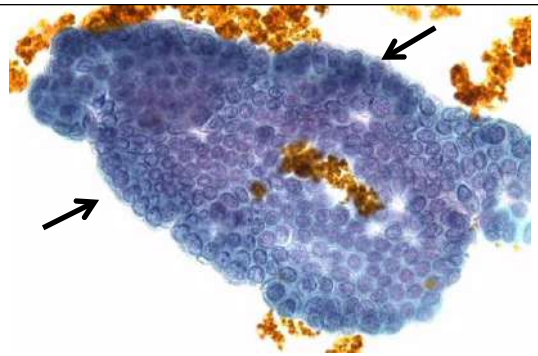
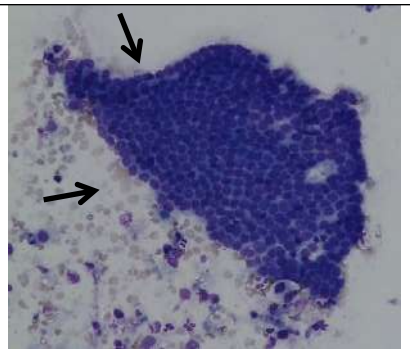
	Nuclear overlap Disorganized arrangement of nuclei in rows and columns with loss of polarity	**3D-cluster** Tightly cohesive cell balls with considerable nuclear overlapping, high N/C ratio	**Acinar arrangement**	**Single malignant cell** Single cell with intact cytoplasm, nuclei occupying at least 50% of the cell area, chromatin changes, irregular nuclear shape with or without prominent nucleoli	**Cytophagy** Epithelial cell-in-cell arrangement in single or cell cluster
PAPA	 x 40	 x 40	 x 40	 x 63	 x 40
MGG	 x 40	 x 40	 x 40	 x 63	 x 40

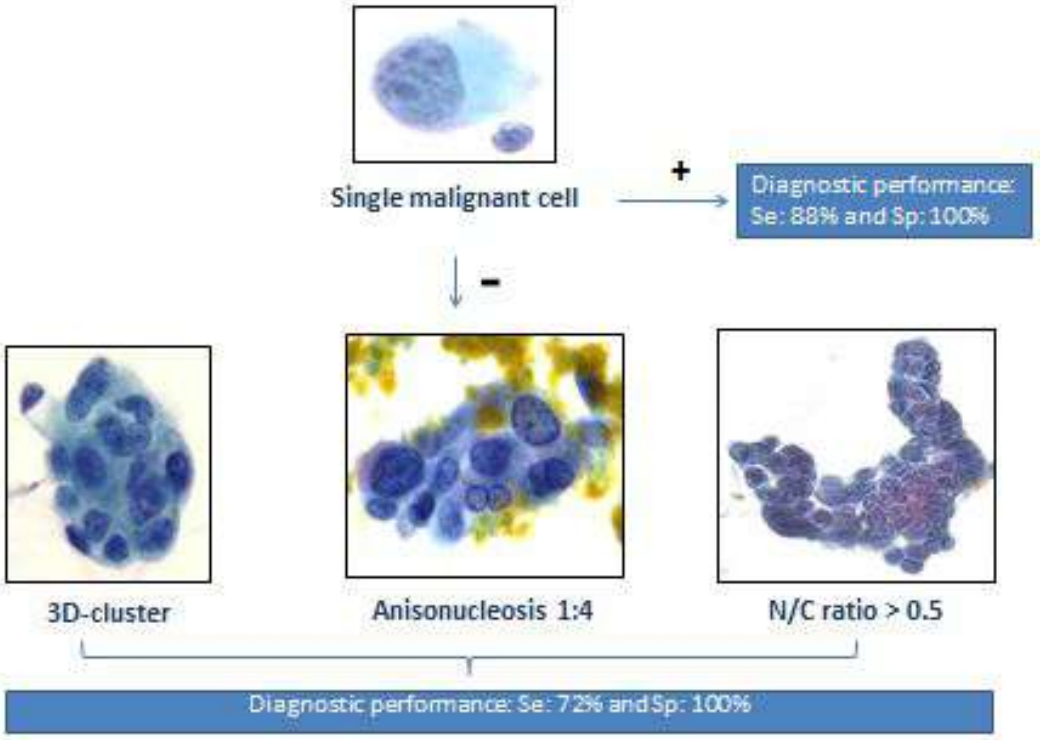
PRESENTATION OF EPITHELIAL CELLS OR CLUSTERS

	Anisonucleosis (≥ 1:4) Size variation with at least 4-fold variation in nuclear size	**Enhanced nuclear membrane** Visible at 20 x objective lens	**Irregular nuclear shape** Including nuclear grooves and flat nuclei	**N/C ratio > 0.5**
PAPA	 x 40	 x 40	 x 40	 x 40
MGG	 x 40	Not seen with MGG staining	 x 40	 x 63

PRESENTATION OF EPITHELIAL CELLS OR CLUSTERS

	Vacuolated cytoplasm	Enhanced cytoplasmic membrane	Neutrophils phagocytosis	Degenerative cell
PAPA	 x 63	 x 40	 x 40	 x 40
MGG	 x 40	 x 40	 x 63	 x 40

CHROMATIN CHANGES CRITERIA (Not seen with MGG staining)				
	Clear chromatin	**Coarse chromatin**	**Hyperchromatism**	**Prominent nucleoli**
PAPA	 x 40	 x 40	 x 40	 x 40
NORMAL BILIARY FLAPS				
All the previous cytological criteria must be compared to the nuclei of the benign ductal epithelium stained by PAPA (x 40 objective lens) and/or MGG (x 20 objective lens) caracterised by large, flat, monolayered cell sheet well-limited by a palissadic border (arrows). The cells are mostly cohesive, with well-defined cell borders and polarity. They possess small, centrally placed, round oval, regular nuclei and abundant cytoplasm. The chromatin is fine and nucleoli inconspicuous.				


Single malignant cell
+
Diagnostic performance:
Se: 88% and Sp: 100%
-
3D-cluster
Anisonucleosis 1:4
N/C ratio > 0.5
Diagnostic performance: Se: 72% and Sp: 100%

Categories	Criteria	First reading k (95% CI)	Second reading k (95% CI)
1-Background	Pigment-rich	0.97 (0.91-1)	1 (1-1)
	Clean	0.54 (0.34 - 0.73)	0.88 (0.8-0.97)
	Bloody	0.84 (0.7-0.99)	0.95 (0.89-1)
	Necrotic	0.58 (0.24-0.91)	0.64 (0.4-0.88)
	Inflammatory	0.66 (0.45-0.86)	0.92 (0.84-1)
	Mucinous	0.33 (0.15-0.5)	0.64 (0.49-0.79)
2-Presentation	Cell density	0.35 (0.19 - 0.52).	0.86 (0.79-0.94)
	Nuclear overlap	0.39 (0.15-0.92)	1 (1-1)
	3D-cluster	0.43 (0.26-0.61)	0.88 (0.79-0.96)
	Acinar arrangement	0.64 (0.46-0.82)	0.69 (0.57-0.81)
	Single malignant cell	0.68 (0.49-0.86)	0.95 (0.9-1)
	Cytophagy	0.12 (0.095-0.33)	0.3 (0.094-0.51)
3-Nuclei	Anisonucleosis	0.90 (0,71-1)	1 (1-1)
	Irregular nuclear shape	0.99 (0.93-1)	1 (1-1)
	Enhanced nuclear membrane	0.31 (0.12-0.5)	0.89 (0.81-0.98)
	N/C ratio > 0.5	0.67 (0.51 – 0.83)	0.99 (0.97-1)
	Clear chromatin	0.74 (0.51-0.98)	0.96 (0.91-1)
	Coarse chromatin	0.18 (0.007-0.35)	0.84 (0.75-0.94)
	Hyperchromatism	0.54 (0.36-0.72)	0.92 (0.82-1)
	Prominent nucleoli	0.18 (0.007-0.35)	0.73 (0.64-0.83)
4-Cytoplasm	Vacuolated cytoplasm	0.54 (0.36-0.72)	0.97 (0.92-1)
	Enhanced cytoplasmic membrane	0.63 (0.48 - 0.79)	0.94 (0.88-1)
5-Accompanying criteria	Neutrophils phagocytosis	0.31 (0.19-0.8)	0.84 (0.73-0.95)
	Degenerative cell	0.25 (0.05-0.44)	0.48 (0.25-0.71)

Categories	Criteria	Malignant	Non-Malignant		Sensitivity (CIs 95%)	Specificity (CIs 95%)
		(N=69)	Atypical (N=11)	Benign (N=55)		
1-Background	Pigment-rich	46 (66%)	5 (45%)	33 (60%)	65% (53-78%)	41% (33-49%)
	Clean	23 (33%)	3 (27%)	19 (34%)	41% (28-53%)	74% (67-82%)
	Bloody	16 (23%)	2 (18%)	10 (218%)	26% (14-38%)	85% (77-93%)
	Necrotic	9 (13%)	1 (9%)	4 (7%)	10% (0-22%)	89% (81-97%)
	Inflammatory	14 (20%)	4 (36%)	14 (25%)	23% (11-35%)	76% (68-83%)
	Mucinous	17 (24%)	5 (45%)	19 (34%)	16% (4-28%)	55% (47-62%)
2-Presentation	Cell density	34 (50%)	4 (36%)	18 (33%)	84% (77-89%)	30% (23-38%)
	Nuclear overlap	67 (97%)	5 (45%)	5 (9%)	97% (93-99%)	79% (71-85%)
	3D-cluster	55 (80%)	1 (9%)	0	79% (71-85%)	98% (94-99%)
	Acinar arrangement	59 (86%)	0	1 (2%)	85% (78-90%)	98% (94-99%)
	Single malignant cell	61 (88%)	0	0	88% (81-92%)	100% (97-100%)
	Cytophagy	24 (35%)	0	0	35% (27-43%)	100% (97-100%)
	Anisonucleosis	62 (90%)	2 (18%)	0	89% (82-93%)	97% (92-99%)
3-Nuclei	Irregular nuclear shape	69 (100%)	7 (64%)	16 (29%)	100% (97-100%)	62% (54-70%)
	Enhanced nuclear membrane	41 (59%)	2 (18%)	0	59% (54-67%)	97% (93-99%)
	N/C ratio > 0.5	68 (98%)	5 (45%)	2 (4%)	98% (94-99%)	89% (83-93%)
	Clear chromatin	45 (65%)	6 (54%)	19 (34%)	86% (73-98%)	83% (76-91%)
	Coarse chromatin	35 (50%)	3 (27%)	14 (25%)	65% (53-78%)	89% (82-97%)
	Hyperchromatism	17 (24%)	0	5 (9%)	30% (18-43%)	98% (91-100%)
	Chromatin changes	69 (100%)	6 (54%)	10 (18%)	100 (97-100%)	75% (67-81%)
	Prominent nucleoli	55 (80%)	5 (45%)	11 (20%)	94% (89-97%)	56% (48-64%)
4-Cytoplasm	Vacuolated cytoplasm	69 (100%)	5 (45%)	16 (29%)	100% (97-100%)	68% (60-75%)
	Enhanced cytoplasmic membrane	34 (49%)	5 (45%)	14 (25%)	49% (41-57%)	71% (63-78%)
5-Accompanying criteria	Neutrophils phagocytosis	25 (36%)	3 (27%)	6 (1%)	36% (28-44%)	86% (79-91%)
	Degenerative cell	17 (25%)	1 (9%)	2 (3%)	25% (13-37%)	95% (87-100%)

Criteria	Regression coefficients	Score
3D-cluster	5.54 (3.48 – 7.60)	6
Anisonucleosis	5.65 (4.04 – 7.26)	6
N/C ratio > 0.5	6.35 (4.23 – 8.48)	6
Enhanced nuclear membrane	3.85 (2.36 – 5.33)	4

Final score	Specificity (95% CIs)	Sensitivity (95% CIs)
> 6	95% (87-98%)	96% (88-99%)
> 10	97% (90-99%)	96% (88-99%)
> 12	98% (92-100%)	86% (75-92%)
> 16	100% (94-100%)	72% (61-82%)

Four retained criteria	Third reading k (95% CIs)
Single malignant cell	0,87 (0,7-1)
3D-cluster	0.80 (0,6-1)
Anisonucleosis (1:4)	0.94 (0,81-1)
N/C ratio > 0.5	0.87 (0,69-1)