

Digital image DNA cytometry: A useful tool for the evaluation of malignancy in biliary strictures

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Abstract. *Background:* Cytologic evaluation of the biliary tract strictures is nowadays widely used for distinguishing between benign and malignant lesions but remains a challenge for some problematic cases. Digital Image cytometry (DNA-cytometry) helps cytopathologists to resolve some unclear situations.

Methods: We have analysed 41 specimens of bile duct brushings obtained from patients during ERCP (11 benign cases, 7 suspicious for malignancy cases and 23 malignant cases) by DNA-cytometry and correlated them with the histological biopsy counterpart.

Results: All eleven cytological and histological benign cases were DNA-diploid and among 22 patients with malignant cytological and histological diagnosis 21 were DNA-aneuploid. One case considered malignant by the cytopathologist revealed DNA-aneuploid but malignancy could not be confirmed by histology. The analysis of the suspicious for malignancy cases revealed that all DNA-aneuploid cases were malignant and all DNA-diploid cases were benign referring to the follow-up of the patients.

The comparison between cytology alone and cytology combined with DNA-cytometry related to the histological diagnosis (gold standard) resulted in a sensitivity of 100% and a specificity of 79% for cytology alone; a specificity of 94% and a sensitivity 92% for DNA-cytometry and a specificity of 93% and a sensitivity of 100% with combined analyses. The positive predictive value was 90% for cytology, 96% for DNA-cytometry and for both analyses. The negative predictive value showed 100% for cytology, 89% for DNA-cytometry and 100% for combined studies.

Conclusions: Despite the limited number of patients involved in the study, the results obtained indicate an increased of specificity and of positive predictive value using DNA-cytometry. These results confirm the pertinence of these method for challenging cases, in conjunction with other available diagnostic tools.

Keywords: Image DNA cytometry, biliary strictures, cytology

1. Introduction

Brush cytology for routine investigations of biliary strictures (BS) has revealed a high degree of specificity but a sensitivity highly variable [6,18,24,29].

In order to improve detection of malignancy in cases with scant cellularity or poor cellular preservation, image cytometry for DNA content has been proposed by some authors [10,19,28]. Sensitivity of DNA-aneuploidy for identification of malignancies in

pleural effusions was evaluated to 83% and specificity of DNA-non-aneuploidy for benignity was estimated to 95% [14]. The positive predictive value of aneuploidy for the occurrence of malignant cells was 97%. Negative predictive value of DNA-non-aneuploidy was 72% [14]. DNA-analysis has been found as a valuable ancillary test in cases of difficult pericardial effusions [5]. In bladder washings the usefulness of flow cytometry has also been reported [4].

Few data underlying the usefulness of DNA-cytometry have been reported in the field of pancreaticobiliary tree. The high prevalence of DNA-aneuploidy in primary sclerosing cholangitis (PSC)-related cholangiocarcinoma and the low prevalence of DNA-aneuploidy

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in benign PSC strictures [17] point to DNA-cytometry as a possible future method for detecting pancreaticobiliary malignancies. Recently few reports tend to emphasize the interest of digital image analysis in endoscopic biliary brush cytology [19,28].

In this study we intend to evaluate the accuracy of DNA-cytometry in order to distinguish between benign and malignant strictures of the biliary tract.

2. Material and method

Forty-one specimens of bile duct brushings obtained from patients during endoscopic retrograde cholangiopancreatography were included in the study. The cytological diagnosis was performed by two cytopathologists (B.R. and M.A.) and for all cases included in the analysis, diagnosis was confirmed by an histological examination (biopsy specimen or surgical resection) (Fig. 1). DNA-cytometry was performed by an experienced cytotechnician (C.L.) aware of the cytological diagnosis but not of the histological diagnosis.

2.1. Specimen preparation

All specimens were obtained during ERCP by brushing the site of stricture. The brush itself was immersed in NaCl solution 0.9% and the product was centrifugated for 10 minutes at 2000 revolutions per minute. The sediment was smeared. Then the preparations were fixed in 95% alcohol for Papanicolaou stain.

The most characteristic Papanicolaou-stained slides were selected for DNA-cytometry. For this purpose, the material was destained in an acid-alcohol solution (1 part chlorhydric acid and 99 parts ethanol 70%), afterwards stained with Feulgen method [7,9,21].

2.2. Cytologic analysis

The smears were analysed by two experienced pathologists [1] and diagnosed according to conventional cytologic criteria. The cases were classified into three categories: negative, suspicious and positive for malignancy.

2.3. DNA-cytometry (DNA digital image analysis)

The analysis has been performed with the Autocyte Quic DNA, version 1.1 (Autocyte Inc., Burlington, NC). For most cases 200–300 nuclei could be analysed for each smear. As external control, 50 to 70 young rats hepatocytes have been used with a coefficient of variation < 5%. In order to avoid a possible influence of destaining process using chlorhydric acid on the final intensity of the Feulgen staining, tissue polymorphonuclear leucocytes were used as internal reference. Histograms were interpreted according to the ESACP recommendations [8] which allow DNA stemlines to be identified as abnormal (or aneuploid) if they deviate more than 10% from the diploid (2c) or tetraploid regions (4c). Rare events in DNA histograms were also considered abnormal cells often called 5c or 9c exceeding events because representing non proliferating abnormal cells with different chromosomal aneuploidies. Considering that in normal tissues and most low-grade or slowly proliferating neoplasms, approximately 85% of the cell population constitute the G0/1 peak and 15% of the cells are in the S-phase and G2/M phases [2,16], we only considered as aneuploid the samples in which more than 15% of the cells had a DNA-index in excess of 1.10.

2.4. Statistical method

Sensitivity and specificity were calculated according to diagnostic tests [3]. The “gold standard” for the evaluation of cytologic/DNA-cytometry diagnoses were the histological diagnoses obtained by biopsies or surgical resection. Cytologically negative were considered cases non-neoplastic or with mild or moderate dysplasia. Were considered cytologically positive cases with severe dysplasia or adenocarcinoma. DNA-cytometric negative diagnoses were DNA-diploid cases and DNA-cytometric positive diagnoses were DNA-aneuploid cases. When both methods, cytology and DNA-cytometry were combined, a case was considered positive for malignancy when malignancy was suggested by one cytology (presence of neoplastic cells) or DNA-cytometry (DNA-aneuploidy) or both techniques.

Specificity was defined as the probability of the test finding no disease among those who do not have the disease or the proportion of patients free of disease who have a negative test. Sensitivity was considered as the probability of the test finding disease among those

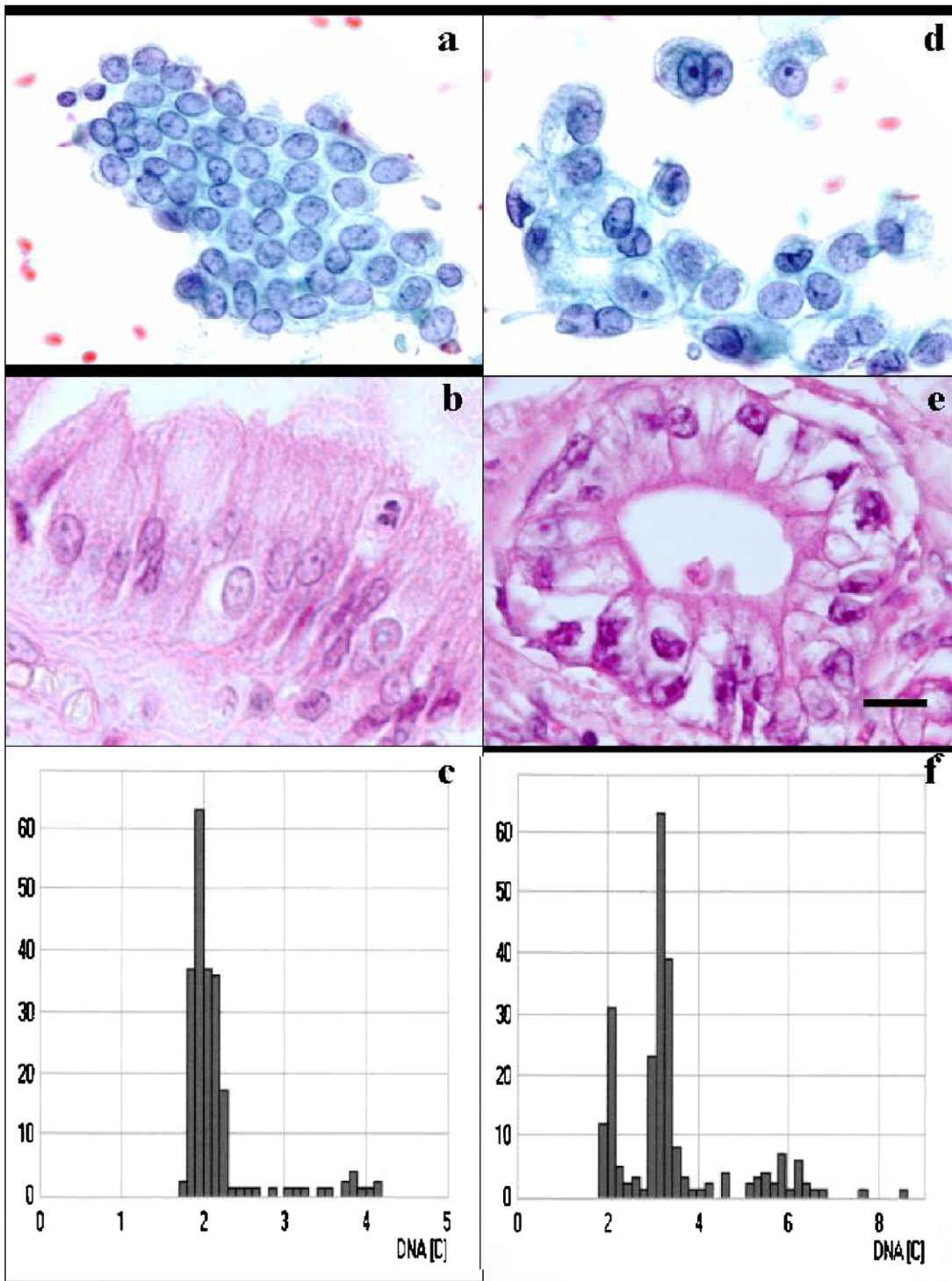


Fig. 1. *Healthy biliary epithelium.* (a) Papanicolaou-stained smear brushing showing benign epithelial cells. (Original magnification, $\times 60$). (b) Hematoxylin and eosin stained section showing healthy epithelium. (Original magnification, $\times 60$). (c) Corresponding DNA histogram shows a diploid pattern. *Neoplastic biliary epithelium.* (d) Papanicolaou-stained smear brushing showing neoplastic epithelial cells. (Original magnification, $\times 60$). (e) Hematoxylin and eosin stained section showing neoplastic epithelium. (Original magnification, $\times 60$). (f) Corresponding DNA histogram shows an aneuploid pattern. Scale bar in lower bottom corner represent $20 \mu\text{m}$.

who have the disease or the proportion of patients with disease who have a positive test result.

Positive Predictive Value (PPV) is the percentage of patients with a positive test result who actually have the disease.

Negative Predictive Value (NPV) is the percentage of patients with a negative test who do not have the disease.

3. Results

Material from 41 patients, 21 women and 20 men was analysed. The mean age at diagnosis was 64y (32y–92y) for women and 66y (46y–89y) for men. Eleven were considered cytologically negative for malignancy, 7 suspicious for malignancy and 23 malignant. For all eleven benign cases histology confirmed the diagnosis. For this material, image cytometry analysis revealed diploid histograms. None of these cases showed cells having a DNA content > 9c (Fig. 1a, b, c).

Malignancy was confirmed histologically in 21 out of the 23 patients with a malignant cytological diagnosis. All were aneuploid except two adenocarcinomas. The aneuploid cases revealed also the presence of more than 1% of cells having a DNA content > 9c (Fig. 1d, e, f). The two remainder cytologically malignant cases corresponded at histological examination for one of them to an adenoma with high grade dysplasia/intramucosal carcinoma and for the other to a duodenal mucosal biopsy with regenerative lesions. For both, image cytometry resulted in aneuploid histograms with more than 1% of cells having a DNA content > 9c.

Finally, the most interesting category was the cytologically suspicious of malignancy group. Out of eight cases included in this series, the histologic examination revealed three adenocarcinomas and 5 nonmalignant cases, one with mild dysplasia and the others displaying inflammation with regenerative changes. All the adenocarcinomas presented aneuploid histograms with more than 1% of cells having a DNA content > 9c. Five cases corresponded to benign lesions with histograms showing diploidy in four cases and aneuploidy in only one case. Histology from later case was rather inconspicuous, composed of limited material, without atypia. Following these results, we have determined sensibility and specificity of cytology and cytometry against histology that was considered as gold standard. For cytology alone, specificity was 79% and sensitivity

100%. With cytometry alone, specificity reached 94% and sensitivity 92%. Cytology and cytometry combined revealed a specificity of 93% and a sensitivity of 100%. The positive predictive value was 90% for cytology, 96% for cytometry and for both analyses. The negative predictive value showed 100% for cytology, 89% for cytometry and 100% for combined studies.

4. Discussion

Biliary and pancreatic duct strictures are most often caused by inflammatory or neoplastic disorders. Surgical treatment depends on whether the stricture is due to a malignant change or not. Brush cytology has revealed a safe and simple way of collecting material during ERCP [25,27]. However, cytological distinction between reactive cellular changes and malignancy can be difficult, particularly when tumors are well differentiated or when there is active inflammation. During the last years, literature concerning cytology of biliary system has evolved and cytological criteria of malignancy in smears from ERCP were determined [4]. But, for cytopathologist, diagnosis of malignancy on a smear rests a great challenge due to the clinical implications which follow this diagnosis. Ancillary techniques [13, 22,23] and particularly nuclear DNA content analysis have been reported susceptible to give more confidence to the cytological diagnosis [11]. Several studies have suggested a real improvement in discrimination between benign and malignant biliary strictures with the combination of cytological examination and DNA-cytometry [11,19,28].

In our study we have analysed only biliary brush cytology specimens with the corresponding histological diagnosis. The DNA-cytometry was performed by an experienced cytotechnician who analysed the atypical epithelial cells from the smears avoiding the inflammatory cells component that may interfere when the analysis is performed with flow cytometry. All cytological and histological benign cases were diploid (100% of concordance between cytology, digital image analysis and histology) and most of cytological malignant cases were aneuploid confirmed by histology. Twenty-one out of 22 aneuploid cases corresponded to adenocarcinomas and one to an adenoma with high grade dysplasia/intramucosal carcinoma. For one case considered as malignant by the cytopathologist, image analysis revealed aneuploidy and corresponded to a normal duodenal mucosa with regenerative modifications but the material was inconspicuous. This dis-

cordance in the group of malignancies with histology may be probably due to a problem of sampling: a biopsy represents only a small part of the stricture compared to a cytological analysis which is able to examine a larger region. The most important interest of the nuclear DNA content analysis method rises from the group of cytological suspicious for malignancy cases. We have analysed 7 cases from this group. Three were aneuploid with a confirmed malignant stricture by histology and 3 were diploid with a benign histology. The last one revealed aneuploidy but histology revealed only few cells without atypia and was not conclusive. Interestingly, in this case, a second biopsy performed one year later revealed malignancy. All these results suggest that brush cytology is a useful diagnostic method, safe, simple and with the great advantage to cover a larger area than sampling biopsy. The results of sensitivity and specificity we have obtained are not consistent with the data from literature. There is no false-negative case when the experienced cytopathologist is confident. In that situation DNA-cytometry can only confirm the diagnosis and cannot be very helpful. But when the cytopathologist diagnoses malignancy with confidence, a negative histology must not rule out malignancy, especially if DNA-cytometry shows aneuploid profile. Finally, the image analysis seems to take all its importance when the cytopathologist is worried about atypias but not enough confident to assume the diagnosis of malignancy. In these cases, aneuploid nuclear DNA content of the cell population analysed might comfort the suspicious of malignancy or at least warn the clinician to explore more carefully the patient.

Specificity in our study was comparable with other results in the literature, but sensitivity rate of cytology was 100% due to the absence of false-negative cases. This may be caused by several factors. First, we have considered positive as well clearly positive cytologic cases as suspicious cytologic cases. Secondly, in order to test the influence of digital image cytometry, we have collected for the study, only the straightforward diagnostic cases for cytology and excluded cases with inappropriate material as well as cases concerning inflammatory lesions as primary sclerosing cholangitis. This selection of cases implied a too small number of cases for significant analyses. It was interesting to determine the usual histograms profile in adenocarcinoma of biliary tract, but the study must be completed by a larger analysis including benign lesions of the biliary tract as inflammatory lesions (primary sclerosing cholangitis) or regenerative epithelial reactions.

In conclusion, and despite the limited number of patients involved in the study, the DNA content us-

ing DNA-cytometry may implement standard ERCP-guided brush cytology studies. For ambiguous cases, this approach should be useful for cytopathologists, together with other available diagnostic tools as FISH or immunohistochemistry in order to achieve the best therapeutic results.

To define the precise role of DNA-ploidy in routine clinical practice, a large number of patients with similar conditions is needed to reach meaningful endpoints and to correlate whatever, with biopsy samples and outcome of these patients.

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