The diagnostic and prognostic value of genetic aberrations in resectable distal bile duct cancer
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Citation for published version (APA):
Chapter 2

Diagnostic Value of DNA Analysis in Effusions by Flow Cytometry and Image Analysis

A Prospective Study on 102 Patients as Compared with Cytologic Examination

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Am J Clin Pathol 1991; 95: 6-12
ABSTRACT

One hundred twenty-six effusion samples from 102 patients were examined by cytology and flow cytometry (FCM). Overall, there was an 84% correlation between cytologic and FCM results. Of the 36 malignant cases determined by cytologic examination, FCM revealed an aneuploid peak in 20 (56%). Image cytometry (ICM) performed on the malignant cytologic cases with a diploid flow pattern detected two additional aneuploid peaks. In addition, FCM indicated three aneuploid cases in which cytologic characteristics were initially interpreted as benign (false negative). Aneuploidy was therefore detected in 64% of the malignant effusion specimens by FCM and ICM. Twenty-three of the total of 24 aneuploid cases detected by FCM were associated with malignancy (predictive value=96%). The one nonmalignant case was that of hemorrhagic pancreatitis with infected pseudocyst.

FCM is an excellent tool when moderate to large numbers of tumor cells are present, whereas use of ICM is advantageous for specimens containing smaller numbers of malignant cells because these can be directly analyzed. When an aneuploid peak is present, a diagnosis of malignancy must be suspected, and, if the initial cytologic screen is negative, a critical review of the cytology slides is justified. In those cases with an equivocal atypical cytology report and an abnormal cytometric histogram, additional investigation is warranted. In some malignancies the tumor cells will be diploid (in this study 36%) and neither FCM nor ICM will add to tumor detection, leaving cytologic examination as the definitive technique.

INTRODUCTION

Cytologic examination has been used successfully in the diagnosis and staging of malignant disease in patients with effusions. However, in a large number of patients the neoplasm may not be detected (i.e., the specificity of the method is high but the sensitivity is low). Therefore, various ancillary methods for the detection of cancer in effusions have been proposed. These include chromosome analysis, tissue culture techniques, and the detection of carcinoembryonic antigen (CEA) levels in fluids, all with varying degrees of success.

Previously, studies of human neoplasms have shown that most contain detectable chromosome abnormalities and this of itself is strong evidence for neoplasia. Flow cytometry (FCM) offers the pathologist/cytologist methods to measure DNA content of a large population of cells in solid tumors and in dispersed cells such as are found in cytologic specimens. It has been demonstrated, in conjunction with cytologic methods, to be useful for the possible confirmation of malignancy in effusions.

Image cytometry (ICM) also analyzes the DNA content of cells, the difference being that it has the capability to analyze individual cells rather than large cell populations. However, it has not been used in the study of effusions. The aim of this study, therefore,
Diagnostic value of DNA analysis was to determine the role of FCM combined with ICM in effusions of a variety of patients in conjunction with the more conventional, but time-proven, cytologic technique and to determine its utility.

MATERIALS AND METHODS

One hundred twenty-six effusion samples from 102 consecutive patients were examined by cytology and FCM. Thirty-one fluid samples (26 malignant and 5 benign) were also examined by ICM. Of the 126 specimens, 75 (60%) were pleural, 46 (36%) peritoneal, and 5 (4%) pericardial effusions (Table 1). Of the 102 patients, 50 were male and 52 female, with an age range of 18-84 and 26-88 years, respectively, and both with a mean age of 57. Patient records were reviewed, and particular attention was paid to subsequent cytology, histology, or radiology response for possible confirmation of the original cytologic diagnosis.

Cytology
Fresh fluids were received in the cytology laboratory, of which 20 mL was spun down in a centrifuge at 500 X g for 5 minutes. From the resulting cell button, five slides were made (three direct smears and two cytospin preparations with the use of the Shandon Cytospin 2® (Shandon, Sewickley, PA) for 3 minutes at 1,250 rpm. The cytospin and two direct smears were fixed in 95% (volume/volume [v/v]) ethanol and stained by Papaincolaou's method, while the remaining direct smear was allowed to air dry and stained with the Diff-Quik® (Baxter Healthcare Corp., McGaw Park, IL) staining set (a modified rapid Wright's stain). Any clotted material or tissue was processed as a cell block by fixing it in 10% (v/v) formalin solution, embedding it in paraffin, and cutting it as a histologic section.

Slides were screened and evaluated routinely as being negative for malignant cells (no neoplasm), atypical (abnormal but not neoplastic), insufficient specimen for diagnosis, suspect for malignant cells (suspect neoplasm but specimen not conclusive), or positive for malignant cells (presence of malignancy is deemed certain).

Flow Cytometry
Fluid specimens received from the cytology laboratory were centrifuged at 500 X g for 5 minutes. Residual red blood cells were lysed with the use of ammonium chloride reagent, and remaining cells were washed twice with phosphate-buffered saline (PBS), pH 7.2. After centrifugation, the cell pellets were resuspended in PBS and the cell count adjusted to 1 x 10^6/L. A 200-µL sample was used for FCM and DNA analysis, using a modified Vindelov technique. A final 1 mL solution containing a combination of propidium iodide (0.05 g/L), RNAse (700 U/L), and NP40 (1 mL/L) was used for DNA staining.
Chicken erythrocyte nuclei were used as an internal standard, being approximately 35% of the normal human diploid DNA value. Normal human whole blood, lysed with ammonium chloride and washed twice with PBS, was used as the assay diploid reference control. After a 30-minute incubation on ice in the dark, the specimens were filtered through 41 μm nylon mesh. The specimens were analyzed on a Becton Dickinson FACScan® (Mountainview, CA) equipped with a 15-mW, 488-nm argon-ion laser. At least 10,000 events were collected with the use of Consort 30® software for data acquisition and the DNA Cell Cycle Analysis® software for DNA data analysis.

The FCM results were categorized as either normal (diploid) or abnormal (aneuploid). Aneuploidy was defined in this study as one or more distinct separate peaks compared with the G₀/G₁ and G₂M peaks of the diploid cell population. The normal diploid cell control G₀/G₁ peak was set at 100 on a linear scale. The coefficient of variation for the normal G₀/G₁ blood cell population used as a control was 2.3%. DNA content was expressed as the DNA index (DI), which is the ratio of the DNA content of the tumor cells in the G₀/G₁ phase to the G₀/G₁ cells of a normal diploid population. By definition, the DI of diploid cells is 1.00.

**Image Analysis**

Nuclear DNA content was determined by ICM with the use of the Cell Analysis Systems (CAS) (Elmhurst, IL) integrated system of slides, reagents, software, and hard-ware. Eighteen of the 31 specimens analyzed were processed directly for Feulgen staining. The fluids (usually 50 mL) were centrifuged at 500 X g for 10 minutes at room temperature, and smears were prepared from the sediment on CAS slides containing predeposited control cells. The slides were fixed in 10% (v/v) buffered formalin for 30 minutes and air dried. Analysis was attempted for the remaining 13 specimens on destained original Papa-nicolao-stained preparations (seven cases) or Wright's-stained slides (six cases) prepared from the specimen processed for FCM. The coverslips were removed in xylene, and these slides were destained in acid-alcohol, rehydrated through graded alcohols to distilled water, dried, and then fixed in 10% (v/v) buffered formalin for 30 minutes, rinsed in distilled water, and air dried. All of the slides were hydrolyzed in 5 mol/L HCL for 60 minutes at room temperature before Feulgen staining with the use of the CAS purified reagents. Each of the directly processed slides was independently calibrated with the use of the predeposited control cells. Broken cells and inflammatory cells were disregarded, and a minimum of 300 intact, noninflammatory cells were analyzed.

Because the destained slides contained no control cells, they were mixed in staining batches containing CAS slides (a minimum of nine slides) and calibrated with the use of the mean calibration value of the appropriate staining batch. Lymphocytes were used as internal DNA diploid standards and their values fell within the previously reported normal range. The destained slides were generally less cellular than the direct preparations, and the number of intact, noninflammatory cells analyzed varied from 100 to 304, with a mean of 244. Proliferating populations with a defined G₀/G₁ population and
an appropriate G<sub>2</sub>M peak were identified in 9 of the 13 cases. One of the specimens contained less than 100 noninflammatory cells and was not destained, one specimen was technically inadequate and, in the remaining two specimens, no defined proliferating populations could be identified and these specimens were considered insufficient. The histograms were interpreted on the basis of the DI of the G<sub>0</sub>/G<sub>1</sub> population and distribution of cells in the histogram. Tumors with DIs indistinguishable from one (0.920-1.08) were considered DNA diploid; those with DIs of two (1.9-2.2) were considered DNA tetraploid; and those with DIs not equal to one or two were considered DNA aneuploid. In one case, the major G<sub>0</sub>/G<sub>1</sub> population had a diploid DNA index with a large (15.7%) G<sub>2</sub>M peak. This tumor was classified as diploid/tetraploid.

RESULTS

As seen in Table 1, 90 effusions from 74 patients were either cytologically benign or atypical (but benign). Eighty-six of these specimens were diploid by FCM.

<table>
<thead>
<tr>
<th>Location</th>
<th>Benign*</th>
<th>Malignant†</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pleural</td>
<td>48</td>
<td>27</td>
<td>75 (60%)</td>
</tr>
<tr>
<td>Peritoneal</td>
<td>41</td>
<td>5</td>
<td>46 (36%)</td>
</tr>
<tr>
<td>Pericardial</td>
<td>1</td>
<td>4</td>
<td>5 (4%)</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>36</td>
<td>126‡</td>
</tr>
</tbody>
</table>

* Forty-six specimens for malignant cells and 44 atypical (benign) specimens.
† Seven specimens suspicious for malignant cells and 29 positive for malignant cells.
‡ There were no insufficient specimens.

Four of these "benign" specimens, however, clearly had aneuploid peaks on the flow histogram, two being from the same patient. The patient with two samples was a 66-year-old woman with carcinoma of the lung with a pleural biopsy that confirmed the presence of neoplasm (see Figures IA-B). The second patient with negative cytologic results but aneuploid FCM results was a 57-year-old woman with metastatic breast carcinoma, whose pleural fluid cytologic findings one year previously contained malignant cells. The fourth fluid (from the third patient) with negative cytologic results but an aneuploid FCM peak (Figure 2) was from a 43-year-old man with hemorrhagic pancreatitis and an infected pseudocyst, who, to date, has no detectable malignancy on follow-up.
Figure 1. A. Pleural cytological specimen from a 66-year-old woman with history of carcinoma of lung. Cytologic diagnosis was atypical (benign). Several clusters of bizarre cells are illustrated. Papanicolaou (x312). B. Abnormal (aneuploid) FCM histogram from same patient.

Figure 2. Aneuploid FCM histogram from a 43-year-old man with hemorrhagic pancreatitis with no malignancy on follow-up.
Table 2. Results of FCM and ICM of 36 cytology specimens, 7 suspicious and 29 positive for malignant cells.

<table>
<thead>
<tr>
<th>Primary origin and type of neoplasm</th>
<th>Case no.</th>
<th>Type of effusion</th>
<th>FCM</th>
<th>ICM</th>
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<tr>
<td></td>
<td>1</td>
<td>Pl</td>
<td>D</td>
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<td>2</td>
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<td></td>
<td>3</td>
<td>Pl</td>
<td>A</td>
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<td></td>
<td>4</td>
<td>Pl</td>
<td>A</td>
<td>DT</td>
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<tr>
<td></td>
<td>5</td>
<td>Pl</td>
<td>D</td>
<td>D</td>
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<td>A</td>
<td>A</td>
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<td></td>
<td>7</td>
<td>Pl</td>
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<td></td>
<td>8</td>
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<td>A</td>
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<td>Lung adenocarcinoma</td>
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<td></td>
<td>9</td>
<td>Pl</td>
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<td></td>
<td>10</td>
<td>Pe</td>
<td>D</td>
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<td>As</td>
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<td></td>
<td>30</td>
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<tr>
<td>Malignant fibrous histiocytoma</td>
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<td>31</td>
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<tr>
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<td>32</td>
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<td></td>
<td>36</td>
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<td>D</td>
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Pl = pleural; As = peritoneal; Pe = pericardial; D = diploid; A = aneuploid; EQ = equivocal; QNS = insufficient material; T = tetraploid; DT = diploid/tetraploid
normal tissue. This, however, is an arbitrary figure, and a comparison between benign and malignant cell populations may not always be valid.

Twenty of the 36 (56%) of the malignant effusions were aneuploid by FCM. However, because three benign effusions were regarded as being false-negative findings, on follow-up the true figure is 23 of 39 (59%), which is similar to that reported in other effusion studies\(^1\)\(^,\)\(^2\)\(^,\)\(^3\)\(^,\)\(^4\)\(^,\)\(^5\)\(^,\)\(^6\)\(^,\)\(^7\)\(^,\)\(^8\)\(^,\)\(^9\)\(^,\)\(^10\)\(^,\)\(^11\)\(^,\)\(^12\)\(^,\)\(^13\)\(^,\)\(^14\)\(^,\)\(^15\)\(^,\)\(^16\)\(^,\)\(^17\)\(^,\)\(^18\)\(^,\)\(^19\)\(^,\)\(^20\)\(^,\)\(^21\)\(^,\)\(^22\)\(^,\)\(^23\)\(^,\)\(^24\)\(^,\)\(^25\)\(^,\)\(^26\)\(^,\)\(^27\)\(^,\)\(^28\)\(^,\)\(^29\)\(^,\)\(^30\). It is noteworthy that 25% of all tumors are truly diploid and 10-15% near diploid\(^3\(^,\)\(^23\). The latter are distinguished with difficulty from the diploid cells in the G\(_0\)/G\(_1\) phase and depend on the number of malignant cells and the coefficient of variation of the peak. In our study the incidence of diploid tumors combining both techniques of FCM and ICM is 36%. Aneuploidy in different tumor types varies, as seen in Table 2. Lung tumors in our study have a lower frequency of aneuploidy (50%) in contrast to those found by others\(^5\)\(^,\)\(^6\)\(^,\)\(^7\)\(^,\)\(^8\)\(^,\)\(^9\)\(^,\)\(^11\)\(^,\)\(^12\)\(^,\)\(^13\)\(^,\)\(^14\)\(^,\)\(^15\)\(^,\)\(^16\)\(^,\)\(^17\)\(^,\)\(^18\)\(^,\)\(^19\)\(^,\)\(^20\)\(^,\)\(^21\)\(^,\)\(^22\)\(^,\)\(^23\)\(^,\)\(^24\)\(^,\)\(^25\)\(^,\)\(^26\)\(^,\)\(^27\)\(^,\)\(^28\)\(^,\)\(^29\)\(^,\)\(^30\). There were no false-positive cytology cases, whereas this did occur in one case with FCM. This was a patient with hemorrhagic pancreatitis who had an obvious aneuploid peak on the flow histogram (Figure 2); follow-up studies have not shown any evidence of malignancy to date. It is well known that benign tumors and occasional disease states may have aneuploid cell populations\(^1\(^,\)\(^7\)\(^,\)\(^11\)\(^,\)\(^15\)\(^,\)\(^21\)\(^,\)\(^28\). Although this is an unusual phenomenon, it signifies that aneuploidy by itself is not a conclusive sign of malignancy. This is similar to cytologic criteria of malignancy, in which no one criterion is conclusive by itself.

Fourteen of the 39 malignant effusions were diploid with FCM. ICM was used in all these cases because it has higher sensitivity for the detection of minor cell subpopulations with aneuploidy than does FCM\(^20\). It is of interest, therefore, that the diploid mode was confirmed in 9 of these 14 cases, whereas aneuploidy was detected in 2 (14%). This corresponds with the 20% found by Koss and associates\(^20\) in bladder washings. In three cases there were not enough cells for evaluation. Therefore, aneuploidy was missed in two cases by FCM. In one of these there were only a few malignant cells, and a hypertetraploid cell population was detected by ICM (DNA index of 2.87), whereas the other contained excess tetraploid cells. A possible explanation for the discrepancy between FCM and ICM is the dilution of the tumor cell population by inflammatory or other nonneoplastic cells (e.g., mesothelial cells) resulting in a "diploid" pattern by FCM.

Despite these considerations, the most important cause for diploidy detected by FCM in malignant tumors in this study is that a definite percentage of tumors are indeed truly diploid and therefore cannot be distinguished from normal (benign) cells by this method. Only 1 of the 23 aneuploid histograms was hypodiploid (case 4, Table 2). Hypodiploidy is regarded by Dewald and associates\(^1\) and Korsgaard and associates\(^27\) as an unreliable indicator for malignancy in pleural effusions, because it may be caused by mechanical disturbance of chromosomes during processing of the specimen. Displacement of one or more chromosomes was seen at times in apparently normal metaphase in their material. Careful examination of the histogram in the case with hypodiploidy (Figure 3) shows that the G\(_0\)/G\(_1\) peak of the hypodiploid cells also has a prominent G\(_2\)M peak. This tends to in-
dicate that the hypodiploid cell population is actively dividing. Other investigators have also regarded hypodiploidy in the FCM as a supportive criterion for the diagnosis of malignancy.

Flow cytometric analysis of effusions can be of particular value in certain situations. When an aneuploid peak is present, a diagnosis of malignancy must be suspected. If the cytometric histogram is abnormal and the cytology screen normal, a reexamination of the cytologic slide is necessary to determine whether malignant cells may have been overlooked. Likewise, in the cytologic atypical case that has an abnormal histogram, a need for a repeat specimen or even a tissue biopsy is probably warranted. If ICM is also available, the detection of abnormal cases will be increased and both procedures should be considered complementary. FCM analyzes thousands of cells and can be performed rapidly. If only small numbers of malignant cells are present, ICM has an advantage because individual cells may be selectively analyzed and the results are not influenced by the presence of inflammatory or other benign cells. Finally, a definite proportion of malignant cells will be diploid and in these instances neither FCM nor ICM will be diagnostic and cytologic examination then is the definitive technique. If these cytometric techniques are not available to aid us, we would subscribe to Koss's dictum that great caution in diagnosis is desirable, because a positive cytologic diagnosis in an effusion not only provides a definitive diagnosis, but also implies a grave prognosis.

REFERENCES


