Automated quantification of DNA aneuploidy by image cytometry as an adjunct for the cytologic diagnosis of malignant effusion

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Abstract: DNA aneuploidy is a cancer biomarker, which may have a potential diagnostic value in body effusion specimen. DNA aneuploidy is determined by measuring the DNA content of tested cells and comparing them with diploid cells (2c). In order to assess the value of automated DNA image cytometry (DNA-ICM) in the cytologic diagnosis of effusion, we measured DNA ploidy using an automated DNA-ICM analysis system in 126 consecutive effusion specimens and followed the cases for histologic diagnosis. Half of each effusion specimen was used to prepare cytologic smears for conventional cytologic diagnosis, while the other half was used to prepare a monolayer slide stained by Feulgen stain for automated ICM. By using Youden index, we found that 4 cells exceeding 2.5c is the optimal cut off value for aneuploidy, which has a sensitivity of 88.3% and specificity of 100% for diagnosis of malignant effusion. We also found that the DNA aneuploidy thresholds used for other types of cytologic specimens cannot be used in the diagnosis of effusion specimens. Our study demonstrated that automated DNA image cytometry is a simple, practical and cost-effective method for adjunct diagnosis of malignant effusion.

Keywords: Aneuploidy, DNA image cytometry, cytology, effusion

1. Introduction

Malignant effusion is the accumulation of exudates in pleural, abdominal or pericardial cavities, characterized by presence of malignant cells within the body cavities. It is commonly seen in carcinomas of lung, breast, stomach, ovary and endometrium, either as a presenting sign or part of advanced metastatic disease. It is also often the presenting symptom of malignant mesothelioma. Prompt and accurate diagnosis of malignant effusion is clinically critical, as it represents cancer in advanced stage and requires immediate treatment [1, 2].

Although cytologic examination of body cavity fluids is a widely used routine clinical diagnostic method for the detection of malignant cells in effusion, the sensitivity of conventional cytology is low, ranging from 30% to 60% [3, 4]. The accuracy of cytologic diagnosis of effusion depends on sampling, technique used for preparation of cytology slide and experience of cytopathologists. Despite the development of new techniques and improvement in immunohistochemistry, distinguishing reactive mesothelial proliferation from mesothelioma and distinguishing mesothelial cells from metastatic adenocarcinoma remain diagnostic problems. While many experimental or molecular techniques may have potential values in assisting the cytologic diagnosis of effusions and increasing its diagnostic accuracy, only a few of them could be applied in the “real world” or in routine clinical practice in cytology laboratory, when costs of equipment...
and reagents, training of staff and efficacy of diagnosis are considered [3]. Automated quantitative image analysis of DNA aneuploidy has been shown to be a simple, practical and effective method for application of the biomarker in cytologic diagnosis [5–7]. DNA aneuploidy is a hallmark of cancer, resulting from genetic and chromosome instability of cancer cells, a common event in cancer carcinogenesis [8]. DNA aneuploidy has been reported as a prognostic and predictive biomarker of cancer. It was shown to be a predictive marker for progression of dysplasia in cervical cancer [9], transformation from dysplasia to malignancy in esophageal adenocarcinoma [10], and requirement of aggressive treatment in breast [11], ovarian [12] and endometrial cancers [13] as it is associated with adverse prognosis in those and other cancers [14]. Several recent studies have shown the value of automated quantitative analysis of DNA aneuploidy by image cytometry (ICM) in the cytologic diagnosis and screening of cervical, esophageal and oral cancer [5–7, 15], among others.

In order to assess the value of DNA aneuploidy determined by automated DNA-ICM in cytologic diagnosis of effusion, we conducted a study of 126 cases of effusion by conventional cytology and automated DNA-ICM, and estimated the optimal diagnostic threshold values of automated DNA-ICM for effusion cytology using follow up histology result as the gold standard.

2. Materials and methods

2.1. Patients and cytology specimens

This study was reviewed and approved by the Ethical Committee, Peking Union Medical Hospital, Beijing. Between December 2010 and October 2011, a total of 126 consecutive patients who had body cavity fluid were included in the study. Among the patients, 75 had pleural effusion, 47 had ascites and 4 had pericardial effusion. 49 patients were male and 77 patients were female. The age distribution of patients ranged from 25 to 82 years, with a median of 59 years old. All of the effusion specimens were received fresh without mixing with anticoagulant, and were processed immediately. The specimens were centrifuged for five minutes at 2500 rpm/min. The supernatant was discarded. Half of the sediment was used to prepare for cytology smears. This was done by transferring the sediment onto glass slide and spreading the sediment evenly between two slides by gently pulling them apart. The cytology smears were stained with hematoxylin and eosin (H/E). The other half of the sediment was placed into an alcohol based fixative. After vortex and centrifugation, an aliquot of 25 microliters of the cell pellet was placed on to a glass slide to make a monolayer slide for DNA staining.

2.2. Cytology diagnosis

The cytology smears stained with H/E staining were used for cytologic diagnosis. We divided the cytologic diagnosis into 4 classes: 1. Negative: effusions without presence of abnormal or malignant cells; 2. Atypical: presence of abnormal but no definite malignant cells; 3. Suspicious: presence of only sparse severely abnormal cells or equivocal malignant cells; 4. Positive: effusions containing unequivocal malignant cells. Cytologic diagnosis was made without knowledge of DNA aneuploidy result. Accuracy of cytologic diagnosis was compared with histologic diagnosis in tissue biopsy and surgical resection specimen from clinical follow-up assessment of all cases for about half a year.

2.3. Automated DNA ICM

DNA staining was carried out using the Feulgen method under temperature-controlled conditions according to the protocol of a DNA quantification kit (Motic, Xiamen, China). In brief, the slides were fixed in 10% buffered formalin, placed in 5 N HCl at 27°C for one hour for acid hydrolysis, then stained in Feulgen solution for another hour. They were then rinsed thoroughly with water for 15 minutes. Measurement of DNA ploidy of cells on the slide was carried out by an automated DNA image cytometry system (MoticClassify system, Motic Medical Diagnostic Service Inc., Xiamen, China). The system consisted of a computer controlled motorized microscope and high-resolution digital camera for image scanning and capturing, and software for controlling of image scanning, processing and analysis. The software used for the automatic classification of cells, measurement of DNA ploidy and analysis of aneuploidy was derived from the British Columbia Cancer Agency, Vancouver, Canada [16]. For each slide, 1000 cell nuclei or all of the cell nuclei on the slide were scanned and
nuclear images were collected into a nuclear DNA image gallery. The nuclei were automatically classified into normal epithelial/mesothelial nuclei, lymphocytes, neutrophils, and abnormal nuclei, according to a set of nuclear parameters, including nuclear morphology, nuclear texture features and nuclear DNA content. The specimens were considered satisfactory if at least 200 nuclei of mesothelial or epithelial cells were collected. Nuclei of rat hepatocytes were used as external control and for calibration of equipment to reach a coefficient of variation of less than 5%. The guidelines in the consensus report on DNA measurements were followed [17, 18]. The DNA content of each cell nucleus was measured and DNA ploidy was recorded as a ‘c’ value. A DNA ploidy of 2c indicates a normal diploid cell, and 4c indicates a tetraploid cell.

The DNA measurement was carried out by a member of study team who had no knowledge of the results of conventional cytologic diagnosis of the cases.

2.4. Estimate of optimal DNA ploidy thresholds for diagnosis of effusion

An aneuploid specimen was defined as a cytology specimen containing more than a predetermined number of cells whose DNA content exceeded a threshold value. We compared the DNA aneuploidy results of each case with its histologic diagnosis in follow-up tissue biopsy or surgical resection specimen, using the latter as the gold standard. We calculated the sensitivities and specificities of DNA aneuploidy for cytologic diagnosis of effusion by varying the threshold for the 2 parameters: the critical DNA content threshold for classifying a single nucleus as aneuploid and the number of aneuploid cells in a sample. Cellular DNA content thresholds ranging from 2.5c to 9c and aneuploid cell count thresholds ranging from 1 to 20 cells were used for the calculation. The optimal thresholds of DNA aneuploidy for the cytologic diagnosis of effusion were identified by analysis of the sensitivity and specificity of the combination of the 2 parameters.

2.5. Comparison with different aneuploidy criteria for cytologic diagnosis of cancer

Previous studies have reported cut off values of 3 cells with DNA ploidy index above 5c for cervical and esophageal cancer detection [5, 16], 3 cells with DNA ploidy index above 4.6c for oral cancer detection [7] as well as 1 cell exceeding 9c for tumor detection by DNA ploidy [19]. Sensitivity, specificity, PPV and NPV of DNA aneuploidy for diagnosis of effusion were also calculated using the previously described criteria of DNA image cytometry and compared with the optimal threshold values we identified in this study.

2.6. Statistical analysis

Sensitivity (Se), specificity (Sp), positive predictive value (PPV), and negative predictive value (NPV) were calculated for different diagnostic criteria using DNA image cytometry. Although the areas under the ROC curve (AUC) is the most commonly used index of diagnostic accuracy, the Youden Index (YI) is also frequently used [20]. The YI is defined as YI = Sensitivity + Specificity – 1 and ranges between 0 and 1. Complete separation of the distributions of the marker values for the diseased and healthy populations results in YI = 1, whereas complete overlap gives YI = 0. In this study, we used the YI as it estimates diagnostic accuracy similar to AUC but also has other attractive features not present in the AUC. YI provides a criterion for choosing the “optimal” threshold value, the threshold value for which Se(c) + Sp(c) - 1 is maximized. YI also requires no additional information such as prevalence rate and decision error cost.

3. Results

3.1. Cytologic diagnosis and follow up histologic diagnosis

Out of 126 cases, 26 showed no presence of abnormal or tumor cells and were diagnosed by conventional cytology as “negative”, which on follow up showed 1 small cell carcinoma and 1 lung adenocarcinoma histologically. 1 case showed an excessive amount of lymphocytes and was diagnosed by conventional cytology as “suspicious”, which on follow up lymph node biopsy was diagnosed histologically as lymphadenitis. 99 cases were diagnosed by conventional cytology as “positive” (Table 1). Among the cytology positive cases, 57 were adenocarcinomas either from ovary or lungs, confirmed by subsequent tissue biopsy or surgical resection; 2 cases were small cell carcinomas confirmed by lung biopsy and 3 cases were lymphomas (2 large B cell lymphoma and 1 T cell lymphoma) con-
firmed by lymph node biopsy. For 3 cases, we applied immunohistochemistry using cell block. One of these was diagnosed by immunohistochemistry as malignant mesothelioma, while the other two were diagnosed as clear cell carcinomas from ovary and uterine cervix, all of them were confirmed in follow-up histologic diagnosis. The remaining 34 cytology positive cases could not be clearly classified or traced to their origins. The sensitivity and specificity of the conventional cytology with the assistance of immunohistochemistry are 98% and 96%, respectively, if the suspicious cytology diagnosis is counted as positive.

3.2. Diagnosis of malignant effusion by DNA aneuploidy

Out of the 126 cases submitted for the DNA ICM analysis, 9 cases were inadequate for the analysis because of insufficient cell numbers. Only 117 cases were included in this study. The cytologic diagnosis for the 117 cases were as follows: 21 negative cases; 96 positive cases which included 58 adenocarcinoma, 3 small cell carcinoma, 2 lymphoma, 1 clear cell carcinoma, 1 mesothelioma and 31 of malignancy of unknown origin.

| Cytologic diagnosis | Numbers of cases |
|---------------------|-----------------|
| Positive            |                 |
| Adenocarcinoma      | 57              |
| Small cell carcinoma| 2               |
| Mesothelioma        | 1               |
| Lymphoma            | 3               |
| Clear cell carcinoma| 2               |
| Unclassifiable      | 34              |
| Suspicious          | 1               |
| Atypical            | 0               |
| Negative            | 26              |
| Total               | 126             |

Fig. 1. Sensitivity (A) and specificity (B) of diagnosis of effusion by DNA ploidy expressed as cut off values of DNA ploidy index (ranging from 2.5c to 9c) and minimum number of cells (ranging from 1 to 20 cells).
unknown primary. Table 2 and Fig. 1 show the sensitivity and the specificity of DNA aneuploidy using various thresholds based on combinations of the 2 parameters of ploidy.

3.3. Optimization of DNA ploidy parameters for diagnosis of malignant effusion

Figure 2 showed that when the number of cells with DNA ploidy 2.5c was more than 4 cells, the YI was maximized, with a corresponding sensitivity and specificity of 83.3% and 100%, respectively. Therefore, the optimal cut off threshold value of DNA image cytometry in effusion diagnosis was 4 cells with DNA ploidy exceeding 2.5c.

3.4. Comparison with different aneuploidy criteria for cytologic diagnosis of malignant effusion

Table 3 lists the cut off threshold values for different kinds of cancers detected by DNA aneuploidy using image cytometry in comparison to the optimal threshold value of DNA aneuploidy for diagnosis of effusion we identified in this study. The result indicates that threshold value of 4 cells exceeding 2.5c has much better sensitivity and specificity as compared to the criteria used for other types of cytology specimens or cancer detection.

4. Discussion

For many years, cytologic examination of effusion cell either by conventional cytology smear or by monolayer preparation has been the principal method for diagnosis of malignant effusion [21]. However, the ability of visual detection of cancer cells in effusion samples is heavily dependent on the diagnostic skill and experience of cytopathologists. Low sensitivity in effusion cytology by conventional cytology in some situations may be due to inexperienced cytopathologists and lack of an adjunct tool for cytologic diagnosis of effusion [22, 23].

As a proven cancer biomarker, DNA aneuploidy has a potential to become an adjunct for routine cytologic diagnosis of effusion. DNA aneuploidy is assessed by measurement of DNA ploidy in cells and by comparing them with diploid cells. Both flow cytometry and image cytometry (ICM) can be used to measure DNA ploidy for the cytometric diagnosis of cancer. Previous studies using DNA ploidy for diagnosis of effusion showed that ICM was more sensitive than flow cytometry [24, 25]. Motherby et al. [25] measured DNA ploidy in 200 effusion cytology specimens and found that the sensitivity and specificity were 75% and 100% by ICM but were 32.1% and 100% by flow cytometry. By using ICM for quantification of DNA aneuploidy, several other studies showed that DNA aneuploidy was more sensitive than conventional cytology for detection of malignant cells in effusion specimen [19, 26, 27]. El-Habashi et al. [27] reported that sensitivity,
Table 2

Sensitivity, specificity and Youden Index of cytologic diagnosis of effusion by DNA ploidy using different DNA ploidy parameters obtained from automated DNA-image cytometry.

| DNA ploidy (C) | Number of cells | TP   | TN   | Se   | Sp   | Youden index |
|----------------|----------------|------|------|------|------|---------------|
| 2.5 ≥ 1        | 91             | 9    | 97.85% | 37.50% | 35.35% |
| 2.5 ≥ 2        | 88             | 15   | 94.62% | 62.50% | 57.12% |
| 2.5 ≥ 3        | 83             | 19   | 88.25% | 79.17% | 68.41% |
| 2.5 ≥ 4        | 77             | 21   | 82.80% | 87.50% | 70.30% |
| 2.5 ≥ 5        | 73             | 23   | 78.49% | 93.83% | 74.33% |
| 2.5 ≥ 10       | 63             | 23   | 67.74% | 93.83% | 63.38% |
| 2.5 ≥ 20       | 53             | 24   | 56.99% | 100.00% | 56.99% |
| 3 ≥ 1          | 89             | 14   | 95.70% | 58.33% | 54.03% |
| 3 ≥ 2          | 88             | 15   | 94.62% | 62.50% | 57.12% |
| 3 ≥ 3          | 83             | 19   | 88.25% | 79.17% | 68.41% |
| 3 ≥ 4          | 77             | 21   | 82.80% | 87.50% | 70.30% |
| 3 ≥ 5          | 73             | 23   | 78.49% | 93.83% | 74.33% |
| 3 ≥ 10         | 63             | 23   | 67.74% | 93.83% | 63.38% |
| 3 ≥ 20         | 53             | 24   | 56.99% | 100.00% | 56.99% |
| 3.5 ≥ 1        | 89             | 14   | 95.70% | 58.33% | 54.03% |
| 3.5 ≥ 2        | 88             | 15   | 94.62% | 62.50% | 57.12% |
| 3.5 ≥ 3        | 83             | 19   | 88.25% | 79.17% | 68.41% |
| 3.5 ≥ 4        | 77             | 21   | 82.80% | 87.50% | 70.30% |
| 3.5 ≥ 5        | 73             | 23   | 78.49% | 93.83% | 74.33% |
| 3.5 ≥ 10       | 63             | 23   | 67.74% | 93.83% | 63.38% |
| 3.5 ≥ 20       | 53             | 24   | 56.99% | 100.00% | 56.99% |
| 4 ≥ 1          | 75             | 21   | 80.65% | 95.83% | 74.33% |
| 4 ≥ 2          | 73             | 21   | 78.49% | 93.83% | 69.99% |
| 4 ≥ 3          | 69             | 21   | 73.08% | 96.87% | 66.97% |
| 4 ≥ 4          | 67             | 22   | 67.74% | 93.83% | 63.17% |
| 4 ≥ 5          | 63             | 23   | 64.29% | 95.83% | 61.12% |
| 4 ≥ 10         | 54             | 24   | 58.06% | 100.00% | 58.06% |
| 4 ≥ 20         | 42             | 24   | 45.16% | 100.00% | 45.16% |
| 4.2 ≥ 1        | 66             | 22   | 70.97% | 95.83% | 65.97% |
| 4.2 ≥ 2        | 57             | 23   | 64.29% | 95.83% | 61.12% |
| 4.2 ≥ 3        | 51             | 24   | 57.31% | 100.00% | 57.31% |
| 4.2 ≥ 4        | 45             | 24   | 48.39% | 100.00% | 48.39% |
| 4.2 ≥ 5        | 41             | 24   | 44.09% | 100.00% | 44.09% |
| 4.2 ≥ 10       | 31             | 24   | 33.33% | 100.00% | 33.33% |
| 4.2 ≥ 20       | 20             | 24   | 21.51% | 100.00% | 21.51% |
| 4.4 ≥ 1        | 62             | 23   | 66.67% | 95.83% | 62.50% |
| 4.4 ≥ 2        | 51             | 24   | 54.84% | 100.00% | 54.84% |
| 4.4 ≥ 3        | 44             | 24   | 47.31% | 100.00% | 47.31% |
| 4.4 ≥ 4        | 37             | 24   | 39.78% | 100.00% | 39.78% |
| 4.4 ≥ 5        | 32             | 24   | 34.41% | 100.00% | 34.41% |
| 4.4 ≥ 10       | 22             | 24   | 23.66% | 100.00% | 23.66% |
| 4.4 ≥ 20       | 10             | 24   | 10.75% | 100.00% | 10.75% |
| 4.8 ≥ 1        | 58             | 23   | 62.37% | 95.83% | 58.20% |
| 4.8 ≥ 2        | 50             | 24   | 53.76% | 100.00% | 53.76% |
| 4.8 ≥ 3        | 43             | 24   | 46.24% | 100.00% | 46.24% |
| 4.8 ≥ 4        | 34             | 24   | 36.56% | 100.00% | 36.56% |
Table 2

| DNA ploidy (C) | Number of cells | TP | TN | Se | Sp | Youden index |
|----------------|-----------------|----|----|----|----|--------------|
| 4.8 ≥5          | 28              | 24 | 100.00% | 38.11% | 0.11% |
| 4.8 ≥10         | 19              | 24 | 100.00% | 20.43% | 0.43% |
| 4.8 ≥20         | 8               | 24 | 100.00% | 8.60%  | 0.60% |
| 5 ≥1            | 57              | 23 | 64.29%  | 95.83% | 57.12% |
| 5 ≥2            | 47              | 24 | 50.54%  | 100.00%| 50.54% |
| 5 ≥3            | 41              | 24 | 44.09%  | 100.00%| 44.09% |
| 5 ≥4            | 28              | 24 | 30.11%  | 100.00%| 30.11% |
| 5 ≥5            | 26              | 24 | 27.96%  | 100.00%| 27.96% |
| 5 ≥10           | 18              | 24 | 19.35%  | 100.00%| 19.35% |

Table 3

| DNA ploidy (c) | Cell numbers | Sensitivity | Specificity | PPV | NPV |
|----------------|--------------|-------------|-------------|-----|-----|
| 2.5            | 4            | 83.3%       | 100.00%     | 100.00% | 53.5% |
| 4.6            | 5            | 33.3%       | 100.00%     | 100.00% | 24.7% |
| 5              | 3            | 42.7%       | 100.00%     | 100.00% | 27.6% |
| 9              | 1            | 26.0%       | 100.00%     | 100.00% | 25.9% |

Diagnostic accuracy using DNA ploidy diagnostic threshold values of different kinds of cytologic specimens.

In our study, we found that DNA threshold value for effusion diagnosis is 2.5c. This is somewhat similar to the DNA Index 1.15 (2.3c) reported by Osterheld et al. [29] in a study using semi-automatic DNA ICM on Feulgen stained cytology slides in 42 effusion specimens. However, our threshold value is quite different from 9c or ≥10% variation in first DNA stem line.
the cut off value reported by Motherly et al. [19] in a study of 310 cases of effusion specimens using manual ICM method. The difference of DNA threshold values between our study and previous studies done by manual methods may be due to differences in cytology slide preparation, DNA staining method used and manual selection of cells for measurement of DNA. Automated DNA ICM on a monolayer cytology slide stained by Feulgen staining described in our study may offer a more reproducible method for aneuploidy quantification as compared to manual DNA ICM on cytology smear of an air dried cytology slide.

In our study, we found that the cut off value of 5c for aneuploidy determined by automated DNA ICM used for cervical and esophageal cancer detection [5, 6, 16] and 4.6c use for oral cancer detection should not be used as threshold values for effusion cytology diagnosis, as it is not as robust as the 2.5c we identified. This is due to the fact that malignant cells in cervical, esophageal and oral cancer are squamous cell carcinoma while malignant cells in effusion are largely adenocarcinoma.

Immunohistochemistry analysis using cellblock is a powerful adjunct tool in effusion cytology. A panel of antibodies including Ber Ep4, MOC 31, calretinin, CK5/6, WT-1 and others has been shown to have high sensitivity and specificity for differential diagnosis between reactive mesothelial cells and metastatic adenocarcinoma in effusion specimen [22, 23]. In our study, we applied immunohistochemistry in 3 difficult cases for differential diagnosis. The high sensitivity and specificity in our cytology diagnosis is the result of a combination of conventional cytology and immunohistochemistry. Indeed, a few earlier studies have found that a combination of Ber EP4 antibody immunostain and DNA aneuploidy analysis significantly increased the sensitivity and specificity of cytologic diagnosis of effusion [30, 31]. However, immunohistochemistry may not be available in some situations, when there is not enough cells present in cytology sample, or when cytology sample is inadequately or poorly fixed before being received by the pathology laboratory, and in most of the small hospitals in China and developing countries, as they do not have automated immunostainers or access to a full list of the antibody panel needed for quality immunohistochemistry. The cost of a full panel of immunohistochemistry for effusion diagnosis is now higher than that of automated ICM. In addition, immunohistochemistry is not useful for distinction of reactive mesothelial cells from malignant mesothelioma. In those situations, DNA aneuploidy analysis could provide a practical and effective adjunct for cytologic diagnosis of effusion.

In conclusion, this study demonstrated that automated DNA ICM is a simple, easy to use and practical method for adjunct cytologic diagnosis of effusion. Optimal threshold value for effusion cytology specimen is different from those used for other cytology specimens. A cut off value of 4 cells with ploidy exceeding 2.5c is the optimal threshold value for the cytologic diagnosis of malignant effusion.

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