Assessment of Plasma DNA Levels, Allelic Imbalance, and CA 125 as Diagnostic Tests for Cancer


Background: Allelic imbalance (AI), the loss or gain of chromosomal regions, is found in many cancers. AI can be detected in genomic tumor DNA released into the blood after necrosis or apoptosis. We evaluated plasma DNA concentration, allelic status in plasma DNA, and serum CA 125 level as screening tests for ovarian and other cancers. Methods: Plasma samples were obtained from 330 women (44 normal healthy control individuals, 122 patients with various cancers, and 164 control patients with non-neoplastic diseases). Plasma DNA concentration was determined in all samples. Allelic status was determined by digital single nucleotide polymorphism (SNP) analysis with eight SNP markers in plasma DNA from 54 patients with ovarian cancer and 31 control patients. CA 125 was determined in 63 samples. Receiver-operating characteristic (ROC) curves were plotted, and the areas under the ROC curves—a measure of the overall ability of a diagnostic test with multiple cutoffs to distinguish between diseased and nondiseased individuals—were determined. Results: The area under the ROC curve for plasma DNA concentration was 0.90 for patients with neoplastic disease versus healthy control individuals and 0.74 for patients with neoplastic diseases versus control patients with non-neoplastic diseases. For control subjects given a specificity of 100% (95% confidence interval [CI] = 92% to 100%), the highest sensitivity achieved was 57% (95% CI = 49% to 67%). AI in at least one SNP was found in 87% (95% CI = 60% to 98%) of patients with stage I/II ovarian cancer and 95% (95% CI = 83% to 99%) of patients with stage III/IV ovarian cancer, but AI was not found in 31 patients with non-neoplastic diseases (specificity = 100%, 95% CI = 89% to 100%). The area under the ROC curve assessing AI was 0.95. Combining the serum CA 125 level with the plasma DNA concentration increased the area under the ROC curve from 0.78 (CA 125 alone) to 0.84. Conclusion: Plasma DNA concentration may not be sensitive or specific enough for cancer screening or diagnosis, even when combined with CA 125. AI was detected with high specificity in plasma DNA from patients with ovarian cancer and should be studied further as a screening tool. [J Natl Cancer Inst 2002;94:1697–1703]
survival rate is 90% for women with tumors confined to the ovary. Thus, the development of a plasma-based molecular diagnostic test will be extraordinarily useful in identifying asymptomatic patients with early and clinically curable neoplastic diseases.

**Materials and Methods**

**Samples and DNA Purification**

A total of 330 plasma samples obtained from females were retrieved from the Gynecological Pathology Tumor Bank and the Division of Clinical Chemistry in the Department of Pathology, The Johns Hopkins University School of Medicine. The waiver of patients’ consent was obtained through the local institutional research board. Plasma samples were obtained from 54 patients with sporadic ovarian cancer, 68 patients with malignant neoplasms with other tissue origins, 164 patients with a variety of non-neoplastic diseases who were admitted to The Johns Hopkins Hospital for treatment, and 44 healthy individuals without known neoplastic diseases who visited The Johns Hopkins Hospital for a routine physical examination. Patients with non-neoplastic diseases were age-matched with the 54 patients with ovarian tumors. Of the ovarian tumors studied, 12 were stage I (the International Federation of Gynecology and Obstetrics Staging System), three were stage II, 36 were stage III, and three were stage IV. A histologic examination classified these tumors as follows: 41 serous carcinomas, six borderline tumors, three endometrioid tumors, two clear cell carcinomas, one granulosa cell tumor, and one immature teratoma. The selection of samples for this study was based on the availability of specimens in the tumor bank but not on the disease category or on other patients’ clinical profiles. The plasma DNA concentration was determined for all 330 patients, and plasma DNA from the 54 patients with ovarian cancer and the 31 patients with benign diseases was analyzed by digital SNP analysis. CA 125 levels were determined for 45 patients with ovarian cancer and benign diseases was age-matched with the 54 patients with sporadic ovarian cancer, 68 patients with non-neoplastic diseases by use of the digital SNP analysis (18–20,25). The sequences of eight SNP markers with a high frequency of allelic losses in ovarian carcinomas (26–29) were retrieved from the National Cancer Institute SNP map (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp). The primers and molecular beacons were synthesized by Gene Link (Thornwood, NY), and their sequences are listed in Fig. 1 (19,20,25).

**Digital SNP Analysis**

AI was assessed in plasma DNA from 54 patients with ovarian tumors and from 31 age-matched women with non-neoplastic diseases by use of the digital SNP analysis (18–20,25). The sequences of eight SNP markers with a high frequency of allelic losses in ovarian carcinomas (26–29) were retrieved from the National Cancer Institute SNP map (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp). The primers and molecular beacons were synthesized by Gene Link (Thornwood, NY), and their sequences are listed in Fig. 1 (19,20,25).

**Fraction of Tumor-Released DNA in Plasma**

Digital SNP analysis was used to determine the fraction of total plasma DNA represented by tumor-released DNA as follows: let A be the number of the abundant alleles in plasma, B be the number of the minor alleles in plasma, n denote DNA derived from normal cells, and t denote DNA derived from tumor cells; then \( A = A_n + A_t \) and \( B = B_n + B_t \). If AI in the primary tumor is homogenous, the fraction of tumor-released DNA in plasma \( f_t \) is equal to \( (A_t + B_t)/(A + B) \). Both alleles are in balance in DNA released from normal cells (i.e., \( A_t/B_n = 1:1 \) and \( A_n = B_n \)), because two alleles in normal cells are composed of one maternal and one paternal allele. In human tumors, AI is most often associated with loss of heterozygosity, i.e., loss of one of the parental alleles present, either the maternal or paternal allele, in the patient’s normal cells (31,32). Therefore, \( B_t = 0 \) and \( B = B_n \). Given that \( A_n = B_n \) and \( B_n = B \), then \( f_t = (A_t + B_t)/(A + B) = (A_t)/(A + B) = (A - A_n)/(A + B) = (A - B)/A \). The numbers of A and B alleles are determined by the digital SNP analysis.
Statistical Analysis

Receiver-operating characteristic (ROC) curves were used to assess the feasibility of using plasma DNA concentration and allelic status as diagnostic tools for detecting ovarian and other cancers. An ROC curve is a graphic representation of the sensitivity plotted against the false-positive rate (i.e., 1 minus specificity), and the ROC curve is used to evaluate the performance of a test at different thresholds of a diagnostic measure. The area under the ROC curve is a measure of the overall ability of a diagnostic test with multiple cutoffs to distinguish between diseased and control individuals.

For the plasma DNA concentration, two separate analyses were performed: one with patients with non-neoplastic disease as control subjects and the other with healthy patients without known major diseases as control subjects. Plasma DNA concentration was evaluated at cutoff values of 5, 10, 15, 23, 30, 40, 60, 100, 200, and 500 ng/mL. For the analysis of allelic status, the allelic proportion (abundant alleles/total alleles) was evaluated at cutoff values of 0.55, 0.60, 0.65, 0.70, 0.75, 0.80, 0.85, 0.90, and 0.95. AI is defined in this system as having at least one informative marker above the cutoff among the eight markers tested. With the use of the ROC curves, we selected the point with the highest sensitivity, given 100% specificity, and calculated the exact binomial 95% confidence interval (CI) for the corresponding sensitivity and specificity. A high specificity is desirable for screening technologies that could be applied to a population of asymptomatic individuals with a very low prevalence of disease.

Logistic regression models were used to evaluate whether the combination of plasma DNA concentration and CA 125 level increased the sensitivity and the specificity for detecting ovarian cancer and to generate ROC curves. CA 125 was used both as a continuous and dichotomous predictor; the standard clinical normal upper limit for CA 125 of 35 IU/mL was applied (21). The areas under the ROC curves with and without the inclusion of plasma DNA concentration were measured. All statistical tests were two-sided.

RESULTS

Quantitation of Plasma DNA

Plasma DNA concentrations were determined for 330 individuals: 122 patients with neoplastic disease, 164 patients with non-neoplastic diseases, and 44 healthy individuals. The specific diagnoses of the patients with neoplastic and non-neoplastic diseases are presented in Table 1. Plasma DNA concentration ranged from 0 to 6707 ng/mL. The median DNA concentrations were as follows: in the 44 healthy patients, 7 ng/mL (10th–90th percentile = 0–20 ng/mL); in the patients with non-neoplastic diseases, 16 ng/mL (10th–90th percentile = 7–71 ng/mL); and in the patients with neoplastic diseases, 59 ng/mL (10th–90th percentile = 10–844 ng/mL) (P < .001, Kruskal–Wallis test). The area under the ROC curve assessing plasma DNA concentration was 0.90 for healthy control subjects and 0.74 for control patients with a non-neoplastic disease (Fig. 2). For healthy control subjects, given a specificity of 100% (95% CI = 92% to 100%), the highest sensitivity that could be obtained was 57% (95% CI = 49% to 67%). The sensitivity of plasma DNA concentration at any given specificity was substantially lower when applied to control patients with a non-neoplastic disease (Fig. 2). Although the number of control patients in each subgroup of benign diseases was small, no association was detected between DNA concentrations and a particular benign disease.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy normal</td>
<td>44</td>
</tr>
<tr>
<td>Non-neoplastic disease</td>
<td>164</td>
</tr>
<tr>
<td>Anemia</td>
<td>8</td>
</tr>
<tr>
<td>AIDS (acquired immunodeficiency syndrome)</td>
<td>10</td>
</tr>
<tr>
<td>Musculoskeletal disease</td>
<td>10</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>7</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>13</td>
</tr>
<tr>
<td>Asthma and pulmonary disease</td>
<td>5</td>
</tr>
<tr>
<td>Essential hypertension</td>
<td>6</td>
</tr>
<tr>
<td>Infectious disease</td>
<td>19</td>
</tr>
<tr>
<td>Autoimmune disease</td>
<td>10</td>
</tr>
<tr>
<td>Status post organ transplant</td>
<td>23</td>
</tr>
<tr>
<td>Liver disease</td>
<td>9</td>
</tr>
<tr>
<td>Trauma</td>
<td>8</td>
</tr>
<tr>
<td>Neurologic disorder</td>
<td>12</td>
</tr>
<tr>
<td>Gynecologic disease</td>
<td>10</td>
</tr>
<tr>
<td>Drug abuse</td>
<td>8</td>
</tr>
<tr>
<td>Others</td>
<td>8</td>
</tr>
<tr>
<td>Neoplastic disease</td>
<td>122</td>
</tr>
<tr>
<td>Ovarian tumor</td>
<td>54</td>
</tr>
<tr>
<td>Endometrial/cervical carcinoma</td>
<td>10</td>
</tr>
<tr>
<td>Head and neck carcinoma</td>
<td>11</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>4</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>8</td>
</tr>
<tr>
<td>Lung carcinoma</td>
<td>11</td>
</tr>
<tr>
<td>Gastrointestinal carcinoma</td>
<td>11</td>
</tr>
<tr>
<td>Brain tumor</td>
<td>5</td>
</tr>
<tr>
<td>Others</td>
<td>6</td>
</tr>
</tbody>
</table>

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Digital SNP analysis was performed to assess AI in plasma DNA from 54 patients with ovarian neoplasms and 31 patients with non-neoplastic diseases who had plasma DNA levels greater than 50 ng/mL. The median number of informative markers per patient was 4 (10th–90th percentile = 2–6). The highest sensitivity and specificity were obtained at a threshold level for allelic proportion of 0.6. The specificity was 100% (31 of 31, 95% CI = 89% to 100%), and the sensitivity was 93% (50 of 54, 95% CI = 82% to 98%). None of the 31 control patients with non-neoplastic diseases had AI. When the cutoff of 0.6 was used to define AI, the sensitivity of AI in patients with early-stage (I/II) ovarian cancer was 87% (13 of 15, 95% CI = 60% to 98%), and the sensitivity of AI in patients with late-stage (III/IV) ovarian cancer was 95% (37 of 39, 95% CI = 83% to 99%). The area under the ROC curve assessing AI was 0.95 (Fig. 3, A).

For comparison, the area under the ROC curve assessing plasma DNA concentration as the diagnostic tool to detect ovarian tumors was 0.75 (Fig. 3, B). Requiring a specificity of 100% (95% CI = 89% to 100%), the highest sensitivity achieved was 54% (95% CI = 40% to 67%) at a cutoff of 60 ng/mL. The sensitivity for early-stage (I/II) ovarian cancer was 47% (7 of 15, 95% CI = 21% to 73%), and the sensitivity for late-stage (III/IV) ovarian cancer was 56% (22 of 39, 95% CI = 40% to 72%).

When digital SNP analysis was used to determine the fraction of tumor-released DNA in total plasma DNA \( f_t \), we found that tumor-released DNA contributed substantially to the total plasma DNA in the majority of samples with an \( f_t \) average of 0.48 (95% CI = 0.43 to 0.53) and a range from 0.26 to 0.89. We
were unable to demonstrate that the $f_l$ was associated with the amount of total plasma DNA because the $r$ (correlation coefficient of linear regression) value was only 0.3.

The digital SNP analysis was validated by using eight representative samples, including four with AI and four with allelic balance, and repeating the digital SNP analysis three times on plasma aliquots from the same patient. The allelic ratios were consistent between the original and the repeated assays in all samples tested (data not shown). To assess the AI pattern in the corresponding primary tumors, we determined the allelic status of the tumors with the same eight SNP markers in 17 representative tumors with available tissue. Among these 17 tumors, 15 had an AI pattern identical to that of the corresponding plasma DNA sample. The remaining two tumors showed a discordant AI pattern in at least one informative SNP marker.

**Combined Analysis of Plasma DNA Concentration and Serum CA 125 Level**

Serum CA 125 data were determined for 63 of the 85 patients with digital SNP analyses: 45 with ovarian cancer and 18 with a non-neoplastic disease. The area under the ROC curve assessing CA 125 was 0.78 when serum CA 125 level alone was used as a continuous measure (Fig. 4, A). When a combination of plasma DNA with serum CA 125 derived from a logistic regression equation was used, the area under the ROC curve assessing the combination of CA 125 and plasma DNA concentration was 0.84 (Fig. 4, B; $P = .08$ for incremental contribution of DNA level). The areas under the curves were similar when the CA 125 cutoff value of 35 IU/ml (the standard clinical cutoff) was used. With this cutoff value, the sensitivity was 67% (95% CI = 51% to 80%) and the specificity was 89% (95% CI = 65% to 99%).

**DISCUSSION**

The results of this study indicate that measurement of plasma DNA concentrations has a sensitivity and specificity for cancer that may be too low for population screening. In contrast, digital SNP analysis, which allows the number of the two parental alleles in plasma to be precisely counted and the allelic status to be unequivocally determined in the background of normal DNA, appears to be a promising method for detecting at least ovarian cancer. This method should be tested more extensively on ovarian and other cancers.

Because tumors release DNA into plasma, we expected that increased amounts of plasma DNA would be detected in the majority of cancer patients and that a simple measurement of plasma DNA would offer a cost-effective approach for population-based cancer screening. In this study, even though there was a highly statistically significant difference in the average plasma DNA levels between cancer patients and both healthy control subjects and control patients with non-neoplastic disease, no cutoff value for plasma DNA concentration produced performance characteristics that would make it a good screening tool for neoplastic disease.

Why plasma DNA levels were not elevated in some patients with neoplastic disease, even when their tumors were large, is unclear. Perhaps minimal cell death resulted in a slow release of tumor DNA and/or the half-life of plasma DNA was short because of a high clearance rate. Digital SNP analysis of plasma DNA from patients with ovarian cancer and control patients with non-neoplastic diseases and with elevated plasma DNA showed that these tumors contributed a substantial fraction of DNA in the majority of patients, even in those with low absolute levels of plasma DNA. The allelic balance identified in the plasma DNA from four cancer patients could indicate that all markers tested in the corresponding primary tumors also showed allelic balance. Alternatively, AI might be masked by the overwhelming amount of normal DNA that may have originated from allelically balanced nontumor cells (3,33,34). The above findings are consistent with the conclusion from a previous report that detected AI with quantitative methylation-specific PCR (3). Although the AI pattern in plasma and in the corresponding tumor was concordant in most patients analyzed, two patients did not show the identical pattern. The discordant allelic pattern in the plasma and the primary tumor has been recently reported, and it likely reflects intratumoral clonal heterogeneity and biased tissue sampling (35).
How does our method compare with current screening approaches for ovarian cancer? Although pelvic and, more recently, vaginal sonography have been used to screen high-risk patients, both techniques lack sufficient sensitivity and specificity to screen the general population (36). CA 125 is used in the postoperative management of patients with ovarian carcinoma, but it lacks sufficient sensitivity and specificity as a screening tool. Specifically, CA 125 is negative in 30%–40% of patients with ovarian carcinomas, and its levels are elevated in a variety of benign diseases (37–39). Combining results of CA 125 and plasma DNA testing did little to enhance the predictive value of either, because they were closely correlated.

There is little evidence that measurement of AI alone would provide tissue-specific information regarding tumor sites because of the high frequency of genetic instability in a variety of cancer types. This problem may limit the usefulness of AI as a clinical diagnostic test for the early detection of a particular type of cancer. There are, however, many existing or emerging biomarkers that are relatively tissue-specific, yet their sensitivity is too low for them to be used as stand-alone diagnostic tests (21). It is possible that a combination of determining AI and testing for one or more tissue-specific serologic biomarkers could produce a diagnostic index that is cancer-type specific and has a much improved sensitivity over the original biomarkers.

This report provides preliminary evidence that AI in plasma DNA can be detected with apparently high specificity by digital SNP analysis in a substantial percentage of patients with potentially curable ovarian carcinomas. This evidence suggests that this technology might be useful in patients who present with adnexal masses. However, before digital SNP analysis becomes a practical cancer screening tool, several issues need to be addressed. In this study, the sensitivity of digital SNP analysis in diagnosing early-stage ovarian cancer was 87% (95% CI = 60% to 98%). Sensitivity might be improved by increasing the number of SNPs in the assay to determine whether sensitivity could be increased without decreasing specificity. Results of a digital SNP analysis could also be combined with results for tests of mutant genes specific for ovarian cancer. The effect on sensitivity of broadening the array of cancers tested will have to be studied.

It should be noted that the specificity and the sensitivity obtained with the optimal cutoff value from this study will probably not be as high when applied to other sets of patients. In addition, the 95% CIs in this study were fairly wide. The lower confidence bound on the 100% specificity estimate was 98%, meaning that more noncancer patients must be studied to increase the precision of that estimate before the test is widely applied. The control population in our SNP study was selected to have a high concentration of plasma DNA. Although we thought that this selection would only decrease specificity, that has not been demonstrated, and it will be important to confirm these numbers with more representative control subjects in prospective cohort studies.

Finally, it should be noted that although the current cost of digital SNP analysis is quite high (> $200 per test), it could be fully automated by using a high-throughput format to reduce labor and reagent cost. Because digital SNP analysis is based on the discrimination of SNP on a single molecule basis, powerful new tools being developed for nanotechnology could be applicable (40,41).

REFERENCES


NOTES

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