Overall, the median concentration of post-TOP fDNA among all patients was not significantly increased over the median pre-TOP fDNA concentration, but our data show an increase in fDNA after TOP only after 8 weeks of gestation. This increase occurred in 11 of 17 patients when pregnancy was later than 9.5 weeks of gestation (Fig. 1). The increase in fDNA at later gestations could be attributable to either an increase in placental size/mass or disruption of the fetomaternal circulation. Using Doppler studies, Jauniaux et al. (7) showed that placental blood flow is not established until 8–9 weeks of gestation. Thus, we would not necessarily expect to find FMH after TOP earlier than this gestational age. In support of this, Leong et al. (8) found no fetal cells in the blood samples of women after surgical TOP at $<$6 weeks menstrual age. We therefore suggest that the source of this DNA may be fetal hematopoietic cells within newly established placental blood vessels that are disrupted as a result of the TOP procedure and that the increased fDNA concentrations may indicate excessive FMH after 9 weeks of gestation.

Plasma fDNA concentrations in many patients, however, unexpectedly decreased after the TOP procedure. Because no intravenous fluid bolus was given to any patient before the procedure, a dilution effect is unlikely. This decrease might be explained instead by the physiologic differences in how fDNA enters the maternal circulation. It is generally believed that in pregnant women the source of cell-free fetal nucleic acids is placentally derived apoptotic cells (9). Some fDNA sequences are detectable in membrane-bound apoptotic vesicles (10). This particle-associated form is believed to protect fetal nucleic acids from degradation by nucleases in maternal blood (11). After elective TOP, fDNA is liberated directly into the maternal circulation from the sudden disruption of the fetomaternal interface; thus it may not be protected in apoptotic bodies. We therefore suggest that unprotected posttermination fDNA sequences are vulnerable to destruction by maternal nucleases, leading to the rapid decrease in these sequences after elective termination in some patients.

Future study should define the association between post-TOP cell-free fDNA concentrations, measured by real-time PCR, and cellular trafficking, determined by the traditional Kleihauer–Betke test, in a large cohort of patients at various gestational ages. In addition, the correlation between the alteration in fDNA concentrations and triggering of the maternal immune response remains to be elucidated. Current measurement of fDNA is based on Y-chromosome-specific sequence detection, which is not applicable to women who carry a female fetus. Therefore, the continued development of fetal gender-independent markers, such as fetal/placental specific mRNA, in the maternal circulation is essential (12). Comparison of fetal globin gene expression with placently derived gene expression may allow us to definitively determine whether the increased fetal nucleic acids seen after 9 weeks of gestation in maternal plasma are from a placental or hematopoietic source (13).

Dr. Wataganara’s maternal-fetal medicine fellowship is supported in part by Anandamahidol Foundation, Thailand. We thank Olivera Vragovic for help in organizing the study and enrolling the patients, and Dr. Dittakarn Boriboonthirumson for insightful comments on the manuscript.

References

Previously published online at DOI: 10.1373/clinchem.2004.042135

Biological Variation of Tumor Markers and Its Application in the Detection of Disease Progression in Patients with Non-Small Cell Lung Cancer, Jaume Trape1,** Joaquim Pérez de Olaguer2, Josep Buxó2, and Laura López1 1Clinical Laboratory and 2 Service of Oncology, Hospital Sant Joan de Déu, Althaia Xarxa Assistencial de Manresa, Barcelona, Spain; * address correspondence to this author at: Clinical Laboratory, Hospital Sant Joan de Déu, Althaia Xarxa Assistencial de Manresa, Dr Joan Soler, s/n, 08243 Manresa (Barcelona), Spain; fax 34-93-8743859, e-mail jtrape@yahoo.es

Patients with advanced non-small cell lung cancer (NSCLC) may benefit from chemotherapy (1, 2). This type of treatment is highly toxic and expensive, and patients should be monitored to detect those in whom the disease progresses despite treatment to avoid ineffective treatment and a loss of quality of life. Tumor markers are
useful for detecting progression in cancer patients undergoing chemotherapy\(^{(3, 4)}\), but there are no uniform criteria establishing how large a difference between two consecutive measurements should be to indicate progression of the disease.

The first step in dynamic assessment of a specific biological constituent is to define the difference between two consecutive results that indicates a change in the patient's health status. The most widely accepted approach for this purpose is the so-called reference change value (RCV), a concept described by Harris and Brown\(^{(5)}\). Using serial analytic results from the same individual, it is possible to calculate the RCV that defines the critical difference between two consecutive results for a constituent at a specific probability. The RCV encompasses both biological and analytical variation and has been determined for several constituents in healthy patients and in some diseases\(^{(6–14)}\).

The aims of this study were\((a)\) to estimate the biological variation (BV) of the tumor markers CYFRA 21-1, CA 125, and carcinoembryonic antigen (CEA) in a control group and in patients with NSCLC;\((b)\) to establish analytical quality goals for CYFRA 21-1 measurements; and\((c)\) to calculate the RCVs for these tumor markers and assess their application for detecting disease progression in NSCLC patients under treatment.

The BV components of CYFRA 21-1, CA 125, and CEA were determined in two groups. The control group included 30 patients\([15\text{ men}; mean (SD) age, 61.1 (7.2) years]\) with colon adenocarcinoma\((\text{Astler–Coller classification stages A or B1})\) treated surgically. Three samples were obtained from each patient every 6 months for 3 years after the procedure. There was no evidence of recurrence\((\text{by clinical examination and imaging methods})\) during the study period and up to at least 1 year after the last determination. The results from this group also were used to establish the analytical quality goals.

The lung cancer group comprised 10 NSCLC patients\([5\text{ men}; mean (SD) age, 59 (8.1) years; 4 with stage IIA and 1 with stage IIIa disease]\) presenting with complete remission treated with chemotherapy and 5 patients\([4\text{ men}; mean age, 57 (5.2) years; 3 with stage IA and 2 with stage IIIa disease]\) who underwent total tumor resection. Tumor markers were measured every 3 months for all patients of this group, with a mean of 4.8 determinations\((\text{range, 2–11})\) per patient. Samples in the treated patients were obtained before administration of chemotherapy. Results of tests performed in the time period from 6 months before the detection of progression to the time of progression were excluded from the BV calculations. Possible significant differences in BV values in this group compared with the control group were assessed with the F-test. The variation obtained in this group included the between-day analytical variation and baseline factors\((\text{e.g., smoking habits})\) that can affect tumor marker concentrations in patients with NSCLC; the values from this group were therefore used to calculate the RCV.

To investigate the usefulness of the tumor marker RCVs for the detection of disease progression, a study was performed in 44 patients\([42\text{ men}]; range in age from 38 to 84 years\(\text{ (mean, 61.8 years)}\). The selection criteria were the following: diagnosis of NSCLC stages IIIA to IV; performance status\((\text{Eastern Cooperative Oncology Group})<3\); and platin-based chemotherapy. The clinical characteristics of the patients are described in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue1/. The mean monitoring period was 13.5 months\(\text{ (range, 2–72 months)}\), and the mean number of tumor marker measurements per patient was 6.9\(\text{ (range, 3–23)}\). Markers were measured every 4 weeks during chemotherapy, every 3 months on completion of chemotherapy over 1 year, and every 6 months thereafter until recurrence. In patients undergoing second-line treatment, monitoring was repeated every 4 weeks. Disease progression was defined as lesion growth >25% on two-dimensional imaging or >40% on volumetric imaging, or the appearance of new lesions.

Tumor markers were measured by an electrochemiluminescent method on an ELECSYS 2010 analyzer\((\text{Roche Diagnostics})\). Control group samples were analyzed in a single run. For the control group, within-run imprecision\((\text{CV}; n=10)\) was 1.8% for CYFRA 21-1\(\text{ (mean concentration, 2.1 µg/L)}\), 2.1% for CEA\(\text{ (2.9 µg/L)}\), and 1.7% for CA 125\(\text{ (21 kilounits/L)}\), obtained with replicates of patient samples. Samples from the NSCLC groups were analyzed on the day of collection. The between-run CVs\((n=620)\), i.e., the mean imprecision for all lots of Precicontrol tumor marker 1\((\text{Roche Diagnostics})\) used during the study, were 4.3% for CYFRA 21-1\(\text{ (2.8 µg/L)}\), 3.9% for CEA\(\text{ (4.8 µg/L)}\), and 4.8% for CA 125\(\text{ (31 kilounits/L)}\).

Variations in tumor marker values around the means in the control and lung cancer groups did not follow a gaussian distribution\((\text{Kolmogorov–Smirnov test, } P<0.2)\); thus, logarithmic transformation was applied\((6)\).

The within-subject BV\((\text{CV}_w)\) was calculated using the formula:

\[
\text{CV}_w = 100(\text{Sw}_w^2 + \text{sa}_w^2)^{1/2}/M
\]

in which \(\text{Sw}_w^2\) is the experimental variation in the results for each group of patients, \(\text{sa}_w^2\) is the analytical variation, and \(M\) is the mean tumor marker value for each group of patients studied.

The between-subject BV\((\text{CV}_b)\) was calculated with the following formula:

\[
\text{CV}_b = 100(\text{St}_b^2 - \text{Sw}_b^2)^{1/2}/M
\]

in which \(\text{St}_b^2\) is the total variation obtained by use of all values from all patients.

The index of individuality\((II)\) was calculated according to the formula:

\[
\text{II} = \frac{\text{S}_w^2}{\text{S}_b^2}/\text{S}_g^2
\]

where \(\text{S}_g^2 = \text{S}_b^2 - \text{S}_w^2\).

The RCV was determined with the following formula:

\[
\text{RCV} = Z(2\text{Sw}_w^2)^{1/2}
\]
where the Z statistic was 1.28, 1.64, and 2.33 for probabilities of 90%, 95%, and 99%, respectively, for a one-tailed test.

There were no significant differences in tumor marker BV values between the control and lung cancer groups: F-test results for CYFRA 21-1, $P = 0.17$; CA 125, $P = 0.28$; and CEA, $P = 0.19$. The BV values for the two study groups are shown in Fig. 1. The II was estimated as 0.55, 0.57, and 0.28 for CYFRA 21-1, CA 125, and CEA, respectively, in the lung cancer patients. The performance of the tumor marker RCVs for the detection of disease progression at the different probabilities is shown in Table 1. There was a decrease in sensitivity and an increase in specificity at increasing probabilities.

Application of the tumor marker RCVs ($P < 0.05$) to the detection of disease progression in a separate cohort of NSCLC patients showed that CYFRA 21-1 provided the highest diagnostic performance, followed by CEA and CA 125. In 18 of the 44 patients (40.9%), disease progression was detected at the first clinical evaluation (3 months after beginning treatment). RCVs for CYFRA 21-1, CA 125, and CEA detected progression at the first clinical evaluation in 7 of 18 (38.8%), 6 of 18 (33.3%), and 10 of 18 (55.5%) patients, respectively, with detection by all three RCVs in 12 (66.6%) patients. The mean lead time of the patients showing progression by RCVs was 2 months (range, 1–6 months), with 11 of 44 (25.0%), 7 of 44 (15.9%), and 15 of 44 (34.1%) detected by CYFRA 21-1, CA 125, and CEA, respectively, and 18 of 44 (40.9%) detected by all three markers. Disease progression was simultaneously detected by imaging methods and tumor marker RCVs in 12 of 44 (27.2%) patients by CYFRA 21-1, 10 of 44 (22.7%) by CA 125, 7 of 44 (15.9%) by CEA, and 15 of 44 (34.1%) by a combination of the three markers. No critical increases in tumor markers were found at the time of imaging progression in 11 of 44 (25.0%) patients. In six cases, the increases were all lower than the RCV, and in five patients values were within the reference intervals at initial diagnosis and showed no increases during the study. On completion of the study, only two patients did not experience disease progression. The $CV_w$ values for CA 125 and CEA in this study were similar to those reported previously (15–17). The components of BV for CYFRA 21-1 in healthy individuals are unknown (15). We established the $CV_w$ of CYFRA 21-1 in the control group at 22.5%. The desirable analytical performance for CYFRA 21-1, defined as $CV_a = 0.5CV_w$ (18–21), was 11.2%.

Previous studies have applied the criterion used in imaging findings (40% increase in tumor volume) to define the critical difference in consecutive analyses. With this criterion, the corresponding RCV for CYFRA 21-1 is closest to the 90% probability and that for CEA is closest to the 99% probability value. If the RCV at the 90% probability value is used for CEA, the critical difference to assess would be 22.6%, and the sensitivity would increase to 52%. Application of the RCV at the 95% probability value (the most widely used value) allows detection of disease progression with a lead time similar to that reported by other authors (3, 4) and a sensitivity slightly lower than for CYFRA 21-1 in other reports (3, 4, 22, 23) and higher for CEA (22).

In conclusion, the criteria we have established for the magnitude of the difference in serial tumor marker values based on BV at 95% probability allowed the detection of 75% of cases of disease progression. Furthermore, when none of the markers increased above the RCV, the probability of progression was low. Monitoring of lung cancer patients undergoing chemotherapy with the use of these three tumor markers is helpful for the detection and exclusion of disease progression and allows a point of reference for detection of progression to be established specifically for each tumor marker.

### Table 1. Diagnostic performance of tumor markers at different probabilities for RCV.

<table>
<thead>
<tr>
<th>RCV at 90% probability ($P &lt; 0.1$), %</th>
<th>CYFRA 21-1</th>
<th>CA 125</th>
<th>CEA</th>
<th>CYFRA 21-1 and/or CA 125</th>
<th>CA 125 and/or CEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity, %</td>
<td>56.8</td>
<td>43.2</td>
<td>52.3</td>
<td>77.3</td>
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</tr>
<tr>
<td>Specificity, %</td>
<td>86.8</td>
<td>85.0</td>
<td>84.4</td>
<td>73.7</td>
<td></td>
</tr>
<tr>
<td>PPV, %</td>
<td>64.1</td>
<td>58.6</td>
<td>62.5</td>
<td>56.5</td>
<td></td>
</tr>
<tr>
<td>NPV, %</td>
<td>85.7</td>
<td>81.0</td>
<td>81.8</td>
<td>94.4</td>
<td></td>
</tr>
<tr>
<td>RCV at 95% probability ($P &lt; 0.05$), %</td>
<td>52.5</td>
<td>53.1</td>
<td>29.4</td>
<td>75.0</td>
<td></td>
</tr>
<tr>
<td>Sensitivity, %</td>
<td>52.4</td>
<td>38.1</td>
<td>50.0</td>
<td>78.9</td>
<td></td>
</tr>
<tr>
<td>Specificity, %</td>
<td>90.8</td>
<td>86.3</td>
<td>87.5</td>
<td>86.3</td>
<td></td>
</tr>
<tr>
<td>PPV, %</td>
<td>70.8</td>
<td>60.7</td>
<td>66.7</td>
<td>60.3</td>
<td></td>
</tr>
<tr>
<td>NPV, %</td>
<td>85.2</td>
<td>81.2</td>
<td>81.6</td>
<td>92.8</td>
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<tr>
<td>RCV at 99% probability ($P &lt; 0.01$), %</td>
<td>74.1</td>
<td>75.1</td>
<td>41.5</td>
<td>62.8</td>
<td></td>
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<tr>
<td>Sensitivity, %</td>
<td>36.4</td>
<td>31.8</td>
<td>36.4</td>
<td>86.2</td>
<td></td>
</tr>
<tr>
<td>Specificity, %</td>
<td>96.1</td>
<td>92.1</td>
<td>92.8</td>
<td>87.5</td>
<td></td>
</tr>
<tr>
<td>PPV, %</td>
<td>80.0</td>
<td>71.1</td>
<td>74.5</td>
<td>66.9</td>
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<tr>
<td>NPV, %</td>
<td>81.1</td>
<td>80.4</td>
<td>79.5</td>
<td>88.5</td>
<td></td>
</tr>
</tbody>
</table>

* PPV, positive predictive value; NPV, negative predictive value.

### References

2. Cesano A, Lane SR, Ross GA, Fields SZ. Stabilization of disease as an


DOI: 10.1373/clinchem.2004.040659