Detection and/or Monitoring of 14 Types of Cancer:

- Lung
- Breast
- Stomach
- Liver
- Colon
- Rectal
- Ovarian
- Esophageal
- Cervical
- Trophoblastic
- Thyroid
- Malignant
- Lymphoma
- Pancreatic

ONKO-SURE
“The Power of Knowing”
US FDA Approved Cancer Test

2010 ONKO-SURE
physician’s desk reference and studies
INTENDED USE  Onko-Sure is a non-invasive blood test for cancer that helps determine the effectiveness of post-surgery therapy and treatments for colorectal cancer (CRC). Onko-Sure is an ELISA-based assay and was evaluated as an informative test for monitoring disease progression in colorectal cancer patients. Onko-Sure is U.S. FDA-cleared for colorectal cancer monitoring. It is used to check for cancer recurrence of disease status during cancer treatment.

ONKO-SURE  the power of knowing.

The test should be used in conjunction with other clinical modalities considered to be the standard of care for CRC disease progression monitoring.

CLINICAL SIGNIFICANCE  Onko-Sure is the first new cancer test to be cleared in the USA for the monitoring of CRC in over 25 years. In early CRC stages (Dukes stages A & B) between 68% and 97% of the biopsy positive patients have negative CEA values and are unable to be monitored with CEA.

ADVANTAGES OVER CEA  Onko-Sure DR-70 (FDP) antigen is freely diffusible in blood and therefore is easy to measure. CEA is normally firmly attached to cancer cells since it is an adhesion molecule and less abundant in blood, therefore more difficult to measure. Onko-Sure also has an advantage over CEA for CRC monitoring, especially in patients with low CEA values. Approximately half of all CRC patients have low CEA values and up to half of those patients will experience cancer recurrence after treatment.

COMPARED TO D-DIMER  D-dimer is not approved for detecting or monitoring cancer. Onko-Sure detects all of the breakdown products of Fibrin and Fibrinogen, including a unique cancer-related breakdown product, Initial Plasmin Degradation Product (IPDP).
ONKO-SURE FOR SCREENING  Because Onko-Sure is a non-invasive blood test there are no side effects to patients using the test. As with other cancer diagnostic products, false positive and false negative test results could pose a small risk to patient health if the physician conducting the test is not vigilant in following the Onko-Sure test results with other clinically-relevant diagnostic modalities. While the Onko-Sure test is helpful in diagnosing whether a patient has cancer, the attending physician should use other testing methods to determine and confirm the type and kind of cancer involved.

HOW ONKO-SURE WORKS  While the production of Fibrin and Fibrinogen Degradation Products (FDP) is limited in healthy individuals, FDP are over produced by cancer cells, which release proteolytic enzymes such as plasmin and thrombin. Current assays for FDP usually measure a specific FDP component, such as D-dimer, as a representative of this group; whereas the Onko-Sure™ test detects the full complement of FDP. Onko-Sure™ acts as a “barometer for cancer” by simultaneously measuring the multiple FDP species that may be underestimated by other tests.

INCREASED POSITIVE CONCORDANCE  50% or more CRC patients have low CEA values (<30). As demonstrated in the graph, Onko-Sure (blue line) showed increased positive concordance, i.e., ability to monitor patients with low CEA values.

According to the National Cancer Society, there are 1.1 million people with Colorectal Cancer in the U.S. and over 10 million people who are CRC survivors.

For CRC survivors, effective monitoring for disease recurrence is perhaps the most important part of an effective post-surgery treatment plan. Onko-Sure is the first new cancer test to be cleared in the USA for the monitoring of CRC in over 25 years. In early CRC stages (Dukes stages A & B), between 68% and 97% of the biopsy positive patients have negative CEA values and are unable to be monitored with CEA.

Onko-Sure is cost-effective for first line cancer screening and detection, and is approved in the U.S., Europe, Canada, Korea, Taiwan, India and Vietnam.

ONKO-SURE the power of knowing.
Onko-Sure® Proposed Mechanism of Action in Patients with Colorectal Cancer

Onko-Sure® is a simple, non-invasive, patent-pending and regulatory-approved in vitro diagnostic (IVD) blood test used both for monitoring colorectal cancer (CRC) during treatment and for post-treatment CRC recurrence. It is an ELISA-based assay that measures the accumulation of Fibrin/Fibrinogen Degradation Products (FDP) in the serum using a polyclonal antibody against the DR-70 tumor marker.

For CRC, early identification of recurrence with prompt treatment can lead to a better survival rate and quality of life for the patients.¹ For the last 25 years, Carcino-Embryogenic Antigen (CEA) has been the only available tumor marker for colorectal cancer (CRC) monitoring; however, similar to any other tumor marker, it has its own limitations.¹ DR-70 is the only other available tumor marker cleared by the US FDA for the monitoring of colorectal cancer treatment.

Mechanism of Action of Onko-Sure®

The production of Fibrin and Fibrinogen Degradation Products (FDP) is restricted in healthy individuals by normal cells. However, cancer cells release proteolytic enzymes such as plasmin and thrombin as they grow and metastasize and they also redirect the coagulation cascade which leads to overproduction of FDP in the process of carcinogenesis.² ⁴

FDP level measurement is routinely performed for the detection of coagolopathies; however, the current assays for FDP measurement usually detect only one out of the many FDP components (D-dimer).⁵ There have been many publications studying the D-dimer level measurement in different malignancies including CRC⁶-⁹; however, it is not approved for detecting or monitoring malignancies.

Onko-Sure® is the first blood test available for the monitoring of colorectal cancer recurrence based on FDP measurement. It is an ELISA-based test that uses DR-70 polyclonal antibody against the full array of FDP. Onko-Sure® detects all of the breakdown products of Fibrin and Fibrinogen, including a unique cancer-related breakdown product, Initial Plasmin Degradation Product (IPDP).¹⁰

Clinical data supports the medical utilization of Onko-Sure® for the monitoring of colorectal cancer. In these clinical studies, Onko-Sure® was used to measure DR-70 levels in 226 patients and the results positively correlated with the progression of CRC.¹⁰-¹² Furthermore, as reported by four other studies, the D-dimer level was also linked to CRC progression in 298 patients.⁶-⁹

Comparison of Onko-Sure® with CEA

CEA, an adhesion molecule, is firmly attached to cancer cells.¹³ Therefore, it is less abundant in blood and more difficult to be measured. However, DR-70 antigen is freely diffusible in blood and therefore easy to measure even in low concentrations. Approximately half of all CRC patients have low CEA values not detectable by CEA test.¹⁴-¹⁶ Likewise, half of the CRC patients will experience
For this very reason, Onko-Sure® is advantageous over CEA in detecting lower levels of tumor marker leading to an early diagnosis of recurrence.

**Figure 1. Comparison of Onko-Sure® with CEA.** Fifty percent or more CRC patients have low CEA values (<30). As demonstrated in the graph below, Onko-Sure® (blue line) showed increased positive concordance, i.e., ability to monitor patients with low CEA (red line) values.

CEA has approximately a 20% chance of false positive in smokers while Onko-Sure® measurements are not affected by smoking.

In general, a combination of several tumor markers provides more accurate information about CRC monitoring. Therefore, it is recommended that both CEA and Onko-Sure™ are used in combination for the monitoring of post-surgery CRC recurrence. More clinical studies are ongoing to verify the beneficial effect of CEA combination with Onko-Sure®. Furthermore, Onko-Sure® should be used in conjunction with other clinical modalities considered to be the standard of care for CRC disease progression monitoring.

**Summary**

Onko-Sure® is a simple, non-invasive, patent-pending blood test used both for monitoring colorectal cancer (CRC) during treatment and for post-treatment CRC recurrence. It is an ELISA-based assay that measures the accumulation of Fibrin/Fibrinogen Degradation Products (FDP) in the serum using a polyclonal antibody (DR-70) as a tumor marker.

For the last 25 years, CEA has been the only available tumor marker for CRC monitoring. DR-70 is the only other available tumor marker approved by the US FDA for the monitoring of colorectal cancer treatment. It is recommended to use these two tests in combination to increase the accuracy of the CRC recurrence monitoring.

The information in this letter is intended for healthcare professionals practicing in the US. It is provided to you as a professional courtesy in response to your specific unsolicited request.
References


Onko-Sure® in International Use

Onko-Sure® is a simple, non-invasive, patent-pending and regulatory-approved in vitro diagnostic (IVD) blood test. It is an ELISA-based assay that measures the accumulation of Fibrin/Fibrinogen Degradation Products (FDP) in the serum using a polyclonal antibody against the DR-70 tumor marker. The test has been approved by the FDA for monitoring colorectal cancer in the USA. In key international markets Onko-Sure® has been granted important approvals and is currently being used for the following diagnostic purposes:

**Lung Cancer Detection and Treatment Monitoring**
- Canada (Health Canada)

Relevant international insert information: The findings from a formal clinical trial evaluation of the utility of DR-70® to detect lung cancer indicate that the test correctly identified 66% of all lung cancers and 91.8% of the normal controls.¹ The test identified all types of lung cancer about equally well (non-small cell and small cell). The test was not influenced by alcohol consumption nor by use of tobacco, nor by gender, nor by chronic obstructive pulmonary disease (COPD). The level of DR-70® was also found to correlate with the stage of disease.

**General Cancer Screen**
- Europe (CE mark)
- Taiwan (FDA)
- India (Ministry of Health)

Relevant international insert information: A subsequent clinical study using DR-70® immunoassay for the detection of 13 different cancers was conducted with 277 healthy subjects and 136 cancer patients.² At a 95% specificity level, the sensitivity of the assay was 87.8%, 92.6%, 65.2% and 66.7% respectively for lung, stomach, breast and rectum cancers. The overall specificity and sensitivity of DR-70® for cancers tested were respectively 95.0% and 83.8%. The predictive values of positive and negative tests were 89.1% and 92.3%, respectively.

**Other Territories**

Onko-Sure® is currently being used extensively in Vietnam. Expected approval by the Vietnamese Ministry of Health will provide access to other ASEAN member countries. Healthcare distribution partners are also working to promote the test in South America, Israel, Australia, Russia, Greece, and Turkey.
Research Paper Compilation

This ODR contains a compiled format of research papers highlighting the various clinical trials undertaken in different parts of the world on 7,469 patients with Onko-Sure. Please read the materials thoroughly and completely. Important items have been highlighted in yellow. We believe this ODR is an effective guide for physicians to understand the value of our unique cancer test, and it provides an understanding of how to perform and interpret the results for our Onko-Sure cancer test.
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<td>1-9</td>
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<td>2) Lee, K-H et al., 2006 &quot;Meaning of the DR-70™ Immunoassay for Patients with the Malignant Tumor,&quot; Immune Network 6, 43-51.</td>
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<td>4) Kerber, A et al., 2004 &quot;The new DR-70 immunoassay detects cancer of the gastrointestinal tract: a validation study.&quot; Ailment Pharmacol Ther 20, 983-987.</td>
<td>29-33</td>
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<td>5) Rucker, P et al., 2004 &quot;Elevated Fibrinogen-Fibrin Degredation Products (FDP) in Serum of Colorectal Cancer Patients.&quot; Analytical Letters 37, 2965-2976.</td>
<td>34-46</td>
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Ovarian Carcinoma: Clinical Validity by Simultaneous Determination of Fibrin Degradation Products with the DR-70™ Immunoassay and CA-125

Abstract

Background and question: Malignant cells characteristically possess high levels of proteases (e.g., plasminogen activator, cathepsin etc.), which should induce fibrinolysis. The DR-70™ Immunoassay quantifies the amount of fibrin degradation products (FDP) in the body. This test system has successfully been used for the detection of a number of cancers including lung, stomach, breast, rectum, liver, colon and uterus cancer. In this paper we tested the diagnostic validity of DR-70™ relative to CA-125 as a tumor marker for the detection of ovarian carcinoma. Methods: We have simultaneously investigated the serum activities of DR-70™ and CA-125 from 61 preoperative patients with histologically confirmed ovarian carcinoma. One hundred healthy blood donors served as control group. We measured the FDP utilizing the DR-70™ ELISA as per the test instructions of its manufacturer AMDL (Tustin, CA, USA). Results: Low levels of FDP measured by DR-70™ have been detected in the serum of 100 apparently healthy persons (mean ± S.D., 0.41 ± 0.15 µg/ml). Among the 61 ovarian carcinoma patients, DR-70™ mean value (± S.D.) was 7.0 ± 10.5 µg/ml in a range of 0.6 to 58.5 µg/ml. The cut-off level for DR-70™ was set at > 1.2 µg/ml. The specificity was 100%, whereas the sensitivity was at 83.6%. Relative to CA-125 a cut-off level of > 65 U/ml was applied. The comparable sensitivity of DR-70™ was higher by 13.1%. Conclusions: Malignant cells with

Zusammenfassung

Fragstellung: Tumorzellen weisen häufig einen hohen Gehalt an Proteasen auf, die entscheidend an der Fibrinolyse beteiligt sind. Der DR-70™-Immunoassay misst mit hoher Spezifität Degradationsprodukte des Fibrins. Dieser Test ist, wenn auch an kleinen Fallzahlen, erfolgreich eingesetzt worden als Tumormarker zur Erfassung einer Reihe von Tumorentitäten. In der vorliegenden Arbeit wird dieser Test beim Ovarialkarzinom mit CA-125, dem „Goldstandard“ beim Ovarialkarzinom, verglichen. Methoden: Im Serum von 61 Patientinnen mit histologisch gesicherter Ovarialkarzinom, aber auch im Serum von 135 Patientinnen mit entzündlichen Erkrankungen bzw. anderen Malignomen bzw. benignen Tumor erkrankungen wurde die Aktivität von DR-70™ im Serum gemessen. Als Kontrollgruppe dienten 100 Blutspenderinnen, die als tumorfreie, gesunde Frauen eingestuft wurden. Der DR-70™ ist ein ELISA, der nach den Vorgaben des Herstellers (AMDL, Tustin, CA, USA) eingesetzt wurde. Ergebnisse: Als Mittelwert ± S.D. in der Kontrollgruppe wurde 0.41 ± 0.1 µg/ml für DR-70™ ermittelt. Im Vergleich dazu lag der Mittelwert der Serumproben von 61 Patientinnen mit Ovarialkarzinom bei 7.0 ± 10.5 µg/ml, bei einer Schwankungsbreite von 0.6 bis 58.5 µg/ml. Wird der Cut-off-Wert für DR-70™ auf > 1.2 µg/ml festgelegt, beträgt die Sensitivität des Testes 83.6%, und der Wert für die Spezifität liegt bei 100%. Verglichen mit
high levels of proteases are significant contributors to fibrinolysis. The DR-70™ immunoassay has been designed to measure the level of FDP in the serum. DR-70™ represents a tumor marker with which ovarian carcinoma can be diagnosed at levels of sensitivity and specificity which compare favorably to those measured with CA-125.

Key words
Ovarian carcinoma - DR-70™ immunoassay - tumor marker

Introduction

DR-70™ immunoassay works on fibrin degradation products. Fibrin is naturally occurring in the body and is involved in the clotting process of blood. Moreover, biochemical evidence has been presented that procoagulants and plasminogen activators regulating coagulation and fibrinolysis are involved in the growth and metastasis of solid tumors [11-61]. As a result, high concentrations of fibrin degradation products can be detected in serum as a sensitive indicator for monitoring the course of tumor disease. Proteases have been implicated in a number of malignant conditions, and researchers have observed increased secretion of proteases into the interstitial fluid around growing tumors [7-9]. These proteases inevitably act on proteins, including those in the coagulation cascade leading to the formation of fibrin [10-15]. DR-70™ assay uses a proprietary antibody which detects the fibrin degradation products. Secondly, DR-70™ can quantify the amount of cancer in a particular blood specimen as the test compares the intensity of a color signal from the DR-70™ test with known cancer specimens.

A clinical study using DR-70™ immunoassay for the detection of 13 different cancers has been conducted with 277 healthy subjects and 136 cancer patients [16]. The test results showed that DR-70™ immunoassay was capable of detecting cancers with a high degree of specificity and sensitivity. At 95% specificity levels, the sensitivity of the assay was 87.8% for lung cancer, 92.5% for stomach cancer, 95.2% for breast cancer and 66.7% for rectum cancer. Furthermore, the test kits were shown to be stable and performed reproducibly [16]. In an attempt to assess the potential of using this assay as an indicator for the presence of various cancers, the DR-70™ immunoassay was also shown to detect a number of other cancers including liver, colon, urothelial, esophageal carcinomas [17].

In this paper we have further investigated the value of fibrin degradation products (FDP) by DR-70™ in peripheral venous blood in women with benign, borderline malignant or malignant ovarian tumors. Among gynecological malignancies, it always has been a major diagnostic problem to clearly identify ovarian carcinoma. It is well known that Bast et al. [18-21] discovered a circulating antigen expressed by human ovarian carcinoma cells. Moreover, they demonstrated that the concentration of this antigen (CA-125) is increased in the serum of 82% of women with epithelial ovarian cancer. Therefore the present study was undertaken to determine whether the concurrent measurement of DR-70™ and CA-125 would provide a more precise correlation with tumor detection for a larger fraction of ovarian cancer patients than could be monitored when using a single assay only.

Material and Methods

We have simultaneously investigated the serum activities of DR-70™ and CA-125 from 61 patients with histologically confirmed ovarian carcinoma before operative therapy. Furthermore, within this group of 61 patients with ovarian carcinoma, serial samples of 12 patients were collected before and after surgery in a period of chemotherapy (a combination of carboplatin and taxol administered at 3 weekly intervals to a total of 6 courses) ranging from 1 to 7 months after initial operative treatment. Furthermore, the study included 23 recurrences of ovarian carcinoma, 5 borderline tumors, 3 teratomas, 70 patients with histologically confirmed benign ovarian tumors, and 34 patients with other gynaecologic tumors. The institutions participating in the study were the Department of Gynecology and Gynecologic Oncology (director Prof. Dr. du Bois) of the Dr. Horst-Schmidt-Klinik (HSK), Wiesbaden, Germany, the Department of Gynecology and Obstetrics of the University of Mainz (director: Prof. Dr. Kolb), Germany, and the Department of Experimental Endocrinology of the University of Mainz (director: Prof. Dr. Pollow), Germany. Sera from 100 apparently healthy blood donors obtained from the Regional Blood Transfusion Centre of the University of Mainz, Germany, were assayed for DR-70™. Sera samples were obtained in the morning before any meal was taken and stored at -20°C until required for analysis.

Levels of CA-125 were determined using a commercially available kit (manufacturer DPC, Germany) according to the manufacturer's instructions. We measured FDP utilizing the DR-70™ kit (manufacturer AMDL, Tustin, CA, USA) according to the instructions of the manufacturer. Briefly, the DR-70™ assay is an ELISA based assay utilizing removable strips in a 96 microwell format. The wells are coated with affinity purified rabbit anti FDP polyclonal antibodies. The FDP in diluted sera (1:200) is captured out of the sera by these antibodies immobilized on the well of a microwell plate. After a wash step, anti FDP antibodies conjugated to horse-radish peroxidase are added to the wells. If antigen is present the anti FDP peroxidase complex will bind to the captured FDP to form an immunological sandwich with the immobilized antibodies. After a second wash step, the enzyme substrate TMB is added to the well. The end point is read in a microtiterplate reader at 450 nm after the reaction has been stopped with 0.1 N HCL.
Table 1  DR-70™ activities in 100 healthy blood donors, 61 patients with histological confirmed ovarian carcinomas, 5 borderline tumors, 3 teratomas, 23 recurrences, 70 benign ovarian tumors, and 34 patients with other tumors

<table>
<thead>
<tr>
<th>DR-70™ (μg/ml)</th>
<th>n</th>
<th>Mean</th>
<th>S.D.</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Percentiles</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5%</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
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<td>0.41</td>
<td>0.19</td>
<td>0.37</td>
<td>0.10</td>
<td>1.11</td>
<td>0.10</td>
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<tr>
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<td>61</td>
<td>7</td>
<td>10.5</td>
<td>3</td>
<td>0</td>
<td>58.5</td>
<td>1</td>
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<tr>
<td>Borderline tumor</td>
<td>5</td>
<td>0.84</td>
<td>0.49</td>
<td>0.6</td>
<td>0.5</td>
<td>1.7</td>
<td>0.5</td>
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<tr>
<td>Teratoma</td>
<td>3</td>
<td>0.73</td>
<td>0.06</td>
<td>0.7</td>
<td>0.7</td>
<td>0.8</td>
<td>0.7</td>
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<tr>
<td>Recidve</td>
<td>23</td>
<td>5.53</td>
<td>5.96</td>
<td>2.7</td>
<td>0.5</td>
<td>18.7</td>
<td>0.64</td>
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<td></td>
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<tr>
<td>Cystic ovary</td>
<td>52</td>
<td>0.73</td>
<td>0.27</td>
<td>0.7</td>
<td>0.3</td>
<td>1.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Cystadenoma</td>
<td>8</td>
<td>1.63</td>
<td>1.43</td>
<td>0.85</td>
<td>0.4</td>
<td>4.5</td>
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<tr>
<td>Dermoid tumor</td>
<td>3</td>
<td>0.83</td>
<td>0.12</td>
<td>0.9</td>
<td>0.7</td>
<td>0.0</td>
<td>0.7</td>
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<tr>
<td>Adenexis</td>
<td>7</td>
<td>1.53</td>
<td>1.2</td>
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<td>3.6</td>
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<td>Other hypogastric tumors</td>
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<td>Endometriosis</td>
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<td>0.66</td>
<td>0.23</td>
<td>0.7</td>
<td>0.3</td>
<td>1.1</td>
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<td>Myxomatosis</td>
<td>5</td>
<td>7.74</td>
<td>5.3</td>
<td>8.7</td>
<td>1.3</td>
<td>13.6</td>
<td>1.3</td>
</tr>
<tr>
<td>Colon carcinoma</td>
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<td>7.6</td>
<td>6.24</td>
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<td>1.8</td>
<td>19.0</td>
<td>1.8</td>
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<tr>
<td>Endometrial carcinoma</td>
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<td>1.76</td>
<td>0.83</td>
<td>1.7</td>
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<td>3.2</td>
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<tr>
<td>Cervix carcinoma</td>
<td>4</td>
<td>2.25</td>
<td>1.58</td>
<td>1.55</td>
<td>1.3</td>
<td>4.6</td>
<td>1.3</td>
</tr>
</tbody>
</table>

All statistical analyses were performed using the SPSS statistical package version 12.0 (SPSS Inc., Chicago, III, USA) for Windows program (Microsoft corporation, Redmond, Wash, USA). Apart from mean value and S.D., the median and extreme values of each corresponding group were compiled as measures of the distribution. The correlation coefficients were considered statistically significant whenever p < 0.05. The coefficient of intra- and inter-assay variations of DR-70™ were 16.7% and 11.8%, respectively.

Results

Low levels of FDPs measured by DR-70™ have been detected in the serum of apparently healthy people donating blood (mean ± S.D.: 0.41 ± 0.19 μg/ml) (Table 1). The choice of limits for normal values is arbitrary. Only one serum sample of 100 normal donors had a DR-70™ level greater than 1 mg/ml. This was the highest value observed in the group of apparently healthy persons. Based on a 95% confidence level, the upper limit for the normal DR-70™ level can be set at ≤1.24 μg/ml. If the mean value plus 2-fold S.D. is applied as cut-off, it would correspond to an upper limit of ≤0.8 μg/ml. For CA-125 the values of ≤ 35 U/ml or, alternatively, ≤65 U/ml is applied as outlined in the literature.

No correlation was observed between the age and the DR-70™ levels in the group of normal donors (r = -0.215, p = 0.032).

Among 61 patients with surgically demonstrable epithelial ovarian carcinoma, DR-70™ mean value (± S.D.) was 7.0 ± 10.5 μg/ml. The values from different patients with ovarian carcinoma ranged from 0.6 to 58.5 μg/ml (preoperative values). There was no statistically significant prevalence for any histological type amongst the patients with ovarian cancer. The mean value of DR-70™ in patients with grade I tumors was lower (but not significantly) than in patients with grade II, III, IV tumors. DR-70™ was also detected in sera from patients with malignant borderline tumors (n = 5), teratomas (n = 3), and recurrence (n = 23). However, the most marked elevations were observed in patients with epithelial ovarian cancer. The mean values (± S.D.) for borderline tumors were 0.84 ± 0.49 μg/ml with a maximum value of 1.7 mg/ml, and for teratomas 0.73 ± 0.06 μg/ml with a maximum value of 0.8 μg/ml, which means that these values correspond to those of normal patients. For recurrences the mean value ± S.D. was 5.63 ± 5.96 μg/ml with a maximum value of 18.7 μg/ml. DR-70™ also detected fibrin degradation products in serum of patients with benign ovarian tumors. The mean value ± S.D. in the group with cystic ovaries was 0.73 ± 0.27 μg/ml with a maximum value of 1.6 μg/ml in the group with cystadenoma 1.83 ± 1.43 μg/ml with a maximum of 4.5 μg/ml, and in serum specimen of patients with adenexis 1.53 ± 1.2 g/ml with a maximum value of 3.6 μg/ml.

In addition, numerous other hypogastric "tumors" were tested, which often impress of being ovarian carcinoma in the clinical check-up. Included in this group are endometriosis, uterus myomatosis, and carcinoma of the colon, endometrium, and cervix. Except for endometriosis, which showed test results in the normal range, the results of all other tumors were in the same range as ovarian carcinoma.

In Table 2 we have compiled the basis data for CA-125 which has been considered the "Gold Standard" tumor marker when testing for ovarian carcinoma. Within the group of malignant ovarian
Table 2  CA-125 activities in 100 healthy blood donors, 61 patients with histological confirmed ovarian carcinomas, 5 borderline tumors, 3 teratomas, 23 recidives, 70 benign ovarian tumors, and 34 patients with other tumors

<table>
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<tr>
<th>CA-125 (U/ml)</th>
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<th>Minimum</th>
<th>Maximum</th>
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<th>95%</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
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<td>8.12</td>
<td>11.5</td>
<td>7.0</td>
<td>38.0</td>
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<td>Ovarian carcinoma</td>
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<td>993</td>
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<td>212</td>
<td>5.7</td>
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<td>55.9</td>
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<td>10.0</td>
<td>11.48</td>
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In evaluating the specificity and sensitivity of the two tests, DR-70™ and CA-125, the sera from 61 patients with ovarian carcinoma as well as 100 apparently healthy blood donors were tested separately using each test respectively (Table 3). The parameters sensitivity and specificity are required to define the accuracy of a laboratory test. In the context of a tumor marker, the term “sensitivity” is used to characterize the incidence of “true positive” results obtained when the assay is applied to patients known to have a cancer. The term “specificity” is used to characterize the incidence of “true negative” results obtained when an assay is applied to subjects known to be free of cancer.

Based on cut-off of > 0.8 µg/ml for DR-70™ the specificity was 95%, whereas the sensitivity was measured at 96.7%. At > 1.2 µg/ ml cut-off, the sensitivity dropped to 83.6% and the specificity increased to 100%. CA-125, which is considered the “standard” tumor marker for ovarian carcinoma and used complementary to DR-70™, yielded sensitivity of 83.6% at > 35 U/ml cut-off and sensitivity of 70.5% at > 65 U/ml cut-off. The specificity was determined as 98% for > 35 U/ml cut-off level, and as 100% for > 65 U/ml cut-off level.

The DR-70™ immunoassay (at a > 1.2 µg/ml cut-off) identified 13 cases as “true positive” which had been identified by CA-125 as “false negative”. Conversely, CA-125 (at a > 65 U/ml cut-off) identified 4 tumors as positive which DR-70™ had identified as negative. The combined application of both tests should be considered an improvement in correctly diagnosing ovarian cancer particularly with regards to the specificity issue.

Fig. 1 shows that 12 patients who had responded to chemotherapy also showed a correlation between DR-70™ and CA-125 serum levels. All patients in this group had increased peripheral blood DR-70™ and CA-125 antigen concentrations when measured in the preoperative treatment phase, ranging from 2.3 to 28.9 µg/ml with DR-70™ and ranging from 137 to 3494 U/ml with CA-125. Radical surgical removal of the malignant ovarian tumors resulted in decreased levels of both DR-70™ and CA-125 antigen. A further decrease took place between the 1st and the 7th month. This decrease was a manifestation of a positive response to chemotherapy. The antigens were leveling off to normal ranges during the phase of treatment.

Eight patients tested for DR-70™ and CA-125 demonstrated a rising antigen level after an initial response to their respective chemotherapeutic treatment (Fig. 2). The ovarian cancer within these patients had continued to progress as determined by repeated clinical examination and biopsy.
Depending on the levels of DR-70™ and CA-125 used as the cut-off values, a range of specificity values and their corresponding sensitivities can be obtained for ovarian cancer. The relationships between series of specificities and sensitivities of both tumor markers can be profiled in ROC (Receiver Operating Characteristic) curves as shown in Fig. 3. The power of discrimination between both tumor markers can be readily observed in the ROC curve. The closer the curve is to the upper right hand corner of the plot, the greater is the discrimination power of the test. A detailed ROC analysis of the data presented in Fig. 3 demonstrates that the sensitivity and specificity of both tests are nearly identical.

**Discussion**

This research attempts to compare the DR-70™ antigen levels by means of test runs and follow-up control in ovarian carcinoma patient serum, and correlate this data with antigen levels of the tumor marker CA-125. The comparability and clinical reliability of tumor marker investigations crucially depend on clearly defined control and patient populations as well as on appraisals of specificity and sensitivity. Serological immunodiagnostics of malignant diseases is necessarily based on quantitative differences since the detection of tumor-associated and oncofetal antigens is neither tumor-specific nor is it confined to patients with malignancies. The objective of achieving as high as possible a degree of sensitivity frequently leads to the use of “lower” limit ranges.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Distribution of the patients and the normal individuals in relation to the cut-off values of DR-70™ and CA-125</th>
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<tr>
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<td>Cervix carcinoma</td>
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</table>
Fig. 2 Serum profiles of DR-70™ and CA-125 in eight patients with ovarian carcinoma demonstrated a rising antigen levels after initial response to their respective chemotherapeutic treatment. All patients received combined adjuvant cytostatic chemotherapy (a combination of carboplatin and taxol administered every 3 weeks to a total of 6 courses).

Fig. 3 Receiver Operating Characteristic (ROC) curves of DR-70™ and CA-125 immunoassays in patients with ovarian carcinoma.

However, in clinical use of antigens as tumor markers it appears to be appropriate to relate the specificity to the general age-related patient population with the given exclusion criteria. The limit values that are predominantly higher in the literature are therefore better suited for the clinical use of tumor markers. Even though this gain in specificity is associated with reduced sensitivity, a higher practicality is attained in clinical terms by the reduction in the number of false-positive cases. It only becomes possible to compare the results of different working groups when the specificity and sensitivity values are specified and the patient groups investigated are clearly defined. Consistent results are reported by a large number of investigators in respect of the pre-eminent suitability of CA-125 for progress control of ovarian carcinoma [19,22–28]. However, there is still disagreement about the most suitable limit (> 35 or > 65 U/ml). How many false-positive results the clinician will accept for the sake of greater sensitivity depends on the specific diagnostic objective.

The preoperative sensitivity of DR-70™ ranges from 83.6% to 96.7% based on a threshold cut-off of 1.2 μg/ml or 0.8 μg/ml, respectively. The preoperative sensitivity of CA-125 ranges from 70.5% to 83.6% based upon CA-125 measurement thresholds of > 65 U/ml and > 35 U/ml, respectively. When considering the lower threshold values for both assays, an improvement in sensitivity of 13.1% for the DR-70™ immunoassay was observed. Publications on CA-125 describe the range of sensitivity threshold values between 54% and 88% in the preoperative treatment phase depending on the stage of malignancy and the cut-off value [18,21,22,29–31]. The specificity of both tumor markers was established by testing blood donors who were apparently healthy. A well-known phenomenon with immunoassays is the fact that lowering the threshold cut-off value simultaneously causes a loss in specificity. The specificity for CA-125 was determined as 98% for > 35 U/ml cut-off values, and as 100% for > 65 U/ml cut-off values, whereas the specificity for DR-70™ was measured as 95% for > 0.8 μg/ml cut-off values and as 100% for > 1.2 μg/ml cut-off values.

To compare the reliability of tumor markers, ROC diagrams [receiver operating characteristic curves [32]] can be used that enable the qualities of different markers to be appraised in terms of their sensitivity and specificity. It is shown that the results of the DR-70™ kit are practically equivalent to those of CA-125 determination (Fig 3). It should not be inferred that CA-125, which is a reliable and generally established tumor marker, should be replaced by DR-70™. However, it can be concluded from these investigations that fibrin is formed and degraded in patients with ovarian carcinoma and that the DR-70™ level in the serum reflects the extent of abdominal spreading of the carcinoma.

Based on the overall number of benign ovarian tumors when calculating the specificity the following can be concluded: 50.0% of all tested patients with benign tumors had increased levels of DR-70™ on the basis of a cut-off values of > 0.8 μg/ml, while these same patients had an increase in CA-125 of 5.7% (cut-off values of > 35 U/ml). When using the higher cut-off levels as a basis for calculation, there is a significant simultaneous drop in positively measured serum (DR-70™ 17.1%, CA-125 2.9%). Therefore we established a cut-off value of > 1.2 μg/ml for DR-70™.
It is remarkable - also with regards to the benign group - that DR-70™ antigen reacts with above average increase of DR-70™ values in cases of infectious processes when compared with CA-125.

When including the remaining hypogastic tumors in this analysis which impress with a look-alike of ovarian tumors, it shows that DR-70™ reacts highly sensitive towards endometrium and cervix carcinoma when compared with CA-125. With colon carcinoma and myoma the degree of sensitivity in both tumor markers is analogous. The missing specificity of CA-125 as a tumor marker has been documented by other authors [18,31].

The cut-off values of >1.2 μg/ml for DR-70™ and >65 U/ml for CA-125 support that postoperative follow-up controls utilizing tumor markers are dependent on the availability of tumor cell specimen. Given the fact that a majority of patients suffer from an advanced stage of ovarian cancer chemotherapy is ensuing. The response and success of such follow-up services and treatments are reflected in the levels of CA-125 and DR-70™. Fully recovered patients, free of relapse, have DR-70™ levels but also CA-125 values in the same range as those for a healthy control group. Once a relapse occurs or malignancy progresses, it can be observed that DR-70™ and CA-125 antigen levels increase.

In conclusion, DR-70™ represents a tumor marker capable of diagnosing ovarian carcinoma with a consistently high degree of sensitivity and specificity in relation to the group of apparently healthy blood donors. This compares favorably with the CA-125 test. Despite its high sensitivity, application of DR-70™ as well as CA-125 in screening procedures for ovarian carcinoma is doubtful, due to lack of specificity. Elevated levels of both tumor markers have been reported in patients with adenitis, endometriosis and in other benign and malignant diseases. DR-70™ seems particularly useful in the phase of postoperative follow-up and recurrence monitoring in order to observe progress and therapeutic response.

The correlation between tumor cell mass and antigen concentration, delimitation of non-specific tumor-associated biochemical processes, the changes in differentiated antigen expression before and after therapeutic measures and the establishment of the diagnosis of ovarian carcinoma from the ascites which shows 10 to 100 times higher levels of cross-linked fibrin derivatives than cirrhosis ascites [2] remain worthwhile proposing objectives of further investigations.

References


Meaning of the DR-70™ Immunoassay for Patients with the Malignant Tumor

Ki-Ho Lee1,2, Dong Hee Cho1, Kwang-Min Kim1, Sang-Man Kim1 and Duck-Joo Lee3
1Yonsei University, Research Institute of Aging Science, Seoul, 2Hanyang University Hospital Health Promotion Center, Seoul, 3Department of Laboratory Medicine, Sungkyunkwan University School of Medicine, Samsung Cheil Hospital, Seoul, 4Department of Family Medicine, Ajou University, Suwon, 5Pochon Cha University Cha Bio Medical Center, Seoul, Korea

ABSTRACT

Background: The DR-70™ immunoassay is a newly developed cancer diagnostic test which quantifies the serum fibrin degradation products (FDP), produced during fibrinolysis, by antibody reaction. The purpose of this study was to evaluate the potential of DR-70™ immunoassay in screening malignant tumor. Methods: Sample subjects were 4,169 adults, both male and female, who visited the health promotion center of a general hospital from March 2004 to April 2005 and underwent the DR-70™ immunoassay test and other tests for cancer diagnosis. The patient group was defined as 42 adults out of the sample subjects who were newly diagnosed with cancer during the same time period when the DR-70™ immunoassay test was performed. Final confirmation of a malignant tumor was made by pathological analysis. Results: The mean DR-70™ level was 0.83±0.65μg/ml (range: 0.00 (0.0001)–7.42 μg/ml) in the control group (n=4,127) as opposed to 2.70±2.33 μg/ml (range: 0.12–9.30 μg/ml) in the cancer group (n=42), and statistical significance was established (p<0.0001, Student t-test). When categorized by the type of malignant tumor, all cancer patients with the exception of the subgroups of colon and rectal cancer showed significantly higher mean DR-70™ levels compared with the control group (p<0.0001, Kruskal-Wallis test). The receiver operating characteristic (ROC) curve analysis revealed 1.091μg/ml as the best cut-off value. Using this cut-off value, the DR-70™ immunoassay produced a sensitivity of 71.4%, a specificity of 70.1%, a positive predictability of 69.4%, and a negative predictability of 69.2% (1). Conclusion: A significant increase in the mean DR-70™ value was observed in the cancer group (thyroidal, gastric, breast, hepatic and ovarian) compared with the control group. In particular, the specificity and sensitivity of the DR-70™ immunoassay was relatively high in the subgroups of breast, gastric, and thyroidal cancer.

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There is need for further studies on a large number of malignant tumor patients to see how the DR-70 level might be changed according to the differentiation grade and postoperative prognosis of the malignant tumor. (Immune Network 2006;6(1):43-51)

Key Words: FDP, DR-70, immunoassay, cancer, tumor marker
Introduction

Haemostasis and angiogenesis function is very strict control system that has physiological process in normal status but most of regulation function is destroyed when the carcinoma cells are growing (2).

Generally, it has known that tumor carry with procoagulants and fibrinolytic factors both. To grow the tumor, invasion and metastasis of tumor, it should be connected extrinsic coagulation system and fibrinolytic cascade both matters, which are creation of topical thrombin and accumulation for fibrin, are most important factors (3-5).

Furthermore, it has reported that cells of malignant tumor is possessed high level of plasminogen activator to induce topical dissolution (6) and these plasminogen activator of malignant tumor can be as prognosis factor (7).

Fibrin degradation Product (FDP) has some functions such as creation blood vessel, chemo- attractants and anti-inflammatory functions (6). It has reported levels of FDP are significant increased in serum that extracted from malignant tumor patients (8-11).

Malignant cells characteristically possess high levels of fibrin degradation products (FDP), and fibrin is occurred in the body and is involved in the clotting process of blood (12-13). This test system will successfully use for the detection as screening test of a number of malignant tumor.

DR-70™ immunoassay is diagnostic test, which quantifies the serum FDP, produced during fibrinolysis, by antibody reaction. There are some research papers to identify DR-70™ immunoassay can diagnose not only lung cancer compared to two groups, lung cancer group and control group with 92% of specificity and 66% of sensitivity (8), also stomach cancer, breast cancer, rectal cancer, colon cancer, liver cancer, ovarian cancer,
esophageal cancer, cervical cancer, especially the screening tool of adenocarcinoma(15). In the study is aim to evaluate latency of newly developed DR-70™ Immunoassay that detect malignant tumor and analyze DR-70™ results as prognosis index of tumor diffusion and growth, it compared to DR-70™ mean value between cancer patients and normal people, both male and female, and age group based on immunological analysis method related to fibrin degradation.

Material and Methods

1. Objects of study

Sample subjects were 4,169 adults, both male and female, who visited the health promotion center of Samsung general hospital from March 2004 to April 2005 and underwent the DR-70™ immunoassay test.

Final research subjects were total 4,169 except the people who diagnosed with benign in hepatitis A, B, C surface antibody, syphilis, AIDS, liver and kidney disorder patients, diabetes, blood coagulation disease, coronary artery disease and arrhythmia patient based on medical examination by interview, physical examination and laboratory test. In addition, subjects are eliminated who can diagnosed with false-positive such as pneumonia, bronchitis or acute infection, autoimmune related diseases and recent trauma history of case and hyperlipidemia that have possibility to diagnose with false-negative among total 4,169 subjects.

The patient group was defined as 42 adults out of the sample subjects who were newly diagnosed with malignant carcinoma such as liver cancer, colon cancer, ovarian cancer, thyroid papillary cancer and breast cancer during the same time period when the DR-
70™ immunoassay test was performed.

The cancer patient group was defined as 19 breast cancer patients, 5 rectal and colon cancer patients, 2 hepatoma patients, 7 ovarian cancer patients, 5 stomach cancer patients and 4 thyroid papillary cancer and confirmed as malignant tumor based on X-ray, computer tomography (CT), endoscopy and final pathological test.

2. Materials and Methods

1) Serum

We took 2ml venous blood that underwent on a fast of 8 hours before having a test and hold the specimens in serum separate tube. We used the serums that were centrifuged for 15 minutes with 1500-rpm speed after kept for 30 minutes in room temperature to analyze.

2) DR-70™ diagnostic kit

The AMDL, Inc. DR-70™ assay is an ELISA based assay utilizing removable strips in a 96 micro well format. The wells are coated with affinity purified rabbit anti- DR-70™ polyclonal antibodies. In addition, it includes a bottle of antibody-peroxidase conjugate, Diluent Buffer Concentrate, TMB Substrate, Stop Solution, Wash Buffer Concentrate, Low Control and High Control, 5 calibrator.

3) AMDL DR-70™ principal

The DR-70™ test is an enzyme liked immunosorbent assay (ELISA) using affinity purified rabbit anti- DR-70™ immobilized on the bottom of the well to capture DR-70™ antigen from the diluted serum. The captured antigens, upon washing, are then complexed by peroxidase labeled anti- DR-70™ conjugate to form an immuno-sandwich. The bound enzyme conjugate is quantitatively measured with TMB substrate. Immediately after stopping the enzymatic
reactions, the absorbance of the solution is read at 450nm.

4) DR-70™ testing procedure

All sera were diluted 200 fold with the diluent solution supplied in the kit. Typically, 10 μl of serum were added to 2000 μl diluent. Upon proper mixing, the diluted serum was added to two adjacent wells of a dilution plate. Each well received 200 μl as duplicate samples. Using an 8-channel pipettor 100 μl of the diluted serum was removed from the dilution plate and 100 μl was delivered to the antibody-coated plate. The plate was sealed with a plate sealer and incubated at room temperature for 15 minutes. The plate was then washed 6 times using 300 μl of wash solution for each wash. The wells were dried with a stream of air for 2 minutes. Then 100 μl of TMB substrate solution were added to each well, protected from direct light and incubated for 10 minutes for color development. Finally, 100 μl stopping solution were added to each well to stop the enzymatic reactions. The absorbance of the solution was read at 450nm in an ELISA reader. From the absorbance of the 5 calibrators, a standard curve was constructed. The DR-70™ level of the serum was read from this standard curve.

5) Data analysis

Each student t-test and ANOVA test were operated to confirm the average difference DR-70™ testing result and average of each age group based on gender. Student t-test and Mann Whitney test were operated to confirm the average difference of DR-70™ testing result between malignant tumor (adenocarcinoma) group and normal control group and one way ANOVA test and Kruskal-Wallis test were operated to confirm the average difference between control group and each tumor. We calculated specificity and sensitivity based on various standard densities and measured most suitable cut-off value to DR-70™ immunoassay using ROC curve. I was considered statically significant when P value was under 0.05 in both examinations. All statistical analyses were performed using the SPSS statistical package.
version for windows 10.0.

Results

The DR-70™ mean value was 0.830±0.649 μg/ml in the control group (n=4,1247 subjects) who have no tumor in their body. (Table 1)(Fig.1.). The mean value was 0.69±0.65 μg/ml in male control group as opposed to 0.84±0.65 μg/ml in female control group, the mean value of female is higher than male control group and the difference was statically significant (P<0.05, Student t-test).

When characterized by age in control group, the mean value was 0.58±0.60 μg/ml in age group 30, 0.55±0.46 μg/ml in 40 age group, 0.80±0.57 μg/ml in 50 age, 1.02±0.71 μg/ml in 60 age, 1.13±0.84 μg/ml in over 70 age group, and the mean value was statically significant in age difference (P<0.0001, One way ANOVA test). When divided into three groups under 40(I group), 50(II group), over 60 ages(III group), the higher age was showed, the higher DR-70™ mean value was statically significant in Turkey multiple comparison.

When characterized by age in male patient group, the mean value was 0.44±0.47 μg/ml, 0.62±0.67 μg/ml in 40 age group, 0.55±0.46 μg/ml in 50 age, 0.66±0.43 μg/ml in 60 age, 1.34±1.07 μg/ml in over 70 age group, and the mean value was statically significant in age difference (P<0.0001, One way ANOVA test), also statically significant prevalence was established only between over 70 age group and other age groups based on Turkey multiple comparisons.

When characterized by age in female control group, the mean value was 0.60-0.62 μg/ml, 0.55±0.45 μg/ml in 40 age group, 0.81±0.57 μg/ml in 50 age, 1.03±0.71 μg/ml in 60 age, 1.12±0.82 μg/ml in over 70 age group, and the mean
value was statically significant in age difference, also statically significant prevalence was established only between over 70 age group and other age groups based on Turkey multiple comparisons. It was very significantly difference((P<0.0001, One way ANOVA test)) and the older age group was, the higher mean value was shown based on Turkey multiple comparisons, when characterized by age that it divided into three groups, under 30 and 40 age group are made as “a”, 50 age group is “b”, 60 and over 70 are “c”(Table III). The DR-70™mean value was no difference both male and female under 40 and over 70 age groups, whereas the female mean value was 0.81±0.57µg/ml and higher than male as 0.55±0.4µg/ml in 50 age group, also the female mean value was 1.03±0.7µg/ml and statically significantly higher than male as 0.66±0.43µg/ml, when compared to the DR-70™ mean value between male and female in each age group(P<0.0001, One way ANOVA test).

Fig.2 showed the comparison of the DR-70 mean value between malignant tumor group and normal control group in each age group. When compared to malignant tumor group and normal control group among subjects(n=4,169), mean value was 0.83±0.65µg/ml(range:0.00(0.0001)~7.42µg/ml) in control group(n=4,127), whereas the mean value in cancer group (n=42) was 2.70±2.33µg/ml(range:0.12 ~9.30), three time higher than control group, and statically significant prevalence(P<0.0001, Student t-test).

When analysed the mean value between cancer group and control group in each age, the DR-70™ mean value was 0.55±0.46µg/ml in 40 age group, even though the mean value was higher than cancer group as 0.75±0.36µg/ml, there was no statically significant(P>0.05, Student t-test). The mean value was 0.80±0.5µg/ml in control group and 2.84±2.44µg/ml in 50 age cancer group, not only the mean value of cancer group was higher as 3.18±2.26µg/ml (P<0.0001, Student t-test) than 1.02±0.71µg/ml
in 60 age control group, but also the mean value was statically significantly higher the mean value 1.13±0.84 μg/ml in control group than the mean value 3.31±2.95 μg/ml as cancer group (P<0.05, Mann-Whitney test).

When compared to the DR-70™ mean value between normal control group and control group in malignant tumor, as we mentioned earlier, whereas the DR-70™ mean value was 0.83±0.65 μg/ml, it was showed rectal and colon cancer (1.38±1.07 μg/ml), hepatoma (2.85±3.31 μg/ml), ovarian cancer (2.39±3.36 μg/ml), stomach cancer (2.97±1.30 μg/ml), thyroid cancer (4.32±3.09 μg/ml), all cancer group with the exception of the sub groups of colon and rectal showed significantly higher mean DR-70™ value compared to control group (Kruscal-Wallis test P<0.001). Especially in Turkey multiple comparison, DR-70™ mean value was statically significantly higher in thyroid cancer compared to other malignant carcinoma and the mean value of hepatoma, ovarian cancer, and stomach cancer was higher than colon cancer, rectal and breast cancer (Table II),(Fig.3).

The receiver operating characteristic (ROC) curve analysis revealed ≤1.091 μg/ml as the best cut-off-value, ≤1.091 μg/ml in field under curve. Using this cut-off-value, sensitivity of diagnosis for malignant tumor was 71.4%, specificity 70.1%, a positive predictability 69.4%. With 95% specificity, the cut-off-value of DR-70™ was 1.854 μg/ml and sensitivity in malignant tumor (p<0.01). Based on a 95% confidence level, the upper limit for the normal DR-70™ level can be set at 1.914 g/ml with 47.6% of sensitivity and 95.4% of specificity. Sensitivity was 100%, 95%, 90%, 85% and 80%, when the mean value was 0.118 μg/ml, 0.222 μg/ml, 0.3095 μg/ml, 0.5095 μg/ml, 0.929 μg/ml with 6.8%, 23.6%, 36.4%, 60.2% of specificity. The specificity was 100%, when the DR-70™ mean value was over 6.13 μg/ml.
Table III showed the sensitivity and specificity of DR-70™ in each benign tumor. In case of breast cancer, based on cut-off of 1.0905 μg/ml for DR-70™ the specificity was 70%, whereas the sensitivity was measured at 73.7%, and 70%, 95% of specificity (DR-70™ = 1.854 μg/ml), sensitivity was 47.4. In case of rectal and colon cancer, sensitivity was 80% with 70.1% in the DR-70™ mean value at 1.0905 μg/ml, and sensitivity was 50% with 70.1%, 95% specificity (DR-70™ = 1.865 μg/ml) in the mean value at 1.0905 μg/ml in hepatoma. The sensitivity was 57.1% with 57.1% specificity at 0.5845 μg/ml and 95% specificity (DR-70™ = 1.854 μg/ml) with 28.6% of sensitivity in ovarian cancer. Based on cut-off of 1.0905 μg/ml for DR-70™ the specificity was 70.1% 95% DR-70™ = 1.854 μg/ml, whereas the sensitivity was measured at 100%, and the sensitivity was 80% with 70.1% of specificity in stomach cancer. At 1.0905 μg/ml of cut-off value, sensitivity was 75%, with 70.1%, 95% specificity, and the sensitivity was 75% at (DR-70™ = 1.854 μg/ml), and the sensitivity and specificity were statically significant prevalence to detect breast cancer, stomach cancer and hepatoma using DR-70™ immunoassay by itself (P<0.01).

**Consideration**

The theory (3- 11) that DR-70™ grows when a tumor occurs is significant that DR-70™ is valuable as an important mark for multiplication of tumors because the creation of fibrin resolution substance is usually related to quantitative growth and number and quantity of transferred parts of tumors reflecting not only for diagnosis of malignant tumors, but also newborn vasomotor operation and diffusion of tumors. Related with this, studies reported that DR-70™ value increases when a differentiation of malignant
tumor is intense (17). In this study, the result of gradational classification and change of
tumors are not presented, so DR-70™ numeral value related to a differentiation of
tumors is not observed and additional studies are needed for this in future.

Gianfranco B. (18) and others reported that the plasma D-Dimer does not have
significant difference for age and gender, but in this study, the average of total female
(n=3,970) was $0.688 \pm 0.648 \mu g/ml$, higher than the DR-70™ average of total male
patients (n=159), $0.688 \pm 0.653 \mu g/ml$ in the control group ($P<0.05$, Student-t test).

Especially the 50 and 60’s female’s average was significantly higher than male’s. Also,
average DR-70™ value of male 70’s was significantly higher than other ages ($P<0.0001$,
One way ANOVA test), and in the case of female, among the 3 groups (group a; 30~40’s,
group b; 50’s, group c; 60’s), average of DR-70™ was higher in older age group, and
statically significant ($P<0.0001$, One way ANOVA test). Related with this, there were
reports that show the value of fibrinolytic parameter (19) including D-dimer was
increase when Hormone Replacement Therapy (HRT) was done to women in the
menopause, and D-dimer, plasminogen (20), and fibrinolytic activity (21) was increased
when taking the pill. The study result that the DR-70™ mean value of 50~60’s women
were higher than other age’s women and same age male presents necessity of
comparison of DR-70™ mean value for HRT to women in the menopause.

As same as previous study results(2,8) analyzed DR-70™, the mean value of the group
without malignant tumors were $0.83 \pm 0.65 \mu g/ml$ (range:0.00~7.42 $\mu g/ml$), and the mean
value of the group with malignant tumors were $2.70 \pm 2.33 \mu g/ml$ (range:0.12~9.30 $\mu g/ml$),
and 3 times higher than the control group, and was statically significant ($P<0.0001$,
Student $t$-test). For the reference, as we see above, DR-70™ mean value of female
group was significantly higher than male group. DR-70™ mean value followed
existence of malignant tumors was compared only in female group.  
DR-70™ mean values for the female control group without malignant tumors (n=3,970) were 0.84±0.65 μg/mL (range: 0.00~7.42 μg/mL) and the group without malignant tumors (40 people) were 2.73±2.37 μg/mL (range: 0.12~9.30 μg/mL), and as a result, the group with malignant tumors had significantly higher mean value. This showed similar result in the total group (Table not shown, P<0.001, t-test).  
Related with DR-70™ mean value was different with ages in the normal control group, when malignant tumor group and control group were comparably analyzed with their ages, the group under 40’s was not statically significant. The average (0.75±0.36 μg/mL) of cancer patients (n=6) was higher than the average (0.55±0.46 μg/mL) of the control group (n=1,122) (p<0.05, Student t-test). The DR-70™ average of 50’s cancer patients (n=18) was 2.84±2.44 μg/mL, and the 50’s control group (n=1,381) had 0.80±0.57 μg/mL. and the average of cancer patients was 3.18±2.26 μg/mL, the control group was (n=1,624) 1.02±0.71 μg/mL in over 60 age group (p<0.0001, Student t-test). The average of cancer patients was higher, and their average value was higher than the normal control group without any relation with their ages. When doing stratification analysis followed to ages based on the standard 1.091 of DR-70™ value as the cut off value, distinguishing existence of malignant tumors compared with this value is higher or not. If DR-70™ value was higher than 1.091, there was statically significant high risk of malignant tumors in the all age groups except 40s (under 40s: P>0.05, 50’s: P<0.0001, 60s: P<0.0001, over 70s: P<0.05, Table not shown, Mantel-Haeszel test).  
Filed and others (2) reported the clinical test result of using DR-70™ for diagnosis of lung cancer in the Cross Cancer Institute in 1995. The result showed that DR-70™ could be used for diagnosis of non-small cell lung cancer and small cell lung cancer
with same level. The sensitivity of non-small cell lung cancer stage IV patient was 83.7%, and the progressive cellular lung cancer was 80.0%. Previous results also presented that DR-70™ could be used for diagnosis of more cancers out of lung cancer (17). This study, same as the previous studies, could confirm that DR-70™ Immunoassay can detect various types of cancer.

Wu and others (8) had study of DR-70™ for lung, stomach, breast, and rectal cancer, and reported that DR-70™ sensitivity was high with stomach cancer (92.6%), and low with colon cancer (66.7%). A Kerber and others (22) reported that cholangiocellular cancer and esophageal cancers were 100%, Hepatocellular carcinoma with 94.7%, pancreatic cancer with 92.3%, stomach cancer with 90%, and colorectal cancer with 80.0%. The previous studies diagnosed DR-70™ with the patients who already had progressive malignant tumors or were diagnosed as cancer, but the diagnosis in this study had been done at the first stage of normal people who had their health test or were doubted as a malignant tumor. Therefore, the meaning of this study can be comparably important to judge the level of sensitivity and specificity of DR-70™ for early diagnosis of malignant tumors.

When comparing the usefulness of DR-70™ for cancer diagnosis with traditional cancer detect method, in the case of breast cancer, comparing with value 1.091, sensitivity was 73.7%, specificity was 70% and 95%, and sensitivity in specificity level (DR-70™=1.865 μg/ml) was 47.4%. In the previous studies, Esklinen and others (23) reported the sensitivity of CEA, CA 15-3, and TPS for breast cancer was 23%, 27% and 18%, and Arslan and others (24) reported that the sensitivity of CA15-2 and CEA before the breast cancer surgery was 23.2% and 95.3%; specificity was 17.4% and 83.7%. Therefore, this can be proved this study is more accurate than above results, and
CA 72-4 with stomach cancer was 68-93% of specificity and 27-84% of sensitivity (24,25). As a result of the research, DR-70™ sensitivity was 100%, 70.1% of specificity and 95% of specificity (1.854 \( \mu g/ml \)) with 80% of sensitivity (P<0.01) and CEA and AFP are widely used for other digestive organ cancers, but high level of sensitivity with stomach cancer that there are no specific cancer detection methods was meaningful result.

In the case of the thyroid cancer, which was not analyzed with previous studies, the sensitivity was 75.5% and specificity was 70.1%. The 95% level of specificity (1.854\( \mu g/ml \)) had 75% sensitivity (P=0.005). In the case of the rectal cancer and colon cancer, the result showed that sensitivity was 80%, specificity was 70.1%, and the level of 95% specificity (1.854) was had 20% sensitivity. This shows the sensitivity is lower than the studies of Wu and others (8), but accurate comparison could not be done because this study result was not statically significant. (P>0.05) Approximately 50% of sensitivity was shown with the liver cancer, and in the case of the ovarian cancer, with the DR-70™ value (0.5845), 57.1% of sensitivity, and 28.6% of sensitivity with 59.5% of level of specificity (DR-70™=1.854\( \mu g/ml \)) were shown, but this is not statically significant (P>0.05).

In this study, the mean value of DR-70™ was higher than the control group with all early cancer. Especially breast, stomach, thyroid and liver cell cancer were significantly higher than the control group, and rectal, colon, and ovarian cancer had higher DR-70™ mean value than the control group, but was not statically significant. Therefore, using DR-70™ as a cancer detection method for specific malignant tumors will be difficult, but this method can be clinically used as an entire early cancer detection method. Especially, DR-70™ Immunoassay can be used for cancer detection test with annual
The cancer transfer risk level for each points of DR-70™ was found with random sampling from 20% (n=835) of the entire control group (n=4,169).

Setting the standard as 1.091 µg/ml which is chosen as best cut-off value by writers, the risk of transferring to malignant tumors was 6 times higher with the subjects who had their DR-70™ mean value higher than 1.091 (Table IV: OR=6.20, 95% C.I.(3.21-12.31), P<0.001, Chi-Square test), and Setting the standard as 1.854 µg/ml with 95% of specificity which is chosen as best cut-off value by writers, the risk of transferring to malignant tumors was 19 times higher with the subjects who had their DR-70™ mean value higher than 1.091 (Table V: OR=6.20, 95% C.I.(9.59-37.92), P<0.001, Chi-Square test).

The limitation of this study can be chosen that the number of malignant tumor patients was fewer than the normal control group. Especially the results of the colon and rectal cancer were not statically significant because of lack of patient number, and there was high difference between the number of male and female subject. Third, DR-70™ value change of tumor differentiation type was not presented and DR-70™ value was not analyzed before and after.

However, this study analyzed DR-70™ value in the normal group and malignant tumor patient group in the large scale of population, not only for eastern people.

It is proven that DR-70™ is more accurate than traditional cancer detection method with some early stage cancers. This study also proven that the thyroid cancer patient who was not studied with previous test had significantly high DR-70™ value.

With strict revision of ages and gender, analyzing concretely for disturbance factors for DR-70™, and setting standard for exclusion of the disturbance factors, the study with
larger scale of malignant tumor patient group should be done.
Also, the study for substances that have high DR-70™ value but malignant tumors should be done in near future.

Conclusion
A significant increase in the DR-70™ mean value was observed in the cancer group such as thyroidal, breast cancer, stomach cancer and liver cancer compared with the control group. As a result of the research, the test will be very useful to detect adenocarcinoma and screening test in malignant tumor to patients who have no acute infection, autoimmune related diseases. In particular, the specificity and sensitivity of DR-70™ immunoassay was relatively higher and considered statistically significant in the subgroups of breast, gastric and thyroidal cancer patients. It means that DR-70™ can use as universal tumor marker or pan tumor marker to detect adenocarcinoma.
There is need for further studies on a large number of malignant tumor patients to see how the DR-70™ value might be changed according to the different grade and postoperative prognosis of the malignant tumor.
Serum concentration of AMDL DR-70 for the diagnosis and prognosis of carcinoma of the tongue

Xiaodan Li\textsuperscript{a}, Zhi Qiao\textsuperscript{b}, Xing Long\textsuperscript{a,\ast}, Jinxiong Wei\textsuperscript{a}, Yong Cheng\textsuperscript{a}

\textsuperscript{a} Department of Oral Maxillofacial Surgery, College of Stomatology, Wuhan University, Key Laboratory for Oral Biomedical Engineering, Ministry of Education, Wuhan, Hubei 430079, PR China
\textsuperscript{b} Department of Microbiology, College of Medicine, Wuhan University, PR China

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Abstract

The aim of this study was to discover the clinical value of the tumour marker AMDL DR-70 in a group of patients with cancer of the tongue. Serum concentrations of AMDL DR-70 were estimated by enzyme linked immuno-sorbent assay in 52 patients with carcinoma of the tongue and compared with 40 controls and 42 patients with benign lesions in the tongue. Thirty-nine patients with carcinoma of the tongue had results above 6 mg/L (75%), compared with 3/40 (7%) in healthy controls and 4/42 (10%) in those with benign tumours. The concentration of AMDL DR-70 in serum correlated significantly with 3-year survival.

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Keywords: AMDL, DR-70; Squamous cell carcinoma; Diagnosis; Prognosis

Introduction

In certain areas of Asia oral cancer accounts for about half of all malignancies, and squamous cell carcinoma (SCC) is the most common malignant tumour of the tongue.\textsuperscript{1} Recent data suggest that the incidence is increasing.\textsuperscript{2,3} Five-year survival of patients with oral SCC have changed little in the past 30 years partly as a result of delayed diagnosis. Early detection of oral cancer involves screening,\textsuperscript{4} but most screening requires specialists’ examinations, which are costly and time-consuming. A rapid and cheap method for the early detection of carcinoma of the tongue is therefore essential.

Increased serum proteolytic activity and activation of parts of the coagulation cascade are associated with the activity of cancer cells. AMDL DR-70 was designed to detect fibrinogen degradation products, and has been reported to be a good screening test for various malignant diseases with good sensitivity.\textsuperscript{5} It has, however, not been used for diagnosis or prognosis of SCC of the tongue.

\textsuperscript{\ast} Corresponding author. Tel.: +86 27 876 46312; fax: +86 27 878 73260. E-mail address: longxing.china@hotmail.com (X. Long).

Material and methods

Patients

We studied 52 patients with biopsy-proved squamous cell carcinoma of the tongue, 42 patients with various benign lesions of the tongue, and 40 healthy people. The patients ranged in age from 24 to 78 years; 20 were women and 32 were men. Of the 52, 33 (63%) were in T1 and T2 stages and 19 (36%) in T3 and T4 stages. They were followed up for 3 years. Of the 42 with benign tumours of the tongue 24 were women and 18 men (age range 13–74 years). Diagnoses included fibroma (n = 8), haemangioma (n = 13), and neurofibroma (n = 21). Sera from 40 healthy donors (26 men and 14 women, aged 18–70 years) were chosen as controls.

Assay of AMDL DR-70 by enzyme-linked immunosorbent assay (ELISA)

A sample of 2 mL whole blood from each patient in the above three groups was collected. Serum was separated and stored at −20 °C. AMDL DR-70 detection kits were bought from the

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AMDL Corporation, USA. Serum concentrations of AMDL DR-70 were measured in accordance with the manufacturers' instructions. In short, samples in a dilute buffer were added in 100-μL amounts to wells of an antibody-coated microwell plate. After 1-h incubation at room temperature the wells were washed then 100-μL aliquots of conjugate were added to each well. The plate was again incubated for 30 min at room temperature, washed and incubated with 100 μL AMDL DR-70 substrate. After 15 min at 25 °C the reaction was stopped by adding 100 μL 0.1N hydrochloric acid into each well. The absorbance at 450 nm was proportional to the concentration of AMDL DR-70.

Statistical analysis

We used Cox's proportional-hazards model to examine the correlation of concentrations of AMDL DR-70 with survival. The covariates we included were age, sex, and stage of tumour. The data were analysed with the statistical software Egret and EPI-Info.

Results

Serum concentrations of AMDL DR-70 in the three groups

Receiver-operating characteristic (ROC) curves were made to assess the relation between the sensitivity and specificity. Using an upper limit of normal corresponding to 92.5% specificity, a sensitivity of 73% was reached for cancers of the tongue. As showed in Table 1, the mean (SD) serum concentration of AMDL DR-70 in the patients with carcinoma of the tongue was 11 (6) mg/L. The serum concentrations of AMDL DR-70 in the patients with benign tumours and normal control subjects were 4 (2) and 4 (1) mg/L. We considered concentrations of AMDL DR-70 of over 6 mg/L as above the reference range. In the cancer group, 39 of 52 (75%) patients had concentrations higher than 6 mg/L, whereas the concentration of AMDL DR-70 in sera of 38 of 42 (90%) patients with benign tumours and 37 of 40 healthy subjects (92.5%) were less than 6.0 mg/L (Table 1). There was no significant difference in concentration of AMDL DR-70 between those with benign tumours and healthy people. High fibrinolytic activation in the sera of patients with carcinoma of the tongue was shown by AMDL DR-70.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cases</th>
<th>Concentration of AMDL DR-70 (mg/L)</th>
<th>0–3.0</th>
<th>3.1–6.0</th>
<th>6.1–10.0</th>
<th>10.1–20.0</th>
<th>&gt;20</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoma of tongue</td>
<td>52</td>
<td></td>
<td>2</td>
<td>11</td>
<td>15</td>
<td>14</td>
<td>10</td>
<td>11 (6)</td>
</tr>
<tr>
<td>Benign tumour</td>
<td>42</td>
<td></td>
<td>13</td>
<td>25</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>4 (2)</td>
</tr>
<tr>
<td>Healthy people</td>
<td>40</td>
<td></td>
<td>16</td>
<td>21</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>4 (1)</td>
</tr>
</tbody>
</table>

Discussion

The 1-, 2-, and 3-year survival of patients with SCC were roughly 94%, 71%, and 65%, respectively. We divided the 52 patients with cancer into two groups: one with AMDL DR-70 concentration less than 11 mg/L (n = 33) and the other with AMDL DR-70 concentration over 11 mg/L (n = 29). The 3-year survival in the group with less than 11 mg/L was 80%, while in the group with more than 11 mg/L it was 45% (p < 0.01). The 3-year survival in patients in T1 and T2 stages was 79%, compared with 37% in patients in stages T3 and T4. Survival of patients in the two groups (more or less than 11 mg/L) were compared in relation to clinical staging. Of 24 patients in stages T1 and T2 and AMDL DR-70 less than 11 mg/L 21 lived (survival 87%), but only five of nine patients with AMDL DR-70 greater than 11 mg/L in T1 and T2 stages survived. For patients in T3 and T4 stages, survival rates were 3/6 when AMDL DR-70 was less than 11 mg/L and 4/13 when they were over 11 mg/L (Fig. 1). There was no correlation between survival and age or sex.

There have been many efforts to facilitate the early diagnosis of cancer by estimation of cancer antigen 125, carcinoembryonic antigen, neuron-specific enolase, and tumour polysaccharide substance in serum. There have been no highly sensitive and specific tumour markers for carcinomas of the tongue up to now. Serum proteases are associated with a number of malignant conditions. These proteases activate...
parts of the coagulation cascade, which results in increased breakdown of certain plasma proteins, such as fibrin. Malignant cells also possess high concentrations of plasminogen activator, which may induce local fibrinolysis. AMDL DR-70 estimates the amount of fibrinogen degradation products in serum. A study of 136 patients showed sensitivities of AMDL DR-70 of 88%, 93%, 65% and 67% for carcinomas in lung, stomach, breast, and colon, respectively. We now report that serum concentrations of AMDL DR-70 were significantly higher in patients with malignant disease of the tongue than in control groups. Diagnostic sensitivity of AMDL DR-70 was 73% and specificity was 93%.

Multiple factors including: size, area, age, sex, treatment, and biological variables are related to the prognosis of patients with carcinomas of the tongue. The 3- and 5-year survival rates were reported to be almost identical (64% and 61%) in patients with oral cancer. We now show that not only late stage of tumour, but also concentration of AMDL DR-70 predict survival.

**INTERESTING CASE: Cervical lymphadenopathy, induced by head lice**

An 8-year-old girl presented with gross cervical lymphadenopathy (Fig. 1). She had originally been referred to the paediatricians with general malaise and concern because of a long history of involvement of social services with her family. On examination, the had bilateral cervical lymphadenopathy, alopecia, infection with head lice and a haemoglobin concentration of 4.6 g/l. She also had a raised white cell count and erythrocyte sedimentation rate but was not unwell. There was no focus of infection or any lymph nodes palpable elsewhere.

Provisional diagnoses were lymphoma, atypical mycobacterial infection, and tuberculoid. She had two units of blood transfused before a lymph node was biopsied. She was given oral iron supplements and the head lice were treated.

**References**


**Fig. 1.**

Histopathological examination showed follicular hyperplasia of the lymph nodes (Fig. 2). No viruses, bacteria or mycoplasma were isolated. These results discounted the provisional diagnosis but following effective treatment of the head lice, iron supplements, and an improved diet, her condition improved spontaneously. There was complete resolution of the lymphadenopathy, improvement of the haemoglobin concentration and hair grew.

A final diagnosis of reactive lymphadenopathy caused by chronic infection with head lice was made.

**Fig. 2.**

P. Scott
L.S. Middelton
G. Fabbri
G.A. Mitchell
The new DR-70 immunoassay detects cancer of the gastrointestinal tract: a validation study

Medical Department II, University Hospital, Frankfurt/Main, Germany

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SUMMARY

Background: Malignant cells characteristically possess high levels of plasminogen activator, which induce local fibrinolysis. The DR-70 immunoassay is a newly developed test, which quantifies fibrin degradation products in serum by a proprietary antibody.

Aim: To evaluate the DR-70 immunoassay as a detection assay for the presence of gastrointestinal cancers.

Methods: We prospectively collected blood sera of 85 patients with histologically proven tumour and 100 healthy blood donors. Ten microlitres of the sera was used for the DR-70 immunoassay. Nineteen patients had a hepatocellular and 10 cholangiocellular carcinoma, 13 cancer of the pancreas, 30 colorectal cancer, 10 stomach cancer and three cancer of the oesophagus.

Results: Receiver–operator curve analysis revealed <0.7 µg/mL as the best cut-off value to distinguish between patients with cancer and healthy controls. Using this cut-off value, the DR-70 immunoassay showed a good clinical performance with a sensitivity of 91% and a specificity of 93%. Patients with advanced tumour spread showed significantly higher DR-70 values than those with early-stage tumours (P < 0.0003).

Conclusion: The DR-70 immunoassay reliably differs between cancer patients and healthy controls. Therefore, it promises to become a useful test for the detection of cancer in clinical practice.

INTRODUCTION

Haemostasis and angiogenesis are tightly regulated physiological processes, which are deregulated in cancer growth. Tumours have been shown to produce procoagulants and fibrinolytic factors. Activation of the extrinsic coagulation system and the fibrinolytic cascade within a tumour is thought to be related with growth, invasion and metastasis. The local thrombin generation and fibrin deposition and dissolution might be important in tumour growth and dissemination. Malignant cells characteristically possess high levels of plasminogen activator, which induces local fibrinoly-

Correspondence to: PD Dr B. Braden, Medical Department II, University Hospital, Theodor Stern Kai 7, 60590 Frankfurt/Main, Germany. E-mail: braden@em.uni-frankfurt.de

Plasminogen activator seems to be a prognostic factor in neoplasms. Fibrin degradation products have been reported to possess angiogenic, chemotactic and anti-inflammatory activities. It is an established fact that fibrin degradation products are elevated in plasma of patients with malignancies. Therefore, the assumption seems to be reasonable that the assessment of fibrin degradation products might be clinically useful as a screening test for cancer.

The aim of this study was to evaluate the potential of the newly developed DR-70 immunoassay, which is based on the immunochemical detection of fibrin degradation products as a detection assay for gastrointestinal cancers. In addition, the quantitative value of DR-70 should be tested as a parameter of tumour load, progression and dissemination.
METHODS

Patients and controls

After obtaining informed written consent, we prospectively collected blood sera of 85 cancer patients (27 females, 58 males; median age: 68 years, range: 37–89 years) with histologically proven malignant tumour and 100 healthy blood donors (32 females, 68 males; median age: 37 years, range: 18–66 years). On admission, all patients underwent biochemical evaluation of the serum (blood cell count, aspartate aminotransferase, alanine aminotransferase, \( \gamma \)-glutamyltransferase, alkaline phosphatase, total bilirubin, albumin and prothrombin activity). Patients with colorectal cancer were tested for carcinoembryogenic antigen (CEA). CEA levels >5 ng/ml were considered pathological. In serum of patients with hepatocellular carcinoma, alpha fetoprotein (AFP) was measured (normal level <10 \( \mu \)g/L). Routine biochemical tests were carried out using commercially available tests. Staging examinations were performed including sonography and computer tomography of the abdomen and chest X-ray. Additionally, patients with oesophageal tumour underwent computer tomography of the chest and endosonography. The tumour extension and spread was classified using the organ-specific TNM classification. All sera were stored at \(-20^\circ\)C until they were analysed using the DR-70 immunoassay.\(^5\)

Nineteen patients had hepatocellular and 10 cholangiocellular carcinoma, 13 cancer of the pancreas, 30 colorectal cancer, 10 stomach cancer and three cancer of the oesophagus. In all 85 patients, the malignancy was assessed histologically after biopsy or after operative resection.

DR-70 immunoassay

The fibrin degradation products were quantitatively measured using DR-70 kits (AMID, Inc., Tuston, CA, USA) according to the manufacturer’s instructions. The DR-70 assay is an enzyme-linked immunosorbent assay (ELISA)-based serological test utilizing removable strips in a 96-microwell format. Briefly, the wells are coated with polyclonal antibodies from the rabbit against DR-70 products of fibrin degradation. One hundred microlitres of patient sera (diluted 1:200) is incubated in the wells for 30 min. After washing, a second anti-DR-70 antibody conjugated to horse-radish peroxidase is added, which binds to the captured tumour marker. After further wash steps, the colour reaction is started by adding 3,3',5,5'-tetramethylbenzidine to the wells. After stopping the reaction with 0.1 \( \text{N} \) hydrogenchloride, the intensity of the colour formed is read in a microplate reader at 450 nm. The concentrations of DR-70 in the sera are obtained from a standard curve, which results from the extinctions of calibrators provided with the kit. The concentrations of these DR-70 standards are 0, 0.625, 2.5, 5.0 and 10 \( \mu \)g/ml.

Statistical analysis

Data are shown as median and range. Statistical analysis between groups was carried out using the Wilcoxon–Mann–Whitney U-test. Sensitivities and specificities of the immunoassay were calculated at various threshold concentrations. Thus, the best cut-off value for the DR-70 immunoassay was obtained performing receiver operating characteristics (ROC) curve analysis; 95% confidence intervals are given. \( P < 0.05 \) was considered to be statistically significant.

RESULTS

The results of the measured amounts of fibrinogen degradation products in patients and controls are given in Figure 1. The DR-70 values in cancer patients (2 \( \mu \)g/ml; range: 0.4–18.6) significantly differed from the values in controls (0.37 \( \mu \)g/ml; range: 0.3–1.11 \( \mu \)g/ml; \( P < 10^{-10} \)). The results in the organ-related subgroups of cancer patients are also presented in Figure 1 and in Table 1.

Receiver operating characteristic curve analysis revealed \( \leq 0.7 \mu \)g/ml (Figure 2) as the best cut-off value to distinguish between patients with cancer and healthy controls. The area under the ROC curve was 0.965177.

Using \( \leq 0.7 \mu \)g/ml as the cut-off value, the DR-70 immunoassay showed a good clinical performance with a sensitivity of 90.6\% (82.3–95.9\%) and a specificity of 93.0\% (86.1–97.1\%). The positive predictive value was 91.7\% (83.6–96.6\%), the negative predictive value was 92.0\% (85.0–96.5\%) and the efficacy was 91.9\% (87.0–95.4\%).

According to the organ-specific TNM classification, 37 cancer patients were categorized as with limited disease (\( T \leq 3, n < 2, M_0 \)). Forty-eight patients presented with advanced cancer stages, i.e. T4 stadium.
sensitivity 89.2% (74.6–97.0%), specificity 93.0% (86.1–97.1%), predictive positive value 82.5% (67.2–92.7%), predictive negative value 95.9% (89.8–98.9%) and specificity 92.0% (86.1–95.9%).

Patients with advanced tumour spread showed significantly higher DR-70 values (median: 2.4 μg/mL, range: 0.3–18.5 μg/mL) than those with early stage-tumours (median: 1.3 μg/mL, range: 0.5–4.4 μg/mL, \( P < 0.0003; \) Figure 3).

Thirteen of 30 patients with colorectal cancer showed elevated CEA values in serum. The sensitivity of the CEA assay to detect colorectal carcinoma was 43.3% (25.5–62.6%).

In 18 of 19 patients with hepatocellular carcinoma, the AFP was elevated. Therefore, the sensitivity of AFP to detect hepatocellular carcinoma was 94.7% (74.0–99.8%).

Table 1. Performance of the DR-70 immunoassay in the groups with different types of cancer

<table>
<thead>
<tr>
<th>Type of carcinoma</th>
<th>( n )</th>
<th>Median</th>
<th>Range</th>
<th>Sensitivity</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocellular</td>
<td>19</td>
<td>2.4</td>
<td>0.7–9.4</td>
<td>94.7</td>
<td>74.0–99.8</td>
</tr>
<tr>
<td>Cholangiocellular</td>
<td>10</td>
<td>2.6</td>
<td>1.3–4.0</td>
<td>100</td>
<td>69.1–100</td>
</tr>
<tr>
<td>Colorectal</td>
<td>30</td>
<td>1.2</td>
<td>0.4–4.0</td>
<td>80.0</td>
<td>61.4–92.3</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>13</td>
<td>2.3</td>
<td>0.3–18.0</td>
<td>92.3</td>
<td>63.9–99.8</td>
</tr>
<tr>
<td>Gastric</td>
<td>10</td>
<td>3.6</td>
<td>0.6–4.9</td>
<td>90.0</td>
<td>55.5–99.8</td>
</tr>
<tr>
<td>Oesophageal</td>
<td>3</td>
<td>2.0</td>
<td>0.8–15.0</td>
<td>100</td>
<td>29.2–100</td>
</tr>
</tbody>
</table>

DISCUSSION

In our findings, DR-70 levels were significantly higher in all types of gastrointestinal cancer tested than in healthy controls. Therefore, DR-70 cannot be considered as an organ-specific tumour marker. But as the immunoassay detects multiple cancers with a high degree of sensitivity and specificity, it could be clinically used as a global serological cancer detection tool, not limited to specific tumour types.

The quantitative comparison of DR-70 levels in patients with early stages of cancer and in those with advanced tumour progression shows that DR-70 levels increase with the stage and dissemination of the malignant disease. The level of fibrin degradation products was positively correlated with tumour load and number of metastatic sites. The association between markers of fibrin degradation and tumour stage suggests that the increase of DR-70 in the individuum is a clinically important marker for progression and points towards a relation between haemostasis and tumour progression.

The results of our study confirm the findings of Wu et al., who tested patients with different types of cancer (lung, breast, stomach and rectum). Wu et al. found the best sensitivities in patients with stomach cancer (92.6%), while the DR-70 test kit performed with lower sensitivity in patients with rectum cancer (66.7%).

In few cases, which were not included in this study, we exemplarily investigated whether other pathological conditions, which also induce fibrinolysis, might result in increased DR-70 levels and false-positive tests. Four patients with a deep vein thrombosis and/or acute lung emboli did not show increased DR-70 levels. Moreover, six patients with inflammatory processes or systemic immunogenic diseases (Crohn’s disease three, diverticulitis one, appendicitis one, lupus erythematoses one) were negative in the DR-70 immunoassay. Although the knowledge about the performance of the DR-70 immunoassay in disorders with activated coagulation and fibrinolysis is still limited, we do not have indications that these conditions might lead to false-positive results. But further studies in patients with dysbalanced coagulation system are required.

The good clinical performance of DR-70 immunoassay and the sharp discrimination between cancer patients and healthy controls in our study might partly be based on the fact that the majority of our patients (56%) presented with advanced tumour disease. For a screening assay, however, reliability in cases with early stages of cancer is important. The purpose of a screening test is to detect subjects, in whom therapeutic consequences will still have an effect on survival, i.e. patients with early-stage tumours.

Considering only patients with early-stage tumours, satisfying test results were obtained using the DR-70 immunoassay in our study (sensitivity 89.2%, specificity 93.0%). But before the DR-70 immunoassay can be recommended as a global screening test for the presence of malignancy, further studies with a higher number of patients in early stages of cancer disease are required.

When we compared the DR-70 immunoassay with conventional tumour markers, DR-70 turned out to be superior to CEA in the detection of patients with colorectal cancers and equivalent to AFP in patients with hepatocellular carcinoma. A recent study by Blackwell et al. demonstrated that circulating D-dimer levels were better predictors of overall survival and disease progression than carcinoembryonic antigen levels in patients with metastatic colorectal carcinoma.

The laboratory performance of the DR-70 immunoassay takes about 2 h and requires skilled technical personnel and equipment found in most hospital and commercial laboratories.

In conclusion, the DR-70 immunoassay reliably differs between patients with cancer of the gastrointestinal tract or the hepatobiliary system and healthy controls. Therefore, it promises to become a useful cancer detection tool in clinical practice. In addition, there is an association between the quantitative DR-70 value and the stage of tumour extension. This points at an applicability of the DR-70 immunoassay as a prognostic factor in the clinical course of malignant diseases.
ACKNOWLEDGEMENT

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REFERENCES

Elevated Fibrinogen-Fibrin Degradation Products (FDP) in Serum of Colorectal Cancer Patients

Perry Rucker,1,* Sheila M. Antonio,2 and Barbara Braden3

1Seashore Tetrachem, Inc., Santa Ana, CA, USA
2AMDL Inc., Tustin, CA, USA
3Medical Dept II, Johann Wolfgang Goethe University, Frankfurt/Main, Germany

ABSTRACT

Serum fibrinogen-fibrin degradation product (FDP) levels of healthy control subjects and of patients with colorectal cancer have been measured by DR-70® ELISA in Frankfurt, Germany (Germany study) and in Tustin, California (U.S. study). Serum FDP levels of patients with colorectal cancer were significantly higher than those of the healthy controls. The median serum FDP levels in healthy control groups of the German and U.S. studies were, respectively, 0.37 μg/mL and 0.61 μg/mL. The median serum FDP levels in the colorectal cancer groups of the German and U.S. studies were, respectively.

*Correspondence: Perry Rucker, Seashore Tetrachem, Inc., 1560 E. Edinger Ave, Suite B, Santa Ana, CA 92705, USA; E-mail: perruc@aol.com.

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1.25 μg/mL and 1.60 μg/mL. The results are consistent with enhanced fibrinolysis in serum of patients with colorectal cancer.

**Key Words:** Colorectal cancer; Fibrin-fibrinogen degradation products (FDP); DR-70® ELISA; CEA.

**INTRODUCTION**

The invasiveness and metastasis of tumors require the degradation of an extracellular matrix and fibrin surrounding the tumor. Locally generated and released proteases must prepare the migration of tumor cells out of the tumor. Malignant cells generally possess high levels of plasminogen activator, which induces local fibrinolysis. Local thrombin generation and fibrin deposition and dissolution appear to be important in tumor growth and dissemination. It has previously been shown that plasma FDP levels, primarily measured as D-dimer, were elevated in colorectal patients and other cancers. Wu et al., using AMDL DR-70® Immunoassay kit, found that serum FDP levels of lung cancer patients were significantly elevated. AMDL DR-70® ELISA is an immunoassay developed to measure serum FDP. The aim of this study was to use DR-70® Immunoassay kit to measure serum FDP levels in patients with colorectal cancer and compare to the levels in healthy individuals.

**EXPERIMENTAL**

Current studies were conducted at two independent sites: Site 1: Frankfurt/Main, Germany; Site 2: Tustin, CA. They are, respectively, referred to as German study and U.S. study.

**Germany Study**

*Healthy Control Subjects:* Sera from 100 healthy blood donors (68 male, 32 female; median age: 37 years; range: 18–66 years) were used as the healthy controls in this study.

*Cancer Patients:* Sera from 30 patients diagnosed with colorectal cancer were collected. There were seven female patients with age ranges from 59–82 years old and 23 male patients with age ranges from 38–82 years old. On admission to the hospital, all patients underwent routine biochemical evaluation of the blood that includes blood cell count, aspartate aminotransferase,
Elevated Fibrinogen-Fibrin Degradation Products (FDP)

alanine aminotransferase, γ-glutamyltranspeptidase, alkaline phosphatase, total bilirubin, albumin, and prothrombin activity. Staging examinations were performed with sonography and computer tomography of the abdomen and chest x-ray. The serum carcinoembryonic antigen (CEA) levels of 24 of the 30 colorectal cancer patients were determined by enzyme-linked immunosorbent assay (ELISA). All cancer patients were histologically proven, with fine needle puncture guided by endosonography or with operative resection, to have malignant tumor.

U.S. Study

Healthy Control Subjects: Sera from 100 healthy volunteers were used as the healthy controls in this study. There were 40 male volunteers with age ranges from 20–69 years old and 60 female volunteers with age ranges from 21–81 years old.

Cancer Patients: Sera from 44 patients diagnosed with colorectal cancer were purchased commercially. There were 20 male patients with age ranges from 22–80 years old and 24 female patients with age ranges from 49–76 years old. The malignancy of these patients was confirmed histologically, and stage of the colorectal cancer was assigned to each patient. There were three patients with Duke’s stage 1 cancer, 17 with Duke’s stage 2 cancer, 19 with Duke’s stage 3 cancer, and five with Duke’s stage 4 cancers.

Sera Collection

All sera were obtained by the following procedures: Approximately 5 mL venous blood was drawn into a serum separation vacutainer tube. (This tube contained SST gel and clot activator, the Tiger Top tube.) The blood in the tube was left at room temperature for 30 min in an upright position, then centrifuged at 2,000 rpm for 15 min. The clear serum was transferred to a separated tube for analysis. When not in use, the sera was aliquoted and stored at −20°C.

Measurement of FDP Using DR-70° ELISA Kit

Serum FDP levels were measured with an ELISA kit (DR-70° ELISA, AMDL Inc., Tustin, Ca 92780) manufactured by AMDL, Inc. The kit contains a microwell plate of 12 × 8 well strips coated with affinity-purified rabbit anti-FDP antibodies, a vial of peroxidase-antibody conjugate, one vial of
each of diluent, substrate solution, stop solution, wash buffer, low control, high control, and five calibrators.

The Assay Principle

DR-70\textsuperscript{®} ELISA used affinity-purified anti-FDP antibodies that were immobilized on the bottom of the microwell to capture FDP in serum. These serum FDPs were products of fibrinolysis and proteolysis, presumably by enzymes secreted by tumor cells. The captured FDPs were then complexed by peroxidase labeled antibodies to form an immuno-sandwich. The bound enzyme-antibody conjugates are quantitatively measured with TMB substrates. The amount of bound enzyme-antibody conjugate is directly proportional to the amount of captured FDP. Upon stopping the enzymatic reactions, the absorbance is read at 450 nm.

Assay Procedures

Serum was diluted 200-fold with diluent solution supplied in the kit. For example, 10\,\mu L of serum was added to 2000\,\mu L diluent. Upon mixing, the diluted serum was added to two adjacent wells of a dilution plate. Each microwell of the dilution plate received 200\,\mu L of the diluted serum in duplicate. 100\,\mu L of the diluted serum from microwells in the dilution plate was transferred to the corresponding microwells of the antibody-coated plate using an eight-channel pipettor. The plate was incubated at room temperature for 30\,min. Then, the microwells were washed six times each with 300\,\mu L of wash buffer, inverting the plate and tapping it on a clean absorbent paper. This was immediately followed by adding 100\,\mu L of peroxidase-antibody conjugate to each microwell using an eight-channel pipettor. The plate was incubated at room temperature for 30\,min. Again, the microwells were washed six times each with 300\,\mu L of wash buffer, inverting and tapping it on a clean absorbent paper. Then, 100\,\mu L of TMB substrate solution was added to each microwell using an eight-channel pipettor. The plate was then covered with a piece of aluminum foil and incubated at room temperature for 15\,min. The reactions in the microwells were stopped by adding 100\,\mu L of stop solution to each microwell using an eight-channel pipettor. The absorbance in each microwell was immediately read at 450 nm. From the absorbance of the five calibrators, a standard curve was constructed. The FDP level of the serum was read from this standard curve.

AMDL DR-70\textsuperscript{®} ELISA kit can also be run in automated ELISA equipment.
Elevated Fibrinogen-Fibrin Degradation Products (FDP)

Statistical Methods

Data are given as median and ranges. Receiver operating characteristic (ROC) curves were performed, calculating sensitivities and specificities for variable cutoff values.

The term “sensitivity” is used in the context of diagnostic immunoassay to characterize the incidence of true positive results obtained when the assay is applied to patients known to have a cancer. The term “specificity” is, on the other hand, used to characterize the incidence of true negative results obtained when an assay is applied to subjects known to be free of cancer.\footnote{Mathematically, the sensitivity and specificity can be presented as follows:

\[
\text{Sensitivity} = \frac{TP \times 100}{TP + FN} \\
\text{Specificity} = \frac{TN \times 100}{FP + TN}
\]

In the above, \(TP\) = true positive = number of cancer patients correctly classified by the test. \(TN\) = true negative = number of noncancer patients correctly classified by the test. \(FP\) = false positive = number of noncancer patients misclassified by the test. \(FN\) = false negative = number of cancer patients misclassified by the test.

Data between groups were compared using the unpaired student \(t\)-test. Results show that \(P \leq 0.05\) was considered statistically significant.

RESULTS

Results of Germany Study

\textit{Healthy Controls}: The serum FDP levels of healthy controls were obtained by measuring the serum FDP level for 100 healthy blood donors using DR-70\textsuperscript{®} ELISA. The assay results showed that the serum FDP level of the healthy blood donor group ranges from 0.1–1.11\(\mu\)g/mL with a median value of 0.37\(\mu\)g/mL.

\textit{Cancer Patients}: The serum FDP levels of patients with colorectal cancer were obtained by measuring the serum FDP levels for 30 patients with histologically proven colorectal cancer using DR-70\textsuperscript{®} ELISA. The results showed that the serum FDP levels of the colorectal cancer range from 0.4–7.8\(\mu\)g/mL with a median value of 1.25\(\mu\)g/mL.
The results from the study of site 1 in Germany on the FDP levels of both healthy controls and colorectal cancer patients are presented as a scattergram in Fig. 1.

**Results of U.S. Study**

*Healthy Controls:* The serum FDP levels for healthy controls were obtained by measuring the serum FDP levels of 100 healthy volunteers using DR-70® ELISA. The assay results showed the serum FDP levels of the healthy group range from 0.25–2.2 µg/mL with a median value of 0.61 µg/mL.

*Cancer Patients:* The serum FDP levels for patients with colorectal cancer were obtained by measuring the serum FDP levels of 30 patients with histologically proven colorectal cancer using DR-70® ELISA. The results showed that the median serum FDP level of the colorectal cancer ranges from 0.31–10 µg/mL with a median value of 1.60 µg/mL.

The results of the study of site 2 in Tustin, California, on the FDP levels of both healthy controls and colorectal cancer patients are presented as a scattergram in Fig. 2.

*Figure 1.* Scattergram for serum FDP level of healthy controls and of patients with colorectal cancer (Germany study). The heavy horizontal line represents a serum FDP cutoff level at 1 µg/mL.
Figure 2. Scattergram for serum FDP levels of healthy controls and of patients with colorectal cancer (U.S. study). The heavy horizontal line represents a serum FDP cutoff level at 1 μg/mL.

The above-mentioned results are summarized in Table 1, which also includes some statistical parameters such as the median values, standard deviations of the median, and the $P$ values for statistical significance.

Table 2 shows the relationship between serum FDP cutoff level and the sensitivity and specificity of the assay. Depending on the level serum FDP used as the cutoff value, a range of specificity values and their corresponding sensitivity values can be obtained for colorectal cancer. The relationship between a series of specificity and sensitivity can be presented as ROC curve, as shown in Fig. 3.

The relationship between the assay sensitivity and the stages of colorectal cancer for results obtained in Tustin, California, are shown in Table 3.

DISCUSSION

Independent studies conducted in Frankfurt, Germany, and Tustin, California, showed that the serum FDP levels in patients with colorectal cancer were significantly higher than the levels in their respective healthy
Table 1. Summary of the serum FDP levels of healthy controls and patients with colorectal cancer.

<table>
<thead>
<tr>
<th>Category</th>
<th>Study in Frankfurt, Germany</th>
<th>Study in Tustin, California</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of subjects</td>
<td>Median FDP level µg/mL</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>100</td>
<td>0.37</td>
</tr>
<tr>
<td>Colorectal cancer patients</td>
<td>30</td>
<td>1.25</td>
</tr>
</tbody>
</table>
Table 2. The specificity and sensitivity of DR-70® ELISA for colorectal cancer at different serum FDP cutoff concentrations.

<table>
<thead>
<tr>
<th>FDP cutoff (μg/mL)</th>
<th>Study in Frankfurt, Germany</th>
<th>Study in Tustin, California</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specificity</td>
<td>Sensitivity</td>
</tr>
<tr>
<td>0.4</td>
<td>57%</td>
<td>100%</td>
</tr>
<tr>
<td>0.5</td>
<td>74%</td>
<td>93%</td>
</tr>
<tr>
<td>0.6</td>
<td>83%</td>
<td>87%</td>
</tr>
<tr>
<td>0.7</td>
<td>93%</td>
<td>80%</td>
</tr>
<tr>
<td>0.8</td>
<td>95%</td>
<td>73%</td>
</tr>
<tr>
<td>0.9</td>
<td>97%</td>
<td>70%</td>
</tr>
<tr>
<td>1.0</td>
<td>99%</td>
<td>70%</td>
</tr>
<tr>
<td>1.1</td>
<td>99%</td>
<td>63%</td>
</tr>
<tr>
<td>1.2</td>
<td>100%</td>
<td>63%</td>
</tr>
<tr>
<td>1.3</td>
<td>100%</td>
<td>50%</td>
</tr>
<tr>
<td>1.4</td>
<td>100%</td>
<td>43%</td>
</tr>
<tr>
<td>1.5</td>
<td>100%</td>
<td>43%</td>
</tr>
</tbody>
</table>

Figure 3. Receiver operating characteristic (ROC) curve for measurements of serum FDP levels using DR-70® ELISA kit.
control groups (Figs. 1,2). The FDP levels for the colorectal cancer patients for both the German and California studies range from 1.25–1.60 μg/mL. In contrast, the corresponding values for healthy controls range from 0.37–0.61 μg/mL. The differences in the median serum FDP levels between the cancer and healthy control groups were highly significant with $P$ values less than 0.0001 in both the German and U.S. studies (Table 1). Based on our current studies, Table 2 was constructed to show the relationship between the specificity and sensitivity of DR-70® ELISA for colorectal cancer testing. At a serum FDP cutoff level of 1 μg/mL, the German study shows a specificity of 99%, whereas the U.S. study shows 88%. The sensitivity obtained from both studies was similar, being 70% and 71%, respectively, for the German and U.S. studies. Fig. 3 shows the ROC curve based on data obtained from the German study. Although the number of colorectal cancer patients was not sufficiently large enough to allow for a definitive conclusion to be made on the sensitivity of DR-70 ELISA for different stages of colorectal cancer, nevertheless, it is of interest to analyze current preliminary results obtained from the U.S. study. Such preliminary analysis showed that the DR-70–ELISA was able to detect all stages of cancer with sensitivity values ranging from 51–71% (Table 3).

Data presented in this report are consistent with the notion that fibrinolysis is often associated with oncogenic transformation[12] and with the findings that plasma D-dimer level, a component of FDP, was elevated in breast cancer and in colorectal cancers.[3,4,6–8] Wu et al.[9,10] and others[11–13] have demonstrated that the serum FDP levels in several different cancers, including colorectal cancer, were also elevated. Furthermore, it has been shown for more than two decades that the measurement of urinary FDP was as accurate or more accurate than other available tests, including urine cytology.[14] Determination of urinary FDP by immunoassay is an efficient,
Elevated Fibrinogen-Fibrin Degradation Products (FDP)

reliable, noninvasive, as well as quantitative or qualitative method that can be a useful adjunct on the surveillance of superficial bladder cancer and for monitoring the course of the disease.[11,15]

In conclusion, we have shown that serum FDP levels, measured by using DR-70® ELISA, were significantly higher in colorectal cancer than those of the healthy control. It is therefore worthy to investigate the potential use of this assay in the management of colorectal cancer.

REFERENCES


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SENSITIVITY & SPECIFICITY OF DR-70™ LUNG CANCER IMMUNOASSAY

Keywords: Cancer test, Lung cancer, Sensitivity and Specificity

Dong Fang Wu¹, Xin Zhou¹, Gina Anderson⁵*, Alicia Fuentes⁵, Lewis M. Slater⁴, Dyer Narinesingh⁵, Paul Jimenez⁵ and Tim Gopoesingh⁵

¹Dept. Clinical Lab., ²Affiliated Hospital, Hubei Medical University, Wuhan, China
²AMDI, Inc., 14272 Franklin Avenue, Suite 106, Tustin, CA 92780
³Dept. Chemistry, The University of the West Indies, St. Augustine, Trinidad
⁴Dept. Medicine, University of California, Orange, CA 92998
⁵Dept. Obstetrics and Gynecology, Eric Williams Medical Sciences Complex, Trinidad

ABSTRACT

A multi-center study in the U.S. and China involving a total of 393 healthy subjects and 203 lung cancer patients was conducted to investigate the utility of DR-70™ immunoassay to detect lung cancer. Interference to DR-70™ test due to different disease states and blood collection procedures were also investigated. Benign and non-cancer diseases that can give rise to elevated DR-70™ levels included pneumonia, lung infection, burns, trauma due to surgery, arthritis, renal failure, sepsis etc. The study also showed that most consistent DR-70™ test results were obtained when blood was collected in vacutainers containing SST gel and clot activator. It is important that the DR-70™ test not be performed on samples of patients suffering from the aforementioned diseases. As these non-cancer disease states and improper blood collection procedures may lead to false positive results.

INTRODUCTION

Fields et al.¹ concluded that the serum DR-70™ level is promising for use as a marker in the assessment of patients with lung cancer. The study showed that normal DR-70™ levels were similar in both male and female subjects. The DR-70™ levels of current smokers and ex-smokers were, respectively, about 15% and 4% higher than non-smokers. The levels were also higher by approximately 15% in controls 65 years of age or older, versus those less than 65 years of age.¹ The mean DR-70™ level of cancer patients was 3.5 fold higher than the mean value of control non-cancer subjects. The sensitivity of the test for lung cancer was 67% and the specificity was 91%¹. Wu et al.² recently reported the successful detection 13 different cancers including lung cancer with DR-70™. The results, based on 277 healthy subjects and 136 cancer patients, showed an overall sensitivity and specificity of 84% and 95%, respectively. Contrary to the studies of Fields et al.¹ and Wu et al.², Stieber et al.³ reported the lack of success in the use of DR-70™ for detecting lung cancer. In order to resolve these diametrically different conclusions on the validity of using DR-70™ as a test for lung cancer, we conducted studies in China and in the U.S. Results of the
investigations are reported herein. Possible factors that may be contributing to the differences between results reported by Fields et al\textsuperscript{1} and Wu et al\textsuperscript{2}, and those of Stieber et al\textsuperscript{3} will be discussed.

CLINICAL SPECIMENS

(a) Study in China

Control Subjects.

The sera were drawn from 335 healthy individuals who attended the Clinic of 2\textsuperscript{nd} Affiliated Hospital of Hubei Medical University for routine check-ups. Sera were obtained by venipuncture and drawn into SST tubes. There were 190 males and 145 females with age range of 18 to 70 years old with an average age of 50.3 years of age. All control subjects gave negative test results for hepatitis surface antigens A, B, C and syphilis. They all have normal functioning livers, kidneys and lungs.

Cancer Patients.

Of the 83 cancer patients, 55 were male and 28 were female. They were all patients of our clinic who sought treatments here. The presence of cancer in these patients were confirmed by X-ray, ultrasound, CT, biopsy and/or surgical procedures. The age of the patients range from 29 to 80 years old.

(b) Study in the U.S.

Control Subjects.

The sera were drawn from 58 healthy volunteers.

Cancer Patients.

Sera from 120 lung cancer subjects were obtained from Austin Medical Ventures.

Patients With Benign, Non-Cancer Conditions.

Sera from patients with other “benign”, non-cancerous conditions were obtained from the Department of Pathology, University of California at Irvine, after approval by the University of California, Irvine, Institutional Review Board.

MATERIALS AND METHODS

Sera

Unless otherwise stated, all sera were obtained by the following procedures:

Approximately five (5) mL venous blood was drawn into a serum separation vacutainer tube (Becton Dickinson, Franklin Lakes, NJ 07417) in the morning before the consumption of food. This tube contained SST gel and clot activator. The blood was left at room temperature for 30 min, then centrifuged at 1500 rpm for 15 min. The resulting clear serum was analyzed. For comparison, sera were obtained blood that was purposely drawn into “red top” tubes, i.e. vacutainer tubes without the SST gel and clot activator.

Plasma

Plasma from healthy volunteers was obtained by drawing the blood into vacutainer tubes containing buffered sodium citrate.

DR-70\textsuperscript{TM} Test Kits

The kits, obtained from AMDL (Tustin, Ca 92780), contained a plate of 12 x 8 well strips coated with affinity-purified rabbit anti-DR-70\textsuperscript{TM} antibodies, a vial of antibody-peroxidase conjugate, one vial each of diluent, substrate solution, stop solution, wash buffer, low serum control, high serum control and 5 calibrators.

DR-70\textsuperscript{TM} Test Principle

DR-70\textsuperscript{TM} test is an enzyme linked immunosorbent assay (ELISA) using
affinity purified anti-DR-70™ immobilized on the bottom of the well to capture DR-70™ antigen from the diluted serum. The captured antigens, upon washing, are then complexed by peroxidase labeled anti-DR-70™ conjugate to form immuno-sandwich. The bound enzyme conjugate is quantitatively measured with TMB substrate. Upon stopping the enzymatic reactions, the absorbance of the solution is read at 450 nm.

**DR-70™ Assay Procedure**

All serums were diluted 200 fold with diluent solution in the kit. Typically, 10 µL of serum were added to 2000 µL diluent. Upon proper mixing, the diluted serum was added to two adjacent wells of a dilution plate. Each well received 200 µL as duplicate samples. Using an 8-channel pipettor, remove 100 µL of the diluted serums from the dilution plate and deliver 100 µL to the antibody-coated plate. The plate was sealed with a plate sealer and incubated at room temperature for 15 minutes. The plate was then washed 6 times with 300 µL wash solution in each wash. Wells were dried by blowing with a stream of air for 2 minutes. All wells were filled with 100 µL of enzyme-antibody conjugate and incubated at room temperature for 15 minutes. The plate was then washed 6 times with 300 µL wash solution in each wash. Wells were dried by blowing with a stream of air for 2 minutes. Then 100 µL of TMB substrate solution was added to each well, covered from direct light and incubated for 10 minutes for color development. Finally, 100 µL stopping solution was added to each well to stop the enzymatic reactions. The absorbance of the solution was read at 450 nm in an ELISA reader. From the absorbance of the 5 calibrators, a standard curve was constructed. The DR-70™ level of the serum was read from this standard curve.

**RESULTS**

**Serum DR-70™ Level in Control Subjects**

In the study conducted both in China and in the U.S., the normal serum DR-70™ level in 393 healthy control subjects ranged from approximately 1 mg/L to less than 9 mg/L.

**Specificity and Sensitivity of DR-70™ Test for Lung Cancer Patients**

Eighty-three lung cancer patients were enrolled in the study conducted in China. Using the DR-70™ test and setting the specificity requirement at 95%, a sensitivity of 87% was obtained by using 335 control subjects and 83 lung cancers (Fig. 1, Table 1). In the U.S. study, 58 healthy controls and 120 lung cancer patients were enrolled in testing the DR-70™ assay. The results showed that at 96% specificity, a sensitivity of 86% was achieved (Figs. 2, 3, Table 2).

**Effects of Vacutainer With and Without SST Gel and Clot Activator on the Values of DR-70™ Measured**

The blood of 12 control subjects was collected in vacutainers with and without SST gel and clot activator, the DR-70™ values in the resulting sera were analyzed immediately and at 2 other time intervals. The overall results of this study suggested that fresh sera collected with and without SST gel and clot activator showed lower values compared to the sera collected, and under identical conditions stored 5 hours at room temperature and 53 hours at room temperature. However, sera obtained from blood collection using vacutainer with SST gel and clot activator showed significantly lower DR-70™ results than the sera obtained from blood collected in vacutainers without SST gel and clot activator (Table 3).

**Conditions Causing Elevation of Serum DR-70™ Level**

Certain benign, non-cancer conditions were found to cause elevation of serum DR-70™ level. They include: pneumonia, sepsis, cellulitis, acute and chronic infection, burns, trauma, surgery, renal insufficiency, jaundice and rheumatoid arthritis. The DR-70™ level in patients with examples of these conditions are listed in Table 4.
**Fig. 1:** Receiving Operating Characteristics (ROC) curve of DR-70™ immunoassay for lung cancer discriminating patients with lung cancer from control healthy subjects based on the study in China.

**Table 1:** The Specificity and Sensitivity of DR-70™ Lung Cancer Immunoassay at Different Cutoff Levels.

<table>
<thead>
<tr>
<th>DR-70™ Cutoff Level (mg/L)</th>
<th>Specificity %</th>
<th>Sensitivity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>97.6</td>
<td>62.7</td>
</tr>
<tr>
<td>5.5</td>
<td>97.6</td>
<td>67.5</td>
</tr>
<tr>
<td>5.0</td>
<td>96.7</td>
<td>73.5</td>
</tr>
<tr>
<td>4.5</td>
<td>94.9</td>
<td>86.7</td>
</tr>
<tr>
<td>4.0</td>
<td>89.0</td>
<td>88.0</td>
</tr>
<tr>
<td>3.5</td>
<td>88.4</td>
<td>89.2</td>
</tr>
<tr>
<td>3.0</td>
<td>86.9</td>
<td>95.2</td>
</tr>
<tr>
<td>2.5</td>
<td>83.9</td>
<td>97.6</td>
</tr>
</tbody>
</table>

Total Number of Lung Cancer Patients: 83  
Total Number of Normals: 335

**Fig. 2:** Receiving Operating Characteristics (ROC) curve of DR-70™ immunoassay for lung cancer in discriminating patients with lung cancer from control healthy subjects based on the study in the U.S.

**Fig. 3:** Scattergram for DR-70™ values of control healthy subjects and lung cancer patients.
Table 2: The Specificity and Sensitivity of DR-70™ Lung Cancer Immunoassay at Different Cutoff Concentration.

<table>
<thead>
<tr>
<th>DR-70™ Cutoff Level (mg/L)</th>
<th>Specificity %</th>
<th>Sensitivity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>100</td>
<td>65</td>
</tr>
<tr>
<td>12</td>
<td>99</td>
<td>76.6</td>
</tr>
<tr>
<td>11</td>
<td>98.1</td>
<td>78</td>
</tr>
<tr>
<td>10</td>
<td>98.1</td>
<td>82.5</td>
</tr>
<tr>
<td>9</td>
<td>96.2</td>
<td>85.8</td>
</tr>
<tr>
<td>8</td>
<td>93.5</td>
<td>86.7</td>
</tr>
<tr>
<td>7</td>
<td>88.8</td>
<td>90.8</td>
</tr>
<tr>
<td>6</td>
<td>72.2</td>
<td>95</td>
</tr>
<tr>
<td>5</td>
<td>45.3</td>
<td>97.5</td>
</tr>
<tr>
<td>4</td>
<td>10.1</td>
<td>98.3</td>
</tr>
</tbody>
</table>

Total Number of Lung Cancer Patients: 120
Total Number of Normals: 108

Table 3: Comparison of the DR-70™ values in sera from blood collected in vacutainer with SST gel and clot activator versus vacutainer without SST gel and clot activator at 2 time intervals. The number of control subjects in this study is 12.

<table>
<thead>
<tr>
<th>Sera collected in vacutainer</th>
<th>Average DR-70™ level (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&quot;Fresh&quot;: 30 minutes at room temperature</td>
</tr>
<tr>
<td>With SST gel and Clot Activator</td>
<td>5.71</td>
</tr>
<tr>
<td>Without SST gel and Clot Activator</td>
<td>5.64</td>
</tr>
</tbody>
</table>

DISCUSSION

A multi-center study to evaluate the clinical utility of DR-70™ for cancer detection has been conducted in the U.S. and China. DR-70™ immunoassay kit detects in serum specific fibrin degradation resulting from a malignancy. Also included in the study were the effects of various non-cancer diseases and laboratory factors that may influence the results of DR-70™ tests and hence their interpretations.

The DR-70™ level of 393 normal, healthy subjects was measured to be in the range of 1 to 9 mg/L. The value for serum DR-70™ level can vary significantly depending on how the serum was obtained. For example, when blood was drawn into vacutainers containing neither SST gel nor clot activator, and left in the vacutainers for 5 hours, the value for the DR-70™ level in the resulting serum increased by 16% and increased further to 20% or more for samples left in the vacutainer tubes for more than 48 hours. On the other hand, under the same conditions, when blood was obtained in vacutainers with SST gel and clot activator, the DR-70™ values increase by only 7% and 11%, respectively.

The influence of a number of non-cancer disease conditions on the value of DR-70™ was also investigated. It is clear from Table 4 that non-cancer diseases such as infection, burns, trauma due to surgery, arthritis, sepsis, etc. are likely to contribute to an increase in the level of serum DR-70™. These effects can lead to false positive results. It is therefore important to note that when DR-70™

Table 4. Conditions causing elevation of serum DR-70™ level.

<table>
<thead>
<tr>
<th>Patient Code</th>
<th>Clinical Diagnosis</th>
<th>DR-70™ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-1</td>
<td>Infection</td>
<td>10.2</td>
</tr>
<tr>
<td>S-19</td>
<td>Neurogenic bladder + chronic infection</td>
<td>11.2</td>
</tr>
<tr>
<td>S-14</td>
<td>Pneumonia &amp; Fasciitis (Necrotizing) Klebsiella</td>
<td>16.6</td>
</tr>
<tr>
<td>S-42</td>
<td>Acute Pneumonia</td>
<td>20.8</td>
</tr>
<tr>
<td>S-17</td>
<td>50% Body burns, 2nd &amp; 3rd degree</td>
<td>8.9</td>
</tr>
<tr>
<td>S-18</td>
<td>Chest trauma-surgery</td>
<td>20.6</td>
</tr>
<tr>
<td>S-20</td>
<td>Renal insufficiency-jaundice</td>
<td>19.4</td>
</tr>
<tr>
<td>S-38</td>
<td>Rheumatoid Arthritis</td>
<td>37.6</td>
</tr>
<tr>
<td>S-45</td>
<td>Rheumatoid Arthritis</td>
<td>8.8</td>
</tr>
<tr>
<td>S-46</td>
<td>Rheumatoid Arthritis</td>
<td>18.4</td>
</tr>
<tr>
<td>S-39</td>
<td>Cellulitis of knee</td>
<td>20.0</td>
</tr>
<tr>
<td>S-48</td>
<td>Sepsis</td>
<td>15.2</td>
</tr>
</tbody>
</table>
Immunohistochemistry is prescribed to detect the presence of lung cancer, the patient should be cleared of the aforementioned diseases and that proper vacutainer and blood collection procedures are used. Interference of a tumor marker test by benign, non-cancer diseases is not uncommon. For example, CYFRA 21-1 was shown to be elevated in chronic obstructive bronchopneumopathy and in chronic renal failure. Similarly, CA 125 test picked up benign pelvic diseases.

When an appropriate reference group, i.e. normal and healthy individuals without the above mentioned non-cancer diseases listed in Table 1 and proper serum collection procedures are used, DR-70™ lung cancer test can perform satisfactorily with good clinical sensitivity and specificity as shown in the ROC curve analysis (Figures 1 to 3). It, therefore, appears that the major factor contributing to the vast differences between our results obtained in a multicenter study and the results of Stieber et al. is most likely due to the difference in the reference group used. Stieber et al. used patients with benign diseases of the lung and smokers. The consequence of using such a reference groups, when the specificity is fixed at 95%, is an increase in DR-70™ cut-off level for "normal" subjects. This, in turn, leads to an artificially low sensitivity as was indicated in Stieber's report.

**CONCLUSION**

When patients with certain benign lung conditions such as infection or trauma and other non-malignant conditions such as burns, arthritis and sepsis are excluded, and proper blood collection and serum processing procedures are used, DR-70™ lung immunoassay is able to give results with good sensitivity and specificity relative to lung cancer. These observations strongly suggest that the DR-70™ assay may be useful as a screening test for lung cancer in asymptomatic individuals.

**REFERENCES**


Received: January 1, 1999
Accepted: February 16, 1999
Application of Tumor Marker of DR-70® in the Diagnosis of Malignant Tumors

Lin Ding, Shen Ping, Yang Jingmei
Central Research Laboratory
Chongqing Cancer Institute
Chongqing, Sichuan
China 400030

Abstract Objective: To evaluate the tumor marker of DR-70® in the diagnosis of malignant tumors. Method: Using DR-70® ELISA kit for detection of DR-70® in serum samples of 20 healthy controls and 59 patients with various malignant tumors. Results: The positive rates of serum DR-70® of the patients were 66.7~100%, all of which were significantly higher than that of normal controls. Conclusion: The detection of serum DR-70® is a valuable tumor marker in the diagnosis of malignant tumors.
Key words: DR-70® Marker of tumor ELISA

Malignant tumor has become one of the most threatening diseases of mankind. According to the data¹, the mortality rate for malignant cancer is 108.39/100,000, which accounts for 17.97% of total death in China. It has become the number two cause of death. If cancer can be detected and treated early, then the cure rate can be greatly increased. Early detection of cancer is currently an important area of medical research. Although China has begun clinical uses of tumor marker, it still has not achieved the international level in terms of usage. This is the first paper reporting the use of DR-70® ELISA test in China for the diagnosis of cancer. The subjects involved in this trial consist of 59 malignant cancer patients and 20 normal persons.

Materials and Methods
Normal subjects: total number is 20 with 11 males and 9 females. Their ages range from 20 to about 45 years old. The samples were obtained from Chongqing Blood Center and all the blood chemistry tests indicated that they are normal blood donors.
Cancer subjects: total number is 59 with 37 males and 22 females. Their ages range from 27 to about 75 years old. There are 20 patients with primary liver cancer (of whom 7 have AFP level of 20 ng/ml), 18 have lung cancer, 7 have ovarian cancer, 6 have breast cancer, 5 have stomach cancer, 2 have malignant lymphoma and 1 has brain cancer.
Test kit was AMDL DR-70® kit, Tustin, USA and was supplied by the Guangzhou Office of US Comet Medical Ltd.

Instrument was a Bio-Rad BR450 ELISA reader.

Methods

The principle of DR-70® test is a double antibody sandwich ELISA. The serum samples were tested on the same day otherwise they were stored at −20º C. Beginning with sample collection to performing the test, the procedures described on the Manual were strictly followed. The operation of the ELISA reader was controlled by a computer running on VISUAL BASIC®.

The standard curves were managed by using Microsoft Excel 5.0. A typical standard curve has the following formula: \( Y=-0.0012X^2-0.1173X-0.2973 \) (\( R^2=99.68 \)) where \( y \) is the absorbance value, \( x \) is the DR-70® concentration (mg/L) and \( R \) is the relative coefficient.

Results

The average value for serum DR-70® in normal subjects is \((x+s)\) 4.41+0.81 mg/L. The average value for 11 male is 4.50+0.83 mg/L and that of 9 females is 4.32+0.93 mg/L. There is not statistically \( (p>0.05) \) difference in DR-70® value between male and female. Using \( x+1.96s \) (95% confidence limit), the serum DR-70® cutoff value is 6.0 mg/L which is similar to the value reported in the literature 3.

Table 1. DR-70® values in the serum of cancer patients.

<table>
<thead>
<tr>
<th></th>
<th>No. of patients</th>
<th>DR-70® Level (mg/L)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>~&lt;6 ~10 ~20 ~&gt;20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver Cancer</td>
<td>20</td>
<td>1 4 8 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>18</td>
<td>3 5 6 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovarian</td>
<td>7</td>
<td>1 1 2 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>6</td>
<td>2 2 1 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>5</td>
<td>1 1 2 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mal. Lymph.</td>
<td>2</td>
<td>1 1 1 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>59</td>
<td>8 14 21 16</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The comparison of serum DR-70® in the normal and cancer subjects is shown in Table 2. Positive DR-70® values in cancer patients are significantly higher than normal subjects (66.7%-100%). The DR-70® values of the normal are significantly different from those of the cancer patients (P<0.001).

Table 2. Comparison of the DR-70® value of the normal and cancer subjects.

<table>
<thead>
<tr>
<th></th>
<th>No. of patients</th>
<th>No. of Positives</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>20</td>
<td>1</td>
<td>5.0</td>
</tr>
<tr>
<td>Cancer</td>
<td>59</td>
<td>51</td>
<td>86.4</td>
</tr>
<tr>
<td>Liver</td>
<td>20</td>
<td>19</td>
<td>95.0</td>
</tr>
<tr>
<td>Lung</td>
<td>18</td>
<td>15</td>
<td>83.3</td>
</tr>
<tr>
<td>Ovarian</td>
<td>7</td>
<td>6</td>
<td>85.7</td>
</tr>
<tr>
<td>Breast</td>
<td>6</td>
<td>4</td>
<td>66.7</td>
</tr>
<tr>
<td>Stomach</td>
<td>5</td>
<td>4</td>
<td>80.0</td>
</tr>
<tr>
<td>Mal. Lymph.</td>
<td>2</td>
<td>2</td>
<td>100.0</td>
</tr>
<tr>
<td>Brain</td>
<td>1</td>
<td>1</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Discussion

Although there have been more than 20 years since the proposal of serum tumor markers, the discovery of tumor marker which existed only in the tumor cell or specifically produced by the tumor cell has not been made. There are now dozens of tumor markers in use for the diagnosis of cancer. Current tumor markers can be detected in 50-70% of the serums from cancer patients. For example, 30-40% of liver cancer cases were negative for AFP, CA 15-3 were negative in 20-30 % of breast cancer patients, 50% of colon cancer patients were negative for CEA, the false negative rate was high.

In 1970, Donald Rounds discovered DR-70® culture medium of cells undergoing malignant transformation. Later ring shaped particles were observed in the serum of cancer patient. To honor him, the first letter of his first and second names and the year of the discovery were used to name the tumor maker DR-70®. In 1991, AMDL, Inc., in the USA, showed that DR-70® is a group of cancer related proteins, and it is a protease secreted by the cancer. Justice et al. first used DR-70® for the diagnosis of breast cancer; later reports continue to show the clinical value of DR-70® in the diagnosis of cancer. In 1996, AMDL, Inc, produced a lung cancer diagnosis test kit.
Wu, D. et al. used DR-70® to determine 13 different cancers in 136 patients. The sensitivity of the test for lung cancer, stomach cancer, breast cancer and colon cancer was, respectively, 87.8%, 92.6%, 65.2% and 66.7%.

The results reported in this paper showed that DR-70® is a pan tumor marker of great value and clinical utility for the diagnosis of cancer. The sensitivity of the test for liver cancer and lung cancer is as high as 95% and 83.3%. Of the 20 liver cancer patients in our center, there are 7 cancer patients who tested negative for AFP and 2 of them gave strong DR-70® reaction with DR-70® level as high as 13.4 and 21.2 mg/L. These results indicated that the use of DR-70® as pan tumor marker and a specific tumor marker can significantly the accuracy of clinical diagnosis. As with other tumor markers, DR-70® test also gave varying degree of false positives, this may be due to fact that DR-70® is not a protein of cancer gene expression, and its relation to cancer needs to be studied. Current investigations showed elevated levels of DR-70® in subjects suffering from hepatitis, lung infection and bronchitis, which may lead to false positive results.

In serum, DR-70® appears as a complex substance; therefore it is critical that the serum sample is properly collected. In order to avoid false positive results, fasting blood samples should be used. Hemolyzed sample tends to give high DR-70® values. To avoid hemolysis, blood samples should be processed 30 minutes after the blood has coagulated. In order to fully preserve the activity of DR-70® and to avoid getting false positive values, those samples which cannot be tested within 24 hours should be stored at −20°C. DR-70® ELISA test is simple to perform, rapid and requires only 1.5 hours to obtain the results. Compared to other ELISA tests that take 6 hours to complete, DR-70® test requires significantly shorter time to complete and therefore can provide clinical report in shorter time.

Performing unit: Chongqing Cancer Research Center (400030)

References
Clinical Performance of the AMDL DR-70™ Immunoassay Kit for Cancer Detection

Dongfang Wu¹, Xin Zhou¹, Guoliang Yang², Yuntao Xie², Mingbail Hu², Zhangqi Wu³, Gang Yang¹, Minxiang Lu¹

¹Department of Clinical Laboratory, 2nd Affiliated Hospital, Hubei Medical University, 29 Donghu Road, Wuchang, Wuhan, China.
²Oncology Institute, 2nd Affiliated Hospital, Hubei Medical University, Wuhan, China
³Wuhan Institute of Virology, Chinese Academy of Science, Wuhan, China

ABSTRACT

A clinical study using DR-70™ immunoassay for the detection of 13 different cancers have been conducted with 277 healthy subjects and 136 cancer patients. The test results showed that the DR-70™ immunoassay kit was capable of detecting cancers with high degree or specificity and sensitivity. At 95% specificity level, the sensitivity of the assay was 87.8%, 92.6%, 65.2% and 66.7%, respectively for lung, stomach, breast and rectum cancers. Furthermore the test kits were shown to be stable and performed reproducibly.

INTRODUCTION

A clinical trial using the DR-70™ immunoassay for the detection of lung cancer was conducted at the Cross Cancer Institute in Edmonton, Alberta, Canada in 1993-95 (1). The trial results, based on 237 cancer patients and 244 normal controls, showed that the DR-70™ immunoassay detected both small cell and non small cell lung cancer with an overall sensitivity of 66% at 92% specificity. In addition to the detection of lung cancer, the DR-70™ immunoassay was also shown to detect a number of other cancers. In an attempt to assess the potential of using this assay as a cancer screening tool, we have conducted a clinical study utilizing the DR-70™ assay for the detection of cancer of the lung, stomach, breast, rectum, colon, liver, ovary, esophagus, uterus, etc. The present study involves 277 healthy individuals and 136 cancer patients. The results of the study, which are encouraging, are the subject of this communication.

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CLINICAL SPECIMENS

CONTROLS

The control sera were drawn from healthy individuals who came to our clinic for routine check-ups. There were 163 males and 114 females with ages ranging from 18 years to 60 years. All the control subjects gave negative test results for hepatitis surface antigen A, B & C and for syphilis. They all had normally functioning livers, kidneys and lungs.

CANCER PATIENTS

Of the 136 cancer patients, 74 were male and 62 were female. They were all patients at our clinic who sought treatment here. The presence of cancer in these patients was confirmed by X-ray, ultra-sound, CT, biopsy and/or surgical procedures. The patients range in age from 1 year to 80 years. The composition of the cancer patients is: 41 lung, 27 stomach, 23 breast, 15 rectum, 6 colon, 6 liver, 6 ovary, 5 esophagus, 3 cervical, 2 trophoblast, 1 thyroid, 1 malignant lymphoma and 1 pancreas.

MATERIALS AND METHODS

Two ml venous blood was drawn into a serum separation tube (Becton Dickinson) in the morning before the consumption of any food. The blood was left at room temperature for 30 minutes, then it was centrifuged at 1500 rpm for 15 minutes. The resulting clear serum was taken for analysis.

DR-70™ TEST KIT

The kits, obtained from AMDL, Inc., contained a plate of 12 X 8 well strips coated with affinity-purified rabbit anti-DR-70™ antibodies, a vial of antibody-peroxidase conjugate, one vial each of diluent, TMB substrate solution, stop solution, wash buffer, low serum control, high serum control and 5 calibrators.
DR-70™ TEST PRINCIPLE

The DR-70™ test is an enzyme linked immunosorbent assay (ELISA) using affinity purified rabbit anti- DR-70™ immobilized on the bottom of the well to capture DR-70™ antigen from the diluted serum. The captured antigens, upon washing, are then complexed by peroxidase labeled anti-DR-70™ conjugate to form an immuno-sandwich. The bound enzyme conjugate is quantitatively measured with TMB substrate. Immediately after stopping the enzymatic reactions, the absorbance of the solution is read at 450 nm.

DR- 70™ ASSAY PROCEDURE

All sera were diluted 200 fold with the diluent solution supplied in the kit. Typically, 10 μl of serum were added to 2000 μl diluent. Upon proper mixing, the diluted serum was added to two adjacent wells of a dilution plate. Each well received 200 μl as duplicate samples. Using an 8-channel pipettor, 100 μl of the diluted serum was removed from the dilution plate and 100 μl was delivered to the antibody-coated plate. The plate was sealed with a plate sealer and incubated at room temperature for 15 minutes. The plate was then washed 6 times using 300 μl of wash solution for each wash. The wells were dried with a stream of air for 2 minutes. Then 100 μl of TMB substrate solution were added to each well, protected from direct light and incubated for 10 minutes for color development. Finally, 100 μl stopping solution were added to each well to stop the enzymatic reactions. The absorbance of the solution was read at 450 nm in an ELISA reader. From the absorbance of the 5 calibrators, a standard curve was constructed. The DR-70™ level of the serum was read from this standard curve.

RESULTS

STANDARD CURVE

The standard curve for DR-70™ was constructed based on the absorbance values obtained from the 5 calibrators supplied with the kit. The concentrations of DR-70™ in the calibrators were 0.0, 1.5, 3.0, 9.0 and 18.0 μg/mL. The resulting absorbance values were 0.020, 0.168, 0.357, 0.962 and 1.650, respectively. A typical standard curve using 5 calibrators is presented in Fig. 1 with a curve fined to a quadratic equation with $r^2 = 0.993$. 
FIG. 1. Standard curve with DR-70\textsuperscript{TM} concentration (mg/L) plotted against absorbance at 450 nm.

DR-70\textsuperscript{TM} LEVELS IN SERUM OF NORMAL CONTROLS AND CANCER PATIENTS

CONTROL SUBJECTS: The DR-70\textsuperscript{TM} level for control subjects was established from the level of 277 controls. The average level was 1.66 mg/L. Based on a 95% confidence limit, the upper limit for the normal DR-70\textsuperscript{TM} level can be set at 4.0 mg/L.

CANCER PATIENTS: A total of 136 cancer patients were enrolled in this study. The DR-70\textsuperscript{TM} level of cancer patients is listed in Table 1. The average DR-70\textsuperscript{TM} level of the cancer patients as a group is 13.31 mg/L. This is more than 3 times the upper limit for the control subjects.

Figure 2 showed the individual values of DR-70\textsuperscript{TM} in both the normal (non-cancer) and cancer patients. The colon and rectum cancer patients were grouped in one column under C/R. Cancers of the trophoblast, thyroid, lymph and pancreas were also grouped in
Table 1: DR-70<sup>®</sup> Level in Serum of Cancer Patients

<table>
<thead>
<tr>
<th>Cancer Category</th>
<th>Number of Cancer Patients</th>
<th>Average DR-70&lt;sup&gt;®&lt;/sup&gt; Conc. (±, mg/ml)</th>
<th>Number of Positive Patients</th>
<th>Sensitivity of DR-70&lt;sup&gt;®&lt;/sup&gt; test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>41</td>
<td>9.65 ± 7.47</td>
<td>36</td>
<td>87.8%</td>
</tr>
<tr>
<td>Stomach</td>
<td>27</td>
<td>11.56 ± 8.53</td>
<td>25</td>
<td>92.59%</td>
</tr>
<tr>
<td>Breast</td>
<td>23</td>
<td>5.81 ± 3.63</td>
<td>15</td>
<td>62.55%</td>
</tr>
<tr>
<td>Reetum</td>
<td>15</td>
<td>8.38 ± 5.98</td>
<td>10</td>
<td>66.66%</td>
</tr>
<tr>
<td>Colon</td>
<td>6</td>
<td>9.23 ± 4.74</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>5</td>
<td>8.37 ± 4.49</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Ovary</td>
<td>6</td>
<td>24.19 ± 13.79</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Esophagus</td>
<td>5</td>
<td>10.44 ± 5.58</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Cervix</td>
<td>3</td>
<td>11.03 ± 8.11</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Trophoblast</td>
<td>2</td>
<td>5.51</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Thyroid</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Malign. Lymphoma</td>
<td>1</td>
<td>11</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>1</td>
<td>49.94</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Individual serum values of DR-70<sup>TM</sup> in control, non-cancer subjects, and cancer patients. The heavy dashed line represents upper normal limit DR-70<sup>TM</sup> value (the cut-off value) which is set at 4 mg/L.
one column under Miscellaneous (Mi). All other cancers were listed in different individual columns.

**RECEIVER OPERATING CHARACTERISTIC (ROC) CURVES**

In addition to analytical precision and accuracy, other parameters such as sensitivity, specificity and predictive values (PV) are required to determine the accuracy of a laboratory test. In the context of an immunoassay for cancer, the term "sensitivity" is used to characterize the incidence of true positive results obtained when the assay is applied to patients known to have a cancer. The term "specificity" is used to characterize the incidence of true negative results obtained when an assay is applied to subjects known to be free of cancer (2). The predictive value can be applied to either positive or negative test results. A positive predictive value indicates the frequency of cancer patients in all patients with positive test results. The negative predictive value indicates the frequency of non-cancer (control) subjects in all subjects with negative test results (3). Therefore, the sensitivity and specificity of an assay are parameters which refer to a homogeneous group of patients, whereas the terms positive or negative predictive values refer to a mixed group. Mathematically, the above parameters can be presented as follows:

\[
\text{Sensitivity} = \frac{TP \times 100}{TP + FN} \\
\text{Specificity} = \frac{TN \times 100}{FP + TN} \\
\text{Positive PV} = \frac{TP \times 100}{TP + FP} \\
\text{Negative PV} = \frac{TN \times 100}{TN + FN}
\]

TP = True Positive = number of cancer patients correctly classified by the test.
TN = True Negative = number of non-cancer patients correctly classified by the test.
FP = False Positive = number of non-cancer patients misclassified by the test.
FN = False Negative = number of cancer patients misclassified by the test.

At a specificity of 95.0%, the sensitivity of the DR-70™ assay was 87.8%, 92.6%, 65.2% and 66.7% for lung, stomach, breast and rectum cancers, respectively. The overall
Table 2: Overall Performance of DR-70™, Cancer Patients vs. Control Subjects

<table>
<thead>
<tr>
<th>Category</th>
<th>Number</th>
<th>Number of Positive Patients</th>
<th>Specificity</th>
<th>Sensitivity</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer Patients</td>
<td>136</td>
<td>114</td>
<td></td>
<td>83.82 %</td>
<td></td>
</tr>
<tr>
<td>Control Subject</td>
<td>277*</td>
<td>14</td>
<td>94.95%</td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Specificity and sensitivity of DR-70™ for cancers tested were respectively 95.6% and 83.8% (Table 2). The predictive values of positive and negative tests are 89.1% and 92.3%, respectively.

Depending on the level of DR-70™ used as the cut-off value, a range of specificity values and their corresponding sensitivities can be obtained for a particular cancer. The relationship between a series of specificities and sensitivities can be profiled in an ROC curve as shown in Fig. 3. The power of discrimination between the cancer patients and the control non-cancer subjects of a test can be readily observed in the ROC curve. The closer the curve is to the upper left hand corner of the plot, the greater is the discrimination power of the test.

PRECISION

The intra-day precision of the DR-70™ kit was studied by measuring high, medium and low control sera 20 times within one day. The results of the study, as presented in Table 3, indicated that the DR-70™ kit provided an acceptable precision with CV values of 4.3%, 7.6% and 11.9% for high, medium and low control sera, respectively.

DISCUSSION

Fields et al. reported in 1995 the results of a clinical trial using DR-70™ for the detection of lung cancer conducted at the Cross Cancer Institute. The trial results indicated DR-70™ detected both non-small cell lung cancer and small cell lung cancers equally well. The level of DR-70™ increases from 9.3 to 31.2 as the stage of the cancer changes from stage I to stage IV. The results also showed 83.7% sensitivity of DR-70™ marker in stage IV patients with non-small cell lung cancer and 80.0% sensitivity in advanced patients with small cell lung cancer. The overall sensitivity of the assay was 66% at a specificity level of 92% (1). Previous results also indicated that DR-70™ was
FIG. 3. Receiver Operating Characteristic (ROC) curves of DR-70™ immunoassay in discriminating patients with various cancer from control, non-cancer patients.

Table 3: Intra-day Precision Study with DR-70™ Kit

<table>
<thead>
<tr>
<th>Control Serum</th>
<th>Average Level (mg/L)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>15.505</td>
<td>4.2753</td>
</tr>
<tr>
<td>Medium</td>
<td>4.98</td>
<td>7.5864</td>
</tr>
<tr>
<td>Low</td>
<td>1.613</td>
<td>11.9404</td>
</tr>
</tbody>
</table>
able to detect a number of cancers other than lung cancer (unpublished data from studies conducted in California, Germany and Brazil). The results from our study confirmed earlier observations that the DR-70™ immunoassay can indeed detect a number of cancers. In our studies, a total of 13 different cancers have been detected with high degree of sensitivity and specificity by the DR-70™ immunoassay kit (Fig. 2). The application of DR-70™ immunoassay to lung cancer detection showed an overall sensitivity of 87.8% when evaluated at 95.0 specificity. A detailed ROC analysis of the data was presented in Fig. 3. The reported sensitivity of CYFRA 21-1 lung cancer immunoassay was 68% is squamous cell carcinoma of the lung when tested at 95% specificity in patients with benign lung disease (4). Our study with 27 stomach cancer patients yielded a test sensitivity of 92.6% and a specificity of 95.0%. In comparison, the reported sensitivity of CA 72-4 for stomach cancer ranged from 27% to 84% at a specificity range of 68-93% (5,6). The results also suggest that this assay may be useful for cancer screening in, for example, an annual physical check-up.

In order to obtain reliable results with the DR-70™ assay, the assay control procedure described in the product insert must be strictly adhered to (including properly washing the microwells). Patients who are suffering from infections should not take the test while the infection is still active and ongoing. Sera must be fresh and well prepared. Plasma, or serum obtained from clotted plasma, cannot be used. Hemolyzed serum, serum from incompletely clotted blood or serum from patients with SLE or suffering from infection with Histoplasma capsulatum, pneumonia, hepatitis and possibly other agents should not be used as they may give false positive test results. Highly lipidic sera may result in false negative tests. Therefore, it is best to obtain the serum sample from the patient in the morning before any meal is taken.

CONCLUSION

When the assay procedure as described in the manual is adhered to, and the patients to be tested are not suffering from acute infection, auto-immune disorders or trauma, this report clearly shows that DR-70™ immunoassay can give reliable results that are useful in the detection, screening, and management of cancer. With lung and stomach cancers, DR-70™ provided test results with very high degrees of sensitivity and specificity. It is also demonstrated that the DR-70™ assay is capable of detecting at least 13 different
cancers. This is consistent with the concept that DR-70™ can serve as a “universal tumor marker” of “pan tumor marker.”

REFERENCES


ADVANTAGES OF THE AMDL-ELISA DR-70 (FDP) ASSAY OVER CARCINOEMBRYONIC ANTIGEN (CEA) FOR MONITORING COLORECTAL CANCER PATIENTS

Andrea L. Small-Howard and Holden Harris
AMDL Inc., Tustin, CA, USA

The DR-70\(^{1}\) (FDP) test was the first cancer test cleared by USFDA for monitoring colorectal cancer (CRC) since Carcinoembryonic Antigen (CEA) in 1982. Conservatively, 50% of biopsy-positive CRC patients have negative CEA values. DR-70 and CEA values were compared for 113 CRC monitoring patients. Total concordance rates for DR-70 and CEA were 0.665 and 0.686, respectively. CRC patient pairs were grouped based on their CEA value to deduce DR-70\(^{1}\)'s effectiveness at monitoring patients with low CEA values. DR-70 had 12% to 100% greater positive concordance rates than CEA in this group. DR-70 is a welcome new option for CRC patients.

Keywords: carcinoembryonic antigen, CEA, colorectal cancer, DR-70\(^{1}\), FDP, fibrin-fibrinogen degradation products

INTRODUCTION

Awareness of the importance of CRC screening\(^{11}\) and early treatment has risen,\(^{2}\) but CRC is presently still a significant health concern in the United States.\(^{3}\) In 2007, the Surveillance, Epidemiology and End Results (SEER) program estimated there were 153,760 new colorectal cancer patients\(^{4}\) with a five year survival prediction of 50%. CRC accounts for approximately 10.6% of all new cancer cases and approximately 10% of all cancer deaths in the United States.\(^{5}\) Colorectal cancer (CRC) remains the second leading cause of cancer death in the United States,\(^{5}\) despite a reported decrease in colorectal cancer mortality over the past forty years. This decreased rate is related to increased screening, intervention, and monitoring programs.

Monitoring programs have emerged as an important tool in enhancing survival in post-operative CRC patients.\(^{6}\) For CRC, approximately half of all patients treated will experience disease recurrence.\(^{7}\) Curative

Address correspondence to Andrea L. Small-Howard, Ph.D., AMDL Inc., 2492 Walnut Ave., Suite 100, Tustin, CA 92780, USA. E-mail: asmallhoward@amdl.com
retratment options exist, and reatreatment options are applied with a modest decrease in CRC mortality (approximately 10–15%).\textsuperscript{[8–10]} However, in order to enhance the survival benefit of CRC monitoring programs, “the availability of sensitive and specific tests to identify recurrences at a treatable stage” needs improvement.\textsuperscript{[6]}

The AMDL-ELISA DR-70 (FDP) test (AMDL Diagnostics, Inc., Tustin, CA) is the first new in vitro diagnostic cancer test to be cleared by the US FDA for monitoring colorectal cancer (CRC) since January 14, 1982 when Carcinomembronytic Antigen (CEA) was approved. CEA has been in routine usage for many years as a blood test for monitoring CEA, but it also has well known limitations that are related to the nature of the tumor marker.\textsuperscript{[6]} CEA has been characterized as an oncofetal marker, which implies that it is only present during cancer progression or normal embryogenesis. Evidence exists that contradicts its classification as a pure oncofetal marker; as there are reports of this antigen’s presence in healthy organs and its elevation due to benign conditions that affect the liver,\textsuperscript{[11–14]} lungs,\textsuperscript{[15]} and the gastrointestinal system.\textsuperscript{[16]} Also, CEA is not a good target for a blood test because CEA is normally firmly attached to cancer cells due to its role as an adhesion molecule.\textsuperscript{[15]} In contrast, the DR-70 (FDP) antigen is freely diffusible in the blood.

For many CRC patients with biopsy confirmed cancer, CEA levels are not measurable above the physiological background. Data was taken directly from three independent studies\textsuperscript{[17–19]} and presented directly in Table 1 below to assess the need for an additional CRC monitoring tool. Approximately 50% of all CRC patients with low CEA values cannot not use the CEA test to monitor their cancer because their CEA levels fall below the manufacturer’s defined physiological background level. In the table below, CEA Low Responders is the term given for biopsy positive cancer patients with low CEA values. All of the numbers in the table have been published in the referenced papers.

The DR-70 test measures both Fibrin and Fibrinogen Degradation Products (referred to collectively as FDP in this paper) in human serum samples. Measuring multiple FDP species prevents the DR-70 (FDP) immunoassay from underestimating the cancer-related levels of FDP.\textsuperscript{[20]} Refer to

<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>Number of Patients</th>
<th>% Below CEA Cut-Off by Duke’s Stage</th>
<th>Overall % CEA Low Responders</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Landenson et al.</td>
<td>1980</td>
<td>203</td>
<td>97</td>
<td>75</td>
</tr>
<tr>
<td>Wang, J.Y. et al.</td>
<td>1994</td>
<td>318</td>
<td>100</td>
<td>68</td>
</tr>
<tr>
<td>Wang, W.S. et al.</td>
<td>2000</td>
<td>218</td>
<td>75</td>
<td>61</td>
</tr>
</tbody>
</table>
**FIGURE 1** Cancer Elevates FDP Levels Through Two Pathways: Coagulation and Fibrinolysis. The AMDL-ELISA DR-70\(^1\) (FDP) test measures the FDP produced by multiple pathways, unlike other FDP assays which only measure one pathway or one pathway product. Researchers have established that cancer causes elevated levels of both urokinase-type plasminogen activator (u-PA)\(^{123-251}\) and tissue factor (TF).\(^{126-281}\) Both the u-PA and TF pathways effect the production of FDP in cancer cells. The u-PA pathway (1A and 1B) activates plasmin by transforming plasminogen, the inactive precursor of plasmin, into functional plasmin.\(^{123-251}\) The TF pathway (2) alters the extrinsic coagulation system causing an activation of thrombin.\(^{126-291}\) Thrombin (3) converts Fibrinogen to Fibrin.\(^{301}\) The type of FDP produced will be different depending upon which of the two substrates is digested by plasmin. When fibrinogen is the substrate for plasmin (4), fragments D and E are the end products with fragments X and Y as intermediate products in this digestion. When fibrin is the substrate of plasmin (5), D-dimer is the end product. As a result of either pathway (4) or (5), cancer will cause an elevation in FDP levels as measured by the DR-70 (FDP). Tests that measure only one of the individual FDP species, i.e., D-dimer tests, will miss up to half of the FDP generated as a result of cancer physiology.

Figure 1 for a schematic describing how the DR-70 (FDP) assay measures FDP generated from all of the major cancer induced FDP production pathways. Researchers have established a strong link between increased FDP levels and cancer.\(^{20-221}\) This strong link is based on multiple factors including: a cancer-caused redirection of the coagulation cascade and a cancer-related increase in proteolysis within tumors as they grow and metastasize.

Because the DR-70 test uses a different tumor marker than the CEA test, physicians have an additional blood test for monitoring CRC patients that may be superior to CEA for many of their patients with low CEA values. The purpose of this study is to determine if DR-70 is effective at monitoring CRC patients with low CEA levels.

**EXPERIMENTAL**

**Description of the Clinical Samples**

The samples for the serial monitoring study were retrospective banked samples that were collected blindly and without bias to include all patients
with diagnosed colorectal cancer in the bank at the time of the collection. The serial monitoring samples for this study were obtained from two retrospective sample banks. Forty-eight serial sets were obtained from Geffen Cancer Center in Vero Beach, FL and sixty-four serial sets were from the serum banks at MD Anderson Cancer Center in Houston, TX. Institutional Review Board Approval for use of the samples and informed consent were available for each patient sample set.

Clinical information detailing the status of each patient’s disease was collected at the time of each sample draw. The clinical diagnoses included Duke’s Stage, grade and type (colon or rectal cancer). None of the patients had a history of malignancy within the past five years of the initial sample draw other than colorectal cancer. A breakdown of the patient series is presented in Table 2. The average number of observations per patient is 4.0.

Of the original 113 patients, one had to be dropped from further analysis due to incomplete clinical records. The 112 evaluable subjects in this CRC monitoring cohort consisted of 44 males and 68 females. The average age of the male patients was 65 while female patients averaged 62 years. The overall average age was 63 years. There was no significant difference between the average age of the males and the females in this cohort based on a student’s t-test analysis for the determination of variances [t = 1.41, p = 0.163 (unequal variances)].

**TABLE 2** Patient Observation Series

<table>
<thead>
<tr>
<th>Number of Samples in Series</th>
<th>Number of Observation Pairs in Series</th>
<th>Total Number of Pairs in Series</th>
<th>Percent of the Total Samples</th>
<th>Cumulative Percent of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>38</td>
<td>33.9</td>
<td>34.8</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>48</td>
<td>42.9</td>
<td>77.7</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>18</td>
<td>16.1</td>
<td>93.8</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>3</td>
<td>2.7</td>
<td>96.5</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>3</td>
<td>2.7</td>
<td>99.2</td>
</tr>
<tr>
<td>8</td>
<td>7</td>
<td>1</td>
<td>0.9</td>
<td>100.0</td>
</tr>
</tbody>
</table>

**TABLE 3** Ethnic Distribution

<table>
<thead>
<tr>
<th>Ethnic Background</th>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>African American</td>
<td>7</td>
<td>6.3</td>
</tr>
<tr>
<td>Asian</td>
<td>2</td>
<td>1.8</td>
</tr>
<tr>
<td>Caucasian</td>
<td>99</td>
<td>88.4</td>
</tr>
<tr>
<td>Hispanic</td>
<td>4</td>
<td>3.6</td>
</tr>
<tr>
<td>Total</td>
<td>112</td>
<td>100.0</td>
</tr>
</tbody>
</table>
The ethnic composition of the cohort is shown in Table 3. Approximately 88% percent of the cohort was Caucasian; approximately 6% of African-American background; approximately 4% of Hispanic descent; and approximately 2% of Asian decent.

Table 4 presents the Dukes stage of the disease at time of diagnosis for 111 of the 112 evaluable serial patients. One patient’s chart did not contain information related to the stage at time of diagnosis.

Table 5 demonstrates the relationship between Dukes Stage at diagnosis and the presence of metastases. As the Stage of the disease progressed, the percentage of patients with metastases increased.

### Statistical Analysis Plan for Association Between DR-70¹ (FDP)/AIA-Pack™ CEA and Disease Status

The initial objective of this analysis is to determine the overall positive, negative and total concordance values of the DR-70 (FDP) and CEA assays. Then, the CRC patients will be grouped based on their CEA values to evaluate the effectiveness of the DR-70¹ (FDP) assay at monitoring CRC patients with low CEA values; defined as a CEA value of 30 or below.

### TABLE 4  Stage of Cancer at Time of Diagnosis

<table>
<thead>
<tr>
<th>Dukes Stage at Diagnosis</th>
<th>Frequency</th>
<th>Percent</th>
<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>B</td>
<td>18</td>
<td>16.2</td>
<td>20.7</td>
</tr>
<tr>
<td>C</td>
<td>39</td>
<td>35.1</td>
<td>55.9</td>
</tr>
<tr>
<td>D</td>
<td>49</td>
<td>44.1</td>
<td>100.0</td>
</tr>
<tr>
<td>Total</td>
<td>111</td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

### TABLE 5  Distribution of Metastases by Stage at Diagnosis

<table>
<thead>
<tr>
<th>Dukes Stage</th>
<th>Known Metastases at Time of Diagnosis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>A</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>16.7%</td>
<td>83.3%</td>
</tr>
<tr>
<td>C</td>
<td>29</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>74.4%</td>
<td>25.6%</td>
</tr>
<tr>
<td>D</td>
<td>49</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>73.0%</td>
<td>27.0%</td>
</tr>
</tbody>
</table>
Defining the Clinical Sample Set
Serial samples were taken from 112 colon cancer patients resulting in a total of 445 paired observations in which a DR-70 (FDP) reading and a determination of disease progression were obtained. In total, there were also 445 paired observations in which an AIA-Pack™ CEA Assay reading and a determination of disease progression were obtained. The sequential draws covered an average longitudinal period of at least nine months. Progression of the DR-70 (FDP) value or AIA-Pack CEA Assay value in the serial monitoring set was evaluated as a percentage change between the current and previous readings (Y). The minimum percentage to specify disease progression in either assay was determined to be 15%, as will be described in detail later. Clinical disease progression (D) was determined by the Subject’s physician based on their office procedures and clinical laboratory based analyses that were the standard of care during the time of the monitoring period.

Monitoring Cases for Response to Therapy
Subjects in the serial monitoring cohort were followed after surgery and or after various types of therapy including chemotherapy and radiation therapy. The response to therapy was evaluated using information provided in the records by the clinicians based on the results of clinical examinations and imaging results (i.e., bone scans, CT scans, magnetic resonance imaging studies, radiography, or ultrasound).

Response to therapy is defined as follows:

- Complete response (CR) or no evidence of disease (NED): The complete disappearance of all clinical and image-measurable disease as evidenced by the clinical exam and imaging or other diagnostic modalities as ordered by the physician.
- Partial Response (PR): In patients with metastases at the time of the original draw, a noticeable reduction in the size of primary metastatic lesions or bone metastases demonstrating at least stabilization as observed on the bone scan.
- Stable Disease (SD): No significant change in the size of primary metastatic lesions or no noticeable increase in the size of primary lesions or no new lesions as evidenced by the clinical exam and imaging or other diagnostic modalities as ordered by the physician.
- Progressive Disease (PD): Clinical or imaging results that clearly indicate the presence of lesions not seen on previous examinations or a significant increase in the size of primary or metastatic lesions.

Definition of Outcome Measure
The outcome measure for this analysis is the determination of progression of disease from time point \( i \) (clinical visit \( i \), \( \frac{i}{13} \) to \( \frac{n - 1}{13} \) to
a succeeding time point $j$ (clinical visit $j$, $j > i$) up to $n$. In this analysis $n$ is
the number of clinical visits for which samples are collected from a Subject
after diagnosis of colorectal cancer and prior to death, loss to follow up or
remission of disease.

Let $D_{ij}$ represent the variable disease progression as measured above
and allow $D_{ij}$ to have the values

$D_{ij} =
\begin{cases}
1 & \text{if there is progression of disease from visit } i \text{ to visit } j; \\
0 & \text{if there is no progression of disease, the disease is either}
\text{stable or responding to therapy, from visit } i \text{ to visit } j; \\
\end{cases}
$

\textbf{Determining Values of} \ D

Disease progression from visit $i$ to visit $j$ will be determined by the
Subject’s physician based on any or all of the following:

. Examination of the subject for clinical signs and symptoms, including the
results of laboratory tests that are current standard of care for the assess-
ment of colorectal cancer disease status.
. Examination of radiographic findings (imaging) that can be used for the
assessment of colorectal cancer disease status. Radiographic findings
include results from CAT scans, PET scans, MRI and X-Ray images.

\textbf{Determination of Clinical Significance in Marker Value Change}

To ensure that the change between the values of the test device over a
time interval could not be attributed to assay variation, a 15\% increase from
the previous visit was determined to be the most appropriate threshold for
significant \% change for the determination of disease progression in the
DR-70 (FDP) assay. The coefficient of variation (CV) used in the calculation
for significant \% change was based on an imprecision study following regu-
ulatory guidelines. In that precision study, the total CV over all runs, days,
and intra-assay was computed for each control specimen analyzed. The
highest CV values were observed for specimens with a low concentration
of (0.21–0.42 mg/mL); however, in a study of cancer progression, such as
the one being reported here, such samples constituted less than 5\% of
the measurements. Over 80\% of the measurements had concentrations of
0.6 mg/mL or higher where the CV is lower. Therefore the CV values for
the lower concentrations will not be used to determine the significant \% change. If the CV values for the highest laboratory specimens with concen-
tration of 1.31 (CV \% 7.85) or 4.11 (CV \% 7.14) mg/mL are averaged, the
mean is 7.495\%. The CV is given by the following formula.
\[
\text{CV} \approx 100 \frac{\sigma}{X}
\]

which is the standard deviation divided by the mean. The proportion difference between two successive measurements is given by the following formula.

\[
p \approx \frac{x_2 - x_1}{x_1}
\]

where \( p \) is the proportion difference from the previous measurement. Normal theory would suggest that 97.5% of the measurements are within the range of the following expression.

\[
x \approx 1.96\sigma
\]

Using the expression for CV, we can solve for \( \sigma \) and obtain an expression that can be used to define the difference between successive measurements.

\[
x \approx 1.96 \frac{\text{CV}}{100} \frac{x}{X}
\]

Assuming that the previous measurement was a mean at that time, less than 2.5% of the time, by chance, will the following expression hold.

\[
x_2 > x_1 \approx 1.96 \frac{\text{CV}}{100} \frac{x_1}{X}
\]

Algebraically rearranging this expression we get the following.

\[
\frac{x_2}{x_1} > 1.96 \frac{\text{CV}}{100}
\]

Alternatively the following can be used.

\[
\frac{x_2}{x_1} > 1 \approx 1.96 \frac{\text{CV}}{100}
\]

Using the value of 7.495 on the previous page, this expression indicates that the proportion of difference must be greater than 7.495 * 1.96 = 100 \( \frac{1}{4} \) 0.1469. For simplicity, this cut off value has been rounded up to 0.15 or 15% or the ratio should exceed 1.15. Thus if a later visit has a value that is greater than 15% higher than the previous value, it will be considered evidence of disease progression.

A 15% increase from the previous visit was also determined as the most appropriate threshold for significant % change for the determination of disease progression using the AIA-Pack™ CEA Assay from TOSOS
Bioscience. The same evaluation, as above, was used with the CV listed in the AIA-Pack™ CEA Assay product insert.

**Definition of Significance in Marker Value Change**

Let $\delta$ equal the significant change in marker value for either assay, which has been determined at 15% for either assay, as described above. Let $x_i$ be the value of the test device obtained from the assay of a blood sample drawn from the Subject at visit $i$ and $x_j$ be the value of the test device obtained from the assay of a blood sample drawn from the Subject at visit $j$.

Define $Y_{ij}$ as

$$Y_{ij} = \begin{cases} 1 & \text{if } \delta x_j \geq \delta x_i \\ 0 & \text{otherwise} \end{cases}$$

**Determining the Association Between D and Y**

With $D_{ij}$ and $Y_{ij}$ defined above for either assay, a 2 × 2 contingency table can be constructed for the analysis of this data. The contingency table has the format of Table 6. In this table the variable $a$ represents the number of $(Y_{ij}, D_{ij})$ pairs that have the value of 1 for both $Y_{ij}$ and $D_{ij}$. The variable $b$ represents the number of $(Y_{ij}, D_{ij})$ pairs that have the value 1 for $Y_{ij}$ and 0 for $D_{ij}$. The variable $c$ represents the number of $(Y_{ij}, D_{ij})$ pairs that have the value 0 for $Y_{ij}$ and 1 for $D_{ij}$. Lastly variable $d$ represents the number of $(Y_{ij}, D_{ij})$ pairs that have the value of 0 for both $Y_{ij}$ and $D_{ij}$. The accrued values of $a, b, c,$ and $d$ are determined over all serial interval values of $Y_{ij}$ and $D_{ij}$. The sum of $a, b, c$ and $d$ is the total number of all $(Y_{ij}, D_{ij})$ pairs for all Subjects. This sum is designated $N$ in Table 6.

From Table 6, sensitivity and specificity are computed as follows:

- **Specificity:** 100 = $\frac{b}{d}$
- **Sensitivity:** 100 = $\frac{a}{c}$

**TABLE 6** Model Contingency Table for D and Y

<table>
<thead>
<tr>
<th>$Y_{ij}$</th>
<th>$D_{ij}$</th>
<th>Disease Progression</th>
<th>No Progression</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\frac{1}{4}$ Significant increase in tumor marker as measured by assay</td>
<td>$\frac{1}{4}$ Significant increase in tumor marker as measured by assay</td>
<td>$a$</td>
<td>$b$</td>
<td>$a \neq b$</td>
</tr>
<tr>
<td>$\frac{1}{4}$ No Significant increase in tumor marker as measured by assay</td>
<td>$\frac{1}{4}$ No Significant increase in tumor marker as measured by assay</td>
<td>$c$</td>
<td>$d$</td>
<td>$c \neq d$</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>$a \neq c$</td>
</tr>
</tbody>
</table>
From Table 6, concordance values are computed as follows:

\[
\text{Positive Concordance } \delta C \left|_{\frac{4}{4}} \right. a = \delta a \ | \ cP \\
\text{Negative Concordance } \delta C \left|_{\frac{4}{4}} \right. d = \delta b \ | \ dP \\
\text{Total Concordance } \delta C P \left|_{\frac{4}{4}} \right. \delta a | \ dP = N
\]

**Justification of Sample Size**

Given the above assumptions and calculations, the minimum sample size for this study was determined to be 70 subjects with an average of 3 draws each.\[^{31}\] The samples are retrospective banked samples collected blindly and unbiased. Out of a total of 445 evaluable observations, there were 112 evaluable patient serial sets with an average of 4 draws each.

**RESULTS**

**General Effectiveness of DR-70 (FDP) or CEA for CRC Monitoring**

The clinical trial results were tabulated, as described above. The results for the DR-70 (FDP) test immediately follow in Table 7 and the results for CEA are found in Table 8. In addition, the following interpretations are provided: Positive Concordance (C|), Negative Concordance (C ), Total Concordance (C), Sensitivity, and Specificity.

Based on these data the concordances for the DR-70 (FDP) vs. Clinical Disease Status are:

\[
C \left|_{\frac{4}{4}} \right. \text{Positive concordance} P \left|_{\frac{4}{4}} \right. 0.652; \\
C \left|_{\frac{4}{4}} \right. \text{Negative concordance} P \left|_{\frac{4}{4}} \right. 0.673 \text{ and} \\
C \left|_{\frac{4}{4}} \right. \text{Total overall concordance} P \left|_{\frac{4}{4}} \right. 0.665:
\]

In estimating the specificity and sensitivity of the DR-70 (FDP) test, using a significant % change value of 15% or a ratio value of 1.15 or higher,

**TABLE 7** Clinical Disease Status vs. AMDL-ELISA DR-70 (FDP)

<table>
<thead>
<tr>
<th>DR-70 (FDP)</th>
<th>Clinical Disease Status</th>
<th></th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Progression</td>
<td>No Progression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significant increase (&gt;15%)</td>
<td>88</td>
<td>65</td>
<td>153</td>
<td></td>
</tr>
<tr>
<td>No significant increase (&lt;15%)</td>
<td>47</td>
<td>134</td>
<td>181</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>135</td>
<td>199</td>
<td>334</td>
<td></td>
</tr>
</tbody>
</table>
the estimated specificity was determined as 67% with an estimated sensitivity of 65%.

Based on these data the concordances for the TOSOH AIA-PACK CEA vs. Clinical Disease Status are:

$C_P \theta$ Positive concordance $\frac{1}{4} 0.739$;

$C \theta$ Negative concordance $\frac{1}{4} 0.653$ and

$C \theta$ Total overall concordance $\frac{1}{4} 0.686$:

In estimating the specificity and sensitivity of the CEA test, using a significant % change value of 15% or a ratio value of 1.15 or higher, the estimated specificity was determined as 73% with an estimated sensitivity of 65%.

### TABLE 8 Clinical Disease Status vs. the TOSOH AIA-PACK CEA

<table>
<thead>
<tr>
<th>CEA</th>
<th>Clinical Disease Status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Progression</td>
</tr>
<tr>
<td>Significant Increase (&gt;15%)</td>
<td>99</td>
</tr>
<tr>
<td>No Significant Increase (&lt;15%)</td>
<td>35</td>
</tr>
<tr>
<td>Total</td>
<td>135</td>
</tr>
</tbody>
</table>
FIGURE 3 Negative Concordance for DR-70 or CEA Grouped by CEA values. Negative progression patient sample pairs were grouped in ascending order based on the CEA value. The % Concordance for DR-70 relative to the clinical findings was graphed in blue for each group. The % Concordance for CEA relative to the clinical findings was graphed in red for each group. There were a total of 199 negative progression patient pair values.

**Effectiveness of DR-70 (FDP) Test in CRC Monitoring Patients with Low CEA Values**

After grouping the CRC patients’ sample pairs in ascending order based on their CEA values, the following relationships were revealed. Positive concordance values of CRC patients measured with DR-70 or CEA in patient groups relative to their CEA values are provided in Figure 2. Negative concordances of CRC patients measured with DR-70 or CEA in groups relative to their CEA values are provided in Figure 3.

In Figure 2, DR-70 had between 12% and 100% greater positive concordance rates than CEA for CRC monitoring patients with low CEA values. In contrast, the negative concordance values of DR-70 and CEA showed less than 10% difference for all CRC patient groups in the trial, as depicted in Figure 3.

**DISCUSSION**

Overall, the results from this trial support the assertion that the AMDL-DR-70 (FDP) test is as good at monitoring CRC patients as CEA.
Based on the results presented in Table 7 and Table 8, the total concordance values for DR-70 and CEA are 0.665 and 0.686, respectively. The total concordance values for DR-70 and CEA differed by only 3.2% in this clinical trial.

In addition, Figure 2 suggests that DR-70 is more effective at monitoring patients whose CEA values are 30 or less. Forty-six (46) of 135 positive progression patient pair values fell in the groups containing CEA values of 30 or less. In this group, DR-70 had between 12% and 100% greater positive concordance rates than CEA for CRC monitoring patients. The negative concordance rates were about the same for both assays across all groups when ordered by the CEA value of the patient. The difference between the negative concordance rates of these assays was less than 10% for all patient groups relative to their CEA values. Additional trials are planned to examine this same sub-group to verify the value of the test for patients with low CEA values.

The results of this trial suggest that DR-70 could have a positive impact on mortality that is associated with CRC recurrence. All of the patients in the reported trial were either post-surgery with no adjuvant therapy or post-therapy, as was described in the methods section. This holds clinical significance because disease progression in these patients would be described as disease recurrence. As reported in the introduction, approximately half of all CRC patients treated will experience disease recurrence. An additional and improved tool for the monitoring of disease recurrence could profoundly impact the mortality rate that is associated with CRC recurrence. Future studies could assess the impact of the DR-70 test on clinical outcomes in a longer term, prospective trial.

FDP has been shown to be valuable as a tumor marker in a number of different cancers.\cite{32-43} FDP levels correlate with cancer occurrence, stage, progression and prognosis. Among these studies, the DR-70 (FDP) assay was used to detect FDP levels in 7,839 patients and the DR-70 (FDP) assay results consistently correlated with either the positive detection or positive progression of a variety of cancers.

Researchers have established a strong link between increased FDP levels and cancer which is based on multiple factors including: a cancer-caused redirection of the coagulation cascade\cite{44-49} and a cancer-related increase in proteolysis within tumors as they grow\cite{50,51} and metastasize.\cite{52-56} Clinical studies reveal that measuring FDP levels, either with the DR-70 (FDP) test or with other related tests, has significant diagnostic value for a variety of cancers. These studies demonstrate that FDP levels correlate with the cancer stage\cite{41-43,57-60} and with the cancer progression,\cite{41,59,61} as quantified by the number of lymph node metastases. Clinical research efforts have shown that pretreatment measurements of FDP levels have prognostic significance for post-treatment survival.\cite{33,60,62-66} In addition to survival
prognoses, pretreatment FDP values may be used to indicate when adjuvant systemic treatments are required for surgical Subjects.\textsuperscript{[64,66]}

Cancer is a disease that is characterized by disregulation at the cellular level. As cancer progresses, the cellular disregulation spreads to the systems level. The coagulation system is one of the first systems affected by cancer-related processes. As referenced in Figure 1, the coagulation pathway may be inappropriately activated in cancer patients either by the activation of the coagulation pathway alone, the fibrinolysis pathway alone, or both pathways simultaneously. Coagulation may be increased due to elevated levels of tissue factor (TF),\textsuperscript{[62,67]} which acts through the extrinsic coagulation system. Alternatively, the fibrinolysis pathway may be mistakenly activated in cancer patients through elevations in the levels of urokinase-type plasminogen activator (u-PA)\textsuperscript{[144]} that activates the protease plasmin. Disregulation of the coagulation system has important adverse affects on cancer patients because the coagulation system plays dual roles in homeostasis and immunity. As the cancer-related disregulation of the coagulation system increases, these clots can lead to heart attack, stroke, or pulmonary embolism. Other FDP-related tests have been helpful in predicting survival outcome based on the often fatal consequences of blood clots in cancer patients.\textsuperscript{[59,62,67]} As the utility of the DR-70 (FDP) assay becomes more widely known, DR-70 should be adapted to help patients with a variety of cancers in different clinical settings.

**CONCLUSIONS**

The DR-70 test appears to have additional benefits in monitoring CRC patients with low CEA values. DR-70 had between a 12% and a 100% greater positive concordance rate than CEA for CRC monitoring patients with low CEA values. Given that 50% is a conservative estimate of CRC patients with low CEA values, physicians and patients could significantly benefit from this new option for monitoring CRC cancer.

**REFERENCES**

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Advantages of DR-70 over CEA for CRC Monitoring


