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A Comparison of CYFRA 21-1, NSE, and CEA for the Serodiagnosis of Lung Cancer

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Abstract: There were 224,210 new cases of lung cancer in the US during 2014, and 159,260 died from lung cancer during that year [1]. Since early diagnosis and treatment leads to a better prognosis, the medical community is actively looking for new, non-invasive diagnostic tests for the disease. This includes the search for new and effective tumor markers. Tumor markers are used in combination with other tests to diagnose cancer. After the diagnosis, they are used to follow a patient's case. The three tumor markers studied were neuron specific enolase (NSE), carcinoembryonic antigen (CEA), and CYFRA 21-1. In this study, the normal reference intervals were developed using sera from healthy adult donors. The analytical properties of the tumor marker assays were tested for and found to be satisfactory. The study was designed to compare the diagnostic and predictive values for the three tumor markers. Preliminary results on 638 patients (76 lung cancer patients, 562 healthy/non cancer patients) included: (1) diagnostic % sensitivity (CEA: 22.37%, NSE: 0.00%, CYFRA 21-1: 18.92%), (2) diagnostic % specificity (CEA: 80.43%, NSE: 99.39%, CYFRA 21-1: 93.16%), (3) %PV+ (CEA: 13.39%, NSE: 4.17%, CYFRA 21-1: 28.00%), (4) %PV- (CEA: 88.45%, NSE: 87.12%, CYFRA 21-1: 89.09%), (5) % efficiency (CEA: 73.51, NSE: 86.65%, CYFRA 21-1: 84.00%). It was hypothesized that CYFRA 21-1 would be superior to CEA and NSE for the sero-diagnosis of lung cancer in a cohort of patients, and the hypothesis was rejected.

Key Words: Cancer, Carcinoembryonic Antigen, Neuron Specific Enolase, CYFRA 21-1, Lung Cancer.

INTRODUCTION

During the past 150 years, infectious diseases have been replaced by arterial disease and cancer as the major causes of death. Today, arterial disease accounts for 50% of all deaths in the US, and cancer accounts for 20% of deaths in the US. Heart attacks and strokes, which are associated with arterial disease, are seen as hazards of old age, lack of exercise, and poor diet. Cancer, though, is thought of as an unpredictable disease. It strikes no matter how old or how fit one is. This seems to be true because cancer can be related to environmental factors (Conklin, 1949) [2].

In the US, there were 1,665,540 cases of all types of cancer in 2014 and 585,720 deaths in 2014 (American Cancer Society, 2014) [1]. Since early diagnosis and treatment leads to a better prognosis, the medical community is actively looking for new non-invasive tests for the disease. This includes the search for new and effective tumor markers. The objective of this study was to compare and evaluate three tumor markers, CYFRA 21-1, carcinoembryonic antigen (CEA), and neuron specific enolase (NSE) for the sero-diagnosis of lung cancer.

The tumor marker CYFRA 21-1 is used to diagnose lung cancer, but it has also proven successful in identifying other tumors. It can be a marker for cancers of the head and neck. It also has proven successful in monitoring tumors of the cervix and has been considered useful in identifying non-small cell lung cancer (NSCLC). This includes squamous cell carcinoma (SCC), adenocarcinoma, and large cell carcinoma. These types of tumors account for 80% of the lung tumors (Nakamura & Wu, 1997) [3].

CEA is a marker that has been used for colorectal cancer, renal cancer, ovarian cancer, and breast cancer. It was first discovered in extracts of colon cancer. It was thought that a tumor specific marker had been found, but it was later discovered that not all colon tumors produced CEA. This is because tumors are very heterogeneous in their composition. Similarly, elevated blood CEA has been observed in heavy smokers who were tumor free. It is used

as a minor marker in lung cancer (Nakamura & Wu, 1997) [3].

NSE is a soluble metal-activated glycolytic metalloenzyme that provides components necessary for aerobic glycolysis. Decreasing values of this enzyme after primary treatment corresponding to the half-life period is the first sign of a good prognosis and good treatment effect. NSE can play no role in the staging of the disease. It was also found unable to differentiate between partial and complete response to treatment (Schneider et al., 2002) [4].

There were 224,210 new cases of lung cancer in the US during 2014, and 159,260 that died during 2014 (American Cancer Society, 2014) [1]. The incidence of lung cancer in western countries is directly proportional to the amount of cigarettes its inhabitants smoked 10 to 20 years earlier. The number of cigarettes smoked concomitantly in the western countries is completely irrelevant to the incidence of lung cancer during that time period. The damage has to have been done to the body years earlier than when the lung cancer first presents/occurs (Cairns, 1975) [5].

Just as the choice to smoke cigarettes influences the chance of someone developing lung cancer years later, a person's occupational choice can have the same effect. Occupational cancers are those that are due to exposure to industrial chemicals (e.g., benzene) while working. These cancers may not appear until 10 to 20 years after the person has retired (Cairns, 1975) [5].

An area of importance when studying lung cancer is the method of diagnosis. Computed tomography is an important form of diagnosis and staging. Computed tomography scanning is based on the measurement of the amount of x-ray weakening as x-rays pass through different tissues within the body. Bone and tissues interact differently with the tomography, producing different attenuation coefficients. Attenuation coefficients characterize how easily a material or medium can be penetrated by a beam of light. Attenuation coefficients can be calculated as a function of the space in the cross sectional area where the x-rays pass. These different functions of space show up on the two-dimensional image as different shades of grey in an area. This creates a two-dimensional image and is generally used for chest x-rays and mammograms. If there is a tumor in the lung or in breast tissue, there will be a different attenuation coefficient as compared to that seen with normal lung and breast tissue. CT scanning is also a type of computed tomography. CT scanning is a cross sectional image obtained by exposure to a thin beam of x-rays throughout a 360 degree rotation. Both x-ray imaging and CT scanning provide exclusively anatomical information (Sherar, 2005) [6].

Another important form of diagnosis and staging is nuclear medicine and bone scans. Nuclear medicine uses radioactive agents to obtain images of tumors in the patient for diagnosis. The radioactive agents are radioactive isotopes. The radioactive isotopes used for diagnostic imaging emit high-energy photons. The photons are detected by a large sodium iodide crystal scanner, which transforms the photons into light signals. The light signals are then detected using a photomultiplier tube. This type of imaging is used commonly for detecting the presence of metastatic disease to the bone (Sherar, 2005) [6].

Magnetic resonance imaging (MRI) along with ultrasound can also be used for diagnosis and staging. Magnetic resonance imaging is based on magnetization of tissues when a patient is placed in a large, externally applied magnetic field contained in a MRI scanner. MRIs have become a commonly used technique for the diagnosis of cancer. MRIs have an excellent soft tissue contrast and resolution. It is excellent for imaging the brain, head, neck, and pelvic region (Sherar, 2005) [6].

The standard B mode ultrasound is used in diagnosis. The imaging projected from this ultrasound is based on the reflection of very high frequency sound signals. The ultrasound uses a piezoelectric crystal that generates a short ultrasound pulse that penetrates the tissue and is reflected by structures with different mechanical properties. The image the ultrasound forms is produced by time-gating the signals scattered back to the transducer. The scattering of ultrasound is different between normal tissues and tumors. An ultrasound is particularly useful for diagnosis in the abdomen and prostate. However, it will not pass through bone well enough for it to provide proper imaging the abdomen (Sherar, 2005) [6].

It was hypothesized that CYFRA 21-1 would be superior to CEA and NSE for the sero-diagnosis of lung cancer in a cohort of patients.

MATERIALS & METHODS

Two of the kits used in this project for the ELISA assays, carcinoembryonic antigen (CEA) and neuron specific enolase (NSE), were acquired from Diagnostic Automation, Inc. (Calabasas, CA). The third kit for the ELISA assay, CYFRA 21-1, was acquired from Fujirebio Diagnostic, Inc. /USA: Immuno-Biological Laboratories, Inc. (Seguin, TX). All the solutions that were used were

prepared using reagents and diluents present in the kits. Tests were performed using ELISA assays. Statistical analyses were performed using SPSS version 22 statistical software. Permission for this study was granted by the University of Southern Mississippi Institutional Review Board (protocol number 13042901) to ensure adherence to stipulated criteria.

Six hundred and thirty- eight patient serum samples were obtained from area hospitals with only a sample code number and the cancer diagnosis provided. Normal serum samples from two hundred and four healthy adult subjects were also obtained from area hospitals. All procedures protecting the confidentiality of the patients and subjects were followed. No information regarding the identification of a patient or subject was released by the hospitals involved. Aseptic techniques were used at all times with the samples. Blood samples were collected by hospital personnel at the respective hospitals, allowed to clot, and were separated before being frozen, given a code number, and packaged in plastic tubes for transport. Before testing, all of the samples were sorted into test tube racks and allowed to reach room temperature by soaking in a water bath at approximately 25°C.

Patient samples were classified by the hospital pathologists as either cancerous or cancer free based on histopathology (Table 1 and Table 7). This diagnosis was provided for comparison only. Similarly, healthy adult control subjects were determined to be disease free by their attending physicians (Table 6).

There were testing procedures included in the assay reagent kits which were followed for each assay (CEA, CYFRA 21-1, and NSE). The results of the assays performed were read with a Beckman Coulter AD 340 (Beckman-Coulter, Brea, CA, USA) microplate reader.

CEA ELISA Assay Kit

The kit's reference number was 5201-16, and the lot# was DA314050802. The kits came from Diagnostic Automation/Cortez Diagnostics, Inc. (Calabasas, CA, USA). Other materials required that did not come in the kits were disposable tips, pipettors of 25 uL and 100 uL, a microwell reader, and deionized water for blanks.

The CEA quantitative test kit is based on a solid phase enzyme-linked immunosorbent assay with a detection range of 0-120 ng/mL. The test requires 50 uL of serum, and it performs with a specificity of 95% and sensitivity of 1.0 ng/mL per the manufacturer. The assay system utilizes one monoclonal anti-CEA antibody for solid phase immobilization and another mouse monoclonal anti-CEA antibody in the antibody-enzyme conjugate solution. The standards and the testing specimens were added to the CEA antibody coated microtiter wells. The CEA antibody labeled with horseradish peroxidase (conjugate) was added. If human CEA was present in the specimen, it would combine with the antibody on the well and the antibody conjugate. The solution was then washed with the wash buffer, which removed any unbound conjugate. The TMB solution was then added. A colorimetric reaction occurs whose final intensity reveals the concentration of CEA present (CEA Package Insert) [7].

When preparing the assay, all the reagents and samples were brought to room temperature (~25°C) and gently mixed. The wash buffer was prepared by adding 15 mL of the washing buffer into 735 mL of distilled water in a large flask. The mixture was capped and inverted several times to mix. The wash buffer was then poured into the wash solution bottle. Blanks (deionized water), calibration solutions, and controls were run in duplicate in the first 14 wells of each plate. The remaining wells contained serum samples and extra controls. A data sheet was kept to identify samples, calibrators, and controls with their locations [7].

NSE ELISA Assay Kit

The kit's reference number was 6334-16, and the lot# was DA314050901. The kit came from Diagnostic Automation/Cortez Diagnostics, Inc. (Calabasas, CA, USA). Other materials required that did not come in the kit were disposable tips, pipettors of 25 uL and 100 uL, a microwell reader, and deionized water for blanks.

The NSE quantitative test kit is based on a solid phase enzyme-linked immunosorbent assay with a detection range of 0-200 ng/mL. The test requires 15 uL of serum, and it performs with a specificity of 98.7% and sensitivity of 1.5 ng/mL per the manufacturer. The assay system utilizes one monoclonal anti-NSE antibody for solid phase immobilization and another monoclonal anti-NSE antibody in the antibody-enzyme conjugate solution. The standards and the testing specimens were added to the antibody coated microtiter wells. If human NSE was present in the specimen, then it would combine with the antibody on the well and the antibody conjugate. The solution was then washed with the wash buffer, which removed any unbound conjugate. The amount of bound peroxidase (enzyme conjugate) was proportional to the concentration of the NSE present in each sample. After addition of the substrate and chromogen, the intensity of

blue color developed in proportion to the concentration of NSE antigen in the samples (NSE Package Insert) [8].

When preparing the assay, all the reagents and samples were brought to room temperature (~25ºC) and gently mixed. The wash buffer was prepared by adding 15 mL of the washing buffer into 735 mL of distilled water in a large flask. The mixture was capped and inverted several times to mix. The wash buffer was then poured into the wash solution bottle. Blanks (deionized water), calibration solutions, and controls were run in duplicate in the first 14 wells of each kit. The remaining wells contained serum samples and extra controls. A data sheet was kept to identify samples, calibrators, and controls with their locations [8].

CYFRA 21-1 ELISA Assay Kit

The kit's number was 211-10, and the lot# was 34112:1. The kits came from Fujirebio Diagnostic, Inc.//USA: Immuno-Biological Laboratories, Inc. (Seguin, TX). Other materials required that did not come in the kits were disposable tips, pipettors of 25 uL and 100 uL, a microwell reader, and deionized water for blanks.

The CYFRA 21-1 quantitative test kit is based on a solid phase enzyme-linked immunosorbent assay with a detection range of 0.5-50 ng/mL. The test sensitivity was 0.12 ng/mL and the % specificity was 98% per the manufacturer. The assay system utilizes one monoclonal anti-CYFRA 21-1 antibody for solid phase immobilization and another mouse monoclonal anti-CYFRA 21-1 antibody in the antibody-enzyme conjugate solution. The standards and the testing specimens were added to the CYFRA 21-1 antibody coated microtiter wells. The CYFRA 21-1 antibody labeled with horseradish peroxidase (conjugate) was added. If human CYFRA 21-1 was present in the specimen, then it would combine with the antibody on the well and the antibody conjugate. The solution was then washed with the wash buffer, which removed any unbound conjugate. The TMB solution was then added. A colorimetric reaction occurs whose final intensity reveals the concentration of CYFRA 21-1 present (Cyfra 21-1 Package Insert) [9].

When preparing the assay, all the reagents and samples were brought to room temperature (~25ºC) and gently mixed. The wash buffer was prepared by adding 50 mL of the washing buffer into 1200 mL of distilled water in a large flask. The mixture was capped and inverted several times to mix. The wash buffer was then poured into the wash solution bottle. Blanks (deionized water), calibration solutions, and controls were run in duplicate in the first 14 wells of each kit. The remaining wells contained serum samples and extra controls. A data sheet was kept to identify samples, calibrators, and controls with their locations [9].

Table 1. Patient Sample Classification

Number of Samples	Cancer Diagnosis
76	**Cancerous**
562	**Cancer Free**
Total number of Patients: 638	

RESULTS

Over the course of the project, there were NSE, CEA, and CYFRA 21-1 quality control samples incorporated into the assays to determine within-run and between-run precision (Tables 2-3). These controls had a known amount of antigen incorporated into the control sample. This determined if the assay was performing correctly. With a percent coefficient of variation (% CV) of less than 10% all three markers had excellent within-run precision (Table 2). Similarly, the between-run precision was excellent for CYFRA 21-1 and CEA but only good for NSE (15.37%) (Table 3).

Serial dilutions of patient samples were used to determine the linearity of the assays (Table 4). These results indicate excellent linearity with R^2 values between 0.94 and 0.99.

The minimum concentration each assay was able to detect (analytical sensitivity) was determined by assaying replicates of a control with no antigen (zero control) and calculating the mean (+/-) two standard deviations ($\bar{X}$ +/- 2SD) (Table 5). Values less than the high end of the range are considered to have no antigen or a value of zero. The analytical sensitivity of NSE was 7.02 ng/mL and those for CYFRA 21-1 and CEA had cut off values of less than 1.0 ng/mL (Table 5).

The normal reference intervals ($\bar{X}$ +/- 2SD) are the reference intervals that were developed from healthy adult control subjects. The healthy adult control subjects were known to have no disease. The normal reference intervals for each of the antigens studied can be seen in Table 6. For tumor markers, the high end of the range represents a possible cut-off value between "presumed healthy" (negative for disease) and "presumed cancerous" (positive for disease). The low end of the range is assumed to imply healthy. Since a variety of factors (e.g. age,

gender, genetics) can affect the normal reference interval for a marker, the manufacturers encourage the lab to adjust the cut-off values to reflect the local population. In determining the negative and positive patient results for this study, the manufacturers' cut-off values were used.

Diagnostic sensitivity is the proportion of individuals with a disease who test positive for the disease. The higher the sensitivity the better the test is. Diagnostic sensitivities of 0.00% (NSE), 18.92% (CYFRA 21-1), and 22.37% (CEA) were obtained (Table 7), suggesting that CEA was slightly better than CYFRA 21-1 and that NSE was not useful. Diagnostic specificity is the proportion of individuals without the disease who test negatively for the disease. Diagnostic specificities of 99.39% (NSE), 93.16% (CYFRA 21-1), and 80.43% (CEA) were obtained. Here the values for NSE were superior and those of CEA and CYFRA 21-1 were excellent (Table 7). Some other parameters that can be evaluated are positive predictive value (PV+ %), negative predictive value (PV- %), and percent efficiency (Efficiency %). Positive predictive value is the fraction of positive tests that are true positives. Negative predictive value is the fraction of negative tests that are true negatives. The percent efficiency is the fraction of all test results that are either true positives or true negatives. The positive predictive values are low, whereas the negative predictive values are high for all three assays. The percent efficiencies are good, as well, for the three assays (Table 7).

Table 2. Within Run Assay Precision for NSE, CYFRA 21-1, and CEA

	n	Mean (ng/mL)	SD	%CV
NSE control	10	7.55	0.21	2.78
CYFRA 21-1 High Control	20	14.17	0.77	5.41
CYFRA 21-1 Low Control	20	4.41	0.28	6.27
CEA High Control	40	64.04	2.79	4.36
CEA Low control	43	4.28	0.29	6.78

Table 3. Between Run Precision for NSE, CYFRA 21-1, and CEA

	n	Mean (ng/mL)	SD	%CV
NSE control	43	7.87	1.21	15.37
CYFRA 21-1 High Control	76	13.97	0.86	6.16
CYFRA 21-1 Low Control	78	4.45	0.50	11.23
CEA High Control	72	62.64	3.40	5.43
CEA Low control	76	4.44	0.37	8.33

Table 4. Assay Linearity for NSE, CYFRA 21-1, and CEA

Assay	R squared
NSE	0.997
Cyfra 21-1	0.992
CEA	0.939

Table 5. Analytical Sensitivity for NSE, CYFRA 21-1, and CEA

	n	Mean (ng/mL)	SD	Range
NSE	10	6.56	0.23	6.10-7.02
CYFRA 21-1	20	0.01	0.03	0.00-0.07
CEA	20	0.00	0.35	0.00-0.70

Table 6. Normal Reference Intervals for NSE, CYFRA 21-1, and CEA

	n	Min	Max	Mean	SD	Range
NSE	174	3.62	8.45	6.61	1.31	3.99-9.23
CYFRA 21-1	189	0.00	82.9	2.21	9.36	0.00-20.93
CEA	204	0.00	16.10	2.40	2.63	0.00-7.66

Table 7. Predictive values for NSE, CYFRA 21-1, and CEA in 638 Patients

	Sensitivity (%)	Specificity (%)	PV+ (%)	PV- (%)	Efficiency (%)	Cut-Off (ng/mL)
NSE	0.00	99.39	4.17	87.12	86.65	15.01
CYFRA 21-1	18.92	93.16	28.00	89.09	84.00	1.82
CEA	22.37	80.43	13.39	88.45	73.51	5.01

DISCUSSION

The analytical parameters for each of the three testing methods were good. The normal reference interval for CYFRA 21-1 was dramatically higher than the reference interval determined by the manufacturer. This is possibly due to geographic location and consequently the mix of healthy adult subjects tested. None of the diagnostic sensitivities were optimal, but of the three examined, CEA was the best predictor of the disease. The sensitivity

would be the most important test result because it demonstrates the ability of the assay to diagnose the presence of disease. The diagnostic specificities obtained for the true negatives were excellent, with NSE having the best specificity at 99.39%. This result greatly differed from the 0% found for the sensitivity of NSE. Mathematically, because of the high percent efficiency of NSE at 86.65%, it appeared to be the best predictor of the disease, but with a diagnostic percent sensitivity of 0%, NSE is only useful to exclude disease. The cut-off points used for all three of the markers were those of the manufacturers. By adjusting the cut-off points one could raise the diagnostic percent sensitivity, but the diagnostic percent specificity would be concomitantly lowered. The manufacturers recommend that each laboratory should determine its own normal/healthy and abnormal/unhealthy ranges so as to account for any environmental factors such as diet or climate and/or the genetic mixtures of patients seen in the area. In this case, changing the cut-off points did not significantly improve the results (data not shown).

A strong point of this study is the small number of people who were directly involved in the testing of the samples. This keeps the relative amount of human error minimal. The age of some of the samples is a possible weakness due to potential sample degradation at minus 20^{o}C. To improve the accuracy of the study, a larger number of subjects could be obtained, and the subjects could be acquired from multiple geographic regions. The samples should also be fresh and only thawed once when tested.

From the data collected, CEA was the most sensitive marker for predicting lung cancer. NSE was the most specific, and CYFRA 21-1 had the next highest sensitivity and specificity. The highest sensitivity is the most important part of a test because it predicts the true positives. CEA, the best predictor of the disease, is one of the oldest tumor markers. It is commonly used in determining other cancers such as colorectal cancer. The CEA subgroup members are cell membrane associated and show a complex expression pattern in normal and cancerous tissues (Hammarstrom, 1999) [10]. This is a strong point for the tumor marker because it has the ability to track cancer formation in different areas of the body in different organs. One objective of a tumor marker is to serve as a non-invasive test to track a patient's health (remission vs relapse) after recovery from cancer. Physicians and researchers are always seeking non-invasive tests, like tumor markers, to make early diagnosis and track a patient's recovery. While arguably it came in a close second, the hypothesis that CYFRA 21-1 would be the most sensitive and specific predictor of lung cancer was rejected because CEA had a higher sensitivity than CYFRA 21-1. However, CYFRA 21-1 is an independent prognostic factor that is useful in the earlier stages of squamous cell lung cancer (SQC) (Kulpa, 2002) [11].

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