ONCOblot Reports

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Incidence of ONCOblot Detection of ENOX2 in Young Adults

D. James Morré, PhD and David J. Taggart, PhD

MorNuCo, Inc. continues its monthly report for participating physicians and health professionals in order to answer common questions relating to the ONCOblot® Tissue of Origin (Cancer) Test.

As a further assessment of the false positive error rate for the ONCOblot® Tissue of Origin Cancer Test, the incidence of ENOX2 presence in the serum of young adults, 20 to 39 years of age, was evaluated.

Methods

Sera of 50 male and 50 female volunteers, without clinical evidence of cancer, between 20 and 39 years of age were analyzed for the presence of ENOX2 proteins by the ONCOblot® Tissue of Origin Cancer Test. Sera were collected by venipuncture, stored and analyzed using IRB approved protocols.

Results

In the age group 20-29 years, none of the 25 females and only one (colorectal) of the 25 males exhibited ENOX2 proteins indicative of cancer presence. Similarly, in the age group 30 to 39 years, one (blood cell) of the 25 females and one (prostate) of the 25 males exhibited ENOX2 proteins. Therefore, the overall incidence of ENOX2 presence within all 100 serum samples analyzed was 3%.

Discussion

The predicted incidence of newly diagnosed cancers within a population of men and women between 20 to 39 years old is approximately 2%, as predicted by NCI's SEER Cancer Statistics Review (1). The difference between the present findings of a 3% incidence of ENOX2 proteins within 100 subjects from this age range and the predicted incidence of newly diagnosed cancers within this population (3% - 2% = 1%) is consistent with the previously estimated false positive rate of <1% for the ONCOblot® Tissue of Origin Cancer Test (2).

Conclusion

The sera from 100 subjects (50 male and 50 female) between the ages of 20 and 39 years were analyzed for ENOX2 presence provide findings consistent with a previous estimate of a false positive incidence of less than or equal to 1% for the ONCOblot® Tissue of Origin Cancer Test.

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Interpretation of ONCOblot Test Results

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MorNuCo, Inc. continues its monthly report for participating physicians and health professionals in order to answer common questions relating to the ONCOblot® Tissue of Origin (Cancer) Test.

The ONCOblot® Tissue of Origin (Cancer) Test detects the presence of protein transcript variants of the ENOX2 cancer marker in blood serum. Based upon the charge (isoelectric point given as pH), size (molecular weight given in kilodaltons) and the number of ENOX2 protein transcript variants detected, the tissue of cancer origin can be identified.

Number of Protein Transcript Variants

The number of ENOX2 protein transcript variants varies according to the tissue of cancer origin. For many tissues of origin (breast, lung, prostate, blood cell, and five others), only one ENOX2 protein transcript variant is produced. These ENOX2 proteins vary in average size and charge (Table 1), and can therefore be differentiated.

Table 1. Tissues of Cancer Origin that Produce One ENOX2 Protein Transcript Variant

Cancer	MW (kDa)	pI (pH)
Blood Cell	34-47	3.5-4.5
Breast	64-69	4.2-4.9
Cervical	90-100	4.2-5.4
Esophageal	42-47	4.6-5.2
Lung	52-56	4.1-5.3
Melanoma	37-41	4.6-5.3
Pancreatic	48-51	3.9-5.4
Prostate	71-88	5.1-6.5
Squamous Cell	57-68	5.0-5.4

For some cancers, two ENOX2 protein transcript variants are produced. These include: ovarian, hepatocellular, uterine, and six others (Table 2).

Table 2. Tissues of Cancer Origin that Produce Two ENOX2 Protein Transcript Variants

	Protein 1		Protein 2	
Cancer	MW (kDa)	pI (pH)	MW (kDa)	pI (pH)
Bladder	63-66	4.2-5.6	42-48	4.1-4.8
Hepatocellular	58-70	4.5-5.0	34-40	4.1-5.2
Mesothelioma	60-68	3.8-4.1	38-44	3.8-4.6
Ovarian	72-90	3.7-5.0	37-47	3.7-5.0
Sarcoma	50-55	5.2-5.6	37-45	4.3-4.9
Testicular Germ Cell	61-62	5.0-5.4	42-45	4.4-4.7
Thyroid Follicular	48-56	4.7-5.1	37-42	4.5-5.2
Thyroid Papillary	56-67	4.5-5.0	37-44	3.2-3.6
Uterine (Endometrial)	67-71	4.2-5.1	41-48	3.7-5.4
Uterine (Unspecified)	63-66	4.2-4.9	41-48	4.4-5.6

For three tissues of origin, three ENOX2 protein transcript variants are produced. These tissues of origin include: stomach, colon/rectum, and renal cell (Table 3).

The detection of four or more ENOX2 protein transcript variants may indicate cancer originating in the brain (Not shown).

Table 3. Tissues of Cancer Origin that Produce Three ENOX2 Protein Transcript Variants

	Protein 1		Protein 2		Protein 3	
Cancer	**MW (kDa)**	**pI (pH)**	**MW (kDa)**	**pI (pH)**	**MW (kDa)**	**pH (pH)**
Colorectal	80-96	4.4-5.4	50-65	4.2-5.3	33-46	3.8-5.2
Gastric (Stomach)	120-188	4.7-5.5	50-62	4.5-5.6	45-53	2.4-3.6
Renal Cell (Kidney)	69-73	4.7-5.4	54-61	4.1-5.2	38-43	3.7-4.3

The number of transcript variants is primarily an indication of the tissue of cancer origin and has no apparent relationship to disease severity, progression or resistance to treatment.

For tissues of origin that produce multiple ENOX2 protein transcript variants (Tables 2 and 3), it is possible for one or two ENOX2 proteins to be either absent or produced below the lower limit of detection of the assay. This occurs in approximately 3% of ONCOblot® tests of clinically-confirmed cancer patients (1).

Molecular Weight

The size of each ENOX2 protein produced by a cancer of any given tissue of origin fall within the ranges indicated above. For any given cancer, the size of each ENOX2 protein is predicted to remain within this range for the duration of the disease. An average variation of ± 2 kilodaltons is expected as the upper and lower limit of accuracy in molecular weight measurements. The molecular weight provides information regarding the tissue of origin and is unrelated to the disease stage, progression or severity.

Isoelectric Point

The charge of an ENOX2 protein transcript variant is measured with an average variation of ± 0.2 pH units and falls within the ranges indicated above for each tissue of origin. The isoelectric points of ENOX2 proteins have not been shown to change during the course of the disease, and these values are unrelated to disease stage, progression or severity.

Absence of ENOX2 Transcript Variants

The absence of ENOX2 protein transcript variants within an ONCOblot® Test indicates that ENOX2 proteins are either not present or produced at concentrations below the lower limit of detection of the test. The current limit of detection of ENOX2 is estimated to be produced by as few as 2 million cancer cells, equivalent to a solid tumor between 0.8 and 1.2 mm in diameter (2).

Summary

The ONCOblot® Tissue of Origin Test confirms cancer presence based upon the detection of one or more ENOX2 transcript variants in a subject's blood serum. Depending on the number of transcript variants, their size and their charge, the tissue of cancer origin can be determined. However, no additional information as to stage, progression, or disease severity can be derived from these values.

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ENOX2: A Potential Target for Early Cancer Intervention

D. James Morré, PhD

Experts agree that early detection is a key component for any strategy to effectively reduce cancer-related morbidity and mortality. By detecting cancer early, the opportunity arises to treat the disease at what is considered the most curative stage, prior to metastatic spread. Unfortunately, other than surgical intervention, there is very little in the current Standard of Care appropriate for early intervention. Out of necessity, current Standards of Care are largely focused on coping with late stage cancer after presentation of clinical symptoms and frequently, after the cancer has spread beyond the primary site. Therefore, there is a great need for the development of safe and effective biological, pharmaceutical, nutraceutical and/or nutritional interventions that lack dose-limiting toxicities and are applicable to the treatment of early stage cancer.

The target molecule detected by the ONCOblot test is the ENOX2 cancer marker. Expression of the ENOX2 protein is restricted to cancer cells and is absent from normal cells and tissues. ENOX2 is currently the only protein marker thus far documented to be consistently produced by all of the most common forms of human cancer. ENOX2 is a cell surface protein that localizes to the outer plasma membrane of cancer cells. However, partially due to the fact that ENOX2 lacks a full transmembrane domain, ENOX2 is shed from cancer cells into the circulation. Interestingly, tissue-specific isoforms of ENOX2 are produced by cancers of different tissues of origin. Consequently, the combination of the number of unique ENOX2 isoforms, and molecular weight and isoelectric point of each ENOX2 isoform present within blood serum are indicative of the cancer tissue of origin. Therefore, the ENOX2 serum marker has utility for identification of the tissue of cancer origin, as demonstrated by the analysis of sera samples from clinically-confirmed cancer patients (1).

When associated with the outer leaflet of a cell plasma membrane, enzymes of the ENOX protein family perform functions that are critical to the growth phase of cell proliferation. For cancer cells, the constitutively active ENOX2 enzyme functionally replaces ENOX1, which is present on all cells and is highly regulated by both growth factors and hormones. The unregulated activity of ENOX2 strongly contributes to the characteristic unregulated growth and invasive phenotype that is common to most, if not all, forms of human cancer. The universal response of cancer cells in tissue culture when the enzymatic activities of ENOX2 are blocked is to undergo programmed cell death (apoptosis). As such, the ENOX2 proteins may serve as ideal molecular targets for early cancer intervention.

There is considerable evidence to support the above concept. For example, when ENOX2

was exogenously produced within MCF-10A (breast) or CHO (kidney) non-cancer cell lines, these cells gained an invasive phenotype (2). In contrast, when ENOX2 expression was silenced within a HeLa (cervical) cancer cell line, the invasive potential of these cancer cells was significantly reduced. These results suggest that ENOX2 expression is both necessary and sufficient for unregulated growth and invasive ability of immortalized cell lines.

In order to proliferate, cells must enlarge. If cells do not reach a critical size, they are unable to pass a checkpoint in G_1 that monitors cell size. Unregulated cancer cells, unlike most normal cells, undergo programmed cell death (apoptosis) if unable to grow and divide within 48 to 72 h (3, 4). Consistently, inhibitors of ENOX2 have been shown to selectively induce apoptosis in cancer cells through blocking the growth-related activities of this cell surface enzyme. Two such ENOX2 inhibitors are Epigallocatechin gallate (EGCg) found in green tea and capsaicin, a pungent molecule produced by chili peppers. EGCg has been shown to inhibit the proliferation of both BT-20 (breast cancer) and HeLa (cervical cancer) cells in culture, and the enzymatic activity of ENOX2 with an IC_{50} in the nanomolar range (4). Likewise, capsaicin inhibits the proliferation of BT-20 (breast cancer) cells, and inhibits ENOX2 activity with an IC_{50} in the nanomolar range as well (5). In contrast, the growth of the non-cancer MCF-10A (breast) cell line was not inhibited by either of these small molecules. Thus, ENOX2 may provide an effective pan-cancer intervention target that can be inhibited by compounds lacking any significant dose-limiting toxicities.

Importantly, not all very early cancers may develop into a life threatening disease. Therefore, aggressive treatment of early cancers remains controversial. While increasing the frequency at which cancers are detected early is predicted to lead to an increased rate of curative treatment with respect to the detection of the same disease at a later stage of disease progression, some very early cancers may not require treatment at all. Presently there is no way of knowing which early neoplasms will progress to life threatening cancers and which will not. With caution as a guiding principal, ENOX2 offers opportunities to develop new and effective intervention strategies designed to eliminate early malignancies. The overall concept is that of Curative Prevention®, where early detection and early intervention would be combined to prevent both invasive spread of the cancer as well as further development of the cancer into a life threatening condition.

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ONCOblot Reports

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Detection of Mesothelioma-Specific ENOX2 Isoforms 4-10 years in Advance of Clinical Symptoms

David Taggart, PhD

Malignant mesothelioma is the most common cancer induced by exposure to asbestos. Recently, a retrospective analysis of banked serum samples collected from individuals who developed asbestos-induced mesothelioma was undertaken to determine if ENOX2 isoforms may serve as biomarkers for this disease and to investigate how long in advance of clinical symptoms ENOX2 proteins could be detected in patient sera. Interestingly, two mesothelioma-specific ENOX2 isoforms were detected in serum samples of asbestos-exposed individuals, 4-10 years in advance of a clinical diagnosis of mesothelioma (1). This is the very first published evidence that the ONCOblot test is able to detect ENOX2 produced by a malignancy in advance of clinical symptoms. This work was a collaboration between MorNuCo Inc. and Drs. Jenette Creaney, A. W. Musk and Bruce Robinson of the National Center for Asbestos Related Diseases and the School of Medicine and Pharmacology at the University of Western Australia.

Detection of Mesothelioma-Specific ENOX2 Isoforms in Patient Sera

Banked serum samples from 17 individuals who were diagnosed with malignant mesothelioma were investigated for the presence of ENOX2 isoforms. Each of these serum samples contained two ENOX2 proteins with average molecular weights of 64 kDa and 41 kDa and average isoelectric points of 3.9 and 4.3, respectively. The simultaneous detection of these two ENOX2 isoforms was mesothelioma-specific and has not been observed within serum samples derived from patients diagnosed with cancers other than mesothelioma. For seven of these individuals, annual serum samples were available for 8-13 years prior to a diagnosis of mesothelioma. For these samples, both mesothelioma-specific ENOX2 isoforms could be detected 4-10 (average 6.2) years prior to clinical diagnosis of mesothelioma. Serum samples of 15 asbestos-exposed individuals currently diagnosed with benign disease (pleural plaques with or without accompanying asbestosis) were also analyzed. Of the serum samples from these subjects, 9 (60%) lacked detectable ENOX2, 5 (33%) contained only one mesothelioma-specific ENOX2 isoform and only 1 (7%) contained both mesothelioma-specific ENOX2 isoforms. It is possible that the presence of ENOX2 within the serum of these subjects diagnosed with benign disease is an early indicator of the development of mesothelioma. However, additional follow-up will be required to test this hypothesis.

Asbestos-Induced Malignant Mesothelioma

Asbestos microfibers are easily aerosolized. Once inhaled, these carcinogenic fibers cling to the respiratory tract and eventually become embedded in soft tissues. The primary respiratory diseases associated with asbestos exposure are lung cancer, mesothelioma, formation of pleural plaques, and asbestosis, a benign, chronic respiratory disease. Importantly, patients diagnosed with asbestosis are at a higher risk of both lung cancer and mesothelioma.

Malignant mesothelioma is an aggressive and almost uniformly fatal cancer. It is a tumor of the mesothelium, predominantly of the pleura, and it is often widespread at the time of presentation. Patients who are treated with supportive care have a median survival time of 9 months. The latency period for asbestos-induced mesothelioma (the time between asbestos exposure and diagnosis) is 10 to 50 years with an average of approximately 35 years. Thus, asbestos-induced mesothelioma develops relatively slowly and often presents clinically only in late stages.

Patient Population Investigated

Crocidolite (blue asbestos) was mined and milled in the town of Wittenoom in Western Australia from 1943 to 1966, primarily by a single company, the Australian Blue Asbestos Company. Beginning in the late 1970's, a cohort of more than 6,000 men and 400 women who were employed in this asbestos mining and milling operation have been followed longitudinally to investigate the prevalence of asbestos-related morbidity and mortality (2, 3). Although asbestos exposure for this population was often brief, with 74% of the workforce employed for less than 1 year and only 5% for 5 years or longer, asbestos exposure was estimated to be high, particularly for mill workers. A subset of these individuals have elected to participate in an ongoing cancer surveillance and prevention program (4, 5). Serum samples from some of these individuals, collected on a yearly basis for over a decade, were utilized in our study.

Summary

Two mesothelioma-specific ENOX2 isoforms were detected in the serum of asbestos-exposed subject 4-10 years in advance of clinical diagnosis. Importantly, asbestos-induced mesothelioma is characterized by a long latency period and often does not induce significant symptoms until later stages, delaying diagnosis. If validated in larger clinical trials, the ENOX2 cancer marker may aid early detection with the goal of reducing mesothelioma-related mortality.

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Estimation of the Accuracy of the ONCOblot® Tissue of Origin Cancer Test

D. James Morré, PhD and David S. Gilmartin

MorNuCo, Inc. is pleased to announce the electronic publication of a new series of monthly reports for participating physicians and health professionals dealing with developing areas and extant questions relating to the ONCOblot® Tissue of Origin (Cancer) Test. In this inaugural issue, the question of the accuracy of the ONCOblot® test is addressed. Future issues will address possible subtypes within major tissues of origin, tissues of origin in relation to distant metastases and clinical evidence from longitudinal studies as well as comments from physicians.

The ONCOblot® Tissue of Origin Cancer Test is an *in vitro* diagnostic intended to measure the degree of similarity between expression of ENOX2, a cancer-specific protein marker shed into human sera, and the ENOX2 protein expression patterns in a data base of serum samples from subjects with cancers clinically diagnosed according to then-current clinical and pathological practice.

The original data base (01/01/2013) contained 804 entries. Data were from sera of clinically diagnosed, primarily late stage, cancer patients from sources including the following: Greater Baltimore (Maryland) Cancer Center, Goshen (Indiana) Cancer Center, Early Detection Research Network of the National Cancer Institute, Novagen (North Ryde, Australia), Illinois Leukemia Society, Indiana University (Indianapolis), Hanau Medical Group (West Lafayette, Indiana) and Horizon Cancer Center (Lafayette, Indiana). Of these serum samples from patients reported to have clinically diagnosed cancer, two failed to yield ENOX2 transcript variants indicative of cancer. Both were reported as breast cancer (2 out of 291 breast cancer entries analyzed or 0.7%). The current data base (4/29/2015) consists of 1587 entries with no additional false negatives.

Definition of a False Positive

A false positive is the detection of an in range ENOX2 transcript variant from a cancer-free individual that fails to appear upon subsequent analyses of the same serum sample. For the most recent 1500 ONCOblots® carried out according to current protocols, only one ONCOblot® result has been confirmed as a false positive (0.06%).

Definition of a False Negative

A false negative is the absence of an in range ENOX2 transcript variant from an individual with clinical symptoms arising from a clinically diagnosed (pathology) cancer, present at the time of testing and at an ENOX2 concentration above the limit of detection of the assay. For the most recent 1500 ONCOblots® carried out according to current protocols, only ten ONCOblot® results have been confirmed or suspected false negatives (0.6%).

Limit of Detection

As with any diagnostic test, there is a lower limit of detection currently set at 200 femtomoles of ENOX2. This amount of ENOX2 is estimated to be produced by about 2 million cancer cells in the body, which is equivalent to a solid tumor 0.8 to 1.2 mm in diameter.

Misidentification of the Tissue of Origin

For several cancers, two or more ENOX2 transcript variants must be present within the ONCOblot® Tissue of Origin Cancer Test to permit the correct identification of the tissue of origin. These include bladder, colorectal, gastric, mesothelioma, ovarian, renal cell and uterine cancer. If one or more ENOX2 transcript variants are absent or below the limit of detection, the tissue of origin of the cancer may be misidentified. For the 1,587 late stage cancers currently in the data base, 53 (3.3%) would have been misidentified on the basis of a missing transcript variant. Missing ENOX2 proteins may be more prevalent with early stage cancers.

There were 32 examples of suspected or documented indications of an incorrect diagnosis out of the most recent 1500 ONCOblots® analyzed under our current protocol for a misidentification rate of 2.8%, very close to that of 3.3% predicted from the estimate based on the data base entries above.

Summary

In this ongoing analysis of the accuracy of the ONCOblot® Tissue of Origin Cancer Test, representing the most recent 1500 tests completed under the current protocol, the incidence of confirmed false positives and confirmed false negatives is low, less than 1% each. The major source of error, approximately 3%, is misidentification of the tissue of origin. The latter is most prevalent with colorectal, ovarian, renal cell and uterine cancers where two or more ENOX2 transcript variants are required for identification of the tissue of cancer origin. Thus, the overall sensitivity of the test with clinically diagnosed cancers may be greater than 95%, but more testing is needed to accurately determine the rate, especially at or near the limit of detection of the assay.

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Primary Tumors and Distant Metastases Produce Identical ENOX2 Protein Transcript Variants that Aid in the Identification of Cancers of Unknown Primary

David J. Taggart, PhD

MorNuCo, Inc. is pleased to continue the electronic publication of a new series of monthly reports for participating physicians and health professionals dealing with developing areas and extant questions relating to the ONCOblot® Tissue of Origin Cancer Test. In this issue, the ability of the ONCOblot® Test to identify the primary tissue of cancer origin with respect to distant metastases and cancers of unknown primary (CUP) is addressed.

The ONCOblot Test Aids Identification of Cancers of Unknown Primary (CUP)

Defining the tissue of origin of a cancer of unknown primary (CUP) is one of the more challenging tasks faced by oncologists (1, 2), especially when pathological examination of the tissue of a poorly differentiated metastatic tumor does not yield a definitive result. In these cases, the ONCOblot® Tissue of Origin Cancer Test may offer significant assistance in the identification of the tissue of origin of a primary cancer through the detection of tissue-specific ENOX2 proteins in patient serum. Based upon the analysis of sera samples from over 800 clinically-confirmed, primarily late stage cancer patients, the ONCOblot test is capable of detecting tissue-specific ENOX2 protein transcript variants that are sufficiently distinct to allow for the identification of 24 separate tissues of primary cancer origin.

Primary and Metastatic Tumors Produce Identical ENOX2 Protein Markers

Metastases occur when malignant cells are shed from a primary tumor and travel through either the blood or the lymph system to distant sites in the body. These malignant cells then possess the potential to establish new tumors, referred to as metastatic tumors. Although metastatic tumors develop at sites distant from the primary tumor, these metastatic tumors retain the phenotype and cell surface markers of the primary tumor (3), as the metastatic tumor cells are initially genetically identical to the cells of the primary tumor (4).

Consistently, both metastatic tumors and the primary cancers from which they are derived produce identical ENOX2 protein transcript variants, which are then shed into blood serum. Therefore, detection of ENOX2 protein transcript variants shed by either a primary cancer or its distant metastases will

yield similar ONCOblot® test results. For example, if a primary breast cancer has metastasized to the lung, only ENOX2 transcript variants indicative of breast cancer will be produced by both the primary and the distant metastasis, which share a common tissue of origin. This observation is supported by the finding that serum from subjects that were clinically diagnosed with either a single primary tumor or a primary tumor with distant metastases only contained ENOX2 transcript variants indicative of the tissue of primary cancer origin (5). Importantly, if two or more primary cancers are simultaneously present within a subject, ENOX2 transcript variants indicative of each tissue of primary cancer origin will be expressed and shed into the blood serum.

As with other serum cancer marker tests, the ONCOblot® test does not reveal if a primary cancer has metastasized or indicate where in the body a metastatic tumor may be located. Instead, the primary role of the ONCOblot® test is to identify the primary tissue of origin of any cancers that are present through the detection of tissue-specific ENOX2 protein transcript variants shed into blood serum.

Summary

Metastatic tumors located at distant sites are derived from cells that were shed from the primary cancer and therefore, produce identical ENOX2 protein transcript variants as the primary cancer. Since the ONCOblot test differentiates among 24 different tissues of origin, the test has the potential to aid in the identification of cancers where the tissue of origin is not known.

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Blood Cell Cancers are Detected but not Identified as to Type or Subtype by the ONCOblot® Tissue of Origin Cancer Test

Katherine C. Parra, PhD.

MorNuCo, Inc. continues its monthly report for participating physicians and health professionals in order to answer common questions relating to the ONCOblot® Tissue of Origin (Cancer) Test. In the current issue, the detection of blood cell cancers by the ONCOblot® Tissue of Origin (Cancer) Test is discussed. Blood cells share a common progenitor, hematopoietic stem cells. Thus, different types of blood cancers produce similar ENOX2 transcript variants, which have yet to be differentiated.

ENOX2 proteins are associated with the cancer cell surface, where they contribute to the uncontrolled growth of cancer cells. Cancers of different tissues of origin produce specific ENOX2 protein transcript variants, which are detected and differentiated by the ONCOblot® Tissue of Origin (Cancer) Test.

Blood is a highly specialized fluid form of connective tissue (1). Cellular elements of the blood are derived from a common cell, the multipotential hematopoietic stem cell (hemocytoblast) located in the bone marrow. Thus red cells, platelets and white cells are produced through the process of hematopoiesis within this single tissue. Blood cancer involves combined defects in cellular maturation and differentiation, mostly of white cells, preventing normal formation or function.

Blood cancers are classified into three main types according to the function of the cells affected; leukemia, myeloma and lymphoma. Within each blood cancer type there are several subtypes that are classified based on the actual onset of the disease.

Leukemia results from the malignant transformation of an early hematopoietic stem cell, together with the expansion and accumulation of malignant white cells in the bone marrow which interferes with the production of red cells and platelets in blood (2). Lymphoma cells are malignant lymphocytes, another type of white cell which may arise from T cells, B cells or from different stages of lymphocyte development (2). Myeloma cells are malignant plasma cells (B-cells), a type of white cell that is responsible for the production and secretion of monoclonal immunoglobulin or M protein (3).

Confirmed cases of different types and subtypes of blood cancers in our database were assayed by the ONCOblot® Tissue of Origin (Cancer) Test and are shown in Table 1. The molecular weights and isoelectric

points of the ENOX2 proteins detected, while not identical, fall into a single category or common range of values for blood cell cancers (Figure 1).

Table 1 Blood cancers detected by the ONCOblot® Tissue of Origin (Cancer) Test.

Type	Subtype
Lymphomas	Hodgkin and non-Hodgkin: Follicular, diffuse large cell and cutaneous.
Leukemias	Chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), acute myelogenous leukemia (AML) and B-cell (ALL).
Myelomas	Myeloma/Multiple Myeloma

For leukemias and lymphomas, the molecular weights of the detected ENOX2 proteins were primarily distributed over a very narrow range of 43 to 45 kDa, which corresponds to the margin of error in their determination by the ONCOblot® Tissue of Origin (Cancer) Test. Isoelectric points varied more widely, but with the majority falling between pH 3.7 and 3.9. Corresponding values for myelomas fell outside (both higher and lower) these ranges with non-overlapping values for myeloma and multiple myeloma.

Summary

The ONCOblot® test detects ENOX2 protein transcript variants produced by cancerous cells, including when the tissue of cancer origin is blood. Different blood cancers exhibit a common range of molecular mass (38-48 kDa) and isoelectric point (pH 3.6-4.5).

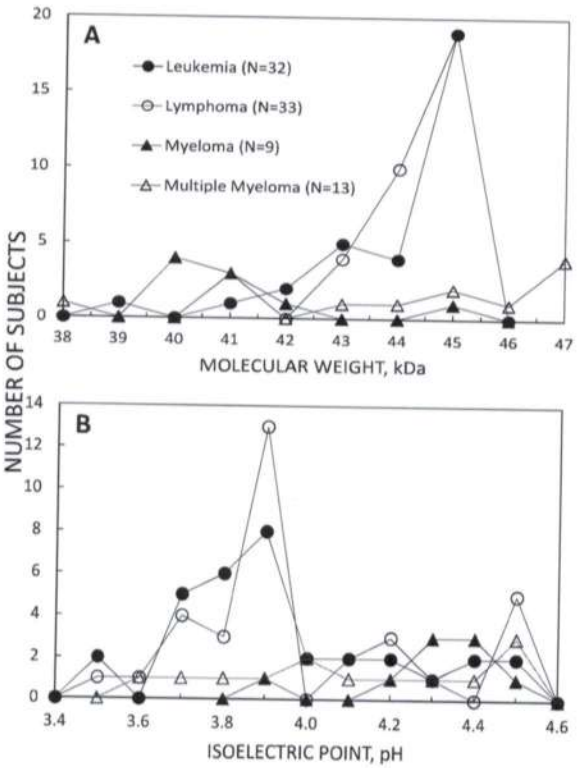

Figure 1 Distribution of ENOX2 molecular weight (A) and isoelectric point (B) determined for blood cancers.

ENOX2 protein transcript variants produced by different types and subtypes of blood cancers may not be identical, but cannot be consistently differentiated due to the overlapping molecular weights and isoelectric points of the ENOX2 proteins produced by each type of blood cancer.

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ONCOblot Reports

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ONCOblot Consistently Detects Stage 0 and Stage I Cancers and Correctly Identifies the Tissue of Origin

D. James Morré, PhD and David J. Taggart, PhD

MorNuCo, Inc. continues its monthly report for participating physicians and health professionals in order to answer common questions relating to the ONCOblot® Tissue of Origin (Cancer) Test.

ONCOblot® differs from early detection strategies based on circulating tumor cells (CTC Tests) that require the cancer to have progressed to the extent that cancer cells are in the blood. ONCOblot® detection is based on the presence of cancer-specific cell surface ENOX2 proteins shed from cancer cells. All that is required is that the cancer has a blood supply.

Stage 0/Stage I Defined

Stage 0 cancers are characterized by abnormal cells that are exclusively confined to the tissue of origin (1).

Similarly for stage I, the cancer has not yet spread outside the tissue of origin or to lymph nodes. Stage I solid tumors are usually less than 2 cm in diameter (1).

Sometimes referred to as carcinoma *in situ*, stage 0 and stage I cancers are usually amendable to surgical intervention.

Methods

Sera from stage 0 patients were purchased from Asterand Bioscience, Detroit, MI.

Sera from stage I patients were collected by MorNuCo, Inc. Stage and tissue of origin were confirmed by biopsy for all participants using IRB approved protocols.

Results

Sera from twenty-five stage 0 cancer patients and twenty-five stage I cancer patients confirmed by biopsy (Table 1) were analyzed by ONCOblot®. For all 25 patients in each category, early cancers were detected by ONCOblot® and correctly identified as to tissue of origin.

With a sample size of 25, values for each of the cancers fell within the pre-determined ranges of values for the characteristic ENOX2 transcript variant(s). Thus, the positive percent agreement observed was 100% (90% confidence interval, 88.7-100%).

Table 1. The tissue of origin of stage 0 and stage I cancers analyzed

Stage 0 Cancers	n	Stage I Cancers	n
Bladder	2	Bladder	1
Blood Cell	3	Blood Cell	2
Breast	6	Breast	16
Cervix	3	Colorectal	5
Colorectal	3	Lung	1
Hepatocellular (Ampullary)	1		
Lung	1		
Melanoma	1		
Renal Cell	2		
Squamous Cell (Vulvar)	2		
Uterine	1		

Discussion

ONCOblot® detects solid and blood cell cancers at both stage 0 and stage I (and beyond) making it possible to determine the tissue of origin of a cancer even if the patient is not showing physical symptoms associated with cancer.

By detecting stage 0 and stage I cancer, in most cases, treatment can be initiated before metastases occur.

DCIS. A Common Example of Stage 0

With breast cancer, ductal carcinoma *in situ* (DCIS), is stage 0 and has not spread outside a breast duct or into the surrounding breast tissue. Not all DCIS progress to become metastatic disease (2). Some never leave the duct. Never-the-less, most specialists recommend that all DCIS be treated with surgery often followed by radiation and hormone therapy (3).

Without mammography, DCIS would be a rare diagnosis. Even if it does not present as a lump, DCIS is frequently detected by the presence of microcalcifications, clusters of white specs of calcium, on the mammogram (4). Currently there appears to be no cancer tests, including ONCOblot®, that can predict with certainty, the risk of development of an invasive cancer from DCIS although this remains an area of active investigation (3).

Summary

The ONCOblot® Tissue of Origin Cancer Test reliably determines the presence and tissue of origin of 26 different cancers based on circulating ENOX2 transcript variants of differing molecular weights and isoelectric points in serum. The test correctly identified cancers as to tissue of origin with both stage 0 and stage I cancers. Often referred to as cancer *in situ*, with stage 0 and stage I cancers, disease has not spread beyond the tissue of origin. Thus, the ONCOblot® Cancer Test may signal cancer earlier than CTC (Circulating Tumor Cell) tests that detect only cancer cells present in the blood.

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Estimation of Lower Limit of Detection of ENOX2 in Serum

David J. Taggart, PhD and D. James Morré, PhD

MorNuCo, Inc. continues its monthly report for participating physicians and health professionals in order to answer common questions relating to the ONCOblot® Tissue of Origin (Cancer) Test.

Based upon detection of purified, recombinant protein, the lower limit of detection of ENOX2 for the ONCOblot® test is <200 femtomoles per assay or a concentration of 1.3 nM of an ENOX2 protein within human serum. This steady-state concentration of ENOX2 within an average adult is estimated to be produced by approximately 2 million cancer cells, which is equivalent to a solid tumor 1.2 mm in diameter. By comparison, while estimates vary, 7 mm diameter breast cancers were detected approximately 50% of the time by mammography whereas tumors larger than 32 mm were detected 100% of the time (1). For comparison, tumors that are 7 mm and 32 mm in diameter are calculated to contain 350 million and 33 billion cells, respectively.

Determination of the lower limit of ENOX2 protein detection

When proteins are separated by two-dimensional (2-D) gel electrophoresis and detected by immunoblot, visualized proteins appear as small circles or ovals termed 'spots'. The average diameter of the spot produced by an ENOX2 protein is proportional to the amount of ENOX2 protein present. To determine the limit of ENOX2 detection by ONCOblot, a standard curve of spot diameter was generated. To this end, a functional, 46 kDa form of human ENOX2 was first produced in *E. coli* and purified to near homogeneity. The complete amino acid sequence of a full-length 72 kDa form of ENOX2 is available from GenBank under Accession No. AF207881. Various amounts of this recombinant ENOX2 protein were then assayed by ONCOblot®. The log of the resulting ENOX2 spot diameter was then plotted against the log of the amount of ENOX2 protein assayed (Fig. 1) and a strong linear correlation (r^2 = 0.95) was found among these values.

The practical lower limit of detection of an ENOX2 protein assayed by ONCOblot® is a spot on the order of 0.25 mm, which by comparison to Figure 1, correlates to the detection of 170 femtomoles (1.0×10^{11} molecules) of ENOX2 per assay. This value is in agreement with the previously reported lower limit of ENOX2 protein detection by ONCOblot® of approximately 100 femtomoles of ENOX2 per assay (2). By comparison, the largest ENOX2 spots produced by the analysis of human sera from late-stage cancer patients are approximately 3 mm in diameter, equivalent to 3.0×10^{13} molecules per assay or 300 times more ENOX2 protein than the lower limit of detection.

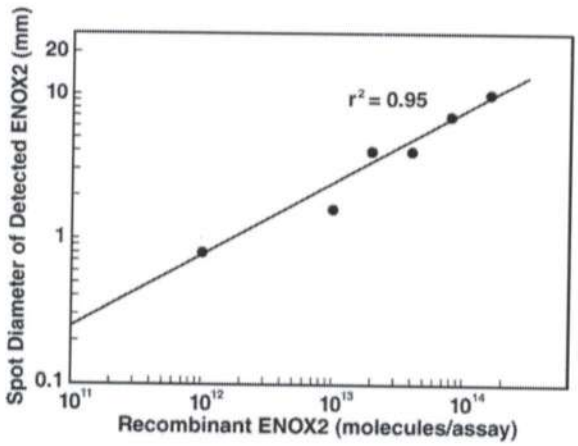

Figure 1. Linear log-log relationship between detected ENOX2 spot diameter and total amount of ENOX2 protein assayed by ONCOblot®.

Estimation of the size of a solid tumor at the lower limit of detection

Previously, ENOX2 proteins within sera from 25 Stage 0 and 25 Stage I cancer patients were detected by ONCOblot® (3). The average concentration of ENOX2 within these sera samples was determined to be approximately 990 femtomoles (6.0 X 10^{11} molecules) per 150 µL assay, by comparison to the standard curve (Fig. 1). Although ENOX2 concentration within blood serum is not a predictive measure of tumor size, this finding can be used to estimate the smallest tumor that can be detected by the ONCOblot test by assuming that ENOX2 production is proportional to tumor size during early stages of disease and tumors possess a uniform blood supply. By definition, Stage 0 and Stage I solid tumors are typically less than 20 mm in diameter (4). If each of these Stage 0 and Stage I tumors were the maximum size of 20 mm in diameter, the lower limit of detection of 170 femtomoles of ENOX2 per assay would then be predicted to be produced by a 3.3 mm cancer. However, if the average Stage 0 or State I tumor was a more common 5 mm to 10 mm in diameter, the lower limit of detection of ENOX2 would be predicted to be produced by a 0.8 mm to 1.6 mm diameter tumor (1.2 mm average). These findings are consistent with a previous estimate of the minimum solid tumor size detected by ONCOblot® of 0.8 mm in diameter (2).

If tumor cells are treated as spheres, then the number of cells in a solid tumor can be calculated by using Equation 1, where V_{tumor} is the volume of the tumor, V_{cell} is the average volume of a cancer cell, D is the tumor diameter and d is the average diameter of a tumor cell. Although the average size of mammalian cells varies according to cell type, if the average diameter of a cancer cell is estimated to be 10 µm, then a 1.2 mm diameter cancer is calculated to contain approximately 2 million (2 X 10^6) cells.

$$\#\ tumor\ cells = \frac{V_{tumor}}{V_{cell}} = \frac{\frac{4}{3}\pi\left(\frac{D}{2}\right)^3}{\frac{4}{3}\pi\left(\frac{d}{2}\right)^3} = \frac{\left(\frac{D}{2}\right)^3}{\left(\frac{d}{2}\right)^3} = \left(\frac{\frac{D}{2}}{\frac{d}{2}}\right)^3 = \left(\frac{D}{d}\right)^3 \quad \text{Eq. 1}$$

Summary

Based on the lower limits of detection of recombinant ENOX2, the lower limit of detection of the ONCOblot® test is approximately 170 femtomoles of ENOX2 protein (1.0 X 10^{11} molecules) in 150 µL of serum. This concentration of ENOX2 is predicted to be produced by solid tumors approximately 1.2 mm in diameter.

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