Slide-based multi-target immunohistochemical and in situ hybridization solid tumor testing utilizing multiple colors with image analysis for superior prognostic/diagnostic profiling

Overall aim/significance

The overall goal of this project is to develop, test and apply a novel approach to the diagnosis of solid tumors that will improve patient care via increased efficiency and decreased cost. STEM majors (sophomores, juniors, and seniors) will first be trained in the necessary scientific background and techniques at Wesley College (Wesley). After receiving pertinent training in chemistry, microbiology, and immunology, they can conduct (paid) internships at Green Clinics Laboratory (GCL), Dover, where they will test and apply a novel approach to the diagnosis of solid tumors.

Collaborating institutions

Wesley is a minority-serving undergraduate institution (MSI) with a total population of 1473 students of which, 39% are Caucasian. In addition, >40% of the Wesley undergraduate population are first in their families to go to college, and ~95% are Pell-eligible.

The STEM faculty adopted a unified undergraduate program of evidence-based high-impact mentoring strategies [1,2]. Since 2003, authentic faculty-mentored undergraduate research projects in a nationally recognized STEM-Directed Research program are coupled with co-curricular endeavors that require literature searches, peer-collaborations, leadership and internship experiences, and career planning [1-5]. All Wesley STEM majors are expected to complete a senior-thesis capstone project [1,2,5].

Dr. Kevin E. Shuman joined the College (fall 2016) as the Assistant Director of STEM Initiatives (staff position) and 50% of his time, is spent mentoring students in microbiology research projects, senior-capstone projects, and in an NSF-supported Cannon Scholars program. Dr. Shuman will serve as PI and as a staff member, he will not receive any ARC-sponsored salary support. Since fall, Dr. Kelly Ann Miller, is on a tenure-track Assistant Professor in Microbiology position. She has agreed to serve as co-investigator on this project. Dr. Miller will mentor the undergraduates in the lab-components of her microbiology and immunology courses and within the mentored directed research and summer internship programs. Dr. Miller has requested a 1.76-month (calendar year) salary for her efforts.

Green Clinics Laboratory (GCL) is located a mile south of the College. It is a dedicated research facility with immunohistochemistry, in situ hybridization, real-time PCR analysis, frozen section computer assisted image analysis, and special stains analytic capabilities. A co-owner of GCL is Ghada Alabed, a 2010 Delaware-INBRE supported biology graduate of Wesley College. The second co-owner is Dr. Fady Gerges, a renowned Dover pathologist. GCL agrees to mentor the Wesley student-interns, provide necessary lab-space and the use of their equipment facilities, and GCL will contribute an in-kind support equivalent of $75K (a signed agreement is included in this proposal).

Prior (Wesley) collaborative work with the two GCL co-owners has resulted in several publications [6-9] and the GAO (Government Accountability Office), used the resultant research outcomes to criticize the US Food and Drug Administration [10].

Training at Wesley tied to coursework and the directed research program

Wesley College biology, biological chemistry, or medical technology majors who have successfully completed microbiology and organic chemistry will be specifically selected to participate in the

research collaboration with the Green Clinics Laboratory. In chemistry, students are exposed to advanced computational chemistry software, extraction, crystallization, chromatography, spectroscopy, and a variety of other relevant qualitative and quantitative methods. In microbiology, students learn aseptic techniques, basic microscopy, and differential staining, all of which are necessary for learning to prepare and analyze tissue samples. In addition, in the microbiology course through differential staining and microscopy, students have experience using two or more dyes, can tell the difference between cells and debris, as well as differentiate cell structures.

On progression to their junior and senior years, students then take genetics and immunology to expand upon knowledge acquired in microbiology and to learn clinical specific techniques that will be needed to conduct the clinical analysis of patient specimens and interpret the results. In genetics, students will learn the concepts underlying the methodology of fluorescent in situ hybridization (FISH). In immunology, students will develop an understanding of ELISA, immunofluorescence, and immunohistochemistry, as well as how to prepare and analyze samples for these procedures.

In this proposed research collaboration, in the fall semester, students chosen will be enrolled in Wesley's Directed Research program. Research projects will be developed to further train students in lab techniques that they were introduced to in microbiology and chemistry, as well as in techniques that they will be introduced to in immunology and genetics.

In Spring, students will be trained in searching for and interpreting the scientific literature to provide necessary background for their senior thesis research and/or their summer internship. This will provide them with the skills needed to find and understand scientific literature needed for their work at GCL. Rising seniors will be encouraged to develop a senior thesis project on a topic that would expand on the necessary background and lab techniques needed for use in a pathology laboratory.

Background

Diagnostic tools in solid tumors have been largely dependent on immunohistochemical analysis and in situ hybridization with a very limited applicability of flow cytometry analysis. The immunohistochemical and in situ hybridization targeting different antigens and DNA/RNA fragments, respectively, is predominantly utilized by using a specific antibody to one target on the tumor cells with single chromogen/color recognition. Image analysis has been used extensively in the past 15 years to help evaluate different patterns of staining for the target antigens and DNA/RNA fragments within solid tumor slide sections. The analysis has been primarily focused on the pattern of staining of a single color/chromogen utilizing specific algorithms that can translate into diagnostic or prognostic indicators. The stains are subsequently interpreted by comparing the original light microscopic hematoxylin and mucin stain slide's evaluation with the pattern of staining in the immunohistochemical/in situ hybridization study. This is followed by comparing the patterns of staining of different immunohistochemical studies across the panel of stains performed. Several imaging modalities have been utilized in the above-mentioned comparison. The double stain antibody allows the investigator to simultaneously see both kappa (M) (brown) and lambda (P) (red) on the same tissue section, thus allowing the end-user a more accurate and easier assessment of both stains (Figure 1). However, comparing more than 2 fields of view simultaneously targeting the same area to evaluate the pattern of staining of these antigens proves to be very challenging technically [11-13].

The ability to run simultaneous immunohistochemical or in situ hybridization studies on the same slide representing a certain solid tumor has several advantages both diagnostically and prognostically. The

ability to use a comparable image analysis software to target more than 2 chromogens/colors on the same slide present a huge advantage by faster more accurate evaluation while lowering the cost and increasing the testing range. Additionally, a significant reduction in cost and testing time is projected. Targeting multiple antigens, as well as RNA/DNA fragments, with different colors has been used; however, certain limitations exist. These include an inability to quantitatively evaluate more than 2 colors/chromogens on the same slide with the naked eye and the inherent problem of overlapping related to antigenic presence in different cellular compartments (nucleus, cytoplasm, cytoplasmic membrane, or nuclear membrane). This problem has been largely solved in suspension based samples by using flow cytometry; however, the limitation in solid tumors has largely been consistent [14-19].

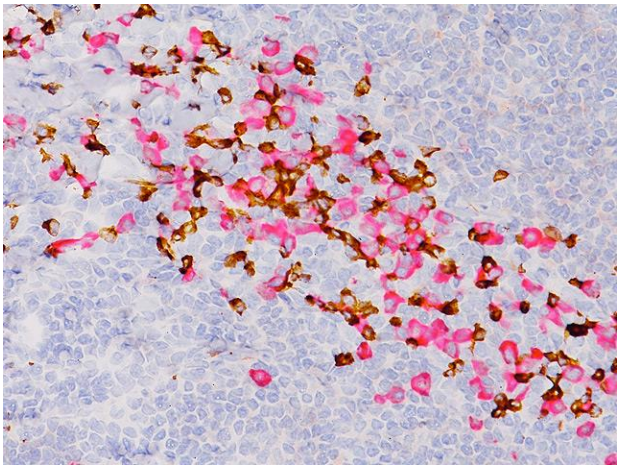

Figure 1. Multiplex Kappa (red) Lambda (Brown) Immuno-histochemical stain.

In the specific instances where a cell suspension can be obtained, a cell block is prepared from the cellular suspension and as such will lend itself easily for immunohistochemical and in situ hybridization studies. This is particularly useful in patients with genitourinary malignancies. These patients who suffer from prostatic, kidney, urethral, or bladder cancers will shed some of these cells in the urinary stream which can be easily harvested from a urine sample [20].

Implementation of techniques at Green Clinical Laboratory

GCL is an anatomic pathology based service that provides services to the state of Delaware and the surrounding states. GCL receives several kinds of solid tumor biopsies including gastrointestinal, lymphoid, genitourinary, and dermatology base tumors. A battery of immunohistochemical and in situ hybridization studies are utilized on some of these tumors especially for posing certain diagnostic or prognostic questions. These studies are vital for the patient treatment and are an essential component of tools used by the hematologist/oncologist for patient treatment.

Identification of the specific solid tumors included in this study will be completed through prospective and retrospective data mining within the GCL patient pool. The identification of the specific solid tumors in question will be done with no reference to patient's demographics to secure anonymity.

Paraffin blocks containing the solid tumor representative tissue will be pulled and assigned generic serial numbers correlated with the diagnosis in question. 25 blank slides will be prepared by the histotechnologist per patient. Samples with cell suspensions (example urine samples from patients with urogenital malignancies) will be subjected to cellblock preparation. 25 blank slides will also be prepared per cell-block.

Using open access immunohistochemical and in situ hybridization analyzers (FDA approved), several antigens, as well as DNA or RNA fragments, will be targeted on the same representative slide of specifically selected solid tumors. Between 3 and 5 antigens are targeted per test using different colors/chromogens. The resulting slides will be scanned by a high-resolution slide scanner (Figure 2). Specific representative fields of view will be selected by the examiner. These representative images will be uploaded into a specific image analyzing software. The software will be specifically written and formulated for the purpose of this project (software development costs are requested in the budget). The images will be analyzed depending on the color as well as shape differential of the cells in question. Upon completion of the image analysis a compiled profile of the solid tumor in question will be retrieved from the analyzer with both qualitative and quantitative measures. Qualitative measures will match or exceed the examiner ability to assess for a positive/negative or equivocal result by naked eye examination of the same slide in question. Quantitative analysis is evaluated as a novel new advantage of this testing modality which is not practically feasible by naked eye examination. The ability of the analyzer to quantitate the number of simultaneous positivity of targeted cells in the solid tumor across the spectrum of the panel of antigen tested will be added to the intensity and pattern of positivity of the cells. These results will be compared to the traditional modalities as related to their diagnostic and or prognostic utility.

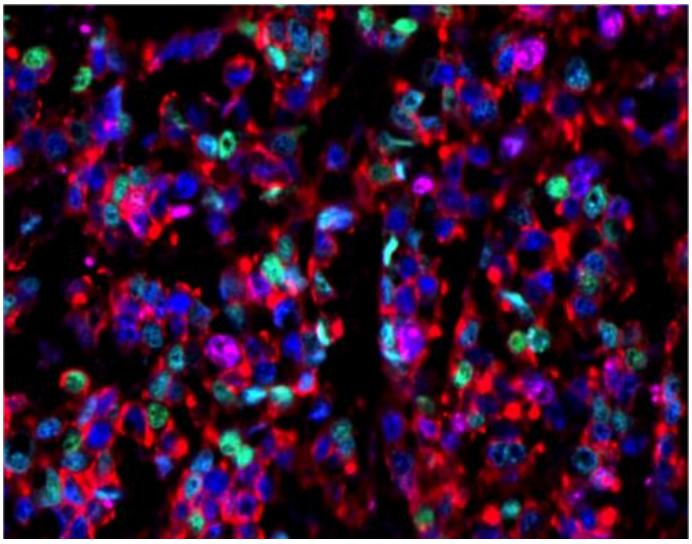

Figure 2. The image represents sensitive and specific fluorescent staining of HER2 (red), Estrogen Receptor (ER, blue), Progesterone Receptor (PR, green) using rabbit monoclonal antibodies and Ki-67 (magenta) using a mouse monoclonal antibody on a single triple-positive breast cancer tissue with detection by Cell IDx's hapten-based technology.

Intended result

This program will develop biomedical talent at an MSI, and the participating STEM majors will be well trained for future clinical positions. A potential advantage of our proposed processes can be realized along all aspects of improved patient care. By running our tests on solid tumors using a well-established platform (immunohistochemical and in situ hybridization analysis) we will be able to produce better diagnostic and prognostic indicator profiles which will translate into better cost-effective patient care. The improved turnaround time will also translate into faster and more reproducible testing. The resulting ability to correlate 3 to 5 different diagnostic/prognostic indicators simultaneously on the same cell within the same in situ setting with the qualitative and quantitative values has not been yet available for solid tumor analysis. Such an advantage will have significant implications in reducing cost while increasing yield in this specific segment of healthcare.

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