ADVANCEMENTS IN COMPUTATIONAL DIGITAL PATHOLOGY FOR EGFR AND PD-L1

by

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A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Bioinformatics and Computational Biology

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DEDICATION

This is dedicated to my loving wife Marian, my four wonderful children Adryn, Natalya, Katia and Sebastian.

ACKNOWLEDGEMENTS

I would like to thank the many friends, relatives, and supporters who have made this happen. My loving wife, Mirian, assisted me in my research. Dr. Aman Ullah, Dr. Donald Seto, and Myoung “Lucy” Nam who are the members of my committee providing invaluable help.

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LIST OF ABBREVIATIONS

Artificial Intelligence……AI
Epidermal growth Factor Receptor……..EGFR
Food and Drug administration .....FDA
Formalin Fixed Paraffin Embedded Tissue……FFPE
Image Analysis……..IA
Immunohistochemistry……IHC
Machine Learning……ML
Next Gen Sequencing……NGS
Non-small cell lung cancer……...NSCLC
Program Death Ligand-1.....PD-L1
Quantification cycle……CQ
Small Cell Lung Cancer……...SCLC
Turnaround Time .....TAT
The Cancer Genome Atlas…… TCGA
Tyrosine Kinase Inhibitors……..TKIs
Whole Slide Images……WSI

ABSTRACT

ADVANCEMENTS IN COMPUTATIONAL DIGITAL PATHOLOGY FOR EGFR AND PD-L1

Nicholas Hoo-Fatt, M.S.

George Mason University, 2022

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Annually Inova Health Care System treats more than two million individuals through their integrated network. With the increasing need for fast and accurate results for its cancer patients, the Inova Laboratories Healthcare System's reference laboratory, has acquired a Rapid Molecular Testing System, called BioCartis Idylla^{tm} which is a real-time PCR bases molecular testing system. This system is user friendly and will help improve the turnaround time for diagnosis and treatment of cancer patients. Most of the existing molecular testing for Next-Generation Sequencing (NGS) platforms such as whole genome sequencing, Exon sequencing and Disease diagnosis panel takes approximately fourteen days or longer to obtain results. This is due to the need for high volume batched samples, specimen procurement, transportation and complex interpretation.

While there have been great expansion of knowledge on molecular changes occurring in the cancer development, clinical utility of molecular tests for the diagnosis and especially for the treatment of cancers have been limited. The FDA has approved some

treatments targeted for specific molecular changes such as EGFR mutation and KRAS mutation in lung cancers and BRAF mutation in melanomas. Molecular testing aids the pathologist to check for certain changes in a gene or chromosome that may cause or affect the chance of developing a specific disease or disorder, in this case lung cancer. Another area of rapid progress is the advancement of immune targeted therapy aimed at PD-L1. Initially applied to lung cancers and some GI cancers, and has been approved by FDA- for multiple cancer types recently. To be able to apply these advanced therapeutics there is a growing need for more efficient targeted biomarker testing, including fast TAT (turnaround time), user friendliness, remarkable accuracy, specificity and sensitivity which are equivalent to the NGS test results

The objective of this project is to determine the use of rapid molecular tests for tumor mutations and the application of AI-based digital scoring methods of IHC (immunohistochemistry) test for PD-L1 for Keytruda® (Pembrolizumab).

CHAPTER ONE: INTRODUCTION

Lung cancer is the second most common cancer in both men and women. According to the American Cancer Society for the year 2022 there is an estimated 236,740 new cases of lung cancer (117,910 in men and 118,830 in women) for the United States. There is a projected to be approximately 130,180 lung cancer deaths as a result of these cases (68,820 in men and 61,360 in women). The average age of people diagnosed with lung cancer is about 70 years of age (15). There are a very small number of people diagnosed each year that are younger than 45 years of age. Lung cancer is the leading cause of cancer death each year among both men and women, this makes up about 25% of all cancer deaths. More people die of lung cancer each year than of colon, breast and prostate cancers combined.

There are two main types of lung cancer. First the Non-small cell lung cancer (NSCLC), which make up 80%-85% of all lung cancer cases. This type of lung cancer has three main sub-types depending on the different type of lung cells. The sub-types of NSCLC are adenocarcinoma, squamous cell carcinoma and large cell carcinoma. A second type of lung cancer is known as small cell lung cancer (SCLC), which makes up 10%-15% of all lung cancers. SCLC typically is more aggressive, growing and spreading faster than NSCLC (1).

In this project we will focus our attention on the NSCLC sub-type adenocarcinoma, which accounts for 80-85% of all lung cancers. This sub-type of NSCLC lung cancer is associated with the epidermal growth factor receptor (EGFR) gene (1). The EGFR is found on the cell membrane with one end of the protein inside the cell and the other end projecting from the outer surface of the cell; this configuration helps the function of the EGFR gene to deliver instructions for making the receptor protein(1). The receptors then bind to other proteins which are called ligands. These ligands are usually outside the cell and enable the cell to receive signals that help in response to its environmental change. Ligands and receptors fit together using the lock and key method. This binding of ligand to epidermal growth factor receptor allows the receptor to attach to another nearby epidermal growth factor receptor protein called Dimerize. Dimerize then activates the receptor complex. This activation causes signaling pathways within the cell to be triggered and promote cell growth and division (proliferation) and cell survival. The epidermal growth factor receptor (EGFR) has been found to bind to at least seven different ligands (2).

In this study we will observe the effects of PD-L1 expression on clinical outcomes in patients with EGFR mutant NSCLC lung cancer using digital scoring methods (Table 1). Genetic mutation in EGFR can occur by deletions in exon 19, arginine for leucine substitution at amino acid 858(L858R) in exon 21 and exon 20 insertions (16). These gene changes cause the receptor protein to be constitutively on which results in receiving signals to proliferate, even when it is not bound to a ligand. As a result tumor formation occurs. One of the proteins that EGFR has been found to be

upregulated is the programmed death-ligand 1(PD-L1) expression in lung cancer cells (1). PD-L1 contributes to the regulation of the body's immune response by stopping its occurrence from happening. This ability to stop the body's immune response plays a critical role in EGFR-driven lung tumors (1).

Digital scoring methods are derived from digital pathology, which involves a digitized glass slide to help, acquire, manage and interpret pathological information. This information aids in primary diagnosis, diagnostic consultation, intraoperative diagnosis, healthcare workers training, both manual and semi-quantitative review of Immunohistochemistry (IHC) and research. Digital pathology allows for immediate web based consultations with other expert pathologist and provides the security of archival pathology data. The use of digital scoring for PD-L1 as a diagnostic corroborating tool will enable a more accurate, reproducible and standardize scoring, as pathologist inter- and intra-variability can affect scoring.

With the adoption of whole slide images (WSI), there has been an increase in the use of the applications of artificial intelligence (AI) and machine learning (ML) to aid in understanding of the data from the WSIs to perform diagnostic tasks. AI is the capability of a computer or robots controlled by a computer to do task that are usually done by humans. Machine learning (ML) is a branch of AI that is the study of computer algorithms that are improved automatically by the use of data and through experience (6). ML can be supervised, unsupervised and semi supervised. These different approaches all utilize the data that it receives into the algorithms for a learning task, but they differ in how the input and output data are defined. In supervised learning, the algorithms received

highly specific, annotated data with specific classifiers which help to define both the input and output data. For unsupervised learning, the algorithms receive raw input data with no classifiers and looks for patterns to help define the output data. Semi-supervised learning is a middle ground between the previously defined two supervisory learning; it provides annotated data to the algorithm for training but lacks classifiers for the output data with the use of ML, computers now can capture and incorporate sub visual morphometric features into algorithms that may be more accurate. This type of image analysis (IA) algorithm can perform complex tasks such as classification of breast cancer stroma, identification of lymph node metastases and predication of non-small cell lung cancer (NSCLC)(6).

In this study we will focus on the AI-based IA algorithm for use in predication of non-small cell lung cancer (NSCLC). The role of using the IA algorithms for NSCLC to aid in PD-L1 scoring has shown considerable promise in recent studies. With the combination of the IA algorithm and immunohistochemistry, we are now able to have an in-depth quantitative analysis of the broader tumor microenvironment. With the use of these results, we can use the IA as a clinical tool to aid the pathologist in care for their patients in a more personalized way.

Table 1. Disease indications, testing requirements and approved diagnostic scoring algorithms for approved programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1) therapies. Udall M, Rizzo M, Kenny J, Doherty J, Dahm S, Robbins P, Faulkner E. PD-L1 diagnostic tests: a systematic literature review of scoring algorithms and test-validation metrics. Diagn Pathol. 2018 Feb 9;13(1):12. doi: 10.1186/s13000-018-0689-9. PMID: 29426340; PMCID: PMC5807740.

ICI	Current indications	Testing requirements	Scoring cut-off	Indications
Pembrolizumab	NSCLC HNSCC UC G/GEJ Adenocarcinoma ESCC Cervical cancer MSI-H[a]	PD-L1 IHC is required (companion)	≥1% TPS ≥1% CPS ≥10% CPS	1L for mNSCLC and unresectable stage III NSCLC 2L for mNSCLC 1L for unresectable HNSCC 2L for recurrent locally advanced or metastatic G/GEJ adenocarcinoma 2L for recurrent or metastatic cervical cancer 1L for locally advanced and metastatic UC 2L for recurrent locally advanced or metastatic ESCC
	Melanoma SCLC cHL PMBCL HCC	Testing is not required		

ICI	Current indications	Testing requirements	Scoring cut-off	Indications
	MCC RCC			
Nivolumab	NSCLC HNSCC UC MSI-H[a]	PD-L1 IHC is suggested (complementary)	≥1, 5 or 10% ≥1%	2L for non-squamous NSCLC 2L for HNSCC 2L for UC
	Cervical cancer HCC cHL RCC SCLC Melanoma	Testing is not required		
Durvalumab	NSCLC UC	PD-L1 IHC is suggested (complementary)	≥1% TCs ≥25% TCs or ≥25% ICs and ICP>1% or ICP = 1% and IC = 100%	2L for unresectable NSCLC that has not progressed on chemoradiation 2L for locally advanced or metastatic UC
Atezolizumab	UC TNBC	PD-L1 IHC is required (companion)	≥1% ICs ≥5% ICs	1L for TNBC 1L for UC
	NSCLC	PD-L1 IHC is suggested (complementary)	≥50% TCs or ≥10% ICs ≥1% TCs or ≥1% ICs	1L for mNSCLC 2L for mNSCLC
	SCLC	Testing is not required		

Table 2. Overview of selected programmed cell death ligand 1 (PD-L1) image analysis (IA) algorithms

Author	ML method	Tumor type	Scoring type	Sample dataset	Relevant data
Koelzer et al.	Random forest/supervised learning	Melanoma	%TC	69 samples of melanoma	Pearson correlation coefficient (*r* = 0.97, *P* <0.0001) between pathologist and IA
Kim et al.	Supervised learning	Gastric cancer	CPS	39 patients with clinical response to pembrolizumab	Correlation of PD-L1 positivity with patient (RFS) outcome [HR 0.536 (95% CI 0.316–0.94), *P* = 0.0294]
Humphries et al.	Supervised learning	TNBC	% positive PD-L1	90 samples with clinical outcome	Correlation of PD-L1 positivity with patient (RFS) outcome [HR 0.536 (95% CI 0.316–0.94), *P* = 0.0294]
Kapil et al	GAN/semi-supervised learning	NSCLC (biopsies)	TPSa	270 needle core biopsies; 60 slides used for concordance of manual to IA scores	IA scoring concordance with visual scores (OPA = 0.88, NPA = 0.88, PPA = 0.85; Lin's CCC = 0.94; Pearson CCC = 0.95)
Taylor et al.	Supervised learning with feedback loop	NSCLC	%TC, %IC	230 cases	Concordance (Lin's CCC) of IA with three pathologists (%TC = 0.81, 0.78, 0.68; %IC = 0.62, 0.53, 0.88)

CHAPTER TWO: METHODOLOGY

This chapter presents the methodology used for collecting the data for this study. First, we will use formalin fixed paraffin embedded (FFPE) tissue in this project to first determine the presence and quantity of the tumor tissue by examining the H&E stain face-cut slide. Second, we will use rapid PCR testing in determine the mutation if present on the formalin fixed paraffin embedded (FFPE) tissue. The determination of the presence or absence and percent positivity and the intensity of the PD-L1 will be done utilizing immunohistochemistry in the third step. We will then score PD-L1 using image analysis (IA) combined with an AI algorithm and compare with the manual scoring results. Patient's pathological lesion will be surgically removed and Formalin fixed paraffin embedded (FFPE) tissue, will be created in the Histology laboratory following multiple steps:

1. Fixation is the process of using chemicals to stabilize proteins and prevent them from undergoing further chemical changes.
2. Processing which dehydrates clears and infiltrates the tissue.
3. Embedding this places, the processed tissue into a special wax call paraffin to hold the tissue and its contents in place.
4. Microtomy is then performed on the embedded tissue. Cut unstained tissue is placed on slide.

Determination of EGFR using rapid molecular testing

The Biocartis Idylla; Jersey city, New Jersey, is a fully automated, real time rapid PCR system. This system was acquired by Inova Health System and the instrument and protocols have been validated using the College of American Pathologist (CAP) guidelines for patient testing. This validation of Accuracy/Precision has been done for BRAF, EGFR, KRAS, MSI and NRAS mutations (17). The Idylla technology is a cartridge based system that uses microfluidics processing with all the reagents readily available within the cartridge. This cartridge requires the user to place macro-dissected FFPE tissue into the chamber for nucleic extraction. This fully automated process homogenizes and lyses the cells by a combination of heat, high-frequency ultrasound, enzymatic and chemical digestion. Both extraction and purification is done by using a silica based compound. The PCR methods used by the Idylla system are based on a real-time fluorophore-based detection system.

The fluorophore-based detection system uses primers that are labeled with a single fluorophore on a base next to the 3' end with no quencher required. At the 5' end a tail of 5-7 nucleotides is added, this forms a "blunt-end" hairpin when the primer is not incorporated into a PCR product. These "blunt-end" hairpins of oligonucleotides are just as efficient as linear primers and provide additional specific to the PCR method by preventing primer-dimers and mispriming. This combination provides a low initial fluorescence of the primers that increases up to 8 fold upon detection of the specific mutation (12).

These steps are used to determine the presence or absence of the mutation of interest. The EGFR mutation results are shown here:

Table 3: Summary of data collected in the EGFR mutation assay study used for validation of rapid PCR found in different tissue types. In accordance with the CAP MOL.31130 and COM.40350 checklists, a minimum of 10 EGFR positive mutation and 10 negative or Wild Type previously tested patient samples and characterized controls will be tested for the Accuracy study.

EGFR non mutated					PDL-1 SCORE
Sample ID	**Expected results**	**Idylla™ Results**	**Specimen Adequacy**	**Other comments**	**(%)**
Patient 1	No mutation detected for EGFR	No Mutation detected for EGFR L858R EXON 19 DELETION, 790M,G719A/C/S, EXON 20,S768I,L861Q,EXON 21	0.8 x 0.5 cm	PANCREAS, TAIL MASS, ENDOSCOPIC ULTRASOUND-GUIDED FINE NEEDLE ASPIRATION	5
Patient 2	No mutation detected for EGFR	No Mutation detected for EGFR L858R EXON 19 DELETION,T790M,G719A/C/S, EXON 20,S768I,L861Q, EXON 21	0.4 x 0.2 x 0.1	BRAIN, RIGHT FRONTAL LOBE MASS:	70
Patient 3	No mutation detected for EGFR	No Mutation detected for EGFR L858R EXON 19DELETION,T790M,G719A/C/S, EXON 20,S768I,L861Q, EXON 21	3.5 x 2.0 x 1.0 cm	B. BRAIN, LEFT OCCIPITAL TUMOR, RESECTION: METASTATIC POORLY DIFFERENTIATED CARCINOMA	1
Patient 4	No mutation detected for EGFR	No Mutation detected for EGFR L858R EXON 19DELETION,T790M,G719A/C/S, EXON 20,S768I,L861Q, EXON 21	1.0 x 0.5 x 0.1 cm	RECTUM, MASS, BIOPSY	0
Patient 5	No	No Mutation detected for EGFR L858R EXON	3.0 x 1.0 x 1.0 cm	RIGHT NASAL POLYP, EXCISION	0

	mutation detected for EGFR	19DELETION,T790M,G719A/C/S, EXON 20,S768I,L861Q, EXON 21			
Patient 6	No mutation detected for EGFR	No Mutation detected for EGFR L858R EXON 19DELETION,T790M,G719A/C/S, EXON 20,S768I,L861Q, EXON 21	5.2 x 4.8 x 4.4 cm	LEFT KIDNEY, ROBOTIC ASSISTED RADICAL NEPHRECTOMY:	5
Patient 7	No mutation detected for EGFR	No Mutation detected for EGFR L858R EXON 19DELETION,T790M,G719A/C/S, EXON 20,S768I,L861Q, EXON 21	0.8 cm and 1.0 cm	PERITONEAL NODULE	N/A
Patient 8	No mutation detected for EGFR	No Mutation detected for EGFR L858R EXON 19DELETION,T790M,G719A/C/S, EXON 20,S768I,L861Q, EXON 21	5.0 x 3.0 cm	T2 VERTEBRAL BODY, CORPECTOMY:	N/A
Patient 9	No mutation detected for EGFR	No Mutation detected for EGFR L858R EXON 19DELETION,T790M,G719A/C/S, EXON 20,S768I,L861Q, EXON 21	0.5 x 0.3 cm	LYMPH NODE, STATION 11R, ENDOBRONCHIAL ULTRASOUND-GUIDED FINE NEEDLE ASPIRATION	95
Patient 10	No mutation detected for EGFR	No Mutation detected for EGFR L858R EXON 19DELETION,T790M,G719A/C/S, EXON 20,S768I,L861Q,EXON 21	6.0 x 3.0 x 2.5 cm	HEAD OF PANCREAS	0

EGFR Mutated					PDL-1 SCORE
Sample ID			**Specimen Adequacy**	**Other comments**	(%)
Patient 11	Mutation Detected for EGFR	Mutation is detected in EGFR Exon 21.	0.1 x 0.3 cm	LYMPH NODE, STATION 4L, ENDOBRONCHIAL ULTRASOUND-GUIDED FINE NEEDLE ASPIRATION	70
Patient 12	Mutation Detected for EGFR	Mutation is detected in EGFR Exon 19.	1.0 x 0.2 cm	LEFT LOWER LOBE TRANSBRONCHIAL BIOPSY	N/A
Patient 13	Mutation Detected for EGFR	Mutation is detected in EGFR Exon 21.	0.5 x 0.3 cm	ENDOBRONCHIAL ULTRASOUND-GUIDED FINE NEEDLE ASPIRATION	2
Patient 14	Mutation Detected for EGFR	Mutation is detected in EGFR Exon 21.	0.1 cm to 0.2 cm	LUNG, LEFT UPPER LOBE LESION, BRONCHOSCOPIC BIOPSY	3
Patient 15	Mutation Detected for EGFR	Mutation is detected in EGFR Exon 21.	0.4 x 0.4 cm	LYMPH NODE, STATION 7, ENDOBRONCHIAL ULTRASOUND-GUIDED FINE NEEDLE ASPIRATION	2
Patient 16	Mutation Detected for EGFR	Mutation is detected in EGFR Exon 21.	0.5 x 0.4 cm	LUNG, RIGHT UPPER LOBE, ENDOBRONCHIAL ULTRASOUND-GUIDED FINE NEEDLE ASPIRATION	0
Patient 17	Mutation Detected for EGFR	Mutation is detected in EGFR Exon 19.	0.2 x 0.2 cm	BRONCHIAL BIOPSY, RIGHT UPPER LOBE	90
Patient 18	Mutation Detected for EGFR	Mutation is detected in EGFR Exon 21.	0.4 x 0.3	LYMPH NODE, STATION 11L, ENDOBRONCHIAL ULTRASOUND-GUIDED FINE NEEDLE ASPIRATION	N/A
Patient 19	Mutation Detected for EGFR	Mutation is detected in EGFR Exon 19.	0.5 x 0.4 cm	BRAIN, RIGHT FRONTAL LESION: METASTATIC ADENOCARCINOMA COMPATIBLE WITH LUNG PRIMARY	5
Patient 20	Mutation Detected for EGFR	Mutation is detected in EGFR Exon 20.	0.4 x .3 cm	LYMPH NODE, MEDIASTINAL STATION 7, ENDOBRONCHIAL ULTRASOUND-GUIDED FINE-NEEDLE	N/A

Table 4: EFR Accuracy/Precision study results for non-mutated EGFR used demonstrate that potential interfering substances will not affect the EGFR assay results, a non-EGFR mutation sample or control.

EGFR Accuracy/Precision Study Results								
EGFR non mutated								
Sample ID	**Expected Result**	**Idylla™ Result**	**Idylla™ Module**	**Sample Type**	**Date**	**USER**	**SIZE (mm)**	**Other comments**
Test Patient 1	NEG	NEG	EGFR-1	Brain-met (multiple fragments)	7/9/2021	NH	5x3	Brain Met(original lung cancer); also-non mutated
Test Patient 2	NEG	NEG	EGFR-2	Met-melanoma	7/9/2021	NH	16x10	Met melanoma (Good neg ctl)
Test Patient 3	NEG	NEG	EGFR-1	Met RCC	7/9/2021	NH	20x16	Met RCC
Test Patient 4	NEG	NEG	EGFR-2	Pancreatic NE cancer	7/9/2021	NH	17x15	Pancreatic NE cancer
Test Patient 5	NEG	NEG	EGFR-1	Met Gastric Cancer	7/9/2021	NH	4x2	Met gastric cancer

Table 5: EGFR Accuracy/Precision study results for mutated EGFR used demonstrate that potential interfering substances will not affect the EGFR assay results, a non-EGFR mutation sample or control.

EGFR Mutated								
Sample ID	**Expected Result**	**Idylla™ Result**	**Idylla™ Module**	**Sample Type**	**Date**	**User**	**SIZE (mm)**	**Other comments**
Test Patient 6	POS	POS	EGFR-1	Tiny tumor Fragment	7/9/2021	NH	1x1	Tiny tumor fragment
Test patient 7	POS (UB cancer) Foundation Medicine data	NEG: Will try another EGFR/NRAS kit; and check the mutation info for both kits and compare with	EGFR-2	Multiple fragments	7/10/2021	NH	30x15	
Test patient 8	Equivocal by Foundation Med	NEG	EGFR-1	B1 Returned block – will repeat on B3,4 or 6	7/10/2021	NH	9x3	
Test patient 9	POS	POS	EGFR-1		7/10/2021	NH	20x5	Large positive control
Test patient 10	POS	POS	EGFR-1	Bloody tiny tumor fragment. Unstained level 1	7/10/2021	NH	6x5	Bloody Tiny Tumor

Table 6: EGFR Accuracy/Precision threshold study results for mutated EGFR used to demonstrate assay, instrument module and technologist reproducibility.

EGFR Mutated								
Sample ID	**Expected Result**	**Idylla™ Result**	**Idylla™ Module**	**Sample Type**	**Date**	**Performed By**	**SIZE (mm)**	**Other comments**
Test Patient 10	POS	POS	EGFR-2	Unstained Level 2	7/12/2021	NH	6x5	
Test Patient 10	POS	POS	EGFR-1	Unstained level 3	7/12/2021	NH	6x5	
Test Patient 10	POS	POS	EGFR-2	Unstained level 4	7/23/2021	NH	6X5	
Test Patient 10	POS	POS	EGFR-1	Unstained level 5	7/23/2021	NH	6X5	

Immunohistochemistry for PD-L1

Immunohistochemistry (IHC) is a laboratory method that uses antibodies to check for certain antigens (markers) in a sample of tissue. The antibodies are usually linked to an enzyme or a fluorescent dye. After the antibodies bind to the antigen in the tissue sample, the enzyme or dye is activated, and the antigen can then be seen under a microscope. Immunohistochemistry is used to help diagnose diseases, such as cancer. It may also be used to help tell the difference between different types of cancer (18).

Immunology, reagents, and the method utilized all influence the outcome of immunohistochemical stains. In this study we will focus on the PD-L1 immunohistochemistry (IHC) stain. PD-L1 plays a critical role in cancer cells by allowing these cells to escape immunity, multiply and development. It has been found that immunotherapy that targets PD-L1 by use of monoclonal antibodies has emerged as a safe and highly effective new treatment for malignant tumors, such as Non-small Cell Lung Cancer (NSCLC)(8). For NSCLC, there have been developments of five different inhibitors each which its own PD-L1 assays and interpretation criteria (19). Of the five, three of the inhibitors have attained clinical approval (19).

The Dako 22C3 pharmDx monoclonal mouse anti-PD-L1 clone was used for this study which has been clinically validated with KEYTRUDA^{TM} (Pembrolizumab) (20). The PD-L1 IHC testing is required for patients with NSCLC. Eligibility of the KEYTRUDA^{TM} (Pembrolizumab) is determined by patients that has more than >=1% of the stained Tumor Cells (TC) present (20).

Digitization and Scoring

After the NSCLC tissue was stained with the immunohistochemistry techniques for PD-L1, the slides are place into the whole slide imaging scanner. Leica Aperio Scan Scope XT was used to obtain the image. Virtual microscopy is the scanning of a whole stained and unstained microscope slide into a single high-resolution file. The single high resolution slide is a combination of numerous small high resolution images called titles, which are placed together to create the full histological image.

This process will be the next step of the project as the algorithm used to prepare the scoring for PD-L1 needs to be purchased and tuned to detect the specific PD-L1 cells we are looking for associated with NSCLC.

CHAPTER THREE: FINDINGS AND CASE STUDIES

With all the advancements in medicine and the rapid development of new therapies, the targeting of inhibitory receptors for patients with NSCLC has transformed cancer treatment (5). These pathways show that there is a correlation between Micro-RNA expression of PD-L1 and immunohistochemistry PD-L1 stains. Some micro-RNA expression showed over expression of PD-L1, which resulted in a PD-L1 IHC score of high. This shows that there is a case for more bio marker studies to aid in the prediction of the response to PD-L1 pathway inhibitors.

This project is based on the combination of three individually influential components for diagnosis and treatment for patients with NSCLC. First we did rapid molecular testing on the Formalin Fixed Paraffin Embed (FFPE) tissue to determine the positivity and accuracy of the genes for NSCLC. These results have proven to be accurate using the rapid EGFR assay given and correlated with the next gen sequencing results performed at the reference laboratories. This study included 20 samples from patients which ten (10) negative patients for NSCLC tested negative that is no-mutation. The remaining ten (10) positive patients gave positive result for EGFR mutations. The results showed that the minimum threshold for the amount of tumor needed was 1 mm^2.

Secondly, PD-L1 (clone 23C3) specific for KEYTRUDA^{TM} (Pembrolizumab) from Agilent (DAKO) Santa Clara, CA immunohistochemistry was also validated and

produced comparable results to the preliminary reports. The PD-L1 positive control using pediatric tonsil seemed to have a high reactivity rate than the use of the placenta tissue. As PD-L1 immunohistochemistry stain fades over time, the use of control as showed fading over time, so a more recent piece of control tissue was found to be more appropriate.

The first two steps of the three steps process to obtain a more personalized medicine for NSCLC patients have been used for clinical trials.

Case Studies: Seven case studies are presented to illustrate that the implementation of immunohistochemistry, rapid molecular testing and digital scoring to obtain fast accurate results are feasible.

First case:

Patient 1 is a 58 year old Asian female, with a positive diagnosis for NSCCA of lung with brain and liver mets. The specimen was obtained through an endobronchial ultrasound guided fine needle aspiration of which showed metastatic poorly differentiated non-small cell carcinoma. The PD-L1 immunohistochemistry test used was in agreement with both outside and in-house labs results to be PD-L1 positive- High at 100%. Rapid molecular testing results for the patient was negative for EGFR- No mutation detected in L858R, Exon 19 deletion, T790M, G719A/C/S, exon 20 insertion, s768I and L861Q with a Cq value of 18.1(Figure 1). PD-L1 tumors that have expression of 50% or greater have been found to respond well to immunotherapy drugs for check point inhibitors. But,

studies of patients treated with first line EGFR-TKIs have shown that high PD-L1 expression is associated with poor treatment response and shorter progression-Free survival regardless of the EGFR mutation sub-types (21).

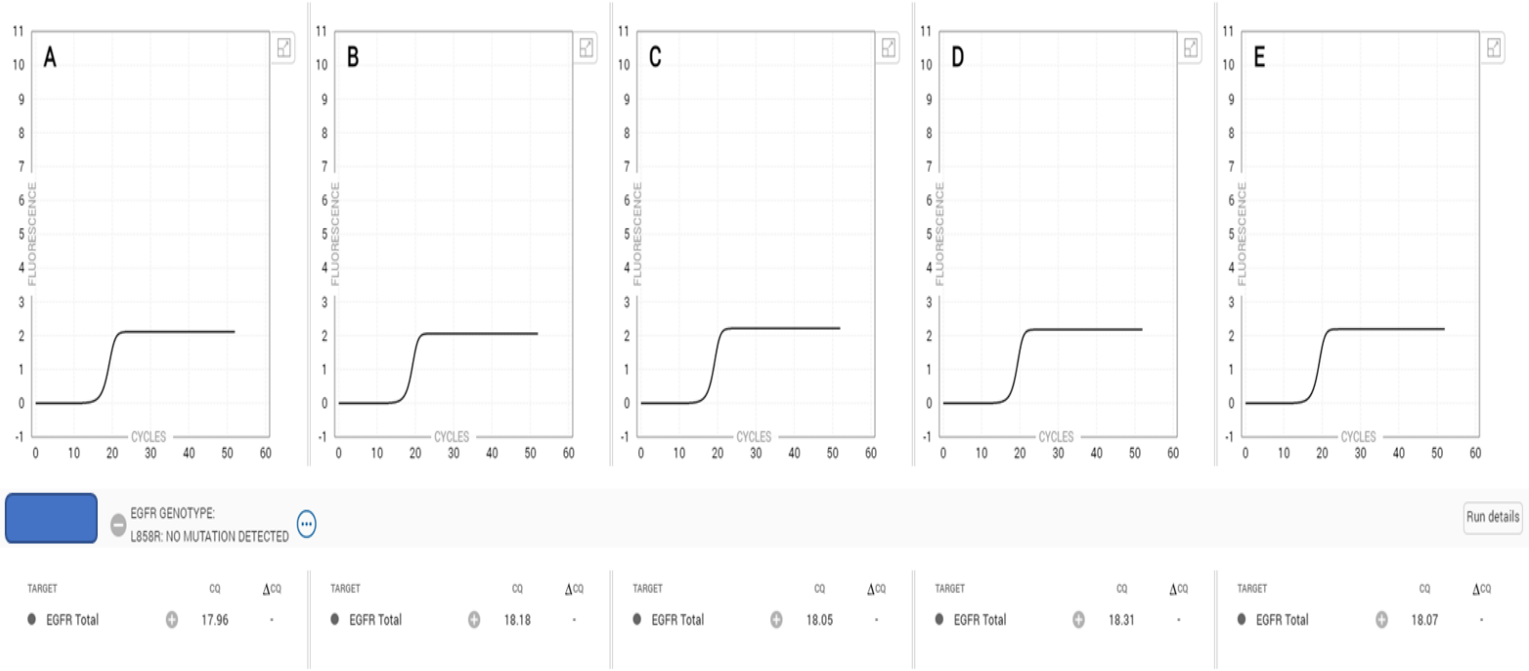

Figure 1: "Rapid PCR results with Cq graphs for Case study One (1)." Patient was negative for EGFR with no Mutation detected and all controls ran correctly indicated by no discrepancy on the curve shown in chambers A-E.

Second case:

Patient 2 is a 66 year old former smoker Asian male. The specimen used was an endobronchial ultrasound guided fine needle aspiration which showed metastatic adenocarcinoma. The PD-L1 immunohistochemistry test results were in agreement with both outside and in-house labs to be PD-L1 positive- High at 70%. To decide if it was primary lung cancer as well as adenocarcinoma sub-type, immunostains for TTF-1, pancytokeratin and p40 was done with positive TTF-1 and negative p40 indicating the adenocarcinoma. Rapid molecular testing results for the patient was negative for EGFR- no mutation detected in L858R, exon 19 deletion, T790M, G719A/C/S, exon 20 insertion, s768I and L861Q with a Cq value of 20.6 (Figure 2).

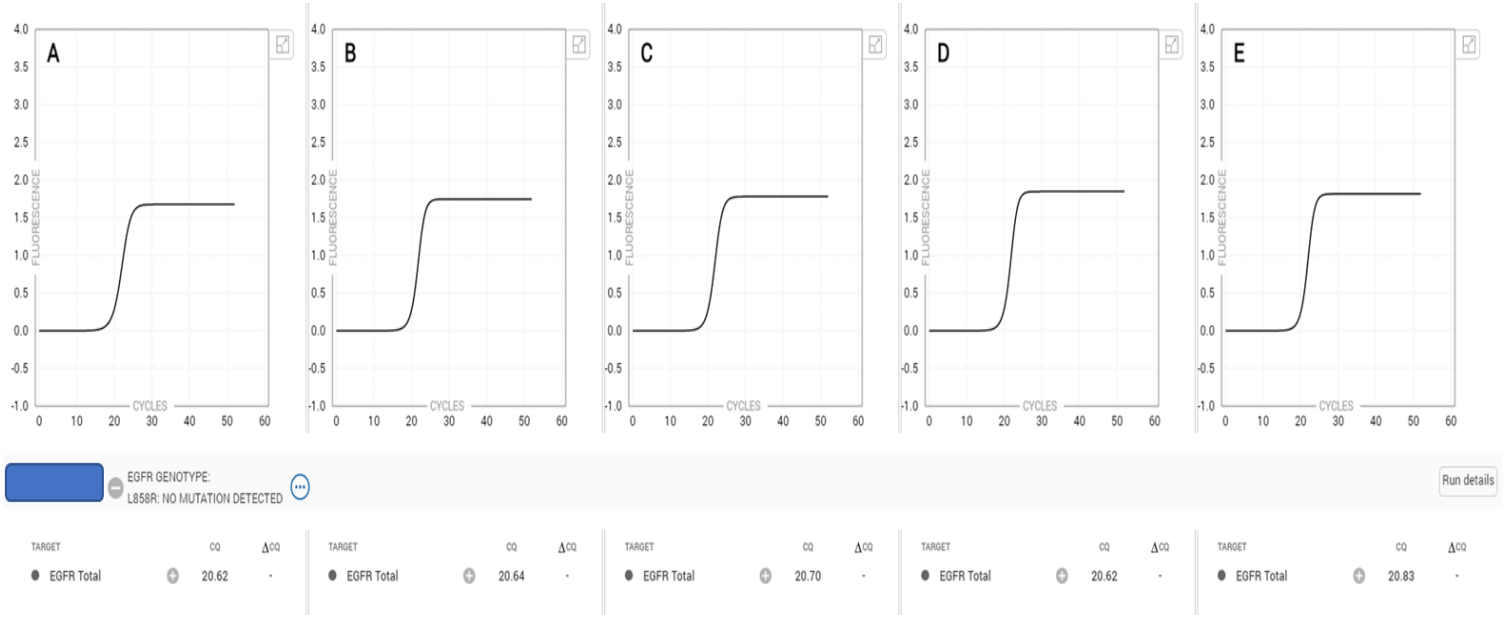

Figure 2: "Rapid PCR results with Cq graphs for Case study two (2)." Patient was negative for EGFR with no Mutation detected and all controls ran correctly indicated by no discrepancy on the curve shown in chambers A-E.

Third case:

Patient 3 is a 75 year old Caucasian non-smoker male with a recent diagnosis of metastatic non-small cell lung cancer and a recent admission to an outside hospital for management of obstructive jaundice. During his admission to the outside hospital he was diagnosed with non-small cell lung cancer after being found to have multiple laboratory abnormalities including elevated LFTs and elevated bilirubin secondary to metastatic liver lesions. The specimen obtained from patient was an ultrasound-guided fine needle aspirate which provided a diagnosis of adenocarcinoma. Rapid molecular tests results were negative for EGFR with a Cq value of 23.5 (Figure 3), which correlated well with the negative results for EGFR, KRAS, NRAS, BRAF, ALK, ROS1, MET and Her2 obtained by Next Gen-sequencing(NGS) . Next-generation sequencing (NGS) of tumor tissue and peripheral blood confirmed the presence of a *KIF5B-RET* gene fusion. KIF5B-RET is a RET proto-oncogene found in advanced NSCLC patients it is detected using Liquid biopsy procedure to detect circulating tumor DNA (CtDNA)(22).

The PD-L1 analysis was also performed and resulted in a negative PD-L1 expression of less than 1 % Tumor Proportion Score (TPS). With these results the patient's treatment program included Selpercatnib (Retevmo), a precision oncology treatment used specifically for patients with certain RET- driven cancers. Patient's outcome had decreased liver function, but stable brain functionality with an increase in bone metastasis.

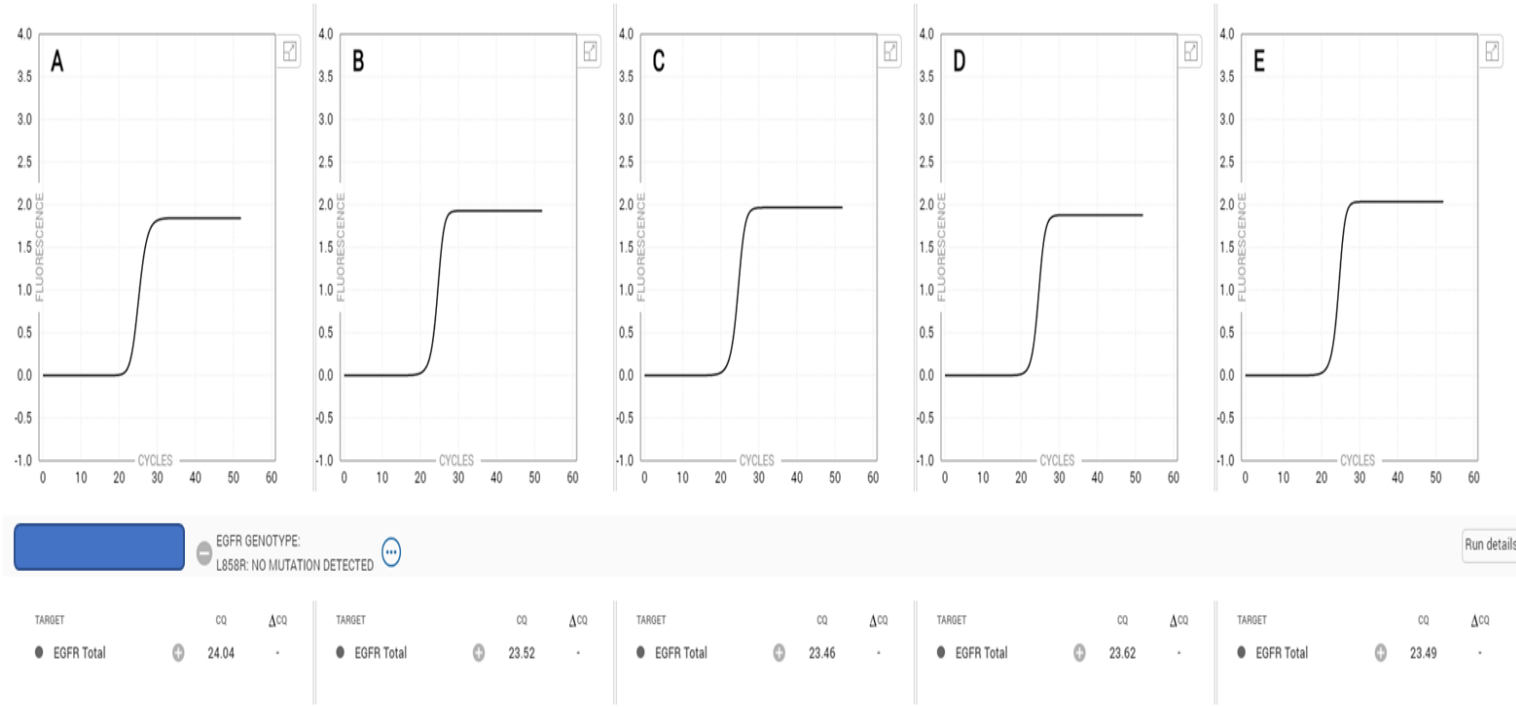

Figure 3: "Rapid PCR results with Cq graphs for Case study three (3)." Patient was negative for EGFR with no Mutation detected and all controls ran correctly indicated by no discrepancy on the curve shown in chambers A-E.

Fourth Case:

Patient 4 is a 45 year old non-smoker Asian female diagnosed with a left-sided lung adenocarcinoma by biopsy of the lung nodule. Preliminary findings from the level 7, 4L and 11L lymph nodes are suspicious for involvement by adenocarcinoma. The specimen obtained was an endobronchial ultrasound-guided fine needle aspirate which provided a diagnosis of metastatic adenocarcinoma. Rapid molecular test results were positive for exon 20 insertion and negative for KRAS mutation with a median Cq score of 19.4 (Figure 4). Outside consult results for Next gen sequencing are pending.

With these results a treatment plan she was not a candidate clinically for TKI-EGFR treatment and a second option for EGFR trails were sought. It was also found that the patient had wide spread mets to brain, bone and abdominal Lymph nodes.

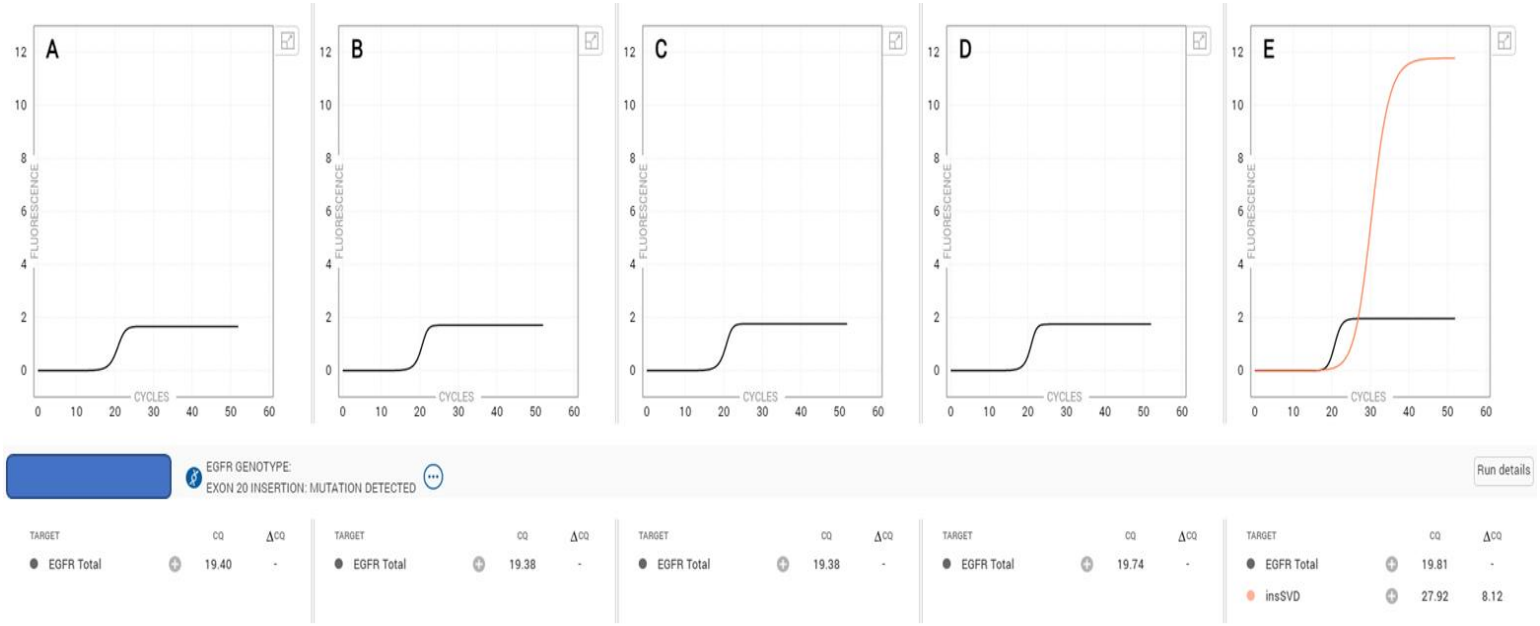

Figure 4: "Rapid PCR results with Cq for Case study Four (4)." Patient was positive for EGFR exon 20 insertion mutation which was detected in chamber 'E' with a Cq value of 27.92. All controls ran correctly indicated by no discrepancy on the control curve shown in chambers A-E.

Fifth Case:

Patient 5 is 52 year old nonsmoker white female. The specimen obtained was an endobronchial ultrasound-guided fine needle aspirates which as diagnosed to be metastatic adenocarcinoma. Rapid molecular test results correlated with NGS results showing both were positive for EGFR mutation in exon 19 deletions with a Cq score of 21.1(Figure 5). The PD-L1 immunohistochemistry test used was in agreement with both outside and in-house labs to be PD-L1 positive at 1%. These results lead to a diagnosis of non-small cell carcinoma.

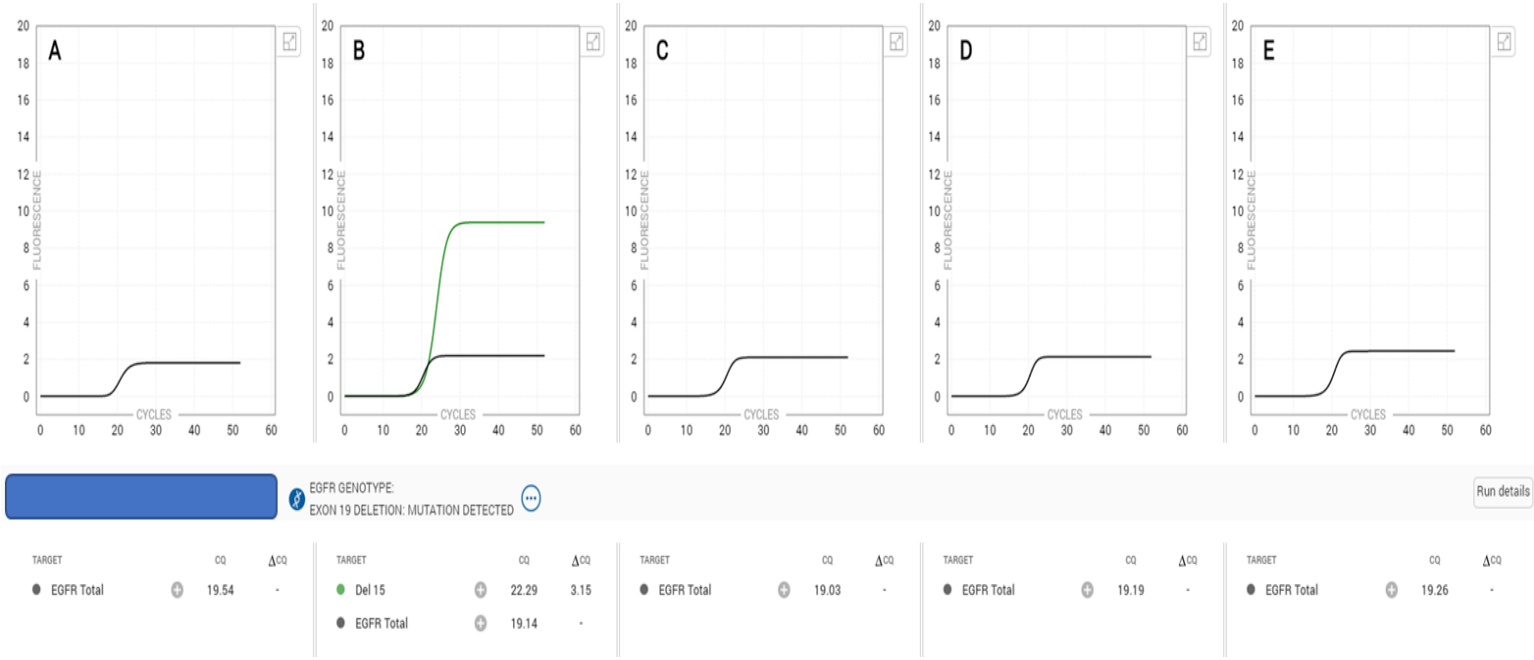

Figure 5: "Rapid PCR results with Cq for Case study Five (5)." Patient was positive for EGFR exon 19 deletion mutation which was detected in chamber 'B' with a Cq value of 19.14. All controls ran correctly indicated by no discrepancy on the control curve shown in chambers A-E.

Sixth Case:

Patient 6 is a 60 year old Hispanic male. The specimen received was left parietal brain tumor resection, which was diagnosed to be metastatic poorly differentiated pulmonary adenocarcinoma. Rapid molecular test and NGS result both showed exon 19 deletion with a Cq of 20.1(Figure 6). To decide if it was primary lung cancer as well as adenocarcinoma sub-types immunostaining was performed for CK AE1.AE3, CK7, TTF-1. All of these showed positive results. Also addition stains used were CK20, PAX-8, GATA3, p63, calretinin, WT1, beta-HCG, S100, uroplankin II, Oli-2 and GFAP showed negative results. The PD-L1 immunohistochemistry test used was in correlation with both outside and in-house labs to be PD-L1 positive- High at 80%. In this particular case due to the fact that the patient had a diagnosis of CNS metastases, a treatment plan of simultaneously administer radiation therapy and tyrosine kinase inhibitor such as KEYTRUDA^{TM} (Pembrolizumab).

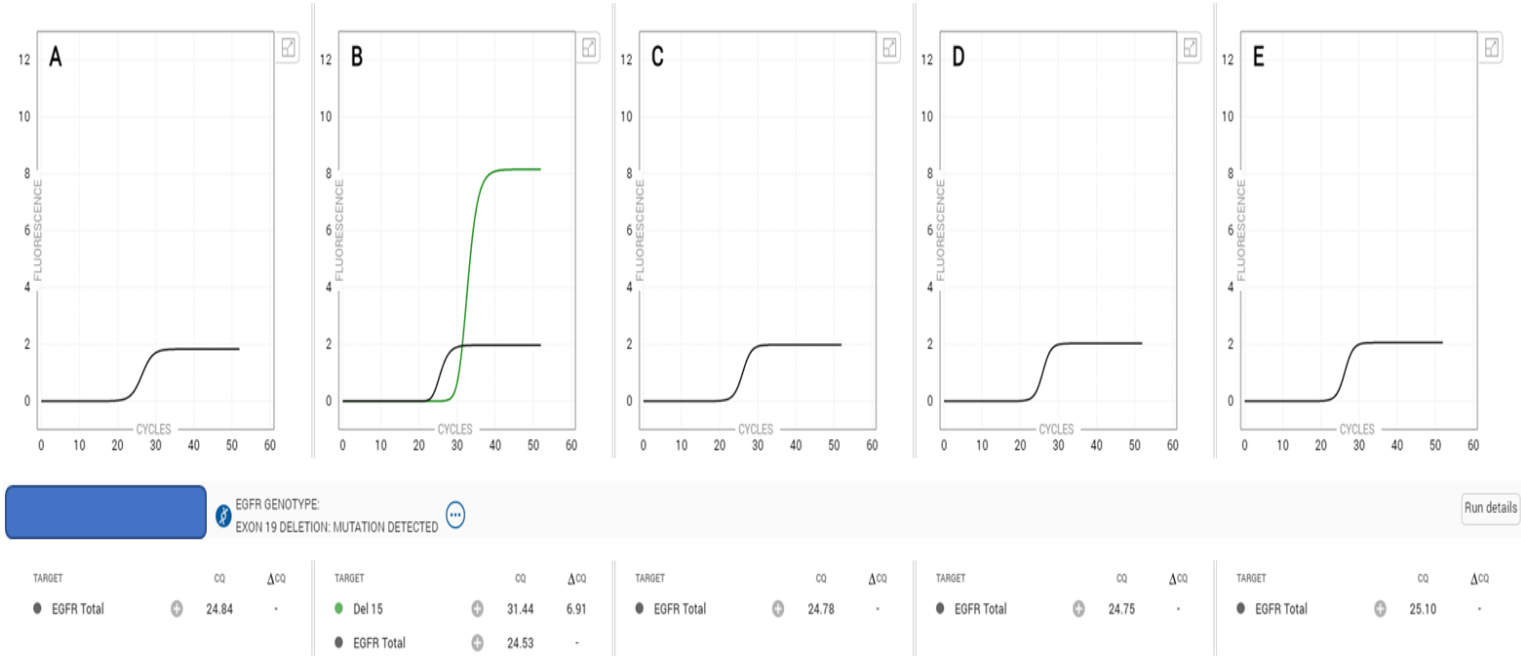

Figure 6: "Rapid PCR results with Cq for Case study six (6)." Patient was positive for EGFR exon 19 deletion mutation which was detected in chamber 'B' with a Cq value of 24.53. All controls ran correctly indicated by no discrepancy on the control curve shown in chambers A-E.

Seventh Case:

Patient 7 is 51 yr old non-smoking Asian male. Who was recently found to have a lung mass with metastasis. Initial biopsy showed adenocarcinoma and the entire paraffin block was sent for Next Gen Sequencing (NGS) and PD-L1 scoring.PD-L1 score was negative (<1%), and after 25 days the tissue results showed inadequacy for NGS.A second biopsy was done to do rapid PCR test for EGFR mutation. Rapid PCR test was then done on the FFPE and in approximately 24 hrs. the results showed that the patient biopsy tissue had EGFR deletion 19 mutation. PD-L1 results were negative and a correlating in house score of less than 1%. Patient is not a candidate for pembrolizumab, but will receive 2^{nd} or 3^{rd} generation EGFR-Tyrosine kinase inhibitor (TKI).

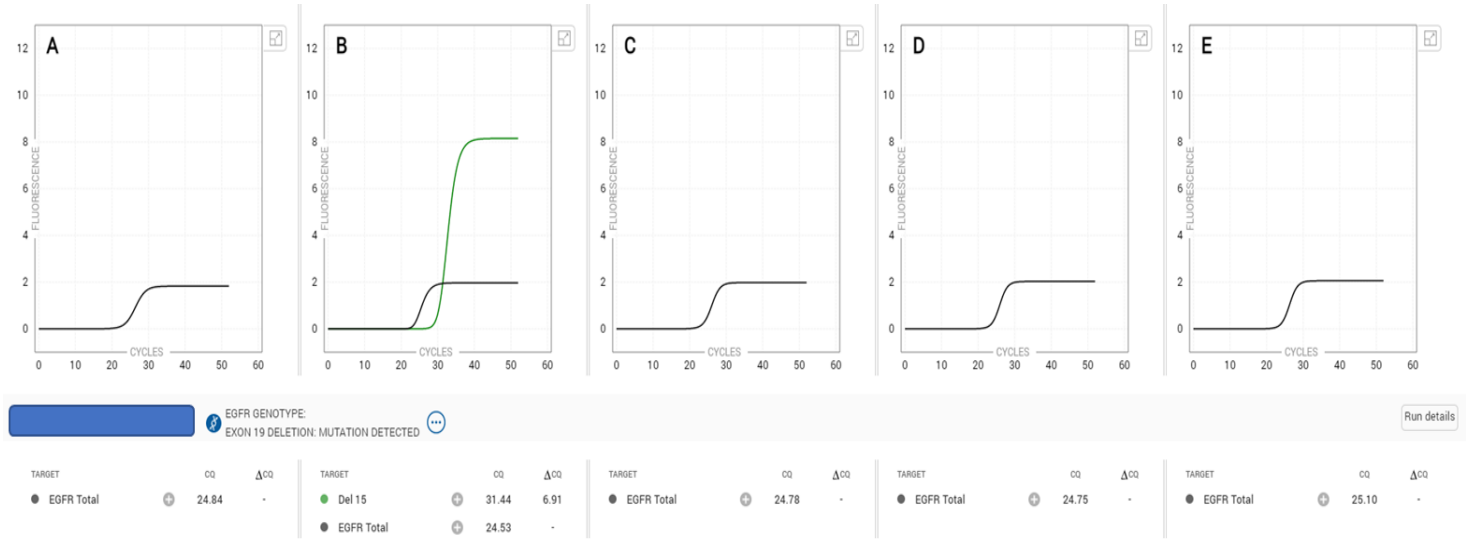

Figure 7: "Rapid PCR results with Cq for Case study seven (7)." Patient was positive for EGFR exon 19 deletion mutation which was detected in chamber 'B 'with a Cq value of 24.53. All controls ran correctly indicated by no discrepancy on the control curve shown in chambers A-E.

The results of the seven cases used in this study showed a good correlation results obtained from rapid PCR and NGS. As the results were the same, but the turnaround time (TAT) for Rapid PCR was exponentially faster by an average of 20 days than that of Next Gen sequencing (NGS)(Figure 8).Results showed that the average turnaround time (TAT) for Rapid PCR was 1.14 days and for NGS was 20.85 days (Table 7). This turnaround time difference is critical for diagnosis and treatment of cancer patients, thus improving patient care and quality of life.

Also, the quantification cycle (Cq) of the specimens used in the assays were within the threshold range of 7-38 cq (23). The threshold line/range is used to aid in detection or to find the point at which a reaction reaches a fluorescent intensity above background levels (Figures 1-7). The quantification cycle (C_q) value is the PCR cycle number at which the reaction curve intersects the threshold line. This value tells how many cycles it took to detect a real signal from the samples. As with each case study the Cq value was well under the 29-threshold value, meaning our case study sample had high amount of the targeted sequences. Had there been a sample above the threshold Cq value of 38, we would have to re-evaluate the tissue sample and detection assay as it would result in low amounts of the targeted sequence.

Case Study	Rapid PCR Test Performed	Rapid PCR Turnaround time(TAT)	Outside NGS lab results	Outside lab NGS Turnaround time	Comments	Send out PDL-1	Send out results for Tumor proportion Score (%):
1	REGFR	24 hrs.	EGFR NEG	11 days		HIGH	100
2	REGFR	24 hrs.	EGFR NEG	28 days	EML4-ALK+, NO BLOCK AVAIBLE	HIGH	70
3	REGFR	24 hrs.	EGFR NEG	22 days		NEGATIVE	less than 1
4	REGFR & RKARS	24 hrs.	EGFR POS	31 days	EXON 20 INSERTION	Pending	pending
5	REGFR	48 hrs.	EGFR POS	19 days	EXON 19 DELETION	Low	1
6	REGFR	24 hrs.	EGFR POS	11 days	EXON 19 DELETION	High	80
7	REGFR	24 hrs.	Inadequate	25 days	EXON 19 DELETION	Unavailable	Unavailable

Table 7: "EGFR Case Study Correlation Table". Data showing the correlation of rapid PCR results with agreement to Next Gen sequencing (NGS) and turnaround time (TAT) difference for each cases study.

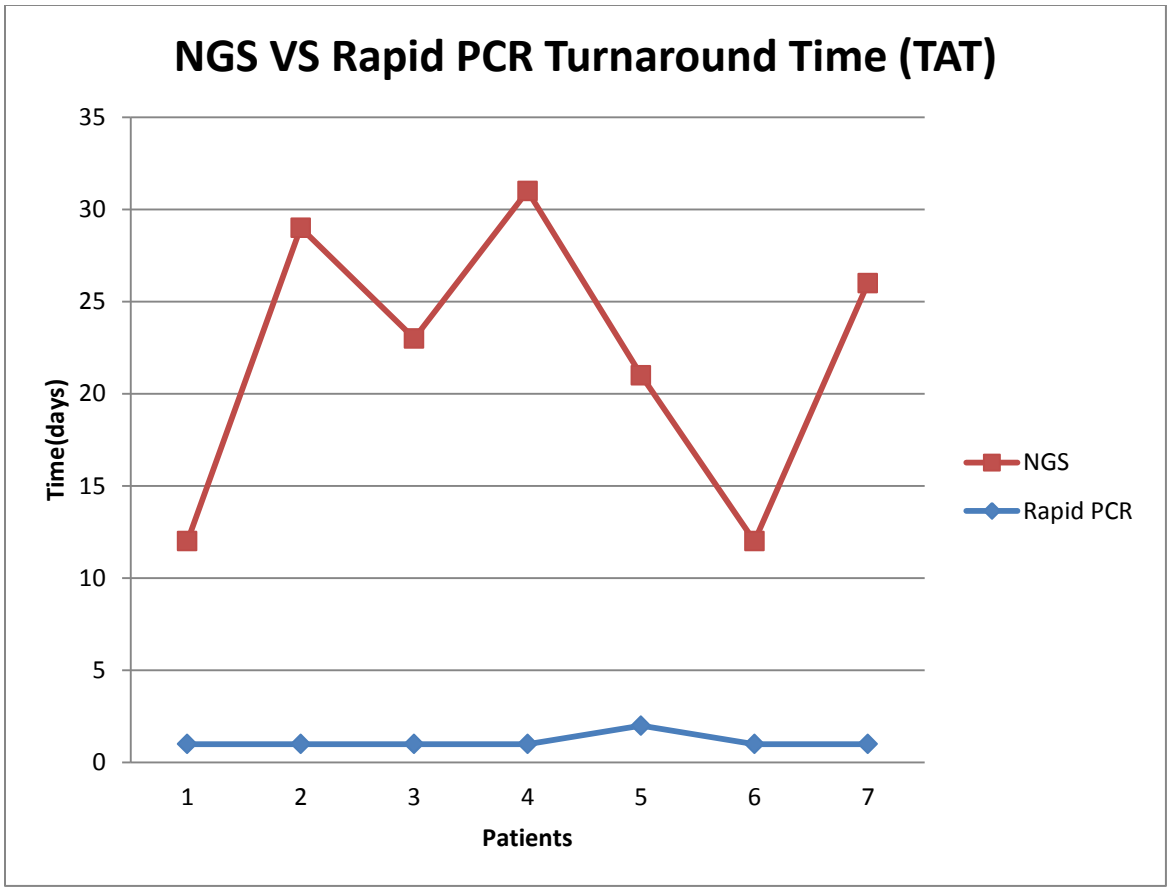

Figure 8: "NGS vs Rapid PCR Turnaround time (TAT)." Turnaround time for Rapid PCR was significantly faster than that of Next Gen Sequencing(NGS).

CHAPTER FOUR

The objective of this study is to determine the use of rapid molecular tests for tumor mutations and the application of digital scoring methods of IHC (immunohistochemistry) test for PD-L1 (clone 22C3) for Keytruda® (Pembrolizumab). This project has demonstrated that effective personalized cancer treatment using rapid efficient testing, with fast TAT (turnaround time), user friendliness, remarkable accuracy, specificity and sensitivity is achievable and can be implemented quickly. This will prove especially useful for those with an advanced stage of cancers.

We have done two of the three stages proposed in the rapid molecular testing and IHC test for PD-L1 for NSCLC. Our next step will be to obtain the digital algorithm to aid in the detection of PD-L1 using whole slide imaging (WSI) techniques. The adaptation of choice will be the addition of AI using supervised learning. This will enable a more robust form of learning for the algorithm, which will lead to a more accurate scoring. It can be seen that patients with NSCLC can benefit from treatment using PD-L1 targeting immunotherapy which are evaluated using approved PD-L1 immunohistochemistry assays. The manual scoring by the pathologist can be difficult due to significant inter-observer variation because the four FDA approved PD-L1 immunohistochemistry assays have different scoring criteria. The AI that will be used for further study has shown high concordant with consensus Tumor proportion score (TPS)

for the 22C3 PD-L1 clone. Once the algorithm is approved by FDA to score PD-L1 in NSCLC in clinical settings, its knowledge will be used as the base for other forms of tumors such as gastrointestinal cancer, Head and neck cancer, Triple negative breast cancer and uterine cancer.

The future direction of this newly formulated application of these techniques will include the use of reflex techniques in the occurrence that EGFR mutation is negative we will automatically test the specimen for KRAS mutation. Should KRAS mutation result in negative mutation and second reflex will be done using the gen fusion assay which include the detection of ALK, ROS1, RET, NTRK1/2/3. KRAS is known as Kirsten rat sarcoma virus, its function is to act as a hub for signals in the cell that causes cell growth, therefore when a mutation occurs it signals the cells to continuously grow. We use the detection of KRAS mutation as a biomarker for patients with non-small cell lung cancer (NSCLC) to determine what type of treatment a patient receives.

Gene fusions have been found to play a pivotal role in non-small cell lung cancer (NSCLC) precision medicine. Several techniques can be used, from fluorescence in situ hybridization and immunohistochemistry to next generation sequencing (NGS) (13).Another future direction of this project is to use a gene fusion assay by rapid PCR for the detection of ALK, ROS1, RET, NTRK1/2/3. Fusion genes are hybrid genes generated by the juxtaposition of two previously independent genes, following structural rearrangements like deletions, inversions, translocations or duplications within the same chromosome or between different chromosomes. Currently, more than 10,000 gene fusions have been identified in human cancers, many of which are strong driver

alterations (14). With the increase use of precision medicine, in particular to non-small cell lung cancer (NSCLC) and FDA approved drugs such as KEYTRUDA™ (Pembrolizumab) to treat patients, finding a way to produce fast, efficient, reproducible molecular results has become mandatory.

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BIOGRAPHY

Nicholas Hoo-Fatt graduated from Campion College, Kingston, Jamaica, in 1997. He received his Bachelor of Science from Nova Southeastern University in 2012. He was employed as the Histology Program Education Coordinator in the Fairfax County for three years and received his Master of Science in Bioinformatics and Computational Biology from George Mason University in 2022.