INVESTIGATING TARGETED DRIVER MUTATIONS AND PD-L1 EXPRESSION FOR IMPROVED THERAPY OF NON-SMALL CELL LUNG CANCER

By

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ABSTRACT

Most lung cancer patients are diagnosed at an advanced stage, limiting their treatment options to chemotherapy with very low response rate or other palliative managements. New therapies that target driver gene mutations (e.g. *EGFR*, *ALK*, *BRAF*), are being used to treat patients who have tumours with these mutations. In addition, a type of immunotherapy called immune checkpoint inhibitor is being used to treat lung cancer patients. For instance, patients with tumours that express PD-L1 may be responsive to anti PD-1/PD-L1 therapy. Thus, being able to identify the presence of driver mutations and PD-L1 in tumours will help patients to benefit from different therapies. A total of 851 cases of non-small cell lung cancer samples have been profiled for the presence of *EGFR*, *KRAS*, *BRAF*, and *PIK3CA* mutations by SNaPshot/sizing genotyping. Immunohistochemistry was used to identify the protein expression of ALK and PD-L1. Histological examination was performed to determine the pathological type, grade, and lymphatic/vascular invasion. Moreover, *PD-L1* mRNA expression was quantified by RT-qPCR in a sub-group of the cohort to assess its correlation with PD-L1 protein level. Statistical analysis revealed correlations between the presence of the mutations, PD-L1 expression, and the pathological data. Specifically, it was determined that women had lung tumours with a significantly greater number of *EGFR* mutations than men. *EGFR* mutations were significantly linked to the absence of vascular invasion and PD-L1, and *KRAS* mutations do not associate with PD-L1 expression. Moreover, we found a positive correlation between mRNA levels of *PD-L1* by RT-qPCR with PD-L1 expression by IHC. Together, these data provide insights into driver gene mutations and immune checkpoint status in relation to lung cancer subtypes and pathological characteristics and provide useful information for clinical implications.

LIST OF ABBREVIATIONS USED

PD-1	Programmed death-1
PD-L1	Programmed death-ligand1
RTKS	Receptor tyrosine kinases
PI3K	Phosphoinositide 3-kinase
RAS	Rat sarcoma
EGFR	Epidermal growth factor receptor
STAT	Signal transducer and activator of transcription
RAF	Rapidly accelerated fibrosarcoma
ERK	Extracellular signal-regulated kinase
MEK	Mitogen-activated protein kinase
ALK	Anaplastic lymphoma kinase
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
GAPS	GTPase-activating proteins
KRAS	Kirsten rat sarcoma
CD	Cluster of differentiation
AKT	Protein kinase B
EML4	Echinoderm microtubule-associated protein-like-4
IHC	Immunohistochemistry
FISH	Fluorescence *in-situ* hybridization
EGF	Epidermal growth factor
RT-PCR	Reverse-transcription polymerase chain reaction
TNM	Tumour, Node, Metastasis
AJCC	American Joint Committee on Cancer
FDA	Food and drug administration
IPASS	Iressa pan-asia study
CMET	Tyrosine-protein kinase Met
MHC	Major histocompatibility complex
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
IGG	Immunoglobulin G
AE	Adverse event
TPS	Tumour proportion score
QEII	Queen Elizabeth
HER2/NEU	Human epidermal growth factor receptor 2/neuro
H&E	Hematoxylin and eosin
IFN-Γ	Interferon gamma
TBP	Tata-box binding protein
RPL13A	Ribosomal Protein l13a
LCA	Leukocyte common antigen

N1	Ipsilateral peribronchial, hilar, and intrapulmonary nodes
N2	Ipsilateral mediastinal and subcarinal nodes
TKI	Tyrosine kinase inhibitor
uL	Milliliter
uM	Micromolar
mRNA	Messenger ribonucleic acid
RNA	Ribonucleic acid
ROS	Reactive oxygen species
GEFs	Guanine nucleotide exchange factors

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CHAPTER 1. INTRODUCTION

1.1 General introduction

Lung cancer is the leading and most common cause of cancer-related death among both men and women; it causes more deaths than breast, prostate, and colorectal cancers combined and accounts for approximately 30% of all cancer deaths[1]. Despite some advancements in therapeutic applications, the overall 5-year survival rate for lung cancer remains only 18% [2]. Thus, new treatment strategies are needed.

One important aspect of lung cancer management is the staging system, the methodology used to determine the extent of cancer within an individual. The staging system is mainly based on location of the primary (original) tumour, tumour size and extent of tumours, lymph node involvement (whether or not the cancer has spread to the nearby lymph nodes) and presence or absence of distant metastasis (whether or not the cancer has spread to distant areas of the body)[3]. This reliance on histopathology alone does not provide sufficient information for establishing a well-suited management strategy.

Lung cancer can be divided into two large groups: non-small cell carcinoma and small cell carcinoma, and the former group can be further categorized into three main subtypes: squamous cell carcinoma (30% of non-small cell lung cancer), adenocarcinoma (almost 50%), and large cell carcinoma (about 15%), which represents a diagnosis of exclusion in the absence of squamous and adenocarcinoma cells[4]. Each disease stage of every lung cancer patient is heterogeneous with respect to treatment response, which suggests that further sub-classifications are possible. In particular, further sub-classification of non-small cell lung cancer into clinically related molecular subsets may lead to promising new treatment strategies.

There is a great degree of molecular heterogeneity across lung cancer patients, with a wide array of specific mutations, chromosomal abnormalities, and deletions of tumour suppressor genes[5]. In lung cancer, many of the relevant mutations occur in genes encoding proteins involved in signaling pathways that are important in cell proliferation and survival. These mutations are known as driver mutations, because they are essential in initiating tumour formation and maintenance of tumours[6]. Identification of targetable driver mutations in tumours may provide the basis for therapies specifically targeted at the relevant mutation.

Another promising avenue for treatment of non-small cell lung cancers is immunotherapy. For instance, checkpoint inhibitors such as those blocking the programmed death-1/programmed death-ligand 1 (PD-1/PD-L1) pathway represent an exciting approach for treating lung cancer, with better overall and progression-free survival than chemotherapy[7]. In this chapter, I will discuss the major driver mutations in non-small cell lung cancer and their clinical significance. In addition, I will discuss the PD-1/PD-L1 pathway and its significance in non-small cell lung cancer.

1.2 Important signaling pathways involved in lung cancer

1.2.1 Receptor tyrosine kinases (RTKs)

Receptor tyrosine kinases (RTKs) play a central role in the interactions between cells and their microenvironments. In addition, RTKs influence cell divisions and apoptosis via downstream signaling such as rat sarcoma (RAS) and phosphoinositide 3-kinase (PI3K) pathways. Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor, transmits growth factors signals into the nuclei of the cell via signal transduction. This results in upregulation of transcription, leading to protein synthesis and changes in cell morphology and functions. Intracellularly, signal transductions occur through many downstream

signaling pathways, such as RAS/RAF/ERK pathway, PI3K/AKT pathway and signal transducer and activator of transcription (STAT) pathway. The end results of the signal transduction is a promotion of cell division or differentiation[8] (Figure 1). Moreover, anaplastic lymphoma kinase (ALK) is another important RTK that plays a crucial role in cell proliferation and apoptosis. It also has effects on the fate of the cell through PI3K and RAS signaling[9]. Thus, aberrations in the *ALK* gene over activity would affect cell division and lead to tumour formation (Figure 2).

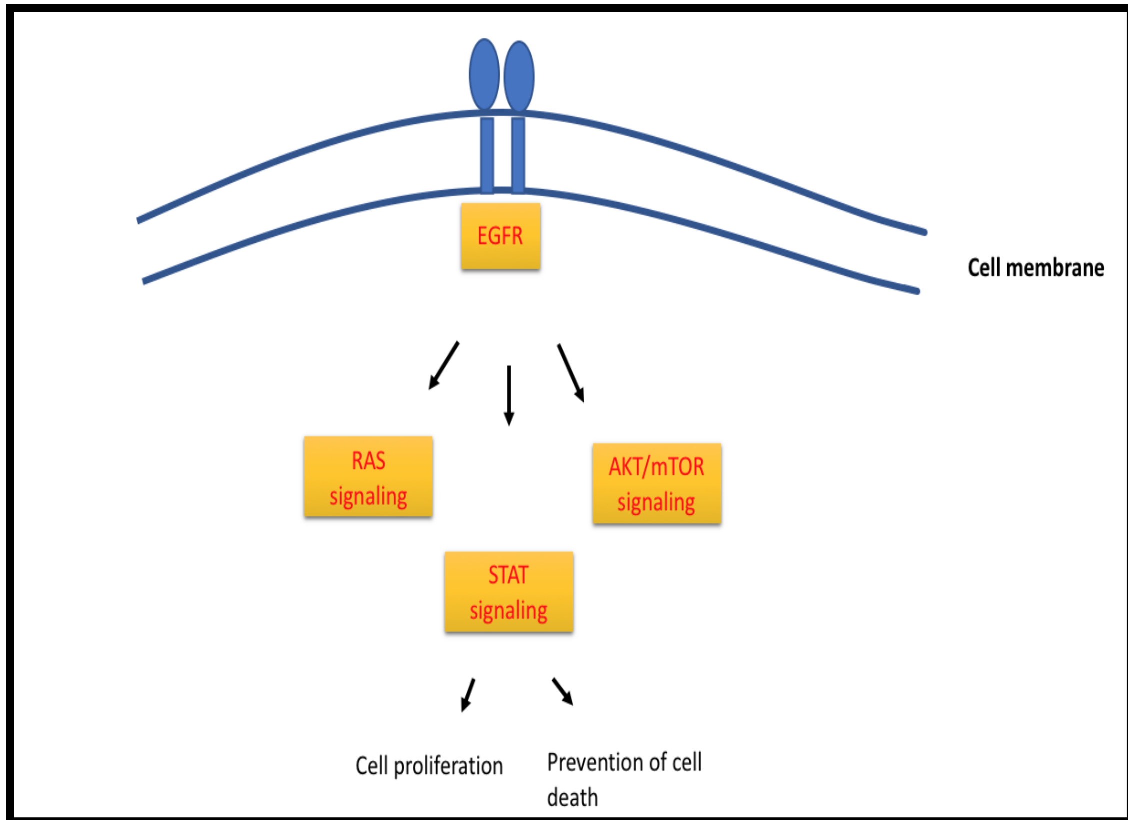

Figure 1: EGFR pathway.

When EGFR gets activated it will affect three major downstream signaling, RAS, STAT and AKT/mTOR signaling pathways. Depending on the pathway, the end result is cell proliferation or cell maintenance by inhibition of apoptosis.

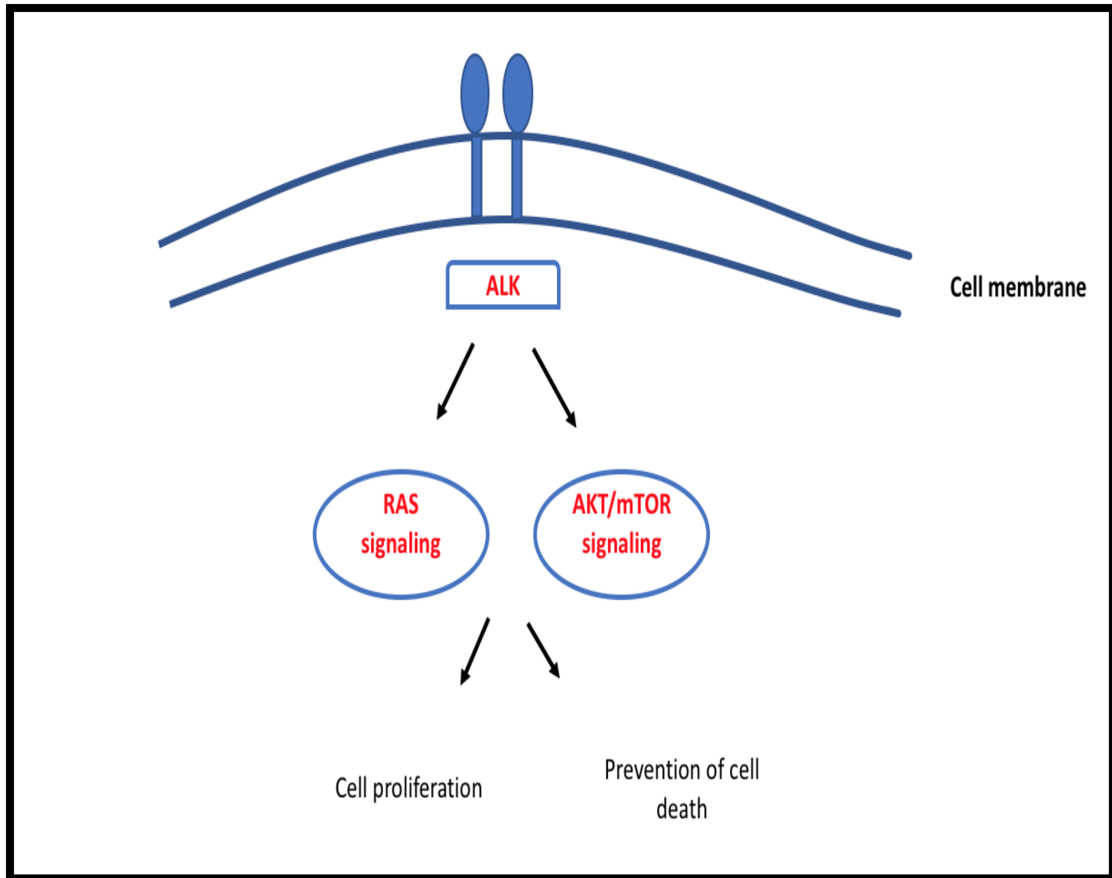

Figure 2: ALK signaling and its effects.

ALK receptor activation influences downstream signaling pathway like RAS and AKT/mTOR which leads to cell division and maintain its survival.

1.2.2 RAS-RAF-MEK-ERK pathway

In the RAS signaling pathway, RAS as a GTPase protein can be either on or off depending on its binding: it is on when bound to guanosine triphosphate (GTP) and off when bound to guanosine diphosphate (GDP). Normally, RAS is bound to GDP and inactive, but in response to extracellular stimuli it becomes active via binding to GTP. Guanine nucleotide exchange factors (GEFs) work by catalyzing the exchange from RAS bound GDP to GTP. However, hydrolysis of GTP to GDP is achieved mainly by GTPase-activating proteins (GAPs). Mutations of Kirsten rat sarcoma (*KRAS*) occurring in cancer cells make KRAS insensitive to GAPs. As consequence, KRAS is always bound to GTP in cancer cells and it becomes always active, causing activation of downstream proteins[10]. Then, oncogenesis is driven by multiple downstream pathways, including the mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) kinase pathway and the PI3K/AKT pathway (Figure 3). Eventually, these downstream pathways lead to prevention of apoptosis, increased cell proliferation, increased angiogenesis and dysfunction of cellular metabolism; all important events will lead to increase tumour progression[10].

Understanding all these pathways is essential for designing drugs against specific targets and therefore may improve patient outcomes. For example, when ERK is activated by upstream effectors, it will phosphorylate a number of substrates that are involved in cell cycle, translation, and apoptosis inhibition. Without understanding these pathways in detail, we will not be able to identify targets that have a potentially positive clinical outcome. Over the past two decades, oncogenic KRAS signaling has been targeted in a

number of ways, including inhibiting KRAS directly or targeting its downstream effectors pharmacologically[11].

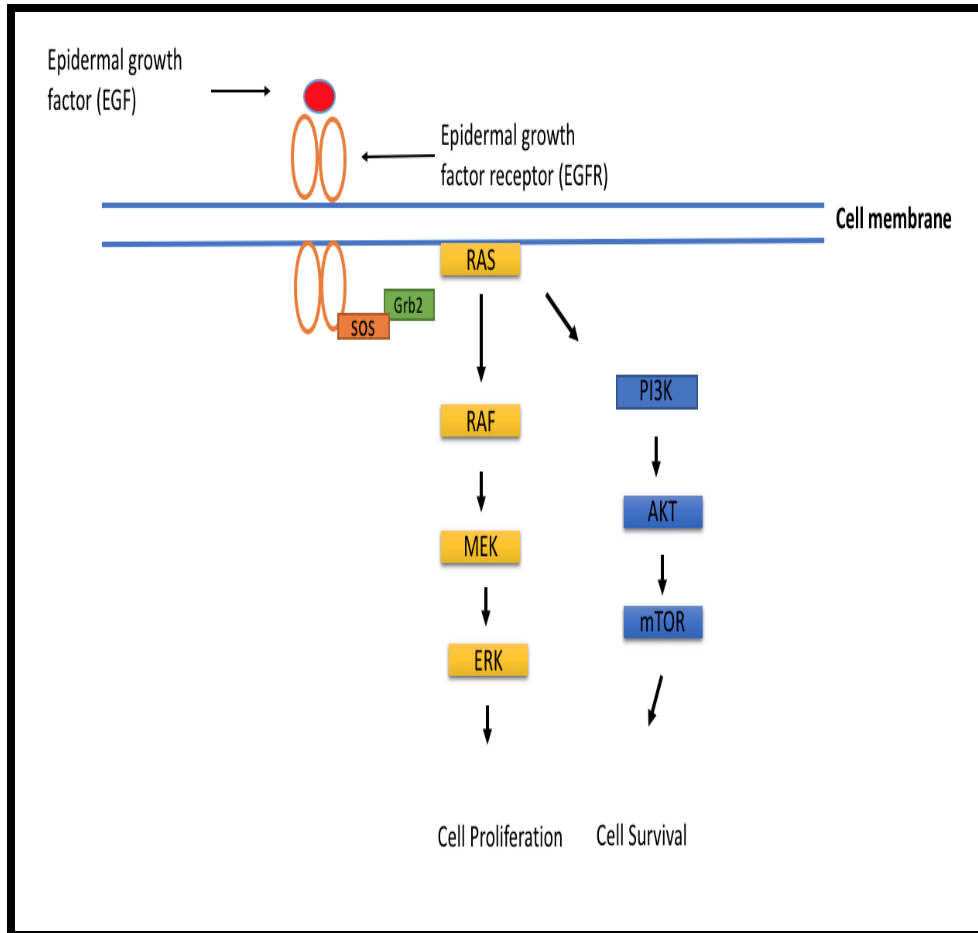

Figure 3: RAS signaling pathway and its effects on RAF-MEK and AKT mTOR pathways.

when RAS protein gets activated, a cascade of protein phosphorylation will be initiated that will lead to activation of the RAF-MEK-ERK pathway and PI3K-AKT-mTOR pathway resulting in promotion of cell proliferation and survival.

1.3 Targetable Mutations in Lung Cancer

1.3.1 *EGFR* mutations

Lung adenocarcinomas often exhibit mutations in *EGFR*, and these mutations make the tumour susceptible to EGFR inhibitors. The deregulation of the ErbB signaling pathway occurs in many malignancies including breast and lung adenocarcinoma. The ErbB family has four main members: HER1 (EGFR/erbB1), HER2 (neu, erbB2), HER3 (erbB3), and HER4 (erbB4)[12]. The deregulation of ErbB leads to uncontrolled cells growth, promotes cell migration, and interferes with programmed cell death.

EGFR is a transmembrane protein that consists of 486 amino acids[13]. The binding of ligand EGF to EGFR causes dimerization that leads to receptor phosphorylation. These dimers might form homodimers or heterodimers with any other member of ErbB family. Both the juxtamembrane domain, which has a tyrosine kinase activity, and the C-terminal domain are intracellular parts of EGFR. ATP affinity is affected by components located in the tyrosine kinase domain. Ligand binding is highly influenced by any alteration within either of these domains. Phosphorylation at the site of tyrosine residues leads to protein-protein interactions, which is important for signal transduction via pathways such as the PI3K or RAS pathways[14].

The frequency of *EGFR* mutations in non-small cell lung cancer in western population is almost 10% but the percentage is higher (35%) in East Asia. The most two common *EGFR* mutations are point mutation in exon 21 resulting in substitution of leucine by arginine at position 858 (L858R) and a deletion in exon 19. These two accounts for approximately 90% of *EGFR* mutations in non-small cell lung cancer[15].

1.3.2 *ALK* rearrangement

Originally, *ALK* rearrangement was identified in anaplastic large-cell lymphoma as a fusion protein encoded by an open reading frame that spans the breakpoint of a (2;5) (p23;q35) chromosomal rearrangement with the *nucleophosmin* gene[16]. In 2007, Soda et al. identified the oncogenic *echinoderm microtubule-associated protein-like-4* gene (*EML4*)-*ALK* fusion transcript. *ALK-EML4* fusion gene was reported in 6.7% of non-small cell lung cancer patient tumours examined in Soda's paper[17]. This fusion results from small inversions within the short arm of chromosome 2[18], and at least nine different variants have been identified[9]. The *ALK-EML4* fusion results in oligomerization and constant activation of the kinase[19]. The *ALK* fusion oncogene plays a critical role in tumour development by activating downstream signaling such as RAS and PI3K pathways, which leads to increased cell divisions and survival[20]. Mice expressing the *ALK-EML4* variant under the control of a lung epithelial cell promoter develop multiple lung adenocarcinoma tumours[21].

In non-small cell lung cancer, *ALK* fusion gene occurs in 3% to 7% of cases[19]. The occurrence of *ALK-EML4* fusion gene in cancer is usually detected via immunohistochemistry (IHC), fluorescence *in-situ* hybridization (FISH), and reverse-transcription polymerase chain reaction (RT-PCR). The tumours of people who never smoked or are light smokers (<15 pack-years) exhibit *ALK-EML4* fusion genes more frequent than current smokers (6% versus 1%)[19].

1.3.3 *KRAS* mutations

KRAS mutations, discovered three decades ago, occurs in about 25% of patients with non-small cell lung cancer[22]. *KRAS* mutations appear to be more common in Caucasian patients than African-Americans: one study observed such mutations in about

17% of African-American patients, whereas another study detected *KRAS* mutations 27% of Caucasian patients[23].

Overall, approximately one third of all cancers, including colon and pancreatic, feature *KRAS* mutations. In several studies, *KRAS* mutation have been clearly associated with adverse prognosis in patients with metastatic disease, and with higher probability of tumour recurrence[24]. Generally, the *RAS* gene has three isoforms (*NRAS, HRAS, KRAS*), and mutations in each of these have been reported in human cancers[22]. The location of *KRAS* mutations associated with lung cancer are mostly at codons 12 and 13, and less often at codon 61[25]. G12C, a mutation highly linked with smoking, is the most commonly seen mutation in lung cancer, making up about 40% of all *KRAS* mutations. The next most common *KRAS* mutations are G12V (22%) and G12D (16%)[26]. Both G12C and G12V are highly engaged in many downstream pathways such as RAL pathway that increase cell proliferation and prevent cell apoptosis, and possibly because of that these mutations are associated with worse outcomes for patients. In contrast, G12D promotes the activation of in the RAF/ERK and PI3K pathways[27]. Losing GTPase activity in these mutations causes oncoproteins to be constantly active, resulting in activation of downstream pathways like MEK-ERK and PI3K/AKT. Thus, many research efforts aim to target and inhibit mutant KRAS.

1.3.4 *BRAF* mutations

BRAF is a member of the RAF family, which consists of serine-threonine protein kinases. RAF has three different isoforms in humans: ARAF, BRAF, and CRAF (also known as RAF-1).

The frequency of *BRAF* mutations is about 50% in malignant melanomas, and about 45% in thyroid cancers. Less than 10% of colorectal, breast, and lung cancers have *BRAF* mutations[28]. *BRAF* mutations have more than 40 different missense mutations, including 24 different codons in human cancer. Most of these mutations are located near the kinase domain and induce kinase activity of BRAF toward MEK. A thymidine to adenosine transversion at nucleotide T1799A at exon 15 is the most common single mutation in the BRAF protein. This transversion results in a valine to glutamate substitution at codon 600 (V600E)[28]. This mutation induces phosphorylation, and BRAF activity is increased by almost 500-fold in comparison with wild-type[29].

The proportion of this specific mutation (V600E) reaches 90% in melanoma and colorectal cancer. Moreover, its frequency in non-small cell lung cancer is 55%[30]. To understand the role of V600E BRAF, researchers introduced the mutation in mice; these mice developed lung cancers similar to those seen in human patients. However, tumour regression was seen when they turned off this transgene expression, along with ERK1 and 2 dephosphorylation. These results imply that BRAF-mutant lung cancer is dependent on the ERK/MEK pathway[31]. It is likely that V600E mutants' mimic phosphorylation and are not dependent on RAS activation. On the other hand, uncommon types of *BRAF* mutations still require interaction with RAS to be activated and phosphorylated[28].

A combination therapy of BRAF inhibitor and MEK inhibitor have shown an overall response rate of 63% in non-small cell lung cancer patients with *BRAF* V600E mutation. These encouraging results has led to Food and drug administration (FDA) approval for the combinational therapy in lung cancer patients with tumour harbouring *BRAF* V600E mutation after progression on platinum chemotherapy[32].

1.3.5 *PIK3CA* mutations

PI3K belongs to the PI3K protein family and is a lipid kinase that typically produces phosphatidylinositol-3-phosphate, which plays a central role mediating growth factor receptors and downstream signaling in the cell[33]. The P110a isoform is encoded by *PIK3CA* gene and is the main catalytic subunit of PI3K[34]. Mutations in the *PIK3CA* gene are not frequent in lung cancer, representing about 2% of non-small cell lung cancer cases; however, *PIK3CA* mutations are present in about 30% of gastric cancers and glioblastomas[34]. Point mutations are the common *PIK3CA* mutations in lung cancer (e.g. mutations in E542K and E545K resulting in glutamic acid to lysine replacement). In the absence of growth factors, mutations of *PIK3CA* will lead to acquire enzymatic function *in* vitro. In addition, PI3K/AKT signaling get activated when growth factors are absent[35]. Oncogenic cellular transformation is also induced by *PIK3CA* mutations[36].

PIK3CA amplifications have been reported in squamous cell carcinoma and smokers[37,38]. Mutant allele do not usually display any kind of gene amplifications in tumours harboring *PIK3CA* mutations[37]. Biologically, *PIK3CA* mutations have not yet shown any oncogenic activity. These mutations can appear in tumours harbouring *EGFR* mutations, and are common in adenocarcinomas and squamous cell carcinomas[39].

Dactolisib, a small molecule targeting PI3K and mTOR proteins inhibits their activity and has antitumour activity in mice[40]. Many PI3K inhibitors are in clinical development, although low response rates have been observed for single agents[41].

1.4 Treatment of non-small cell lung cancer

1.4.1 Overview of lung cancer treatment

Lung cancer treatment relies on the clinical stage and the health condition of the patient. Clinical staging of lung cancer is defined based on TNM system (Tumour, Node,

Metastasis) developed by Pierre Denoix in 1942 and revised by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC). Lung cancer patients are usually treated according to their cancer staging status. In general, surgery is the treatment of choice for early stage patients (such as stage I and II) while multimodality treatments such as chemotherapy, radiation or driver gene targeted therapy, and immunotherapies are used for late stage of the disease (such as stage III and/ or IV) (Table 1).

Table 1: Current treatment recommendation of non-small cell lung cancer patients

Stage	General treatment recommendations
IA	Surgical resection
IB	Surgical resection, can consider adjuvant chemotherapy in selected cases (e.g. tumour size > 4 cm)
IIA	Surgical resection followed by adjuvant chemotherapy
IIB	Surgical resection followed by adjuvant chemotherapy
IIIA	Multimodality treatment: chemotherapy, radiation, +/− surgery
IIIB	Multimodality treatment: chemotherapy and radiation
IV	Chemotherapy, consider targeted therapies according to driver mutations and immune checkpoint inhibitors

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1.4.2 Targeted Therapy

1.4.2.1 Targeting *EGFR* Mutations

The importance of targeted therapy for EGFR has increased when *EGFR* mutations were found in many lung tumours. Gefitinib, which is a small molecule that inhibits EGFR, has been tested in clinical trials and the drug showed modest success in unselected lung cancer patients who had tumour progression after chemotherapy. However, the drug got accelerated approval from FDA for locally advance non-small cell lung cancer patients as at that time there was no third line therapy for advanced non-small cell lung cancer. In that trial, the median duration of response for gefitinib was 7 months and the overall objective response was 10.6% (15 out of 142 patients)[42]. Erlotinib, another EGFR inhibitor, had similar results in the BR.21 trial that led to FDA approval on lung cancer patients who did not respond well to standard chemotherapy[43]. Interestingly, a small group of non-small cell lung cancer who have specific genetic mutation were more responsive to gefitinib[44]. These results transformed treatment approaches in lung cancer patients to be more based on the genetic make-up of the patient. Also, it encouraged researchers to investigate more biomarkers that have the potential to be targeted pharmacologically.

The results of Iressa Pan-Asia Study (IPASS) trial were revolutionary as it introduced the targeted therapy with gefitinib as a first line of treatment option for lung adenocarcinoma patients[45]. The trial was undertaken in East Asia, never and light smoking lung adenocarcinoma patients were recruited to receive gefitinib or chemotherapy. The results showed that patients who had tumours with an *EGFR* exon 19 deletion or *EGFR* L858R point mutation that received the gefitinib treatment arm had increased progression free survival if they compared to those who received chemotherapy.

Even though first-generation EGFR inhibitors worked more effectively than cytotoxic chemotherapies, they failed to provide a durable response. T790M point mutation at exon 20 in the *EGFR* gene is the leading cause of EGFR inhibitor resistance (seen in 50% of relapsed patients). Thus, second generation EGFR inhibitors were developed to overcome this problem.

In pre-clinical studies, second generation EGFR inhibitors were shown to be active against T790M mutant protein, but the toxicity that resulted from the inhibition of the wild type EGFR was a limiting factor in the clinical use of these[46]. Osimertinib is a third-generation EGFR inhibitor that was developed to overcome this problem. The drug had accelerated approval from the FDA to treat lung cancer patients who have the T790M mutation and are resistant to tyrosine kinase inhibitors. Osimertinib has shown more efficacy than a combination of two chemotherapies (pemetrexed with carboplatin or cisplatin) in advanced stage lung cancer patients who had the T790M mutation, as their progression free survival was 10.1 versus 4.4 months for patients who received chemotherapy[47]. Nevertheless, some patients were resistant to osimertinib due to a C797S mutation in their *EGFR* gene, which disrupts the covalent bond formation between EGFR and osimertinib[48]. There are other third generation EGFR inhibitors similar to osimertinib under investigation and some of them such as olmutinib are against EGFR C797S (depends on residue of C797 for irreversible binding)[49].

1.4.2.2 Targeting *ALK* Rearrangement

Crizotinib, which is a drug initially developed to target tyrosine-protein kinase Met (cMET), was found to be effective against tumours with *ALK* rearrangement after the discovery of *EML4-ALK* fusion. The drug was tested in clinical trials to treat non-small

cell lung cancer patients and surprisingly showed promising results. Overall response rate was 61% of 149 pretreated lung cancer patients with *ALK* fusion and progression free survival for 10 months led the FDA to accelerate approval of the drug in the treatment of patients with advanced *ALK* fusion positive lung cancer[50]. Moreover, crizotinib was found to be better than chemotherapy in the first line setting with a median progression free survival of 10.9 months and overall response rate 74%[51]. Nevertheless, resistance mechanisms to crizotinib have been observed in lung cancer patients. There are several reasons behind the resistance, such as the presence C1156Y and L1196M point mutations. In addition, poor penetration of the crizotinib across the blood brain barrier led to brain metastasis[52].

Ceritinib, which is a second generation ALK inhibitor, was developed to overcome some of these resistance mechanisms. First, the drug is more potent than crizotinib. Second, it has the capability of blocking some of the resistance mechanisms, such as L1196M, and to cross the blood brain barrier. ASCEND-1 clinical trial showed encouraging results that led ceritinib to obtain FDA approval. The overall response rate and the progression free survival was 72% and 18.4 months respectively in ALK inhibitor naïve patients whereas ALK inhibitor pretreated patients treated with ceritnib had 6.9 months progression free survival and 56% overall response rate[53].

A resistance mechanism to ALK inhibitors is a bypass of signaling pathway activation. This kind of mechanism occurs either through mutations in *RAS*, *MEK1* and *EGFR* or amplification of MET, or even via transformation to small cell lung cancer. Essentially, driver oncogenic mutations are mutually exclusive in non-small cell lung cancer, but in the resistant setting some of these mutations occur together[54]. Therefore, it

is crucial to investigate the efficacy and possibility of combination therapy, such as ALK inhibitor and MEK inhibitor to overcome the resistance.

The *ALK-EML4* fusion gene can also be found in tumours in the absence of *EGFR* and *KRAS* mutations. Tumours with the *ALK-EML4* fusion gene are highly responsive to drugs that inhibit hyperactivity of the *ALK-EML4* fusion gene[9]. In contrast, such tumours show resistance to other types of drugs, such as the EGFR tyrosine kinase inhibitors gefitinib and erlotinib[9].

1.4.3 Immunotherapy for Lung Cancer

1.4.3.1 PD-1/ PD-L1 in Lung Cancer

Immunotherapy represents an exciting new approach in cancer treatment. Checkpoint inhibitors, in particular, are currently being explored for use in lung cancer treatments. The main goal of immunotherapy is to boost the immune system by activating the immune cells and inducing them to recognize and kill tumour cells. T cells play a critical role in many immunotherapies, and their activation depends on three key signals. First, the interaction between the T cell receptor and the antigenic peptide-major histocompatibility complex (MHC). The second one is antigen–independent costimulatory signals, which involve an activating signal like CD28, and an inhibitory signal, such as the PD-1 and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) receptor pathways. The third one is cytokines, such as interferon gamma (IFN-γ), which is secreted by immune cells, and induces the expression of PD-L1. PD-l protein has two ligands, PD-L1 and PD-L2, and those ligands belong to the B7 family. However, the major ligand is PD-L1 and it is expressed on many normal cells such as hematopoietic cells including macrophages and mast cells, as well as epithelial cells. Many tumour cells that develop from organs such as

lung, head and neck, colon, stomach and skin, express PD-L1[55]. Tumour cells evade immune surveillance via the interaction between PD-1 and PD-L1, which supress the activation of T cells. Blocking PD-1 and PD-L1 has shown greater impact than targeting PD-L2 in vivo. Generally, the interaction of PD-1 and PD-L1 plays a role in the inhibition of cell apoptosis, suppression of immune reaction to tumours and tumour evasion of the immune system[56]. There are several reasons why inhibitors of PD-1/PD-L1 interaction are particularly promising anti-cancer immunotherapies. First, tumour infiltrating lymphocytes and circulating tumour specific T cells exhibit high expression of PD-1[57]. Second, the correlation between the expression of PD-L1 and the prognosis of many cancers suggests that the expression of PD-L1 is a tumour mechanism for the evasion of immune surveillance[58,59].

There is controversy regarding the role of PD-L1 expression in tumour prognosis, as some studies have shown inferior outcomes when correlating with prognosis[60], and in another study, superior outcomes were observed[61]. However, in preclinical studies of PD-1 and PD-L1 blockade, no noticeable adverse immune related events were observed. Based on the existing evidence, PD-1 and PD-L1 inhibitors may play a role in breaking some of the multiple layers of immune inhibition and inducing an effective T cell response against tumours. Tumour cells with positive PD-L1 have noticeably higher PD-L1 expression in comparison with adjacent lung parenchyma[62]. Additionally, PD-L1 expression is associated with poor prognosis and short overall survival[63].

1.4.3.2 PD-1 and PD-L1 inhibitors

A number of preclinical and clinical studies with checkpoint inhibitors such as PD-1 and PD-L1 inhibitors have shown promising results, with robust immune responses. In

lung cancer, these inhibitors represent a beneficial tool for use in combination with targeted therapy, chemotherapy and radiotherapy.

Nivolumab is a human immunoglobulin G4 (IgG4) antibody that blocks the receptor for PD-1. The outcome of the clinical trial phase I with nivolumab was encouraging, with an objective response rate of 17% in 129 patients diagnosed with advance non-small cell lung cancer that were previously treated with chemotherapy. Furthermore, the progression-free survival in this study was 2.3 months, and 9.9 months was the median of overall survival, reflecting a clear, sustained benefit for the patients. After completion of nearly 96 weeks of continuous therapy, many patients remained in remission, and the two-year survival rate was 24%[64].

In another study, nivolumab was used in combination with platinum-based chemotherapy as a first line treatment in non-small cell lung cancer. The overall response rate was 33%, and the adverse event (AE) was grade 3 or 4 for 49% of patients. Most of these adverse effects were linked to the platinum-based chemotherapy[65].

Nivolumab has been approved for use in the treatment of non-small cell lung cancer patients with advanced stage disease that have progressed after or during platinum-based chemotherapy. This approval was based on international randomized study of 582 participants with advanced stage non-small cell lung cancer. All patients were treated either by nivolumab or docetaxel. Patients treated with nivolumab lived an average of 12 months in comparison to 9 months for those treated with docetaxel. FDA approved a test for detection of PD-L1 called IHC 28-8 pharmDx test since an evaluation of a small group of patients from the study suggested that PD-L1 expression detect by IHC in patient tumours could be helpful to predict the drug response[66].

Pembrolizumab, a human (IgG4) antibody that targets and blocks PD-1 receptor, was initially used to treat metastatic melanoma. Recently, FDA and Health Canada approved pembrolizumab as a first line treatment in patients with PD-L1-positive metastatic lung cancer that have no *EGFR* or *ALK* mutations. In a phase III clinical trial, such patients were assigned to receive either pembrolizumab or platinum-based chemotherapy. The outcome of this trial showed superiority of anti-PD-1 therapy over a platinum-based chemotherapy. This PD-1 inhibitor was superior as first line treatment in non-small cell lung cancer patients with tumour proportion score (TPS) of cell surface PD-L1 expression of least 50% of tumour cells. Pembrolizumab as a first line treatment resulted in a significantly longer overall survival (Figure 4) and progression-free survival than did platinum-based chemotherapy[7]. Additionally, the drug is used as a second line treatment for metastatic non-small cell lung cancer when TPS is equal or greater than 1%. When combined with chemotherapy to treat metastatic non-squamous, non-small cell lung cancer, no PD-L1 test is required.

Atezolizumab is another checkpoint inhibitor approved by the FDA to treat patients with metastatic non-small cell lung cancer who have disease progression during or after platinum containing chemotherapy. Atezolizumab is a humanized IgG1 monoclonal antibody against PD-L1 protein. The FDA approval was granted based on two international clinical trials that demonstrated consistent results. Atezolizumab treatment in comparison with docetaxel in patient population resulted in a 2.9 and a 4.2 month improvement in overall survival[67].

1.4.3.3 Targeted therapy and checkpoint immunotherapy for lung cancer treatment

The use of tyrosine kinase inhibitors to target oncogenic driver mutations have shown promising results. These therapies play a role in suppressing tumour growth and therefore boost the quality of life of cancer patients. However, patients treated with selective inhibitors experience tumour progression because of the secondary mutations that lead the resistance to the primary therapy[54]. However, the efficacy of checkpoint inhibitors is not active in all cancer patients; only 10 to 26% had clinical response[68]. Therefore, combinatorial therapies, driver mutation agents and check point inhibitors, could hold a promise to achieve a durable clinical response for cancer patients.

In non-small cell lung cancer, Akbay and his colleagues showed that *PD-L1* is upregulated by activation of the EGFR pathway. In addition, EGFR inhibitors reduced the expression of PD-L1 in activated EGFR non-small cell lung cancer cell lines[69]. However, Chen et al. demonstrated that combination therapy of EGFR inhibitors and PD-1 inhibitor did not show synergistic effects on killing tumour cells in a pre-clinical study[70]. It is likely that the immune escape in EGFR mutant non-small cell lung cancer is mostly regulated by the upregulation of *PD-L1* via EGFR activation. Thus, PD-1 inhibitor and EGFR inhibitor have similar but not additional effects on releasing the break of PD-1/PD-L1 pathway in EGFR mutant lung cancer. Retrospective analysis on lung cancer patients who harboured mutant EGFR or *ALK* rearrangement treated with PD-L1 inhibitors revealed that the objective response rate for those patients was very low. Patients with tumours harbouring *EGFR* mutations achieved 3.6% objective response rate while in contrast, patients harbouring tumours with *ALK* positive mutations demonstrated 23.3% objective response[71]. However, having low response of immune checkpoint blockade in EGFR

mutant lung cancer patients could be due to two reasons. First, PD-L1 expression and infiltrating T cell were very low in those patients. Second, usually lung cancer patients who exhibit *EGFR* mutations, have low mutational load and that could explain the low response to PD-1/PD-L1 pathway inhibitors[72]. Therefore, it could be hypothesized that patients with high expression of PD-L1 whose tumours harbour *EGFR* mutation, benefit from combinatorial strategy of immune checkpoint antagonist and oncogenic driver mutation inhibitors.

1.4.3.4 PD-L1 expression and tumour microenvironment

Tumour cells and other immune cells that express PD-L1 protein can be up-regulated through different mechanisms. For instance, constitutive oncogenic signaling pathways causes the up-regulation of PD-L1[68]. Another mechanism of up-regulating the PD-L1 is called adaptive immune resistance. Nemanja and his colleagues, have proven that the adaptive immune resistance in which PD-L1 expression is induced in melanoma. They have shown the PD-L1 expression on melanocytes is geographically associated with tumour infiltrating lymphocytes secreting IFN-γ in melanocytic lesions of various histologies and stages. Those findings suggest that immune cells in the tumour microenvironment that secrete molecules like IFN-γ contribute to the expression of PD-L1. Therefore, tumour infiltrating lymphocytes down regulate their own activation by inducing the expression of PD-L1[61]. In melanoma, adaptive immune resistance is well characterized. However, in non-small cell lung cancer, such assessments are still limited.

1.5 Rationale, aims and significance of the project

As mentioned, lung cancer is a leading cause of death, killing more people than breast, prostate, and colorectal cancers combined[1]. The five-year survival rate for all stages in lung cancer is only about 18%, which is lower than the other types of cancer mentioned

above. Unfortunately, more than 50% of lung cancer patients die within one year of diagnosis[2]. Even in localized lung cancer, the five-year survival is only about 55% suggesting that biomarker testing in the early stages of the disease has the potential to make a major improvement in the disease control and management. The advancements in molecular profiling in lung cancer has provided powerful tools for implementing new treatments for lung cancer, such as EGFR and ALK tyrosine kinase inhibitors. Along with the new emerging checkpoint inhibitors in lung cancer, it is expected that boosting the overall survival rate associated with lung cancer therapy will involves detecting particular gene mutations that can be targeted, as well as PD-1/PD-L1 expression. *EGFR* mutations is thought to be linked with good prognosis in lung cancer patients[73], and several reports have also shown association between the expression of PD-L1 and poor survival rate in lung cancer patients[74-75]. It is not quite clear whether RT-qPCR can be used as an alternative diagnostic method to detect PD-L1 expression in lung cancer patients. Thus, we hypothesize that the membranous expression of PD-L1 on lung tumour cells using 22C3 antibody and/ or the absence of *EGFR* mutations will be associated with unfavorable pathologic characteristics, and that *PD-L1* mRNA expression by RT-qPCR will correlate with PD-L1 protein expression using 22C3 Anti-PD-L1 by IHC. My studies were focused on three main aims. **The first aim**, was to investigate the link between the most common targetable driver mutations, such as *KRAS*, *EGFR* and *BRAF*, and clinicopathological data in a Nova Scotia cohort of lung cancer patients. **The second aim**, was to assess the relationship between clinicopathological data, druggable driver mutations and PD-L1 expression in lung cancer patients. Understanding the relationships between these factors would have clinical implications for the design of combinatorial therapies in lung cancer

patients. **Lastly, the third aim of the project**, was to assess the possibility of using RT-qPCR as a method to detect PD-L1 positivity in patient samples in comparison with IHC data.

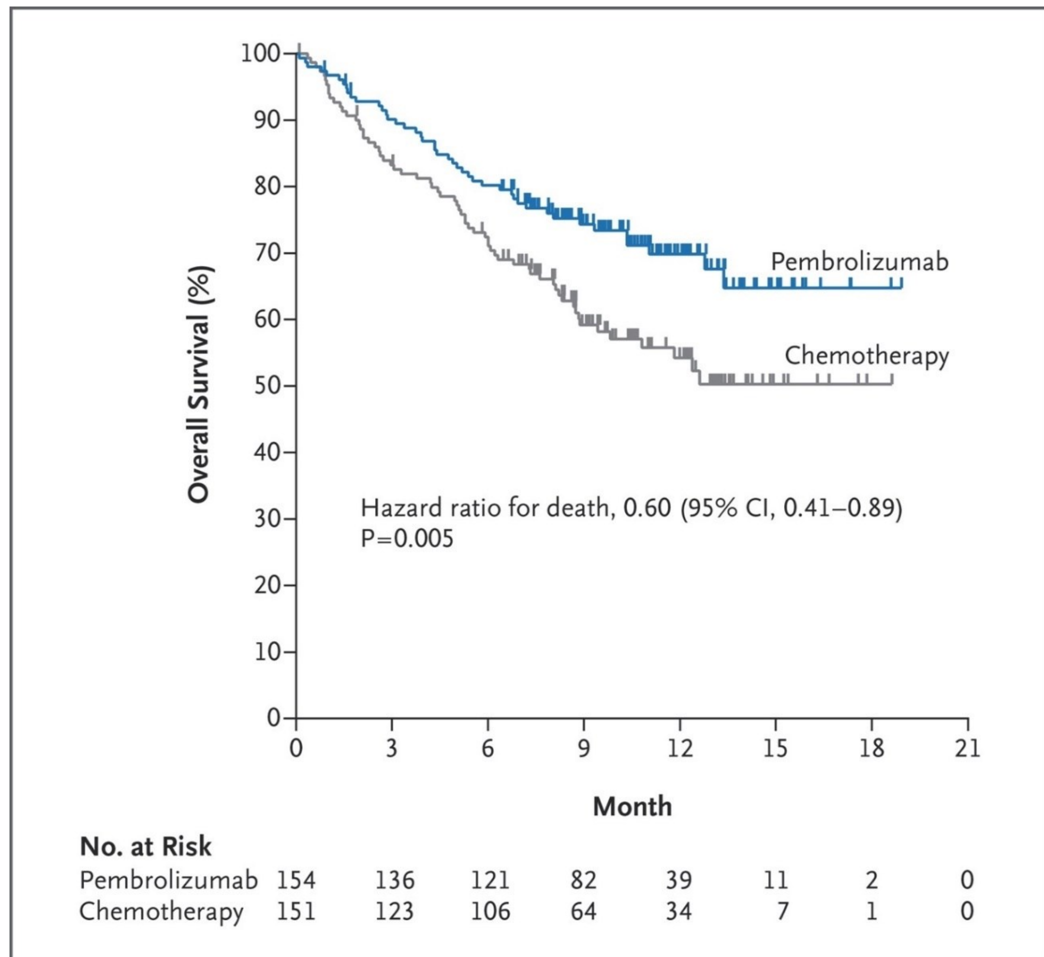


Figure 4: Overall Survival in the Intention-to-Treat Population.

Shown are Kaplan–Meier estimates of overall survival, according to treatment group. Tick marks represent data censored at the last time the patient was known to be alive. The intention-to-treat population included all patients who underwent randomization. Adopted from M Reck et al. N Engl J Med 2016; 375:1823-1833.

CHAPTER 2. MATERIALS AND METHODS

2.1 Study population

Samples from patients who underwent surgical resection for lung cancer from 2005 to 2017 at Queen Elizabeth II (QEII) Health Sciences Centre, were enrolled in the study. Nova Scotia Health Authority's Research Ethics Board approved the study and all patients provided written informed consent. A total of 851 cases formed the study cohort. All the samples that have been taken from lung cancer patients were fixed with formalin and embedded in paraffin (FFPE). In addition, fresh samples of tumour tissues were available and included in the study (**Table 2**). A 4µm-thick section from each block was mounted on a slide and stained with hematoxylin and eosin (H&E). H&E stain is a routine stain used in clinical practice for pathological assessment of tumour classification and pathological characteristics and an appropriate tissue block was chosen for further studies. All the study cases had undergone molecular profiling using two set tests. First, a multiplex polymerase chain reaction based-assay (SNaPshot platform) to detect a panel of point mutations in commonly mutated genes, including *EGFR*, *KRAS*, *BRAF* and *PIK3CA*. Second, quadruplex fragment analysis genotyping to detect deletion and insertion mutations at exon 19 and 20 in *EGFR* gene.

Demographic information, clinicopathological data including age, sex, cancer subtype, vascular invasion, lymphatic invasion, lymph node metastasis, staging, smoking history and mutational status were retrieved from lab files and medical records.

All of the patients who are included and formed the study have FFPE tumour samples. A subset of the cohort, a total number of 232 of FFPE lung tumour samples were used to quantify PD-L1 protein utilizing IHC and a total number of 49 fresh tumour

samples were used to quantify certain immune related genes utilizing real time quantitative polymerase chain reaction (RT-qPCR).

Table 2: Details of sample types included in the study

Type of test	Type sample	Technique	n
Mutational analysis	FFPE	SNaPshot; Quadruplex fragment analysis genotyping	851
PD-L1 protein expression	FFPE	IHC	232*
LCA expression	FFPE	IHC	36*
Immune related gene expression	Fresh samples	RT-qPCR	49 tumour samples*

* All these samples are part of our total number 851.

2.2 Real Time – Quantitative Polymerase Chain Reaction

2.2.1 RNA extraction

RNA from the fresh tumour samples was extracted using TRIzol (Invitrogen) and the Purelink RNA kit (Invitrogen). All 49 fresh samples were transferred into a 1.5 mL tube and 1 mL of TRIzol was added. The tube was vortexed vigorously for 15 second, and then it was incubated for one hour at 37ºC degree. After that 400µL chloroform was added to each sample, mixed vigorously by hand until it becomes cloudy, allowed to stand for 10 minutes, and centrifuged (10,000xg, for 10 minutes, at room temperature). The aqueous phase (the top colourless layer) was collected, with care taken not to collect the phenol and interface layers. An equal volume (500µL) of 70% ethanol was added to the collected layer (500µL), and then 500 µL of the combined solution was transferred into the spin cartridge column, centrifuged (10,000xg, for 1 minute), and the flow through was discarded. The rest of the mixture was loaded into the spin cartridge column, centrifuged (10,000xg, for 1 minute), and the flow through was discarded. Next, 500 µL of wash buffer I was added to the spin cartridge column, centrifuged (10,000xg, for 1 minute), and the flow through was discarded. For DNA digestion step, PureLink™ DNase (1500 U, lyophilized) (Invitrogen) was used and reconstituted by 550 uL of RNAase/DNAase free water. A 40 uL DNase solution was prepared (4 uL of DNase, 4 uL of DNase buffer, and 32 uL of RNAase/DNAase free water per sample), transferred into the spin cartridge column, allowed to stand for 10 minutes, and centrifuged (10,000xg, for 1 minute). Again, wash buffer I was added twice with the same volume and the same speed and time of spinning. After that, wash buffer II was loaded into the spin cartridge column, centrifuged (10,000xg, for 1 minute), and the flow through was discarded (this step occurred twice). The column was transferred into new collection tube, and the flow through was discarded by spinning

(10,000xg, for 2 minute) in order to fully remove any remaining of the wash buffer. The column was put in a new 1.5 mL RNase free-tube, and 40uL of RNAase/DNAase free water was added; after 10 minutes, the tube was centrifuged (10,000xg, for 2 minute). RNA was quantified by Spectramax DNA/RNA reader (Molecular Devices). The solution contains the RNA that was used to quantify expression of immune related genes utilizing RT-qPCR.

2.2.2 Complementary DNA synthesis (cDNA)

The total RNA extracted from the fresh lung tumour samples was used to generate cDNA using iScript cDNA synthesis kit (Bio-Rad). Briefly, three solutions were used to make up a 10 uL of cDNA out of the isolated RNA. The three solutions were RNAase/DNAase free water, 5x iScript supermix (2uL) and the purified RNA. RNA concentrations were equalized to 250 ng. Thus 250 ng was divided by RNA concentration to get RNA volume. After RNA volume is determined, the volume of RNAase/DNAase free water is calculated by extracting the volume of the 5x iScript supermix and purified RNA from 10 uL. The reaction mixture was then gently spun in a microcentrifuge for a few seconds and put in a thermal cycler machine for 5 minutes at 25ºC, 30 minutes at 42ºC, 5 minutes at 85ºC, then held at 4ºC.

2.2.3 RT-qPCR

Real time quantitative polymerase chain reaction was performed using SsoAdvanced Universal SYBR supermix (Bio-Rad). All primers, listed in **Table 3**, were custom designed using an online tool (NCBI-Primer-BLAST). The reaction mixture for RT-qPCR contains 5 uL of 2x SYBR supermix, 1 uL of the primer (Forward and Reverse, 4uM stock) and 4 uL of the diluted cDNA (diluted by adding 90 uL of RNAase/DNAase

free water to the 10ul cDNA), were loaded into 384-well qPCR plate in duplicate. The qPCR plate was sealed and briefly centrifuged. The reaction mix was incubated at 95ºC for 30s, 40 cycles of incubation at 95ºC for 10s and 60ºC for 30s, and 65ºC for 5s utilizing CFX Touch Real-Time PCR Detection System (Bio-Rad). Standard curves were generated to incorporate primer efficiencies and relative levels of mRNA were calculated utilizing internal reference genes TATA-Box Binding Protein (*TBP*) and Ribosomal Protein L13a (*RPL13A*).

Table 3: List of primers used in the study

Primers	Nucleotide sequence (5’ → 3’)
PD-L1	Forward: TATGGTGGTGCCGACTACAA Reverse: TGCTTGTCCAGATGACTTCG
PD-1	Forward: AAACTGGTACCGCATGAGCC Reverse: TTGTGTGACACGGAAGCGG
CD45	Forward: CCTTCCCCCACTGGATTGAC Reverse: CTTTCAAAGGTGCTTGCGGG
CD8	Forward: TTCTCGGGCAAGAGGTTGG Reverse: CAGGGCCGAGCAGAAATAGTA
CD3	Forward: CACCTGTTCCCAACCCAGAC Reverse: AGATGCGTCTCTGATTCAGGC
TBP	Forward: GGCACCACTCCACTGTATCC Reverse: GCTGCGGTACAATCCCAGAA
RPL13A	Forward: TTGGACTTTCCACCTGGTCATAT Reverse: GTGTACAACAGCAAGCTCATGCT

2.3 Immunohistochemistry

For PD-L1 expression analysis, immunohistochemistry using an automated stainer (link 48, Dako), was performed on 5µm sections cut from archival (FFPE) tumour samples from 232 patients diagnosed with non-small cell lung cancer that were retrospectively selected from the QEII Health Sciences Centre. PD-L1 IHC using the PD-L1 22C3 pharmDx kit on the Dako platform was performed according to manufacturer recommendations[76]. The positive and negative controls were from a known PD-L1 IHC positive and negative cases confirmed by IHC testing. The pharmaDx kit (Dako) is designed to perform the staining using a linker and a chromogen enhancement reagent. Pre-treatment of the slides including deparaffinization, and rehydration was performed using PT Link machine. Next, specimens were incubated with monoclonal mouse IgG antibody to PD-L1. After that, specimens were incubated with a mouse linker followed by incubation with a ready-to-use Visualization Reagent consisting of Goat secondary antibody molecules against mouse immunoglobulin and horseradish peroxidase molecules coupled to a dextran polymer backbone. Then, chromogen and chromogen enhancement reagents were added; these result in a visible brown color at the site of the antigen. All slides were cover slipped and visualized using a light microscope.

2.4 Interpretation of PD-L1 expression by immunohistochemistry

All immunstained slides for PD-L1 were evaluated. Every PD-L1 stained slide had a paired H&E slide from the same block in order to identify the tumour cells if it was not clear in PD-L1 stained slide. PD-L1 protein expression is determined by using Tumour Proportion Score (TPS), which is the percentage of viable tumour cells showing partial or complete membrane staining.

2.5 Evaluating tumour associated lymphocytes using Leukocyte common antigen (LCA) immunohistochemistry

A subset of the cohort, 36 non-small cell lung cancer samples, were evaluated via immunohistochemical for the presence of tumour infiltrating lymphocytes using LCA (CD45) marker. All slides were semi-quantitatively evaluated and given a percentage based on positively stained lymphocytes compared with the total number of nucleated cells in the tumour area. Furthermore, positivity of LCA marker was evaluated in normal tissue areas using the same evaluation method for LCA presence in the tumour area.

2.6 Statistical Analysis

The association between the gene mutations and clinical and pathological features, as well as the association between PD-L1 expression and clinicopathological data, molecular alterations, and LCA was evaluated. Statistical analysis was performed using SAS 9.3 software (version 14.0; StataCorp, College Station, TX). Categorical variables were compared using the Pearson x^2 or Fisher' s exact tests, as appropriate, and continuous variables were analyzed using a Wilcoxon rank sum test (Mann-Whitney U test). Statistical comparisons were made by a two-tailed Student's t-test, Spearman correlation using GraphPad Prism software (GraphPad, San Diego, USA). All hypothesis tests were two-sided, and a p value less than 0.05 was considered statistically significant.

CHAPTER 3. RESULTS

3.1 Patient characteristics

The clinical and mutational characteristics of lung cancers from the Nova Scotia region were assessed and compared with another cohort. A total of 851 eligible patients with non-small cell lung cancer were enrolled in the Nova Scotia cohort between 2005 and 2017 at the QE II Health Sciences Centre, Halifax, Nova Scotia, Canada. The vast majority of patients had adenocarcinoma histology (65%). The rest were divided between squamous cell carcinoma (24%), large cell carcinoma (6%), and rare subtypes (5%) (Figure 5). Most of the patients were stage I (56%), followed by stage II (26%), and stage III (16.3%), and IV (1.4%), respectively (Figure 6). Men and women represented equal proportions (49% and 51%, respectively). The mean age at diagnosis was 66 years (range, 34-90).

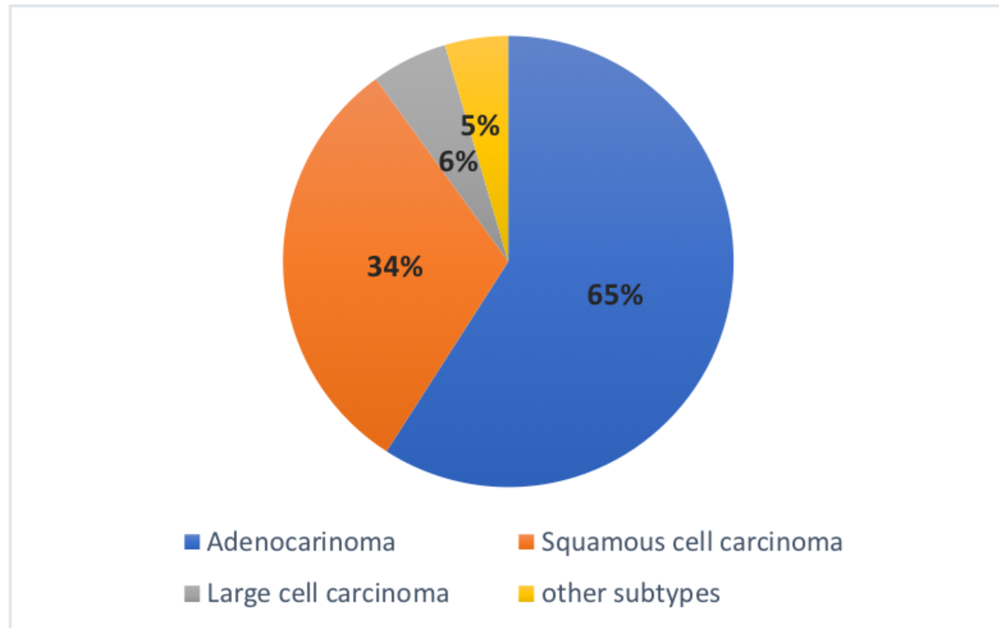


Figure 5: Percentages of lung cancer subtypes in the cohort.

This pie chart shows only the percentages of non-small cell lung cancer subtypes in the Nova Scotia cohort. Lung cancer has two types, small cell and non-small cell lung cancer. The later has three major subtypes, adenocarcinoma, squamous cell carcinoma and large cell carcinoma which all are represented in the pie chart.

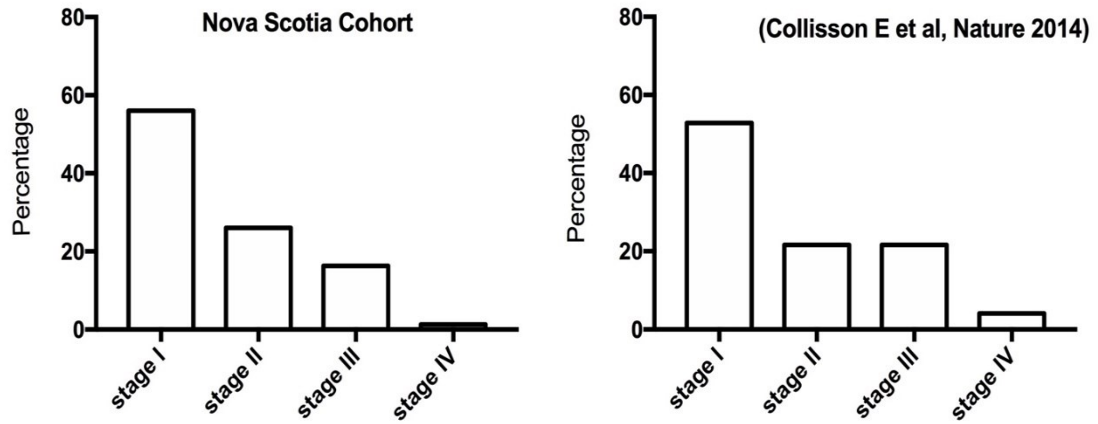


Figure 6: Percentages patient tumours that were associated with a specific clinical stage in the Nova Scotia and Collisson E et al. Nature 2014 cohorts.

For simplicity, these bar graphs were made to show only four clinical stages. Clinical stage was determined at the time of diagnosis. The total number of patients in Nova Scotia cohort is 718, and the TCGA cohort (Collison E et al. Nature 2014) is 230 patients (data accessed through cBioportal).

3.2 Correlation between molecular alterations and clinicopathological characteristics

3.2.1 The frequency of gene mutations in non-small cell lung cancer in two different cohorts

The frequency of specific gene mutations in the Nova Scotia cohort was compared with another cohort obtained from cBioportal (Collisson E et al, Nature 2014). In the Nova Scotia cohort, of 851 lung cancer patients, 553 were lung adenocarcinoma, specific gene mutations were identified in 271 (48.9%) of lung adenocarcinoma cases. These consisted of 199 *KRAS* mutations, 56 *EGFR* mutations, 6 *PIK3CA* mutations, 9 *BRAF* mutations and one *ALK* rearrangement. The details of those molecular alterations in Nova Scotia cohort including all lung cancer subtypes are described in **Table 4**. Two patients exhibited two mutations (*EGFR* & *PIK3CA* and *KRAS* & *PIK3CA*, respectively). Moreover, in the other cohort consisting of 230 lung cancer patients (Collisson E et al, Nature 2014), specific gene mutations were identified in 102 (44%) of the cohort, consisting of 69 *KRAS* mutations, 18 *EGFR* mutations, 8 *PIK3CA* mutations, 4 *BRAF* mutations and three *ALK* rearrangements (Figure 7). In both cohorts, mutations in *KRAS*, followed by *EGFR*, were more common, while *PIK3CA*, *BRAF* and *ALK* mutations were rarer.

Table 4: Details of molecular alterations in Nova Scotia cohort

Mutation	N, (%)
KRAS mutations	212 (72.4)
G12X	
EGFR mutations	59 (20.1)
L858R	25
Exon 19 deletions	31
Exon 20 insertions	3
BRAF mutations	
V600E	9 (3.1)
PIK3CA mutations	12 (4.1)
E545K	6
E542K	6
ALK rearrangements	1 (0.3)
Total	293

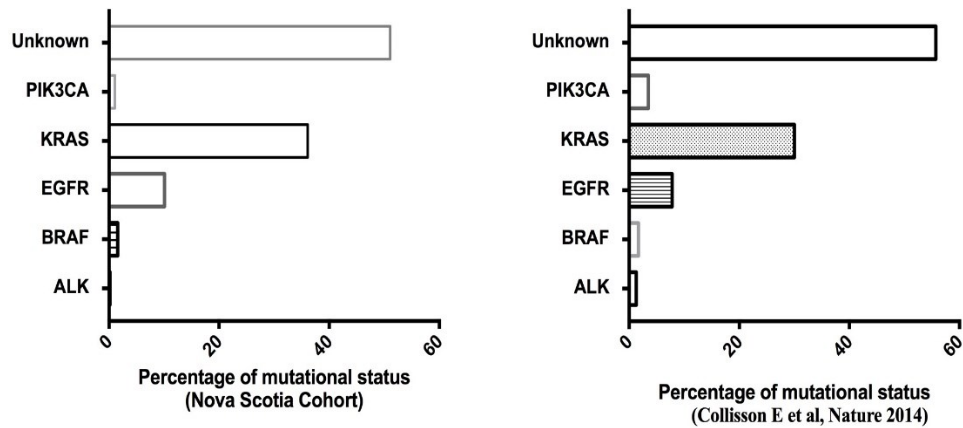


Figure 7: Frequency of gene mutations in lung cancer patient tumours in the Nova Scotia and Collisson E et al. Nature 2014 cohorts.

Lung cancer patients were screened for five molecular biomarkers. This bar graph only shows the percentage of each one. Unknown bar represents the negative result out of those five biomarkers. TCGA data were obtained from cBioportal (Collisson E et al, Nature 2014).

3.2.2 The association between molecular alterations and clinical variables

Clinicopathological characteristics of the Nova Scotia cohort were correlated with *KRAS* and *EGFR* mutations. *EGFR* mutations were significantly associated with female versus male patients (p<0.001). *KRAS* mutations were more prevalent in the younger group, ranging from 34 to 59 years (p=0.03). In addition, never smokers with non-small cell lung cancer were significantly associated with *EGFR* mutations (p<0.001). These clinical variables are summarized in Figure 8. Moreover, in the other cohort consisting of 230 lung cancer patients obtained from cBioportal (Collisson E et al, Nature 2014), *EGFR* and *KRAS* mutations did not correlate with sex (p=0.75) or age (p=0.60). However, *EGFR* mutations significantly correlated with never smokers with lung cancer (p<0.001, Figure 8). Collison et al. used exome sequencing technologies for molecular profiling of lung adenocarcinoma, the comparison was performed between the mutual mutations in the two cohorts, as described in our cohort in **Table** 4.

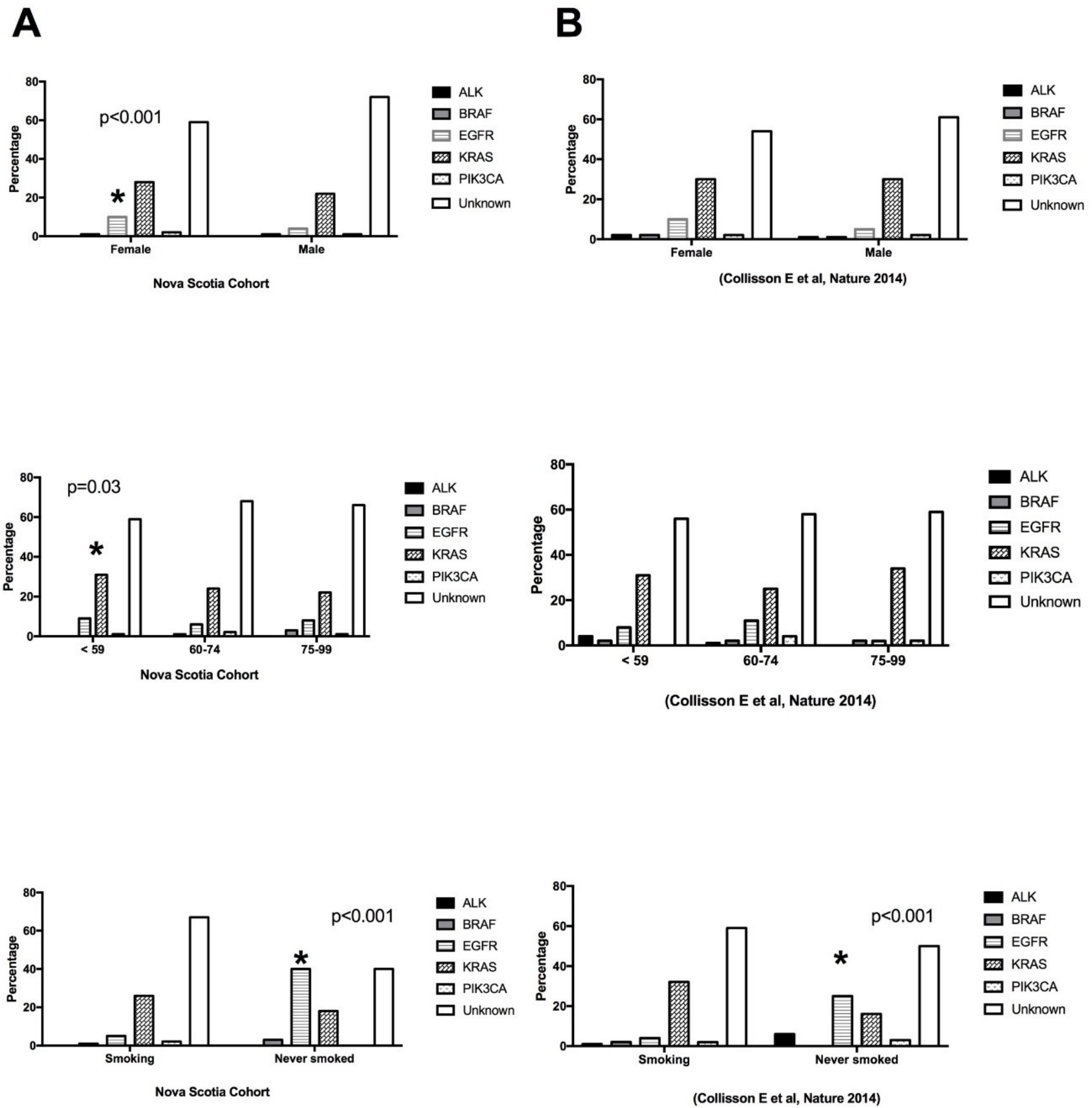
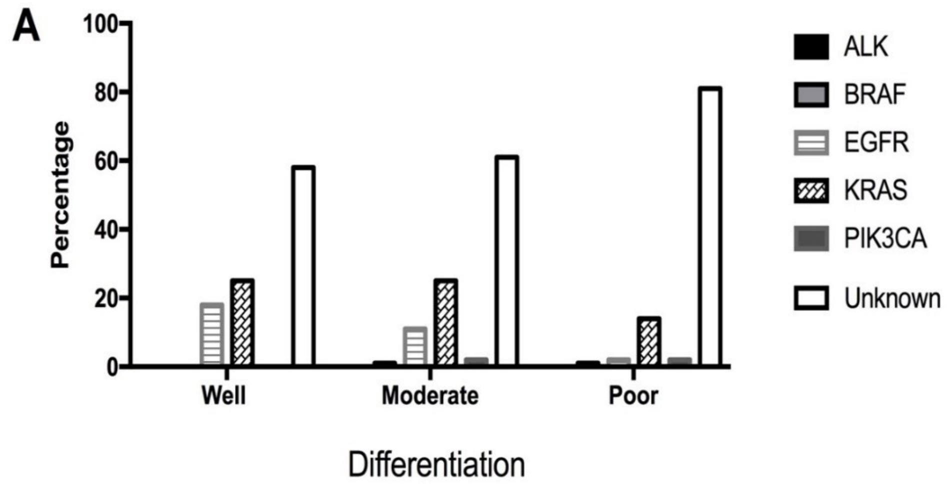


Figure 8: *EGFR* tumour mutations are associated with female and never smokers while *KRAS* tumour mutations are associated with younger ages.

(A) The association between molecular alterations and clinical parameters in the Nova Scotia cohort shows *EGFR* mutations are associated with female and never smoker patients ($p<0.001$, $p<0.001$) and *KRAS* mutations are associated with younger patients ($p=0.03$). **(B)** In the Collison et al. Nature 2014 cohort, only *EGFR* mutations are associated with never smoker patients ($p<0.001$) while there is no association between mutations and sex or age.

3.2.3 The association between the molecular alterations and pathological variables

I determined if these specific gene mutations are associated with specific histopathological data, such as the tumour grade, or pleural, vascular, lymphatic invasion and lymph node metastasis. A significant association between mutations and the presence of the tumour cells in vascular/lymphatic channels or the tumour grade could indicate a poor or good prognostic status. Well differentiated histology was significantly associated with *EGFR* tumour mutations, but not for *KRAS* mutations ($p<0.001$). Poorly differentiated histology was significantly associated with the absence *EGFR* and *KRAS* mutations ($p<0.001$). Patients who had lung adenocarcinoma histology were significantly associated with *KRAS* and *EGFR* mutations ($p<0.001$), but other subtypes such as squamous cell and large cell carcinomas, were associated with the absence of *KRAS* and *EGFR* mutations ($p<0.001$) (**Table** 5). The absence of vascular invasion was associated with *EGFR* mutations ($p<0.01$), but no mutations were significantly associated with pleural or lymphatic invasion and lymph nodes metastasis. All pathological features are shown in Figure 9. **Table** 5 shows only the significant association between the variables and gene mutations.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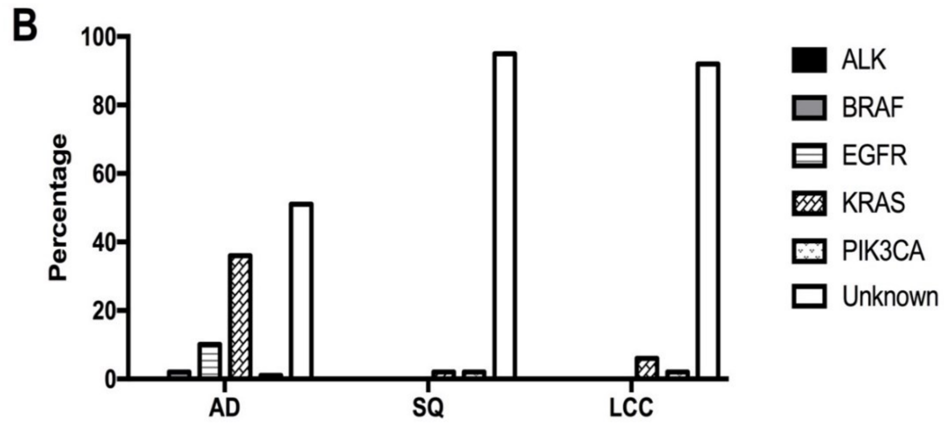
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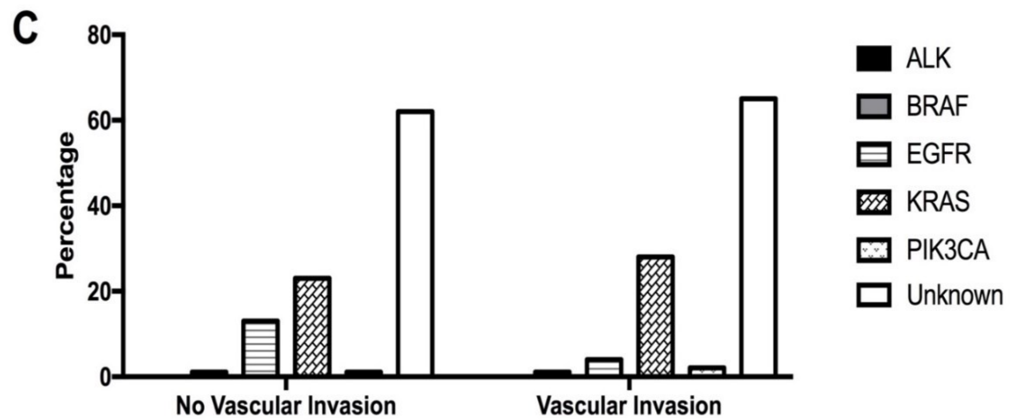
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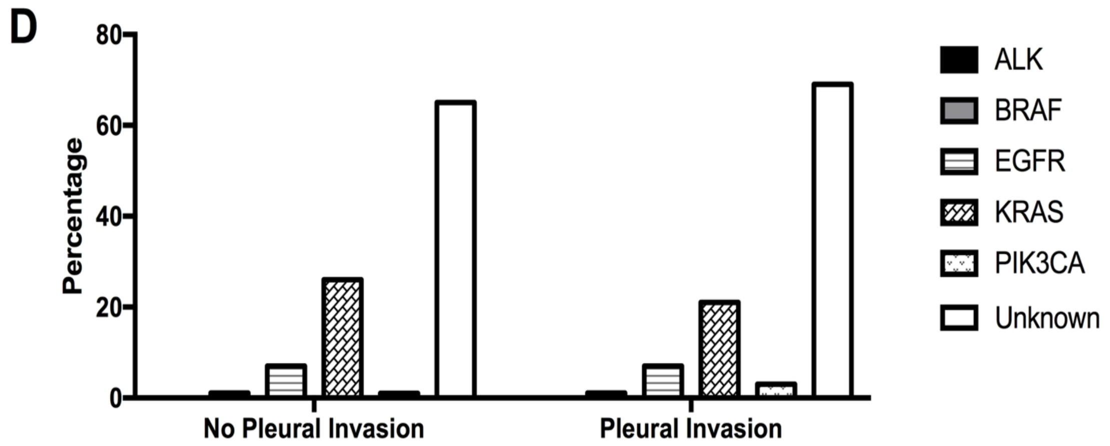
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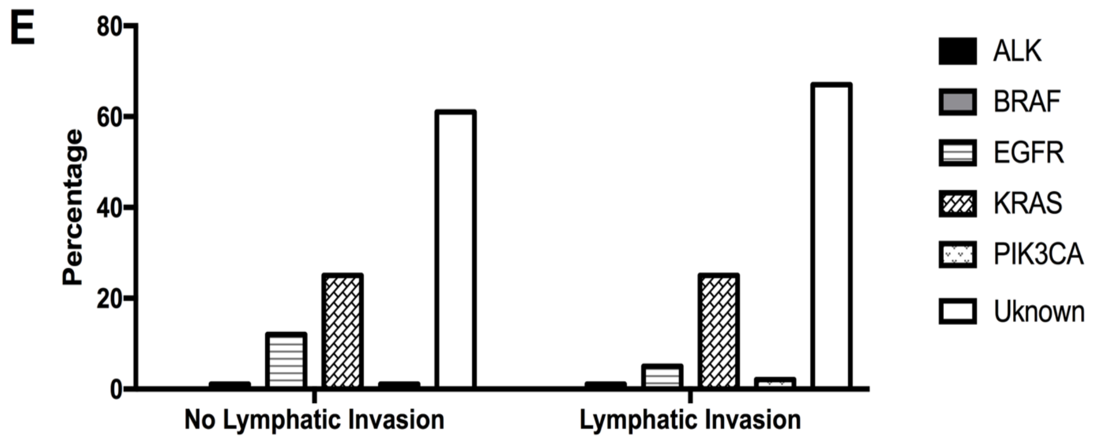
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PIK3CA
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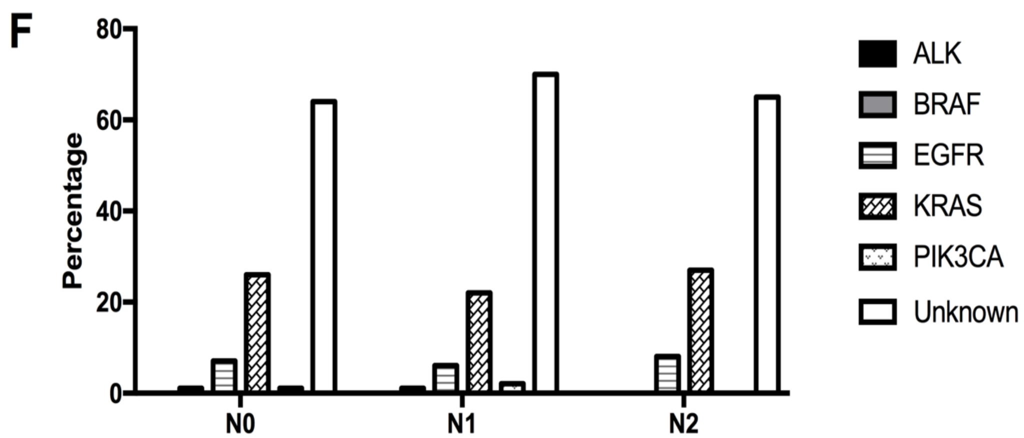

F
80
60
40
20
0
Percentage
N0
N1
N2
ALK
BRAF
EGFR
KRAS
PIK3CA
Unknown

Figure 9: *EGFR* mutations are associated with the absence of vascular invasion.

Pearson's coefficient x^2 test was done to assess the association between each variable and gene mutation. **(A)** The association between molecular alteration and the degree of lung histological differentiation. *EGFR* mutations were associated with well differentiated histology ($p<0.001$). Poorly differentiated histology was associated with the absence of both *EGFR* and *KRAS* mutations ($p<0.001$). **(B)** The association between molecular alteration and the type of lung tumour histology. AD, SQ and LCC stand for adenocarcinoma, squamous cell carcinoma and large cell carcinoma. *EGFR* and *KRAS* mutations is significantly associated with AD and negatively with SQ carcinoma ($p<0.001$). **(C, D, E)** The association between poor prognosis factors (pleural, vascular, lymphatic invasion) and molecular alterations. *EGFR* mutations are negatively associated with vascular invasion. **(F)** The association between molecular alterations and lymph node metastasis. N0= no presence of tumour cells in the lymph nodes. N1= tumour cells present in ipsilateral peribronchial, hilar, and intrapulmonary nodes, N2= tumour cells present in ipsilateral mediastinal and subcarinal nodes.

Table 5: A summary of all significant association between variables and gene mutations in Nova Scotia Cohort

		EGFR mutations			*KRAS* mutations		
	N	Obs*	Exp*	P	Obs	Exp	P
Age < 59	179	16	12.4		55	44.6	*
Male	416	15	28.8	***	92	103.6	
Female	435	44	30.2	***	120	108.4	
Vascular invasion	362	14	25.1	**	101	90.2	
No vascular invasion	489	45	33.9	**	111	121.8	
Smoked	668	27	41.2	***	175	170.3	
Never smoked	46	17	2.87	***	7	11.7	
Adenocarcinoma	552	56	38.5	***	199	137.0	***
Squamous cell	205	1	14.3	***	4	50.9	***
LCC	51	0	3.6		3	12.7	**
Well differentiated	85	15	5.9	***	21	16.3	
Moderately differentiated	320	34	22.3	**	81	61.3	***
Poorly differentiated	441	10	30.8	***	60	84.4	***

* $P < 0.05$ (two-tail); ** $P < 0.01$ (two-tail); *** $P < 0.001$ (two-tail) - Agresti Z-test based on the standardized residual.
* Observed
* Expected

3.3 Correlation of PD-L1 membranous protein expression with clinicopathological characteristics

To determine if PD-L1 protein expression on tumour cells correlates with clinicopathological characteristics we performed IHC on a portion of lung cancer patient tumour samples. Of the 232 lung cancer cases (100 males and 132 females with the median age of 67), 114 (49%) cases demonstrated PD-L1 membranous staining on tumour cells using 1% as a cutoff (almost half of patients) and 118 (51%) showed PD-L1 expression < 1% (Figure 10). Therefore, 1% cutoff represents the median for PD-L1 membrane staining in the cohort.

Out of 232 patients, pathologic staging was available only for 163 patients and smoking data were available for 162 patients. One hundred and fifty-four (95%) of patients were smokers. Stage I disease occurred in 92 (56.4%) while stage II and III occurred in 40 (24.5%) and 28 (17.2%) respectively. Only 3 (1.8%) patients were at stage IV. Some of the clinicopathological features of the cohort were correlated with PD-L1 expression using 1% cutoff. There was no significant association between PD-L1 expression and age, sex, pathological stage and smoking status. Greater than 1% PD-L1 membranous expression on tumour cells was significantly associated with vascular invasion (p=0.035), but not for pleural, lymphatic invasion and lymph nodes metastasis. All those variables and others are shown in **Table** 6. This suggests that PD-L1 expression on tumour cells is associated with more invasive disease.

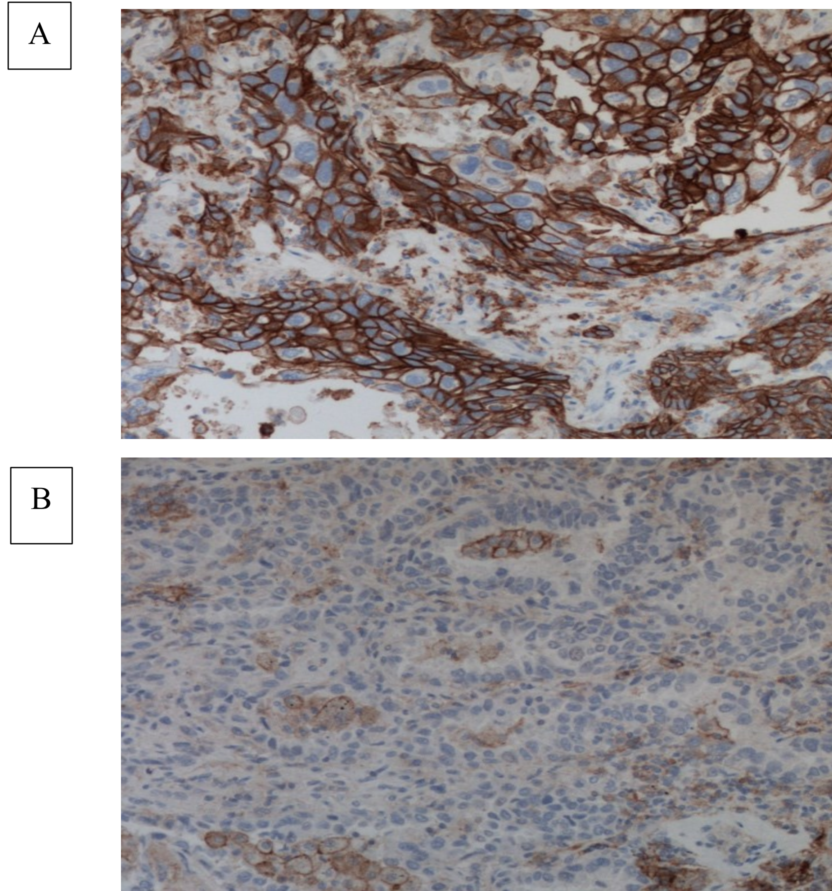


Figure 10: PD-L1 expression on lung tumour tissue.

(A) Representative histological image of a positive PD-L1 expression on lung tumour tissue. (B) Representative histological image of a negative PD-L1 expression on lung tumour tissue.

Table 6: Clinicopathological characteristics and molecular alterations of lung adenocarcinoma patients stratified by PD-L1 expression on tumour cells

	PD-L1 expression (≥1% vs. <1%)		
Variable	PD-L1+ no (%)	PD-L1- no (%)	P
All patients	114	118	
Sex			0.069
Female	58 (51)	74 (63)	
Male	56 (49)	44 (37)	
Age			0.902
< 60	23 (20)	25 (21)	
60-74	68 (60)	67 (57)	
>75	23 (20)	26 (22)	
Smoking			0.065
Never	1	7	
smoked	77	77	
Tumour size in cm (IQR)[1]	2.4	2	0.851
T status (pT)[2]			0.255
T1	41 (36)	49 (41)	
T2	50 (44)	53 (45)	
T3	18 (16)	9 (8)	
T4	5 (4)	7 (6)	
N status (pN)[3]			0.856
N0	79 (71)	78 (68.4)	
N1	19 (17)	23 (20.2)	
N2	13 (12)	13 (11.4)	
Pathologic Stage			0.830
I	44 (56)	48 (56)	
II	21 (27)	19 (22)	
III	12 (15)	16 (19)	
IV	1 (1)	2 (2)	
Pleural invasion[4]			0.060
0	72 (37)	88 (75)	
1	42 (63)	30 (25)	
Lymphatic invasion			0.057
0	68 (61)	85 (72)	
1	45 (39)	33 (28)	
Vascular invasion			0.035
0	47 (41)	65 (55)	
1	67 (59)	53 (45)	

[1] interquartile range

[2]T_1= tumour 3 cm or less; $T_{2=}$ tumour more than 3 cm but ≤ 7 cm; T_3=tumour more than 7 cm; T_4=tumour of any size that invades any of the following: mediastinum, heart, great vessels, trachea

[3] N0= no tumour cells in lymph nodes. N1= tumour cells present in ipsilateral peribronchial, hilar and intrapulmonary nodes, N2= tumour cells present in ipsilateral mediastinal and subcarinal nodes.

[4] 0= absent; 1= present.

3.4 Correlation between PD-L1 expression and major driver mutations

We also assessed if there is an association between PD-L1 membranous staining on tumour cells using 1% cutoff and the presence of the *EGFR* and *KRAS* mutations. This will help determine if there is a relationship between PD-L1 and *EGFR* and *KRAS* gene mutations, which may have implications for the design of combinatorial therapies in non-small cell lung cancer. Molecular alterations were identified in 114 (49%) of the PD-L1 stained sub-cohort, consisting of 78 *KRAS* mutations, 23 *EGFR* mutations, 5 *BRAF* mutations and 8 *PIK3CA* mutations. PD-L1 expression was present in 44 (56%) of the *KRAS* mutants. Conversely, PD-L1 expression was present only in 6 (26%) cases of the *EGFR* mutants. Therefore, *EGFR* mutations were significantly associated with the absence of PD-L1 expression (p=0.02) (Figure 11). However, there was no association between *KRAS* mutations and the expression of PD-L1 (p=0.10) (Figure 11). However, in the Collisson E et al, Nature 2014 dataset accessed through cBioportal, *KRAS* and *EGFR* mutations were observed in 75 (32%), 33 (14%) of the patients respectively. Low and high PD-L1 was obtained by taking the median of *PD-L1* mRNA expression. Low *PD-L1* mRNA expression was present in 20 (8%) cases of *EGFR* mutants, and 32 (13%) cases of *KRAS* mutants. High *PD-L1* mRNA expression was present in 13 (5%) cases of *EGFR* mutants, and 43 (18%) cases of *KRAS* mutant. However, there was no correlation between the status of high or low *PD-L1* mRNA expression and the presence of *EGFR* and *KRAS* mutations (Figure 12). The lack of PD-L1 protein expression data in the Collison et al. Nature 2014 cohort and the use of *PD-L1* mRNA expression by using the median as cutoff between low and high *PD-L1* mRNA expression, could explain the lack of correlation between PD-L1 and the presence of *EGFR* and *KRAS* mutations.

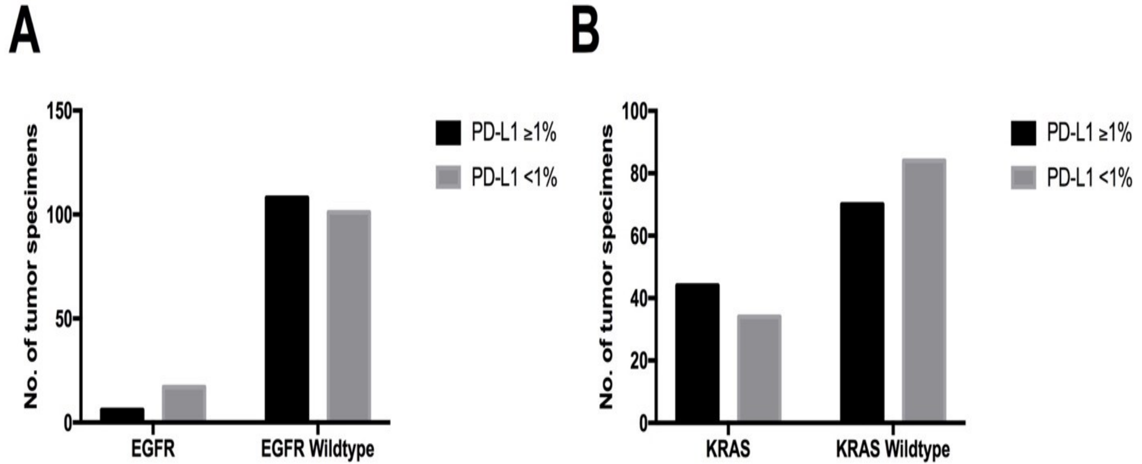


Figure 11: *EGFR* but not *KRAS* was negatively correlated with PD-L1 membranous protein expression in the Nova Scotia cohort.

A total of 232 lung tumours were evaluated for PD-L1 expression on tumour cells. All those patients were screened previously for molecular alterations. (A) PD-L1 and *EGFR* were observed in 49% and 10% of the cases respectively, and there was a negative correlation between *EGFR* and PD-L1 (p=0.02). (B) *KRAS* and PD-L1 were observed in 33% and 46% respectively, but there was not a significant correlation between these two parameters.

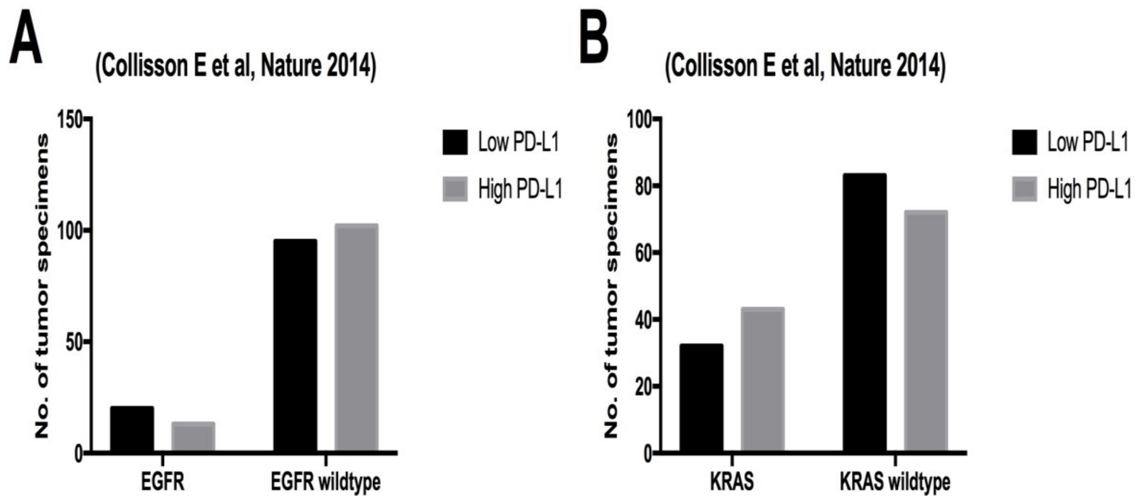


Figure 12: *EGFR* and *KRAS* do not correlate with *PD-L1* mRNA expression in Collisson E et al. Nature cohort.

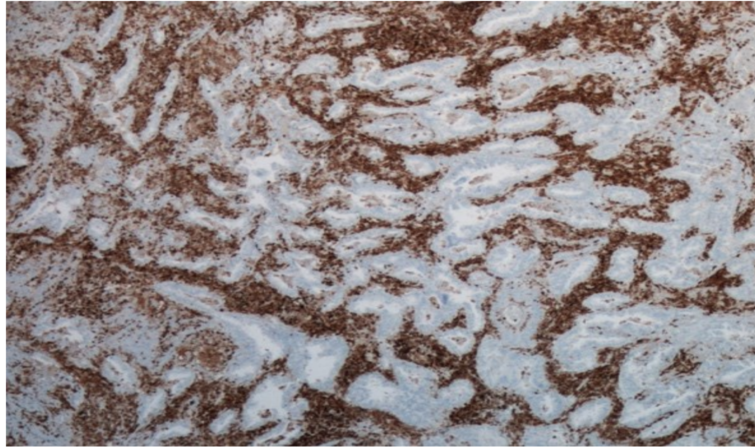
230 lung cancer patients were stratified based on *PD-L1* mRNA expression and the presence of *EGFR* or *KRAS* mutations. (A) *EGFR* mutations shows an insignificant correlation with *PD-L1* mRNA expression (p=0.25). (B) *KRAS* mutations do not correlate with the mRNA expression of *PD-L1*. Low and high *PD-L1* cases were determined by taking the median of PD-L1 expression, where half the cases are designated as high and other half as low.

3.5 Leukocyte cell abundance is correlated with PD-L1 expression in tumour tissues

We were interested to investigate the relationship between tumour infiltrating lymphocytes and PD-L1 in lung tumours, since high PD-L1 in tumours is reportedly associated with increased immune cell infiltration of tumours[77]. Thirty-six lung samples were evaluated for the presence of leukocyte cell abundance in tumours and adjacent non-tumour tissues using anti-leukocyte common antigen (LCA) antibody, which detects CD45 expression (Figure 13); the same samples were also evaluated for PD-L1 expression by IHC. Then, the relationship was assessed between lymphocyte cell abundance in normal and tumour tissues based on PD-L1 protein expression on tumour cells or on both tumour and immune cells.

Leukocyte cell abundance was significantly higher in tumour tissues than in adjacent non-tumour tissues, regardless of whether PD-L1 was present only on tumour cell or inclusive of immune cells ($p=0.004$, $p=0.01$, Figure 14). However, lymphocyte cell abundance was not significantly higher in tumour tissues than in adjacent non-tumour tissues in the absence of PD-L1 expression on either only tumour cells, or inclusive with immune cells ($p=0.62$, $p=0.44$, Figure 15). In addition, the relationship was assessed between lymphocyte cell abundance in the absence and the presence of PD-L1 membranous protein expression on tumour cells only or inclusive with immune cells. In both situations, lymphocyte cell abundance was not significant ($p=0.10$, $p=0.18$, Figure 16). This observation could suggest that the induction of PD-L1 expression is influenced by the presence of lymphocytes within tumour microenvironment, which secrete cytokines such as IFN-γ, or alternatively, that the infiltration of immune cells within tumour is promoted by PD-L1 expression on tumour cells.

A



B

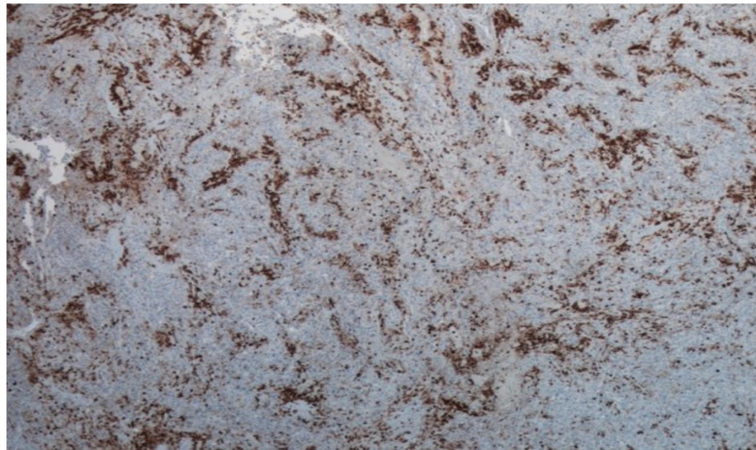


Figure 13: Leukocyte common antigen expression on immune infiltrating lymphocytes within lung tumour tissue.

(A) Representative histological image of strong expression of LCA. (B) Representative histological image of weak expression of LCA.

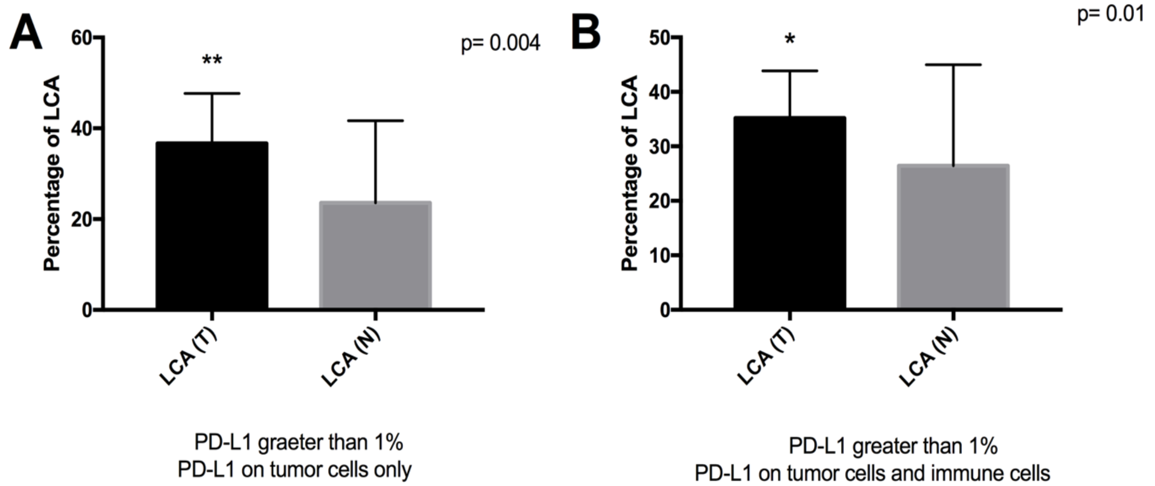


Figure 14: Leukocyte abundance was significantly more in tumour tissue than in adjacent non-tumour tissue in the presence of PD-L1.

A total of 35 lung cases were evaluated for leukocyte common antigen (LCA) on tumour tissue (T) and adjacent non-tumour tissue (N). PD-L1 expression was evaluated on tumour cells only, and on both tumour and immune cells. LCA^+ cells were assessed within the tumour tissue and adjacent non-tumour tissue and given a percentage compared with the total number of nucleated cells within tumour tissue and adjacent non-tumour tissues. The results represent the average of percentages of LCA^+ cells and the error bars represent the standard deviation.

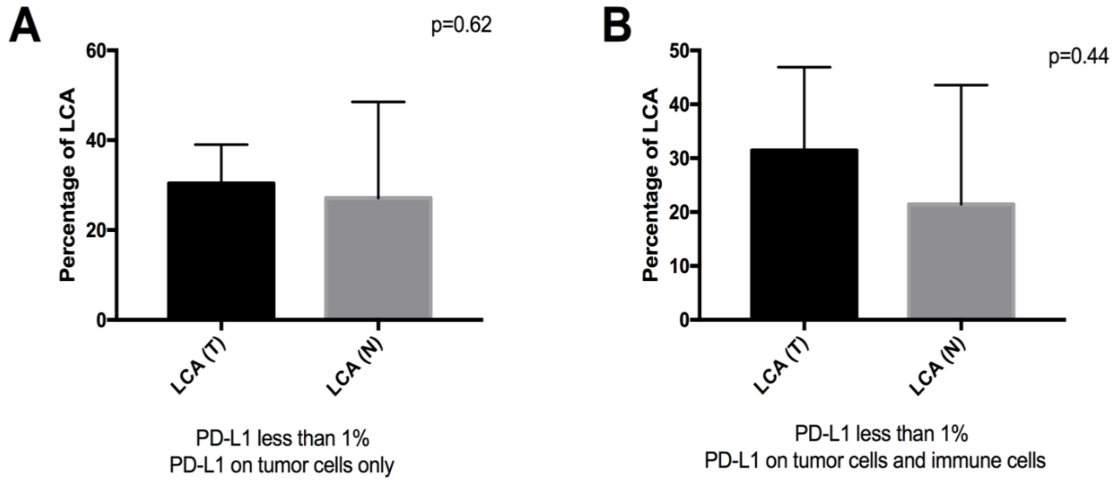


Figure 15: In the absence of PD-L1 membranous protein expression, leukocyte abundance was not significantly more in tumour tissues than adjacent non-tumour tissues.

A total of 35 of lung cases were evaluated for leukocyte common antigen (LCA) on tumour tissues (T) and adjacent non-tumour tissue (N). PD-L1 expression was evaluated on tumour cells only, and on both tumour and immune cells. LCA^+ cells were assessed within the tumour tissue and adjacent non-tumour tissue and given a percentage compared with the total number of nucleated cells within tumour tissue and adjacent non-tumour tissues. The results represent the average of percentages of LCA^+ cells and the error bars represent the standard deviation.

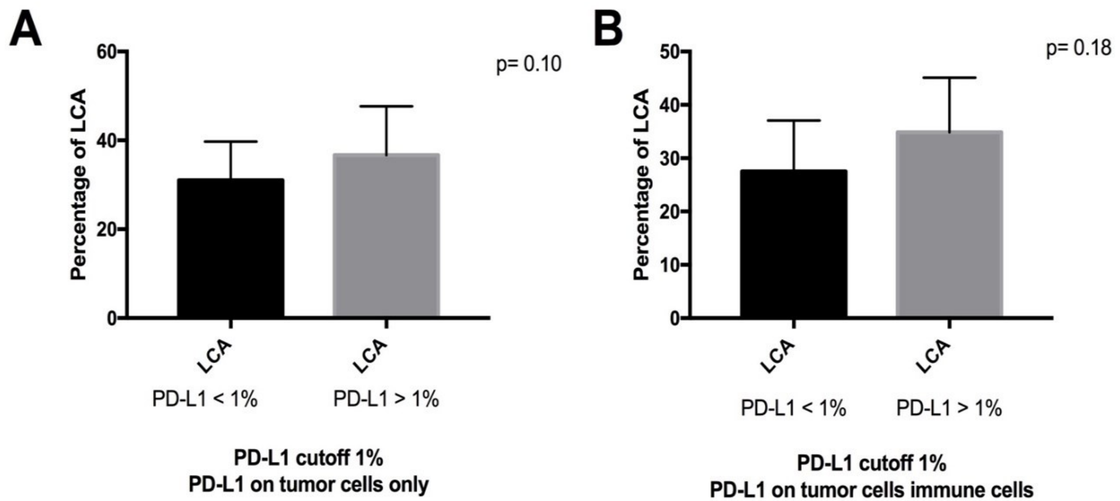


Figure 16: leukocyte cell abundance in the absence and the presence of PD-L1 expression on only tumour cells, or inclusive of immune cells was not significant.

A total of 36 lung cancer cases were evaluated for PD-L1 and for lymphocyte common antigen. PD-L1 expression was evaluated on tumour cells only, and on both tumour and immune cells. LCA^+ cells were assessed within the tumour area and given a percentage compared with the total number of nucleated cells within tumour area. The results represent the average of percentages of LCA^+ cells and the error bars represent the standard deviation.

3.6 PD-L1 membranous protein detected by IHC is correlated with mRNA levels by RT-qPCR

Here, we aimed to investigate the feasibility of using RT-qPCR as a diagnostic tool in the quantification of PD-L1; also, immune relating genes (*CD3*, *CD8* and *CD45*) and its correlation with PD-L1. The first objective is to investigate the expression of PD-L1 and other immune related genes by RT-qPCR in fresh lung samples obtained from lung cancer patients at the QEII Health Sciences Centre, Halifax, Canada. The second objective is to see if the levels of PD-L1 and immune related markers detected by RT-qPCR correlates with PD-L1 by IHC. Forty-nine of lung tumour samples were quantified for *PD-L1* mRNA transcriptional levels and three other immune related genes (*CD3*, *CD8* and *CD45*) utilizing RT-qPCR. All these forty-nine tumour samples were previously quantified for PD-L1 membranous protein utilizing IHC (**Table** 2). Comparing IHC of PD-L1 protein on tumour cells and RT-qPCR of *PD-L1* mRNA level demonstrated a good correlation (Spearman, $r = 0.29$, $p = 0.03$). In addition, correlation between IHC of PD-L1 on tumour cells including immune cells and RT-qPCR of *PD-L1* mRNA level was higher (Spearman, $r = 0.31$, $p = 0.02$) (Figure 17). This suggests the possibility of using RT-qPCR as an alternative method for detection of PD-L1 in non-small cell lung cancer patients.

Looking at the correlation with other markers (CD45, CD3, CD8) and levels of PD-L1 by IHC could help identify a significant marker that has a role in predicting response to checkpoint inhibitors along with PD-L1. CD45, which is a general biomarker for leukocytes, including T and B cells, showed insignificant correlation with PD-L1 detected by IHC for 1% and 50% cutoffs (p=0.49; p=0.12). Moreover, CD3, which is a general marker for T cells including T helper cells and T cytotoxic cells, demonstrated insignificant correlation with PD-L1 detected by IHC for 1% and 50% cutoffs (p=0.47; p=0.25).

However, CD8 which is a specific biomarker for T cytotoxic cells, was correlated with PD-L1 by IHC for 50% ($p=0.04$) cutoff but not for 1% cutoff ($p=0.57$) (Figure 18).

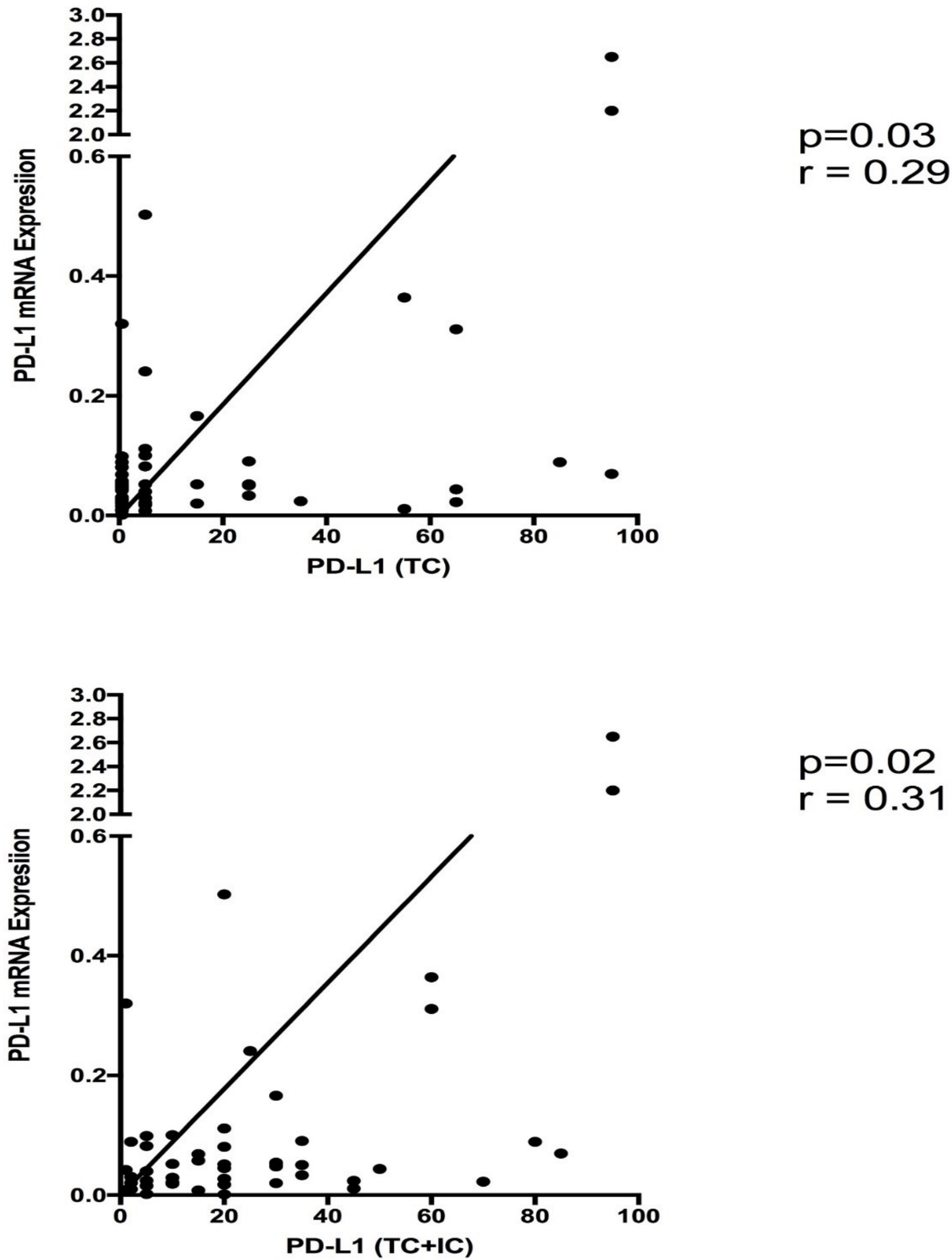
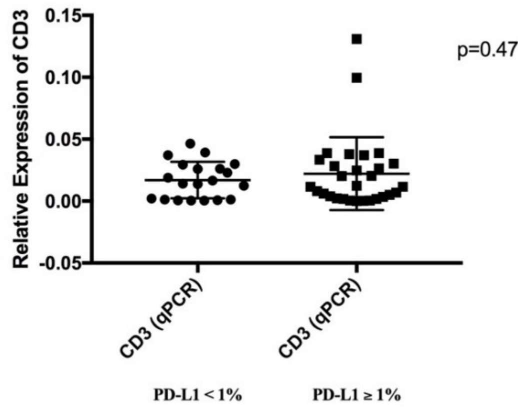
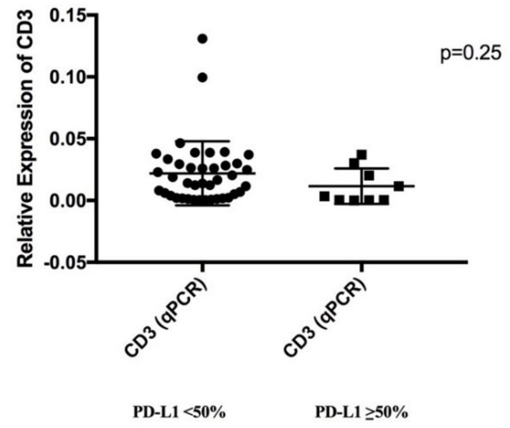
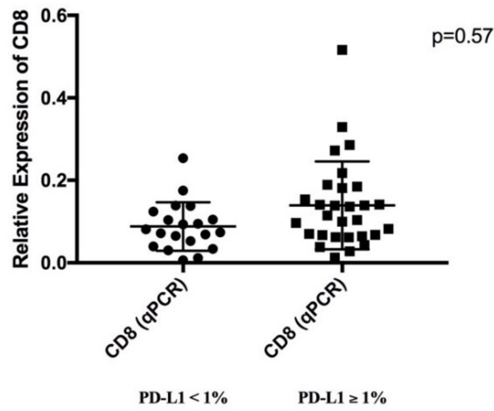


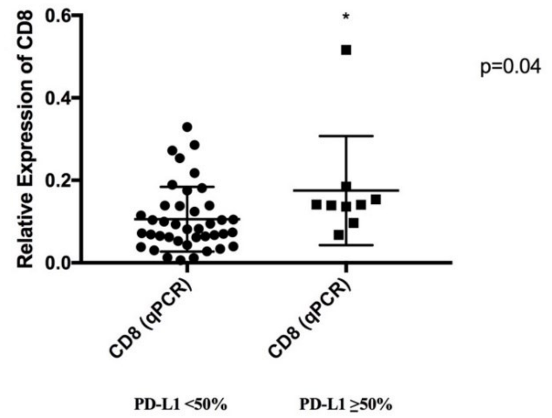
Figure 17: PD-L1 expression by IHC correlates with *PD-L1* mRNA expression by qPCR.

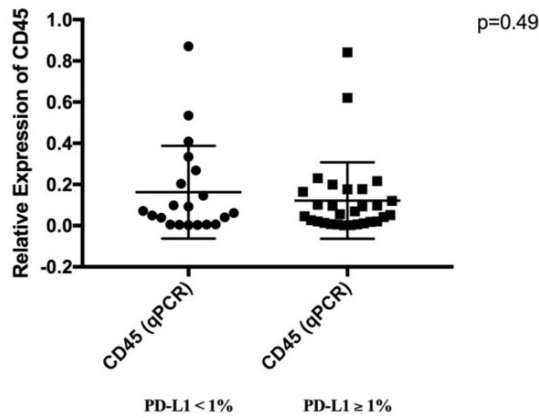
A total of 49 fresh lung tumours were evaluated for PD-L1 expression by IHC as well as quantified for *PD-L1* mRNA by RT-qPCR. PD-L1 expression was evaluated on tumour cells only (TC), and on both tumour and immune cells (TC+IC). (A) PD-L1 expression on tumour cells (IHC) is significantly correlated with *PD-L1* mRNA expression (qPCR). (B) Also, PD-L1 expression on tumour and immune cells (IHC) is significantly correlated with *PD-L1* mRNA expression (RT-qPCR).


Relative Expression of CD3
0.15
0.10
0.05
0.00
-0.05
p=0.47
CD3 (qPCR)
CD3 (qPCR)
PD-L1 < 1%
PD-L1 ≥ 1%


Relative Expression of CD3
0.15
0.10
0.05
0.00
-0.05
p=0.25
CD3 (qPCR)
CD3 (qPCR)
PD-L1 <50%
PD-L1 ≥50%


Relative Expression of CD8
0.6
0.4
0.2
0.0
p=0.57
CD8 (qPCR)
CD8 (qPCR)
PD-L1 < 1%
PD-L1 ≥ 1%


Relative Expression of CD8
0.6
0.4
0.2
0.0
*
p=0.04
CD8 (qPCR)
CD8 (qPCR)
PD-L1 <50%
PD-L1 ≥50%


Relative Expression of CD45
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
p=0.49
CD45 (qPCR)
CD45 (qPCR)
PD-L1 < 1%
PD-L1 ≥ 1%

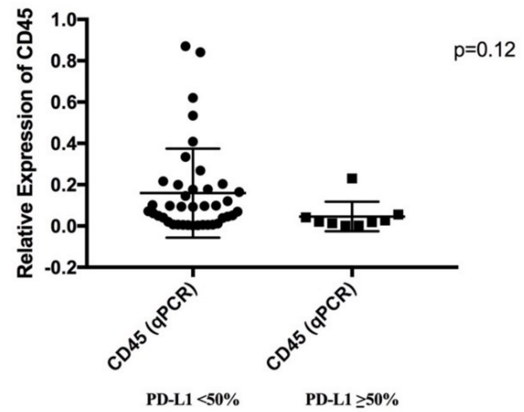

Relative Expression of CD45
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
p=0.12
CD45 (qPCR)
CD45 (qPCR)
PD-L1 <50%
PD-L1 ≥50%

Figure 18: *CD8* expression by qPCR correlates with PD-L1 expression by IHC for 50% cutoff.

A total of 49 fresh lung tumours were evaluated for PD-L1 expression by IHC as well as quantified for *CD8*, *CD3* and *CD45* mRNA by RT-qPCR. (A) *CD45* (RT-qPCR) did not correlate with PD-L1 (IHC, 1% and 50% cutoff). (B) Also, *CD3* marker (qPCR) was not significantly correlated with PD-L1 (IHC, 1% and 50% cutoff). (C) *CD8* marker (RT-qPCR) was significantly correlated with PD-L1 (IHC) for 50% cutoff but not for 1% cutoff.

CHAPTER 4. DISCUSSION

4.1 General Discussion

Lung cancer has the highest mortality rate among all tumours and is leading in terms of increasing incidence rate worldwide. The increasing number of new cases of lung cancer is generally attributed to long-term tobacco smoking (85%)[78]. However, 10%-15% of lung cancer patients have never smoked. Those "never smoker" patients may have had exposure to air pollution, asbestos, radon gas and/ or second-hand smoke[79]. Generally, lung cancer patients are diagnosed at late stage, which limits their treatment options to chemo and radiotherapy. Those conventional therapies have low cure rates and major side effects, resulting in miserable experiences for patients. Now, with the advancement in the discovery of driver gene mutations, targeted therapies against those driver mutations have shown better treatment efficacy and lower side effects for lung cancer patients. Thus, studying and analyzing those driver mutations in early stage lung cancer patients will guide efforts to develop targeted therapies. In addition, checkpoint inhibitors have shown promising results in lung cancer patients. Those inhibitors prevent the binding between PD-1 and PD-L1. The ligand PD-L1 is usually expressed on tumour cells and binds with PD-1 receptors on T-cells, leading to their inactivation. Therefore, increased levels of PD-L1 is a potential biomarker for lung cancer patient treatment options. Pembrolizumab, is an anti-PD-1 inhibitor, which has been approved by the FDA and Health Canada with a companion test, the PD-L1 IHC 22C3 pharmDx, for determining PD-L1 expression. Therefore, quantifying PD-L1 in a large cohort of molecularly annotated lung cancer patients in early disease stage will impact therapeutic strategies.

In our study, reflex molecular testing for specific gene mutations (described in **Table** 4) was applied to non-small cell lung cancer patient tumours. In addition, PD-L1 was quantified using FDA approved diagnostic test anti-PD-L1 22C3 PharmDx (Agilent Technologies, Santa Clara, CA) by IHC. We found 35% of non-small cell lung cancer patients in our cohort were positive for these specific gene mutations. Some of these mutations have currently approved targeted therapies such as *EGFR*, *BRAF*, and *ALK*. The other mutations have targeted therapies under clinical development. Moreover, we found 49% of lung cancer patients were positive for PD-L1 protein of at least 1%. Patients with tumours with at least some PD-L1 positive tumour cells may benefit from anti-PD1/PD-L1 checkpoint therapies.

4.2 The frequency of molecular alterations in the cohort

For maximizing the results, certain exon mutations were chosen for molecular analysis, and the details of these mutations were shown in Chapter 3 (**Table** 4). The mutations were selected based on their frequency and actionability. For example, a point mutation of *KRAS* exon 2 at codon 12 is the most frequent mutation in *KRAS* gene and usually linked to poor prognosis[80-81]. Point mutations such as E542K and E545K at exon 9 of the *PIK3CA* gene are highly frequent[80]. Also, the *EGFR* gene has two mutations that are the most prevalent (*EGFR* exon 19 deletion and *EGFR* exon 21-point mutation)[82]. Tumours with these two mutations in particular respond well to EGFR tyrosine kinase inhibitors (TKI)[83]. While *EGFR* exon 20 insertion mutation is relatively less frequent than the others and tumours with this mutation are typically resistant to EGFR TKI treatments[84].

Our study demonstrates data on the frequency of *KRAS* and *EGFR* mutations in a large cohort of patients diagnosed with non-small cell lung cancer that underwent surgical resection treatment over a defined period in Halifax, Canada. The frequency of *EGFR* mutations in our study was reported at 7%. This rate was different than other studies reported in the literature. For instance, an *EGFR* mutational rate of 16.6% was reported in a cohort consisting of 2105 lung cancer patients from 126 hospitals in Spain, where an extensive study analyzed the frequency of *EGFR* mutations during the period of 2005-2008[85]. One possible explanation for the higher rate of *EGFR* mutation could be differences in histological subgroups proportions, as the study demonstrated up to 78% of adenocarcinoma subgroup in comparison with our cohort that reported 65%. Considering that *EGFR* mutations are more common in adenocarcinomas and our cohort reported more than 90% of *EGFR* mutations in adenocarcinoma. Furthermore, another possible explanation is that many of the lung cancer patients in the Spanish cohort were diagnosed at later stage and biopsy specimens were used for the molecular alterations analysis, while lung cancer patients enrolled in our study were at relatively early stages and only surgical resections were used for assessment. With respect to *KRAS* mutational rate, our cohort reported 25%, which appears to be comparable with the Sequist et al. cohort study published on lung cancer patients and with other studies as well[86-87]. Therefore, our *KRAS* mutations frequency is consistent with published reports.

The frequency of some of the molecular alterations in our cohort is relatively low. For instance, we have only one patient tumour out of 851 lung cancer patients that exhibited *ALK* rearrangement (0.12%), while other studies report a frequency of 3 to 6 %[88]. In addition, our cohort has only 1.1% *BRAF* mutations which is considered to be a low

percentage in comparison with other studies[89-90]. Those low frequencies of *ALK* rearrangement and *BRAF* mutations could be attributed to the type of samples in our study, as we only have surgical resection samples and most of the patients were at early stages of lung cancer. Thus, the frequency of these mutations could increase if we include lung cancer patients from all stages, not only patients who treated with surgery at early stages. Additionally, regarding *BRAF* mutations, in our cohort, we only screened for V600E mutation which accounts for about 50% of all mutations in *BRAF* gene[30].

4.3 Association between molecular alteration and clinicopathological features

Our study shows a number of associations between clinicopathological variables and molecular alterations such as *EGFR* and *KRAS* mutations. We found that *EGFR* mutations are significantly associated with tumours from women. This result is in consistent with those published in the literature[91-92-93]. Furthermore, in regard to the presence of vascular invasion in lung cancer patients, we find that *EGFR* mutations are associated with the absence of vascular invasion and this observation was also consistent with two cohorts in the literature[91-93]. One of the possible explanation for this observation is that vascular invasion is a sign of tumour cells that could metastasize through a haematogenous route and *EGFR* mutation is thought to be linked with good prognosis[73], which may explain the negative association between vascular invasion and *EGFR* mutations.

Although lung cancer patients who never smoked in our cohort are few, we found a significant association between never smokers and *EGFR* mutations. This observation is expected as many studies have shown the association between these two variables[92-93-91]. Regarding *KRAS* mutations and smoking status of lung cancer patients, our study demonstrates no significant relationship between smokers with lung cancer and *KRAS*

mutations. This finding contradicts studies in the literature that have shown significant association between smoking and *KRAS* mutations[94,92].Thus, the strong correlation reported in the literature between *KRAS* and smoking support the impact of tobacco as carcinogens in lung cancer etiology. The exact reason of this inconsistency is unclear. However, one possible explanation for the differing results could be that the proportion of lung cancer patients who never smoke is larger than our cohort, which may have influenced the results.

4.4 PD-L1 status and its association with molecular alterations and clinicopathological features

In this study with surgically resected lung cancer patients at QEII Health Sciences Centre, we showed that membranous PD-L1 on tumour cells was associated with vascular invasion and marginally associated with pleural and lymphatic invasion. The presence of tumour cells in pleura, blood vessels, or lymphatics is an indication of poor prognosis and may contribute to metastases. Indeed, there have been several reports that indicate the association between PD-L1 and poor overall survival in non-small cell lung cancer[74-95-75].

There are two major mechanisms of PD-L1 over-expression in tumour cells; a) innate immune resistance and b) adaptive immune resistance[68]. In innate immune resistance, PD-L1 expression can be upregulated on tumour cells by constitutive oncogenic signaling independent of inflammatory signals in tumour microenvironment. Non-small cell lung cancer models that harbour *EGFR* mutations and *ALK* rearrangements have demonstrated induction of PD-L1 expression and reduction of PD-L1 when treated with targeted therapies such as EGFR and ALK inhibitors[96-97]. Furthermore, several clinical studies reported the association between PD-L1 expression and *EGFR* mutations and *ALK*

fusions[97-98-99]. However, Zhang and colleagues showed that there was not an association between *EGFR* mutations and *ALK* rearrangements and PD-L1 expression[74].

In our cohort, we found that the presence of PD-L1 in at least some tumour cells was associated with the absence of *EGFR* mutations. Furthermore, leukocytes were more frequent in the presence of tumours with at least some PD-L1 expression in comparison with its absence. This observation is consistent with the previously mentioned adaptive immune resistance, where the induction of PD-L1 expression is influenced by cytokines such as IFN-γ that is secreted from lymphocytes within the tumour microenvironment[77].

It is worth noting that due to low number of patients harbouring *BRAF*, *ALK* and *PIK3CA* mutations in their tumours, we could not analyze the association between PD-L1 expression and those mutations.

4.5 Correlation between RT-qPCR and IHC for detecting PD-L1 in lung cancer patients

Another aspect of this project is the comparison between mRNA-based and protein-based methods for the quantification of PD-L1 in lung cancer patient samples. In the clinical setting, the current method used for the detection of PD-L1 is IHC. In fact, among several agents targeting the PD-1/PD-L1 pathway, pembrolizumab is the only drug which has been approved to treat non-small cell lung cancer patients in association with a companion diagnostic test, the anti-PD-L1 immunohistochemical (IHC) 22C3 PharmDx (Agilent Technologies, Santa Clara, CA) using the Dako Autostainer (Dako, Carpinteria, CA). In our study, we aimed to evaluate the possibility and the feasibility of using RT-qPCR to determine *PD-L1* mRNA expression in comparison with the IHC FDA approved diagnostic test. As RT-qPCR could offer an efficient, cost-effective method that provides information on the level of expression of PD-L1.

Our results show that PD-L1 expression in tumour samples correlates significantly between RT-qPCR and IHC quantification methods. Fresh lung tumour samples were used to quantify PD-L1 by RT-qPCR. Therefore, for all lung cases included in RT-qPCR project, PD-L1 expression was reported in tumour cells alone, and in tumour and immune cells. As fresh samples contain tumour and non-tumour cells, PD-L1 expression determined by RT-qPCR could be coming from the tumour and non-tumour cells as well. Our study finds that PD-L1 by RT-qPCR is significantly correlated with PD-L1 expression on tumour cells by IHC ($p=0.03$, $r=0.29$). Furthermore, PD-L1 by RT-qPCR is also significantly correlated with PD-L1 expression on tumour and immune cells by IHC ($P=0.02$, $r=0.31$). As reported, the correlation is stronger when PD-L1 expression on tumour and immune cells is considered, hence the fresh sample contains tumour and non-tumour cells. Le Goux, C. et al. shows a correlation between mRNA and protein expression of PD-L1 in bladder urothelial carcinoma[100].

Significant correlation between PD-L1 protein expression by IHC and mRNA by RT-qPCR in bladder urothelial carcinoma has previously reported, using ant-PD-L1 E1L3N antibody[100], indicating a strong biological link between mRNA and protein expression regardless of the variation in the methodologies. To the best of our knowledge, our study is the first to show a significant correlation between mRNA expression of *PD-L1* utilizing RT-qPCR and protein expression utilizing anti-PD-L1 22C3 PharmDx (IHC). Our finding highlights the feasibility of using RT-qPCR as a potential method to detect PD-L1 in order to help the selection of non-small cell lung cancer patient for pembrolizumab treatment.

Moreover, immune related genes such as *CD45*, *CD3* and *CD8* was quantified in lung cancer fresh samples by RT-qPCR. We found that CD8 expression was significantly correlated with PD-L1 expression by IHC. However, CD45 and CD3 were not correlated with PD-L1. One possible explanation for these results is that CD8 is a more specific marker for cytotoxic T cells which express PD-1 and bind to PD-L1 in PD-1/PD-L1 pathway (Figure 19).

Our study has some limitations, one of the limitations is that we were selective in analyzing various mutations in each gene. For example, PI3KCA (E542K and E545K) point mutations are the only two-point mutations that have been analyzed while there are many other mutations in the same gene was not analyzed such as H1047L and H1047R. The same issue applies to the other gene of interest like *KRAS* and *EGFR*. As a result of limited funding and resources, we were unable to screen for all common mutations in each gene, thus only the most important previously mentioned mutations described in Chapter 2 were analyzed. Furthermore, most of the lung cancer patients in our cohort are diagnosed at early stage and receiving surgical resection as a standard treatment. Therefore, we do not have anti-PD-L1 response clinical data yet that will enable us to determine the best cutoff for qPCR PD-L1 data.

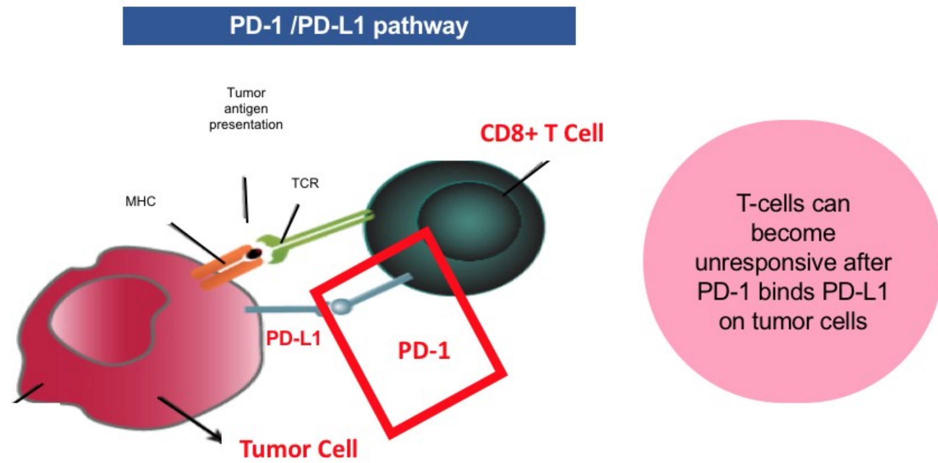


Figure 19: Programmed death -1/ Programmed death ligand-1 pathway.

One of the common mechanisms of tumour cell escape from immunosurveillance is to express negative regulator protein such as PD-L1. PD-L1 binds to its co-inhibitory receptor PD-1 on T cells and provides protection from the immune cells.

4.6 Conclusion and Future insights

Our study has analyzed the most important molecular alterations in non-small cell lung cancer patient tumours at early stages of diagnosis in Nova Scotia. Having this information from patient tumours at early disease stages will provide preliminary data for future studies in the same population and guide the efforts to develop targeted therapies, which will hopefully lead to an increase in the success lung cancer treatments.

This study highlights the expression of PD-L1, a potential biomarker for predicting response to immune checkpoint therapies, in a portion of the Nova Scotia cohort. In addition, our study has shown a significant correlation between PD-L1 and the absence of *EGFR* mutation. PD-L1 expression was also significantly correlated with vascular invasion and marginal correlation with plural and lymphatic invasion which might link the status of PD-L1 to poor prognosis. Furthermore, we have shown a significant correlation between *PD-L1* mRNA expression by RT-qPCR and PD-L1 expression by IHC, indicating the possibility of using RT-qPCR for detection for PD-L1 as an alternative method. RT-qPCR is a fairly economical method and has the capability to quantify many tissue samples at once. In addition, it is an automated method less likely to expose to human error and subjectivity. However, RT-qPCR relies on the quality of the samples and targeting transcripts that might prone to changes in posttranscriptional processes.

One of the future directions of this project is to measure 5-year survival of lung cancer patients involved in the study for gene mutations such as *EGFR* and *KRAS* and PD-L1 expression. A retrospective study could reveal which patients benefited from anti-PD-1/anti-PD-L1 therapy and if this correlated with protein and/or mRNA levels. Furthermore, it would reveal if the PD-L1 expression in immune cells is an important predictor of response. It would be interesting to confirm the makers that we investigated at the mRNA

level, such as CD8 and CD3 by IHC. Moreover, we would assess the possibility of using multiple markers such as CD45, CD8 and PD-L1 as indicators of PD-1 blockade.

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