THE EXPRESSION OF PROGRAMMED CELL DEATH LIGAND 1 IN ESOPHAGEAL CARCINOMA PATIENTS AT MUHIMBILI NATIONAL HOSPITAL

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THE EXPRESSION OF PROGRAMMED CELL DEATH LIGAND 1 IN ESOPHAGEAL CARCINOMA PATIENTS AT MUHIMBILI NATIONAL HOSPITAL

By

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Masters of Medicine in Anatomic Pathology at

Muhimbili University of Health and Allied Sciences

October 2024

CERTIFICATION

The undersigned certify that they have read and hereby recommend for examination by Muhimbili University of Health and Allied Sciences a dissertation entitled; **''The expression of programmed cell death ligand 1 in esophageal carcinoma patients at Muhimbili National Hospital'',** in (partial) fulfillment of the requirements for the degree of Master of Medicine (Anatomical Pathology) of Muhimbili University of Health and Allied Sciences.

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DECLARATION AND COPYRIGHT

I, **Doreen Gerion Gilbert**, declare that this **dissertation** is my original work and that it has not been presented and will not be presented to any other University for a similar or any other degree award.

Signature…… **Date**……...….....

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DEDICATION

I dedicate this work to my parents in appreciation of their unwavering patience, devotion, and support. I am grateful that you have always supported me, encouraged me, and given me the strength I needed along the journey. It makes me happy to witness your pride and happiness in all I accomplish. To myself, for tenacity and the conviction that, with constant attention and faith in God, I can do anything and, yes, He did truly provide for me.

ABSTRACT

Background: Esophageal carcinoma (EC) is a highly aggressive cancer with poor prognosis, which had 511,000 new cases reported globally in 2022. In Tanzania the estimated annual incidence and mortality rates of EC was 7.9% and 11.3% respectively for both sexes, ranking it 2nd among top 3 leading causes of cancer related deaths in the country with a <20% 5-year survival rate. Treatment of EC using Immune checkpoint inhibitors (ICIs') has demonstrated excellent outcomes in the resolution of tumors when used as monotherapy or in conjunction with radiotherapy and/or chemotherapy medications. The expression of Programmed cell death ligand-1 (PD-L1) on tumor cells is now used as a biomarker to identify patients who might benefit from immunotherapy. Thus, based on EC, investigating PD-L1 immunohistochemistry (IHC) expression in our settings can help identify and evaluate therapeutic candidates and their outcomes as there are no data reported as yet on this area.

Broad objective: This study aimed to determine the proportion of PD-L1 IHC expression and its association with clinicopathological factors among patients with EC at Muhimbili National Hospital (MNH).

Materials & Methods: This study was a descriptive cross-sectional evaluation of 136 EC cases from January 2019 to January 2023 at MNH in Dar es Salaam, Tanzania. Data regarding age, sex, histological subtypes, tumor location and grade were retrieved from the pathology laboratory archives. Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) 27.0. The association between PD-L1 IHC expression and clinicopathological factors was assessed using Fisher's exact test. $P \leq 0.05$ was considered statistically significant.

Results: The mean age of the patients was 58 ±14 years and males accounted for majority 66.9% (91/136) of the study population. Most 56.6% (77/136) of the tumors involved middle third of the esophagus and the predominant (89.0%) histological subtype was Esophageal squamous cell carcinoma (ESCC) (121/136), with majority (55.1%) of the tumors being grade 2 (75/136). PD-L1 IHC expression was observed in 5.1% (7/136) of the cases. No significant association was observed between PD-L1 IHC expression and any clinicopathological factors.

Conclusion: This study has shown a low proportion of PD-L1 IHC expression including its lack of association with clinicopathological factors. The variation observed in comparison

to other studies may be partly explained by several factors, including differences in the assays and antibodies used across studies, as well as sample variability. It is noteworthy however, that in our current study, all PD-L1 positive cases were ESCC and not adenocarcinoma or other subtypes. Thus, these results help inform patients' selection for PD-L1 IHC staining and subsequently targeted therapy.

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LIST OF ABBREVIATIONS

AC	Adenocarcinoma
B7-H1	B7 homolog 1
CD	Cluster of differentiation
CPL	Central pathology laboratory
CPS	Combined positive score
CTLA-4	Cytotoxic T- lymphocytes-associated protein 4.
DAB	Diaminobenzidine
DC	Dendritic cells
EC	Esophageal carcinoma
EGFR	Epidermal growth factor
ESCC	Esophageal squamous cell carcinoma
FISH	Fluorescence in situ hybridization
GERD	Gastroesophageal reflux disease
HRP	Horseradish peroxidase
ICIs	Immune check point inhibitors
IHC	Immunohistochemistry
IL	Interleukin
MNH	Muhimbili National Hospital
NK	Natural killer cells
PD-1	Programmed cell death protein 1
PDL1	Programmed cell death ligand 1
RNA	Ribonucleic acid
SOP	Standard operating procedure
SPSS	Statistical Package for Social Sciences
TLR	Toll- like receptors
TPS	Tumor proportion score
VISTA	V-domain immunoglobulin suppressor of T cell activation
WHO	World Health Organization

DEFINITION OF TERMS

A ligand is a molecule that binds to a receptor to form a complex, which serves a biological purpose.

PD-1 is a transmembrane protein predominantly expressed on antigen-experienced memory T cells in peripheral tissues and less commonly on B cells, activated monocytes, dendritic cells (DC), and natural killer (NK) cells.

PD-L1 is a type 1 transmembrane protein receptor, a member of the B7 family that functions as an inhibitor of the adaptive immune system. It is expressed in many types of cells including antigen-presenting cells (APCs), T cells, B cells, monocytes, and epithelial cells.

Immunotherapy is the treatment of diseases by activating or suppressing the immune system.

Checkpoint inhibitor therapy is a type of cancer therapy that targets immune checkpoints, such as LAG-3, VISTA, CTLA4, and PD-1.

Immunohistochemistry is a technique for identifying antigens in cells of tissues by utilizing the idea of antibodies binding uniquely to antigens in biological tissues.

Immunoreactivity refers to the magnitude of the immune response triggered by an antigen.

DAB: A benzene derivative most often used in immunohistochemistry staining as a chromogen.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Esophageal carcinoma (EC) is a highly aggressive malignancy with poor prognosis. There were approximately 511,000 new cases and 445,000 deaths of esophageal cancer reported worldwide in GLOBOCAN report of 2022(1) The highest incidence rates of EC have been reported in Eastern Asia, particularly China, which reported 224,012 new cases and 187,467 deaths in 2022 (2,3). In North America, particularly in the United States, the incidence was relatively lower but still noteworthy, with 21,888 new cases and 19,116 deaths reported (2,4). In Africa, EC represents a significant health burden, with the continent experiencing some of the highest incidence rates globally. Malawi shows the highest incidence and mortality rates worldwide by 9.4% and 12.6% in both sexes respectively (1,5). In Tanzania the estimated annual incidence and mortality rates of EC was 7.9% and 11.3% respectively for both sexes, ranking it 2nd among top 3 leading causes of cancer related deaths in the country. (6)

Histologically, EC is divided into several subtypes, with the two primary ones being esophageal squamous cell carcinoma (ESCC), which constitutes 85% of all EC cases, and esophageal adenocarcinoma (EAC), which makes up 14%. The remaining 1% covers other subtypes such as adeno-squamous carcinoma, adenoid cystic carcinoma, carcinoma undifferentiated, neuroendocrine carcinoma, Not otherwise specified (NOS) and others (2,7). Each subtype has distinct etiological and pathological features for example, ESCC has been linked to alcohol and tobacco use while EAC has been linked to gastro-esophageal reflex disease (GERD) and obesity(8). Significant geographical variations in incidence with each subtype has notably been observed for example, ESCC is more prevalent in Eastern Asia, Eastern and Southern Africa, and parts of South America, while EAC is more common in Western countries, with both subtypes being highly associated with advanced age. The highest occurrence is seen in the age group of 50 to 70 years, with males being affected more than females [approximate ratio of 3 to 5:1] (9,10)

The management of EC involves a multidisciplinary approach that includes surgery, chemotherapy, radiotherapy, targeted therapy, and palliative care. The treatment strategy depends on the stage of the disease, the location of the tumor, the patient's overall health, and other individual factors but, is often diagnosed at advanced stages due to lack of specific early symptoms. This late presentation, together with limited widespread screening programs for EC, particularly in high-risk populations as seen for breast or cervical cancer, it significantly reduces the effectiveness of treatment options and worsens prognosis with a known mortality rate of 8.3 and 3.2 per 100,000 males and females respectively (11–13). Therefore, the application of immune targeted therapies with an example of pembrolizumab for EC patients has been an important discovery with several benefits such as improving survival rates, enhancing response rates as well as creating potential for combination therapies(14–18).

Programmed cell death ligand 1 (PD-L1) is a protein found on the surface of different cells, including some cancer cells. It binds to the programmed death-1 receptor (PD-1) on T cells and sends an inhibitory signal to the T cell, reducing its ability to attack the cells expressing PD-L1 which is a crucial component of the immune system responsible for identifying and destroying cancer cells and pathogens (19). The expression of PD-L1 on tumor cells is used as a biomarker to identify patients who might benefit from immunotherapy. Immunotherapy drugs known as immune checkpoint inhibitors, block the interaction between PD-1 and PD-L1, thereby enhancing the immune system's ability to fight cancer, using drugs such as pembrolizumab and nivolumab which that have been approved for treating various cancers, including EC (16). These drugs can lead to significant improvements in survival and quality of life for patients with PD-L1 positive tumors. In EC particularly in advanced or metastatic cases, PD-1 inhibitors like pembrolizumab have been used effectively (20,21). The presence of PD-L1 on tumor cells is often assessed through several methods such as immunohistochemistry (IHC), flow cytometry, *in-situ* hybridization and RNA sequencing to determine eligibility for these therapies(13,20–24).

Several studies have shown variations in the association of PD-L1 expression with various clinicopathological features such as age, degree of differentiation, tumor location, tumor grade, and nodal involvement (20,25,26). There is no data PD-L1expression among patients

with EC in Tanzania. Most existing studies on PD-L1 expression in EC are based on Western or Asian population,(26,27) which may not be directly applicable in our population due to genetic, environmental, and lifestyle differences. Therefore, investigating PD-L1 expression in our setup can help identify candidates for these therapies and evaluate their therapeutic outcomes in the local context.

This study aimed to determine the proportion of PD-L1 immunohistochemical expression and its associated clinical and pathological factors among patients with EC at Muhimbili National Hospital.

1.2 Problem Statement

Esophageal carcinoma (EC) remains a significant public health challenge in Tanzania, particularly due to its late-stage diagnosis and poor prognosis. The expression of PD-L1 has emerged as a critical biomarker in various cancers, offering potential for improved prognostic evaluation and targeted immunotherapy. However, there is a substantial gap regarding proportion of PD-L1 expression among Tanzanian patients with EC as there are no researches available on this context thus impeding the application of Immune checkpoint inhibitors (ICIs) which have shown a prolonged overall survival in patients as compared to chemotherapy as a second line management for advanced stages(16).

1.3 Rationale of the study

The data provided from investigating PD-L1 IHC expression in patients with EC will be essential for several reasons. Firstly, it will inform on the feasibility and potential impact of introducing PD-L1-targeted therapies, such as pembrolizumab, nivolumab (16,28) which have shown promise in other populations but have not yet been studied in Tanzania. Secondly, by providing data on proportion of PD-L1 IHC expression we can identify individuals who can benefit from these therapies and thereby improving their clinical outcomes.

1.4 Research Questions

1.4.1 General Research Question

What is the proportion of PD-L1 IHC expression among patients with EC and their associated clinicopathological factors at MNH?

1.4.2 Specific Research Questions

1. What is the proportion of PD-L1 IHC expression among EC patients at MNH?
2. Is there an association between PD-L1 IHC expression and clinicopathological factors including age, sex, tumor location, and histological subtypes and grade among patients with EC at MNH?
3. What type of cells express PD-L1 by IHC including their staining pattern and intensity among patients with EC at MNH?

1.5 Objectives

1.5.1 Broad Objectives

To determine the proportion of PD-L1 IHC expression among EC patients and their associated clinicopathological factors at MNH.

1.5.2 Specific Objectives

1. To determine the proportion of PD-L1 IHC expression among patients with EC at MNH.
2. To determine the association of clinicopathological factors (age, sex, tumor location, histological subtypes, and histological grade) with PD-LI IHC expression among patients with EC at MNH.
3. To determine the types of cells, their PD-L1 IHC staining pattern and intensity among patients with EC at MNH.

‘

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Programmed cell death-ligand 1

PD-L1 is a member of the B7 family of proteins also known as B7-H1 or CD274 that interacts primarily with programmed cell death protein 1 (PD-1), a receptor expressed on T cells (29). The binding of PD-L1 to PD-1 results in the inhibition of T cell activity, proliferation, and cytokine production, which helps maintain immune homeostasis and prevent autoimmunity under normal physiological conditions (30). However, this pathway can be co-opted by tumor cells to evade immune surveillance, allowing them to proliferate unchecked.

The expression of PD-L1 on tumor cells and tumor-infiltrating immune cells has been extensively studied across various cancer types. PD-L1 expression can be induced by inflammatory signals such as interferon-gamma, which are part of the body's response to cancer cells. High levels of PD-L1 expression are associated with poor prognosis in several cancers, including melanoma, lung cancer, and renal cell carcinoma (31), this may be due to immune suppression that is brought about by T cell activation and proliferation through PD-L1 binding to its receptor PD-1 on T cells hence allowing tumor cells to proliferate. The discovery of the PD-1/PD-L1 pathway has revolutionized cancer treatment, leading to the development of ICIs. These therapies block the interaction between PD-1 and PD-L1, thereby reactivating T cells to recognize and destroy cancer cells (18). Several clinical trials done in Japan, China and America such as KEYNOTE-181 and CheckMate 577 have demonstrated significant benefits of ICIs in various cancers, including improved survival rates and durable responses in patients with PD-L1-positive tumors (16,17,21,23,32,33) .No clinical trials done in Africa or East Africa have yet been reported.

2.2 PD-L1 immunohistochemical expression in esophageal carcinoma

Most studies on PD-L1 IHC expression in EC have been conducted in Asia, America, and Europe, while research in Africa remains limited (26,31). Most of these studies were in large scale hence provided a larger landscape in providing prevalence of PD-L1 IHC expression in their population. The proportion of PD-L1 IHC expression varies greatly across studies for example, a study in Beijing, China by Rong *et al* showed that 29.9% of ESCC patients

expressed PD-L1 (26). Huang *et al* showed 45% PD-L1 expression in 533 EC patients, and another study in Japan showed an expression of PD-L1 observed in 18.9 % (33) . This wide variation in PD-L1 IHC expression together with controversial prognostic significance of PD-L1 expression in ESCC had led to meta-analysis studies in order to address the issue where in one study PD-L1 was over expressed in 43.7% of ESCC patients (14)

This difference in proportions may brought about by several factors such as geographic and ethnic variability within these study populations, sample size and composition, technical differences in PD-L1 testing (antibody clones and detection methods, IHC scoring methods (CPS, TPS), study designs and methodology (retrospective vs prospective) or single center vs multi-center studies and lastly biological behaviors example inflammatory microenvironment where the local tumor microenvironment, including presence of inflammatory cytokines like interferon gamma, can influence PD-L1 expression.

Currently, there are no known publications or studies on PD-L1 expression by routine IHC among patients with EC in both Africa and Tanzania, but an ongoing study by Buckle *et al* aims at examining the therapeutic landscape of ESCC patients and compare their current treatment outcomes in East Africa, hence greatly supporting the seriousness of this disease and giving room for new therapies such as ICIs to combat this malignancy (7)

2.3 Factors associated with PD-L1 expression among esophageal carcinoma patients

2.3.1 Age

EC has typically been linked to older age, with people over 55 years of age having a higher risk of developing the disease. The relationship between age and PD-L1 IHC expression in EC has shown contradicting results. For example, a study at Wenzhou medical University by Jiang *et al* showed that most 19% (48/246) positive cases were <65 years, similar to Kaiyan *et al* 41.4% (163/394) while studies by Rong *et al* and Chen *et al* showed that most of the positive PD-L1 IHC cases occurred ≥ 60(15,25,26,34). This contradiction may be brought by factors such as immunosenescent, where as individuals age there is a gradual decline in immune function, including reduced T-cell activity and alterations in cytokine production (20). Chronic inflammation mostly seen in old age but also observed in individuals with other comorbidities such as HIV/AIDS, diabetes and hypertension cause an upregulation of immune check point molecules such as PD-L1 in tumors hence an age range

variation seen. These changes can create a tumor microenvironment that promotes cancer progression and can influence PD-L1 expression. Despite the contradictions seen in the mentioned studies, majority do not show any association between age and PD-L1 IHC expression such as Huang *et al* (p=0.651) thereby highlighting the need for further investigations to clarify this association (15,23,33).

2.3.2 Sex

EC is mostly seen in male than female patients (35) . This can be explained by several factors such as behavioral and lifestyle factors example alcohol and tobacco use, hormonal influences where testosterone is believed to have immunosuppressive effects while estrogen is associated with immunoenhancement effects by modulating immune responses hence prevent malignant transformation (36). Immune cells, like dendritic cells, macrophages, natural killer T-cells (NKT), T cells and B cells respond to sex hormones. For example, the activity of macrophages for destroying dead or old tissues in the body and antigen presentation by dendritic cells is greatly assisted by estrogens.

Therefore, based of PD-L1 IHC expression in patients with EC, there are no studies that showed an association between PD-L1 expression with sex, despite Rong *et al* who showed higher PD-L1 expression in female (33.8%) than male (29.0%) while Kaiyan *et al* showed a higher PD-L1 expression in male (41.6%) than female [40.3%] (26,34) . This disparity can be attributed to hormonal influences, differences in immune system activity, and the fact that men are more frequently exposed to predisposing risk factors such as smoking, which further amplifies the development of ESCC between the sexes. This indicates that more research is required to understand this relationship fully.

2.3.3 Tumor location

Anatomically, the esophagus can be categorized into three parts; upper (cervical), middle (thoracic) and lower (abdominal) esophagus. While EAC occurs more frequently in the lower third and gastroesophageal junctions in comparison to other parts of the esophagus, ESCC commonly affects primarily middle and upper thirds of thoracic esophagus (37). This has an impact on tumor biology such as PD-L1 expression depending on the distinctive anatomy and histology of these areas. There have been variations in results reported on this area example studies done by Kaiyan Chen *et al* and Rong *et al* showed a higher PD-L1

expression in the lower esophagus by 35.1% and 33.3 % respectively (15,26) while, Jiang *et al* and Wakita *et al* showed that there was an equal distribution in the middle and lower parts by (39/142) and (7/15) respectively (23,25).

There are no reports that show a high PD-L1 IHC expression in the upper part. Therefore, variations seen in these results may reflect the distinct molecular and environmental etiologies of these tumor locations such as tobacco smoke and alcohol. Also, the esophagus has a unique immune system as well as microbiome whereas it lacks organized secondary lymphoid tissues for antigens to be sampled and presented to T cells rather it's the dendritic cells that phagocytose antigens directly from the lumen (38). These can influence the regulation of immune checkpoints like PD-L1, leading to variability in its expression in different regions of the esophagus.

2.3.4 Histological subtypes

According to 2019 WHO classification of tumors of the digestive system, ESCC and EAC remain as two major histologic subtypes (35). The role of environmental factors such as alcohol and tobacco smoking are still major drivers for ESCC development with evidence that TP53 and SMAD4 mutations being responsible for epithelial proliferation and development of invasive carcinoma respectively (39), with all this the pathogenesis of precursor lesions is yet understood.

It is to the best of our knowledge that reported studies on PD-L1 IHC expression among patients with EC show that ESCC is the predominant histological subtype that show a high PD-L1 IHC expression. This may be attributed that >85% of all EC are ESCC to begin with hence it is the most reported (7,23,26). A study by Huang *et al* who studied 5 histologic subtypes yet it was ESCC that showed more positive cases by 94.2% (226/483) but statistically insignificant [p=0.082], (33). Despite this lack of association, the disease showed a trend of occurring more in ESCC than other histologic subtypes. This can be attributed by difference in tumor pathogenesis such as gastroesophageal reflux disease as seen in lower esophagus and environmental factors such as tobacco smoking in the upper and middle esophagus (8). More studies are required to understand this phenomenon fully.

2.3.5 Tumor grade

Tumor grade, reflects the differentiation status of the tumor cells, indicating how much the tumor cells resemble normal cells. Tumor grade is a crucial prognostic factor in EC, with higher grades generally associated with poorer outcomes (40) this can be attributed to poor cellular differentiation that leads to a poor oncologic staging at the time of diagnosis. Studies have been conducted in exploring the association of tumor grades and PD-L1 IHC expression where, Kaiyan *et al* demonstrated a positive association (p=0.010) with more patients seen in grade 2 43.8% [165/377] (15). Rong *et al* showed that 41.8% (38/91) patients who were of grade 3 showed a high PD-L1 expression and this was also statistically significant [p=0.018] (26). But other studies despite showing similar trends of higher expression seen in higher grades there are some that showed no association as portrayed by Huang Z *et al* with grade 2 52.9% (127/289) positive cases [p=0.846] (33).

This suggests that higher-grade tumors may have a more immunosuppressive microenvironment, contributing to increased PD-L1 expression or that the aggressive nature of poorly differentiated tumors might drive PD-L1 upregulation as a mechanism to evade the immune response. It indicates that PD-L1 could serve as a prognostic biomarker in this subset of patients. The association between higher tumor grade, increased PD-L1 expression, and worse outcomes underscores the importance of understanding the tumor's immunobiology for developing targeted therapies.

2.3.6 TNM stage

The stage of the tumor, indicates the extent of disease spread, is a crucial factor in determining prognosis and treatment strategies in EC (40). Tumor staging involves 3 components T stage (extent of tumor), N stage (extent of regional node involvement) and, M stage (distance metastasis). With respect to PD-L1 IHC expression there have been some variations seen in terms of its association with TNM staging. Huang Z *et al* showed that most 40.9% (218/533) patients were diagnosed at stage III (p=0.060) but as they individually analyzed the TNM components they saw that PD-L1 IHC expression was associated with T stage (p=0.031) as most patients were diagnosed with T3 stage 61.7% (329/533) with no association seen in regional node involvement or metastasis [p=0.086, p=0.761 respectively] (33).

Similarly, Rong *et al* showed an association of advanced tumor staging with PD-L1 expression as majority of the patients were diagnosed in T3 and T4 stages 33.1% (91/275) (p=0.028) and but they further showed an association with N stage (p=<0.001) where most positive cases were in N3 stage 47.6% (10/21) and hematogenous metastasis 43.1% (28/65) [p=0.012] (26). In contrast with other studies Wakita *et al*, showed that there is an association between high PD-L1 expression and TNM staging (p=0.029) but, here most positive cases were in IB (5/15) with no associations seen with T stage or N stage of the tumor (p=0.174, p= 0.525 respectively) (23). However, a meta-analysis study done by Guo *et al* which involved 13 studies showed that there was no association seen between TNM stage (OR = 0.99, 95% CI 0.72–1.38; *P* = 0.97) as well individual analysis of T stage and N stages with PD-L1 expression (p= 0.86, p=0.31 respectively) rather an association was seen with distant metastasis [odds ratio [OR] = 1.58, 95% CI 1.03–2.42; *P* = 0.04] (14) .

This pattern reflects the adaptive upregulation of immune checkpoint pathways in more advanced disease to facilitate immune evasion and tumor progression as tumors progress. Advanced-stage tumors, which are typically larger and more invasive, are more likely to be infiltrated by immune cells that secrete cytokines, thereby upregulating PD-L1 along with other factors (26). With all this, it is still not known whether expression of PD-L1 results in advanced TNM stage or whether an advanced TNM stage promotes PD-L1 expression hence more studies required.

2.3.6 Vascular invasion

One of the key pathological features that can influence the progression and outcome of EC is vascular invasion, where tumor cells invade the lymphatic vessels, facilitating metastasis. When it comes to this parameter most studies show no association between PD-L1 expression and vascular invasion despite differences observed in their trend of occurrence. Example, Rong *et al* showed that most positive cases had vascular invasion 30.2% (48/159) but no association (p=0.915) while Huang Z *et al* showed there was an association as majority of the cases had vascular invasion 66.3% [159/240] [p=0.029] (26,33).

The up-regulation of PD-L1 in tumors with vascular invasion might result from different mechanisms which could include hypoxia and the presence of other pro-inflammatory cytokines such as IFN-γ within the tumor microenvironment. Hypoxia, a typical

characteristic of tumors with vascular invasion, it activates PD-L1 through hypoxia-inducible factors (HIFs) (41). Besides, pro-inflammatory cytokines may enhance PD-L1 expression thereby promoting immune evasion and metastasis.

2.4 The Types of Cells, their staining patterns and intensities with PD-L1 IHC Among Esophageal Carcinoma Patients at Muhimbili National Hospital

PD-L1 IHC expression has been demonstrated on tumor cells in 41.4% (222/536) ESCC patients, Kaiyan *et al* and tumor infiltrating immune cells such as lymphocytes and macrophages(15,21,29). It should be noted that literature shows stromal cells such as fibroblasts and other inflammatory cells such as neutrophils, plasma cells and eosinophils may show positivity but, they are usually not included in the scoring for patients who will be eligible for targeted therapy (42). Its expression on tumor cells directly contributes to the inhibition of cytotoxic T lymphocytes activity, facilitating immune escape and tumor progression (29) . Several studies have further shown that high PD-L1 expression on tumor infiltrating immune cells has been associated with prolonged overall survival of the patients (p=0.023) while no association was seen with tumor cells, Svensson M *et al* (43).

The presence of PD-L1-positive immune infiltrating immune cells has been associated with better responses to immune checkpoint inhibitors, as these cells can serve as a reservoir for PD-L1 blockade (15,22). Most studies as shown above, PD-L1 IHC expression on either tumor cells or immune cells has been linked to either disease free survival or overall survival of these patients. This is attributed to the interaction of the PD-1/PD-L1 that plays a significant role in modulating the immune response within the tumor microenvironment.

In both studies such as Rong *et al* and literature show that cells to be considered positive should have partial or complete membranous staining and/or cytoplasm staining because PD-L1 is a transmembrane protein with two extracellular domains, IgV like and IgC-like domains; a transmembrane domain; and a cytoplasmic domain (26,29).

It is to the best of our knowledge that very few studies applied the concept of PD-L1 intensity with specific intensions for example, Ito *et al* who considered PD-L1 high expression if the grading intensity score was ≥7. This was essential in their study as they applied Allred scoring that required adding proportion scores and intensity scores (20).

CHAPTER THREE

3.0 MATERIALS AND METHODOLOGY

3.1 Study Design

This was a descriptive cross-sectional study that used archived tissue blocks from patients with EC at MNH-CPL, through retrospective data collection.

3.2 Study Population

Patients who were diagnosed with EC from January 2019 to January 2023 at MNH-CPL in Dar es Salaam. Medical records, patient request forms, logbooks for registering specimens housed in the MNH-CPL unit, and electronically stored records through the Jeeva system were also used to collect data.

3.3 Sample Size and Selection

3.2.1 Sample size estimation

Based on different studies, several have shown positive expression of PD-L1 among patients with EC in relation to different clinical pathological features and further quantitatively shown its prognostic importance(19,20,25,26), based on these studies sample size was obtained from simple random sampling of all EC cases from January 2019 to January 2023 with an estimated sample size of 192 cases.

Through the formula;

$$N = \frac{Z^2 \times (100 - p)}{\varepsilon^2}$$

Where:

n= Sample size

z (standard normal deviate) =1.96 for 95% confidence level

p = 50% (probability of outcome/characteristic occurrence)

ε (margin of error) =5%

The estimated sample size for a 95% confidence level is 384.16.

Sample size adjusted for population

n2=n1/1+((n1-1)/N)

Where: n2= adjusted sample size, n1= initial unadjusted sample size, N= finite population size

n2= 384/ 1+((384-1)/384)

The adjusted sample size of 192.

3.4 Inclusion and Exclusion Criteria

3.4.1 Inclusion criteria

(i) EC tissue blocks with H&E slides that are adequate for IHC at MNH-CPL.

(ii) Endoscopic tissue biopsies.

3.4.2 Exclusion criteria

(i) EC cases with prior treatment example chemotherapy/radiotherapy.

(ii) Esophagectomy specimens.

(iii)Metastatic carcinomas to the esophagus.

3.5 Work flow plan

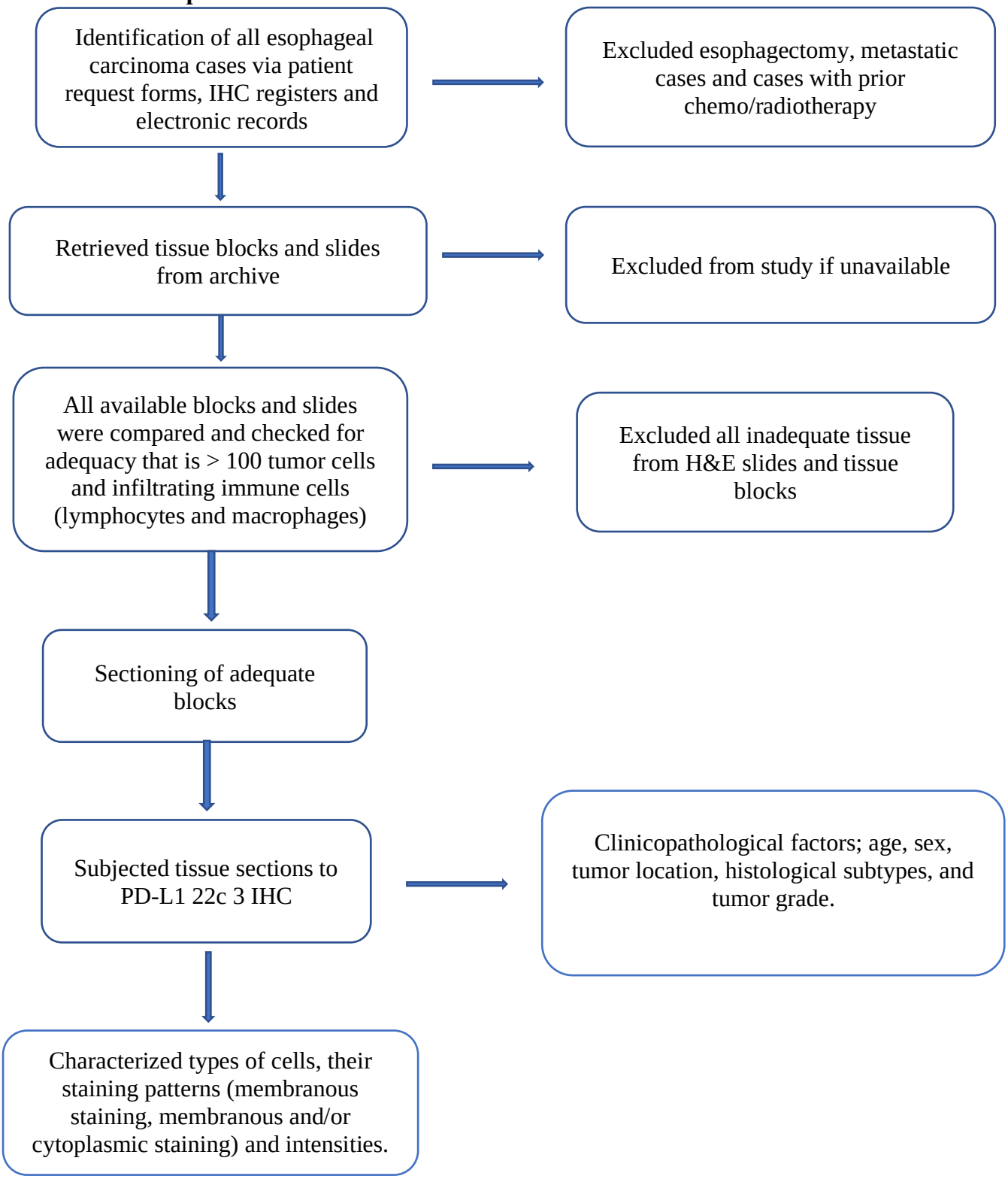


Figure 1: Work flow plan.

Key; PD-L1- Programmed cell death ligand 1, IHC-Immunohistochemistry, H & E- Haematoxylin and eosin

3.6 Study Variables

3.6.1 Independent variables

Age, sex, tumor location, histological subtypes and tumor grade.

3.6.2 Dependent variables

PD-L1 IHC expression status (positive or negative) and staining pattern

3.7 Data Collection Methods

Data were manually obtained by the principal investigator from laboratory registers including patients' request forms, and electronic records (Jeeva system) at MNH-CPL, Dar es Salaam, Tanzania using paper-based data collection tool in (**Appendix 1**) which included patients' demographic information. Each case was then given a special study code and all other variables under study (staining cells, pattern, intensity and PD-L1 status) were then computerized in data excel sheet, which was attached as (**Appendix 2**).

3.7 Laboratory methods

3.7.1 Tissue blocks and slides for IHC

Laboratory registers were used to identify all cases of EC that had occurred between January 2019 and January 2023. The corresponding H & E slides and formalin-fixed paraffin-embedded (FFPE) tissue blocks were obtained from archive for adequacy assessment. These were reviewed by principal investigator and 2 pathologists. For cases where H&E slides were lost, new slides were prepared and re-stained to ensure that cases were representative, adequate, and validated for IHC slide preparations and interpretation.

3.7.2 Immunohistochemistry Procedure for PD-L1

Tissue blocks were sectioned at a thickness of 3µm and mounted on positively charged slides Dako Flex IHC microscopic slides (code K8020) then heated at 55^{o} C in hot air oven for 2 hours before staining. Deparaffinization, rehydration and antigen retrieval were performed on the Ventana Benchmark GX automated slide Stainer (VSS v12.5.4 software) using EZ prep (Ventana 950-102), reaction buffer (Ventana 950-300) and CC1 (Ventana 950-124) solutions respectively, antigen retrieval was performed for 64 minutes at 100^{o} C.

Slides were incubated with primary Anti-Human PD-L1 monoclonal Mouse antibody (clone 22C3, Agilent Technologies, Stevens Creek Blvd, CA 95051, US) using a concentration of 1:50 diluted with Ventana antibody Diluent (251-018, Ventana Medical Systems. Inc.

Tucson, Arizona) for 32 minutes at 37^{o} C. Visualization was done with Ventana Optiview DAB IHC detection kit (760-700, Ventana Medical Systems Inc. Tucson, Arizona) followed by counterstaining with Ventana Hematoxylin II reagent (Ventana 790-2208) for 8 minutes and bluing in bluing reagent (Ventana 760-2037) for 4 minutes. Slides were then dehydrated in ethanol, cleared in xylene and cover slipped with Sakura SC 200 cover slipper. Each IHC run contained a positive control (on-slide known positive PD-L1 tonsil and bladder tissue) and a negative control (placenta tissue) slide in which primary antibody was substituted with buffer solution (**Appendix 3**).

3.7.3 Interpretation of the PD-L1 IHC stained tumor sections

There is no a standardized scoring system, this is due to several factors. First, Different studies use different cut off points based on clinical relevance (eligibility of patients to certain immune check point inhibitors) as shown by Huang Z *et al* who used CPS scores targeting the use of pembrolizumab (33). Second, variability of cutoff points between different cancer types as well as lack of standardized cut off point within similar cancers as shown by Rong *et al* and Ito *et al* (20,26). Lastly scoring may also differ based on assay and antibody used as shown by Huang Z *et al*, Rong L *et al* and Jiang C *et al* (PD-L1 22C3, PD-L1 SP142 and PD-L1 E1L3N clones respectively) (25,26,33)

Therefore, the interpretation of PD-L1 in this study adopted few key points on staining cells, staining pattern and intensities from PD-L1 IHC 22C3 pharm Dx Interpretation manual-Triple negative breast cancer (42). With no cut offs used, all cases that showed perceptible and convincing partial or complete membranous staining that was distinct from cytoplasmic staining at any intensity in the of presence of positive internal control on tumor cells were classified as positive for PD-L1, while diffuse or finely granular cytoplasmic staining alone was considered PD-L1 negative.

While membranous and/or cytoplasmic staining of lymphocytes and macrophages within tumor nests and/or adjacent supporting stroma were considered PD-L1 staining cells. Staining intensities were computed based on observed guided imaging from the manual, where 0= Negative, +1= Weak intensity, +2= Moderate intensity, +3= Strong intensity. The supervisors corroborated the results of the lead investigator's evaluation of all stained slides for both positivity and negativity.

3.8 Data Analysis

Data were analyzed using SPSS version 27. Descriptive statistics were summarized using proportion and frequency tables. Chi-square or Fishers exact tests were used where appropriate to assess the association between clinicopathological characteristics with PD-L1IHC expression. However, binary logistic regression was not performed as variables were not statistically significant ($p<0.05$) from Fisher's exact test and no single variable had $p<0.2$.

3.9 Ethical Consideration

The proposed study was presented for approval to the MUHAS Ethical Clearance Committee, **(MUHAS-REC-12-2023-1984**). Following the approval, permission from the Management and Head of Histopathology at MNH-CPL to conduct the study was sought with reference number **MNH/CRTCU/Perm/2023/574.** Since data was collected from archived specimens retrospectively without direct patient contact, informed consent was waived. Furthermore, data extraction did not include any identifiers, instead special codes were assigned to cases.

CHAPTER FOUR

4.0 RESULTS

4.1 Selection process of cases in the study

A total of 192 patients diagnosed with EC whose records were retrieved from the archived documents. Upon review of the cases by the investigator and pathologists, an exclusion of esophagectomy cases to avoid bias in the results as well as cases with missing blocks and those with tissues inadequate for PD-L1 IHC staining was made (**Figure 2**).

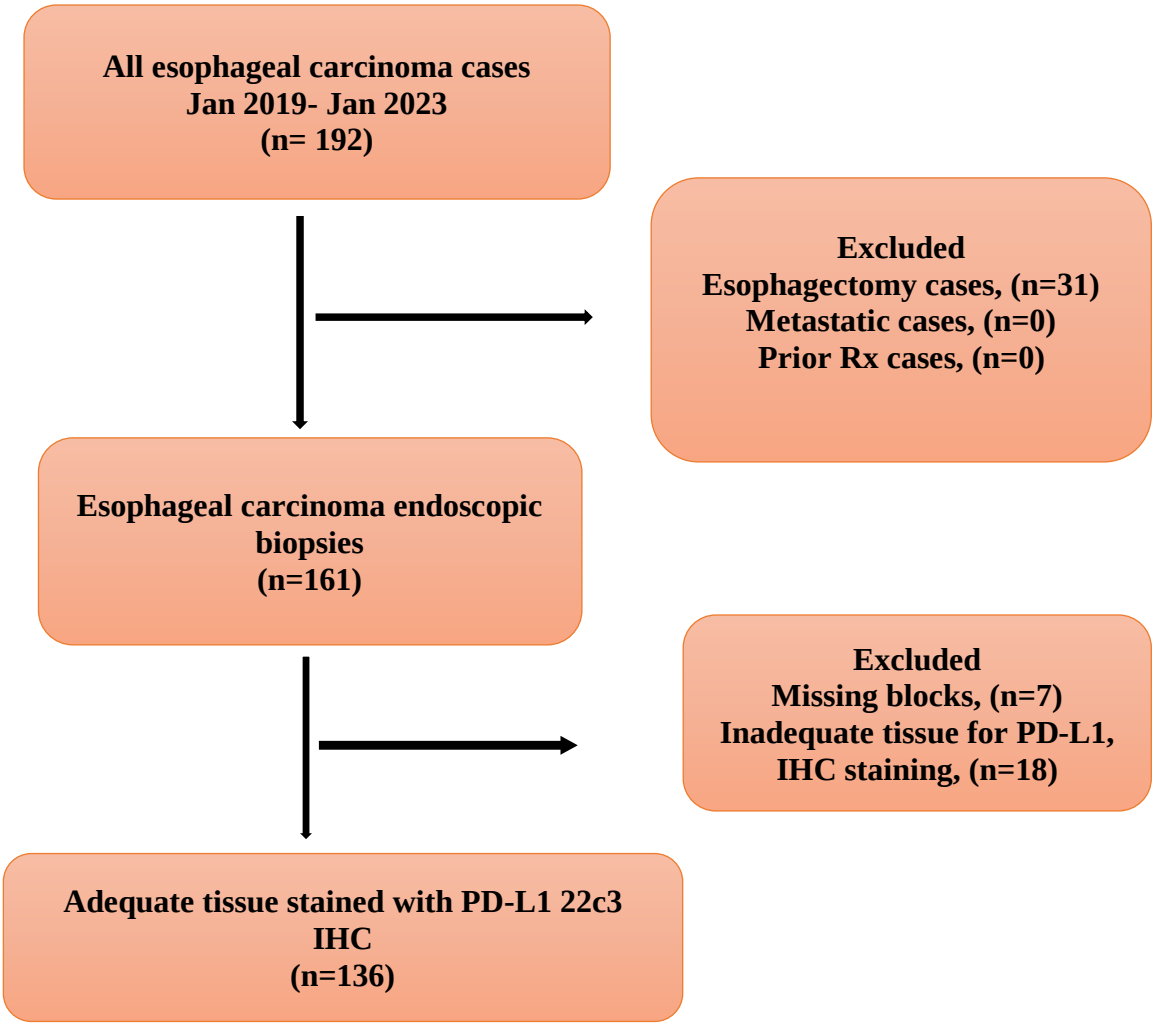


Figure 2: Data flow chart

Key; PD-L1- Programmed cell death ligand 1, IHC- Immunohistochemistry, Rx - Treatment

4.2 Demographic and clinicopathological characteristics

From the selected cohort, most of the patients were <60 years that is 57.4% (78/136) with a mean age of 58.0±14.0 years. Where 66.9% (91/136) were male and most of the tumors were located in the middle third by 56.6% (77/136). The predominant histological subtype was ESCC by 89.0% (121/136) with one adenosquamous cell carcinoma and one poorly differentiated carcinoma. Most of the tumors were grade 2 55.1% (75/136) **(Table 1).**

Table 1: Demographic and clinicopathological characteristics of the studied cases N = 136

Variables	Frequency (n)	Percentage (%)
Age (years)		
< 60	78	57.4
≥ 60	58	42.6
Sex		
Male	91	66.9
Female	45	33.1
Tumor location		
Upper	25	18.4
Middle	77	56.6
Lower	34	25.0
Histological subtypes		
Invasive squamous cell carcinoma	121	89.0
Adenocarcinoma	13	9.6
Adenosquamous cell carcinoma	1	0.7
Poorly differentiated carcinoma	1	0.7
Tumor grades		
Grade 1	48	35.3
Grade 2	75	55.1
Grade 3	13	9.6

4.3 Proportion of PD-L1 immunohistochemical expression among patients with esophageal carcinoma patients at Muhimbili National Hospital

Among the 136 patients which were subjected to PD-L1 immunohistochemical staining using clone 22C3, 7 cases (5.1%) stained positively while 129 cases (94.9%) were negative. **(Figure 3).**

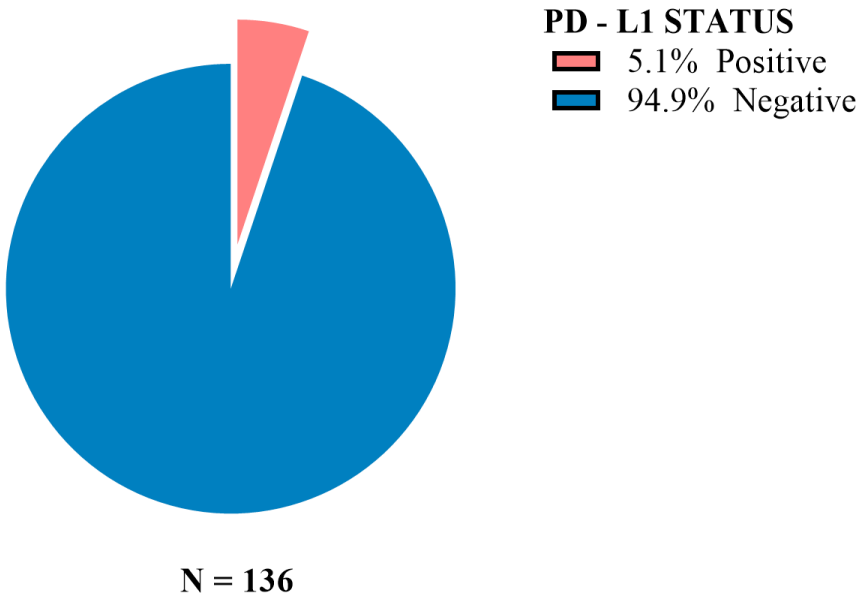


Figure 3: Prevalence of PD-L1 IHC status among EC patients at Muhimbili National Hospital

4.4 The types of cells, their staining patterns and intensities for PD-L1 immunohistochemistry among patients with esophageal carcinoma at Muhimbili National Hospital

The study showed that all positive cases (7/7) had PD-L1 IHC expression on tumor cells and inflammatory cells. Both cell types exhibited cell membranous staining with most cases showing weak intensity (5/7) **(Table 2).**

Table 2: Types of cells and their staining pattern with PD-LI immunohistochemistry among esophageal carcinoma patients at Muhimbili National Hospital

	PD-L1 Status	
Variables	**Positive (n)**	**Negative (n)**
Staining cells		
Tumor cells only	0	0
Tumor cells and inflammatory cells	7	0
No staining cells	0	129
Staining pattern		
Membranous staining	7	0
Membranous and cytoplasmic staining	0	0
Cytoplasmic staining	0	0
Negative	0	129
Staining intensity		
Strong intensity	0	0
Moderate intensity	2	0
Weak intensity	5	0
Negative	0	129

(Figures 4 – 7) are photomicrographs showing PD-L1 IHC sections of positive and negative control tissues of a high grade urothelial carcinoma and placenta tissue respectively. The positive controls show +3, +2 and +1 intensities respectively.

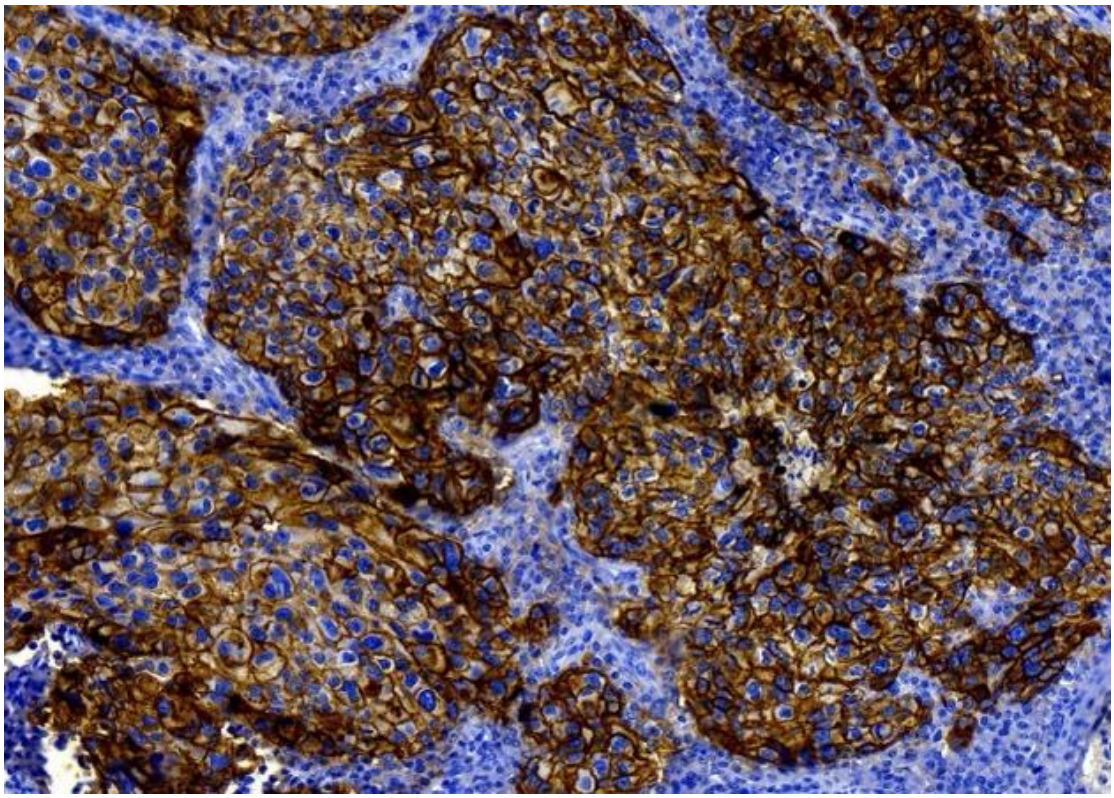


Figure 4: A photomicrograph of positive control for PD-L1 IHC expression from a case of high-grade urothelial carcinoma with staining intensity of +3 (PD-L1 IHC staining, x200)

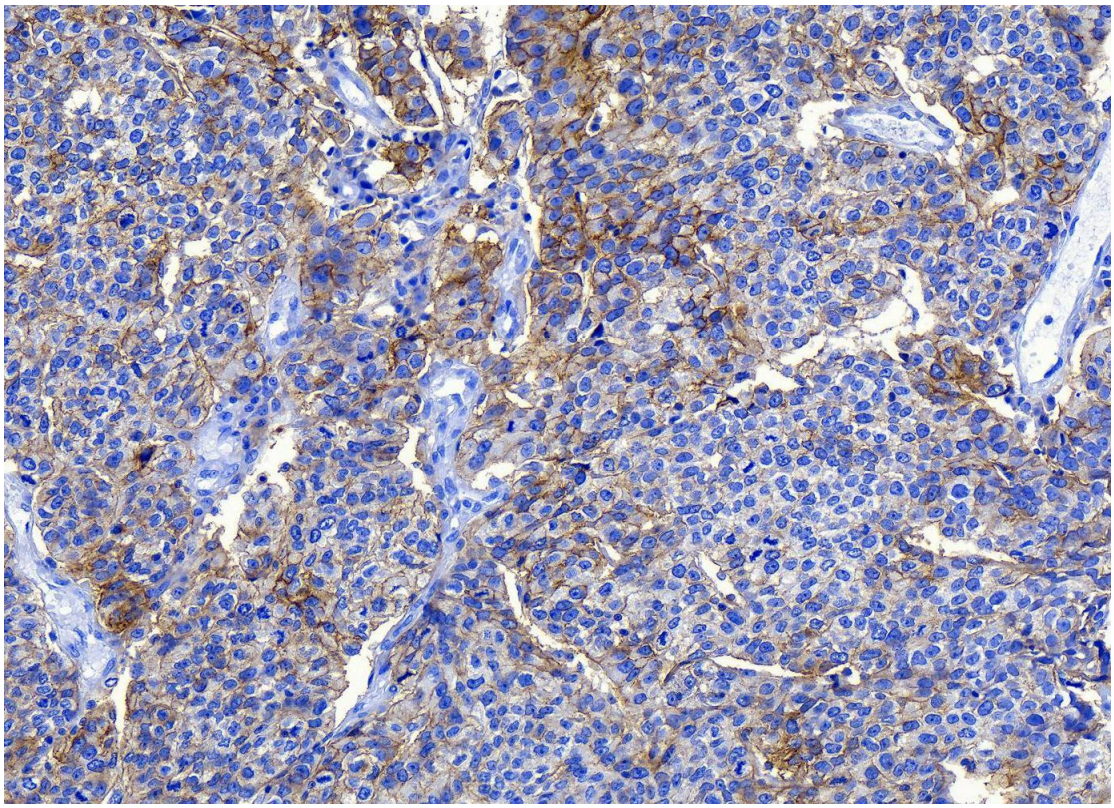


Figure 5: A photomicrograph of positive control for PD-L1 IHC expression from a case of high-grade urothelial carcinoma with staining intensity of +2 (PD-L1 IHC staining, x200)

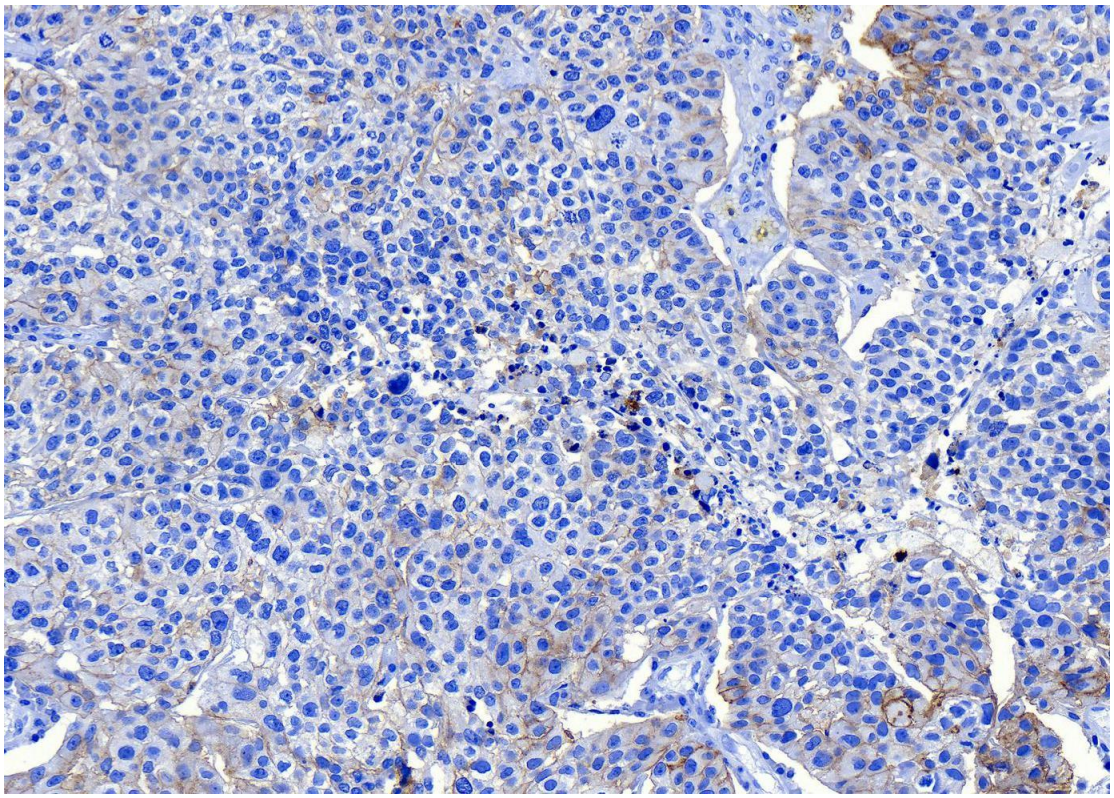


Figure 6: A photomicrograph of positive control for PD-L1 IHC expression from a case of high-grade urothelial carcinoma with staining intensity of +1 (PD-L1 IHC staining, x200)

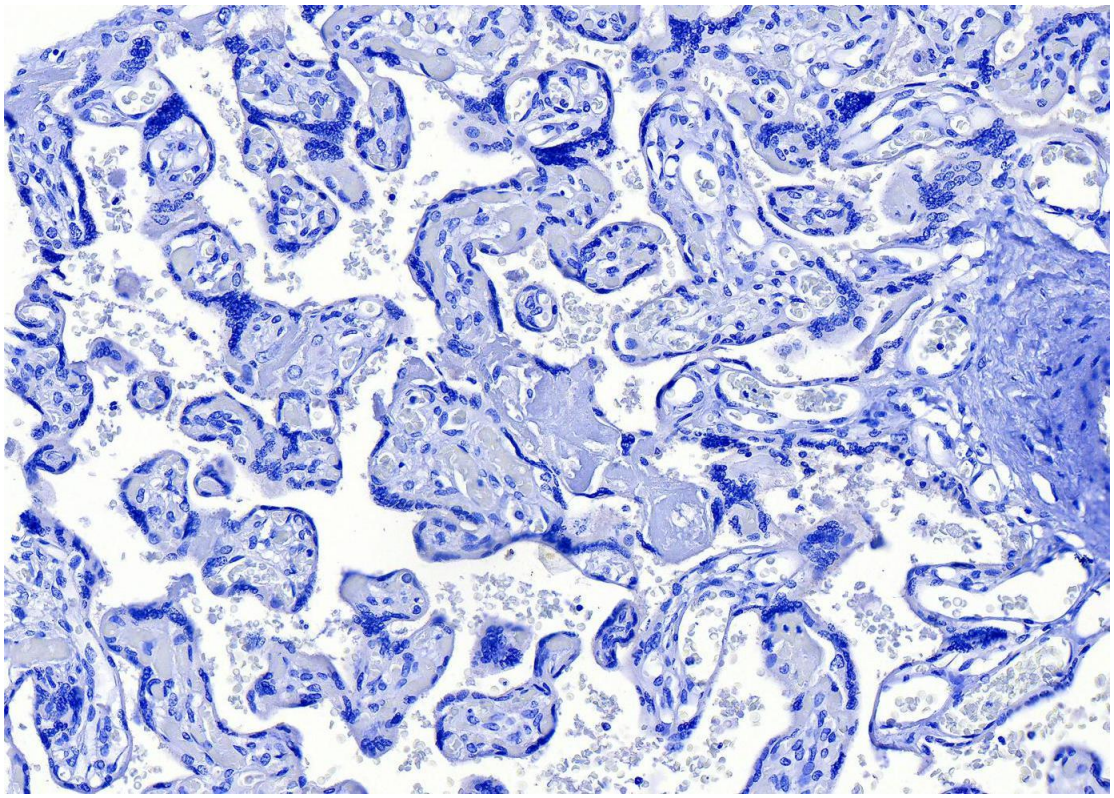


Figure 7: A photomicrograph of negative control for PD-L1 IHC expression from a placenta tissue (PD-L1 IHC staining, x200)

(Figures 8 – 10) are photomicrographs showing PD-L1 IHC of positive and negative EC cases. The positive cases are grade 1 and 2 ESCC with +2 and +1 staining intensities respectively. The negative case is a grade 2 adenocarcinoma.

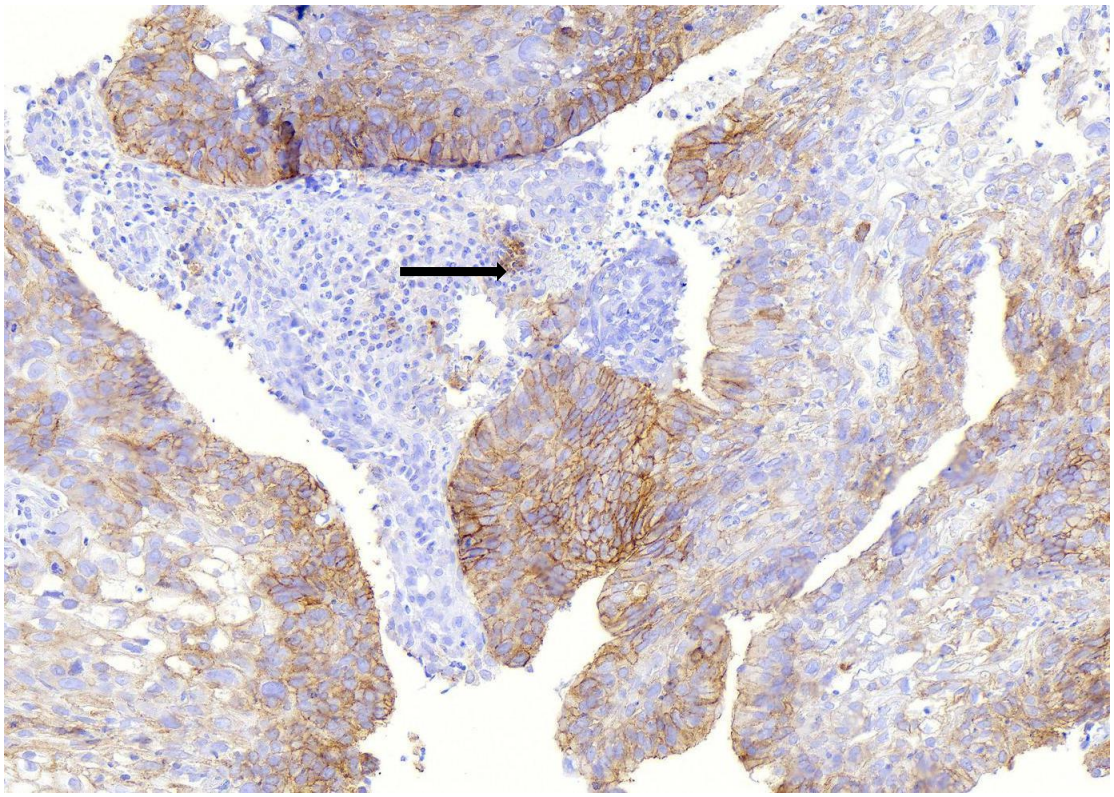


Figure 8: A photomicrograph of positive grade 2 ESCC with staining intensity of +2 that shows few staining inflammatory cells in the stroma (arrow) (PD-L1 IHC staining, x200)

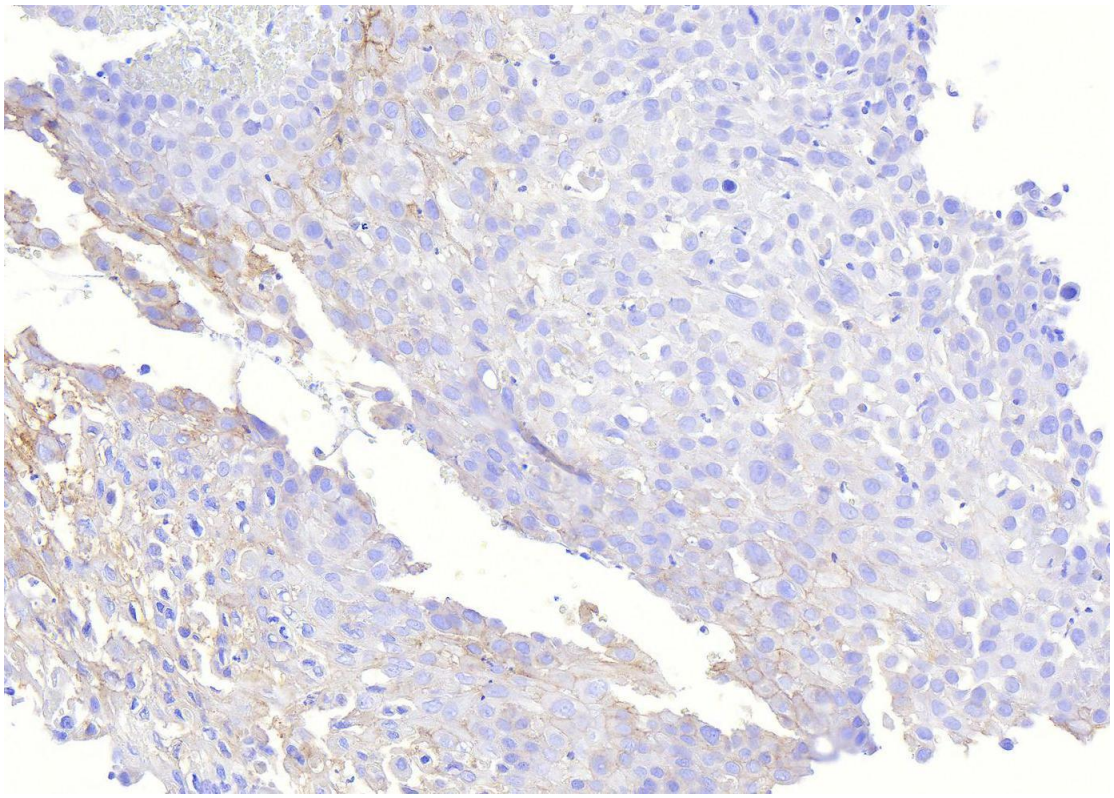


Figure 9: A photomicrograph of positive grade 1 ESCC with staining intensity of +1 (PD-L1 IHC staining, x200)

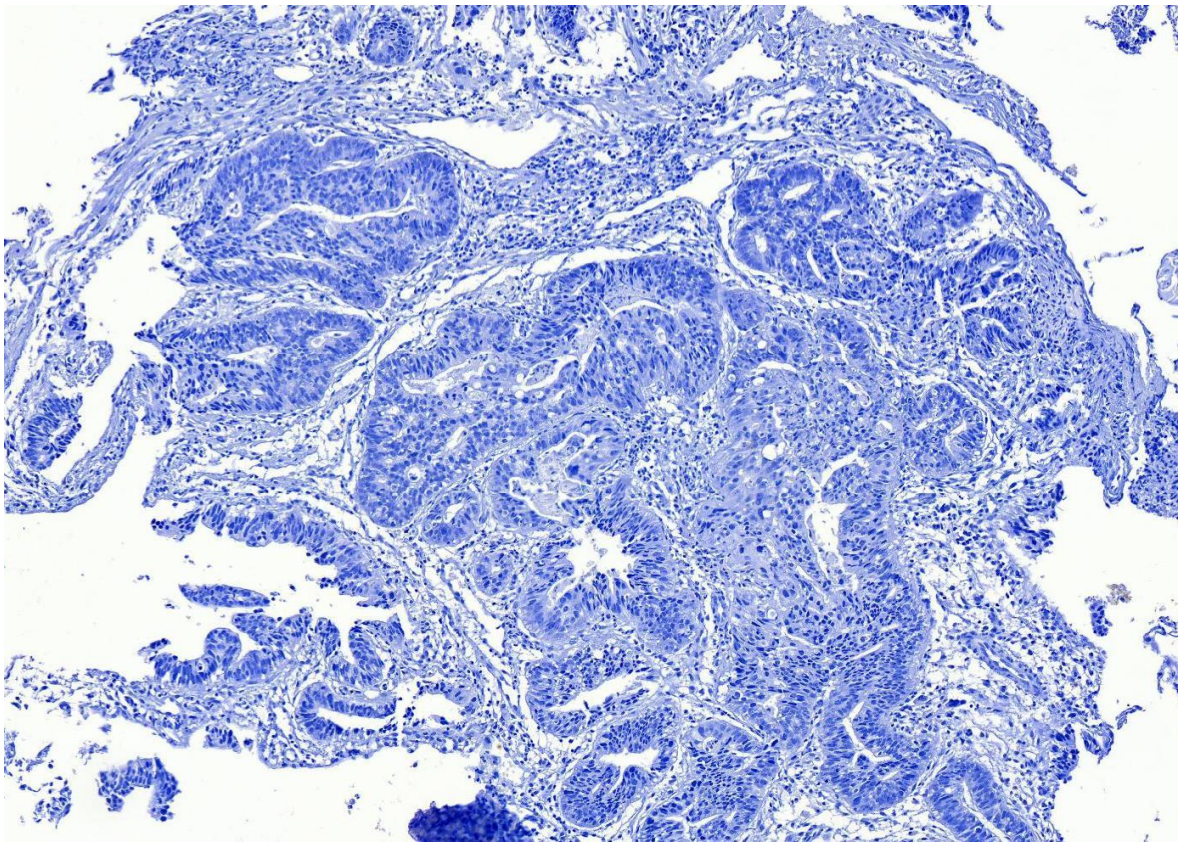


Figure 10: A photomicrograph of negative grade 2 adenocarcinoma (PD-L1 IHC staining, x200)

4.5 Association between clinicopathological features with PD-L1 immunohistochemistry expression among esophageal carcinoma patients at Muhimbili national hospital

In this study using Fisher's exact test, there was no difference seen in PD-L1 IHC expression between the 2 age groups (p=0.459) however, most positive cases were ≥ 60 years 6.9% (4/58) compared to < 60 years by 3.8% (3/78). Similarly with sex, there was no difference seen between male and females (p=0.685) where females had 6.7% (3/45) more positive cases compared to males 4.4% (4/91). On tumor location, the upper esophagus had 8.0% (2/25) positive cases, followed by middle part with 5.2 %(4/77) and then lower esophagus 2.9% (1/34) but this was yet not statistically significant (p=0.755). All positive cases were ESCC 5.8% (7/121) but not statistically significant (p=>0.999). Lastly, there was no association seen among tumor grades (p=0.856) despite more positive cases seen in grade 2 by 6.7% (5/75) followed by grade 1 4.2% (2/48) and 0% for grade 3. **(Table 3)**.

Table 2: The association between clinicopathological features with PD-LI IHC expression among esophageal carcinoma patients

Variables	PD-L1 Status Positive (%)	Negative (%)	p - value
Age (years)			
< 60	3(3.8)	75 (96.2)	0.459
≥ 60	4 (6.9)	54 (93.1)	
Sex			
Male	4 (4.4)	87 (95.6)	0.685
Female	3 (6.7)	42 (93.3)	
Tumor location			
Upper	2 (8.0)	23 (92.0)	0.755
Middle	4 (5.2)	73 (94.8)	
Lower	1 (2.9)	33 (97.1)	
Histological subtypes			
Invasive squamous cell carcinoma	7(5.8)	114 (94.2)	>0.999
Adenocarcinoma	0 (0.0)	13 (100.0)	
Others	0 (0.0)	2 (100.0)	
Tumor grade			
Grade 1	2 (4.2)	46 (95.8)	0.856
Grade 2	5 (6.7)	70 (93.3)	
Grade 3	0 (0.0)	13 (100.0)	

CHAPTER FIVE

5.0 DISCUSSION

In the recent decade, great focus has been dedicated to understanding the tumor microenvironment, which has helped scientists understand how cancers might enter the immune system undetected. It therefore gave rise to ICIs for multiple solid tumors. With a focus on PD-L1 as one of the checkpoints targeted by ICIs. This study aimed to determine the proportion of PD-L1 IHC expression, its association with clinicopathological characteristics and the types of cells with their staining patterns and intensities among patients with EC. The study showed a proportion of 5.1% with no association seen between PD-L1 IHC expression with the clinicopathological characteristics. Tumor cells and infiltrating immune cells showed cell membranous staining and most had weak intensity.

5.1 The proportion of esophageal carcinoma patients with PD-L1 expression by Immunohistochemistry at Muhimbili National Hospital

In this study, PD-L1 was expressed in 5.1% of all the cases analysed. In comparison with other studies done by Kaiyan Chen *et al* which showed a proportion of 41.1%, *Lulu Rong et al* (29.9%) and 18.9% which was reported in the study of Ito *et al* (23,26); the current study had a lower prevalence of PD-L1 IHC expression. This difference could be explained by several factors. First, is the use of different assays and antibodies across the studies for example, Rong *et al* who used PD-L1 clone SP142 while Jiang C *et al* used PD-L1 E1L3N clone and current study applied PD-L1 22C3 (25,26). By using different clones, there is a concept of epitope specificity whereby, different clones of an antibody can recognize different epitopes on the same antigen (44). Variability in epitope recognition can affect the binding affinity and specificity of the antibody thereby giving different level of PD-L1 expression.

Second, the difference in immunohistochemistry techniques such as antigen retrieval time, antibody dilution ratios and the incubation periods (23,26). These directly affects PD-L1 expression as some cases may be false negatives if the antigen retrieval time was too small or over dilution of the antibodies (45). Third, pre analytical factors such as tissue fixation, tissue blocks storage conditions in terms of temperature and humidity. Preservation of cellular details is a crucial step for immunohistochemistry. Therefore, as a retrospective

study there is lack of control for proper fixation techniques which ultimately could cause loss of antigenic sites on cells hence affecting PD-L1 IHC expression.

Fourth, is tumor heterogenicity, that is by using these small endoscopic biopsies it reduces the ability to have a definitive conclusion on PD-L1 IHC expression due to lack of access to the entire tumor. This because late stage EC patients tend to have unresectable tumors hence the difference in tumor biology and tumor microenvironment within the same tumor influences the IHC expression (46). Fifth, is population variability, most studies on PD-L1 IHC expression in EC were done in Asia and Europe (20,24–26,28). Therefore, the difference in the genetic pool with their associated mutations between theses continents and Africa may influence PD-L1 IHC expression.

Sixth, was that some studies applied more sophisticated detection methods such as PD-L1 mRNA as shown by Svensson *et al* where for primary tumors they had a prevalence of 23% on tumor cells, 41.2% on tumor infiltrating immune cells and 84% when it was coupled with lymph node metastasis hence an increase in their detection ability (43). This implies that despite a low PD-L1 IHC expression in our study population, there is a room for improvement by ensuring prevention of pre analytical, analytical and post analytical errors as well adding more advanced tests in the detection of PD-L1.

5.2 The association between clinicopathological features with PD-L1 expression among esophageal carcinoma patients at Muhimbili National Hospital

Regarding the association between age and PD-L1 IHC expression, this study showed no significant difference elderly patients showing more PD-L1 expression. This finding was in agreement with most studies example, Kaiyan C *et al* had 41.5% positive PD-L1 IHC patients ≥ 65 and 41.4% < 65 years like in current study despite lack of association (15). In contrast to this was Rong *et al* who found an association between age and PD-L1 IHC expression among patients who were ≥ 60 by 34.6% and 25.3% < 60 years (26). This could be attributed to the use of a different antibody clone (PD-L1 SP142) which may have a different epitope sensitivity.

The discrepancy in results seen for these studies could be attributed by several factors such as, age related immune system changes a term known as “immunosenescence”. This phenomenon involves a complex changes in the adaptive and innate immune function hence leading to immunodeficiency (47). It also leads to a reduction in the capability to cope with a variety of stressors and a progressive increase in the pro-inflammatory status which may explain PD-L1 over expression seen among the elderly.

Cumulative exposure to environmental risk factors both through occupational health hazards and social activities such alcohol consumption, cigarette smoking and others that further led to immune mediated conditions and chronic inflammation (48,49). Therefore, it influences tumor microenvironment through modulating PD-L1 expression in patients with EC as some studies showed the higher the inflammatory cells the higher is PD-L1 expressed (26). This implies that despite lack of association seen between age and PD-L1 IHC expression in most studies including this study, its occurrence among the elderly gives a direction towards subjecting this age group to the test as they are more likely to be eligible for immune targeted therapies.

Regarding the association between sex with PD-L1 IHC expression, this study showed no significant difference between the sexes, despite female showing more PD-L1 IHC expression. Previous studies are in agreement with this study in terms of no association seen between PD-L1 IHC expression and sex but, these studies showed a trend towards males having a higher expression of PD-L1 IHC than female. For example, Kaiyan *et al* 41.6% male with positive PD-L1 IHC expression while 40.3% female, similarly to Wakita *et al* 86.7% males showing positivity for PD-L1 IHC and 13.3% female (15,23). It is to the best of our knowledge there is no current study which shows a trend towards female having a higher PD-L1 IHC than men like current study.

Our current findings show that females were more frequently associated with PD-L1 expression although this was not statistically significant. This finding is not in concordance with previous studies on the distribution of PD-L1 IHC expression with sex and this discrepancy can be partly explained by several reasons as below: First, tumor microenvironment which includes immune cells, cytokines and other factors which may differ between male and female. In response to antigenic challenges, females display more

robust innate, cell-mediated, and humoral immune responses than males (50). This can lower pathogen load and speed up pathogen clearance, but it can also increase immune-related pathologies such inflammatory or autoimmune illnesses. Therefore, this may cause an upregulation of PD-L1 expression in females than male (51).

Secondly, genes and genetic polymorphism, only 100 genes are found on the Y chromosome, compared to over 1000 on the X chromosome, many of which are immune-related genes that code for proteins involved in immunological processes such as TLR7 and TLR8 common to receptors such as IL-2, IL-4 (50). Therefore, lack of balance such as mutations may alter these proteins and further cause an upregulation of PD-L1 expression in females than male.

Consequently, PD-L1 expression, which is influenced by interactions between tumor cells and the immune microenvironment, could exhibit comparable levels regardless of sex or unlike other cancers where sex hormones (like estrogen and testosterone) might influence tumor biology and immune responses (36). EC may not be significantly affected by these hormones in terms of PD-L1 regulation supporting the lack of association that we see throughout all studies including this. This implies that studies are required to explain the reasons behind this controversy seen in results among studies in the context of PD-L1 expression.

Concerning the association between tumor location and PD-L1 IHC expression, this study was statistically insignificant despite most positive cases being located on the upper third 8%. This was similar with many studies in terms of no association seen between tumor location with PD-L1 IHC expression example, Rong *et al* despite their demonstration of most positive cases in lower third by 33.3% (26). Similarly, Jiang *et al* who showed most cases in the middle (39/246) (25), and Wakita *et al* who showed that there was an equal distribution in the middle and lower parts by (7/15) patients on each part (23). But Kaiyan *et al* showed that there was an association seen with tumor location ($p=0.037$) with most cases seen in lower third (45.1%).

These variations can be explained by the difference in tumor microenvironment based on several points. First, the upper esophagus may have higher levels of tumor-infiltrating lymphocytes and other inflammatory cells compared to the middle and lower esophagus

(37,38). Therefore, increased immune cell infiltration and activation can lead to higher PD-L1 expression on tumor cells as an immune evasion mechanism. Secondly, the upper, middle, and lower esophagus have distinct blood supply, lymphatic drainage, and tissue architecture (37). These anatomical factors can influence the recruitment and activation of immune cells, which in turn can impact PD-L1 expression.

Third, genetic or molecular differences such as microsatellite instability (MSI) whereby esophageal tumors with high MSI often exhibit higher PD-L1 expression (43).This is because EC located in different parts of the esophagus may have distinct mutational profiles and dysregulated molecular pathways. Certain genetic alterations such as DNA methylation and histone modifications can regulate the expression of PD-L1 in EC (8) or activation of signalling pathways such as PI3K/Akt seen in the development of ESCC may induce PD-L1 expression (52).This suggests a potential role for PD-1/PD-L1 targeted therapy in upper esophageal tumors.

Regarding the association between histological subtypes and PD-L1 IHC expression, this study showed no significant difference among histological subtypes despite all positive cases were ESCC. This result was consistent with the study by Huang Z *et al* that reported on 5 histological subtypes whereby ESCC showed more positive cases by 94.2%, and no significant association was seen (33). It should be noted that majority of the studies were based on patients with ESCC only (20,21,25,26), hence lacking good study comparability on this category.

The high PD-L1 IHC expression seen in ESCC may be explained by genomic alterations such as NOTCH1/NOTCH3 and EGFR mutations which have been overexpressed in ESCC than EAC and other subtypes (8). Taking EGFR as example, its over expression has been linked to suppressing immune response through either activating regulatory T cells or reducing the level of T-cell chemoattractant therefore inducing PD-L1 expression (53). This implies that there is a greater role of genomics and molecular characterization of PD-L1 expression in patients with EC and that patient with ESCC may be more eligible for PD-1/PD-L1 targeted therapy.

In the association between tumor grades and PD-L1 IHC expression this study showed no association, but most positive cases were of grade 2. There has been variations seen across studies when it comes to the association between tumor grade and PD-L1 expression example, Huang *et al* and Wakita *et al* both were in agreement with this study showing no association in tumor differentiation (tumor grade) and both showing more positive cases in grade 2 (23,33). In contrast to this were Rong *et al* and Kaiyan Chen *et al* with more positive grade 3 (41.8%) and 1 (54.3%) tumors respectively ,where together showed a positive association with tumor grade [p=0.018 and 0.010 respectively] (15,26).

This variability seen across many studies including current study may be attributed by several factors. First, tumor microenvironment, higher-grade EC often have increased immune cell infiltration, leading to higher levels of immune activation and upregulation of PD-L1 expression (26). Second, inflammation and cytokines, higher-grade EC are associated with increased inflammation and higher levels of inflammatory cytokines, such as IFN-γ, which can drive the expression of PD-L1 (21). Third, hypoxic conditions, higher-grade EC are generally more hypoxic as they have faster proliferation rates, leading to rapid growth that outpaces the available oxygen supply, resulting in the development of hypoxic regions within the tumor and hypoxia is a known inducer of PD-L1 expression (41). This implies that high grade tumors are a potential for immune targeted therapies.

5.3 The types of cells expressing PD-L1 with their staining patterns and intensities at Muhimbili National Hospital

In this study, PD-L1 IHC positivity in staining was seen on tumor cells and infiltrating immune cells which were seen in an extremely small amount. This was a general anticipated expression which was supported by the scientific reasoning behind PD-L1 expression as it involves the interactions between tumor cells and its microenvironment (activated T cells) where pro-inflammatory mediators are released causing an upregulation of PD-L1 on tumor cells (29).

Similar studies done elsewhere example Rong *et al* who quantified both tumor cells and tumor infiltrating immune cells differently and achieved 29.9% and 40.2% positivity respectively (26). Wakita *et al* demonstrated PD-L1 IHC positivity on cancer cells only which were 15/72 (23). It should be noted that literature shows stromal cells such as

fibroblasts and inflammatory cells such as neutrophils, plasma cells and eosinophils may show positivity but, they are usually not included in the scoring for patients who will be eligible for targeted therapy [despite scoring was not applied in current study, only tumor cells, lymphocytes and macrophages are included] (26,42).

All positive cases in this study had a complete or partial membranous staining pattern that was distinct from the cytoplasm. This was supported by literature as cells to be considered positive should have partial or complete membranous staining and/or cytoplasm staining because PD-L1 is a transmembrane protein with two extracellular domains, IgV like and IgC-like domains; a transmembrane domain; and a cytoplasmic domain (29). This was seen throughout studies such as Rong *et al* who demonstrated positivity on tumor cells as partially or completely staining on the cell membrane or cell membrane and cytoplasm while on immune cells was defined as positive cells with membranous or cytoplasmic staining (26,42).

On the aspect of staining intensity, this study showed that most of the cases had weak intensity (+1). It is to the best of our knowledge that very few studies applied PD-L1 intensity with specific intensions for example, Ito *et al* who considered PD-L1 high expression if the grading intensity score was ≥7. This was essential in their study as they applied Allred scoring that required adding proportion scores and intensity scores (20). Similarly, Jiang *et al* who evaluated PD-L1 using H score that required combining staining intensity and the percentage of stained tumor cells (25). In both of the studies above they did not elaborate on what intensities were achieved in their studies therefore, this maybe the first study elaborating on this aspect. Staining intensity alone, as applied in the current study, holds no significant importance in determining the cases. Meaning cases can be called positive at any intensity (42).

5.4 Strengths and Limitations of the Study

The study strengths include; First, this study was able to achieve an introductory glimpse of PD-L1 IHC expression among patients with EC in our settings. Second, this is clinically relevant as examining PD-L1 IHC expression has potential implications for immunotherapy. Third, the study was replicated from other studies under optimization of few conditions using

a consistent staining protocol and automatically stained using Ventana Gx hence reducing human error. Combined with the use of positive and negative controls, it ensures consistency and reproducibility as well as a reliable data source for further studies.

However, the study did encounter certain limitations. First, the low prevalence of PD-L1 IHC expression might have limited the statistical power to detect associations with clinicopathological characteristics. Second, this study was a cross-sectional study design, it limits the ability to infer causality. Others include, small sample size, which may not be representative and could affect the generalizability of the findings. Missing data, such as tissue blocks, hence reducing our sample size which may have introduced bias.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study has shown a low prevalence of PD-L1 IHC expression among patients with EC at MNH. This could partly be attributed by several factors including, the use of different assays and antibodies across studies and population sample variability as most previous studies were from Asia. Our current study has also shown an apparent lack of association seen between PD-L1 IHC expression and the clinicopathological characteristics. Despite this, an important finding was that all positive cases were ESCC. Apparently, our current results may help formulating a diagnostic algorithm which includes PD-L1 IHC staining, so that patients with ESCC may be prioritized for IHC in order to allow judicial use of rather scarce resources available in our settings.

6.2 Recommendations

Future prospective studies are recommended where pre-analytical, analytical, and post-analytical errors can be controlled and thus potentially improving PD-L1 tissue detectability. Secondly, the application of more sophisticated detection methods such as PD-L1 mRNA, and FISH is recommended in order to increase the detection rate of PD-L1 IHC expression in the local context. Lastly, larger and, multicentric studies are recommended to accurately map PD-L1 IHC expression across our country. This will enable capturing a true prevalence of PD-L1 IHC expression in the local context.

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APPENDICES

Appendix 1: Data collection tool

HP/MR NUMBER	STUDY CODE	AGE	GENDER	TUMOR LOCATION	HISTOLOGICAL SUBTYPES	TUMOR GRADE

Appendix 2: Computer based Data collection Sheet in excel

STUDY CODE	AGE	GENDER	TUMOR LOCATION	HISTOLOGICAL SUBTYPES	TUMOR GRADE	STAINING CELLS	STAINING PATTERN	STAINING INTENSITY	PD-L1 STATUS
D28	**57**	**Male**	**Upper 1/3**	**Invasive squamous cell carcinoma**	**Grade2**	**No staining cells**	**Negative**	**Negative**	**Negative**

Appendix 3: Immunohistochemistry staining protocol Ventana Benchmark GX

Detailed procedures

- Deparaffinization using EZ prep solution for 8 minutes at 75 ^{0}C
- Cell conditioning using CC1 for 32 minutes. (For individual antibody protocol retrieval time refer Ventana benchmark GX protocol)
- Pre-primary Peroxidase inhibit for 4 minutes at 37 ^{0} C
- Primary antibody incubation (for primary antibody titration apply hand titration once the machine stops for titration)

  NB: Refer Ventana benchmark GX protocols for individual antibody and incubation time

If Post fixative selected

- Apply Opti-view HQ Linker for 8 minutes
- Opti-view HRP multimer for 8 minutes
- Apply one drop of DAB chromogen and one drop of hydrogen peroxide and incubate for 8 minutes
- Apply one drop of copper and incubate for 5 minutes
- Counter stain with hematoxylin solution for 8 minutes
- Post counterstain with bluing reagent for 4 minutes
- Remove slides from Ventana benchmark GX and wash with running tap water for 5 minutes
- Dehydrate in graded alcohol, clear in xylene and mount the slides.

Appendix 4: Ethical Clearance

UNITED REPUBLIC OF TANZANIA

MINISTRY OF EDUCATION, SCIENCE AND TECHNOLOGY

MUHIMBILI UNIVERSITY OF HEALTH AND ALLIED SCIENCES

OFFICE OF THE DIRECTOR – RESEARCH, PUBLICATIONS AND INNOVATION

Ref. No.DA.282/298/01.C/1984 Date: 04/12/2023

MUHAS-REC-12-2023-1984
DOREEN GERION GILBERT
COLLEGE OF MEDICINE
MUHAS

RE: APPROVAL FOR ETHICAL CLEARANCE FOR A STUDY TITLED: THE EXPRESSION OF PROGRAMMED CELL DEATH LIGAND 1 IN ESOPHAGEAL CARCINOMA PATIENTS AT MUHIMBILI NATIONAL HOSPITAL

Reference is made to the above heading,

I am pleased to inform you that the Chairman has on behalf of the University Senate, approved ethical clearance of the above-mentioned study, on recommendations of the Senate Research and Publications Committee meeting accordance with MUHAS research policy and Tanzania regulations governing human and animal subjects research.

APPROVAL DATE: 04/12/2023
EXPIRATION DATE OF APPROVAL: 04/12/2024

STUDY DESCRIPTION:
Purpose:

The purpose of this descriptive cross-sectional study is to determine the proportion of cases with PD-L1expression among esophageal carcinoma patients and their associated clinicopathological features at Muhimbili National Hospital.

The approved protocol and procedures for this study is attached and stamped with this letter, and can be found in the link provided:
https://irb.muhas.ac.tz/storage/Certificates/Certificate%20-%2014801.pdf and in the MUHAS archives.

The PI is required to:

1. Submit bi-annual progress reports and final report upon completion of the study.
2. Report to the IRB any unanticipated problem involving risks to subjects or others including adverse events where applicable.
3. Apply for renewal of approval of ethical clearance one (1) month prior its expiration if the study is not completed at the end of this ethical approval. You may not continue with any research activity beyond the expiration date without the approval of the IRB. Failure to receive approval for continuation before the expiration date will result in automatic termination of the approval for this study on the expiration date.
4. Obtain IRB amendment (s) approval for any changes to any aspect of this study before they can be implemented.
5. Data security is ultimately the responsibility of the investigator.
6. Apply for and obtain data transfer agreement (DTA) from NIMR if data will be transferred to a foreign country.
7. Apply for and obtain material transfer agreement (MTA) from NIMR, if research materials (samples) will be shipped to a foreign country,
8. Any researcher, who contravenes or fail to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine as per NIMR Act No. 23 of 1979, PART III section 10 (2)
9. The PI is required to ensure that the findings of the study are disseminated to relevant stake holders.
10. PI is required to be versed with necessary laws and regulatory policies that govern research in Tanzania. Some guidance is available on our website https://drp.muhas.ac.tz/.

Dr. Nahya Salim Masoud,

Chairperson, MUHAS Research and Ethics Committee

Muhimbili University of Health and Allied Sciences
DIRECTOR
Research, Publications & Innovations
Box 65001

Appendix 5: Introduction letter

UNITED REPUBLIC OF TANZANIA

MINISTRY OF EDUCATION, SCIENCE AND TECHNOLOGY

MUHIMBILI UNIVERSITY OF HEALTH AND ALLIED SCIENCES

OFFICE OF THE DIRECTOR – POSTGRADUATE STUDIES

In reply quote;
Ref. No. HD/MUH/T.20/2021

12th November, 2023

THE EXECUTIVE DIRECTOR,
MUHIMBILI NATIONAL HOSPITAL,
P.O BOX 65000,
DAR ES SALAAM.

MUHIMBILI NATIONAL HOSPITAL
RECEIVED
12 DEC 2023
REGISTRY
File No:.....

RE: INTRODUCTION LETTER OF DOREEN GERION GILBERT

We would like to inform you that the carrier of this letter is Doreen Gerion Gilbert with registration number HD/MUH/T.20/2021 a student in the School of Diagnostic Medicine at Muhimbili University of Health and Allied Science (MUHAS), pursuing MMed. Anatomical Pathology.

As part of her studies, she will be doing an elective research titled **"THE EXPRESSION OF PROGRAMMED CELL DEATH LIGAND 1 IN ESOPHAGEAL CARCINOMA PATIENTS AT MUHIMBILI NATIONAL HOSPITAL".**

She is being supervised by Prof. Amos Mwakigonja from Anatomical Pathology Department, School of Diagnostic Medicine at MUHAS and Dr. Advera Ngaiza from Anatomical Pathology Department at Muhimbili National Hospital.

The Ethical Clearance for doing the said research has been given by the University.

Kindly request your assistance and cooperation to our student.

Yours Sincerely,

Ms. Victoria Mwanilwa
For: DIRECTOR, POSTGRADUATE STUDIES

cc: Doreen Gerion Gilbert

9 United Nations Road; Upanga West; P.O. Box 65001, Dar Es Salaam: Tel. G/Line: +255-22-2150302/6; Ext. 1015; Direct Line:+255-22-2151378;Telefax:+255-22-2150465;E-mail:dpgs@muhas.ac.tz;Web:https://www.muhas.ac.tz

Appendix 6: Permission to collect data at MNH

THE UNITED REPUBLIC OF TANZANI

MINISTRY OF HEALTH

MUHIMBILI NATIONAL HOSPITAL

In reply please quote;

Ref. No.: MNH/CRTCU/Perm/2023/574 Date: 06th May, 2024

Head of Department
Central Pathology Laboratory

Block Manager,
Maternity I
Muhimbili National Hospital

RE: PERMISSION TO COLLECT DATA AT MNH

Name of Student	**Doreen G. Gilbert**
Title	**"The Expression of Programmed Cell Death Ligand 1 in Esophageal Carcinoma Patients at Muhimbili National Hospital"**
Institution	Muhimbili University of Health and Allied Sciences
Supervisor	**Prof. Amos R. Mwakigonja (Muhas)**
Co-supervisor	**Dr. Advera Ngaiza** (MNH)
Period	14th December, 2023 to 01st February, 2024

Approval has been granted to the above principal investigators to collect data at MNH. Dissemination of study findings at MNH shall be overseen by **Dr. Advera Ngaiza (MNH)** supervisor.

Kindly ensure the named principal investigator abide to the ethical principles and other conditions of the research approval.

Sincerely,

HEAD, TEACHING, RESEARCH & CONSULTANCY UNIT
P. O. Box 65000
DAR-ES-SALAAM

Dr. Sima Rugarabamu
For: Head of Clinical Research, Training and Consultancy Unit

c.c DCSS, DNS
c.c Doreen G. Gilbert
c.c Dr. Advera Ngaiza (MNH)

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