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腫瘤免疫微環境在食道鱗狀細胞癌預後的意義

Tumor Immune Microenvironment in the Prognosis of Locally Advanced Esophageal Squamous Cell Carcinoma

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論文中文題目

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論文英文題目

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本論文係黃大成君（學號D01453002　在國立臺灣大學腫瘤醫學研究所完成之博士學位論文，於民國114年06月07日承下列考試委員審查通過及口試及格，特此證明

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謝辭

洞悉真理是一條漫長的路，而科學研究是披荊帶棘走出這條路的一個方法。希望所開出的這條路能逐漸走成康莊大道，進而能幫忙與改善臨床上照顧的病人。今天能取得學位也只是這條路上的一座里程碑，是一段旅程的結束也是下一段旅程的開始。

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中文摘要

關鍵詞: 食道鱗狀細胞癌，前輔助同步化放療，第三級淋巴結構，PD-L1，腫瘤免疫微環境，化療抗藥性

食道鱗狀細胞癌是亞洲的一個主要癌症，佔比全球食道癌超過 85%。大部分的病人在診斷時已是局部晚期，而前輔助同步化放療加上隨後的根除性食道切除術是目前局部晚期食道鱗狀細胞癌的標準治療。然而，仍有大約 70%的病人會遭受癌症復發。因此，我們需要一個有效的預後生物標記，才能將病患依復發風險加以分層、甚至施以不同的治療，希望能進一步改善這群病人的預後。

人體對抗腫瘤的免疫力可以降低癌細胞的侵略性與生長，這也是包括化學治療與放射治療等許多抗癌治療策略，能達到治療腫瘤療效的重要關鍵之一。因此，免疫相關的生物標記是許多癌症重要的研究主題；針對食道鱗狀細胞癌，過去也有許多相關的研究。PD-L1 是 PD-1 (為一個免疫檢查點)的配體，可以造成 T 淋巴細胞的衰竭進而抑制免疫。PD-L1 在腫瘤中的表現不但代表對腫瘤免疫力的拮抗、也代表著免疫力的存在。過去關於 PD-L1 的表現在食道鱗狀細胞癌預後意義雖有許多研究，其結果並不十分一致。另外，第三級淋巴結構是在慢性發炎的組織(包括腫瘤，也可視為一種慢性發炎組織)發生、由免疫細胞組成的一種淋巴組織。一般認為，這種淋巴組織在腫瘤中具有對抗腫瘤的免疫力。目前關於第三級淋巴結構在食道鱗狀細胞癌預後的研究仍相當有限。本論文研究針對接受前輔助化放療的局部晚期食道鱗狀細胞癌病患，聚焦其治療前的食道癌腫瘤免疫微環境，首先探討腫

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瘤細胞 PD-L1、或免疫細胞 PD-L1 的表現，接著探討第三級淋巴結構的形成與成熟，對局部晚期食道鱗狀細胞癌病患的預後意義。

本論文研究的第一個部分探討 PD-L1的預後意義。這一部分研究收納了 100 位接受以每週太平洋紫杉醇和鉑金化療藥物為處方前輔助同步化放療的局部晚期食道鱗狀細胞癌病患。以治療前上消化道內視鏡取得的食道鱗狀細胞癌組織，進行 PD-L1 的免疫組織化學染色，以半定量的方式分析 PD-L1 表現量為 0、1+、2+或 3+：在免疫細胞上的表現量 0 或 1+定義為低表現、表現量 2+或 3+則為高表現；在腫瘤細胞上的表現量 0 定義為無表現、表現量 1+、2+或 3+則為有表現。我們發現免疫細胞 PD-L1 表現的高低與病患的無惡化存活以及整體存活都有強烈的正相關，而腫瘤細胞 PD-L1 表現的有無則與無惡化存活以及整體存活有顯著的負相關。多變項分析也確認了上述的發現。

本論文研究的第二部分，根據第一部分的研究結果提出了一個假說：腫瘤細胞表現的 PD-L1 與對抗癌藥物的抗藥性有相關性。我們測試五株食道鱗狀細胞癌細胞株對鉑金化療藥物的藥物敏感性與這些細胞株上 PD-L1 表現量的相關性。我們發現 KYSE150 是一株帶有 PD-L1 基因重複（重複數：7）的細胞株，其 PD-L1 表現量是五株細胞株最高的；KYSE150 也五株細胞株中是對鉑金化療藥物抗藥性最高的細胞株。KYSE510 是 PD-L1 表現量最低的，同時也是對鉑金化療藥物最敏感的細胞株。這五株細胞株 PD-L1 的表現量與其在一系列白金濃度下存活的比例，具有中等度的線性相關性。這個結果支持了 PD-L1 的表現與化療抗藥性相關的假

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說。然而，詳細的機轉還需要更進一步的研究。

本論文研究的第三部分探索局部晚期食道鱗狀細胞癌黏膜層的第三級淋巴結構的預後意義。這一部分研究收納了一組 137 位接受以每週太平洋紫杉醇和鉑金化療藥物為處方前輔助同步化放療的局部晚期食道鱗狀細胞癌病患。以治療前上消化道內視鏡取得的食道鱗狀細胞癌組織，進行 CD20 以及 CD23 的免疫組織化學染色，分析第三級淋巴結構的成熟狀態。我們發現成熟的第三級淋巴結構和對前輔助化放療的完全病理反應成顯著的負相關（p=0.031）。雖然在單變項分析中，成熟的第三級淋巴結構和不良的整體存活間只存在不顯著的相關趨勢，但在多變項分析中，成熟的第三級淋巴結構成為了獨立而顯著的不良預後因子（HR: 2.91, p<0.001）。這個結果與我們進行本研究之前的假說並不吻合。

本論文研究的第四個部分，我們嘗試驗證以內視鏡切片檢體判讀第三級淋巴結構狀態的可信度。我們利用 nanoString 平台的 Human Pan-Cancer Immune Panel 來分析 44 位局部晚期食道鱗狀細胞癌病患的腫瘤檢體，探討第三級淋巴結構中，無、不成熟與成熟三種狀態下，腫瘤免疫微環境的差異。我們發現第三級淋巴結構的形成與成熟相關於較活躍的免疫環境，包括較少的衰竭 CD8 T 細胞和較高的白血球、B 細胞、T 細胞、NK 細胞、細胞激素、化學激素、腫瘤壞死因子、toll-like 受器以及抗原呈現等免疫功能。我們接著利用對比成熟第三級淋巴結構與無第三級淋巴結構的腫瘤組織中免疫基因表現的差異，發展出第三級淋巴結構的基因標記（gene signature）。我們首先針對 TCGA 資料庫中食道鱗狀細胞癌的族群（82 人），

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證實這個基因標記可以有效地分別出腫瘤組織中淋巴濾泡組織的有無。我們也針對一個接受免疫檢查站抑制劑治療的轉移復發食道鱗狀細胞癌族群（35 人），證實這個基因標記可以有效地預測免疫檢查站抑制劑治療的療效。這些發現都支持我們使用內視鏡切片的腫瘤檢體所進行的分析方式，可以有效地區分食道癌腫瘤粘膜第三級淋巴結構的成熟狀態。

總結來說，PD-L1 在免疫細胞或者腫瘤細胞的表現、粘膜中成熟的第三級淋巴結構是接受前輔助同步化放療局部晚期的食道鱗狀細胞癌獨立的預後生物標記。PD-L1 在腫瘤細胞的表現和化療的抗藥性有正相關，這可能是 PD-L1 在腫瘤細胞的表現導致不良預後的機轉之一。然而，同步放化療是否對第三級淋巴結構造成傷害而導致不良的預後，則需要進一步的研究。

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Abstract

***Keywords**:* esophageal squamous cell carcinoma, tertiary lymphoid structure, PD-L1, chemoresistance, tumor immune microenvironment, chemoradiotherapy

Esophageal squamous cell carcinoma (ESCC) is a major cancer in Asia, and accounts for more than 85% of global burden of patients diagnosed with esophageal cancer. Most ESCC patients had locally advanced cancer at diagnosis. Neoadjuvant chemoradiotherapy (CRT) followed by radical esophagectomy is a current standard treatment for locally advanced ESCC. However, about 70% of patients suffered from recurrence. Efficient prognostic biomarkers are thus important for helping stratify patients with different risks of recurrence and for developing novel treatment strategies to improve their outcome.

Anti-tumor immunity hinders the aggressiveness and growth of cancer and may contribute to the efficacy of various anti-cancer therapy, including chemotherapy and radiotherapy. Immune-related biomarkers have been investigated for their prognostic significance in patients with locally advanced ESCC before. PD-L1, a ligand of an immune checkpoint, programmed cell death protein 1, suppresses immunity by causing exhaustion of T cells. The expression of PD-L1 in tumor, an antagonist of anti-tumor immunity, may represent the existence of anti-tumor immunity. Multiple previous studies focusing on the expression of PD-L1 in tumors yielded conflicting results in patients with

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ESCC. Tertiary lymphoid structure (TLS)—aggregates of immune cells forming lymphoid structures in or around tumor tissues—is considered as an origin of antitumor immunity. The prognostic significance of TLS has been only reported in a limited number of studies involving patients of locally advanced ESCC. The current thesis, focusing on tumor immune microenvironment (TME) of ESCC, has explored the prognostic significance of PD-L1 expression on immune cells (ICs) or tumor cells (TCs) and the prognostic significance of mucosal TLS in locally advanced ESCC patients treated with neoadjuvant CRT with weekly paclitaxel/platinum chemotherapy.

For the first part of the thesis, the prognostic significance of PD-L1 expression was investigated. A total of 100 locally advanced ESCC patients treated with neoadjuvant CRT with weekly paclitaxel/cisplatin chemotherapy were enrolled. Their pre-treatment ESCC tumor tissues, obtained by endoscopic biopsy, were analyzed for the expression of PD-L1 by immunohistochemistry (IHC) and scored semiquantitatively. PD-L1 expression on ICs (PD-L1 IC) was defined as low (0 or 1+) vs high (2+ or 3+), and PD-L1 expression on TCs (PC-L1 TC) was defined as negative (0) vs positive (1+~ 3+). We found that high PD-L1 IC expression was strongly associated with better progression free survival (PFS) (HR: 0.44, p=0.0025) and overall survival (OS) (HR: 0.44, p=0.0024), and positive PD-L1 TC was significantly associated with worse PFS (HR: 1.7, p=0.029) and OS (HR: 1.63, p=0.035). Multivariate analysis demonstrated both PD-L1 TC and PD-L1

doi:10.6342/NTU202502233

IC as independent prognostic factors.

For the second part of the thesis, we hypothesized that PD-L1 expression on TCs may contribute to chemoresistance, thus underlying the finding of positive PD-L1 TC as a poor prognostic factor in patients with locally advanced ESCC treated with neoadjuvant CRT. We tested the chemosensitivity to cisplatin and the expression level of PD-L1 in 5 human ESCC cells lines. We found that KYSE150, which has PD-L1 amplification (copy number=7), had higher expression of PD-L1 compared to other cell lines and was more resistant to cisplatin than other cells. KYSE510 had the lowest baseline expression among the 5 cell lines and was the most chemosensitive cell line. Linear relationships can be identified between PD-L1 expression level and survival percentage of the 5 cell lines under serial concentrations of cisplatin. These findings support our hypothesis about the association of PD-L1 expression on tumor cells with resistance to chemotherapy. However, the detailed mechanisms warrant additional studies.

For the third part of the thesis, to investigate the prognostic significance of mucosal TLS in locally advanced ESCC, we recruited a cohort of locally advanced ESCC patients who received neoadjuvant CRT with weekly paclitaxel/platinum chemotherapy (n= 137). Pretreatment endoscope-biopsied primary esophageal tumor tissues were analyzed for the maturation status of TLS according to the IHC-defined expression of CD20 and CD23. The status of mature TLS had significant negative association with pathological complete

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response to neoadjuvant CRT (p=0.031). Although mature TLS only had a trend of association with worse OS (HR: 1.45, p=0.15) in univariate analysis, mature TLS turned out to be a strong independent unfavorable prognostic factor in the multivariate cox regression analysis (HR: 2.91, p<0.001). The result rejected our initial hypothesis that TLS is a favorable prognostic factor.

For the fourth part of the thesis, in order to validate the reliability of TLS status identified by endoscope-biopsied tissues, we explored the TME in tumors with no, immature, or mature TLS status of patients with locally advanced ESCC. We investigated the expression profiles of immune-related genes of ESCC tumor tissues by Pan-Cancer Immune Panel of nanoString® (n=44). We found that the maturation of TLS was associated with more active TME, including less exhausted CD8 T cells and more immune functions of leukocytes, B cells, T cells, NK cells, cytokines, chemokines, tumor necrosis factors, toll-like receptors, and antigen presentation. Based on genes with highly differentiated expression between ESCC tumors with mature TLS vs no TLS, we constructed a TLS signature and demonstrated that this TLS signature can significantly differentiate tumor tissues with from without lymphoid follicles in the ESCC cohort of TCGA database (n=82); the TLS signature can also significantly differentiate ESCC patients benefited from anti-PD-1 based immunotherapy in our cohort of metastatic or recurrent ESCC patients (n=35). These findings support that the TLS status identified by

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IHC on pre-treatment endoscope-biopsied tumor tissues is accurate.

Overall, we found that PD-L1 IC, PD-L1 TC, and mucosal mature TLS were independent prognostic biomarkers for locally advanced ESCC patients who received neoadjuvant CRT. Positive PD-L1 TC is associated with chemoresistance, which could lead to unfavorable prognosis. It needs further investigations whether the damage from CRT changes the TME of TLS and leads to unfavorable prognosis.

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Contents

doi:10.6342/NTU202502233

doi:10.6342/NTU202502233

doi:10.6342/NTU202502233

List of Figures

doi:10.6342/NTU202502233

List of Tables

doi:10.6342/NTU202502233

Chapter I Background

1.1 Introduction of esophageal cancer

1.1.1 Epidemiology

Esophageal cancer (EC) ranks as one of the leading causes of cancer-related deaths. According to the GLOBOCAN 2020 database, there are an estimated 604,100 new cases of EC and 544,100 deaths attributed to it each year, positioning EC as the eighth most frequently diagnosed cancer and the sixth leading cause of cancer mortality globally. EC is primarily categorized into two histological subtypes: esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). The majority of patients, approximately 85%, are diagnosed with ESCC, while 14% are identified as having EAC (Morgan et al, 2022).

ESCC and EAC exhibit different geographic incidence and distribution patterns. The highest rates of ESCC occur in Eastern Asia, followed by Southern and Eastern Africa, while EAC shows its highest incidence in highly developed nations such as Australia, Canada, various countries in Northern and Western Europe, and the United States (Morgan et al, 2022).

Moreover, ESCC and EAC are associated with distinct risk factors. ESCC is linked to tobacco use, alcohol intake, achalasia, caustic injuries, a history of thoracic radiation, low socioeconomic status, inadequate oral hygiene, nutritional deficiencies, and non-epidermolytic palmoplantar keratoderma. In contrast, EAC is related to symptomatic gastro-esophageal reflux disease, Barrett's esophagus, obesity, tobacco use, a history of thoracic radiation, older age, male gender, a diet low in fruits and vegetables, and medications that relax the lower esophageal sphincter (Pennathur A et al, 2013).

Additionally, the genetic alterations in ESCC and EAC differ significantly. In ESCC, common and recurrent mutations involve genes such as TP53, NFE2L2, MLL2,

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ZNF750, NOTCH1, and TGFBR2, whereas EAC frequently shows alterations in TP53, CDKN2A, ARID1A, SMAD4, and ERBB2. Genomic amplifications of CCND1, SOX2, TERT, FGFR1, MDM2, NKX2-1, and/or TP63, along with deletions of RB1, VGLL4, and ATG7, are often observed in ESCC. Conversely, EAC more commonly exhibits amplifications of ERBB2, VEGFA, GATA4, and GATA6. The genetic profile of ESCC resembles that of head and neck squamous cell carcinoma, while EAC's genetic alterations are akin to those found in the chromosomal instability subtype of gastric cancer (The Cancer Genome Atlas Research Network, 2017). Ultimately, ESCC and EAC represent two distinct disease entities.

1.1.2 Clinical Presentation and Diagnosis

The primary symptom associated with EC is dysphagia, while some patients who do not exhibit symptoms are identified through surveillance endoscopy conducted for different reasons. Patients with ESCC are more likely to experience weight loss (Pennathur A et al, 2013), and additional symptoms may include gastrointestinal bleeding, recurrent aspiration, and vomiting (Obermannová R et al, 2022). Diagnostic and staging methods encompass endoscopy with biopsy, bronchoscopy, computed tomography, endoscopic ultrasound, and positron emission tomography (Obermannová R et al, 2022).

The American Joint Committee on Cancer (AJCC) staging system is the most frequently utilized method for staging EC, specifically for epithelial cancers of the esophagus and esophagogastric junction (Rice TW et al, 2017). The categories for EC include primary tumor (T), regional lymph node (N), and distant site (M) based on anatomical considerations. This staging system offers distinct classifications for clinical (cTNM), pathologic (pTNM), and post-neoadjuvant therapy (ypTNM) stage groups (Rice TW et al, 2017).

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1.1.3 Treatment

Surgical resection is the primary treatment for early-stage disease. Endoscopic en bloc resection or esophagectomy serves as the therapeutic alternative. For patients with fewer risk factors for lymph node metastasis, endoscopic resection is preferred. Conversely, for those at high risk for lymph node metastasis, surgical resection is the recommended approach (Obermannová R et al, 2022). Minimally invasive esophagectomy has emerged as the standard treatment for patients undergoing esophagectomy (Mariette C et al, 2019).

For patients with locally advanced disease, the primary treatment can be categorized based on histology. Those diagnosed with ESCC may undergo neoadjuvant chemoradiotherapy (CRT) followed by esophagectomy (van Hagen P et al, 2012; Yang H et al, 2018) or definitive CRT (Herskovic A et al, 1992), as recommended by most global treatment guidelines (Obermannová R et al, 2022). Conversely, patients with EAC may receive perioperative chemotherapy combined with esophagectomy (Al-Batran SE et al, 2019) or neoadjuvant CRT followed by esophagectomy (van Hagen P et al, 2012). Overall, the neoadjuvant CRT utilizing the CROSS regimen, which includes weekly doses of carboplatin (with an area under the curve of 2 mg per milliliter per minute) and paclitaxel (50 mg per square meter of body surface area) over a period of 5 weeks, along with concurrent radiotherapy (41.4 Gy delivered in 23 fractions, 5 days a week), followed by esophagectomy, has been recognized as the standard treatment for locally advanced EC of all histological types globally since 2012 (van Hagen P et al, 2012). In Taiwan, a neoadjuvant CRT approach involving twice-weekly administration of paclitaxel combined with cisplatin, along with concurrent radiotherapy and subsequent esophagectomy, has been studied and shown to be an effective treatment for locally advanced EC (Lin CC et al, 2007; Guo JC et al, 2015).

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The advantages of palliative chemotherapy for metastatic EC, have not been established prior to 2019 (Lordick F et al, 2016). The combination of cisplatin and fluorouracil has been utilized for locally advanced unresectable and recurrent or metastatic EC based on consensus, despite the absence of large-scale clinical trials to validate the benefits for OS. In cases of advanced EC where platinum-based chemotherapy has failed, taxanes and other medications have been employed as second-line systemic therapies, although these have only been documented in phase II studies (Kitagawa Y et al, 2019). Immune checkpoint inhibitors, either alone or in combination with chemotherapy have been established as a new standard treatment for metastatic or recurrent EC.

Adverse outcomes in patients with EC are primarily due to the majority being diagnosed at a locally advanced or metastatic stage, compounded by the absence of a screening program for early detection and treatment. Additionally, the high recurrence rate associated with non-metastatic disease and the limited options for systemic therapy contribute to this issue. The 5-year OS rate varies between 15% and 25% (Pennathur A et al, 2013; Guo JC et al, 2015), highlighting a significant unmet medical need to enhance the prognosis for individuals diagnosed with EC.

1.2 Esophageal cancer in Taiwan

The incidence of EC has risen over the past few decades. According to the Taiwan Cancer Registry Center, the age-adjusted incidence rate increased from 3.95 per 100,000 in 1980 to 7.36 per 100,000 in 2019. Additionally, the number of newly diagnosed EC patients rose from 478 annually in 1980 to 2,833 annually in 2019. In Taiwan, over 90% of EC patients are male, with the predominant histology being ESCC,

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which accounts for more than 90% of cases. The male-to-female incidence ratio for EC exceeds 10. Besides alcohol consumption and cigarette smoking, which are well-established risk factors for ESCC, betel nut chewing also significantly contributes to the development of ESCC in Taiwan (Wu IC et al, 2006). The expected years of life lost for patients diagnosed with stages I-V between 2008 and 2014 were 15.8, 17.5, 20.5, and 22.5, respectively. It appears that the incidence of EC in Taiwan is shifting towards younger age groups, and the rates continue to rise (Lai WW et al, 2020). The prognosis for patients with ESCC is grim, with a median survival of less than one year and a five-year OS rate for all stages of less than 17% (Cheng YF et al, 2018).

The available systemic treatment options for patients with EC or ESCC in Taiwan are restricted. Among the standard chemotherapeutic drugs, only cisplatin and fluorouracil are covered by our National Health Insurance. Nivolumab monotherapy is reimbursed as a second-line treatment for patients whose tumors exhibit positive PD-L1 expression and whose disease has advanced following first-line chemotherapy with cisplatin and fluorouracil. (Muro K et al, 2019).

1.3 Neoadjuvant CRT followed by esophagectomy for locally advanced EC

Since the 1980s, numerous randomized clinical trials have been carried out to evaluate the effectiveness of neoadjuvant CRT in the treatment of EC. However, the majority of these trials, which utilized a regimen of cisplatin combined with fluorouracil, lack adequate statistical power to provide a conclusive outcome. A meta-analysis conducted by Sjoquist KM et al. in 2011 reviewed 13 randomized clinical trials of neoadjuvant CRT and found that the hazard ratios (HRs) for mortality were 0.78 (95% CI: 0.70-0.88), 0.80 (95% CI: 0.63-0.93), and 0.75 (95% CI: 0.59-0.95) for the overall

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population, the squamous cell carcinoma group, and the adenocarcinoma group, respectively, when neoadjuvant CRT was compared to surgery alone. Consequently, neoadjuvant CRT followed by surgical intervention is widely recognized as a standard treatment approach for locoregional EC.

In addition to the metaanalysis, there are two significant randomized controlled trials that utilized a regimen of platinum combined with a microtubule-acting agent, demonstrating that neoadjuvant CRT followed by surgery is a global standard of care for patients with locally advanced EC, particularly for ESCC. The CROSS trial, which included patients with EAC or ESCC from various European countries, showed a statistically significant survival advantage for neoadjuvant CRT that employed a combination of weekly paclitaxel and carboplatin (van Hagen P et al, 2012). Importantly, the survival benefit associated with this paclitaxel/carboplatin neoadjuvant CRT was found to be more significant in ESCC patients compared to EAC patients in a subgroup analysis. The second randomized trial, conducted by Yang H et al, involved ESCC patients from several centers across China (Yang H et al, 2018). This study revealed that neoadjuvant CRT using vinorelbine, another microtubule-acting agent, in combination with cisplatin resulted in a higher pathological complete response (pCR) rate and a statistically significant survival benefit for patients with localized ESCC. Nevertheless, a 10-year follow-up report from the CROSS study indicated that the recurrence of distant metastasis was not significantly improved by the neoadjuvant CRT (Eyck BM et al, 2021). There is a pressing need for effective prognostic biomarkers to stratify patients based on their varying risks of recurrence to enhance patient survival.

1.4 Tumor immune microenvironment of ESCC

The immune landscape of the ESCC tumor immune microenvironment (TME)

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indicates that exhausted T and NK cells, regulatory T cells (Tregs), alternatively activated macrophages, and tolerogenic dendritic cells are prevalent within the TME, as demonstrated by single-cell RNA sequencing (Zheng Y et al, 2020). Transcriptional profiling and T cell receptor sequencing uncover lineage relationships among T cell populations. The immunosuppressive TME in ESCC is influenced by exhausted CD4, CD8 T, and NK cells, along with the interactions between macrophages and Tregs (Zheng Y et al, 2020). ESCC tumors exhibit a high presence of Tregs and exhausted T cells, and the immunosuppressive TME deteriorates with tumor progression (Zhang X et al, 2021). Furthermore, the interactions among various immune cell subtypes also play a role in the immunosuppressive TME of ESCC (Zhang X et al, 2021). Reinvigorating exhausted T cells could potentially boost antitumor immunity and enhance the prognosis for patients suffering from advanced ESCC.

The resurgence of cancer immunotherapy, particularly the blockade of immune checkpoints, has been the focus of intense research in recent decades. The upregulation of cytotoxic T-lymphocyte antigen 4 (CTLA-4) on the surface of T cells occurs when they are activated by antigen-presenting dendritic cells, serving as a regulatory feedback mechanism to reduce T cell activation during the priming and activation phases. Programmed cell death-1 (PD-1) represents another immune checkpoint that plays a role in various stages of anti-cancer immunity, including its upregulation on activated T cells, followed by the inhibition of T cell function within the tumor microenvironment, where programmed death ligand-1 (PD-L1) is typically expressed by infiltrating immune cells or tumor cells (Chen DS et al, 2013). Monoclonal antibodies that target CTLA-4, PD-1, and PD-L1 have been developed, leading to the reinvigoration of antitumor immunity and transforming the landscape of systemic therapy for numerous cancer types, including EC.

doi:10.6342/NTU202502233

1.5 Prognostic significance of PD-L1 expression in locally advanced ESCC

PD-L1 and its receptor, PD-1, constitute an immune checkpoint pathway that modulates T cell activation and functionality. When PD-1 on T cells binds to PD-L1, it transmits inhibitory signals that assist in preserving the balance of the adaptive immune system. The signaling pathways of PD-L1 and PD-1 can become dysregulated in various human diseases, particularly cancers. Since 2014, the inhibition of the PD-L1/PD-1 immune checkpoint has emerged as a significant approach in cancer treatment. By the end of 2020, several anti-PD-1/PD-L1 inhibitors had received approval for treating over 20 different cancer indications, including advanced ESCC (Kato et al, 2019; Kojima et al, 2020).

PD-L1 expression has been extensively studied as a predictive biomarker to assist in identifying cancer patients who are likely to gain from anti-PD-L1/PD-1 immune checkpoint blockade (Song et al, 2019; Aguiar et al, 2016). Nonetheless, the expression of PD-L1 on immune cells and tumor cells may possess different biological and clinical implications due to the various and complex mechanisms that underlie PD-L1 expression. It is thought that PD-L1 expression on immune cells and tumor cells is primarily influenced by interferon-gamma released by infiltrating T cells after activation. However, the expression of PD-L1 on tumor cells can also be influenced by oncogenes and may play a role in cancer invasiveness (Chen et al, 2014), metastasis, antiapoptosis (Azuma et al, 2008; Chen et al, 2016), resistance to chemotherapy and radiation (Black et al, 2016; Chen et al, 2016), epithelial–mesenchymal transition (Alsuliman et al, 2015; Kim et al, 2016), cancer stemness (Almozyan et al, 2017), antiautophagy (Clark et al, 2017), and aerobic glycolysis (Chang et al, 2015).

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The prognostic significance of PD-L1 expression in locally advanced ESCC has been documented (Guo et al, 2018), yet the findings are contradictory. Some research indicates that PD-L1 expression on tumor cells correlates with poor prognostic outcomes (Lim et al, 2016), while other studies suggest that PD-L1 expression on immune cells is associated with favorable prognostic outcomes (Hatogai et al, 2016; Zhang et al, 2017). Therefore, for patients diagnosed with ESCC, the disparity in prognostic value of PD-L1 expression between tumor cells and immune cells remains ambiguous.

In this research, we examined the expression of PD-L1 on tumor and immune cells sourced from pretreatment esophageal tumor tissues of patients with locally advanced ESCC undergoing neoadjuvant CRT. We identified the unique prognostic significance of PD-L1 expression on both tumor and immune cells, and we developed a combined index to enhance prognostic forecasting.

Investigations into the prognostic implications of PD-L1 on tumor and immune cells for locally advanced ESCC (Table 8) have yielded varying outcomes, potentially due to differences in the populations studied. Hatogai et al. and Jesinghaus et al. included patients who underwent surgery alone, while Lim et al. and Zhang et al. included patients who received neoadjuvant or adjuvant therapies alongside surgery. Additionally, these studies employed different antibodies for the immunohistochemical assessment of PD-L1 expression and utilized varying cutoff values for defining high or positive expression. Research involving patients who had surgery alone indicated that PD-L1 expression on tumor cells correlated with favorable prognosis (Hatogai et al., 2016; Jesinghaus et al., 2017); conversely, Lim et al. found it linked to poor survival outcomes. Elevated PD-L1 expression on immune cells was associated with improved survival in two studies (Hatogai et al., 2016; Zhang et al., 2017).

doi:10.6342/NTU202502233

Recent meta-analyses have highlighted the varying prognostic significance of PD-L1 expression in tumor versus immune cells. A 2017 meta-analysis encompassing 60 clinical studies with a total of 10,310 patients across 15 different cancer types found that elevated PD-L1 expression on tumor cells is typically linked to a worse prognosis (Wang et al, 2017). Similarly, another meta-analysis involving 2,877 patients with esophageal squamous cell carcinoma (ESCC) from 6 studies yielded comparable findings (Guo et al, 2018). In contrast, a separate meta-analysis of 18 studies with 3,674 patients across 12 cancer types assessed the prognostic implications of PD-L1 expression on tumor-infiltrating immune cells (Zhao et al, 2017). The authors of this study concluded that increased PD-L1 expression on these immune cells correlates with a reduced risk of mortality. The differing prognostic relevance of PD-L1 expression in tumor and immune cells may stem from their unique biological characteristics. PD-L1 expression in tumor cells can be influenced by intrinsic oncogenic activation, which may enhance tumor aggressiveness, contribute to drug resistance, and facilitate metastasis. These factors could elucidate the negative prognostic implications of PD-1 expression in tumor cells. Conversely, PD-L1 expression in tumor-infiltrating immune cells likely indicates a pre-existing adaptive anticancer immune response within the tumor microenvironment. Numerous studies have shown that PD-L1 on immune cells, particularly macrophages and other myeloid cells, plays a role in immune evasion and influences the effectiveness of PD-1/PD-L1 blockade immunotherapy (Tang et al, 2018; Noguchi et al, 2017; Lau et al, 2017; Tang et al, 2018; Lin et al, 2018).

1.6 Prognostic significance of tertiary lymphoid structure in ESCC

Tertiary lymphoid structures (TLSs)—clusters of immune cells, primarily T and B

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cells, that form lymphoid structures in non-lymphoid tissues at sites of inflammation, such as tumors—can facilitate prolonged antitumor immunity. The development of TLSs initiates with early TLSs, which are marked by clusters of B cells, advances to primary follicle-like TLSs featuring follicle-like clusters of B cells mixed with dendritic cells, and ultimately reaches secondary follicle-like TLSs that exhibit germinal center formation. Secondary follicle-like TLSs are recognized as mature TLSs, while early and primary follicle-like TLSs are deemed immature TLSs. (Sautes-Fridman C et al, 2019; Fridman WH et al, 2022; Schumacher TN et al, 2022)

The clinical importance of TLSs in the context of immunotherapy has been thoroughly examined. (Sautes-Fridman C et al, 2019; Fridman WH et al, 2022; Vanhersecke L et al, 2021; Petitprez F et al, 2020) The results indicate that TLSs correlate with the effectiveness of immune checkpoint inhibitors (ICIs) across various cancers, including ESCC. (Sautes-Fridman C et al, 2019; Fridman WH et al, 2022; Vanhersecke L et al, 2021; Petitprez F et al, 2020; Hayashi Y et al, 2023) Additionally, the existence of TLSs is linked to improved postoperative outcomes in patients with different cancers, (Petitprez F et al, 2020; Yu A et al, 2023; He W et al, 2020; Li Q et al, 2020; Cabrita R et al, 2020; Vayrynen JP et al, 2014; Silina K et al, 2018; Lin Q et al, 2020; Sofopoulos M et al, 2019; Li H et al, 2020; Hiraoka N et al, 2015) including ESCC. (Hayashi Y et al, 2023; Li R et al, 2022) In patients with localized ESCC who undergo direct esophagectomy, a greater density of TLSs is linked to improved survival rates. (Hayashi Y et al, 2023; Li R et al, 2022) Nevertheless, it remains uncertain whether TLSs can serve as a prognostic indicator for patients with locally advanced ESCC receiving trimodal therapy. Consequently, this study was initiated to explore the role of TLSs in predicting the outcomes of neoadjuvant CRT followed by esophagectomy in patients with locally advanced ESCC.

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Chapter II Materials and Methods

2.1 Investigating the prognostic significance of PD-L1 expression on tumor cells or immune cells in locally advanced ESCC patients who received neoadjuvant CRT

We hypothesized that PD-L1 expression on immune cells (ICs) is a favorable prognostic factor and PD-L1 expression on tumor cells (TCs) is an unfavorable prognostic factor for locally advanced ESCC patients who received neoadjuvant CRT.

2.1.1 Patients

This research involved patients diagnosed with locally advanced ESCC from three prospective clinical trials carried out at our institute between 2000 and 2015 (refer to Table 1). The design of the study, treatment protocol, and outcomes have been previously published (Lin et al, 2007; Huang et al, 2018). The three trials shared comparable inclusion and exclusion criteria. In summary, patients with locally advanced ESCC, classified as T3 or N+ based on the AJCC staging system (6th or 7th edition), who exhibited sufficient liver, renal, and bone marrow functions, along with an adequate performance status, were included. All participants underwent neoadjuvant CRT with a regimen of paclitaxel and cisplatin, receiving a total radiation dose of 40 Gy delivered in 20 fractions. Esophagectomy was conducted 4 to 6 weeks following the completion of neoadjuvant CRT. Those patients who did not undergo esophagectomy were given a second round of CRT, reaching a cumulative radiation dose of 66 Gy. Patients with ESCC who had archival esophageal tumor tissues available for analysis were retrospectively included in this study, and their clinical stages were re-evaluated according to the AJCC (7th edition). Additional clinical characteristics documented for analysis encompassed age, sex, Estern Cooperative Oncology Group performance status (ECOG-PS), the site of the primary esophageal tumor, whether

doi:10.6342/NTU202502233

radical esophagectomy was performed, pCR status, progression-free survival (PFS), and OS.

2.1.2 Immunohistochemistry and Analysis

All analyzed tissues were collected through endoscopic biopsy prior to the initiation of treatment. Following an examination of the relevant hematoxylin–eosin stains, sections of formalin-fixed paraffin-embedded (FFPE) tissues were obtained from our institute's Department of Pathology. Each tissue slide was verified to contain appropriate tumor lesions, with more than 50% of the tissues showing positive results based on the hematoxylin–eosin staining. The FFPE tissue slides underwent deparaffinization and rehydration. Antigen retrieval was conducted using Tris-EDTA buffer (pH 9.0) in a pressure cooker. A dual endogenous enzyme block (DakoCytomation, Glostrup, Denmark) was applied prior to the overnight incubation with the primary antibody against PD-L1 (dilution 1:100; clone SP142; Ventana, Arizona, USA) at 4°C. Following this, incubation with the secondary antibody (Dako real envision HRP rabbit/mouse) was carried out for 30 minutes, after which diaminobenzidine and hematoxylin were applied. The expression of PD-L1 on ICs and TCs was independently evaluated by two pathologists (Liang and Li) using a published scoring system, with any discrepancies resolved through consensus (Fehrenbacher et al. 2016). PD-L1 expression on TCs was classified as TC0 (<1%), TC1 (≥1% and <5%), TC2 (≥5% and <50%), and TC3 (≥50%). For ICs, PD-L1 expression was categorized as IC0 (<1%), IC1 (≥1% and <5%), IC2 (≥5% and <10%), and IC3 (≥10%). PD-L1 expression on TCs was deemed positive (TC-positive: TC 1-3) or negative (TC-negative: TC0). PD-L1 expression on ICs was classified as high (IC-high: IC2/3) or low (IC-low: IC0/1). The optimal cut-off values were established based on survival curves of TC and IC subgroups.

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2.1.3 Outcomes and Definitions

The results of the study included pCR, PFS, and OS. pCR was characterized as the complete elimination of cancer cells in the esophagectomy samples, which encompassed the dissected esophagus and lymph nodes, following neoadjuvant CRT. PFS was defined as the duration from the initiation of treatment until the first indication of tumor progression, recurrence, or death, whichever happened first. OS was defined as the period from the commencement of treatment to the time of the patient's death.

2.1.4 Statistical Analysis

The pCR rates across various groups were analyzed through a chi-square test. Survival curves were generated using the Kaplan–Meier method and compared among patients exhibiting different levels of PD-L1 expression via a log-rank test. For the multivariate analysis, Cox univariate and stepwise regression models were employed.

2.2 *in vitro* ESCC cell line model for the association of PD-L1 expression on tumor cells with chemoresistance

We hypothesized that PD-L1 expression on TCs associated with chemoresistance of ESCC.

2.2.1 Cell lines

Five ESCC human cell lines, KYSE 70, KYSE150, KYSE270, KYSE510, and TE5 were used for the *in vitro* experiment.

2.2.2 Sensitivity to cisplatin measured by MTT assay

ESCC cells (6,000 cells/well) were cultured in 96-well plates with a total volume of 200 μL per well on Day 0. On Day 1, Cisplatin was introduced to each well to achieve final concentrations of 0, 2, 5, and 10 μM. Cell viability was evaluated by counting cells

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using methylene blue exclusion in the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. On Day 5, 50 μL of 2 mg/mL MTT was added to each well, and the plates were incubated for 2 hours at 37°C, after which the absorbance was measured at 540 nm.

2.2.3 PD-L1 membrane expression by flow cytometry

Suspensions of ESCC cells were created. A fluorescently labeled anti-PD-L1 antibody (clone MIH, BD Pharmagen, New Jersey, USA) was introduced into the cell suspensions. The stained cells were then analyzed using a flow cytometer to measure the fluorescence intensity of each individual cell. The proportion of cells expressing PD-L1, which reflects the PD-L1 expression levels in each ESCC cell line, was evaluated. Additionally, the survival rate of each cell line at varying concentrations of cisplatin was compared with the PD-L1 expression levels of those cell lines.

2.3 Investigating the prognostic significance of the maturation of mucosal TLS in locally advanced ESCC patients who received neoadjuvant CRT

We hypothesized that the maturation of mucosal TLS is a favorable prognostic factor for locally advanced ESCC patients who received neoadjuvant CRT.

2.3.1 Patients

We conducted a retrospective analysis of pertinent data from patients with locally advanced ESCC who underwent paclitaxel/platinum-based neoadjuvant CRT prior to esophagectomy at two leading referral medical centers in Taiwan: National Taiwan University Hospital (NTUH) and Linkou Chang Gung Memorial Hospital (LK-CGMH). A group of 70 patients from NTUH was selected from our earlier research; this group included participants from three phase II clinical trials carried out between 2002 and 2015. (Huang TC et al, 2022; Huang TC et al, 2019; Lin CC et al, 2007) These patients

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received paclitaxel/cisplatin-based neoadjuvant CRT, with a total radiation dose of 40 Gy administered in 20 fractions. The current study focused exclusively on patients who had undergone esophagectomy following neoadjuvant CRT and had archival primary esophageal tumor tissues available for examination. Additionally, a cohort of 67 patients was retrospectively extracted from the LK-CGMH database. These patients were treated with the CROSS regimen—weekly paclitaxel/carboplatin CRT (total radiation dose: 41.4 Gy delivered in 23 fractions)—prior to esophagectomy between 2012 and 2018.

2.3.2 Immunohistochemistry

Prior to treatment, tissue samples were obtained via endoscopic biopsy and stained with hematoxylin/eosin. FFPE tissue sections were sourced from the pathology departments of NTUH and LK-CGMH. A pathologist (Liang) verified that all tissue samples had sufficient tumor lesions, constituting over 50% of all samples based on hematoxylin/eosin staining. Tissue slides for immunohistochemical staining were prepared following a previously established method.(Huang TC et al, 2022) The samples underwent blocking with Dual Endogenous Enzyme Block (DakoCytomation, Glostrup, Denmark) before being incubated with primary antibodies targeting CD20 (clone L26; Zytomed, Berlin, Germany), CD23 (clone DAK-CD23; Agilent Dako, Santa Clara, CA, USA), or PD-L1 (dilution 1:100; clone SP142; Ventana, AZ, USA). This was succeeded by washing and a 30-minute incubation with the appropriate secondary antibodies (Dako Real Envision HRP rabbit/mouse). After washing, the samples were treated with diaminobenzidine and hematoxylin. All samples underwent immunohistochemical staining for CD20 and PD-L1. CD20 expression was evaluated independently by two pathologists (Liang and Li) using a four-level scoring system: no (<1%), scanty (≥1% and <5%), moderate aggregation (≥5% and <10%), and strong

doi:10.6342/NTU202502233

aggregation (≥10%). Any differences in scoring between the pathologists were resolved through discussion until a consensus was reached. Tumors showing moderate or strong aggregation of CD20 expression were further evaluated for CD23 expression. Likewise, CD23 expression within CD20 aggregations was scored independently by the pathologists, with any discrepancies resolved through discussion until a consensus was achieved. TLSs were categorized as immature TLSs (moderate or strong aggregation of CD20 expression without CD23 expression) or mature TLSs (moderate or strong aggregation of CD20 expression along with CD23 expression within the CD20 aggregation). The patients were categorized based on their TLS status into three distinct groups: no TLS (indicating the absence of both immature and mature TLSs in the tissue), immature TLS (characterized by the presence of immature TLSs while mature TLSs are absent in the tissue), and mature TLS (defined by the presence of mature TLSs in the tissue). Additionally, the levels of PD-L1 expression on TCs and ICs were evaluated independently by pathologists following a standardized scoring system. (Fehrenbacher L et al, 2016) Any differences in scoring were addressed through discussions until a consensus was reached. The PD-L1 expression on TCs was classified as TC0 (<1%), TC1 (≥1% and <5%), TC2 (≥5% and <50%), or TC3 (≥50%), and was further categorized as positive (TC1-3) or negative (TC0). For ICs, PD-L1 expression was scored as IC0 (<1%), IC1 (≥1% and <5%), IC2 (≥5% and <10%), or IC3 (≥10%), and classified as high (IC2/3) or low (IC0/1).

2.3.3 Study outcomes

The results of the study included pCR and OS. pCR was characterized as the lack of cancer cells in samples obtained during esophagectomy (for instance, dissected esophagus and lymph node samples) from patients who underwent neoadjuvant CRT. OS was defined as the duration from the start of treatment until the patient's death.

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2.3.4 Statistical analysis

Key clinicopathological features, including age, sex, primary location of the esophageal tumor, and clinical T and N stages, were summarized through descriptive statistics. The levels of PD-L1 expression on TCs and ICs were assessed due to their prognostic significance in patients with locally advanced ESCC undergoing neoadjuvant CRT (Figure 8).(Huang TC et al, 2022) Clinical stages were reclassified according to the AJCC guidelines (seventh edition). The relationship between TLS status and age was evaluated using the analysis of variance test. The correlations of TLS status with sex, primary tumor location, clinical T stage, clinical N stage, PD-L1 expression on TCs, and PD-L1 expression on ICs were examined using the chi-square test. The pCR rate in patients categorized by TLS status was calculated using the chi-square test. Survival curves were created using the Kaplan-Meier method and compared among the TLS groups through a log-rank test. Univariate and multivariate analyses for pCR and OS were conducted using logistic regression and Cox regression, respectively.

2.4 Investivating immune-related gene expression in TME of ESCC in correlation with the maturation of TLS

We hypothesized that IHC of CD20 and CD23 preformed on endoscope-biopsied ESCC tissues can accurately identify the maturation of TLS.

2.4.1 TME exploration

To investigate the TME of ESCC in relation to the development of TLS, 44 FFPE ESCC tumor samples were analyzed. This included 17 samples without TLS, 15 with immature TLS, and 12 with mature TLS. The expression of immune-related genes was assessed using the Human Pan-Cancer Immune Panel from nanoString®. Through CIBERSORT analysis utilizing the nSolver system, the TME was evaluated, focusing on

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the relative quantities of different immune cells and their functions across the various stages of TLS development and maturation: no TLS, immature TLS, and mature TLS.

2.4.2 ESCC specific TLS gene signature

The expression levels of 800 genes were analyzed in ESCC tissues lacking TLS compared to those with mature TLS. The target genes exhibiting the most significant differential expression were chosen based on the following criteria: 1. The fold change in expression, comparing mature TLS to no TLS, is equal to or greater than 3; 2. The p-value indicating the differences is less than 0.01.

2.4.3 Validation of the ESCC specific TLS signature

The TLS signature specific to ESCC was validated using two cohorts. One cohort consisted of 82 patients with ESCC from the TCGA database, which was publicly accessible, and included the status of tumor-related lymphoid follicles identified through hematoxylin and eosin staining. (Barros et al, 2020) The lymphoid follicles were classified as TLS, and a comparison was made between tumors with and without these lymphoid follicles using our ESCC specific TLS signature. Additionally, another cohort of 35 patients with recurrent or metastatic ESCC, who were treated with either anti-PD-1 monotherapy or combination therapy at NTUH, provided gene expression profiles via the Human Pan-Cancer Immune Panel of nanoString®. (Guo JC et al, 2022) TLS is widely recognized as a predictor for the efficacy of anti-PD-1 immunotherapy across various cancers. Our ESCC specific TLS signature was used to compare tumors that exhibited clinical benefits from anti-PD-1 immunotherapy against those that did not.

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Chapter III Results

3.1 PD-L1 expression on immune cells as a favorable prognostic factor and PD-L1 expression on tumor cells as an unfavorable prognostic factor in locally advanced ESCC patients treated with neoadjuvnat CRT

3.1.1 Study population

Data from 100 patients diagnosed with locally advanced ESCC with available and sufficient pretreatment archival ESCC tissues were included in the study. The median age of the patients was 56 years, with the majority (89%) being men. Their baseline characteristics are detailed in Table 2. There were no significant differences in baseline characteristics among the three cohorts (Table 3). A total of 70 patients underwent esophagectomy after receiving neoadjuvant CRT. The median follow-up period was 99 months (ranging from 47 to 176 months), with median PFS and OS recorded at 14 months and 22 months, respectively (Figure 1).

3.1.2 PD-L1 expression on the tumor cells and immune cells of pretreatment ESCC tissues

The expression of PD-L1 on tumor and immune cells is illustrated in Figure 2. In terms of tumor cells, PD-L1 expression levels were categorized as TC0, TC1, TC2, and TC3, with frequencies of 45, 24, 27, and 4 patients, respectively. For immune cells, the PD-L1 expression levels were classified as IC0, IC1, IC2, and IC3, corresponding to 27, 43, 24, and 6 patients, respectively. There was no significant correlation found between the PD-L1 expressions on tumor cells and those on immune cells ($\chi 2 = 1.34$, $P = .51$; Table 4). Additionally, PD-L1 expression on either tumor or immune cells showed no correlation with age, sex, clinical stage, tumor location, or performance status (Table 5).

3.1.3 PD-L1 expression and pCR

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Out of the 70 patients who underwent esophagectomy, 29 (41%) attained a pCR. The pCR rate was greater in patients with high immune cell (IC) status compared to those with low IC status (57% versus 35%), although this difference was not statistically significant. Additionally, the pCR rates for patients with negative tumor cell (TC) status and those with positive TC status were comparable (42% versus 41%) (Table 6).

3.1.4 PD-L1 expression and patient survival

Patients with TC-positive status experienced significantly shorter progression-free survival (PFS) (HR: 1.7, P = .029) compared to those with TC-negative status (Figure 3a). Conversely, patients with IC-high status demonstrated significantly longer PFS (HR: 0.44, P = .0025) than their IC-low counterparts (Figure 3b). In the univariate analysis using the Cox regression model, ECOG-PS 0 (as opposed to 1 or 2), esophagectomy (compared to no esophagectomy), and IC-high status (versus IC-low status) were significantly linked to longer PFS; TC-positive status (in contrast to TC-negative status) was significantly correlated with shorter PFS (Table 7). In the multivariate analysis, factors such as sex, ECOG-PS, esophagectomy, PD-L1 TC expression, and PD-L1 IC expression continued to show significance for PFS.

Patients with TC-positive status also had significantly shorter overall survival (OS) (HR: 1.63, P = .035) than those with TC-negative status (Figure 4a). On the other hand, patients with PD-L1 IC-high status had significantly improved OS (HR: 0.44, P = .0024) compared to those with IC-low status (Figure 4b). The univariate analysis via the Cox regression model indicated that ECOG-PS performance status 0 (versus 1 or 2), esophagectomy (as opposed to no esophagectomy), and PD-L1 IC-high status (compared to IC-low status) were significantly associated with enhanced OS, while PD-L1 TC-positive status (in contrast to TC-negative status) was significantly linked to poorer OS (Table 8). In the multivariate analysis, sex, ECOG-PS, esophagectomy, and

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PD-L1 IC expression remained significant predictors for OS.

3.1.5 Combined predictor of PD-L1 expression on immune cells and tumor cells

The analyses revealed that PD-L1 expression might exhibit varying prognostic impacts between tumor and immune cells. Consequently, we integrated the PD-L1 expression levels from both tumor and immune cells to enhance predictive accuracy. As illustrated in Figure 5, we categorized the patients into four subgroups based on their PD-L1 expression in tumor and immune cells. Our findings indicated that patients with TC-negative and IC-high status experienced the best OS with a median of 130 months, while those with TC-positive and IC-low status had the poorest OS, with a median of 15 months (P = .0037; Figure 5).

3.1.6 Conclusions

We found that PD-L1 expression on ICs is a strong favorable prognostic factor and PD-L1 expression on TCs is a moderate unfavorable prognostic factor, especially for PFS, for locally advanced ESCC patients who received neoadjuvant CRT.

3.2 PD-L1 expression on tumor cells in association with chemoresistance to cisplatin of ESCC cell lines

3.2.1 Chemosensitivity to cisplatin for 5 human ESCC cell lines

We discovered that KYSE150 exhibited the highest level of chemoresistance to cisplatin, while KYSE510 showed the greatest chemosensitivity to the drug. The other 3 cell lines had moderate resistance to cisplatin. The MTT assay results for each cell line are illustrated in Figure 6.

3.2.2 Membranous expression levels of PD-L1 in 5 human ESCC cell lines

The PD-L1 expression was found to be the highest in KYSE150 and the lowest in

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KYSE510 (Figure 7). The other 3 cell lines had moderate expressions of PD-L1.

3.2.3 Correlation of membranous expression levels of PD-L1 and resistance to cisplatin in 5 human ESCC cell lines

Figure 8 illustrates moderate correlations in the linear relationships between PD-L1 expression and the percentage of cell survival for each cell line when exposed to 2, 5, and 10μM of cisplatin. Our findings indicate that PD-L1 expression in ESCC tumor cells has moderate positive correlation with chemoresistance to cisplatin.

In this study, we demonstrated an association between membranous PD-L1 expression and resistance to cisplatin among 5 independent ESCC cell lines.

3.3 Mucosal mature TLS as an unfavorable prognostic factor in locally advanced ESCC patients treated with neoadjuvnat CRT

3.3.1 Study population

This part of research involved 137 patients who received paclitaxel/platinum doublet chemotherapy-based neoadjuvant CRT for their locally advanced ESCC (median age: 54 years; male: 91%). Their initial clinicopathological features are detailed in Table 10 and Table 11. Based on clinical staging, the patients were categorized into stage IIA (n = 4), stage IIB (n = 11), and stage III (n = 121) groups. In terms of treatment results, a pCR was attained in 54 patients (39%). With a median follow-up period of 86 months, the median OS for the entire patient group was 37 months (range: 3 to 176+) (Figure 9).

3.3.2 Pretreatment mucosal TLS status and PD-L1 expression

Figure 10 illustrates the outcomes of immunohistochemical staining for CD20 and CD23, highlighting both mature and immature TLSs. The groups without TLS, with

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immature TLS, and with mature TLS included 64, 40, and 33 patients, respectively (Table 12). Our analysis showed no significant relationship between TLS status and factors such as age, sex, tumor location, clinical T stage, or clinical N stage. Nevertheless, a notable correlation was found between the existence of mature TLSs and the advanced clinical T4 stage (P =.0039; Table 13).

Additionally, we evaluated the expression of PD-L1 in ESCC tissues. The PD-L1 expression on TCs was categorized as TC0, TC1, TC2, and TC3 in 69, 36, 28, and 4 patients, respectively. In total, 68 patients (50%) were found to be positive for PD-L1 expression on TCs. The PD-L1 expression on ICs was classified as IC0, IC1, IC2, and IC3 in 32, 56, 35, and 14 patients, respectively. A total of 49 patients (36%) demonstrated elevated levels of PD-L1 expression on ICs. The status of TLS was associated with PD-L1 expression on ICs, but not with PD-L1 expression on TCs. The level of PD-L1 expression on ICs was significantly higher in the mature TLS group compared to the no TLS group (P =.0044; Table 12).

3.3.3 Mucosal TLS status and pCR

Out of 137 patients, 54 (39%) reached pCR. The pCR rates for the no TLS, immature TLS, and mature TLS groups were 47%, 40%, and 24%, respectively. The rate in the mature TLS group was significantly lower compared to the no TLS group (P = .031). Univariate logistic regression for pCR (Table 14) showed that male patients had a lower pCR rate than female patients (35% vs 83%, respectively; P = .053) in the mature TLS group compared to the no TLS group (P = .034). Multivariate analysis identified male sex (OR: 0.092; P = .0050) and the presence of mature TLSs (OR: 0.26; P = .023) as significant factors predicting a reduced likelihood of achieving pCR.

3.3.4 Mucosal TLS status and OS

The median OS durations for the no TLS, immature TLS, and mature TLS groups

doi:10.6342/NTU202502233

were 46, 45, and 27 months, respectively. A noticeable trend indicating a shorter OS duration was found in the mature TLS group (HR: 1.45; P = .15; Figure 11) when compared to the OS in the no TLS group. Cox regression analysis for OS revealed that a high level of PD-L1 expression on immune cells (ICs) was the most significant predictor of a favorable prognosis (HR: 0.45; P < .001; Table 15). In the multivariate analysis, a high level of PD-L1 expression on ICs continued to be the strongest predictor of a favorable prognosis (HR: 0.30; P < .0001), and the presence of mature TLSs was identified as the strongest predictor of a poor prognosis (HR: 2.96; P = .0008). Other notable predictors of a poor prognosis included male sex (HR: 2.88; P = .021) and PD-L1 expression on tumor cells (TCs) (HR: 1.62; P = .039). Nevertheless, the presence of tumors in the lower esophagus suggested a trend towards a favorable prognosis (HR: 0.57, P = .096).

3.3.5 Conclusions

We found that the maturation of mucosal TLS is a strong independent unfavorable prognostic factor forlocally advanced ESCC patients who received neoadjuvant CRT, which was opposed to our initial hypothesis.

3.4 Construction and validation of a TLS-specifc gene signature derived from endoscopic biopsy ESCC tissues harboring mature TLS

3.4.1 TME of ESCC regading the maturation of TLS

By comparing the expression levels and profiles of immune-related genes from ESCC tissues without TLS vs those with immature TLS vs those with mature TLS, we were able to reveal the immunological chaacteristics and the potential underlying mechanisms that help shape the maturation of TLS in TME of ESCC. The development

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of TLS in ESCC tissues was observed to correlate with a higher presence of tumor-infiltrating lymphocytes, particularly B cells, and a reduction in exhausted CD8 T cells and Tregs (Figure 12). Additionally, the maturation of TLS in ESCC tissues was linked to enhanced functions of leukocytes, T cells, NK cells, B cells, as well as increased activity in antigen presentation, chemokines, cytokines, tumor necrosis factor, complement, and toll-like receptors (Figure 13). These results suggest that the maturation of TLS is associated with an active immune environment, aligning with the established understanding of TLS.

3.4.2 ESCC specific TLS signature and the validation

In order to contrust a robust gene signature that represent mature TLS, we select those genes that were highly statistically differentially expressed genes between our ESCC tumor tissues with mature vs no mature TLS. Eighteen genes meeting the criteria were then slected to construct the TLS signature (Figure 14). To validate that this TLS signature derived from ESCC patients defined by IHC study of CD21 and CD23 using endoscopic biopsy tumor tissue, we first used the ESCC cohort from the TCGA database which included a total of 82 ESCC patients treated with surgery and the presence of lymphoid follicles in their surgical pathology specimens was known. We found that our ESCC-specific TLS signature can effectively distinguish between ESCC tissues with and without lymphoid follicles (Figure 15). To verify whether this ESCC-specific TLS signature can help identify patients who exhibit objective tumor response or prolonged disease stabilization following immune check point inhibitors, an observation that had been reported in several cancer types recently (Sautes Fridman C et al, 2019; Fridman WH et al, 2022; Vanhersecke L et al, 2021; Petitprez F et al, 2020; Hayashi Y et al, 2023), we enrolled a group of recurrent or metastatic ESCC patients receiving anti-PD-1 immunotherapy. We found that the ESCC-specific TLS signature

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can significantly differentiate patients who experience clinical benefits from the treatment *vs* those who expericne no clinical benefit (Figure 15).

3.4.3 Conclusions

We found that that the mature TLS identified by the CD21 and CD23-IHC study bsed on endoscopic biopsy ESCC tumor tissue was associated with an immune TME characteristic of known features of TLS. The derived ESCC-specific TLS signature ws further validated to be useful in identifying ESCC tumors with lymphoid follicle, and in associating with advanced ESCC patients with clinical benefit to anti-PD-1 immunotherapy.

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Chapter IV Discussion

The current thesis work demonstrated that the PD-L1 expression on immune cells or tumor cells, and the TLS in the pre-treatment endoscopic biopsy ESCC tumor tissues are of prognostic significance in locally advanced ESCC patients receiving neoadjuvant CRT. In the PD-L1 study part, our research revealed contrasting impacts of PD-L1 expression on prognosis in tumor versus immune cells: a TC-positive status correlated with poorer survival rates, while an IC-high status was linked to better survival outcomes.

Research investigating the prognostic significance of PD-L1 on tumor and immune cells in locally advanced ESCC (Table 9) has yielded inconsistent findings, potentially due to variations in study populations. Hatogai et al and Jesinghaus et al focused on patients who underwent surgery alone, while Lim et al and Zhang et al included patients who received neoadjuvant or adjuvant therapies alongside surgery. Additionally, these studies employed different antibodies for the immunohistochemical assessment of PD-L1 expression and utilized varying cutoff values to define high or positive expression. The investigations involving patients who had surgery alone indicated that PD-L1 expression on tumor cells correlated with favorable prognosis (Hatogai et al, 2016; Jesinghaus et al, 2017); however, Lim et al found it to be linked with poor survival outcomes. Conversely, high PD-L1 expression on immune cells was associated with improved survival in two studies (Hatogai et al, 2016; Zhang et al, 2017). Unlike previous studies, we included patients with locally advanced ESCC who were treated with neoadjuvant CRT using paclitaxel/platinum, a standard of care recognized by current treatment guidelines globally. Consequently, our findings may be more pertinent and valuable to contemporary clinical practice.

Recent meta-analyses have highlighted the varying prognostic significance of PD-

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L1 expression in tumor versus immune cells. A 2017 meta-analysis encompassing 60 clinical studies with a total of 10,310 patients across 15 different cancer types found that elevated PD-L1 expression on tumor cells is typically linked to a worse prognosis (Wang et al, 2017). Similarly, another meta-analysis involving 2,877 patients with ESCC from 6 studies yielded comparable findings (Guo et al, 2018). In contrast, a separate meta-analysis of 18 studies with 3,674 patients across 12 cancer types assessed the prognostic implications of PD-L1 expression on tumor-infiltrating immune cells (Zhao et al, 2017). The authors concluded that increased PD-L1 expression on these immune cells correlates with a reduced risk of mortality. The differing prognostic relevance of PD-L1 expression in tumor and immune cells may stem from their unique biological characteristics. PD-L1 expression in tumor cells can be influenced by intrinsic oncogenic activation, which may enhance tumor aggressiveness, contribute to drug resistance, and facilitate metastasis. These factors could elucidate the negative prognostic implications of PD-L1 expression in tumor cells. In our study, we demonstrated that the membranous expression level of PD-L1 on 5 different ESCC cell lines correlated with their respective resistance to cisplatin. However, we did not investigate the correlation with the resistance to paclitaxel or radiation, and we did not go further for the mechanistic investigations, which are warranted for future studies. Conversely, PD-L1 expression in tumor-infiltrating immune cells likely indicates a pre-existing adaptive immune response against cancer within the tumor microenvironment. Numerous studies have shown that PD-L1 on immune cells, particularly macrophages and other myeloid cells, plays a role in immune evasion and influences the effectiveness of PD-1/PD-L1 blockade immunotherapy (Tang et al, 2018; Noguchi et al, 2017; Lau et al, 2017; Tang et al, 2018; Lin et al, 2018).

We showed that the combination of PD-L1 expressions on both tumor and immune

doi:10.6342/NTU202502233

cells could enhance predictive performance. In particular, patients with TC-negative and IC-high status exhibited the longest OS at a median of 130 months, while those with TC-positive and IC-low status experienced the shortest OS at a median of 15 months. If confirmed, this index could assist in classifying patients with locally advanced ESCC for tailored therapies.

Our work on the prognostic significance of PD-L1 expression in locally advanced ESCC patients has a number of limitations. Firstly, our sample size was smaller than that of other studies; however, our cohort displayed relatively uniform clinical and treatment characteristics compared to others. Our patients were prospectively enrolled in three clinical trials at a single institution. All patients were set to receive neoadjuvant CRT, which was consistent across the cohorts and aligned with current standards. Nonetheless, there was slight variability in treatment among the three clinical trials. The phase II trial titled "Phase II Study of Metabolic Response to One-Cycle Chemotherapy in Patients with Locally Advanced Esophageal Squamous Cell Carcinoma" included one cycle of induction chemotherapy prior to neoadjuvant CRT, aiming to assess the predictive value of metabolic response to this single cycle of chemotherapy. However, the addition of one cycle of chemotherapy to neoadjuvant CRT did not enhance the pCR rate or patient outcomes (Huang et al, 2018). Secondly, we did not utilize an independent cohort to validate our results, nor did we assess the prognostic sensitivity or specificity of PD-L1 expression on tumor and immune cells. Thirdly, we employed the PD-L1 antibody clone SP142, which is not standard practice for ESCC. Currently, clones 28-8 and 22C3 are utilized for ESCC patients treated with nivolumab and pembrolizumab, respectively. However, comparisons among other PD-L1 antibody clones have not been conducted for ESCC patients.

In the study of the prognostic significance of mucosal TLS, we assessed the

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effectiveness of mucosal TLSs in forecasting the outcomes of paclitaxel/platinum-based neoadjuvant CRT in patients with locally advanced ESCC. Our results showed that the presence of mature TLSs in the mucosa of primary esophageal tumors correlated with a lower pCR to neoadjuvant CRT and a shorter OS duration.

Two retrospective cohort studies have linked the presence of TLSs to a positive prognosis in patients with ESCC. These studies involved patients at clinical stages I to IV who underwent direct esophagectomy without prior chemotherapy or CRT. Hayashi et al (Hayashi Y et al, 2023) characterized TLSs using CD21 and CD23 immunohistochemistry, while Li et al (Li R et al, 2022) utilized hematoxylin/eosin staining for their definition. Both research teams measured the number of TLSs in the tumor area of surgically removed specimens and determined that TLS density is an independent predictor of a favorable prognosis through multivariate analysis. Our results indicate that the presence of mature TLSs acts as an independent predictor of an unfavorable prognosis, which contrasts with the findings of the aforementioned studies. This difference may stem from variations in patient demographics and treatment approaches between our study and the previous ones. Our research focused on patients primarily at clinical stage III who had received neoadjuvant CRT prior to esophagectomy, in contrast to the earlier studies that included patients who had esophagectomy without preoperative chemotherapy or CRT. Although both our study and the previous ones employed immunohistochemical staining or conventional hematoxylin/eosin staining to identify TLSs, we applied different criteria for defining TLS status. We were only able to identify TLSs—whether immature or mature—through endoscopic biopsy, but we could not quantify their prevalence as was done in the earlier studies, which assessed tumor specimens collected during esophagectomy. Another important reason is that our ESCC tissues were obtained by endoscope-biopsy,

doi:10.6342/NTU202502233

which represented tumor tissues in mucosal area, in contrast to the whole layer of ESCC tumor tissues represented by surgical excisions in the previous studies.

The negative prognostic impact of TLSs in our cohort may be attributed to the influence of neoadjuvant therapy on TLSs, which can modify their prognostic significance. Research has investigated the impact of radiation on antitumor immunity, particularly concerning the drainage lymph nodes near tumors. In a study utilizing a mouse model of head and neck SCC, Saddawi-Konefka et al (Saddawi-Konefka R et al, 2022) found that the surgical or radiative removal of drainage lymph nodes diminished the tumor's response to ICIs, consequently leading to poorer OS. The authors linked this outcome to the radiation-induced loss of conventional type I dendritic cells in the drainage lymph nodes, which are essential for the tumor's reaction to ICIs. In a similar vein, Darragh et al (Darragh LB et al, 2022) employed another mouse model of head and neck SCC to show that radiation aimed at drainage lymph nodes weakened the systemic immune response by inhibiting antigen-specific T cells and epitope spreading, thus promoting the growth of both local and metastatic tumors. These findings indicate that drainage lymph nodes are crucial in mediating antitumor immunity and improving the effectiveness of anticancer treatments. Furthermore, the studies underscored that radiotherapy targeting these lymph nodes could negatively impact cancer therapy. Nevertheless, the potential influence of radiation on TLSs and TLS-mediated antitumor immunity is still not fully understood.

In our earlier research, the expression of PD-L1 on ICs was identified as a significant prognostic indicator for patients with locally advanced ESCC undergoing neoadjuvant CRT. (Huang TC et al, 2022) Consequently, we examined this parameter in the current study. The findings reaffirmed that PD-L1 expression on ICs serves as a predictor of a positive prognosis. Importantly, the existence of mature TLSs was

doi:10.6342/NTU202502233

recognized as a strong predictor of a negative prognosis after integrating the PD-L1 expression on ICs into our multivariate analysis model. This observation implies that the detrimental prognostic impact of mature TLSs may be obscured in a univariate analysis due to its robust correlation with elevated PD-L1 expression on ICs, which is a strong indicator of a favorable prognosis.

Our work on the prognostic significance of TLS in locally advanced ESCC patients has a number of limitations. Firstly, the tissue samples consisted of small biopsied tissues, in contrast to the larger surgically resected tissues utilized in other studies. The analysis on TLS in small biopsied tumor tissues has not been reported before; therefore, the accuracy of identifying TLS by analyzing small biopsied tumor tissues has not been validated. Besides, sampling bias has always been an important issue in analyzing small biopsied tumor tissues. In our research, due to the limitation of small biopsied tissues, we could only classify TLS status as absent, immature, or mature TLS, rather than measuring it as a continuous variable like TLS density. However, small biopsied tissues do present certain benefits. They more accurately reflect the live TME compared to surgically resected tissues, which may experience artificial alterations due to hypoxia and surgical stress. Our focus was on a significant group of patients with locally advanced ESCC who underwent neoadjuvant CRT prior to esophagectomy. It is essential to evaluate small biopsied tissues to investigate pretreatment TLS status in this demographic. In the forth part of our research, we validated the accuracy of identifying TLS in small biopsied tumor tissues by showing the compatibility of TME in ESCC tissues with mature TLS, validating by TCGA database, and predictability of response to ICIs. Although the accuracy of TLS identification was validated, the sampling bias is still an inevitable llimitation. Secondly, while the sample size in this study was smaller than in other research, our patient cohort, drawn from two major medical

doi:10.6342/NTU202502233

centers, was relatively uniform. All patients received paclitaxel/platinum-based neoadjuvant CRT, in accordance with current treatment guidelines. This uniformity bolsters the reliability of our results within this particular treatment framework. Thirdly, although we did not utilize an independent cohort to validate our results, we incorporated medical center as a variable in our multivariate analysis. This approach may simulate the effect of external validation by addressing potential intercenter differences. Since the release of the CROSS study findings in 2012, paclitaxel/platinum-based neoadjuvant CRT has established itself as the global standard-of-care regimen for locally advanced ESCC. Therefore, it is crucial for researchers to persist in exploring the prognostic significance of TLSs and other biomarkers in patients with ESCC undergoing neoadjuvant CRT.

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Chapter V Future Perspectives

In my thesis, I investigated the prognostic significance of PD-L1 expression and TLS in patients with locally advanced ESCC who underwent neoadjuvant CRT. I demonstrated that PD-L1 expression on immune cells serves as a positive prognostic indicator and PD-L1 expression on tumor cells is associated with a negative prognosis. The integration of PD-L1 expressions from both tumor and immune cells could enhance prognostic accuracy, and this combined metric warrants validation in larger studies. I aslo found that the existence of mature TLSs serves as an independent poor prgonsotic factor. This result highlights the necessity for further studies in independent patient cohorts receiving trimodality therapy for locally advanced ESCC, to explore the significance of TLS status within this patient group.

Additional immune-related biomarkers require further investigation in the future. Potential candidates for these biomarkers include tumor-associated macrophages. Our initial analysis, utilizing nanoString data from a cohort of 44 patients, compared gene expression between those with the best and worst survival outcomes, revealing several macrophage-related genes such as CSF3, IL6, IL23A, C1QA, and C1QB. These results necessitate further validation.

Considering PD-L1 expression on immune or tumor cells, the maturation of TLS, and the need for validation of more immune-related biomarkers, an effective prognostic model should be developed and validated in the future. This model aims to enhance the overall outcomes for locally advanced ESCC patients receiving neoadjuvant CRT by stratifying the risks of recurrence.

Regarding exploring the impact of radiation on TME of ESCC, investigation on the TME, including TLS, of the post-neoadjuvant CRT ESCC tissues, is crucial. Currently, the study of immune TME of post-CRT esophageal or ESCC tumor tissues

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is ongoing.

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Chapter VI References

- Aguiar PN, Jr., Santoro IL, Tadokoro H, de Lima Lopes G, Filardi BA, Oliveira P et al (2016) The role of PD-L1 expression as a predictive biomarker in advanced non-small-cell lung cancer: a network meta-analysis. Immunotherapy 8: 479-488. https://doi: 10.2217/imt-2015-0002.
- Al-Batran SE, Homann N, Pauligk C, Goetze TO, Meiler J, Kasper S, Kopp HG, Mayer F, Haag GM, Luley K, Lindig U, Schmiegel W, Pohl M, Stoehlmacher J, Folprecht G, Probst S, Prasnikar N, Fischbach W, Mahlberg R, Trojan J, Koenigsmann M, Martens UM, Thuss-Patience P, Egger M, Block A, Heinemann V, Illerhaus G, Moehler M, Schenk M, Kullmann F, Behringer DM, Heike M, Pink D, Teschendorf C, Löhr C, Bernhard H, Schuch G, Rethwisch V, von Weikersthal LF, Hartmann JT, Kneba M, Daum S, Schulmann K, Weniger J, Belle S, Gaiser T, Oduncu FS, Güntner M, Hozaeel W, Reichart A, Jäger E, Kraus T, Mönig S, Bechstein WO, Schuler M, Schmalenberg H, Hofheinz RD; FLOT4-AIO Investigators. Perioperative chemotherapy with fluorouracil plus leucovorin, oxaliplatin, and docetaxel versus fluorouracil or capecitabine plus cisplatin and epirubicin for locally advanced, resectable gastric or gastro-oesophageal junction adenocarcinoma (FLOT4): a randomised, phase 2/3 trial. Lancet. 2019 May 11;393(10184):1948-57.
- Almozyan S, Colak D, Mansour F, Alaiya A, Al-Harazi O, Qattan A et al (2017) PD-L1 promotes OCT4 and Nanog expression in breast cancer stem cells by sustaining PI3K/AKT pathway activation. Int J Cancer 141: 1402-1412. https:// doi: 10.1002/ijc.30834.
- Alsuliman A, Colak D, Al-Harazi O, Fitwi H, Tulbah A, Al-Tweigeri T et al(2015) Bidirectional crosstalk between PD-L1 expression and epithelial to mesenchymal

doi:10.6342/NTU202502233

transition: significance in claudin-low breast cancer cells. Mol Cancer 14: 149. https:// doi: 10.1186/s12943-015-0421-2.

- Azuma T, Yao S, Zhu G, Flies AS, Flies SJ, Chen (2008) B7-H1 is a ubiquitous antiapoptotic receptor on cancer cells. Blood 111: 3635-3643. https:// doi: 10.1182/blood-2007-11-123141.
- Barros LRC, Souza-Santos PT, Pretti MAM, Vieira GF, Bragatte MAS, Mendes MFA, De Freitas MV, Scherer NM, De Oliveira IM, Rapozo DCM, Fernandes PV, Simão TA, Soares-Lima SC, Boroni M, Ribeiro Pinto LF, Bonamino MH. High infiltration of B cells in tertiary lymphoid structures, TCR oligoclonality, and neoantigens are part of esophageal squamous cell carcinoma microenvironment. J Leukoc Biol. 2020 Oct;108(4):1307-1318. doi: 10.1002/JLB.5MA0720-710RRR. Epub 2020 Aug 22. PMID: 32827331.
- Black M, Barsoum IB, Truesdell P, Cotechini T, Macdonald-Goodfellow SK, Petroff M et al (2016) Activation of the PD-1/PD-L1 immune checkpoint confers tumor cell chemoresistance associated with increased metastasis. Oncotarget 7: 10557-10567. https:// doi: 10.18632/oncotarget.7235.
- Cabrita R, Lauss M, Sanna A, Donia M, Skaarup Larsen M, Mitra S, et al. Tertiary lymphoid structures improve immunotherapy and survival in melanoma. Nature. 2020;577(7791):561-5.
- Chang CH, Qiu J, O'Sullivan D, Buck AD, Noguchi T, Curtis JD et al (2015) Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. Cell 162: 1229-1241. https:// doi: 10.1016/j.cell.2015.08.016.
- Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013 Jul 25;39(1):1-10.
- Chen MF, Chen PT, Chen WC, Lu MS, Lin PY, Lee KD (2016) The role of PD-

doi:10.6342/NTU202502233

L1 in the radiation response and prognosis for esophageal squamous cell carcinoma related to IL-6 and T-cell immunosuppression. Oncotarget 7: 7913-7924. https:// doi: 10.18632/oncotarget.6861.

- Chen L, Deng H, Lu M, Xu B, Wang Q, Jiang J et al (2014) B7-H1 expression associates with tumor invasion and predicts patient's survival in human esophageal cancer. Int J Clin Exp Pathol 7: 6015- 6023.
- Chen J, Jiang CC, Jin L, Zhang XD (2016) Regulation of PD-L1: a novel role of pro-survival signalling in cancer. Ann Oncol 27: 409-416. https:// doi: 10.1093/annonc/mdv615.
- Cheng YF, Chen HS, Wu SC, Chen HC, Hung WH, Lin CH, Wang BY. Esophageal squamous cell carcinoma and prognosis in Taiwan. Cancer Med. 2018 Sep;7(9):4193-201.
- Clark CA, Gupta HB, Curiel TJ (2017) Tumor cell-intrinsic CD274/PD-L1: A novel metabolic balancing act with clinical potential. Autophagy 13: 987-988.
- Darragh LB, Gadwa J, Pham TT, Van Court B, Neupert B, Olimpo NA, et al. Elective nodal irradiation mitigates local and systemic immunity generated by combination radiation and immunotherapy in head and neck tumors. Nat Commun. 2022;13(1):7015.
- Eyck BM, van Lanschot JJB, Hulshof M, van der Wilk BJ, Shapiro J, van Hagen P, et al. Ten-Year Outcome of Neoadjuvant Chemoradiotherapy Plus Surgery for Esophageal Cancer: The Randomized Controlled CROSS Trial. J Clin Oncol. 2021;39(18):1995-2004.
- Fehrenbacher L, Spira A, Ballinger M, Kowanetz M, Vansteenkiste J, Mazieres J et al (2016) Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicentre, open-label, phase 2

doi:10.6342/NTU202502233

randomised controlled trial. Lancet 387: 1837-1846. https:// doi: 10.1016/S0140-6736(16)00587-0.

- Guo JC, Huang TC, Lin CC, Hsieh MS, Chang CH, Huang PM et al (2015) Postchemoradiotherapy Pathologic Stage Classified by the American Joint Committee on the Cancer Staging System Predicts Prognosis of Patients with Locally Advanced Esophageal Squamous Cell Carcinoma. J Thorac Oncol 10: 1481-1489. https:// doi: 10.1097/JTO.0000000000000651.
- Fehrenbacher L, Spira A, Ballinger M, Kowanetz M, Vansteenkiste J, Mazieres J, et al. Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicentre, open-label, phase 2 randomised controlled trial. Lancet. 2016;387(10030):1837-46.
- Fridman WH, Meylan M, Petitprez F, Sun CM, Italiano A, Sautes-Fridman C. B cells and tertiary lymphoid structures as determinants of tumour immune contexture and clinical outcome. Nat Rev Clin Oncol. 2022;19(7):441-57.
- Guo JC, Hsu CL, Huang YL, Lin CC, Huang TC, Wu IC, Lin CY, Lien MY, Kuo HY, Cheng AL, Hsu CH. B Cells in Tumor Microenvironment Associated With The Clinical Benefit to Programmed Cell Death Protein-1 Blockade Therapy in Patients With Advanced Esophageal Squamous Cell Carcinoma. Front Oncol. 2022 Jun 29;12:879398.
- Guo W, Wang P, Li N, Shao F, Zhang H, Yang Z et al (2018) Prognostic value of PD-L1 in esophageal squamous cell carcinoma: a meta-analysis. Oncotarget 9: 13920-13933. https:// doi: 10.18632/oncotarget.23810.
- Hatogai K, Kitano S, Fujii S, Kojima T, Daiko H, Nomura S et al (2016) Comprehensive immunohistochemical analysis of tumor microenvironment immune status in esophageal squamous cell carcinoma. Oncotarget 7: 47252-

doi:10.6342/NTU202502233

47264. https:// doi: 10.18632/oncotarget.10055.

- Hayashi Y, Makino T, Sato E, Ohshima K, Nogi Y, Kanemura T, et al. Density and maturity of peritumoral tertiary lymphoid structures in oesophageal squamous cell carcinoma predicts patient survival and response to immune checkpoint inhibitors. Br J Cancer. 2023;128(12):2175-85.
- He W, Zhang D, Liu H, Chen T, Xie J, Peng L, et al. The High Level of Tertiary Lymphoid Structure Is Correlated With Superior Survival in Patients With Advanced Gastric Cancer. Front Oncol. 2020;10:980.
- Herskovic A, Martz K, al-Sarraf M, Leichman L, Brindle J, Vaitkevicius V, Cooper J, Byhardt R, Davis L, Emami B. Combined chemotherapy and radiotherapy compared with radiotherapy alone in patients with cancer of the esophagus. N Engl J Med. 1992 Jun 11;326(24):1593-8.
- Hiraoka N, Ino Y, Yamazaki-Itoh R, Kanai Y, Kosuge T, Shimada K. Intratumoral tertiary lymphoid organ is a favourable prognosticator in patients with pancreatic cancer. Br J Cancer. 2015;112(11):1782-90.
- https://www.esmo.org/guidelines/guidelines-by-topic/esmo-clinical-practice-guidelines-gastrointestinal-cancers/clinical-practice-guideline-oesophageal-cancer
- Huang TC, Hsu CH, Lin CC, Tu YK (2015) Systematic review and network meta-analysis: neoadjuvant chemoradiotherapy for locoregional esophageal cancer. Jpn J Clin Oncol 45: 1023-1028. https:// doi: 10.1093/jjco/hyv119.
- Huang TC, Liang CW, Li YI, Guo JC, Lin CC, Chen YJ, et al. Prognostic value of PD-L1 expression on immune cells or tumor cells for locally advanced esophageal squamous cell carcinoma in patients treated with neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2022;148(7):1803-11.

doi:10.6342/NTU202502233

- Huang TC, Lin CC, Wu YC, Cheng JCH, Lee JM, Wang HP et al (2018) Phase II study of metabolic response to one-cycle chemotherapy in patients with locally advanced esophageal squamous cell carcinoma. J Formos Med Assoc. 118:1024-1030. https:// doi: 10.1016/j.jfma.2018.11.003.
- Jesinghaus M, Steiger K, Slotta-Huspenina J, Drecoll E, Pfarr N, Meyer P et al (2017) Increased intraepithelial CD3+ T-lymphocytes and high PD-L1 expression on tumor cells are associated with a favorable prognosis in esophageal squamous cell carcinoma and allow prognostic immunogenic subgrouping. Oncotarget 8: 46756-46768. https:// doi: 10.18632/oncotarget.18606.
- Kelly RJ, Ajani JA, Kuzdzal J, Zander T, Van Cutsem E, Piessen G, et al. Adjuvant Nivolumab in Resected Esophageal or Gastroesophageal Junction Cancer. N Engl J Med. 2021;384(13):1191-203.
- Kim S, Koh J, Kim MY, Kwon D, Go H, Kim YA et al (2016) PD-L1 expression is associated with epithelial-to-mesenchymal transition in adenocarcinoma of the lung. Hum Pathol 58: 7-14. https:// doi: 10.1016/j.humpath.2016.07.007.
- Kitagawa Y, Uno T, Oyama T, Kato K, Kato H, Kawakubo H, Kawamura O, Kusano M, Kuwano H, Takeuchi H, Toh Y, Doki Y, Naomoto Y, Nemoto K, Booka E, Matsubara H, Miyazaki T, Muto M, Yanagisawa A, Yoshida M. Esophageal cancer practice guidelines 2017 edited by the Japan esophageal society: part 2. Esophagus. 2019 Jan;16(1):25-43.
- Kojima T, Shah MA, Muro K, Francois E, Adenis A, Hsu CH et al (2020) Randomized Phase III KEYNOTE-181 Study of Pembrolizumab Versus Chemotherapy in Advanced Esophageal Cancer. J Clin Oncol 38: 4138-4148. https:// doi: 10.1200/JCO.20.01888.
- Lai WW, Lin CN, Chang CC, Wang JD. Lifetime risks, expected years of life lost,

doi:10.6342/NTU202502233

and cost-per-life year of esophageal cancer in Taiwan. Sci Rep. 2020 Feb 28;10(1):3722.

- Lau J, Cheung J, Navarro A, Lianoglou S, Haley B, Totpal K et al (2017) Tumour and host cell PD-L1 is required to mediate suppression of anti-tumour immunity in mice. Nat Commun. 8:14572. https:// doi: 10.1038/ncomms14572.
- Li H, Wang J, Liu H, Lan T, Xu L, Wang G, et al. Existence of intratumoral tertiary lymphoid structures is associated with immune cells infiltration and predicts better prognosis in early-stage hepatocellular carcinoma. Aging (Albany NY). 2020;12(4):3451-72.
- Li Q, Liu X, Wang D, Wang Y, Lu H, Wen S, et al. Prognostic value of tertiary lymphoid structure and tumour infiltrating lymphocytes in oral squamous cell carcinoma. Int J Oral Sci. 2020;12(1):24.
- Li R, Huang X, Yang W, Wang J, Liang Y, Zhang T, et al. Tertiary lymphoid structures favor outcome in resected esophageal squamous cell carcinoma. J Pathol Clin Res. 2022;8(5):422-35.
- Lim SH, Hong M, Ahn S, Choi YL, Kim KM, Oh D et al (2016) Changes in tumour expression of programmed death-ligand 1 after neoadjuvant concurrent chemoradiotherapy in patients with squamous oesophageal cancer. Eur J Cancer 52: 1-9. https:// doi: 10.1016/j.ejca.2015.09.019.
- Lin CC, Hsu CH, Cheng JC, Wang HP, Lee JM, Yeh KH et al (2007) Concurrent chemoradiotherapy with twice weekly paclitaxel and cisplatin followed by esophagectomy for locally advanced esophageal cancer. Ann Oncol 18: 93-98. https:// doi: 10.1093/annonc/mdl339.
- Lin H, Wei S, Hurt EM, Green MD, Zhao L, Vatan L et al (2018) Host expression of PD-L1 determines efficacy of PD-L1 pathway blockade-mediated tumor

doi:10.6342/NTU202502233

regression. J Clin Invest. 128:805–15. https:// doi: 10.1172/JCI96113.

- Lin Q, Tao P, Wang J, Ma L, Jiang Q, Li J, et al. Tumor-associated tertiary lymphoid structure predicts postoperative outcomes in patients with primary gastrointestinal stromal tumors. Oncoimmunology. 2020;9(1):1747339.
- Lordick F, Mariette C, Haustermans K, Obermannová R, Arnold D; ESMO Guidelines Committee. Oesophageal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2016 Sep;27(suppl 5):v50-7.
- Mariette C, Markar SR, Dabakuyo-Yonli TS, Meunier B, Pezet D, Collet D, D'Journo XB, Brigand C, Perniceni T, Carrère N, Mabrut JY, Msika S, Peschaud F, Prudhomme M, Bonnetain F, Piessen G; Fédération de Recherche en Chirurgie (FRENCH) and French Eso-Gastric Tumors (FREGAT) Working Group. Hybrid Minimally Invasive Esophagectomy for Esophageal Cancer. N Engl J Med. 2019 Jan 10;380(2):152-62.
- Morgan E, Soerjomataram I, Rumgay H, Coleman HG, Thrift AP, Vignat J, Laversanne M, Ferlay J, Arnold M. The Global Landscape of Esophageal Squamous Cell Carcinoma and Esophageal Adenocarcinoma Incidence and Mortality in 2020 and Projections to 2040: New Estimates From GLOBOCAN 2020. Gastroenterology. 2022 Sep;163(3):649-58.
- Muro K, Lordick F, Tsushima T, Pentheroudakis G, Baba E, Lu Z, Cho BC, Nor IM, Ng M, Chen LT, Kato K, Li J, Ryu MH, Zamaniah WIW, Yong WP, Yeh KH, Nakajima TE, Shitara K, Kawakami H, Narita Y, Yoshino T, Van Cutsem E, Martinelli E, Smyth EC, Arnold D, Minami H, Tabernero J, Douillard JY. Pan-Asian adapted ESMO Clinical Practice Guidelines for the management of patients with metastatic oesophageal cancer: a JSMO-ESMO initiative endorsed by CSCO, KSMO, MOS, SSO and TOS. Ann Oncol. 2019 Jan 1;30(1):34-43.

doi:10.6342/NTU202502233

- Noguchi T, Ward JP, Gubin MM, Arthur CD, Lee SH, Hundal J et al (2017) Temporally distinct PD-L1 expression by tumor and host cells contributes to immune escape. Cancer Immunol Res. 5:106–17. https:// doi: 10.1158/2326-6066.CIR-16-0391.
- Obermannová R, Alsina M, Cervantes A, Leong T, Lordick F, Nilsson M, van Grieken NCT, Vogel A, Smyth EC. Oesophageal cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. Ann Oncol. 2022 Jul 29;S0923-7534(22)01850-6.
- Oppedijk V, van der Gaast A, van Lanschot JJ, van Hagen P, van Os R, van Rij CM et al (2014) Patterns of recurrence after surgery alone versus preoperative chemoradiotherapy and surgery in the CROSS trials. J Clin Oncol 32: 385-391. https:// doi: 10.1200/JCO.2013.51.2186. Epub 2014 Jan 13.
- Pennathur A, Gibson MK, Jobe BA, Luketich JD. Oesophageal carcinoma. Lancet. 2013 Feb 2;381(9864):400-12.
- Petitprez F, de Reynies A, Keung EZ, Chen TW, Sun CM, Calderaro J, et al. B cells are associated with survival and immunotherapy response in sarcoma. Nature. 2020;577(7791):556-60.
- Rice TW, Ishwaran H, Ferguson MK, Blsckstone EH, Goldstraw P. Cancer of the Esophagus and Esophagogastric Junction: An Eighth Edition Staging Primer. J Thorac Oncol. 2017 Jan;12(1):23-42.
- Saddawi-Konefka R, O'Farrell A, Faraji F, Clubb L, Allevato MM, Jensen SM, et al. Lymphatic-preserving treatment sequencing with immune checkpoint inhibition unleashes cDC1-dependent antitumor immunity in HNSCC. Nat Commun. 2022;13(1):4298.
- Sautes-Fridman C, Petitprez F, Calderaro J, Fridman WH. Tertiary lymphoid

doi:10.6342/NTU202502233

structures in the era of cancer immunotherapy. Nat Rev Cancer. 2019;19(6):307-25.

- Schumacher TN, Thommen DS. Tertiary lymphoid structures in cancer. Science. 2022;375(6576):eabf9419.
- Silina K, Soltermann A, Attar FM, Casanova R, Uckeley ZM, Thut H, et al. Germinal Centers Determine the Prognostic Relevance of Tertiary Lymphoid Structures and Are Impaired by Corticosteroids in Lung Squamous Cell Carcinoma. Cancer Res. 2018;78(5):1308-20.
- SJOQUIST KM, BURMEISTER BH, SMITHERS B M, et al. Survival after neoadjuvant chemotherapy or chemoradiotherapy for resectable oesophageal carcinoma: an updated meta-analysis. Lancet Oncol 2011; 12: 681-692.
- Sofopoulos M, Fortis SP, Vaxevanis CK, Sotiriadou NN, Arnogiannaki N, Ardavanis A, et al. The prognostic significance of peritumoral tertiary lymphoid structures in breast cancer. Cancer Immunol Immunother. 2019;68(11):1733-45.
- Song Y, Li Z, Xue W, Zhang M (2019) Predictive biomarkers for PD-1 and PD-L1 immune checkpoint blockade therapy. Immunotherapy 11: 515-529. https:// doi: 10.2217/imt-2018-0173.
- Tang H, Liang Y, Anders RA, Taube JM, Qiu X, Mulgaonkar A et al (2018) PD-L1 on host cells is essential for PD-L1 blockade-mediated tumor regression. J Clin Invest. 128:580–8. https://doi: 10.1172/JCI96061.
- Tang F, Zheng P (2018) Tumor cells versus host immune cells: whose PD-L1 contributes to PD-1/PD-L1 blockade mediated cancer immunotherapy? Cell Biosci. 2018 May 2;8:34. doi: 10.1186/s13578-018-0232-4.
- The Cancer Genome Atlas Research Network. Integrated genomic characterization of oesophageal carcinoma. Nature. 2017 Jan 12;541(7636):169-

doi:10.6342/NTU202502233

75.

- Tsutsumi S, Saeki H, Nakashima Y, Ito S, Oki E, Morita M et al (2017) Programmed death-ligand 1 expression at tumor invasive front is associated with epithelial-mesenchymal transition and poor prognosis in esophageal squamous cell carcinoma. Cancer Sci 108: 1119-1127. https:// doi: 10.1111/cas.13237.
- van Hagen P, Hulshof MC, van Lanschot JJ, Steyerberg EW, van Berge Henegouwen MI, Wijnhoven BP et al (2012) Preoperative chemoradiotherapy for esophageal or junctional cancer. N Engl J Med. 2012 May 31;366(22):2074-84.
- Vanhersecke L, Brunet M, Guegan JP, Rey C, Bougouin A, Cousin S, et al (2021) Mature tertiary lymphoid structures predict immune checkpoint inhibitor efficacy in solid tumors independently of PD-L1 expression. Nat Cancer. 2021;2(8):794-802.
- Wang Q, Liu F, Liu L (2017) Prognostic significance of PD-L1 in solid tumor: An updated meta-analysis. Medicine (Baltimore) 96: e6369. https:// doi: 10.1097/MD.0000000000006369.
- Wu IC, Lu CY, Kuo FC, Tsai SM, Lee KW, Kuo WR et al (2006) Interaction between cigarette, alcohol and betel nut use on esophageal cancer risk in Taiwan. Eur J Clin Invest. 2006 Apr;36(4):236-41.
- Yang H, Liu H, Chen Y, Zhu C, Fang W, Yu Z et al (2018) Neoadjuvant Chemoradiotherapy Followed by Surgery Versus Surgery Alone for Locally Advanced Squamous Cell Carcinoma of the Esophagus (NEOCRTEC5010): A Phase III Multicenter, Randomized, Open-Label Clinical Trial. J Clin Oncol. 2018 Sep 20;36(27):2796-803.
- Zhang W, Pang Q, Zhang X, Yan C, Wang Q, Yang J et al (2017) Programmed

doi:10.6342/NTU202502233

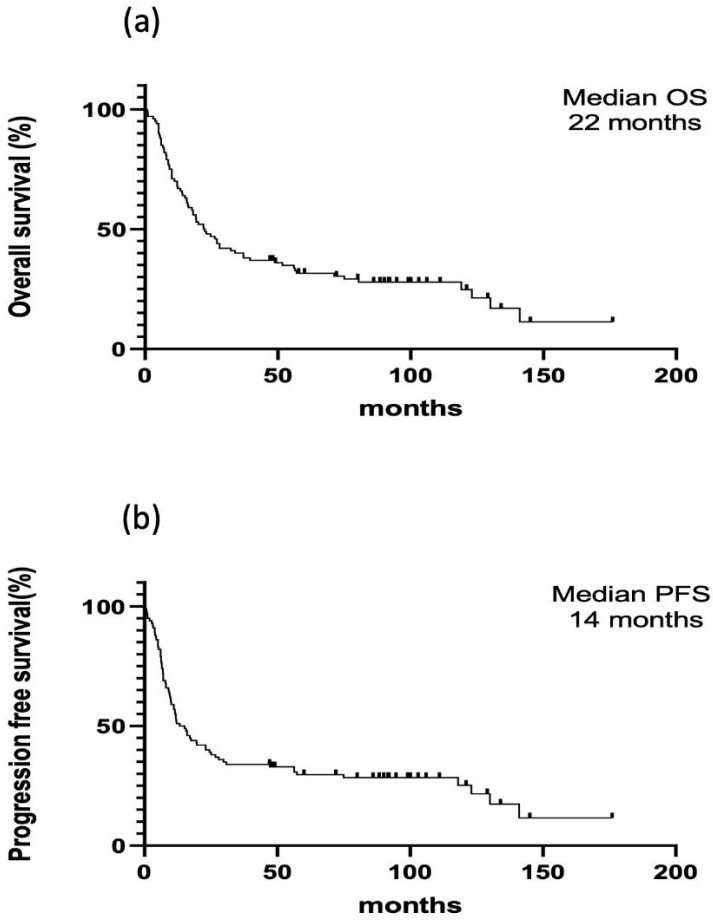
death-ligand 1 is prognostic factor in esophageal squamous cell carcinoma and is associated with epidermal growth factor receptor. Cancer Sci 108: 590-597. https:// doi: 10.1111/cas.13197.

- Zhang X, Peng L, Luo Y, Zhang S, Pu Y, Chen Y et al (2021) Dissecting esophageal squamous-cell carcinoma ecosystem by single-cell transcriptomic analysis. Nat Commun. 2021 Sep 6;12(1):5291.
- Zheng Y, Chen Z, Han Y, Han L, Zou X, Zhou B et al (2020) Immune suppressive landscape in the human esophageal squamous cell carcinoma microenvironment. Nat Commun. 2020 Dec 8;11(1):6268

doi:10.6342/NTU202502233

Chapter VII Figures

Figure 1. (a) OS and (b) PFS of the whole cohort of PD-L1 study.



(From *Prognostic value of PD-L1 expression on immune cells or tumor cells for locally advanced esophageal squamous cell carcinoma in patients treated with neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2022 Jul;148(7):1803-1811.)*

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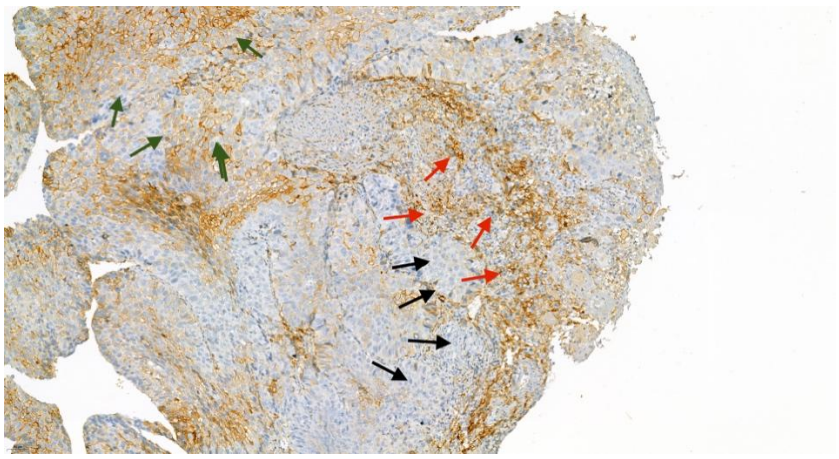
Figure 2. Immunohistochemical staining of PD-L1 with membranous expression on tumor cells (green arrows) and immune cells expression infiltrating tumor tissues (red arrows). Regions of negative staining are indicated by black arrows.



(From *Prognostic value of PD-L1 expression on immune cells or tumor cells for locally advanced esophageal squamous cell carcinoma in patients treated with neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2022 Jul;148(7):1803-1811.)*

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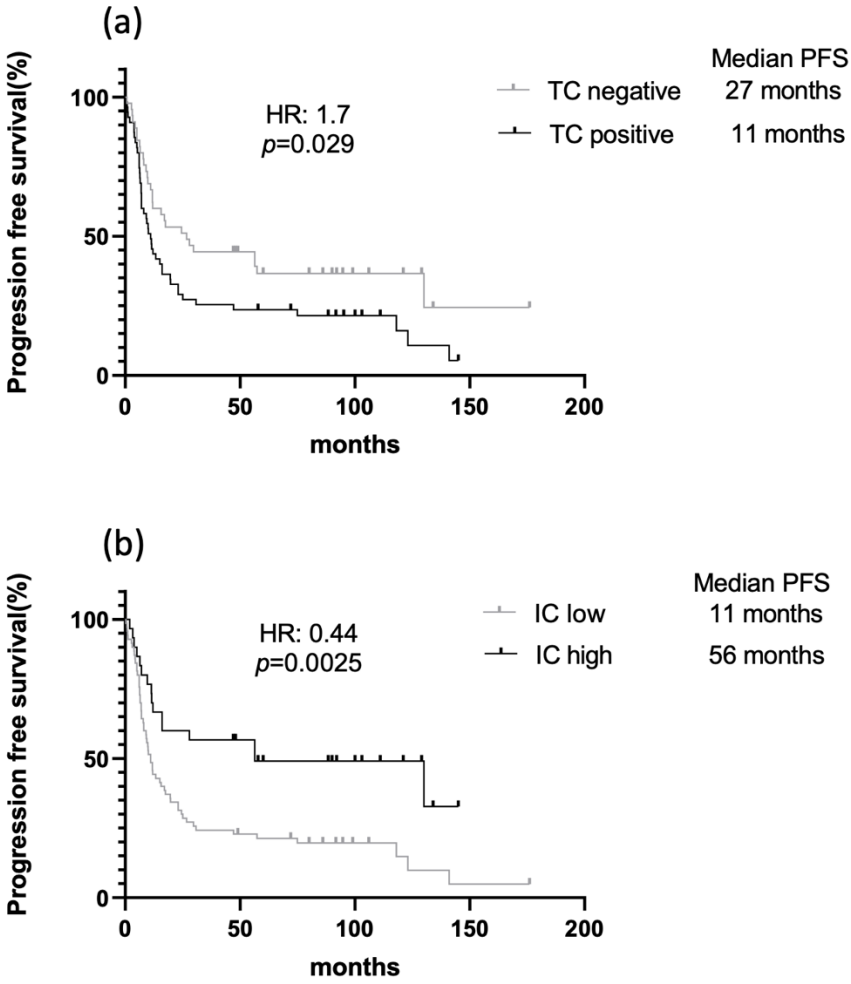
Figure 3. PFS curves of patients with (a) TC positive versus TC negative and (b) IC high versus IC low. The survivals were compared by log rank test.



(From *Prognostic value of PD-L1 expression on immune cells or tumor cells for locally advanced esophageal squamous cell carcinoma in patients treated with neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2022 Jul;148(7):1803-1811.)*

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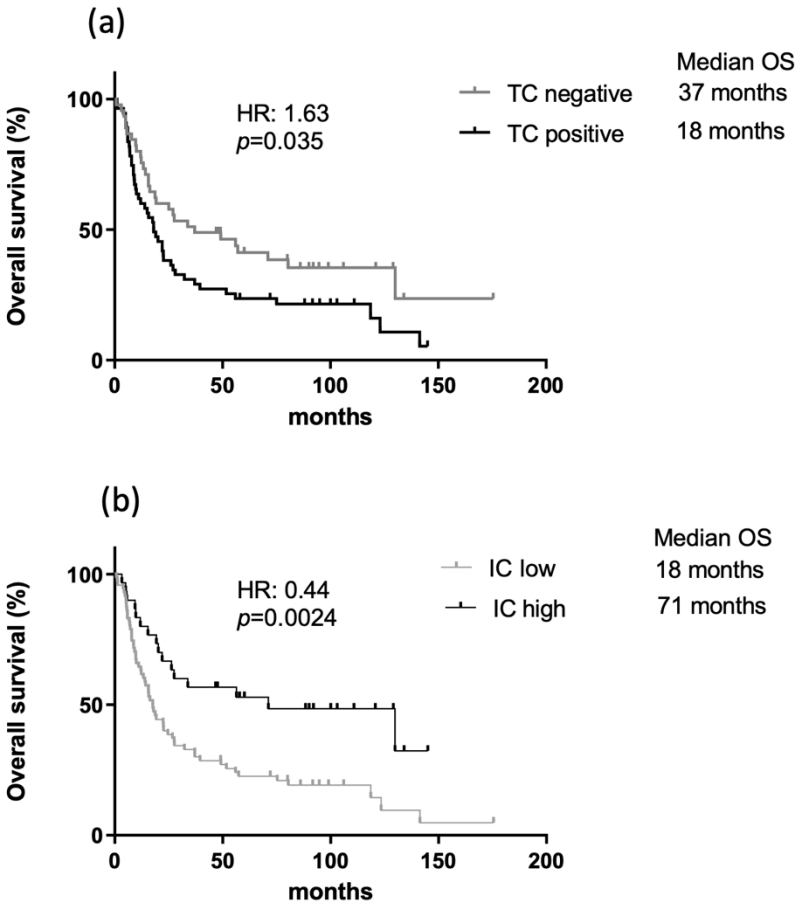
Figure 4. OS curves of patients with (a) TC positive versus TC negative and (b) IC high versus IC low. The survivals were compared by log rank test.



(From *Prognostic value of PD-L1 expression on immune cells or tumor cells for locally advanced esophageal squamous cell carcinoma in patients treated with neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2022 Jul;148(7):1803-1811.)*

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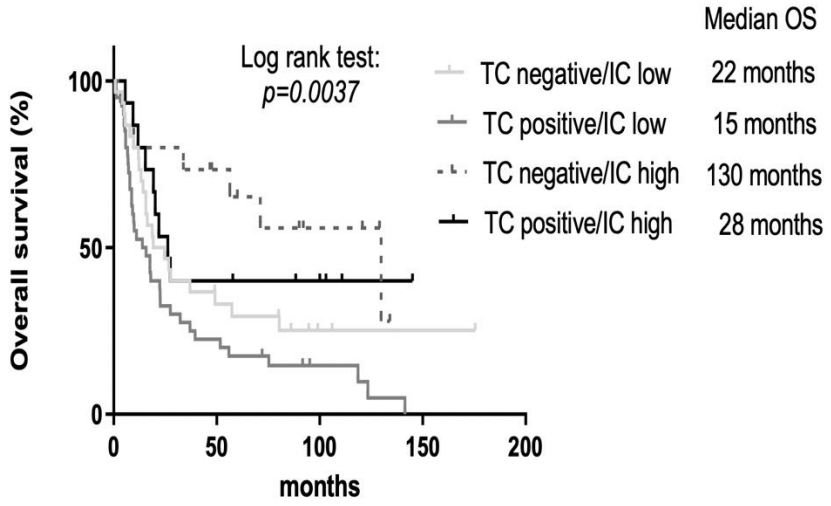
Figure 5. OS curves of 4 subgroups: TC negative/IC low, TC positive/IC low, TC negative/IC high, and TC positive/IC high. The survivals were compared by log rank test.



(From *Prognostic value of PD-L1 expression on immune cells or tumor cells for locally advanced esophageal squamous cell carcinoma in patients treated with neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2022 Jul;148(7):1803-1811.)*

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Figure 6. Chemosensitivity of each ESCC cell line by MTT assay

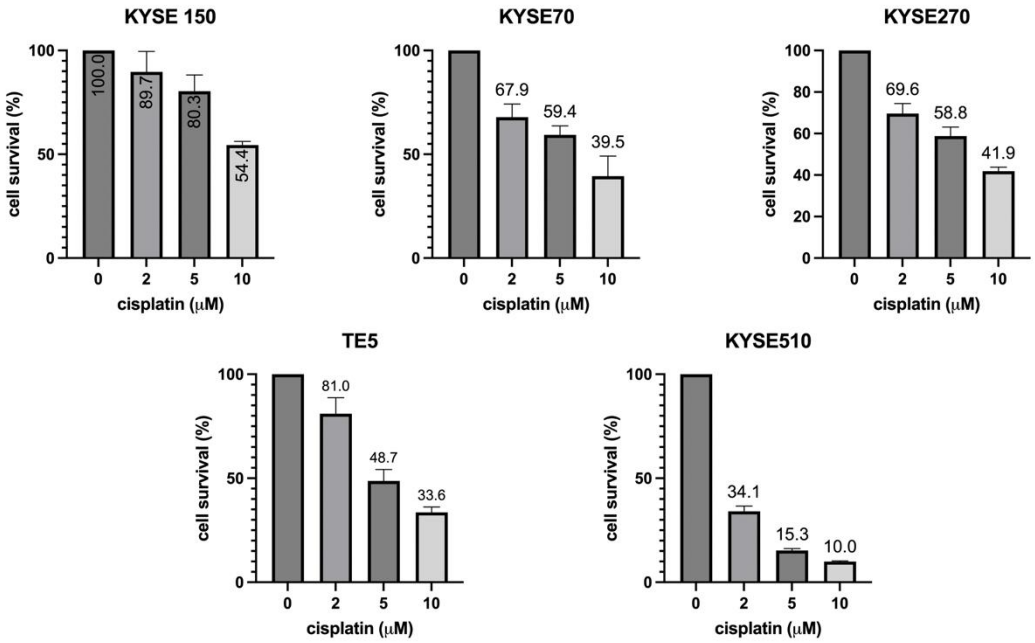

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Figure 7. Membrane expression of PD-L1 of each cell line by flow cytometry.

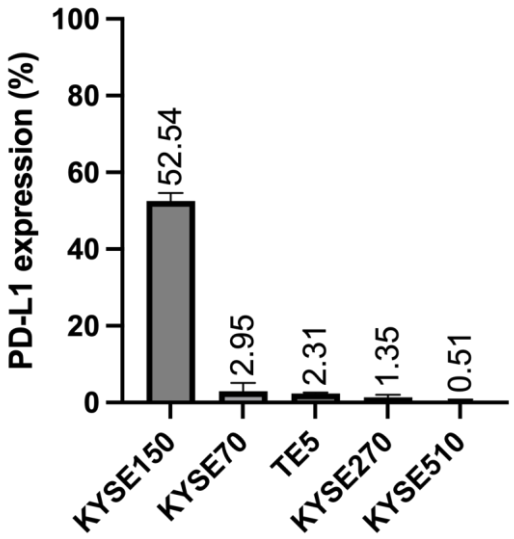

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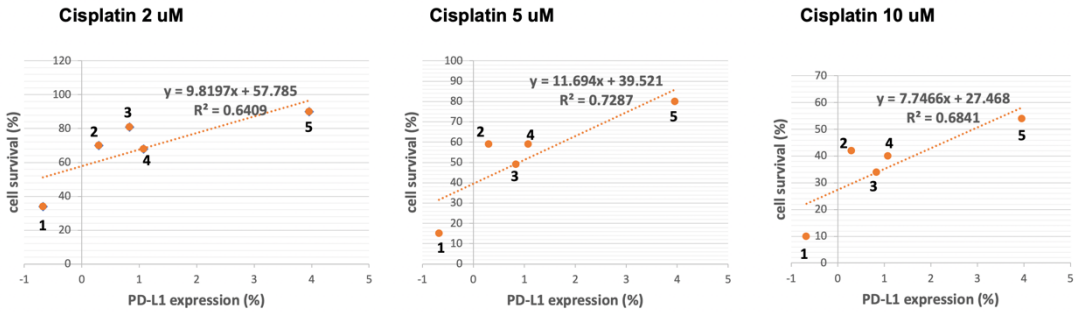
Figure 8. Linear relationship between PD-L1 expression and chemosensitivity to cisplatin among the 5 cell lines under a serial of cisplatin concentrations.



1: KYSE510; 2: KYSE270; 3: TE5; 4: KYSE70; 5: KYSE150

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Figure 9. OS curve of the 137 patients of the cohort in TLS study.

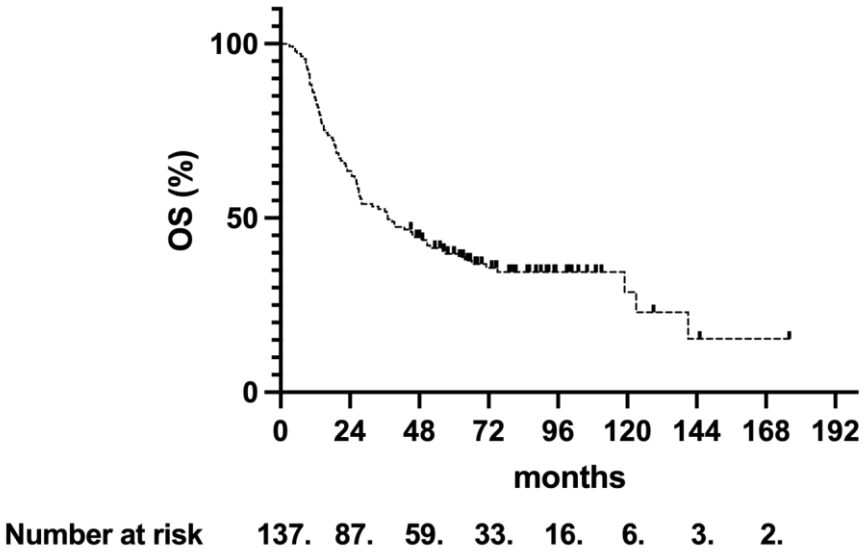

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Figure 10. Results of the Immunohistochemical Staining of CD20 and CD23 Stratified by TLS Status.

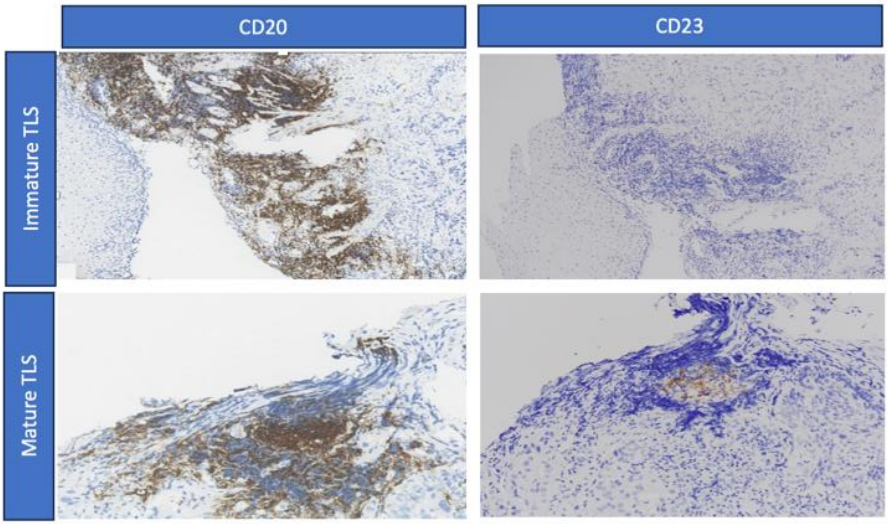

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Figure 11. Curves Depicting OS in Patients Stratified by TLS Status. TLS status: no, immature, or mature TLS. Comparisons between patients with no TLS and those with mature TLS were performed using a log-rank test.

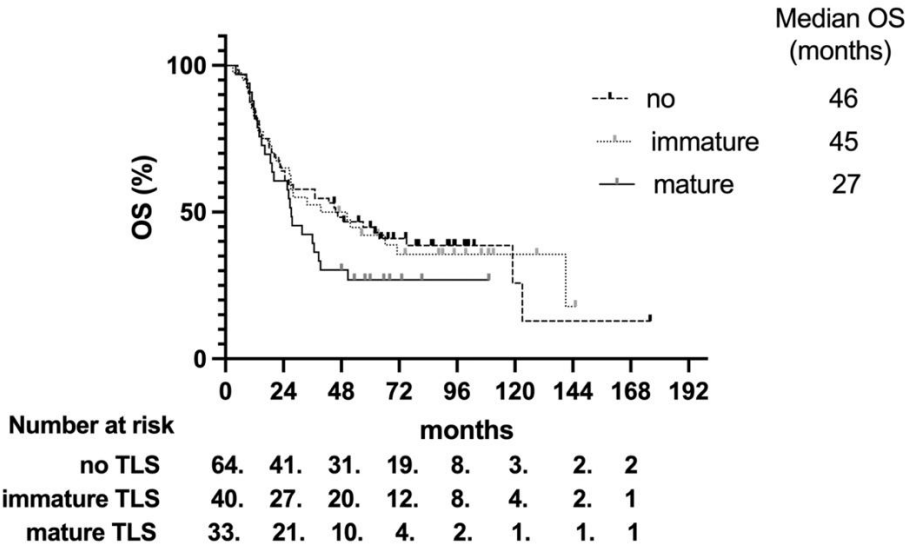

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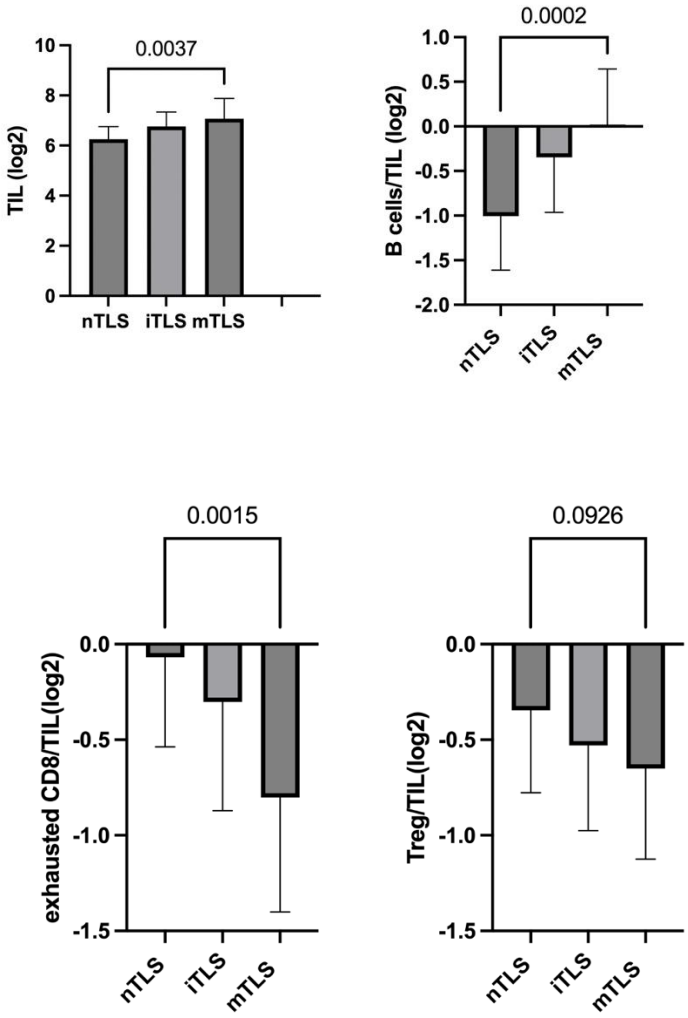
Figure 12. The amount of immune cells was compared along ESCC cells with no, immature, and mature TLS by CIBERSORT analysis, showing that tumor infiltrating lymphocytes (TILs) and B cellswere increasing while exhausted CD8 T cells and Tregs were decreasing with the maturation of TLS.



nTLS: no TLS; iTLS: immature TLS; mTLS: mature TLS

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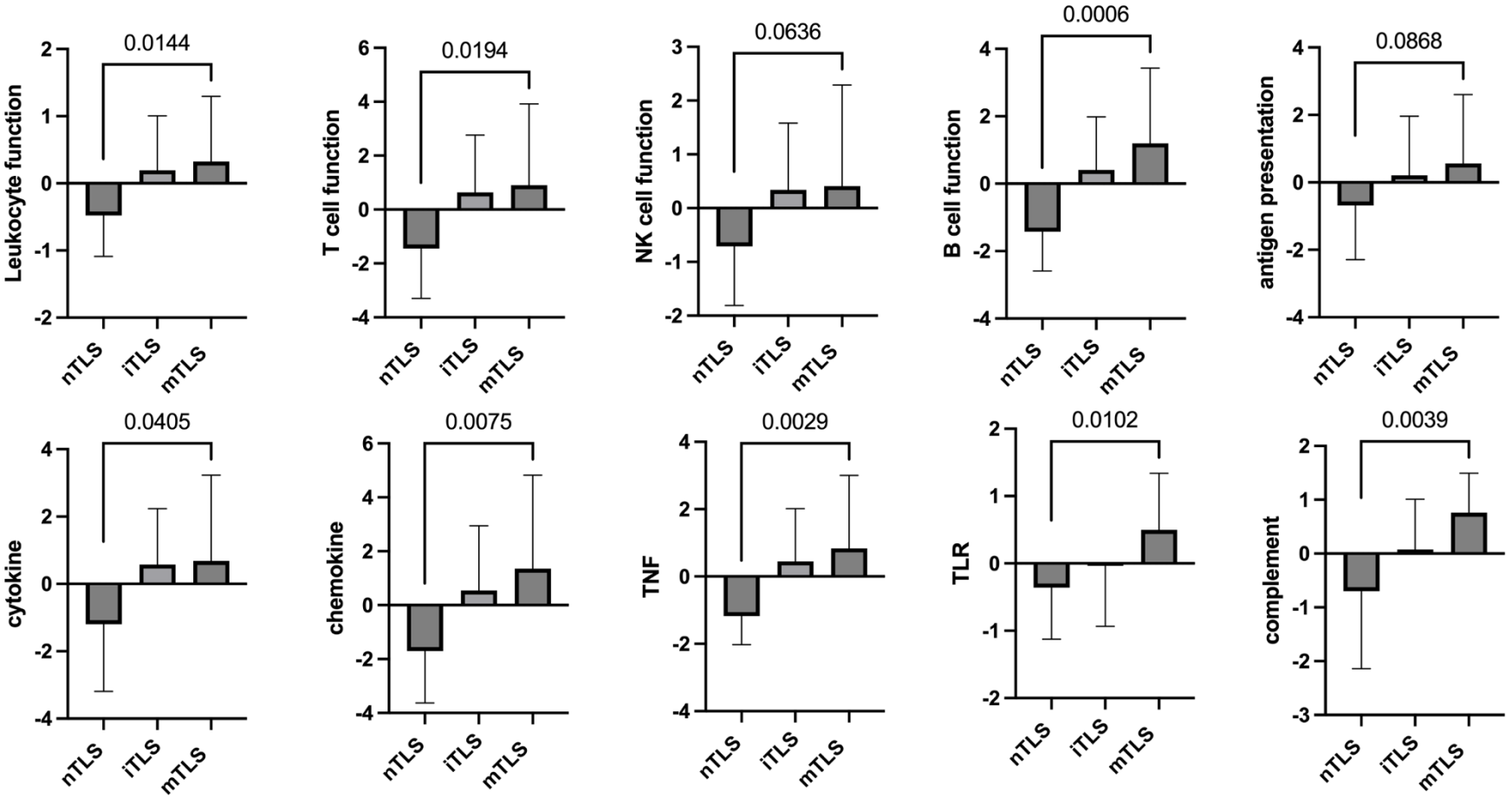
Figure 13. Various functions of immunity were compared along ESCC tissues with no, immature, and mature TLS.



nTLS: no TLS; iTLS: immature TLS; mTLS: mature TLS

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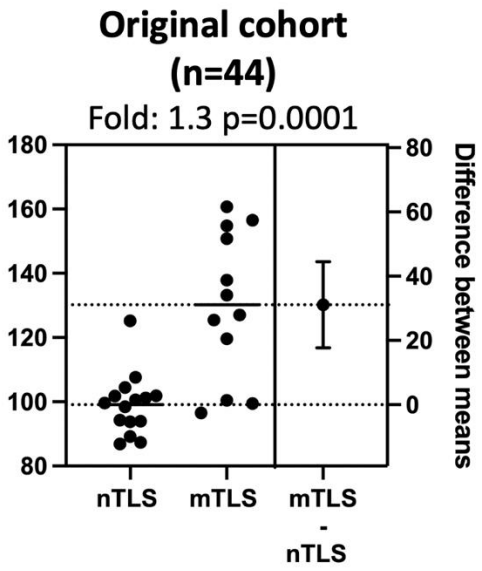
Figure 14. The 18 genes selected by ranking criteria for the ESCC specific TLS signature, and the difference of the signature between ESCC tissues with no TLS and with mature TLS in the original training cohort.

Gene	folds	P value
CD20	8.4	0.0062
CD19	5.1	0.0026
CD79a	6.1	0.00025
CD79b	5.4	0.0050
CD22	6.1	0.0081
CD27	4.8	0.00049
IRF4	5.1	0.00095
TNFRSF13b (TACI)	3.8	0.0031
TNFRSF13c (BAFF-R)	3.3	0.0059
TNFRSF17 (BCMA)	3.8	0.00047
CXCL12	3.6	0.0042
CXCL13	3.5	0.0093
CCR7	3.0	0.0011
LTB	3.5	0.0035
CR1	3.1	0.0072
TLR10	3.2	0.0066
GZMK	3.1	0.0040
ST6GAL1	3.0	0.000072



nTLS: no TLS; iTLS: immature TLS; mTLS: mature TLS

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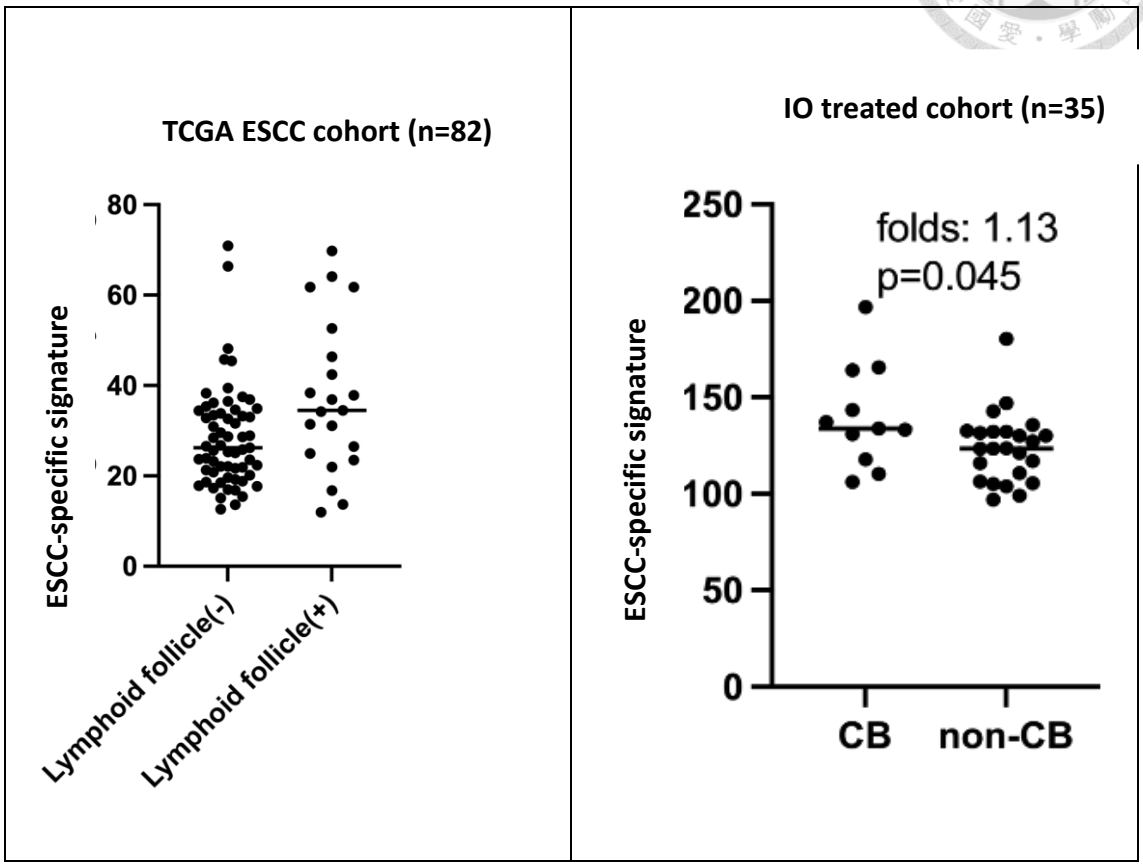
Figure 15. Validation with TCGA ESCC cohort and anti-PD1 immunotherapy treated cohort



IO: immunotherapy; CB: clinical benefit

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Chapter VIII Tables

Table 1. The three clinical trials, from which patients were retrospectively enrolled in PD-L1 study

Trial	Year	Patient No.	Population	Treatment
A	2000~2005	97	ESCC, EAC T3N0-1M0, T1-3N1M0 (AJCC 5th ed.)	[a]Neoadjuvant twice weekly TP-CRT, followed by esophagectomy
B	2008~2012	66	ESCC: T3N0M0, T1-3N1 M0, T1-3N0-1M1a (AJCC 6th ed.)	One cycle of [b]TP-HDFL, followed by [a]neoadjuvant twice weekly TP-CRT, followed by esophagectomy
C	2013~2015	30	ESCC: T3N0M0, T1-3 N1-3M0 (AJCC 7th ed.)	[c]Neoadjuvant weekly TP-CRT, followed by esophagectomy

[a]Paclitaxel 35mg/m^2, d1,4, qwk, for 4 wk; Cisplatin 15mg/m^2, d2,5, qwk, for 4 wk; R/T: 2Gy x 20fx

[b]Paclitaxel 70mg/m^2, d1,8; cisplatin 30mg/m^2, d2,9; 5-fluorouracil 2,000mg/m^2 and leucovorin 300mg/m^2, 24 hour infusion, d2,9

[c]Paclitaxel 50mg/m^2, cisplatin 30mg/m^2, weekly for 4 wk; R/T: 2Gy x 20fx

(From *Prognostic value of PD-L1 expression on immune cells or tumor cells for locally advanced esophageal squamous cell carcinoma in patients treated with neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2022 Jul;148(7):1803-1811.)*

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Table 2. Baseline clinical characteristics of the cohort of PD-L1 study

Parameters	Patients (n=100)
Age (years)	
Median (range)	56 (36~76)
Gender	
Male	89
Female	11
ECOG-PS	
0	29
1, 2	71
Stage (AJCC 7th ed.)	
II	10
III	90
Tumor site	
Upper	27
Middle	49
Lower	24
Surgery	
Yes	70
No	30

(From *Prognostic value of PD-L1 expression on immune cells or tumor cells for locally advanced esophageal squamous cell carcinoma in patients treated with neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2022 Jul;148(7):1803-1811.)*

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Table 3. Baseline characteristics of the three cohorts in the PD-L1 study

Parameters	Cohort A enrolled (n=30)	Cohort B enrolled (n=50)	Cohort C enrolled (n=20)
Age (years)			
Mean±SD	56±8	58±10	59±8
Gender	*P*=0.89		
Male	26 (87%)	45 (90%)	18 (90%)
Female	4 (13%)	5 (10%)	2 (10%)
ECOG-PS			
0, 1	28 (93%)	50 (100%)	20 (100%)
2	2 (7%)	0	0
Stage	*P*=0.63		
II	2 (7%)	5 (10%)	3 (15%)
III	28 (93%)	45 (90%)	17 (85%)
Tumor site	*P*=0.81		
Upper	10 (33%)	12 (24%)	5 (25%)
Middle	12 (40%)	27 (54%)	10 (50%)
Lower	8 (27%)	11 (22%)	5 (25%)
Surgery	*P*=0.82		
Yes	20 (67%)	35 (70%)	15 (75%)
No	10 (33%)	15 (30%)	5 (25%)

Cohort A, B, and C are from trial A, B, and C in Table S1, respectively.

(From *Prognostic value of PD-L1 expression on immune cells or tumor cells for locally advanced esophageal squamous cell carcinoma in patients treated with neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2022 Jul;148(7):1803-1811.***)**

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Table 4. Association of PD-L1 expression between immune cells and tumor cells

X^2=1.34 *p*=0.51		Immune cells	
		low	high
Tumor cells	negative	30	15
	positive	40	15

Tumor cells negative: <1%, positive: ≥1%; Immune cells low: <5%, high: ≥5%.

(From *Prognostic value of PD-L1 expression on immune cells or tumor cells for locally advanced esophageal squamous cell carcinoma in patients treated with neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2022 Jul;148(7):1803-1811.***)**

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Table 5. Association of PD-L1 expression on tumor cells and immune cells with baseline characteristics

	Tumor cells			Immune cells		
	negative	positive	*p*	low	high	*p*
Age Mean(sd)	56 (9)	58 (9)	0.22	57 (8)	57 (10)	0.74
Gender						
male	42	47	0.21	61	29	0.15
female	3	8		9	1	
Location						
upper	10	17	0.28	24	3	0.039*
middle	26	23		30	19	
lower	9	15		16	8	
Stage						
II	5	5	0.74	6	4	0.47
III	40	50		64	26	
ECOG-PS						
0	13	16	0.98	20	9	0.89
1, 2	32	39		50	21	

(From *Prognostic value of PD-L1 expression on immune cells or tumor cells for locally advanced esophageal squamous cell carcinoma in patients treated with neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2022 Jul;148(7):1803-1811.***)**

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Table 6. Association of PD-L1 expression on tumor cells and immune cells with pCR

Parameters		pCR	*p* value
Tumor cells	negative	15/36 (42%)	1
	positive	14/34 (41%)	
Immune cells	low	17/49 (35%)	0.11
	high	12/21 (57%)	

Tumor cells negative: <1%, positive: ≥1%; Immune cells low: <5%, high: ≥5%.

(From *Prognostic value of PD-L1 expression on immune cells or tumor cells for locally advanced esophageal squamous cell carcinoma in patients treated with neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2022 Jul;148(7):1803-1811.)*

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Table 7. Regression analysis for PFS in TLS study

Variables	Patients (n=100)	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Age (year)	100	1.02 (1.00~1.05)	0.08	1.00 (0.96~1.03)	0.74
Gender					
F vs M	11 vs 89	0.78 (0.53~1.16)	0.22	0.33 (0.14~0.78)	0.011*
ECOG-PS					
0 vs 1,2	29 vs 71	0.72 (0.55~0.96)	0.023*	0.40 (0.22~0.73)	0.003*
Stage					
II vs III	10 vs 90	0.92 (0.62~1.36)	0.67	1.20 (0.52~2.78)	0.66
Location					
Middle vs Upper	49 vs 27	0.69 (0.41~1.17)	0.17	1.07 (0.61~1.88)	0.81
Lower vs Upper	24 vs 27	0.83 (0.45~1.53)	0.55	0.89 (0.47~1.70)	0.73
Surgery					
Yes vs No	70 vs 30	0.66 (0.52~0.84)	0.001*	0.42 (0.25~0.71)	0.001*
PD-L1 expression on TC					
Positive vs Negative	45 vs 55	1.29 (1.02~1.63)	0.031*	1.69 (1.01~2.83)	0.044*
PD-L1 expression on IC					
High vs Low	30 vs 70	0.66 (0.50~0.87)	0.003*	0.34 (0.18~0.63)	0.001*

Tumor cells negative: <1%, positive: ≥1%; Immune cells low: <5%, high: ≥5%.

(From *Prognostic value of PD-L1 expression on immune cells or tumor cells for locally advanced esophageal squamous cell carcinoma in patients treated with neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2022 Jul;148(7):1803-1811.)*

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Table 8. Regression analysis for OS in TLS study

Variables	Patients (n=100)	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Age (year)	100	1.03 (1.00~1.05)	0.05*	1.00 (0.98~1.04)	0.72
Gender					
F vs M	11 vs 89	0.80 (0.54~1.18)	0.25	0.39 (0.17~0.91)	0.030*
ECOG-PS					
0 vs 1,2	29 vs 71	0.73 (0.55~0.97)	0.027*	0.47 (0.26~0.85)	0.012*
Stage					
II vs III	10 vs 90	0.93 (0.63~1.38)	0.73	1.17 (0.51~2.66)	0.72
Location					
Middle vs Upper	49 vs 27	0.63 (0.37~1.07)	0.087	0.89 (0.51~1.55)	0.68
Lower vs Upper	24 vs 27	0.79 (0.43~1.45)	0.45	0.88 (0.46~1.67)	0.69
Surgery					
Yes vs No	70 vs 30	0.70 (0.55~0.89)	0.003*	0.51 (0.31~0.85)	0.010*
PD-L1 of TC					
Positive vs Negative	45 vs 55	1.28 (1.02~1.62)	0.036*	1.62 (0.97~2.71)	0.068
PD-L1 of IC					
High vs Low	30 vs 70	0.66 (0.50~0.87)	0.003*	0.38 (0.21~0.69)	0.001*

Tumor cells negative: <1%, positive: ≥1%; Immune cells low: <5%, high: ≥5%.

(From *Prognostic value of PD-L1 expression on immune cells or tumor cells for locally advanced esophageal squamous cell carcinoma in patients treated with neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2022 Jul;148(7):1803-1811.)*

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Table 9. Published studies on the prognostic value of PD-L1 expression on tumor cells and immune cells for locally advanced ESCC

Study	Patient Number	Treatment	PD-L1 IHC antibody	PD-L1(+) percentage	OS (HR)
Hatogai 2016	196	surgery	E1L3N	IC: 60.7%	IC:0.59, p=0.01*
				TC: 18.4%	TC:0.46, p=0.016*
Lim 2016	73	NAC/NCRT + surgery	5H1	IC: 60%	IC:1.44, p=0.82
				TC: 56%	TC:2.29, p=0.023*
Zhang 2017	344	surgery +/- RT	SP142	IC: 25%	IC:0.72, p=0.015*
				TC: 15%	TC: ns
Jesinghaus 2017	125	surgery	SP263	IC: 30%	IC: ns
				TC: 29%	TC:0.56, p=0.026*

NAC: neoadjuvant chemotherapy; NCRT: neoadjuvant chemoradiotherapy; R/T: adjuvant radiotherapy; ns: not significant

(From *Prognostic value of PD-L1 expression on immune cells or tumor cells for locally advanced esophageal squamous cell carcinoma in patients treated with neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2022 Jul;148(7):1803-1811.*)

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Table 10. Baseline clinicopathological characteristics of ESCC patients enrolled in the TLS study.

Parameters	Patient number[#]
Total	137 (100%)
Age (years)	
Median (range)	54 (36~84)
Gender	
Male	125 (91%)
Female	12 (9%)
Location	
upper	35 (26%)
middle	71 (52%)
lower	31 (23%)
cT stage	
1	1 (0.7%)
2	8 (6%)
3	115 (84%)
4	12 (9%)
cN stage	
0	8 (6%)
1	58 (42%)
2	51 (37%)
3	19 (14%)
cSatge[$]	
I	0
IIA	4 (3%)
IIB	11 (8%)
IIIA	51 (37%)
IIIB	43 (31%)
IIIC	27 (20%)
Chemotherapy regimen in neoadjuvant CRT	
Paclitaxel/cisplatin	70 (51%)
Paclitaxel/carboplatin	67 (49%)
pCR	54 (39%)

Abbreviations: CRT, chemoradiotherapy; PD-L1 TC, PD-L1 expression on tumor cells; PD-L1 IC, PD-L1 expression on immune cells; pCR, pathological complete response.

[#] All data except age are expressed in "number (percentage)".

[$]The clinical stage was determined by AJCC 7th edition.

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Table 11. Baseline clinicopathological characteristics of ESCC patients enrolled in the two medical institutes in TLS study

Parameters	NTUH cohort	LK-CGMH cohort
Patient number	70 (100%)[#]	67 (100%)
Age (years)		
Median (range)	54 (36~73)	55 (38~84)
Gender		
Male	62 (89%)	63 (94%)
Female	8 (11%)	4 (6%)
Location		
upper	18 (26%)	17 (25%)
middle	36 (51%)	35 (52%)
lower	16 (23%)	15 (22%)
cT stage		
1	0	1 (1%)
2	3 (4%)	5 (7%)
3	66 (94%)	49 (73%)
4	0	12 (18%)
cN stage		
0	7 (10%)	1 (1%)
1	37 (53%)	21 (31%)
2	20 (29%)	31 (46%)
3	5 (7%)	14 (21%)
cSatge[$]		
I	0	0
IIA	3 (4%)	1 (1%)
IIB	6 (9%)	5 (7%)
IIIA	35 (50%)	16 (24%)
IIIB	20 (29%)	23 (34%)
IIIC	5 (7%)	22 (33%)
pCR	29 (41%)	25 (37%)

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Table 12. TLS status and PD-L1 expression levels of ESCC patients enrolled in current study

	No TLS	Immature TLS	Mature TLS	Total
PD-L1 IC	Chi-square test: *P*=0.0044			
high	15	15	19	49
low	49	25	14	88
PD-L1 TC	Chi-square test: *P*=0.46			
positive	32	22	13	67
negative	32	18	20	70
Total	64	40	33	

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Table 13. The association of baseline characteristics with TLS status.

	No TLS	Immature TLS	Mature TLS	
N	64	40	33	
Age				ANOVA test
Median (range)	55 (38-75)	55 (36-71)	54 (44-84)	*P*=0.97
Sex				Chi-Square test
male	57	37	31	*P*=0.68
female	7	3	2	
Tumor location				
upper	14	14	7	Chi-square test
middle	39	16	16	*P*=0.19
lower	11	10	10	
Clinical T stage				Chi-square test
1	1	0	0	*P*=0.064
2	5	3	0	
3	54	35	26	
4	4	1	7	*P*=0.0039*
Clinical N stage				
0	2	3	3	Chi-squre test
1	28	21	9	*P*=0.16
2	22	13	16	
3	12	2	5	

*Comparing patients with clinical T stage 4 with other patients with clinical T stage 1, 2, and 3.

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Table 14. Univariate and multivariate analysis for pCR in TLS study

Variable	Univariate		Multivariate	
	OR	p	OR	p
Age				
<50	ref	-	ref	-
50~64	0.74	0.48	0.66	0.39
≧65	1.19	0.76	1.43	0.58
Gender (male vs female)	0.11	0.0053	0.092	0.0050
Institute NTUH vs LK-CGMH (ref)	1.19	0.62	1.00	0.99
Location				
Upper	ref	-	ref	-
Middle	0.98	0.96	0.78	0.62
Lower	0.95	0.91	1.04	0.95
cT stage				
1, 2	ref	-	ref	-
3, 4	0.50	0.32	0.82	0.81
cN stage				
0	ref	-	ref	-
1	0.57	0.46	0.47	0.40
2	0.82	0.80	1.11	0.91
3	0.46	0.37	0.61	0.64
PD-L1 on tumor cells	1.48	0.26	1.10	0.81
PD-L1 on immune cells	1.43	0.33	2.15	0.084
TLS				
No TLS	ref	-	ref	-
Immature TLS	0.76	0.49	0.81	0.65
Mature TLS	0.36	0.034	0.26	0.023

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Table 15. Univariate and multivariate analysis for OS in TLS study

Variable	Univariate		Multivariate	
	HR	p	HR	p
Age				
<50	ref	-	ref	-
50~64	1.03	0.91	0.81	0.46
≧65	1.25	0.51	0.79	0.53
Gender male vs female (ref)	1.47	0.28	2.88	0.021
Institute NTUH vs LK-CGMH (ref)	1.49	0.043	1.32	0.30
Location				
Upper	ref	-	ref	-
Middle	0.78	0.29	1.26	0.43
Lower	0.51	0.035	0.57	0.096
cT stage				
1, 2	ref	-	ref	-
3, 4	2.78	0.079	2.06	0.24
cN stage				
0	ref	-	ref	-
1	1.10	0.71	0.95	0.91
2	0.93	0.90	0.66	0.43
3	0.97	0.96	0.64	0.44
PD-L1 on tumor cells	1.37	0.093	1.62	0.039
PD-L1 on immune cells	0.45	<0.001	0.30	<0.0001
TLS				
No TLS	ref	-	ref	-
Immature TLS	1.05	0.81	0.99	0.96
Mature TLS	1.29	0.29	2.96	0.0008

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Chapter IX Appendix

Papers

- **Huang TC**, Liang CW, Li YI, Guo JC, Lin CC, Chen YJ, Cheng AL, Hsu CH. Prognostic value of PD-L1 expression on immune cells or tumor cells for locally advanced esophageal squamous cell carcinoma in patients treated with neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2022 Jul;148(7):1803-1811.
- **Huang TC**, Lin CC, Wu YC, Cheng JCH, Lee JM, Wang HP, Huang PM, Hsu FM, Yeh KH, Cheng AL, Tzen KY, Hsu CH. Phase II study of metabolic response to one-cycle chemotherapy in patients with locally advanced esophageal squamous cell carcinoma. J Formos Med Assoc. 2019 Jun;118(6): 1024-1030.
- Guo JC, Hsu CL, Huang YL, Lin CC, **Huang TC**, Wu IC, Lin CY, Lien MY, Kuo HY, Cheng AL, Hsu CH. B Cells in Tumor Microenvironment Associated with the Clinical Benefit to Programmed Cell Death Protein-1 Blockade Therapy in Patients with Advanced Esophageal Squamous Cell Carcinoma. Front Oncol. 2022 Jun 29;12:879398. doi: 10.3389/fonc.2022.879398.

Abstracts

1. **Huang TC**, Guo JC, Lin CC, Kuo HY, Chuang CH, Cheng JCH, Hsu FM, Lee JM,

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Huang PM, Hsu CH. Exploration of potential biomarkers associated with treatment outcomes in locally advanced esophageal squamous cell carcinoma patients receiving neoadjuvant nivolumab plus paclitaxel and cisplatin chemoradiotherapy. AACR 2025; abstract #6510.

2. **Huang TC**, Liang CW, Li YI, Guo JC, Lin CC, Lee JM, Chao YK, Hsu CH. Tertiary lymphoid structure associates with poor prognosis in locally advanced esophageal squamous cell carcinoma treated with neoadjuvant chemoradiotherapy. Cancer Research (2024) 84(6_Supplement):7654-7654.
3. **Huang TC**, Liang CW, Li YI, Guo JC, Lin CC, Lee JM, Chao YK, Hsu CH. The prognostic role of tertiary lymphoid structure (TLS) in locally advanced esophageal squamous cell carcinoma (ESCC). Cancer Research (2023) 83(7_Supplement):2211-2211.
4. **Huang TC**, Liang CW, Lin CC, Chen YJ, Lin KL, Cheng AL, Hsu CC. Upregulation of Programmed death-ligand 1 (PD-L1) expression after a single-cycle of chemotherapy is associated with inferior survival in patients with locally advanced esophageal squamous cell carcinoma treated with preoperative chemoradiotherapy. Cancer Research (2017) 77 (13_Supplement): 2616.
5. **Huang TC**, Lin KL, Cheng AL, Hsu CC. Upregulation of PD-L1 expression by cisplatin in esophageal squamous cell carcinoma cells is independent of

doi:10.6342/NTU202502233

interferon/JAK/STAT pathway. Cancer Research (2016) 76 (14_Supplement): 4980.

doi:10.6342/NTU202502233