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Fluorescent In Situ Hybridization Assessment of PD-L1 in Endometrial

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Fluorescent In Situ Hybridization Assessment of PD-L1 in Endometrial Endometrioid Carcinomas: Correlation with Clinicopathological Parameters

M.Sc. THESIS IN MOLECULAR MEDICINE PROGRAM

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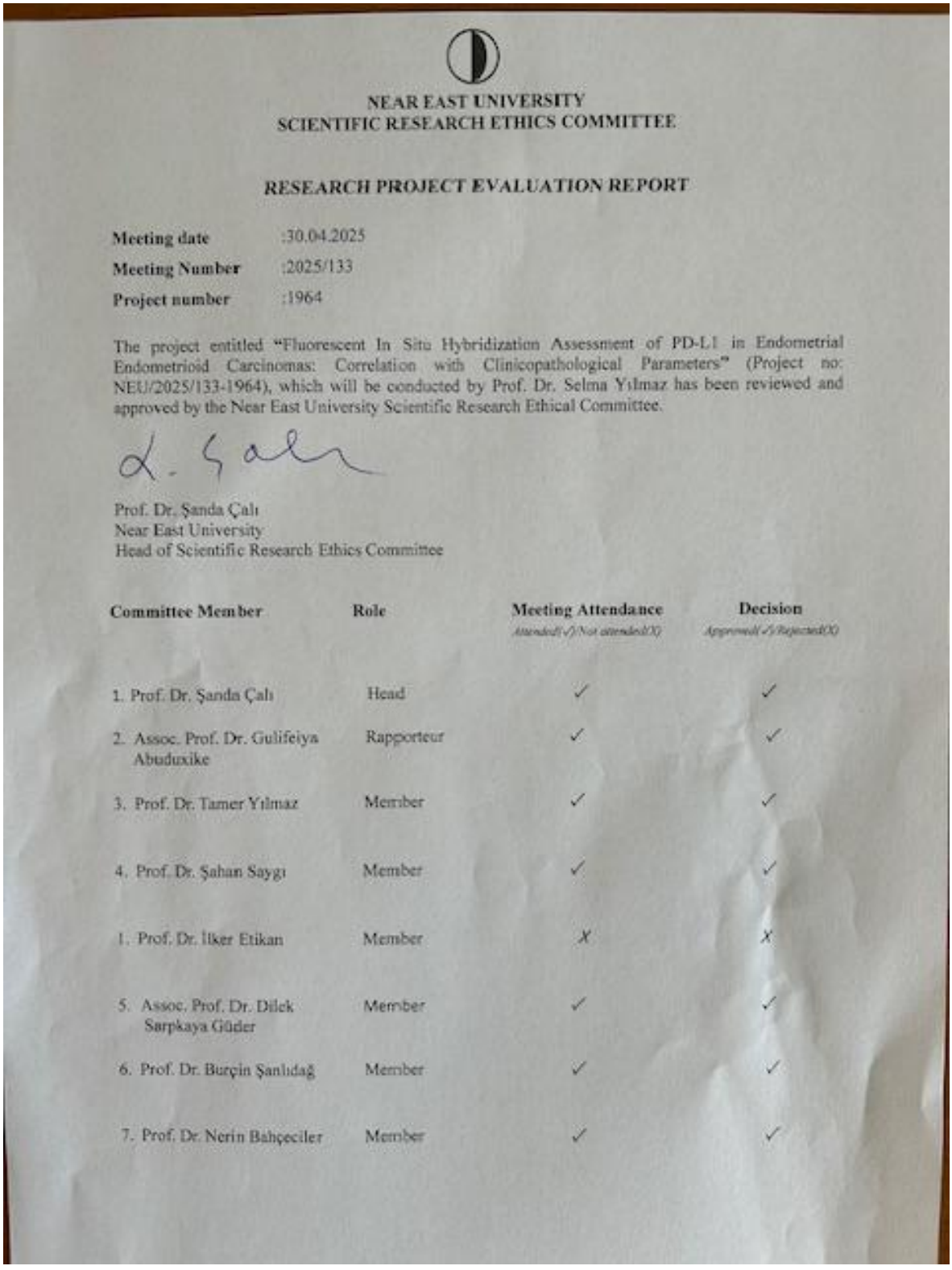

NEAR EAST UNIVERSITY
SCIENTIFIC RESEARCH ETHICS COMMITTEE

RESEARCH PROJECT EVALUATION REPORT

Meeting date :30.04.2025
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The project entitled "Fluorescent In Situ Hybridization Assessment of PD-L1 in Endometrial Endometrioid Carcinomas: Correlation with Clinicopathological Parameters" (Project no: NEU/2025/133-1964), which will be conducted by Prof. Dr. Selma Yılmaz has been reviewed and approved by the Near East University Scientific Research Ethical Committee.

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Declaration

I hereby declare that all information, documents, analysis, and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of the Faculty of Medicine, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

Ghazale Rezaei

27/06/2025

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GHAZALE REZAEI

Abstract

Fluorescent In Situ Hybridization Assessment of PD-L1 in Endometrial Endometrioid Carcinomas: Correlation with Clinicopathological Parameters

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MSc, Department of Medical Biology/Molecular Medicine Program

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Introduction: Endometrioid carcinoma is the most prevalent histological subtype of endometrial cancer, typically presenting as estrogen-dependent (Type I) and often diagnosed at an early stage, particularly in postmenopausal women. Molecular classification by the World Health Organization (WHO) has further refined the prognostic stratification into four subgroups: POLE-ultramutated, microsatellite instability, NSMP, and TP53-mutated tumors. Advances in the understanding of molecular pathways have led to the exploration of targeted therapies, including immune checkpoint inhibitors such as PD-1/PD-L1 blockers and agents targeting ARID1A deficiency, PI3K, MAPK, and HER2 pathways. The PD-1/PD-L1 axis plays a crucial role in immune evasion by tumors, yet emerging evidence suggests that PD-L1 gene alterations, such as amplification or translocation, may occur independently of protein expression. While PD-L1 gene amplification has been reported in non-small cell lung cancer, its role in endometrial cancer remains unclear. This study investigates the presence and potential clinical relevance of PD-L1 gene translocation in endometrioid carcinoma, aiming to uncover novel mechanisms of immune escape and expand the understanding of its pathogenesis.

Methodology: Endometrial carcinoma and endometrial hyperplasia cases were retrospectively identified using electronic health records between 2016 and 2025. One representative formalin-fixed, paraffin-embedded tissue block with adequate tumor tissue was selected per case. Fluorescence in situ hybridization (FISH) analysis was performed on 4 μm tissue sections using a dual-color break-apart PD-L1 probe. Slides were deparaffinized, pretreated, and hybridized with PD-L1 probes following standard protocols. Detection was carried out using a ZEISS Imager.Z2 fluorescence microscope. Green and red signals targeting distinct regions on chromosome 9p24.1 were evaluated manually to detect PD-L1 gene translocations, with a control probe on 9q12 serving as a reference. Descriptive statistics were applied due to the

limited sample size, summarizing patient demographics, tumor features, and PD-L1 gene status.

Results: A total of 26 female patients were included. The cohort comprised 13 cases of endometrioid carcinoma and 13 cases of endometrial hyperplasia. Among the 13 cases of endometrial hyperplasia analyzed, the majority (10 cases; 76.9%) were diagnosed as simple hyperplasia without atypia. Complex hyperplasia without atypia was identified in 2 cases (15.4%), while simple hyperplasia with atypia was observed in only 1 case (7.7%). With a mean age of 59.2 The carcinoma group had a mean age of 59.7 years, with most tumors graded as Grade 2 (46.15%), followed by Grade 1 (30.7%), and Grade 3 (15.38%). Tumor size was available in six cases (mean 28.7 mm). FISH analysis identified PD-L1 gene translocation in one case (3.84 overall), occurring in a 55-year-old patient diagnosed with grade 2 endometrioid carcinoma. All other samples demonstrated no gene rearrangements. Signal separation was confirmed in over 15% of nuclei for the translocation-positive case. The remaining 25 cases showed fused signals.

Discussion: PD-L1 expression in endometrial endometrioid carcinoma has been reported with considerable variability, ranging from 31.5% to 59%, potentially due to underlying molecular alterations. To explore this heterogeneity, we investigated the presence of PD-L1 gene translocation using FISH in 26 cases comprising 13 cases of endometrioid carcinoma and 13 cases of endometrial hyperplasia. Only one case (3.84 overall) with a moderately differentiated carcinoma with superficial myometrial invasion demonstrated PD-L1 gene translocation. Our findings suggest that PD-L1 gene translocation is a rare event in endometrioid carcinoma. While our study highlights the potential of PD-L1 gene alterations as biomarkers for immunotherapy responsiveness, limitations such as small sample size and lack of immunohistochemical evaluation call for further validation in larger, multi-modal studies.

Keywords*:* Endometrioid carcinoma, PD-L1, FISH analysis, translocation, histopathology

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List of Abbreviations

1. CML: Chronic myeloid leukemia
2. FFPE: Formalin-fixed, paraffin-embedded
3. FISH: Fluorescence in situ hybridization
4. IFN-γ: Interferon-gamma
5. LI: Labelling index
6. MMR: Mismatch repair deficiency
7. MSI: Microsatellite instability
8. NSCLC: Non-small cell lung cancer
9. PD: Programmed death
10. PD-L1: Programmed death-ligand 1
11. WHO: World Health Organization

CHAPTER I

Introduction

Endometrioid carcinoma represents the most common histological subtype of endometrial cancer and is categorized as Type I, which is estrogen-dependent and typically associated with a more favorable prognosis than Type II tumors. The majority of endometrial cancer cases approximately 75% occur in postmenopausal women, with endometrioid carcinoma being the predominant form in this group. In a cohort study involving 357 patients, 84.3% were diagnosed with Stage I disease, reflecting the high incidence of early-stage detection for this subtype [1,2,3]. According to the latest World Health Organization (WHO) classification, epithelial tumors of the uterine corpus are categorized based on their combined molecular and histological characteristics. These are divided into four molecular subgroups: Group 1 includes POLE-ultramutated tumors, which are associated with a favorable prognosis; Group 2 consists of microsatellite instability tumors, which are linked to an intermediate prognosis; Group 3 comprises tumors with no specific molecular profile (NSMP), also associated with an intermediate prognosis; and Group 4 includes TP53-mutated tumors, which are characterized by poor clinical outcomes [4].

Numerous alterations in biological pathways have been identified in endometrial cancer, prompting the development of novel therapeutic strategies and the search for predictive biomarkers. These include immune checkpoint inhibitors targeting the programmed cell death protein 1 (PD-1) and its ligand programmed death-ligand 1 (PD-L1 also written as PD L1), as well as agents such as PARP inhibitors, EZH2 and ATR inhibitors particularly in the context of ARID1A deficiency and synthetic lethality. Additional therapeutic targets under investigation involve alterations in the MAPK, PI3K, and HER2 signaling pathways, as well as angiogenic pathways regulated by VEGF, bFGF, PDGF, and HNF1β [5,6]. These discoveries are anticipated to greatly influence treatment strategies and management approaches for endometrial cancer.

T lymphocytes (T cells) are essential elements of the adaptive immune system, where they play a central role in mediating cell-based immune responses that protect the host from a wide spectrum of diseases [7]. Nonetheless, when T cells become excessively or improperly activated, they can target healthy tissues, contributing to autoimmune conditions. To maintain immune homeostasis and prevent such harmful responses, several coinhibitory immune checkpoint proteins such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), PD-1

(encoded by the *PDCD1* gene), and PD-L1 (encoded by the *CD274* gene) serve as critical regulators of T cell activity under normal physiological conditions. PD-L1 is a 33-kDa type I transmembrane glycoprotein composed of 290 amino acids, characterized by extracellular immunoglobulin (Ig) and IgC-like domains [2]. The PD-1/PD-L1 signaling axis plays a pivotal role in establishing and maintaining immune tolerance within the tumor microenvironment. The binding of PD-1 to its ligands PD-L1 or PD-L2 modulates key T cell functions, including activation, proliferation, and cytotoxic activity. This interaction ultimately contributes to the downregulation of anti-tumor immune responses, facilitating immune evasion by tumor cells [8].

The role of PD-L1 in helping tumors grow and evade the immune system has been studied in several cancers, including breast, ovarian, non-small cell lung cancer (NCCLC), and head and neck cancers. In one study focusing on squamous cell carcinoma of the lung, PD-L1 gene amplification was found in about 4.5% of the cases that could be evaluated. Interestingly, this amplification did not seem to be linked to the tumor's overall mutation profile, suggesting that the two are unrelated. Even more notably, 37% of the tumors with PD-L1 amplification did not show any detectable PD-L1 protein. These findings suggest that PD-L1 gene amplification could be a separate way that tumors avoid immune detection in NSCLC, even when the protein itself is not overexpressed [9].

In this study, we aim to investigate the presence and significance of PD-L1 gene translocation in endometrioid carcinoma, a common histological subtype of endometrial cancer. While PD-L1 expression has been widely studied in various cancers, its genetic translocation and potential biological and clinical impact in endometrial cancer remain largely unexplored**,** particularly in the context of endometrioid carcinoma.

CHAPTER II

Literature Review

1) Epidemiology of Endometrial Cancer

As of 2020, endometrial cancer ranked as the second most frequently diagnosed gynecologic malignancy worldwide and stood as the fourth leading cause of death among cancers affecting the female reproductive system [10]. In 2020, there were an estimated 417,367 new diagnoses of endometrial cancer worldwide, representing approximately 2.2% of all newly reported cancer cases and making it the sixth most common cancer overall. During the same year, about 97,370 deaths were linked to this disease, accounting for roughly 1% of all cancer-related fatalities. The global age-standardized incidence rate was recorded at 8.7 per 100,000 individuals, while the mortality rate reached 1.8 per 100,000. Notably, the occurrence of endometrial cancer is significantly more frequent in developed or high-income nations [11]. Metabolic syndrome characterized by factors such as obesity, insulin resistance, dyslipidemia, and hypertension is recognized as a key risk factor in the development of endometrial cancer, particularly the endometrioid type. Moreover, it may impact the prognosis and overall clinical outcomes of affected patients [12].

2) Etiology of Endometrioid Carcinoma

Endometrioid carcinoma typically appears as a well-differentiated tumor and is frequently linked to metabolic disorders such as obesity and hyperlipidemia [12]. Endometrial hyperplasia is widely recognized as a precursor lesion to endometrioid carcinoma, reflecting an early structural alteration in the endometrium that has the potential to develop into cancer if not properly managed [13,14]. Endometriosis, especially in the context of elevated estrogen levels, is believed to play a role in the malignant transformation leading to endometrioid carcinoma. Moreover, the risk of developing this cancer subtype rises with age, particularly after menopause, likely due to the gradual buildup of cellular and molecular changes in the endometrial tissue over time [15,16]. The molecular pathogenesis of endometrioid carcinoma is influenced by several genetic changes, including mutations in *PTEN* and *KRAS*, along with

deficiencies in the DNA mismatch repair (MMR) system, all of which play a key role in its development [17].

3) Histopathology of Endometrioid Carcinoma

Endometrioid carcinoma constitutes the most prevalent subtype of endometrial carcinoma, accounting for up to 80% of cases. It frequently develops in the background of endometrial hyperplasia, which results from prolonged exposure to unopposed estrogen stimulation [18]. Endometrioid carcinoma may occasionally originate from adenomyosis, although such cases are rare. Histopathologically, the tumor may exhibit squamous differentiation and is often characterized by a distinctive architectural pattern namely, confluent or back-to-back glands with little or no intervening stroma. Additionally, the neoplastic glands typically display tubular structures lined by stratified epithelial cells, with oval-shaped nuclei aligned perpendicularly to the basement membrane [19]. The histological grading of endometrioid carcinoma is determined by the proportion of solid, non-glandular growth within the tumor. Grade 1 lesions exhibit ≤5% solid components, Grade 2 tumors demonstrate 6% to 50%, while Grade 3 tumors are defined by having more than 50% solid growth. This grading system reflects the degree of glandular differentiation and is an important prognostic indicator [20]. Endometrioid carcinomas of higher histological grades typically exhibit poorly differentiated tumor cells arranged in dense, solid sheets, frequently occurring in conjunction with an atrophic endometrium [21]. Endometrioid carcinomas may exhibit deficiencies in MMR proteins, such as MLH1, MSH2, MSH6, and PMS2, alterations that are commonly implicated in Lynch syndrome [22]. Endometrioid carcinoma frequently harbors mutations in CTNNB1, the gene responsible for encoding beta-catenin-interacting protein 1, as well as in the tumor suppressor gene PTEN. Alterations in the KRAS oncogene are also commonly observed, while mutations in TP53 are relatively uncommon in this specific subtype [23].

4) Diagnostic Evaluation of Endometrioid Carcinoma

The diagnostic workup for endometrioid carcinoma frequently begins with an assessment of patient-reported symptoms and clinical presentation. Postmenopausal bleeding is the most common initial manifestation of this malignancy. Additional clinical features may include abnormal uterine bleeding, vaginal discharge, pelvic or abdominal pain, abdominal distension, early satiety, and alterations in bowel or bladder habits, particularly in cases with advanced

disease [24]. Histopathological analysis of surgical specimens is an essential component of the diagnostic process, providing definitive confirmation of carcinoma and enabling tumor grading in accordance with FIGO and WHO criteria [25]. Assessment of pelvic and para-aortic lymph nodes is vital, given that nodal involvement substantially influences both prognosis and therapeutic management [26]. Tissue biopsies are critical for histopathological confirmation, particularly when tumors arise in uncommon sites, such as the urethrovaginal septum [27]. Immunohistochemical analysis plays a key role in distinguishing endometrioid carcinoma from other carcinoma subtypes, utilizing markers including p53, MSH6, PMS2, alongside POLE mutation assessment [28]. These immunohistochemical markers are instrumental in differentiating endometrial adenocarcinomas from endocervical adenocarcinomas, which often share overlapping histopathological characteristics [29].

5) Risk Classifications in Endometrioid Carcinoma

Risk stratification in endometrioid carcinoma predominantly relies on tumor grade, the extent of myometrial invasion, and the presence of lymphovascular space invasion. Tumor grade is a key determinant, with high-grade lesions, especially Grade 3 tumors, correlating with an increased likelihood of recurrence and distant metastases. Grade 3 histology has been consistently associated with a heightened risk of extrapelvic relapse. Additionally, deep myometrial invasion defined as infiltration of 50% or more of the myometrial thickness constitutes a significant adverse prognostic indicator [30,31,32].

6) *PD-L1* Gene

The PD-L1 gene plays a pivotal role in immune regulation by serving as an inhibitory checkpoint that suppresses T cell activity, thereby preventing immune-mediated damage to normal tissues. Upon binding of PD-1 to PD-L1, T cell activation is attenuated, promoting immune tolerance and preventing autoimmunity. This PD-1/PD-L1 interaction is critical for maintaining the balance between immune surveillance and immune escape, as it inhibits T cell proliferation and differentiation, facilitating tumor evasion. Therapeutic blockade of the PD-1/PD-L1 axis disrupts this inhibitory signaling, enhancing antitumor immune responses and restoring effective immune-mediated tumor control [33]. Immune checkpoint inhibitors targeting the PD-1/PD-L1 pathway, including PD-1 inhibitors such as nivolumab and pembrolizumab, and PD-L1 inhibitors like atezolizumab, avelumab, and durvalumab, have

been developed to reinvigorate immune function and enhance antitumor activity in cancer therapy [34]. PD-L1 expression levels have been identified as a prognostic biomarker, with elevated expression correlating with adverse outcomes in various malignancies, including gastric cancer and lung adenocarcinoma [35]. Additionally, PD-L1 expression has been associated with tumor radiosensitivity, influencing the effectiveness of radiotherapy in cancer treatment [36].

7) PD-L1 Protein

PD-L1 is a transmembrane protein integral to immune regulation, exerting its effects through interaction with PD-1, an immune checkpoint receptor primarily expressed on activated T lymphocytes [37]. The principal role of PD-L1 within the immune system is to suppress T cell–mediated immune responses, thereby facilitating tumor immune evasion [38]. PD-L1 expression may be upregulated in response to proinflammatory cytokines, particularly interferon-gamma (IFN-γ), which activates the STAT signaling cascade, resulting in immune suppression. Tumor cells can express PD-L1 via intrinsic oncogenic pathways or as part of adaptive immune resistance mechanisms mediated by T cells and IFN-γ [39]. The interaction between PD-1 and PD-L1 leads to the suppression of cytotoxic T-lymphocyte activity, enabling tumor cells to evade immune surveillance and destruction. PD-L1 is expressed on a diverse range of cell types, including immune cells, tumor cells, epithelial cells, and endothelial cells, underscoring its broad role in modulating immune responses. In cancer treatment, inhibition of the PD-1/PD-L1 axis through immune checkpoint blockade restores T cell activity and potentiates antitumor immune responses [40].

8) PD-L1 Test (Labelling Index) Measured by Immunohistochemical Staining

The PD-L1 assay quantifies the Labelling Index (LI) via immunohistochemical staining to evaluate PD-L1 expression in both tumor and immune cells. This LI serves as a vital parameter in understanding tumor biology and predicting potential responsiveness to immunotherapy, as it reflects the dynamic interaction between PD-L1 and the immune microenvironment. The LI provides a quantitative measurement of PD-L1 expression, which is essential for determining patient eligibility for anti-PD-L1 targeted therapies. PD-L1 expression is assessed using specialized assays such as SP142 and 73-10, both of which employ primary rabbit monoclonal antibodies specific to PD-L1. Notably, the SP142 assay targets the C-terminal cytoplasmic domain of PD-L1, whereas the 73-10 assay binds to the intracytoplasmic domain, resulting in

variations in immunoreactivity and, consequently, differences in the LI outcomes. The Labelling Index is calculated by determining the percentage of positively stained cells, thereby providing an estimate of PD-L1 expression levels. Variations in antibody binding sites can lead to discrepancies in positive immunoreactivity, which directly influences the accuracy of the LI calculation. Ultimately, the LI is a critical factor in tumor characterization and immunotherapy planning, given that PD-L1 plays a central role in the negative regulation of T cell activity during tumor development and progression [41].

9) Other Promising Biomarkers in Endometrioid Carcinoma

HOXA5 has been identified as a novel prognostic biomarker for uterine corpus endometrioid carcinoma, indicating its potential utility in predicting patient outcomes and disease progression [39]. Increased levels of CD146 have been associated with higher tumor grade and deeper myometrial invasion in endometrioid carcinoma. Conversely, PTEN expression is markedly decreased in this cancer type relative to other endometrial conditions, underscoring the diagnostic and therapeutic relevance of both markers [40]. The loss of PAX2 expression is considered an important biomarker for endometrioid intraepithelial neoplasia and endometrioid carcinoma, though its exact function in the molecular development of these conditions is still not completely understood [41]. Mutations in genes such as CTNNB1, RHPN2, SF1, and SQSTM1 have been recognized as prognostic markers in patients with low-risk, early-stage endometrial endometrioid carcinoma, providing valuable information that could guide decisions regarding adjuvant therapy [42]. Tie-2, granulocyte colony-stimulating factor, and leptin have been suggested as valuable diagnostic biomarkers for endometrial cancer, offering potential roles in early diagnosis and personalized therapeutic strategies [43].

CHAPTER III

Methodology

Patients and Clinicopathological Information

Endometrial endometrioid carcinoma and endometrial hyperplasia cases were retrospectively identified using electronic health records with typing keywords, e.g., “endometrial endometrioid carcinoma," "endometrial hyperplasia”, between January 2016 and April 2025. All available glass slides of identified cases were retrieved from the archive of the Near East University Hospital, Pathology Laboratory and then reviewed by a pathologist blinded to the FISH results. One representative formalin-fixed, paraffin-embedded (FFPE) tissue block was selected per case and retrieved from the archive. The cases without sufficient quality and quantity of FFPE blocks were excluded. The cases with diagnostic uncertainty were excluded after microscopic review.

Histological grading was performed according to the International Federation of Gynecology and Obstetrics (FIGO) three-tiered system, based on the percentage of solid, non-squamous growth. Grade 1 lesions contained 5% or less solid components, Grade 2 tumors exhibited between 6% and 50%, while Grade 3 tumors were defined by having more than 50% solid growth [44].

Application of Fluorescence In Situ Hybridization (FISH)

4-micron-thick sections were precisely cut from the selected tissue blocks and mounted onto slides. Each section was labeled at the base using a diamond-tipped pen for identification purposes. The slides underwent deparaffinization by incubation in xylene within a 70°C water bath for one hour. This was followed by a rehydration process involving sequential immersion in absolute ethanol and 70% ethanol at room temperature for three minutes each. Afterward, the slides were rinsed in distilled water for two minutes and subjected to heat-induced antigen retrieval using the FISH pretreatment solution which includes a combination of ethanol, acetic acid, and chloroform, as seen in Modified Carnoy's solution II (MC II), used in conjuction with enzymatic treatments, such as RNase A and pepsin solutions, to remove RNA and proteins from the slides, respectively, at 95°C for 40 minutes [45]. Following an additional rinse in distilled water After rehydration, the tissue is treated with CytoCell’s pretreatment solution at 80–90 °C for 15–30 minutes to soften the tissue and enhance probe accessibility. Once cooled and rinsed, enzymatic digestion is carried out by applying a pepsin solution and incubating the

slides at 37 °C for 10–15 minutes. Each step must be performed separately to maintain enzyme activity and ensure optimal tissue preparation for successful hybridization. The samples were then washed in phosphate-buffered saline (PBS) at room temperature for three minutes, dehydrated in graded ethanol (70% and 100%) for seven minutes each, and air-dried completely. A PD-L1-specific FISH probe (CytoCell PD-L1 break apart probe, cat. no. LPH 096) was applied to each tissue section in a 7 µL volume. The slides were then subjected to denaturation at 84°C for 13 minutes, followed by overnight hybridization at 37°C in a humidified chamber. On the following day, the coverslips and adhesive materials were carefully removed, On the following day, coverslips and adhesive materials were carefully removed, and the slides were washed in 0.4× saline-sodium citrate (SSC) buffer at 73 °C for 2 minutes. A second wash was performed using 2× SSC containing 0.05% Tween-20 at room temperature for 2 minutes. After washing, 7 µL of DAPI counterstain was applied to each slide, and coverslips were mounted. The prepared slides were stored in the dark and examined the next day using a Zeiss Imager fluorescence microscope equipped with appropriate filter sets for signal detection and analysis.

Analysis of FISH Results

The green fluorescence-labeled probes were designed to hybridize to the genomic region spanning nucleotide positions 5,147,781 to 5,470,705, based on the GRCh37/hg19 genome assembly. while the red-labeled probes were designed to bind a downstream segment between positions 5,517,365 and 5,819,059, both located within the 9p24.1 region of chromosome 9. These probe sets correspond to distinct portions of the PD-L1 and PD-L2 gene loci, enabling the identification of structural alterations or gene rearrangements. The dual-color labeling strategy (green and red) facilitates the distinction between subregions, thereby enhancing the detection of abnormal hybridization signals under fluorescence microscopy. In addition, an aqua-labeled reference probe targeting the 9q12 region was included to assess chromosome 9 copy number integrity. The slides were scanned using a 20× objective lens and cell images were automatically captured by the fluorescence microscope (Zeiss Imager.Z2). The Zeiss imaging system is equipped with softwares, MetaFluor and Zen, by which we were able to manually count the hybridization signals in the cell nuclei. Each red or green signal observed under the fluorescence microscope represents one copy of the specific gene region targeted by the probe. When the red and green signals appeared separated, or when only one of the colors was visible without the other, this was interpreted as a potential indicator of a gene

translocation. Signal counting was done manually, focusing only on clearly defined and non-overlapping nuclei. For each case, at least 50 nuclei were analyzed to ensure reliable results. A case was classified as positive for PD-L1 gene translocation if more than 15% of the nuclei analyzed showed a split-signal pattern or single-color signal, in line with commonly accepted FISH interpretation guidelines. All observations were carried out using the appropriate fluorescence filters to allow for accurate interpretation of the signals.

Statistical Analysis

Due to the limited sample size in this study, descriptive statistical methods were used to summarize the patient demographics and tumor-related features. Categorical variables such as histopathological diagnosis, tumor grade, type of biopsy, and *PD-L1* gene status were reported using frequencies and percentages. Patient age was presented as the mean along with the range to reflect the overall distribution.

CHAPTER IV
Results

This study was approved by the Noninterventional Ethics Board of the Near East University with meeting number 2025/133 and project number 1964 on 30/4/2025.

Patients and Clinicopathological Information:

Case IDs were documented, and the corresponding tissue blocks along with their hematoxylin and eosin (H&E) stained slides were retrieved from the pathology archives. Among the initially selected 30 cases, 4 were excluded from the study due to specific issues identified during the review process. One case originally classified under the endometrioid carcinoma group was excluded after further examination revealed a serous carcinoma, which does not fall within the intended scope of this study. Another endometrioid carcinoma case was excluded because the corresponding tissue block could not be found. Additionally, two cases from the hyperplasia group were excluded. One of these turned out to be secretory endometrium upon histological review, rather than hyperplasia. The second one was removed because the available tissue block contained an insufficient amount of tissue for analysis.

The study cohort consisted of 26 female patients, ranging in age from 47 to 83 years. The first group comprised 13 cases of endometrial hyperplasia. Except for a single case (7,7) that underwent total abdominal hysterectomy with bilateral salpingo-oophorectomy (TAH + BSO), all other specimens in this group were obtained via curettage**.** The mean age of patients in the hyperplasia group was 59.2 years**,** with a range of 49 to 73 years. Among these**,** 10 cases (76.9%) were diagnosed as simple hyperplasia without atypia (Figure 1), 2 cases (15.4%) as complex hyperplasia without atypia, and 1 case (7.7%) as simple hyperplasia with atypia. Notably, two cases arose from polyps**,** indicating some variation in the underlying tissue context. The second group comprised 13 cases of endometrial carcinoma. Specimen types varied, with 7 (54%) cases obtained through curettage and 6 (46%) through radical surgery. The patients' ages ranged from 47 to 83 years. Tumor size was reported in 6 cases, ranging from 20 mm to 40 mm, with a mean of approximately 28.7 mm. The depth of myometrial invasion was available for 6 cases and 5 (83.3%) showed less than 50% invasion. Tumor grade was available for all cases except one: 6 (46.15) were grade 2, 4 (30.7%) were grade 1, and 2 (15.38 %) were grade 3 (Figure 2). Clinicopathological data for both endometrial hyperplasia

and endometrioid carcinoma are summarized in Table 1 and Table 2, respectively. The results of PDL-1 translocation analysis are shown in Table3.

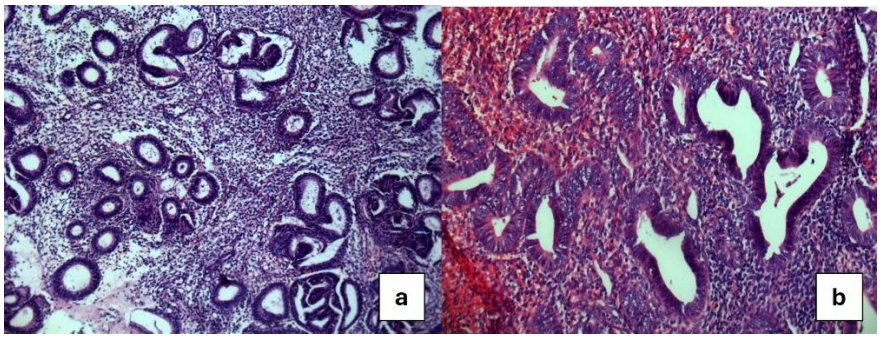

Figure 1) Simple hyperplasia without atypia (H&E, 10X magnification) **b)** Complex hyperplasia without atypia (H&E, 20X magnification).

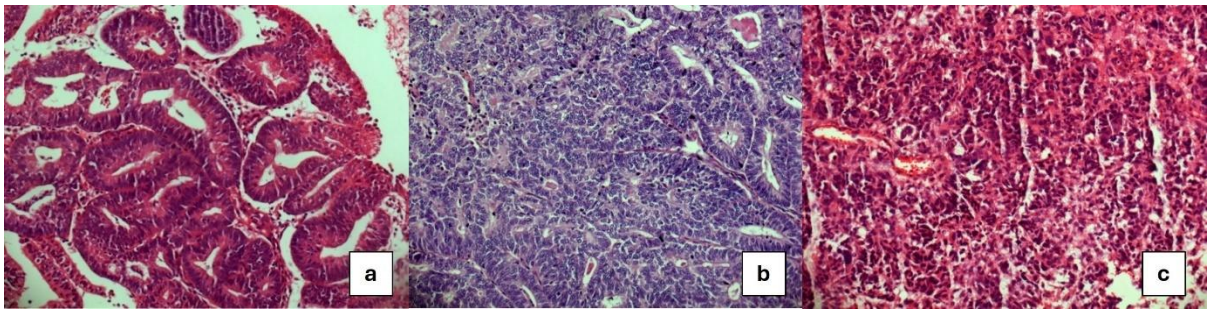

Figure 2) a) Grade 1 endometrioid carcinoma (well-differentiated) is characterized by cells that form distinct, well-organized glandular structures, with minimal nuclear atypia and a low number of mitotic figures (H&E staining, 20× magnification). b) Grade 2 endometrioid carcinoma (moderately differentiated) shows more noticeable nuclear atypia and a moderate increase in mitotic activity (H&E staining, 20× magnification). c) Grade 3 tumors (poorly differentiated) display marked nuclear atypia, a high frequency of mitotic figures, and are composed of solid sheets or poorly developed glandular formations (H&E staining, 200× magnification).

Table 1: Clinicopathological data from 13 cases of endometrial hyperplasia

Case No	Age	Specimen Type	Diagnosis	Note
1	73	Curettage	Simple hyperplasia without atypia	
2	49	Curettage	Simple hyperplasia without atypia	
3	61	TAH+BSO	Simple hyperplasia with atypia	
4	58	Curettage	Complex hyperplasia without atypia	
5	55	Curettage	Simple hyperplasia without atypia	Arising from a polyp
6	62	Curettage	Complex hyperplasia without atypia	Arising from a polyp
7	67	Curettage	Simple hyperplasia without atypia	
8	53	Curettage	Simple hyperplasia without atypia	
9	60	Curettage	Simple hyperplasia without atypia	
10	48	Curettage	Simple hyperplasia without atypia	
11	57	Curettage	Simple hyperplasia without atypia	
12	59	Curettage	Simple hyperplasia without atypia	
13	68	Curettage	Simple hyperplasia without atypia	

1) curettage
2) TAH+BSO: total abdominal hysterectomy and bilateral salpingo-oophorectomy

Table 2: Clinicopathological data from 13 cases of endometrioid carcinoma

Case No	Age	Specimen Type	Tumor Size (mm)	Depth of Myometrial Invasion	Tumor Grade
1	59	Curettage	–	–	1
2	59	Curettage	–	–	3
3	64	TAH+BSO	22	<50%	2
4	47	Curettage	–	–	1
5	64	Curettage	–	–	1
6	56	Curettage	–	–	-
7	53	TAH+BSO	20	<50%	2
8	68	TAH+BSO	25	<50%	2
9	65	TAH+BSO	35	>50%	2
10	83	Curettage	–	–	3
11	55	TAH+BSO	30	<50%	2
12	54	Curettage	–	–	1
13	49	TAH+BSO	40	<50%	2

TAH+BSO: total abdominal hysterectomy and bilateral salpingo-oophorectomy

PD-L1 Translocation Analysis:

Translocation of the *PD-L1* gene was evaluated using FISH in a cohort of 26 cases, comprising both endometrioid carcinoma and endometrial hyperplasia. Among endometrioid carcinoma cases, *PD-L1* gene translocation was identified in only one case (3,84 overall). The case was a 55-year-old woman who underwent radical surgery. The tumor measured 30 mm in size, exhibited less than 50% myometrial invasion, and was histologically graded as grade 2. In this translocation-positive case, clearly separated red and green signals were observed in more than 15% of the nuclei, confirming a true gene rearrangement. All other cases showed fused signals, indicating no evidence of *PD-L1* gene translocation (Figure 3).

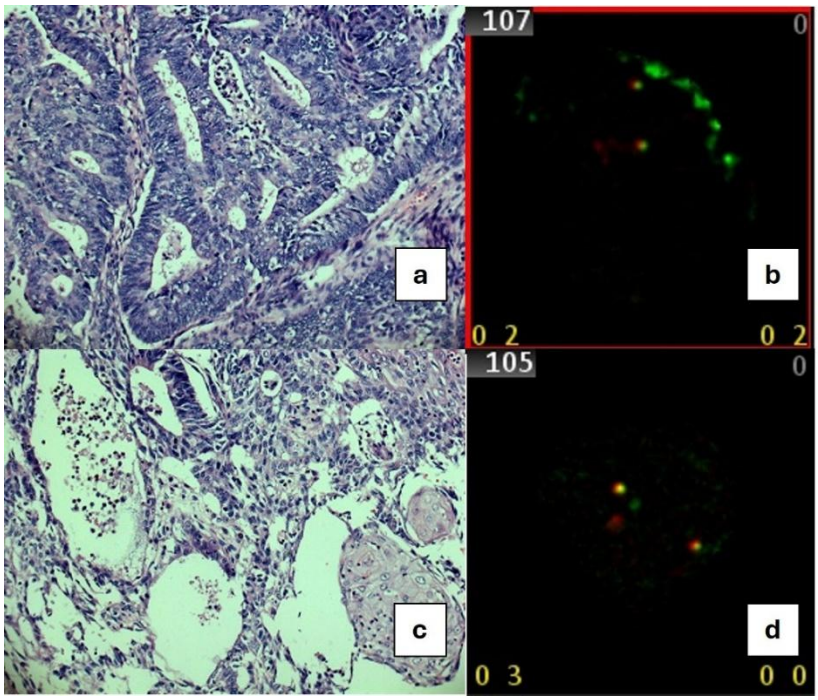

Figure 3. a) H&E of PD-L1 translocated case **b)** PD-L1 translocation **c)** H&E of non-translocated case (H&E staining, 200× magnification) **d)** The same case as in c showing no translocation in FISH test

CHAPTER V

Discussion

In this study, we investigated the frequency of *PD-L1* gene translocations in a cohort of endometrial endometrioid carcinoma and endometrial hyperplasia cases using the FISH method. Our aim was to correlate the presence of this structural genetic alteration with the clinical and pathological features. Interestingly, *PD-L1* gene translocation was identified in only one case of endometrioid carcinoma, highlighting the rarity of this event within our sample. This finding suggests that *PD-L1* translocations may be very uncommon in endometrioid carcinomas. Therefore, no correlation was observed between the presence of *PD-L1* gene translocation and clinicopathological features including age, tumor grade, and depth of myometrial invasion. Our results suggest that *PD-L1* gene translocation may not be linked to tumor features or specific clinical outcomes and it is not typically found in endometrial hyperplasia. However, the rarity of this alteration and the relatively small sample size warrant cautious interpretation.

Several studies have demonstrated that PD-L1 expression is significantly associated with clinicopathological features indicative of an unfavorable prognosis in endometrioid carcinomas. Notably, two independent investigations reported elevated PD-L1 expression within the epithelial component of endometrioid tumors. This heightened expression was closely linked to advanced disease stage and poor histological differentiation, both of which are known to adversely affect disease-free survival and overall prognosis [46,47]. Elevated PD-L1 expression in endometrial cancer has been strongly correlated with advanced tumor stages (III and IV), observed in both tumor cells and infiltrating immune cells. Research has also more frequently reported PD-L1 gene amplification in serous and other high-grade subtypes of endometrial carcinoma. In contrast, one study found no significant relationship between PD-L1 expression and factors such as patient age, histological subtype, or the extent of myometrial invasion [48]. Furthermore, elevated PD-L1 protein expression has been linked to mismatch repair deficiency (MMRd) and the high microsatellite instability (MSI-H) phenotype [49]. Tumors characterized by MMRd and MSI-H typically display a high tumor mutational burden, primarily resulting from the accumulation of genetic alterations, especially frameshift mutations [50].

In various other malignancies, the reported frequency of PD-L1 gene translocations and protein expression ranges from approximately 31.5% to 59%. [51]. The observed variability in PD-L1 expression across different cancer types can be attributed to several factors, including differences in the tumor microenvironment, underlying genetic regulation, and prior treatment history. Additionally, PD-L1 expression levels may vary between primary tumors and their corresponding metastatic lesions [52]. Non-small-cell lung cancer (NSCLC) demonstrates a notable incidence of PD-L1 gene translocations, especially in tumors harboring ROS1 rearrangements [53]. Among the various breast cancer subtypes, triple-negative breast cancer (TNBC) exhibits the highest rate of PD-L1 positivity [54]. PD-L1 expression shows considerable variation among thyroid cancer subtypes, with the highest levels detected in anaplastic thyroid cancer. In this aggressive form, elevated PD-L1 expression has been associated with reduced progression-free survival, highlighting its potential both as a prognostic indicator and as a promising target for immunotherapeutic interventions [55]. Another study examined various rare cancers, including penile, vulvar, and anal carcinomas, and found that PD-L1 expression is present in these tumor types, although the percentage of positive cells varies significantly across cases. These findings underscore the importance of spatially-resolved immune profiling in rare malignancies, as the tumor microenvironment and immune checkpoint expression can differ not only between cancer types but also within individual tumors [56].

PD-L1 translocation has been investigated extensively for its involvement in tumor progression and immune escape mechanisms. The PD-L1 protein can translocate to the plasma membrane, where it binds to PD-1 receptors on T cells, thereby suppressing their cytotoxic function and enabling the tumor to evade immune surveillance [46, 47]. PD-L1 translocation is a key factor in the progression of multiple cancer types, affecting both tumor characteristics and immune system interactions. Notably, nuclear localization of PD-L1 has been observed in cancers such as renal cell carcinoma, lung cancer, hepatocellular carcinoma, and colorectal cancer. In these cases, nuclear PD-L1 expression has been linked to tumor advancement and patient survival, indicating its promise as a valuable prognostic biomarker [48]. Elevated PD-L1 expression has been consistently linked to poorer prognosis and reduced overall survival across a range of cancer types [49].

In examining our control group of benign endometrial hyperplasia, we found no evidence of *PD-L1* gene translocations. This absence is noteworthy, as it suggests that such genetic

alterations though rare even in malignant samples are likely associated specifically with the malignancy of endometrial tissue rather than being incidental or background events. The lack of *PD-L1* translocations in these non-cancerous cases supports the idea that *PD-L1* gene rearrangements may be tied to tumorigenic processes, rather than appearing as random genetic changes in benign conditions.

Biologically, our findings support that high PD-L1 expression in endometrial endometrioid carcinoma is more likely driven by factors such as the tumor's inflammatory microenvironment and intrinsic genomic alterations such as microsatellite instability (MSI) rather than by structural changes like PD-L1 gene translocations. This suggests that immune-related signaling pathways and genomic instability may play a central role in regulating PD-L1 expression in these tumors, rather than chromosomal rearrangements. Clinically, this has important implications: despite the availability of FISH as a tool for detecting gene rearrangements, our study does not support its use for routine evaluation of PD-L1 in endometrioid carcinomas. The absence of *PD-L1* translocations in our cases indicates that FISH testing for *PD-L1* break-apart signals offers no additional diagnostic or predictive value in this context. Therefore, using FISH to confirm PD-L1 status or to guide immunotherapy decisions in endometrioid carcinoma patients is not justified. Instead, clinical decisions should continue to rely on established and validated biomarkers, such as PD-L1 protein levels determined by immunohistochemistry (IHC) and MMR/MSI testing, which better reflect the tumor biology and immune responsiveness.

One of the key strengths of our study lies in its methodological approach. We directly examined the structural status of the *PD-L1* gene using a validated FISH technique. This allowed us to reliably assess the presence or absence of gene translocations in a way that bypasses the interpretive variability sometimes seen in protein-level assays like IHC. By focusing specifically on gene structure, our study offers clear, definitive insight into how often *PD-L1* translocations actually occur in endometrial endometrioid carcinoma, answering an important question that had previously remained underexplored. However, like any research, this study has its limitations. Because it was retrospective in nature and based on a relatively modest number of cases, it may lack the statistical power to detect extremely rare events. In other words, while we only observed one *PD-L1* translocation in our cohort, we cannot entirely rule out the possibility that such alterations might occur at a very low frequency in the broader population. Additionally, since the study was conducted at a single institution, the results may

not fully reflect the diversity of endometrioid carcinoma cases seen in other clinical settings, which limits generalizability. Another important limitation is that we did not perform a direct comparison between our FISH findings and other relevant biomarkers, such as PD-L1 protein expression by IHC or MSI/MMR status. Including these correlations would have allowed for a more integrated understanding of the mechanisms driving immune checkpoint activity in these tumors. Future studies incorporating these complementary markers across multi-center datasets will be essential to validate and expand upon our findings.

Future research in this area should focus on validating our findings in larger, more diverse patient cohorts. Expanding the study population will help determine whether rare *PD-L1* gene translocations may still exist at low frequencies and whether they have any clinical significance. The upcoming studies should take a more integrated approach by combining FISH-based structural analysis with other key biomarkers such as PD-L1 protein expression, MSI testing, MMR status, and even genomic profiling through next-generation sequencing. This kind of multi-layered analysis will offer a more complete picture of the biological mechanisms regulating immune checkpoint activity in endometrial endometrioid carcinoma and may help refine the criteria for selecting patients who are most likely to benefit from immunotherapy. In summary, our study offers evidence that *PD-L1* gene translocation may be an uncommon event in endometrial endometrioid carcinomas and hyperplasia. These findings shift the focus of both research and clinical practice away from structural gene rearrangements and toward more established and informative pathways such as PD-L1 protein overexpression, gene amplification, and mismatch repair deficiency. By building on these foundations, future work can contribute to more accurate patient stratification and improved outcomes in the era of personalized cancer immunotherapy.

CHAPTER VI

Conclusion

In conclusion, our study reveals that PD-L1 gene translocation is an uncommon event in endometrioid carcinoma and is absent in endometrial hyperplasia. Through detailed analysis of 26 cases using FISH, only one carcinoma sample exhibited *PD-L1* rearrangement, with no significant association to tumor grade or histological subtype. These findings suggest that unlike other cancers where PD-L1 genetic alterations contribute to immune evasion, such rearrangements do not appear to play a major role in endometrial cancer. However, given the growing importance of PD-L1 as a biomarker and therapeutic target, further research into its genetic and protein expression profiles is warranted. Expanding our understanding of PD-L1 alterations may ultimately improve patient selection for immunotherapies and guide more personalized treatment approaches in endometrial cancer.

Recommendations

1) Investigate *PD-L1* gene rearrangements, including amplification, deletion, and copy number variations, using techniques such as FISH, quantitative PCR (qPCR), or array comparative genomic hybridization.
2) Explore mutations in regulatory regions of the PD-L1 gene, including promoter mutations, enhancer hijacking, and fusion genes, through whole exome sequencing to uncover novel mechanisms of PD-L1 dysregulation.
3) Quantify PD-L1 mRNA levels using RT-qPCR or RNA-seq and assess their correlation with genetic alterations (e.g., translocation, amplification) and clinical outcomes.
4) Evaluate PD-L1 protein expression by IHC. Multiplex IHC can be employed to simultaneously detect immune-related markers such as CD8, FOXP3, and PD-1, providing insights into the tumor immune microenvironment.
5) Determine the impact of promoter methylation on PD-L1 expression using bisulfite sequencing or methylation arrays to understand epigenetic silencing or activation mechanisms.
6) Conduct chromatin immunoprecipitation followed by sequencing (ChIP-seq) to identify histone modifications (e.g., H3K27ac) at the PD-L1 locus or enhancer regions that may influence gene expression.

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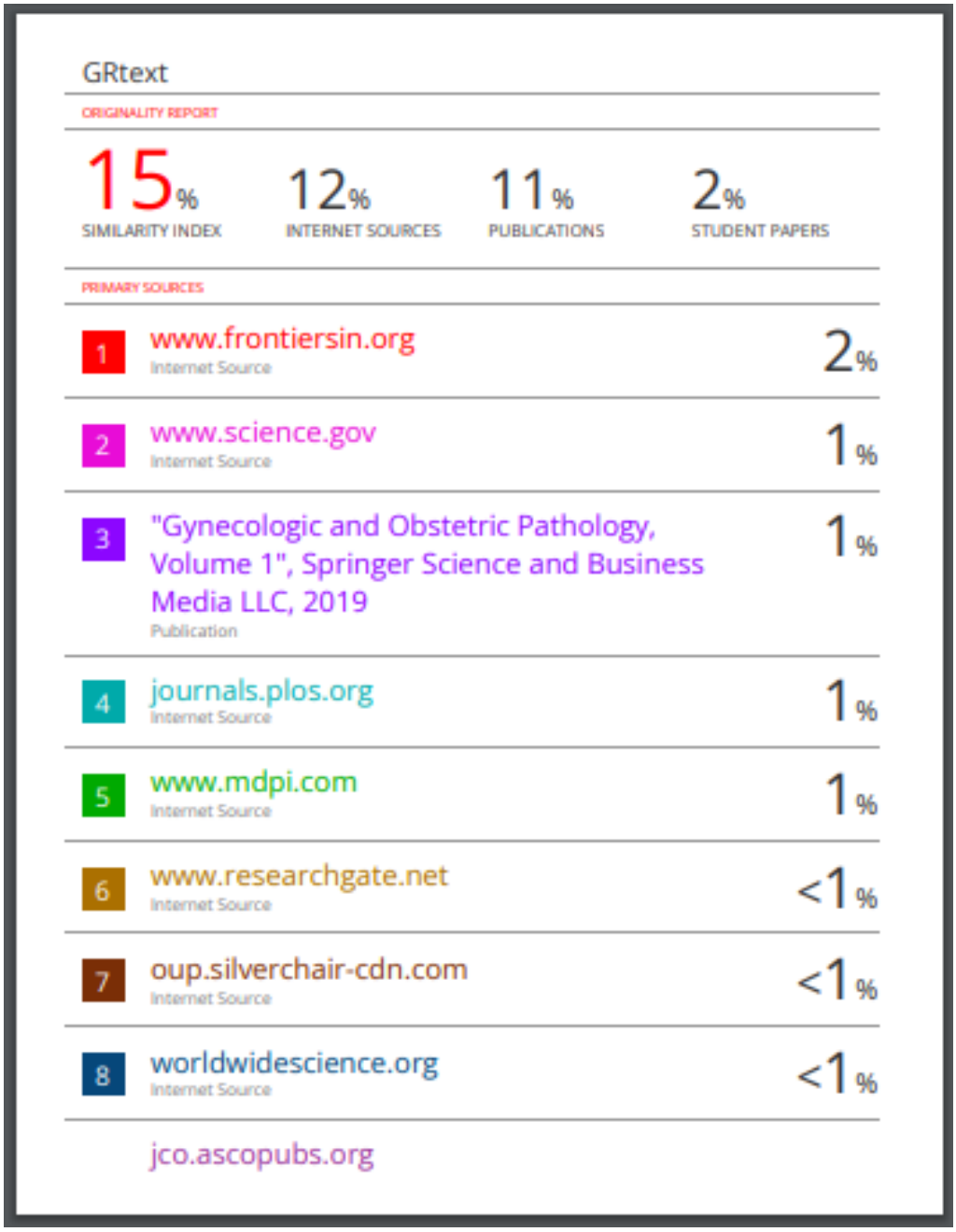

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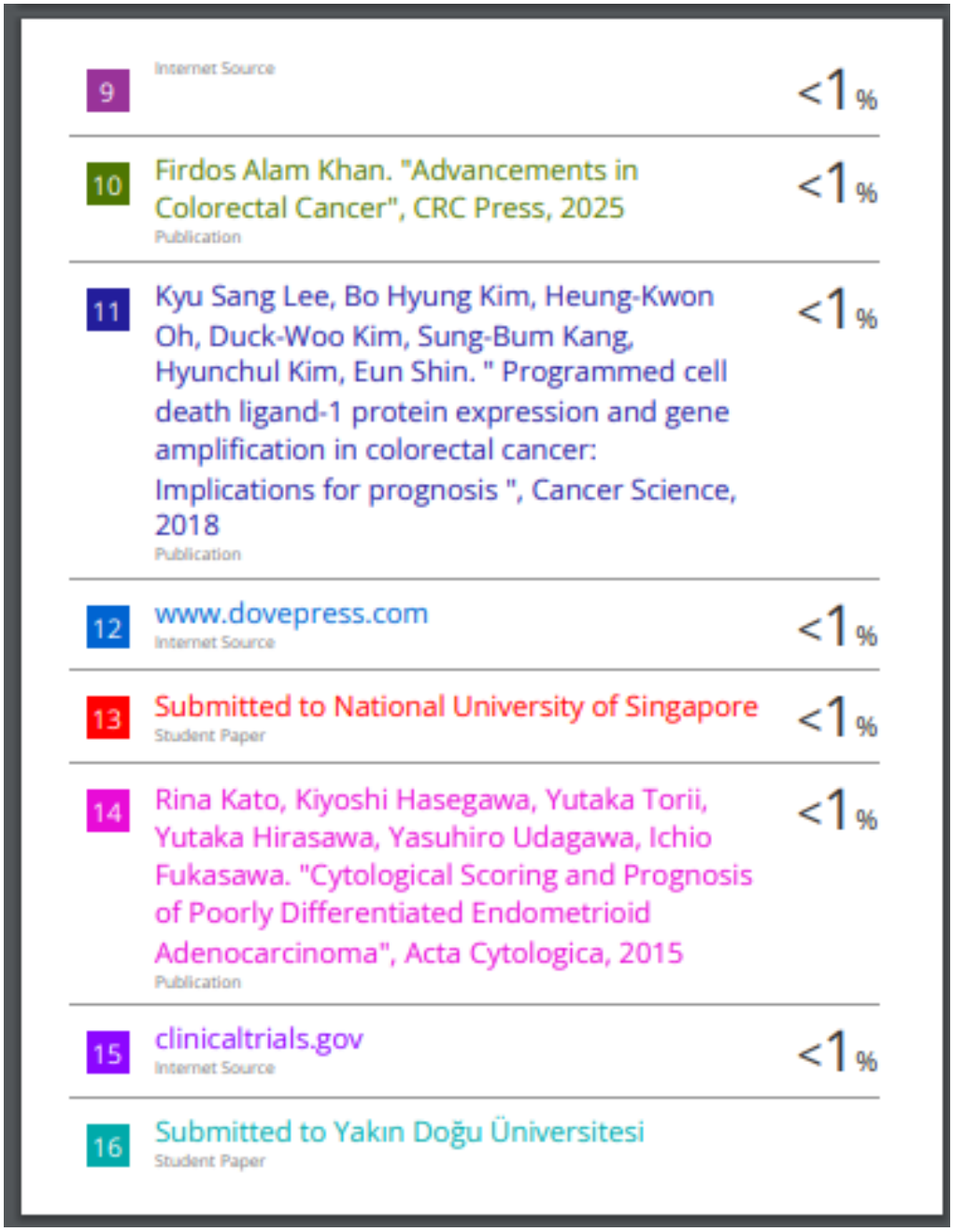

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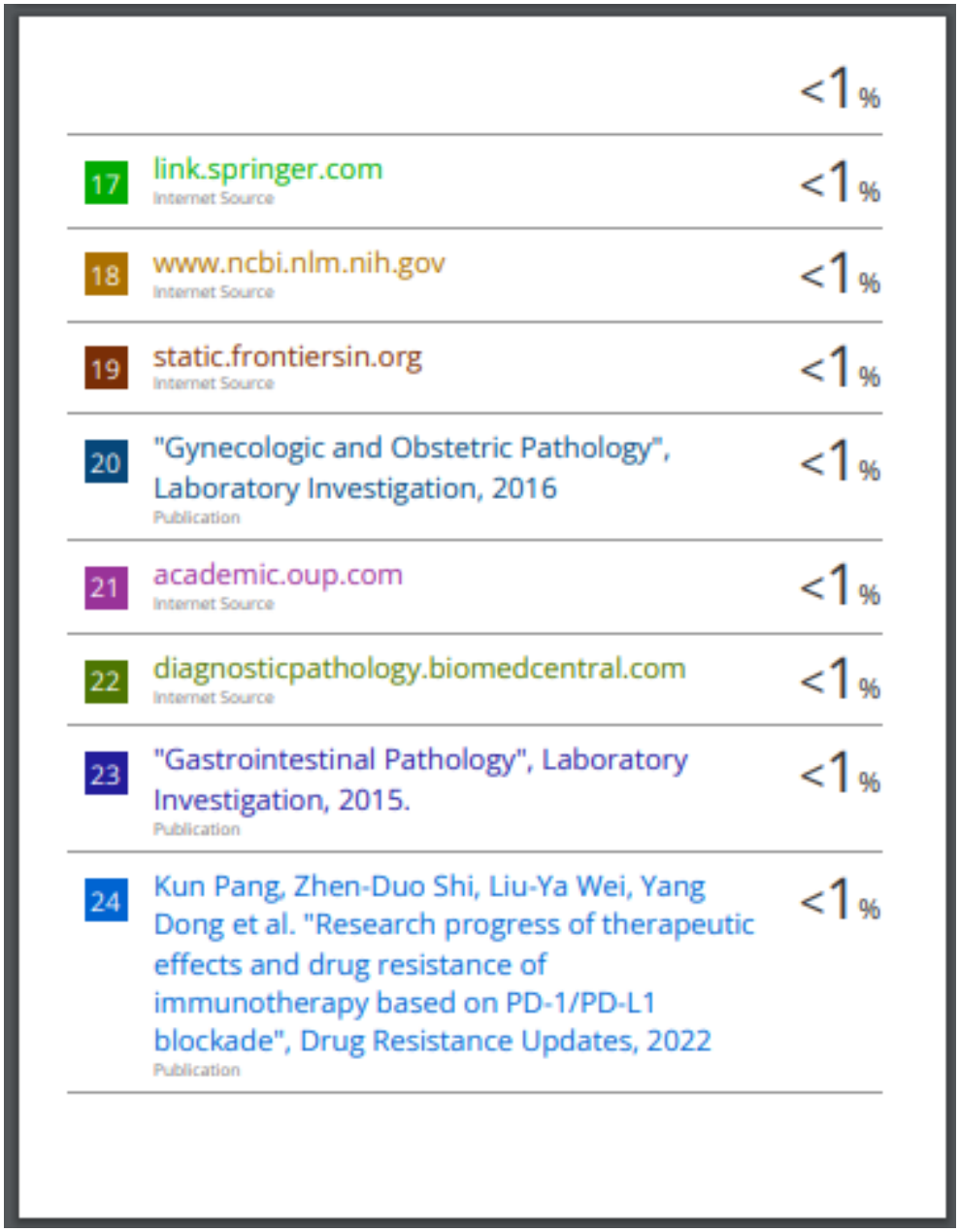

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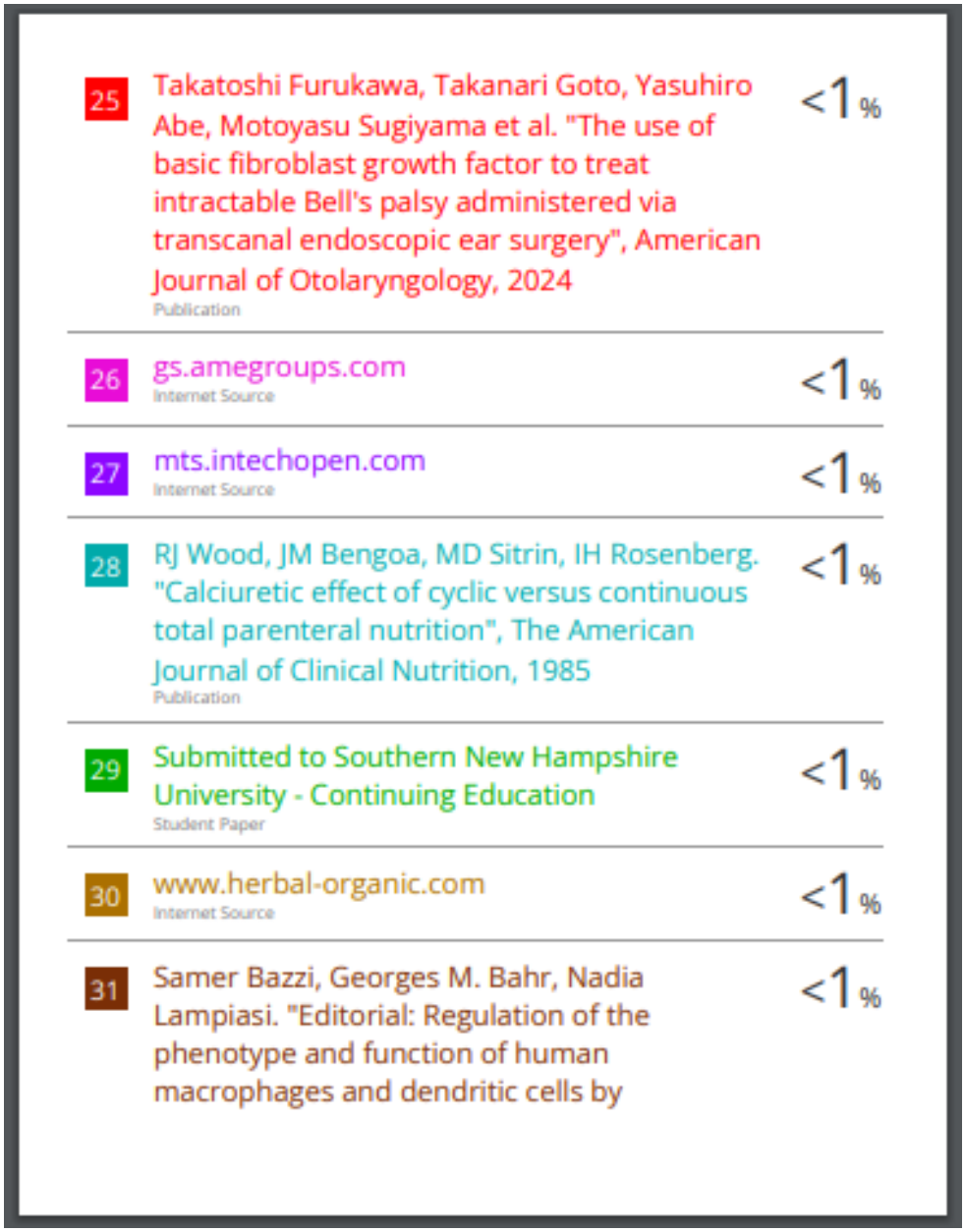

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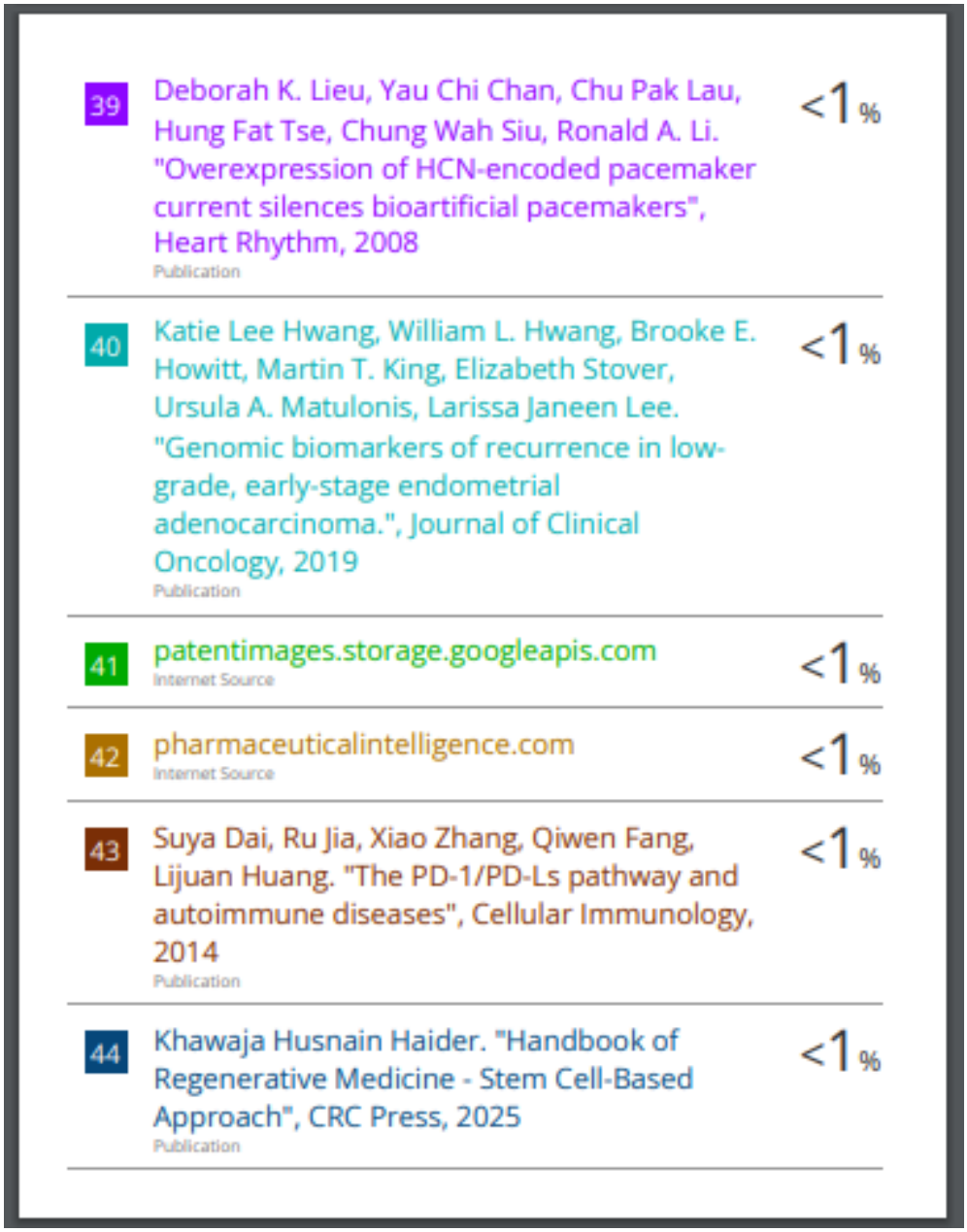

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