Original paper

Unravelling the link between mismatch repair protein deficiency and immune checkpoint markers – programmed death ligand 1 and galectin-9 expression in malignant melanoma

Gizem Teoman[1], Mustafa Emre Ercin[2], Safak Ersoz[1], Savas Yayli[3], Murat Livaoglu[4]

[1]Karadeniz Technical University, Faculty of Medicine, Department of Pathology, Trabzon, Turkey
[2]Lokman Hekim University, Department of Pathology, Ankara, Turkey
[3]Koç University Hospital, Department of Dermatology, Istanbul, Turkey
[4]Karadeniz Technical University, Faculty of Medicine, Department of Plastic and Reconstructive Surgery, Trabzon, Turkey

Malignant melanoma is an aggressive skin cancer, with immune evasion mechanisms contributing to tumour progression. This study evaluated the relationship between mismatch repair (MMR) protein loss and the expression of immune checkpoint molecules programmed death ligand 1 (PD-L1) and galectin-9.
Ninety melanoma cases (60 primary, 30 metastatic) were analysed by immunohistochemistry for MMR proteins, PD-L1, and galectin-9. Associations with clinicopathological features and overall survival (OS) were assessed.
Mismatch repair protein loss occurred in 5% of primary and 16.7% of metastatic melanomas ($p = 0.015$). Programmed death ligand 1 was positive in 18.8% of cases, with higher expression in metastatic tumours, but this was not statistically significant ($p = 0.106$). All PD-L1 positive tumours retained MMR proteins. Galectin-9 expression tended to be higher in tumours with MMR loss and in PD-L1-positive cases, but correlations were not significant. Median OS was 26.0 months, and no variable significantly affected survival in multivariate analysis.
Mismatch repair loss was more frequent in metastatic melanomas and associated with higher galectin-9 expression, whereas PD-L1 showed no clear link with MMR status. None of the associations reached statistical significance, emphasising the descriptive and exploratory nature of the study.
These findings outline biomarker expression patterns in melanoma and support further investigation in larger cohorts, including patients treated with immune checkpoint inhibitors, to clarify their potential clinical relevance.

Key words: malignant melanoma, mismatch repair proteins, PD-L1, galectin-9, immune checkpoint inhibitor molecules.

Introduction

Malignant melanoma is a highly aggressive form and one of the most common skin cancer types that arises from the uncontrolled growth of pigment-producing cells called melanocytes. In recent years, the incidence of malignant melanoma has risen dramatically worldwide. Although only 3% of all skin cancers are melanoma, it is the reason behind 65% of deaths due to skin cancer [1].

Even if the frequency of melanoma is relatively rare, its high mortality rate makes it of great importance. Therefore, it is essential to avoid melanoma occurrence, and early diagnosis is vital to reach

the necessary treatment. Malignant melanoma has a very high metastasis potential and is often resistant to standard treatment regimens. There is a wide variety of treatment modalities in malignant melanoma that tumour-infiltrating lymphocytes also contribute to. For advanced stage 3 or 4 patients, chemotherapy, palliative radiotherapy, and immunotherapy have recently emerged as promising treatment options [2, 3]. Over the years, extensive research has been conducted to understand the molecular mechanisms underlying melanoma progression better and identify potential therapeutic targets. One area of focus has been the investigation of immune checkpoints and their interactions with various signalling molecules in the tumour microenvironment [4].

Mismatch repair proteins are a group of enzymes responsible for maintaining genomic stability by correcting errors that occur during DNA replication. Defects in DNA genes related to mismatch repair (MMR) can lead to the accumulation of DNA mutations and microsatellite instability (MSI), contributing to tumour development and progression [4]. Different repair genes (*MSH2*, *MSH6*, *MLH1*, and *PMS2*) are related in this work and exist as heterodimers [5]. The interpretation of MMR protein expression by immunohistochemistry (IHC) can provide valuable information about the functional status of the MMR system in a tumour. In IHC, the expression of specific proteins or biomarkers is evaluated using antibodies that bind to the target proteins within the tissue sample. The commonly used immunohistochemical markers in MMR deficiency are the 4 MMR proteins: MLH1, MSH2, MSH6, and PMS2. Evaluating MMR protein expression by IHC is a practical and widely used approach to identify tumours with potential MMR deficiency [6, 7]. However, since MMR status is not solely dependent on protein loss but also on underlying genetic alterations, next-generation sequencing (NGS)-based panels are increasingly being used to detect mutations in MMR genes or identify MSI. Next-generation sequencing can reveal germline or somatic mutations in MMR genes, thereby validating IHC findings and providing more comprehensive molecular-level information [8].

Recent studies have highlighted the potential association between MMR protein expression and the immune response in cancer, including melanoma. One of the key immune checkpoints involved in melanoma is programmed cell death-ligand 1 (PD-L1), which is expressed on tumour cells and interacts with the programmed cell death protein 1 (PD-1) receptor on immune cells. This interaction suppresses the immune response, allowing tumour cells to evade immune surveillance [9].

Programmed death ligand 1 expression has been shown to correlate with poor prognosis in melanoma patients, and targeting the PD-1/PD-L1 axis with immune checkpoint inhibitors has demonstrated promising results in clinical trials [10].

Galectin-9 is another immune checkpoint molecule defined initially as a ligand for T-cell immunoglobulin and mucin domain-containing molecule 3 (TIM-3). It belongs to a family of carbohydrate-binding proteins and is involved in regulating the functions of immune cells. Galectin-9 has been shown to have both pro- and anti-tumourigenic properties, depending on the context and cell types involved. In melanoma, galectin-9 expression has been associated with immune evasion and resistance to immunotherapy, although the underlying mechanisms are not yet fully understood [11].

The study aimed to investigate the relationship between the expression of MMR protein, PD-L1, and galectin-9, which are immune checkpoint inhibitor (ICI) molecules in malignant melanoma.

Material and methods

Case selection

The study included 90 cases of malignant melanoma (60 primary and 30 metastatic melanoma) diagnosed 2010–2017 at the Department of Pathology, Faculty of Medicine, Karadeniz Technical University.

Cases diagnosed with primary or metastatic malignant melanoma histologically and having sufficient clinical and pathological information were included in the study. Clinical data of the cases were obtained through the hospital system. A pathologist reviewed the formalin-fixed paraffin-embedded (FFPE) melanoma specimens, re-evaluated haematoxylin and eosin-stained sections, and selected representative tumour blocks for immunohistochemical analysis.

Immunohistochemical scoring

Assessment of mismatch repair protein expression

Epidermal keratinocytes and immune cells served as internal controls, while normal colon mucosa was used as an external control for MLH1, MSH2, MSH6, and PMS2. Tumours were initially classified as mismatch repair deficient (dMMR) if there was a complete loss of nuclear staining in tumour cells for one or more MMR proteins. Tumours with retained nuclear expression in all markers were considered MMR proficient (pMMR). For cases showing partial or heterogeneous loss of MMR protein expression, NGS analysis of MMR genes was performed to confirm dMMR status, providing molecular validation of the IHC findings [12].

Assessment of programmed death ligand 1 expression

The 28-8 clone of the PD-L1 antibody was used. The percentage of complete circumferential or partial

Table I. Details of antibodies used in immunohistochemistry

Antibody	Clone	Vendor	Dilution	Method	Control tissue
MLH-1	M1	Roche	Ready to use	Automated	Colon
MSH-2	G219–1129	Cell marque	Ready to use	Automated	Colon
MSH-6	44	Roche	Ready to use	Automated	Colon
PMS2	EPR3947	Cell marque	Ready to use	Automated	Colon
PD-L1	28–8	Abcam	2 μg/ml	Automated	Tonsil
Galectin-9	21B5	Lifespan	1/150	Automated	Colon adenocarcinoma

linear plasma membrane staining in 4 areas was averaged at 20´ magnification. Cytoplasmic and ground staining were not evaluated. As a threshold value, according to previous studies and the melanoma evaluation guide of the commercial kit, staining below 1% was considered negative. Cases with PD-L1 ≥ 1% staining were considered positive. Tonsillar tissue was used as the external positive control [13].

Assessment of galectin-9 expression

Clone 21B5 of galectin 9 antibody was used. The percentage of cytoplasmic staining and staining intensity was evaluated using the H-score in 4 areas at 40´ magnification. Each slide was examined by dividing it into 4 quadrants for the H-score method, and staining intensity and percentage of all slides were evaluated. The intensity of staining was scored semiquantitatively as [3+(strong), 2+ (moderate), 1+ (weak), and 0 (absent) intensity]. The percentage of staining is the ratio between the number of stained cells and the total number of cells in the same area. This was followed by a calculation of the histoscore (H-score) according to the following formula: H-score = 1*(%cell "1+") +2*(%cell "2+") +3*(%cell "3+"). Four separate H-scores (0–300) were determined for each case, and the average was taken [14]. Colon adenocarcinoma was used as the external positive control. Galectin-9 expression was evaluated exclusively by IHC due to the lack of available tissue material and experimental resources for additional functional assays, such as immune cell infiltration analysis or RNA profiling. While functional validation could provide further mechanistic insights, immunohistochemical evaluation allowed us to reliably assess the expression patterns of galectin-9 in our malignant melanoma cohort. Details of antibodies used in IHC are given in Table I.

Next-generation sequencing-based microsatellite instability analysis

Microsatellite instability status was evaluated by examining the following loci: *BAT40(T)37*, *MONO-27 (T)27*, *BAT26(A)27*, *NR24(T)23*, *BAT25(T)25*, *NR22(T)21*, *HSP110-T17(T)17*, *NR21(A)21*, and *BAT34C4(A)18*. DNA was extracted from FFPE tumour tissues using the QIAGEN GeneRead DNA/RNA FFPE Kit. DNA quantity was measured using Qubit 4, ensuring sufficient material for downstream analysis, while quality was assessed via gel electrophoresis using the Qiaxcel system. Next-generation sequencing was performed on the Illumina NovaSeq platform. Bioinformatic analysis was conducted using Qiagen Clinical Insight Interpret and CLC Genomic Workbench. For FFPE-derived DNA, variants were considered reliable if they had a minimum read depth of 500×, an allele frequency of ≥ 5%, and a quality score > 200. Detected variants were classified according to the Tier classification system, and those deemed pathogenic or likely to be pathogenic were considered clinically significant. This approach enabled the molecular confirmation of MSI status, particularly in cases with partial or heterogeneous loss of MMR protein expression identified by IHC, thereby providing robust validation of dMMR status.

Statistical analysis

All obtained data will be entered into the SPSS database (IBM SPSS Statistics, version 27.0). Descriptive statistics of the evaluation results will be presented as follows: number and percentage for categorical data and mean, minimum, and maximum for interval data. The normal distribution of interval data will be examined using the Kolmogorov-Smirnov test. Comparisons of interval data between independent groups will be evaluated using Student's *t*-test if the normal distribution condition is met and the Mann-Whitney *U* test if not. The differences between the H-scores of the groups were studied using the non-parametric Kruskal-Wallis one-way analysis test and then the Mann-Whitney *U* test. The χ^2 test was used to compare the percentage data in the subgroups. Overall survival (OS) was calculated from the date of diagnosis to the date of death, or to the date of the last follow-up if the patient was still alive. For survival analysis, univariate analysis was performed using the Kaplan-Meier method, and statistical significance was evaluated using the log-rank test. Independent prognostic factors for OS were evaluated using multivariate Cox proportional hazards regression analysis.

The hazard ratio (HR) was used to evaluate the effect of variables on overall survival. For all variables, 95% confidence intervals (CI) were calculated and properly presented when applicable. A p-value of less than 0.05 was considered statistically significant.

Results

Patient characteristics

Our study included 90 cases of malignant melanoma, comprising 60 primary and 30 metastatic melanomas, diagnosed in the period 2010–2017. The mean age of the patients was 62.2 years (range 17–92 years). Female patients accounted for 44.4% (n = 40), and male patients for 55.6% (n = 50). The anatomical distribution of melanoma was as follows: 44.4% in the head and neck, 17.8% in the trunk, 8.9% in the upper limb, and 28.9% in the lower limb.

Among primary melanomas, histological subtypes included acral (n = 14, 15.5%), nodular (n = 31, 34.4%), mucosal (n = 12, 13.3%), superficial (n = 1, 1.1%), and lentigo maligna melanoma (n = 2, 2.2%). Of the 30 metastatic melanomas, 19 involved lymph nodes, 1 involved the adrenal gland, 4 involved the brain, 3 involved the femur, 2 involved the mandible/maxilla, and 1 involved the soft tissue surrounding the femur.

Mismatch repair protein expression

Immunohistochemical evaluation revealed that MMR protein expression was preserved in 82 of 90 cases (91.1%). The distribution of MMR loss was as follows: isolated MSH-6 loss in 3 cases (3.3%), combined MSH-2/MSH-6 loss in 3 cases (3.3%), combined PMS2/MLH-1 loss in 1 case (1.1%), and loss of all 4 proteins in 1 case (1.1%). Overall, complete MMR loss was observed in 6 cases, while partial loss (MSH-2/MSH-6) was observed in 2 cases. Subsequent NGS analysis confirmed MSI-high in both partially lost cases, indicating molecular evidence of MMR deficiency.

Mismatch repair protein loss was detected in 5% of primary melanomas (3/60) and 16.7% of metastatic melanomas (5/30), with a statistically significant difference between primary and metastatic cases (p = 0.015). The loss of MMR protein expression was 8.8% (8/90) in malignant melanoma cases (Figure 1).

Programmed death ligand 1 expression

Programmed death ligand 1 was negative in 73 cases (81.1%) and positive (≥ 1%) in 17 cases (18.8%) (Figure 2). Among primary melanomas, 8/60 cases were PD-L1 positive, whereas 9/30 metastatic melanomas were PD-L1 positive. Programmed death ligand 1 expression was higher in metastatic melanomas compared to primary cases, although this difference did not reach statistical significance (p = 0.106). All PD-L1-positive cases had intact MMR protein expression, and no significant correlation was observed between MMR protein loss and PD-L1 expression (p = 0.345).

Galectin-9 expression

The average H-score for galectin-9 was higher in cases with MMR protein loss. Specifically, cases with isolated MSH-6 loss had an average H-score of 79.6%, while cases with combined loss of 2 or more MMR proteins had an average H-score of 36% (Figure 3). Cases without MMR protein loss had an average H-score of 37.5%. However, no statistically significant correlation was observed between galectin-9 expression and MMR protein loss (p = 0.116).

Galectin-9 expression was also higher in PD-L1-positive cases (mean H-score: 48.4%) compared to PD-L1-negative cases (36.6%), but this difference was not statistically significant (p = 0.371).

Survival analysis

Kaplan-Meier survival analysis demonstrated that the median OS for the cohort was 26.0 months (SE = 6.34; 95% CI: 13.60–38.44), indicating that half of the patients survived beyond this time point (Figure 3). The mean OS was 43.9 months (SE = 5.10; 95% CI: 33.90–53.87 months), reflecting the average survival duration for the entire study population.

The association between PD-L1 expression, MMR status, galectin-9 expression, and median OS is summarised in Table II.

In multivariate Cox regression analysis, none of the evaluated factors reached statistical significance. Programmed death ligand 1-positive cases showed a HR of 0.588 (95% CI: 0.272–1.273; p = 0.178) compared to PD-L1-negative cases. Similarly, dMMR cases had an HR of 0.432 (95% CI: 0.151–1.236; p = 0.118) relative to pMMR cases. Galectin-9 H-score ≥ 100 was associated with an HR of 1.298 (95% CI: 0.545–3.089; p = 0.555) vs. H-score < 100. Tumour localisation in the head and neck vs. trunk/extremity showed HRs of 1.463 (95% CI: 0.682–3.136; p = 0.328) and 0.582 (95% CI: 0.316–1.070; p = 0.082), respectively. Advanced-stage tumours demonstrated an HR of 1.399 (95% CI: 1.097–4.903; p = 0.269) compared to early-stage tumours. These findings are summarised in Table III.

It should be noted that ICI therapy was not administered to any patients in this cohort, as ICIs were not reimbursed under the national healthcare system at the time of the study. Consequently, it was not possible to evaluate the relationship between MMR or galectin-9 expression and response to ICI therapy in this population.

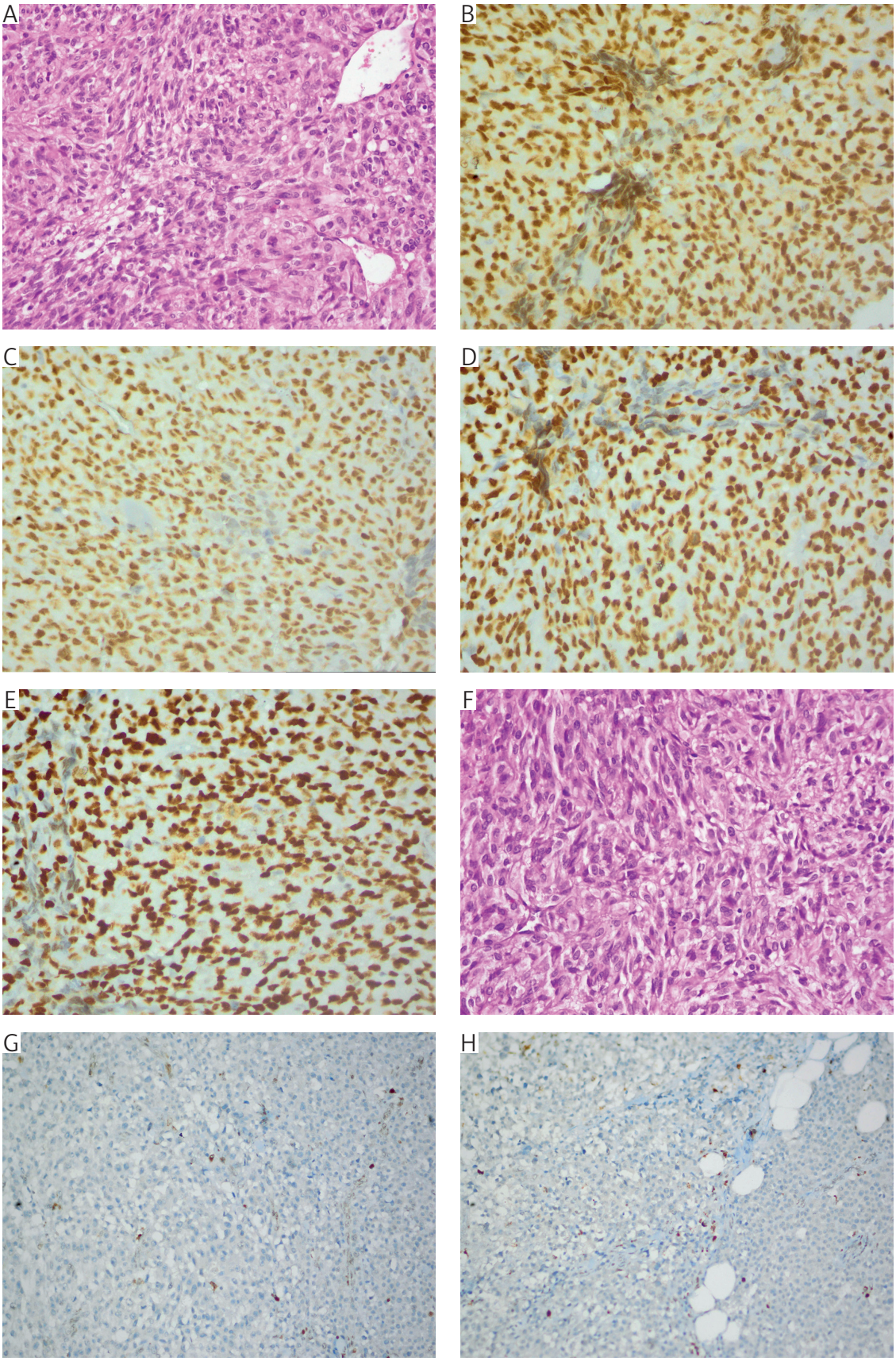

Figure 1. **A**) Haematoxylin and eosin-stained section of melanoma at 400× magnification. **B–E**) MLH1, PMS2, MSH2, and MSH6 immunohistochemical staining with no expression loss are shown at 400× magnification. **F–H**) In another case, haematoxylin and eosin-stained section together with MLH1 and PMS2 loss are shown at 400× magnification (Nikon Eclipse E200 light microscope)

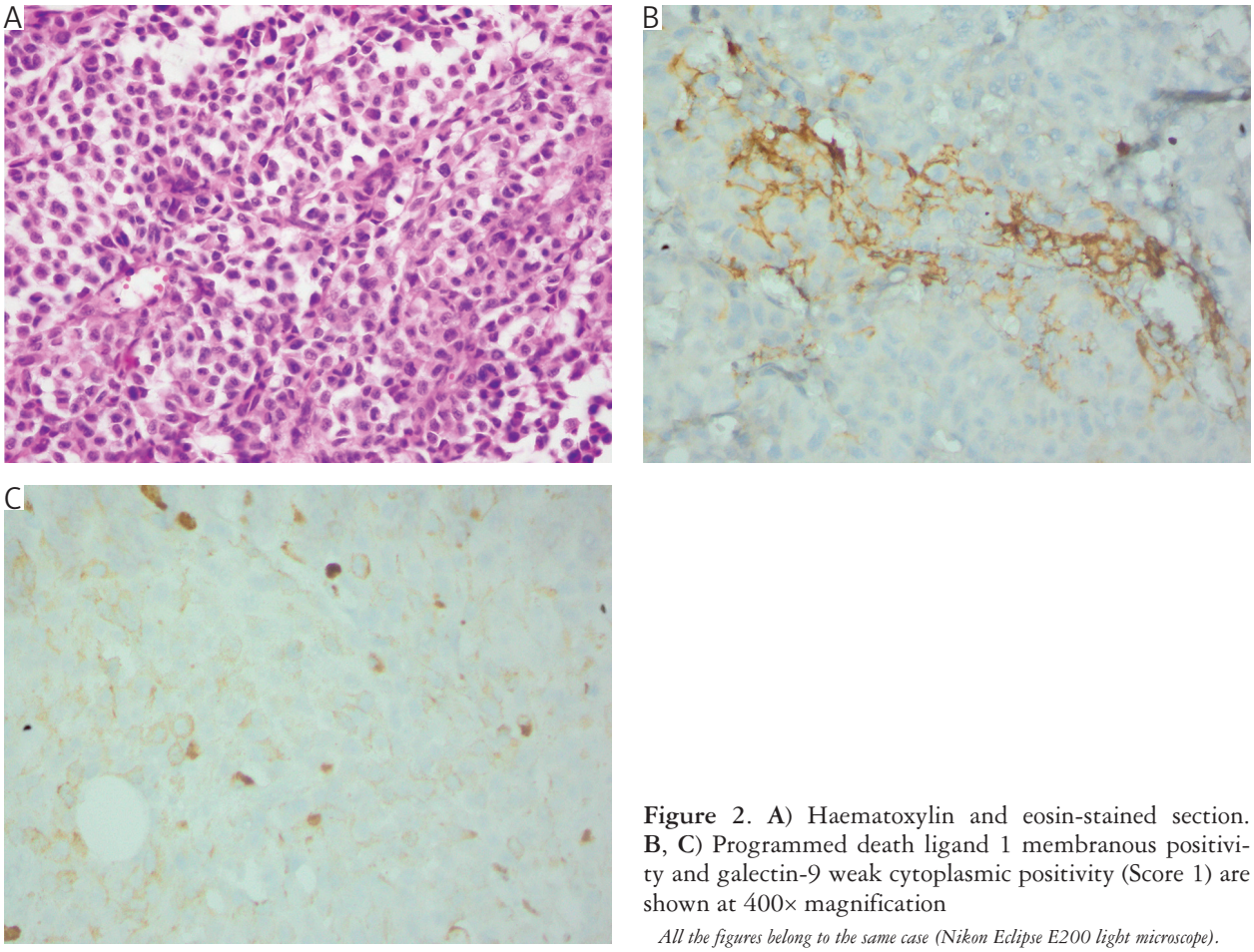

Figure 2. **A**) Haematoxylin and eosin-stained section. **B**, **C**) Programmed death ligand 1 membranous positivity and galectin-9 weak cytoplasmic positivity (Score 1) are shown at 400× magnification

All the figures belong to the same case (Nikon Eclipse E200 light microscope).

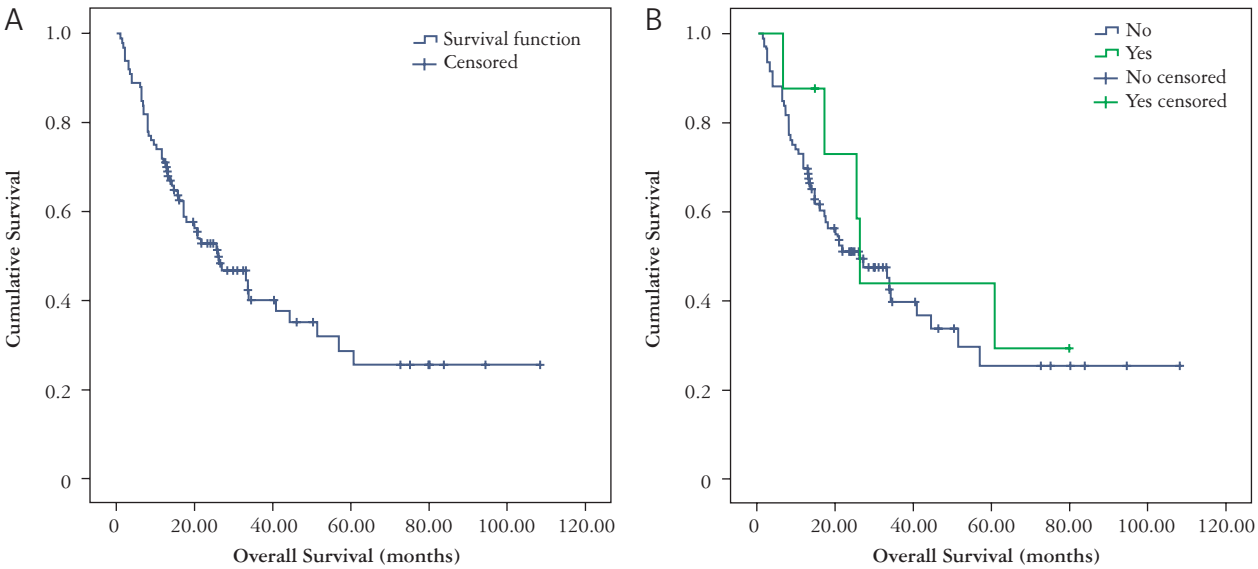

Figure 3. Kaplan-Meier overall survival (OS) curve for the study cohort (left) and Kaplan-Meier OS curves stratified by mismatch repair status (right)

Discussion

The pathophysiologic and molecular mechanisms of cancer have been increasingly studied to identify potential targets for diagnosis and therapy. Mismatch repair proteins and immune checkpoint molecules are of particular interest in melanoma research and may represent potential avenues for further study.

In this study, MMR protein expression loss was observed more frequently in metastatic melanoma cases than in primary tumours. This finding aligns

with previous reports suggesting that metastatic lesions may acquire additional mutations and genetic alterations during tumour progression, resulting in a more diverse genetic landscape [15]. Loss of MMR proteins could lead to increased MSI, which may influence tumour-immune interactions, although this study does not evaluate clinical outcomes.

The tumour microenvironment is known to influence immune responses, and metastatic melanomas often exhibit distinct microenvironmental features compared to primary tumours. These features include increased infiltration of immunosuppressive cells and altered cytokine profiles, consistent with prior literature [16].

In our cohort, cases with MMR protein loss were classified as PD-L1 negative, indicating an absence of detectable PD-L1 expression. This observation is consistent with previous studies reporting variable associations between PD-L1 and MMR/MSI status across different tumour types [17]. Similarly, higher galectin-9 expression was observed in metastatic tumours, including those with MMR protein loss, suggesting potential roles for alternative immune regulatory pathways, as previously reported in melanoma [18].

Importantly, falectin-9 has been shown to interact directly with PD-1 and TIM-3, thereby regulating T-cell apoptosis and contributing to tumour immune evasion [19]. This mechanistic evidence supports our observation that galectin-9 expression tended to be higher in PD-L1-positive cases, although not statistically significant, and suggests that galectin-9 may function as a complementary or compensatory pathway when PD-L1 is not dominant.

Recent clinical data have also highlighted the prognostic role of galectin-9 in melanoma. For example, Díaz-García *et al*. demonstrated that circulating levels of galectin-9 and TIM-3 independently predicted mortality in melanoma and lung cancer patients with obstructive sleep apnoea [20]. Although our cohort was not designed to evaluate circulating biomarkers, the higher galectin-9 scores in metastatic melanomas may reflect similar systemic immunoregulatory activity, which warrants further investigation.

Table II. Median overall survival according to biomarker status

Biomarker	Subgroup	Median OS (months)	*p*-value
PD-L1 expression	Positive	44	0.416
	Negative	20	
MMR status	dMMR	26	0.356
	pMMR	20	
Galectin-9 expression	H-score > 100	17	0.521
	H-score < 100	21	

dMMR – deficient mismatch repair, MMR – mismatch repair, OS – overall survival, PD-L1 – programmed death ligand 1, pMMR –proficient mismatch repair

Earlier studies also support the immunomodulatory role of galectin-9. Enninga *et al*. reported that galectin-9 promotes Th2/M2 polarisation, thereby creating a more immunosuppressive microenvironment, and linked this to survival outcomes in metastatic melanoma [21]. While we did not observe statistically significant associations with overall survival, our findings of elevated galectin-9 expression in advanced disease stages are consistent with the concept that galectin-9 contributes to immune escape and tumour progression.

In addition, Wang *et al*. summarised the diverse and sometimes paradoxical roles of galectin-9 in skin disorders, including both pro-inflammatory and immunosuppressive functions [22]. These dual effects may partially explain why, in our study, galectin-9 expression was higher in certain subgroups (e.g., dMMR- or PD-L1-positive tumours) but without significant prognostic impact. This complexity underscores the importance of studying galectin-9 not in isolation but in combination with other checkpoint molecules such as PD-1, PD-L1, and TIM-3.

No statistically significant association was observed between MMR status, PD-L1, or galectin-9

Table III. Multivariate Cox regression analysis of factors affecting overall survival

Factor	Multivariate analysis		
	Hazard Ratio (log rank)	95% CI	*p*-value
PD-L1 status (positive/negative)	0.588	0.272–1.273	0.178
MMR status (dMMR/pMMR)	0.432	0.151–1.236	0.118
Galectin-9 H-score (≥ 100/< 100)	1.298	0.545–3.089	0.555
Localisation (Head and neck/trunk/extremity)	1.463 0.582	0.682–3.136 0.316–1.070	0.328 0.082
Stage (advanced/early)	1.399	1.097–4.903	0.269

dMMR – deficient mismatch repair, MMR – mismatch repair, PD-L1 – programmed death ligand 1, pMMR –proficient mismatch repair

expression and OS in this cohort. It should be emphasised that none of the patients received immune checkpoint inhibitors, limiting the ability to draw predictive or prognostic conclusions. Therefore, the findings are descriptive and exploratory.

Overall, this study describes patterns of MMR, PD-L1, and galectin-9 expression in primary and metastatic melanomas, highlighting the complexity of immune checkpoint regulation. Mismatch repair loss was more frequent in metastatic melanomas, while PD-L1 and galectin-9 expression did not significantly correlate with MMR status and were not independent prognostic factors in this untreated cohort.

This study has some limitations. The relatively small sample size may reduce statistical power, and the absence of immunotherapy-treated cases prevents assessment of treatment responses. Despite these limitations, the findings provide a foundation for further research, particularly in cohorts treated with immune checkpoint inhibitors, to clarify the clinical relevance of these biomarkers.

Conclusions

This study provides a descriptive and exploratory analysis of MMR, PD-L1, and galectin-9 expression in melanoma. Mismatch repair deficiency was more frequently observed in metastatic tumours, whereas PD-L1 and galectin-9 expression did not show a clear association with MMR status. None of the evaluated markers were significantly associated with survival in this cohort of patients who did not receive immunotherapy, indicating that the findings are primarily descriptive. These results underscore the complexity of immune checkpoint regulation in melanoma and highlight the need for further studies with larger patient cohorts, including those treated with immune checkpoint inhibitors, to better understand the potential clinical relevance and prognostic value of these biomarkers.

Disclosures

1. Institutional review board statement: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The confirmation was granted by the Ethics Committee of the Faculty of Medicine (Date: 02/03/2018, Protocol Number: 2018/36). The authors confirm that human research participants ensured learned permission to publish the images of specimens obtained from them. Informed consent was obtained from all the patients included in this study.
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4. Conflicts of interest: None.

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Address for correspondence

Assistant Professor Dr. **Gizem Teoman**
Department of Pathology
Karadeniz Technical University
Faculty of Medicine
Trabzon, Turkey
e-mail: dr.gizemcivelek@gmail.com