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RESEARCH

PD-L1, MMR, and EGFR expression in gastrointestinal neuroendocrine tumors

Gastrointestinal nöroendokrin tümörlerde PD-L1, MMR ve EGFR ekspresyonu

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Abstract

Purpose: There are limited studies on gastrointestinal neuroendocrine tumors (NETs) in the literature. This study aimed to determine PD-L1 and EGFR expression in primary G1 and G2 NETs and neuroendocrine carcinoma located in the gastrointestinal system, explore the relationship between grades, and investigate the loss of DNA mismatch repair (MMR) protein expression and its association with PD-L1 expression.

Materials and Methods: All patients diagnosed with primary gastrointestinal NETs between January 2017 and January 2021 were included in this study. The study evaluated the protein expression of PD-L1, EGFR, MLH1, MSH2, MSH6, and PMS2 by immunohistochemistry. A total of 30 patients were included in the study.

Results: PD-L1 expression was detected in tumor cells and/or tumor microenvironment immune cells in 8 cases (28%), consisting of four G1, two G2, and two NEC cases. There was no significant relationship between histological grade and PD-L1 expression. A loss of expression of at least one MMR protein was noted in 16 cases (53%). A loss of MMR protein expression was detected in five of the eight cases with PD-L1 expression. EGFR expression was not detected in any of the cases.

Conclusion: The study revealed a loss of MMR protein expression in 53% and PD-L1 expression in 27% of gastrointestinal NETs. This study might be a pioneer for future studies on immune checkpoint inhibitors in microsatellite-unstable NETs, thereby contributing to providing a treatment alternative for this group of patients.

Keywords: Neuroendocrine tumors, PD-L1, MMR, EGFR, immunohistochemistry

Öz

Amaç: Literatürde gastrointestinal nöroendokrin tümörlerle (NET) ilgili kısıtlı çalışma bulunmaktadır. Bu çalışmada gastrointestinal sistem yerleşimli G1, G2 ve G3 nöroendokrin tümörlerde PD-L1 ve EGFR ekspresyonunu saptamak, gradeler arasındaki ilişkiyi incelemek, MMR protein kaybını ve bunun PD-L1 ile ilişkisini araştırmayı amaçladık.

Gereç ve Yöntem: Bu çalışmaya Ocak 2017 ile Ocak 2021 tarihleri arasında primer gastrointestinal NET tanısı konulan tüm hastalar dahil edildi. PD-L1, EGFR, MLH1, MSH2, MSH6 ve PMS2'yi immünohistokimya ile değerlendirdik. Çalışmaya toplam 30 hasta dahil edildi.

Bulgular: Olgular mide, pankreas, kolon, apendiks ve karaciğer yerleşimli idi. Olguların 14 (47%) G1, 7(23%) si G2, 9 (30%) u G3 nöroendokrin tümördü. Tümör ve/veya tümör mikroçevresi immün hücrelerde(TMIC) G1 dört, G2 iki, G3 iki olguda olmak üzere toplam 8 olguda (28%) PD-L1 ekspresyonu saptandı. Histolojik grade ile PD-L1 ekspresyonu arasında anlamlı ilişki saptanmadı. Olguların 16'sında (53%) en az bir MMR ekspresyonunda kayıp izlendi. PD-L1 ekspresyonu olan 8 olgunun beşinde MMR kaybı saptandı, üçünde izlenmedi. Hiçbir olguda EGFR ekspresyonu saptanmadı.

Sonuç: Gastrointestinal NET'lerin %53'ünde MMR protein kaybı ve %27'sinde PD-L1 ekspresyonu gözlemledik. Çalışmamızın, Mikrosatellit instable NET'lerde bir tedavi alternatifi olabilecek immün kontrol noktası inhibitörleri ile ilgili gelecekteki çalışmalara öncü olabileceğini düşünmekteyiz.

Anahtar kelimeler: Nöroendokrin tümör, PD-L1, MMR, EGFR, immunohistokimya

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INTRODUCTION

Neuroendocrine tumors (NETs), defined as a neoplasm consisting of cells with a neuroendocrine phenotype, are classified as Grade 1 (G1), 2 (G2), or 3 (G3, well-differentiated) based on pathological features according to the 2019 WHO classification. Poorly differentiated NETs are referred to as neuroendocrine carcinomas (NECs). There are two subtypes of poorly differentiated NECs, small cell and large cell[1,2]. Owing to their rarity, there is only limited data on these tumors in the literature and their treatment often relies on data from small-cell lung cancer studies. Gastrointestinal NETs deserve more attention since there is limited knowledge concerning the epidemiological and clinical characteristics of these tumors.

Programmed death ligand-1 (PD-L1) is an immunomodulatory glycoprotein that cannot be detected in normal human tissues but may be selectively expressed in various malignancies[3]. The binding of programmed cell death protein 1 (PD-1) and PD-L1 reduces the proliferation and survival of T-cells. This allows the blockade of the PD-1/PD-L1 pathway used in the treatment of various malignant tumors that have PD-L1 expression[4]. PD-L1 is expressed on tumor cells and/or tumor microenvironment immune cells (TMIC), i.e. macrophages and lymphocytes[5]. Co-analysis of PD-L1 expression in tumor cells and the microenvironment may help identify patients who are likely to benefit from immunotherapy. There are literature reports on patients who benefit from treatment due to PD-L1 expression in TMIC despite having only a low level of expression in their tumor cells[6]. While there is only limited and inconsistent data from small series in the literature concerning PD-L1 expression in NETs, there is no data available on tumor response to immunotherapy in such cases[6,7,8]. The high proliferative activity of NECs causes a rapid emergence of neoantigens, rendering these tumors highly immunogenic. Therefore, it seems plausible to assume that NECs may be a suitable target for successful treatment with immune checkpoint inhibitors[9].

In association with their repetitive structure, microsatellites are responsible for repairing replication errors in DNA sequences. Microsatellite instability (MSI) refers to an increased mutation load in certain tumors, typically resulting from a defective DNA mismatch repair (MMR) apparatus[2]. The loss of at least one of the main components of the MMR apparatus (MLH1, PMS2, MSH2, or MSH6), which can be easily analyzed by immunohistochemistry (IHC), indicates MSI and thereby the mutation load in the tumor[10]. The prognosis differs between MS-stable and MS-unstable tumors[11]. MSI has been observed in more than 5% of cancer types in the Cancer Genome Atlas[12].

Studies have shown that a group of EGFR-mutant lung adenocarcinomas transform into small-cell neuroendocrine carcinoma following the loss of EGFR expression. This transformation leads to a decreased expression of the EGFR protein[13]. To date, there have been no studies investigating EGFR expression in gastrointestinal NECs.

Developing new therapeutics for the treatment of NECs is of critical importance. Due to the small number of cases, there is only a limited number of studies investigating gastrointestinal NET development and aiming to identify the relevant mutations. This leads to a lack of knowledge on molecules that may predict the prognosis of these tumors, posing a challenge to identifying target molecules for treatment research.

Herein, this study aimed to determine PD-L1 and EGFR expression and loss of MMR protein expression (mismatch repair deficient (MMRd)) in G1 and G2 primary neuroendocrine tumors and carcinomas located in the gastrointestinal system, explore the relationship between grades, and investigate the association between MMRd and PD-L1. This study may reveal molecules that may change the treatment approach in neuroendocrine tumors located in the gastrointestinal tract.

MATERIALS AND METHODS

Sample

The data of 30 patients, who were diagnosed with primary NETs located in the gastrointestinal system in the Pathology Laboratory of Tekirdağ Namık Kemal University Hospital between January 2017 and January 2021, were retrospectively analyzed via digital archive records. The patients' slides were reviewed and suitable tumor tissues were determined by two experienced pathologists. All patients whose blocks and slides were available in the hospital archive were included in the study. Patients who did not have enough tumor cells in their blocks were excluded from the study. Patients' age, tumor localization,

tumor grade, and Ki67 percentage were retrieved from medical archives records. The study was approved by the Tekirdağ Namık Kemal University Non-Interventional Clinical Trials Ethics Committee (approval number: 2021.94.04.12).

Immunohistochemistry

Paraffin-embedded tissues and H&E-stained slides were retrieved from the archive of the hospital's pathology laboratory. Suitable tumor tissues were determined and sections were taken with a microtome by two pathology technicians. Immunohistochemical staining was performed on the sections taken from these blocks. After deparaffinized the sections, they were placed in a BenchMark XT device. Staining was performed using the antibodies MLH1 (Ventana, RTU), MSH2 (Ventana, RTU), PMS2 (Ventana, RTU), MSH6 (Ventana, RTU), PD-L1 (SP263, Ventana, RTU), and EGFR (Ventana, RTU). Normal myometrium was utilized as an external control. All staining procedures were conducted by two experienced pathologists and two pathology technicians in the Pathology Department at Tekirdağ Namık Kemal University Hospital. The IHC staining results were evaluated by two pathologists with an Olympus BX51 microscope.

The tumor proportion score system was used to evaluate PD-L1 staining of tumor cells. The tumor cells with membranous PD-L1 staining were scored regardless of the staining extent. The proportion of PD-L1-positive cells in the entire tumor area was recorded for each sample and staining greater than 1% was regarded as positive expression[2]. The same method was applied for TMIC. Lymphocytes were identified microscopically and counted manually. The expression of MMR proteins was assessed in tumor cells as preserved (any nuclear staining) or lost (complete absence of nuclear staining compared to internal controls that show appropriate staining)[14]. To evaluate EGFR, the membranous staining pattern was scored on a scale of 0-3 and the scores of 1, 2, and 3 were accepted as positive and 0 as negative[15].

Statistical analysis

All analyses were performed using the IBM SPSS Statistics Version 24.0 statistical software package (IBM Corp. Released 2016, IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp). Shapiro-Wilk test was used to analyze the normality of continuous variables. Categorical data were expressed as numbers (*n*) and percentages (%). Continuous variables were presented as median (minimum-maximum) or mean ± standard deviation, according to the normality. Pearson chi-square test was used to compare categorical variables between groups (G1, G2, and NEC). The α-type error probability, effect size f^2 value, and power of the study were 0.05, 0.21, and 0.12, respectively.

RESULTS

A total of 30 patients diagnosed with primary NETs located in the gastrointestinal system were included in the study. All cases stained diffuse positive with synaptophysin and/or chromogranin. Tumor locations included the stomach (9 cases), pancreas (6 cases), colon (8 cases), appendix (5 cases), and liver (2 cases). The mean age of the patients was 64.5±11.4 years (median, min-max; 64.5, 37-90 years).

Out of the 30 patients, NETs were observed as G1 in 14 (47%), G2 in seven (23%), and small-cell type NEC in nine (30%) patients. The mean rate of Ki67 staining was 1.2% in G1, 8% in G2 tumors, and 60% in NEC.

Table 1 shows PD-L1 positivity in tumor cells and/or TMIC according to tumor grade. PD-L1 expression was observed in tumor cells and/or TMIC in eight cases with NETs. There was no significant correlation between tumor grade and PD-L1 staining.

Table 1. PD-L1 expression in different grades of NETs and/or TMIC (n)

	PD-L1 Expression			
	Positive	**Negative**	**Total**	**p-value**
Histological Grade				
G1	4	10	14	
G2	1	6	7	0.67
NEC	3	6	9	
Total	8	22	30	0.67

NET, neuroendocrine tumor; **TMIC**, tumor-infiltrating immune cells

No loss of MMR proteins was detected in 14 of the patients. Loss of expression of at least one MMR protein was noted in 16 patients. There was a loss of all MMR proteins in one NEC and one G1 case. No significant difference was found in the loss of MMR expression across the tumor grades. Table 2 shows the loss of expression of MLH1, MSH2, PMS2, and MSH6 according to tumor grade of the cases.

Table 2. Loss of MMR expression according to the tumor grade of the cases (n)

	MLH1	MSH2	MSH6	PMS2	Total number of cases with any MMR loss
G1	4	2	10	3	10
G2	1	0	2	0	2
NEC	3	3	1	4	4
p values	0.677	0.196	0.01	0.11	0.083

MMR, mismatch repair; NEC, neuroendocrine carcinoma

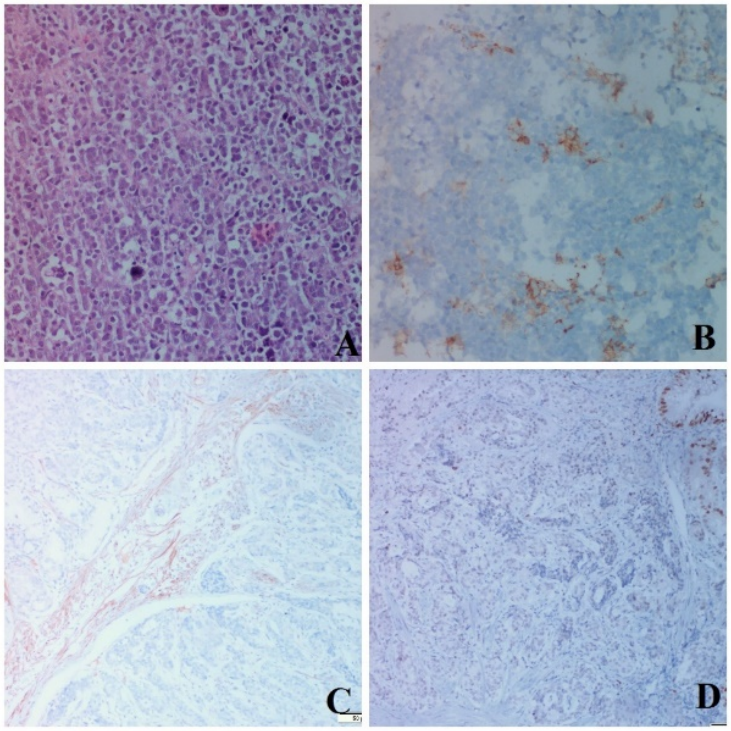

Figure 1 A). Neuroendocrine carcinoma, H&E 100x, B). PD-L1 positive NEC case in tumor cells 100x, C). PD-L1 positive case in tumor microenvironment immune cells 200x, D) MLH1 loss of expression 100x

Loss of MMR proteins was detected in five of the eight cases with PD-L1 positivity. There was no significant relationship between PD-L1 expression and loss of MMR proteins. None of the cases had EGFR expression. PD-L1 staining and loss of expression of MLH1 in NET are presented in Figure 1.

DISCUSSION

The present study investigated PD-L1 and EGFR expression and the loss of MMR protein expression using IHC in gastrointestinal NET. Several studies in the literature show PD-L1 expression is associated with histopathological grade and poor prognosis in malignancies such as hepatocellular carcinoma, renal cell carcinoma, and gastric, esophageal, and pancreatic cancer[16,17]. In light of this information, the study investigated the relationship between histopathological grade and PD-L1 and EGFR expression, the loss of MMR proteins, and the relationship between PD-L1 staining and MMR in

NET. There are no other studies in the literature analyzing PD-L1 expression as well as EGFR expression and the presence of MMRd in NET. The study detected PD-L1 staining in tumor cells and/or TMIC in eight cases (28%), and loss of expression of at least one MMR protein in 16 cases (53%). Taking into account the limited number of studies on this subject matter, the findings of this study may contribute to the relevant literature.

Yang et al. investigated PD-L1 in tumor cells and tumor-infiltrating lymphocytes (TIL) in NECs and detected PD-L1 positivity in 21 cases out of 43. In addition, as part of their study, they identified an increased copy number of PD-L1 in NEC compared to normal tissue in 6 patients in a cohort of 19 cases with gastrointestinal NECs and advocated that the PD-L1 copy number correlated with protein expression[3]. In another study, Cavalcanti et al. investigated PD-L1 expression in the first case of gastroenteropancreatic (GEP) NET in the literature and found a positive correlation between PD-L1 staining and grade, reporting an increase in PD-L1 expression in G3. They found a PD-L1 positivity rate of 28% in NETs. The authors claimed that NETs up-regulate PD-L1 and inhibit TIL, thereby providing resistance to immune surveillance[18]. Kim et al. detected PD-L1 expression in tumor tissue in 7 (21.9%) out of 32 patients with metastatic GEP-NET, demonstrating a significant relationship between PD-L1 expression and histopathological grade (p=0.008). In a study consisting of 116 cases with GEP-NET, Sampedro-Nunez et al. detected PD-L1 positivity using IHC at a rate of 6% in tumor tissues and 8% in peritumoral tissues[19].

This study detected PD-L1 positivity in tumor tissue (five cases) and/or TMIC (seven cases) in a total of eight patients out of 30 cases with NETs. To obtain more robust data concerning the role of immunomodulation in NETs, the researchers investigated PD-L1 staining in tumor cells and TMIC. There was no significant relationship between histological grade and PD-L1 expression. There are inconsistent findings in the literature owing to tumor heterogeneity, location of intratumoral expression, different clones used for IHC methods, the various cut-off values used for positive and negative expression, and the limited number of studies conducted with small samples. In the present study, the SP263 clone was used. The findings revealed PD-L1 staining in 27% of NETs, similar to the findings reported by Cavalcanti et al. All these studies strongly indicate that the PD-1/PD-L1 immune checkpoint system is expressed in a significant proportion of NETs.

Microsatellite instability can be demonstrated by the loss of expression in MLH1, PMS2, MSH2, or MSH6 proteins using IHC, which is a convenient and readily available method. Studies show that the response to anti-PD-1 therapy is variable in the MSI group of unresectable or metastatic solid tumors, and what drives this variable response remains largely unknown[20]. Moreover, there is considerably limited data on MMR in gastrointestinal NETs. In a study on small-cell NECs of the cervix, PD-L1 expression was observed in 70% of the cases, while the loss of MMR expression was detected in 33%[14]. These patients may be eligible for treatment with immune checkpoint inhibitors, which appear to be a promising option for this aggressive disease. There are limited studies that have shown MSI tumors among NECs of the stomach, small intestine, and colorectum[21,22]. In a large series that included 89 gastrointestinal NECs and mixed adenocarcinomas investigated by Sahnane et al., MSI was detected in 11 (12.8%) cases[21]. In another large-scale study conducted by Salem et al., which included 4,125 cases with gastrointestinal carcinoma, the MSI rate in gastrointestinal NECs was 4% and the authors observed PD-L1 expression in all of these cases[23]. Olevian et al. detected loss of MMR expression in 93% of poorly differentiated colorectal NECs[22]. The inconsistency of literature findings may be explained by the fact that different tumor groups are evaluated in different studies and the studies are conducted only in NECs or mixed adenocarcinoma-NEC cases. In the present study, MMR protein loss in 53% of gastrointestinal NETs was detected. A loss of the MMR protein expression was detected in five of the eight cases with PD-L1 staining. To the best of our knowledge, the present study is the first to investigate MMR and its relationship with PD-L1 expression in NETs of all grades.

Park et al. reported pathogenic EGFR mutations in five cases in a study where they utilized next-generation sequencing (NGS) in 87 NETs located in the gastrointestinal system (rectum, stomach, and appendix) to identify pathways that may indicate targets for treatment[24]. On the other hand, Chen et al. did not detect mutant EGFR in cell cultures of small-cell NECs of the lung[25]. Apart from these, no other study investigating EGFR expression in gastrointestinal NETs was found in the literature. None of the cases included in the study's cohort had

EGFR expression. Further studies investigating EGFR expression with more sensitive methods such as PCR and NGS, as is the current practice in lung cancers, are warranted in a greater number of cases with these malignancies.

This study has several limitations. Although all cases from a single center were included in the study, the number of cases was very limited. In addition, the number of NECs was very low in this limited number of cases. Another limitation was the absence of prognostic data.

In summary, although this study did not find a significant relationship between PD-L1 positivity and MMRd, the researchers observed MMRd in 53% and PD-L1 expression in 27% of gastrointestinal NETs in the study. In conclusion, this study might serve as a starting point for future studies that investigate the effect of MMR on prognosis in patients with NETs compared to those with microsatellite-stable disease and thereby contribute to providing alternative treatments for this group of patients. This study will shed light on future studies adding immune checkpoint inhibitors to the treatment of patients with PD-L1 expression.

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