MICROSATELLITE INSTABILITY IN COLORECTAL NEUROENDOCRINE NEOPLASMS

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Microsatellite instability (MSI) characterizes a special molecular genetic subtype of malignancies and is associated with the deficiency of mismatched DNA repair. There are no reliable data on the frequency of MSI in colorectal neuroendocrine neoplasms due to the relative rarity of this cancer type. The prognostic significance of MSI is debatable. The aim of this study was to investigate the frequency of the MSI phenotype among colorectal neuroendocrine neoplasms (NENs) with different primary location, grade and stage. Twenty-nine patients (15 men and 14 women, mean age: 62.5 years) included in the study underwent surgery for colorectal neuroendocrine tumors between 2015 and 2018. The mean follow-up period was 3.8 years. Colorectal NENs were grouped by primary location and stage. The majority of the patients (52%) had stage III cancer at diagnosis. The microsatellite stability (MSS) phenotype was confirmed in 24 patients (83%), whereas the MSI phenotype was observed in 5 patients (17%). All MSI-positive tumors were stage I well-differentiated grade G1 or G2 neuroendocrine tumors (NETs) of the rectum. Overall survival was 50% for patients with stage II MSS-positive NENs of the colon and rectum, 33% for stage III and 0% for stage IV. For patients with stage I MSI-positive NENs of the rectum, overall survival was 100%. Thus, the frequency of MSI-positive colorectal NENs was estimated.

Keywords: colorectal neuroendocrine neoplasms, microsatellite instability, overall survival, prognosis

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Compliance with ethical standards: the study was approved by the Ethics Committee of the National Medical Research Centre for Oncology (Protocol № 3 dated February 9, 2021); informed consent was obtained from all study participants.

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МИКРОСАТЕЛЛИТНАЯ НЕСТАБИЛЬНОСТЬ В НЕЙРОЭНДОКРИННЫХ НОВООБРАЗОВАНИЯХ ТОЛСТОЙ КИШКИ

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С нарушением работы системы репарации неспаренных оснований ДНК связано понятие о микросателлитной нестабильности (MSI), характеризующей особый молекулярно-биологический подтип злокачественных опухолей. Достоверные данные о частоте встречаемости MSI в нейроэндокринных новообразованиях толстой кишки отсутствуют, что связано с относительно небольшим числом пациентов. Сведения о прогностической значимости MSI также противоречивы. Целью исследования было изучение частоты встречаемости MSI в нейроэндокринных новообразованиях (НЭН) толстой кишки в зависимости от локализации, степени дифференцировки опухоли и стадии заболевания. Включенные в исследование 29 пациентов были прооперированы в период с 2015 по 2018 г. по поводу нейроэндокринных новообразований толстой кишки (мужчины — 15 человек, женщины — 14 человек, средний возраст постановки диагноза — 62,5 лет). Средний срок наблюдения составил 3,8 лет. НЭН толстой кишки были распределены по локализациям, а также по стадиям заболевания. У большинства пациентов, включенных в исследование, была диагностирована III стадия заболевания (52%). Статус микросателлитной стабильности (MSS) был подтвержден у 24 пациентов (83%), тогда как MSI-статус — у пяти (17%) соответственно. Все случаи MSI-позитивных новообразований соответствовали высокодифференцированным G1 и G2 нейроэндокринным опухолям прямой кишки на I стадии заболевания. Общая выживаемость пациентов с MSS-позитивными НЭН толстой кишки составила на II стадии 50%, на III — 33%, на IV стадии — 0%. Общая выживаемость пациентов с MSI-позитивными НЭН прямой кишки на I стадии составила 100%. Таким образом, была определена частота встречаемости MSI-позитивных НЭН толстой кишки.

Ключевые слова: нейроэндокринные новообразования толстой кишки, микросателлитная нестабильность, общая выживаемость, прогноз

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DNA mismatch repair is a unique biological mechanism for repairing DNA damage occurring during cell division [1]. Proteins involved in DNA mismatch repair are encoded by 6 key genes: *MSH2*, *MLH1*, *PMS2*, *MSH3*, *MSH6*, and *MLH3*. Inherited or sporadic mutations, as well as other epigenetic events like *MLH1* promoter hypermethylation, can inactivate any of these genes and disrupt the normal functioning of the entire mechanism [2]. This results in the accumulation of multiple unrepaired mutations in the genome and changes to the length of microsatellites, which are 2–9 bp-long sequences in the euchromatin portion of the genome [3]. Consequently, if the microsatellite is located within an intron region, a reading frame shift may occur in the coding sequence, followed by the inactivation of various genes. Thus, defects in the DNA mismatch repair mechanism give rise to a genomic phenotype known as microsatellite instability (MSI) [1].

MSI-positive tumors are less aggressive than those that do not have the MSI phenotype, presumably due to a high

mutation rate resulting in increased neoantigen load, which stimulates antitumor immune response. Logically, tumors with the MSI phenotype should respond better to checkpoint inhibitor (CPI) immunotherapy. This hypothesis has been confirmed for melanoma, gastric cancer and colorectal cancer (CRC). However, the prognostic significance of the MSI status in the late (IV) stages of CRC remains controversial [4].

Today, MSI is recognized as an important predictor of tumor response to immunotherapy regardless of the primary tumor site [1].

Due to the versatility of this marker, which expands indications for CPI therapy, testing for the patient's MSI status is becoming an essential diagnostic procedure approved unanimously by the leading cancer research communities, including ASCO, ESMO, NCCN, and RUSSCO [2]. The MSI status affects the choice of treatment strategy for patients with early-stage CRC, which is being reflected in contemporary clinical guidelines [5].

So far, there have been no randomized clinical trials evaluating the effectiveness of different immunotherapy regimens for colorectal neuroendocrine neoplasms (NENs). Besides, the prevalence of MSI-positive colorectal and gastrointestinal NENs remains understudied, as is the impact of the MSI status on the clinical outcome.

We hypothesize that MSI-positive colorectal NENs are a separate group of tumors with a different clinical presentation and prognosis. Colorectal NENs are relatively rare, so enrolling a large number of patients in the clinical trial may pose a problem. In the largest molecular genetic studies of colorectal NENs conducted so far, the average number of patients did not exceed 100. In earlier publications estimating the frequency of the MSI phenotype among neuroendocrine carcinomas (NECs; *n* = 53) and mixed gastrointestinal adenoneuroendocrine carcinomas (*n* = 36), 12.4% of the tumors (11/89), including NECs of the colon, stomach and the duodenum, were MSI-positive [6]. The authors of the publication identified a few clinicopathological and molecular genetic features of MSI-positive tumors. Briefly, MSI-positive carcinomas had higher methylation levels than MSI-negative tumors (40.6% vs 20.2%); the most frequently methylated genes were *MLH1*, *p16*, *PAX6*, *PAX5*, *THBS1*, *TP73*, *DAPK1*, *MGMT*, *PYCARD*, *CDH13*, *HIC1*, and *TIMP3*. The MSI status was correlated to the presence of mutations in the BRAF gene [6]. These findings show that the MSI-status of the tumor is associated with a specific set of its molecular characteristics, which, in our opinion, may hold promise for future research. The aim of our study was to evaluate the MSI status in colorectal NENs with different primary tumor location, grade and stage.

METHODS

The study included 29 patients undergoing surgical treatment for colorectal NENs at the National Medical Research Centre for Oncology (Rostov-on-Don) between 2015 and 2018. Of them, 15 were men and 14 were woman. The mean age at diagnosis was 62.5 years. The mean follow-up period was 3.8 years. The following inclusion criteria were applied: expression of neuroendocrine differentiation markers (chromogranin A, synaptophysin) confirmed by immunohistochemistry; informed consent to participate in the study. All tissue specimens were analyzed using the 2019 WHO classification criteria.

DNA was isolated from the paraffinized tissue samples obtained during surgery. Briefly, 10 slices were prepared from the paraffin-embedded tumor or seemingly healthy tissue using a microtome; then, they were deparaffinized in xylol. The samples were incubated with a lysis buffer in the presence of proteinase K at 58 °C for 6–12 h until complete tissue lysis. After that, total DNA was isolated and purified using a DNA-sorb-B kit (AmpliSens; Russia) following the manufacturer's protocol. DNA concentrations were measured with a Qubit 2.0 fluorometer (LifeTechnologies; USA).

Five monomorphic microsatellite loci (NR21, NR24, NR27, BAT25 and BAT26) were tested for their MSI status by means of fragment analysis. Each of the obtained total DNA templates were PCR-amplified in 5 reactions. Each 20 µl PCR reaction contained 10–20 ng of DNA, 0.175 µM of each primer, 2 mM of dNTP, 15 mM of $MgCl_2$ and 0.5 un. of Taq-polymerase. The following PCR protocol was applied: initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, primer annealing at 59 °C, extension at 72 °C and final extension at 72 °C for 45 s. The size of the PCR product ranged from 50 to 350 pn.

Detection of the fluorescently tagged PCR products was performed by means of fragment analysis. Briefly, 1 µl of the obtained PCR products was combined with 19µl of Hi-Di formamide and 0.5 µl of GeneScan™ 600 LIZ® Size Standard (Thermo Fisher; USA). The samples were incubated in a CH-100 heating/cooling dry block thermostat (Biosan; USA) at 95 °C for 5 min and processed in an ABI PRISM 3500 genetic analyzer (Aplied Biosystems; USA) following the manufacturer's protocol. The obtained data were analyzed in GeneMapper Software (Thermo Fisher; USA). The peak detection value was set to 50 relative fluorescence units (RFU). MSI was concluded

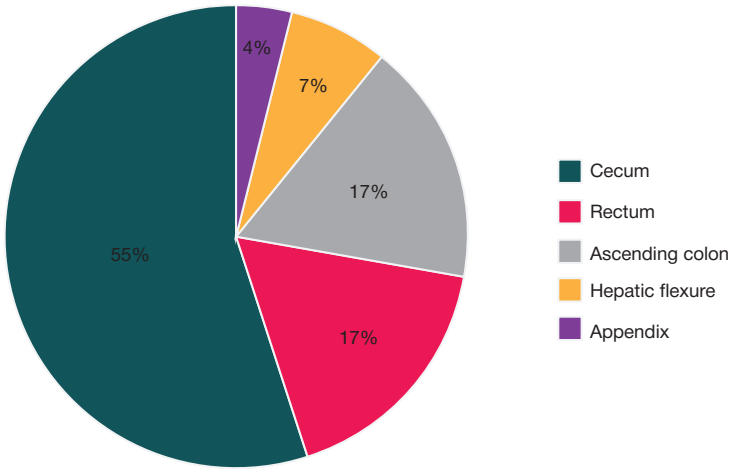

Fig. 1. Distribution of colorectal NENs by primary tumor location

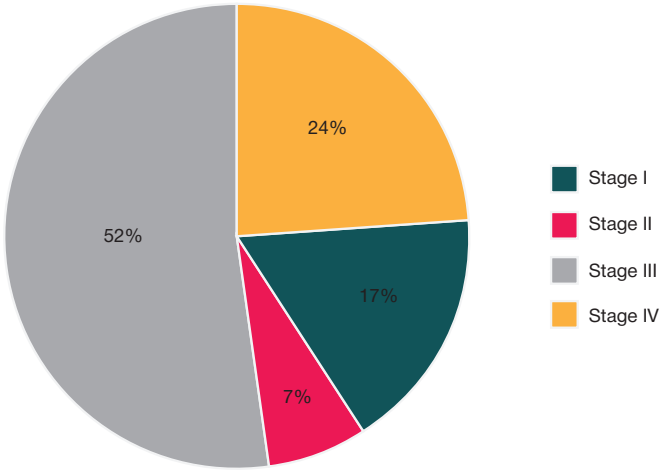

Fig. 2. Distribution of colorectal NENs by stage

if 2 or more of the studied loci were polymorphic. The level of MLH1 methylation was determined by bisulfate-converted DNA pyrosequencing using a PyroMark Q24 CpG MLH1 assay according to the manufacturer's protocol (QIAGEN; Germany).

Statistical analysis was carried out in Statsoft Statistica 10.0 (StatSoft; USA) for Windows 10. Primary data analysis was performed using descriptive statistics (central tendencies and measures of variability).

RESULTS

Colorectal NENs were grouped by primary location. Of them, 55% (n = 16) were cecal, 17% (n = 5) were NENs of the rectum, 17% (n = 5) were tumors of the ascending colon, 7% (n = 2) were localized to the right hepatic flexure, and 4% (n = 1) were appendiceal (Fig. 1).

The majority of the patients included in the study had stage III cancer (52%, n = 15) (Fig. 2).

In our cohort of patients, NENs of the colon occurred more frequently than other histological subtypes (55%, n = 16), and their frequency was directly correlated with the stage of the disease. Patient distribution by the histological subtype and stage of cancer is shown in Fig. 3.

Microsatellite stability (MSS) was confirmed in 83% of cases (n = 24); 17% (n = 5) of the tumors were MSI-positive. All MSI-positive tumors were well or moderately differentiated stage I cancers: two of them were G1 and 3 were G2 neuroendocrine neoplasms of the rectum. All of those 5 tumors had microsatellite instability in all of the 5 studied STR loci.

MLH1 was hypermethylated in all MSI-positive specimens (Me = 20%, range: 14–42%) and hypomethylated in all samples with the MSS phenotype (Me = 4%, range: 4–14%). Based on the obtained data, we concluded that the leading cause of the MSI-status in colorectal NENs was inhibition of transcription of the key mismatch repair system genes caused by hypermethylation of their promoter. In the literature, this mechanism was previously described for sporadic colorectal adenocarcinomas.

The distribution of colonic NENs with the confirmed MSS phenotype by primary location and stage is shown in Fig. 4 and 5.

For our patients with MSS-positive NENs of the colon, the three-year survival rate was 50% for stage II, 33% for stage III, and 0% for stage IV. For the patients with stage I MSI-H NENs of the rectum, the three-year survival rate was 100%.

DISCUSSION

Our findings on the prevalence of microsatellite instability in colorectal NENs were compared to the published data on colorectal cancer. In a recent study of Russian researchers conducted in a Russian cohort of patients with CRC (n = 359), the MSI phenotype was observed in 6.4% of cases (23/359) and was correlated with younger age (p = 0.023), the presence

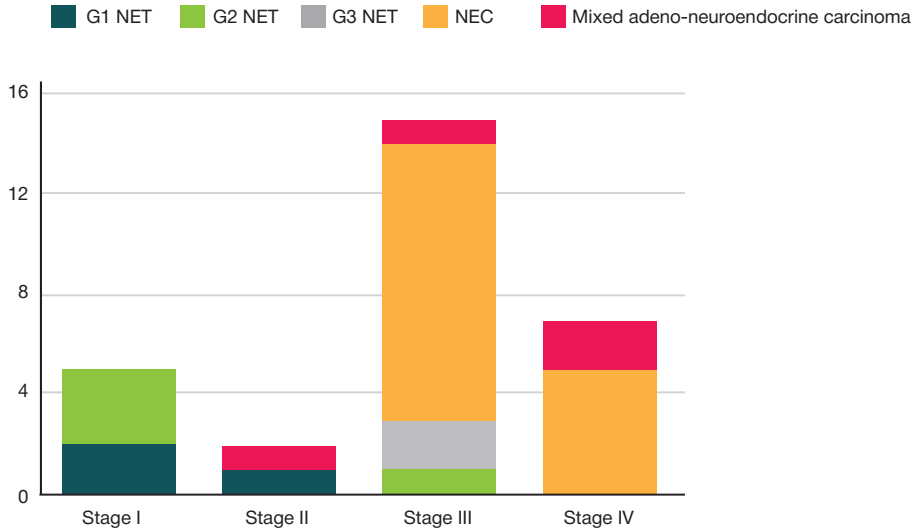

Fig. 3. Patient distribution by the histological subtype of NEN and cancer stage

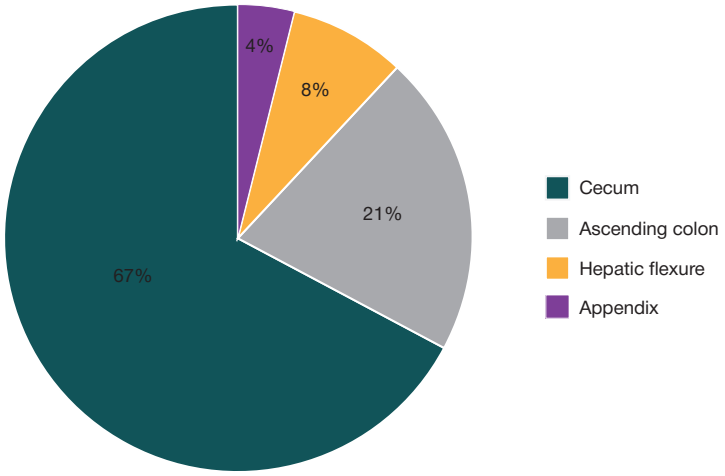

Fig. 4. Distribution of colorectal NENs with the confirmed MSS phenotype by primary location and stage

of multiple primary lesions ($p = 0.0299$), mucinous component ($p < 0.0001$), high grade ($p = 0.0025$) and right-sided location of the tumor in the colon ($p < 0.0001$) [7]. In our study, all MSI-positive NENs ($n = 5$) were low-grade and localized to the rectum.

According to the contemporary literature, there is a clear trend that the rate of MSI detection is inversely correlated with the stage of the disease [1, 3–5]. In our study, all MSI-positive NENs of the colon were stage I cancers. A larger patient sample with adequate representation of every stage of the disease is needed to check this trend for NENs of the colon.

The analysis of microsatellite instability patterns and frequency of occurrence in well-differentiated (G1/G2) neuroendocrine pancreatic tumors studied in our previous publication and NENs of the colon reveals certain differences. The MSI-positive phenotype was observed in 14% of pancreatic NENs [8] vs 17% of NENs of the colon. Besides, in pancreatic NENs, the MSI-positive phenotype was not associated with MLH1 promotor methylation. By contrast, in NENs of the colon the MSI-positive phenotype was associated only with MLH1 hypermethylation. Perhaps, this epigenetic mechanism typical for adenocarcinomas and NENs of the colon is related to their colorectal origin.

Cellular differentiation is a very important criteria in the context of gastrointestinal NENs, which is reflected in the 2019 updated WHO classification. High- and low-grade NENs are different groups of tumors completely heterogenous in terms of their genetic characteristics. Besides, NENs originating in different organs differ in their basic molecular markers. This raises the question of whether there is a link between the degree of cellular differentiation in NENs (NETs and NECs), primary tumor location and MSI pattern distribution. The data provided in the literature is controversial. For example, of 239 studied gastrointestinal and pulmonary NENs, only 4 specimens (1 G3 NETs of the pancreas and 3 NECs of the colon) were MSI-positive [9]. In another study the prevalent primary NEC location were the stomach ($n = 21$) and the pancreas ($n = 6$), and MSI was detected in none of the total 33 lesions [10]. At the same time, according to the meta-analysis of 33 retrospective studies and 8 case reports, MSI was observed in approximately 10% of gastric and colonic NECs [11]. The MSI phenotype was not confirmed in any of 56 well-differentiated NETs of the rectum ($n = 56$), small bowel ($n = 14$), colon ($n = 38$), and pancreas ($n = 16$) [9, 12–15]. According to other reports, 10–33% of pancreatic NETs are MSI-positive [9]. In our study the majority of the specimens (55%, $n = 16$) were represented by NECs of the colon, but their MSI status was negative. However, there are reports confirming the MSI phenotype in 16%, 7%, 10%, and 14% of NECs of the colon, respectively [6, 9, 17–21]. The analysis of the relevant literature suggests that the frequency of MSI could be higher among poorly differentiated NENs. Still, our findings demonstrate the opposite: in our study, MSI was confirmed for well-differentiated grade G1 and G2 NENs of the rectum. That said, only a larger patient sample will drawing reliable conclusions about the pattern of MSI distribution in NENs of the colon.

CONCLUSIONS

The analysis of frequency of microsatellite instability in colorectal NENs depends on the tumor grade, primary location and stage

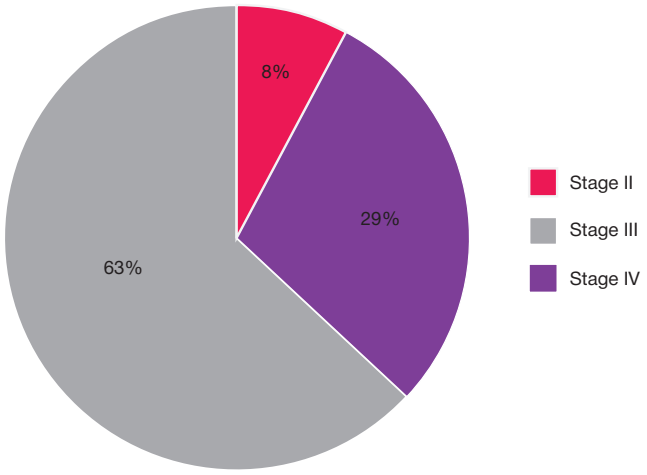

Fig. 5. Distribution of colorectal NENs with the confirmed MSS phenotype by stage

revealed that 17% the samples were MSI-positive. All of them were stage I well-differentiated G1 and G2 NENs located in the rectum. The calculated three-year survival rates demonstrate a direct correlation between the frequency of MSI occurrence in colorectal NENs and the stage of the disease. According to the currently held research, the prevalence of MSI among NENs is similar to the prevalence of MSI among adenocarcinomas of the same organ. Comparison of our findings to the frequency of MSI in CRC reveals that well-differentiated NENs of the colon may be characterized by a higher rate of the MSI-positive phenotype (17% vs 10–15%). Notably, testing patients with NENs for MSI is not part of the standard diagnostic protocol, which we believe is wrong. We are cautiously optimistic in suggesting that novel immunotherapies may be effective against this class of tumors.

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