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Microsatellite Instability in Greek Colorectal Carcinoma Patients: Clinicopathological and Molecular Correlations

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Abstract. *Background/Aim: In the present retrospective study, we assessed the molecular profile and clinicopathological correlations of Greek colorectal carcinoma (CRC) patients. Patients and Methods: Data from 157 CRC patients were collected. High Resolution Melting Analysis and Pyrosequencing/Sanger sequencing were applied to identify KRAS, BRAF, NRAS mutations and microsatellite instability (MSI) status. Immunohistochemistry was performed to characterize the associated Mismatch Repair Protein loss. Statistical calculations were performed using the statistical package SPSS v21.0. Results: KRAS mutations were detected in 39.3% of cases, BRAF in 10.9% and NRAS in 4.9%. MSI status was recognized in 11.5% of CRC patients and was associated with right colon tumors. MSI phenotype was inversely correlated with stage, N status and KRAS mutations and positively correlated with BRAF mutations. Conclusion: MSI positive CRCs in the Greek population are more often right-sided, free of metastasis, KRAS wild type and BRAF mutated. Providing more detailed clinicopathological and molecular data for specific populations will enable better clinical management and individualized therapy in the future.*

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the Western world (1, 2) and remains one of the leading causes of death worldwide. The incidence of CRC in younger population (<50 years of age) has increased, despite the fact that during the previous decade a decline by 3% per year has been observed in the general population, mainly due to the decrease of recto-sigmoid tumours (3). Reports from a variety of countries and ethnicities suggest that population and regional characteristics such as local environment and life habits may contribute to the development and course of CRC. According to the latest Eurostat report in July 2018, CRC deaths in the European Union (EU) accounted for 11.3% of all deaths from cancer and 3% of the total number of deaths, irrespective of cause. Among 28 member states, the proportion of CRC deaths varied from 4% (Croatia) to 2.3% (Greece, Finland and Bulgaria).

The pathogenetic basis of CRC is a combination of various somatic and germline mutations as well as epigenetic alterations. The activation of oncogenes such as *BRAF, KRAS, NRAS* and the inactivation of tumour suppressor genes by hypermethylation of CpG islands in the methylator phenotype (CIMP) constitute distinct pathways underpinning the development of CRC. Gene mutations have been widely studied in CRC Western populations (US and EU) as well as in some Eastern populations (China and Korea). The frequency of microsatellite instability (MSI) was approximately 15-20% in the Western populations (4-9) and 9.6-13% in the Eastern populations (10-14). *KRAS* mutations are considered the most frequent molecular alteration in colon cancer with a range of 22-46.7% in Western populations (5, 6, 15-18) and 19.7-43.9% in the Chinese population. Moreover, the frequency of *BRAF* mutations ranges from 5 to 21.8% in Western populations (15, 19-23) and from 1.7 to 25.4% in the Chinese population (24-30). NRAS mutations occur rarely in CRC with a frequency of 2.2% in the West (31) and up to 3.4% in China (32).

In the era of personalized medicine, tumour molecular characteristics determine prognosis and guide treatment of cancer patients. Testing CRC tumour tissue for MSI phenotype, *KRAS* and *BRAF* mutations has been suggested in a routine clinical setting since 2010 (33). Patients in early stage who are DNA mismatch repair deficient (dMMR) have a favorable prognosis, with longer disease-free survival

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(DFS) and overall survival (OS) (34-38). Concerning chemotherapy, MSI has been the target of different therapeutic protocols due to the poor benefit of MSI positive CRC patients from chemotherapeutics such as pyrimidine analogues (cisplatin, temozolomide and procarbazine) and fluorouracil-based adjuvant chemotherapy (34, 35, 39). Clinical trials have shown that CRC patients with *KRAS* mutations fail to respond sufficiently to anti-epidermal growth factor receptor (EGFR) agents, a major therapeutic target for CRC, exhibiting additionally serious side effects (40-46). Consequently, at present, testing of *KRAS* mutations is imperative for the proper treatment of CRC patients. Recently, the MSI status has been linked to anti-PD1 therapy (47, 48). Moreover, *BRAF* mutations are considered a poor prognostic factor because of their higher incidence in patients with metastatic disease and advanced TNM stage (19).

In the present retrospective study, we gathered data from 157 CRC Greek patients from the pathology database of our Department (First Department of Pathology, University of Athens), a referral laboratory performing diagnosis, consultation and molecular analysis. The purpose of the study was to assess the frequency of MSI phenotype, *KRAS, NRAS* and *BRAF* mutations in the Greek population and examine possible intra-racial differences based on origin, considering that geographical and ethnic differences in molecular profile should be taken into account in the prognostic and therapeutic approach of patients with CRC.

Patients and Methods

Ethics statement. The present study was approved by the University of Athens Ethics Committee (Protocol No. ES1106/212-10-02). Since this was a retrospective study, the Ethics Committee waived the need for an informed consent, and a policy of strict anonymity and confidentiality was adopted. All patient data were anonymized and de-identified in a confidential manner. All information included in the data set was used exclusively for the purpose of this study, and was not shared with other individuals or organizations. Consecutive cases with available material in the First Department of Pathology, University of Athens Medical School, Greece database were included.

Patients. Data were collected from 157 patients, 60.5% males and 39.5% females (males=95, females=62, median age=67 years; range=34-89 years), for whom archival material was available in our Department. The cohort included patients with either primary (right colon 29.3%, left colon 5.7%, rectosigmoid 37%) or metastatic CRC (28%) in liver, lung or lymph nodes. The distribution according the 8th edition of CRC staging was as follows: 8.9% stage I, 40.8% stage II, 22.3% stage III, 28% stage IV.

Genomic DNA isolation. DNA was extracted from formalin fixed paraffin embedded tissues. Ten μm thick sections were cut from tissue blocks after macrodissection under the light microscope. DNA was extracted from the selected tissue areas following a standard DNA extraction kit protocol (NucleoSpin tissue, Macherey–Nagel, Duren, Germany). The concentration of the extracted DNA was measured using a Nanodrop Microliter spectrophotometer.

Molecular analysis. In order to examine the mutational profile of the patients, as far as the MAPK signaling pathway is concerned, High Resolution Melting Analysis (HRMA) on a Light Cycler 480 (Roche Diagnostics, GmbH, Mannheim, Germany) in duplicate. Pyrosequencing/sanger sequencing were applied to confirm mutations in *KRAS*/*NRAS* (exons 2,3,4), and *BRAF* (exon 15).

HRMA: Each PCR reaction consisted of 20 ng DNA, 0.3 μM of each primer, 10 μl LightCycler 480 HRM Master Mix (Roche Diagnostics, GmbH, Mannheim, Germany), 3.5 mM $MgCl_2$ in a total volume of 20 μl. The thermal profile used in the Light Cycler was: 95˚C for 10 min, followed by 50 cycles at 95˚C for 10 sec, with annealing temperatures at 56˚C–*KRAS*, 60˚C–*BRAF*, 64˚C–*NRAS* for 15 sec, and a final extension stem at 72˚C for 7 sec. The sequences of the primers for *BRAF, KRAS* have been published previously (49). For MSI status, a panel of mononucleotide microsatellite markers (BAT25, BAT26, NR21, NR24) was analysed by HRM analysis, performed as reported previously (50, 51).

Pyrosequencing/Sequencing: Alterations in *KRAS* exons 2, 3, 4, *NRAS* exons 2, 3 and *BRAF* exon 15 observed by HRMA, were confirmed by Pyrosequencing using the Pyromark Gold Q24 Reagent kit with the Q24 Pyrosequencer (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Sanger Sequencing was used to confirm mutations in *BRAF* (except codon 600) and in exon 4 of *NRAS*. Briefly, PCR products positive by HRMA were sequenced using the BigDye terminator cycle sequencing kit (Applied Biosystems, CA, USA) in order to confirm the presence of mutations. The sequencing products were analysed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). PCR primers were also used for sequencing analysis.

Immunohistochemistry. Immunohistochemistry for Mismatch Repair (MMR) proteins was performed on 4μm formalin-fixed paraffin embedded tissue sections from 18 patients with MSI molecular phenotype in order to identify the associated protein loss. Detection of MMR proteins was performed using the Leica Polymer Refine Detection kit on a Leica Bond-III Automated Immunohistochemistry stainer (Leica Biosystems Newcastle Ltd, Newcastle, UK). The antibodies applied were MLH1 (clone ESO5, Leica), PMS2 (clone MOR4G, Leica), MSH2 (clone 25D12, Leica) and MSH6 (clone PU29, Leica), at dilutions 1:90, 1:50, Ready to use and 1:300, respectively.

Normal colonic crypt epithelium, lymphocytes and other stromal cells adjacent to cancer cells served as internal positive control. Tumour was defined as deficient when tumour cells showed complete absence of nuclear staining in contrast to non-neoplastic cells which maintained nuclear protein expression, and intact, if tumour cells showed nuclear positivity.

Statistical analysis. Statistical analysis was performed in order to correlate MSI status with mutational status and other parameters such as gender, histological grade, stage *etc*., using Pearson's Chi square and Fisher's exact test where appropriate. Age groups were divided into two categories, above and below the median age. Statistical calculations were performed using the statistical package

Table I. *Clinicopathological data of studied CRC patients*.

Characteristics	Number (n)	Percentage (%)
Gender		
Male	95	60.5%
Female	62	39.5%
Location		
Right colon	46	29.3%
Left colon	9	5.7%
Rectosigmoid	58	37%
Metastasis	44	28%
Tumour grade		
G1	3	1.9%
G2	103	65.6%
G3	51	32.5%
Tumour stage		
I	14	8.9%
II	64	40.8%
III	35	22.3%
IV	44	28%
Vascular/lymphatic invasion		
Presence	41	29.7%
Absence	97	70.3%
N/A	19	
Tumour-infiltrating lymphocytes (TILS)		
Presence	101	72.7%
Absence	38	27.3%
N/A	18	
Ulceration		
Presence	82	59%
Absence	57	41%
N/A	18	
Desmoplastic stromal reaction		
Presence	95	68.3%
Absence	44	31.7%
N/A	18	
Tumour growth on a preexisting adenomatous polyp		
Yes	21	13.5%
No	135	86.5%
N/A	1	
Necrosis		
Yes	50	36%
No	89	64%
N/A	18	

N/A: Not available.

Table II. *Alterations in KRAS, BRAF, NRAS genes and MSI status*.

Molecular analysis	Number (n)	Percent (%)
MSI status		
MSI	18	11.5%
MSS	139	88.5%
KRAS		
Wild type	88	60.7%
Mutant	57	39.3%
N/A	12	
NRAS		
Wild type	137	95.1%
Mutant	7	4.9%
N/A	13	
BRAF		
Wild type	131	89.1%
Mutant	16	10.9%
N/A	10	

N/A: Not available.

SPSS v21.0 for Windows. All results with a two-sided p-value <0.05 were considered significant. Associations of mutational status with TNM stage were limited to surgical samples.

Results

Patient characteristics. The following clinicopathological features were available for statistical analysis: patients' age, gender, tumour stage defined as early (stage I and II) and advanced (stage III and IV), pTNM classification based on the 8th edition of American Joint Committee on Cancer (AJCC), histological tumour grade, tumour location (right, left colon, rectosigmoid), vascular/lymphatic invasion, necrosis, presence of tumour-infiltrating lymphocytes (TILS), ulceration, desmoplastic stromal reaction and tumour growth on a preexisting adenomatous polyp. Clinicopathological data of all studied CRC patients are summarized in Table I.

Alterations in KRAS, BRAF, NRAS genes and MSI status. KRAS mutational analysis was performed in 145 samples of which 39.3% (57/145) were mutated (Table II, Figure 1). *KRAS* mutations affected mainly codon 12 (40.4%) and the most frequent mutations were p.Gly12Asp (21% of mutant cases) and p.Gly13Asp (17.5%). In particular, nine different mutations were identified, five of which at codon 12: p.Gly12Asp, p.Gly12Cys, p.Gly12Val, p.Gly12Ala, p.Gly12Ser, one at codon 13 (p.Gly13Asp), two at codon 61 of exon 3 (p.Glu62Gln, p.Glu61Lys) and one at codon 146 of exon 4 (p.Ala146Thr). Analysis of *BRAF* gene exon 15 (activation segment) revealed mutations in 10.9% of the cases (16 out of 147) all identified as T to A transitions at nucleotide 1799 causing substitution of Valine by Glutamine at codon 600, (p.Val600Glu) (Table II, Figure 1). NRAS mutations were found in 4.9% of the samples (7 out of 144) and were identified as p.Gly12Val in exon 2 and p.Ala59Thr, p.Gln61Leu, p.Gln61Arg in exon 3 (Table II, Figure 1).
Microsatellite instability status was detected in 11.5% (18 out of 157 cases) in our cohort (Table II). These cases were further confirmed by immunohistochemistry for MMR proteins; 17 cases displayed MLH1/PMS2 loss and 1 case showed MSH2/MSH6 loss.

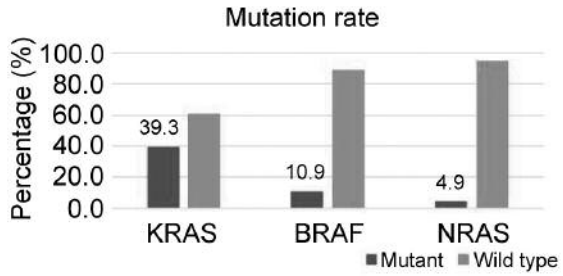

Figure 1. *KRAS, BRAF and NRAS mutational analysis.*

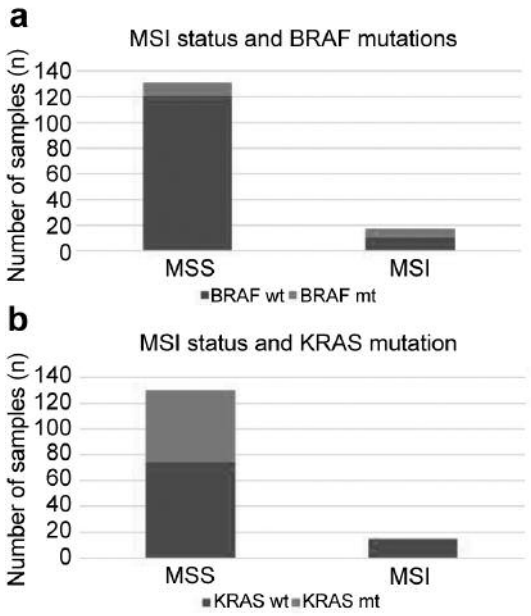

Figure 2. *Correlation of MSI with a) BRAF and b) KRAS.*

Correlations among various clinicopathological parameters. Male patients presented CRC at a younger age (<67 years) compared to female patients (p=0.021). In addition, tumours arose on a preexisting adenomatous polyp more often in females than in males (p=0.001). Desmoplastic stromal reaction was significantly more frequently observed in males compared to female patients (p=0.007) and was associated with the presence of TILS (p<0.0001).

We found a correlation between vascular/lymphatic invasion and stage of disease. Vascular or lymphatic invasion was more frequently found in advanced disease stage compared to early stage (p=0.001). Necrosis was present at higher percentage in advanced stage tumours than at early stage tumours (p=0.034). A positive correlation was also seen between necrosis and the presence of vascular /lymphatic invasion (p=0.001). Moreover, vascular/lymphatic invasion was found at a higher rate in grade 3 tumours than in grade 1&2 tumours (p=0.003). Grade was marginally positively associated with stage (p=0.060) and negatively associated with the presence of TILs (p=0.029).

Correlation of MSI status with clinicopathological parameters. MSI status was inversely correlated with stage (p=0.002). In particular, early stage tumours showed MSI at a frequency of 19.2% (15 out of 78 stage I&II) compared to advanced stage tumours (MSI 9.1%, 3 out of 79 stage III&IV). MSI status was related to tumour location; patients who displayed MSI tended to present with tumours in the right colon (p<0.0001). Furthermore, MSI status was negatively associated with N status (p=0.043).

Correlation of MSI with molecular alterations. MSI tumours were positively correlated with *BRAF* (p<0.0001), (Figure 2a) and negatively correlated with *KRAS* mutations (p<0.006) (Figure 2b).

Correlation of molecular alterations (KRAS/NRAS, BRAF mutations) with clinicopathological features. Tumours growing on a preexisting adenomatous polyp displayed *KRAS* mutations at higher frequency (p=0.012). *KRAS* and *BRAF* mutations were mutually exclusive (p=0.001). Finally, *BRAF* mutations were more frequently encountered in tumours arising in the right colon (p=0.020).

Discussion

In the present study, several clinicopathological features of colon cancer patients of Greek origin were analysed. In particular, our study addressed the characteristics of Greek CRC patients including gender, age, pathological grade and stage, location of the tumour, *KRAS, NRAS, BRAF* gene mutations, the presence of MSI, and other histological findings.

CRC patients younger than 67 years of age tended to be more often males than females. Gender differences with regard to CRC incidence diminished among older patients. Similar results have been presented by Abotchie *et al.* who investigated 373.956 patients and showed that CRC incidence was higher in men than women, particularly in patients below 60 years of age (52).

Our findings do not support gender and age differences among Greek CRC patients concerning *KRAS, BRAF* mutations and MSI phenotype. This is in agreement with reports on American and European (Netherlands) patients that did not observe statistically significant relationships between *KRAS* mutations and gender or age (53, 54). However, four different research groups studying Chinese patients found an association between *KRAS* mutations and gender, but not age (26, 28), while Gao *et al.* have observed

associations between *KRAS* mutations and gender, age as well as tumour differentiation (55). Concerning *BRAF* mutations and MSI phenotype, our results are in disagreement with previous studies that detected MSI and *BRAF* mutations more frequently in females and older CRC patients (19, 56-58).

In our study, *KRAS* mutation rate was 39.3% similar to that reported in other ethnic groups such as Swiss, Spanish, Czech, British, Chinese, Russian, and US populations (6, 15-18, 28, 53, 55, 59, 60-67). No association between metastatic CRC and *KRAS* mutations was determined in the present study. This finding is in accordance with the current treatment strategy which excludes anti-EGFR agents in Stage IV patients with *KRAS* mutated CRC (68).

In the present cohort, *KRAS* mutations did not differ between tissue samples from various metastatic sites. Additionally, *KRAS* mutant tumours in Greek CRC female patients showed a statistically significant association with a preexisting adenomatous polyp. Interestingly this association was not demonstrated in the male population. Upon review of the pathology reports, it was not clear whether these patients had a prior colonoscopy with or without polypectomy. In the female group, our results are in accordance with the findings of two studies, one from Spain and the other from the UK, where adenomatous polyps with *KRAS* mutations showed an increased risk of developing advanced neoplasia (69, 70).

KRAS and *BRAF* mutations in CRC patients were found to be mutually exclusive in this and many other studies from different countries (15, 59, 71, 72), whereas a single survey carried out in Chinese patients has reported the coexistence of *KRAS* and *BRAF* mutations in 24% of cases (57).

BRAF mutation rate in our CRC series was 10.9%, significantly different from that recorded in studies from Russia (4.1%), Japan (3%), Israel (5%) and China (7%); albeit comparable to that reported in studies from the USA and Europe (5, 6, 22, 28-30, 65, 72-74). We suggest that there are considerable ethnic variations regarding *BRAF* mutations. *BRAF* mutations have also been associated with the location of the tumour, showing increased frequency in right-sided tumours, in accordance with previous reports (19, 71). Additionally, MSI tumours were positively correlated with *BRAF* mutations. In the recent literature, *BRAF* mutations have been associated with sporadic MSI CRC, occurring rarely in Hereditary non-polyposis colorectal cancer (HNPCC) - Lynch Syndrome (75, 76).

In the present report, a strong negative correlation between MSI and lymph node metastasis emerged as the vast majority of patients who had MSI did not exhibit lymph node metastasis. The correlation between MSI and DFS, OS and lymph node involvement has been evaluated in previous studies showing that a higher number of negative lymph nodes is associated with MSI positive status (77). MSI positive early stage CRCs demonstrate a more favorable prognosis (78-82). It is hypothesized that this occurs due to an increased immune response regarding these tumours (82). MSI positive CRCs have distinctive clinicopathological characteristics compared to MSI negative ones. In our analysis, the frequency of MSI positivity rose to 11.5%. In other Western countries, this frequency ranges from 15 to 20% (5-8, 17, 34, 39). In the Chinese population, the frequencies of MSI positive CRCs, displaying at least two or more microsatellite markers, was 11.9-13% (5-9, 15, 20). The same reports have shown that MSI positive tumours were more commonly seen in Stage II rather than Stage III disease. We found a similar correlation of MSI with stage in the Greek population so that early stage (I/II) tumours were more often MSI positive than late stage (III/IV) ones (19.2% *vs*. 9%).

The current study analysed the largest series of Greek CRC patients with regard to MSI status by molecular testing which is the direct proof of MSI in the tumour sample. We also performed MMR immunohistochemistry in MSI positive patients to identify the specific gene responsible for mismatch deficiency through protein loss (83). MSI positive CRCs in our samples had a strong correlation with right colon location, while 77.8% of MSI positive CRCs had a right colon location in concordance with most previous investigations (84). In these studies, tumours with increased TILs had MSI positive status suggesting that MSI could lead to the formation of new epitopes responsible for the upregulation of the immune response. This is the explanation for the recent use of PD-1 inhibitors in MSI positive CRCs (47).

In conclusion, the present study demonstrated the frequency of MSI, KRAS, NRAS, BRAF molecular markers in the Greek CRC patients. In Greece, as well as in other countries, male patients show an earlier onset of disease in comparison to female patients. MSI positive tumours were more often right colon located, free of metastasis, KRAS wild type and *BRAF* mutated. Additional epidemiological data should be evaluated in order to establish the geographic and ethnic variability in patients with CRC. Providing more detailed clinicopathological and molecular data for specific populations may facilitate their clinical management and individualized therapy in the future.

Conflicts of Interest

The Authors of this study have no conflicts of interest to declare.

Authors' Contributions

P Korkolopoulou coordinated the present study and edited the manuscript to be submitted. AA Saetta conceived the idea, planned the molecular analysis, interpreted the molecular data and supervised the molecular findings of this work. I Thymara commented on the manuscript. I Giannopoulou carried out the immunohistochemical analyses, interpreted the immunohistochemical results, wrote the

related part and commented on the manuscript. I Chatziandreou carried out the molecular and statistical analyses of the data, performed the experiments, contributed in writing the description of the methods and designed the figures. S Sakellariou worked on the manuscript critically for important intellectual content and helped in the sequence alignment of the text as well as the verification of the results. P Katafygiotis conceived the study, reviewed the literature, collected the clinical and the experimental data and wrote the manuscript.

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