Averroes European Medical Journal
Open Access Publisher
ISSN: 0772-9200
ISSN (print): 2593-1431

March 2020 • Volume 2 • Number 2

Microsatellite instability (MSI) and braf mutation in colorectal carcinomas

Daniel Lerda[1], Edith Illescas[2], Jorge Labrador[2]

ABSTRACT: Background: approximately 15% of colorectal carcinomas (CRC) display high microsatellite instability (MSI-H).
These originate when the DNA mismatchrepair system, known as the MMR system, is dysfunctional, which results in the accumulation of mutations caused by DNA polymerase, like point mutations, deletions and insertions.
Although they are heterogeneous, MSI-H colorectal carcinomas display different biological characteristics when compared to stable or low MSI CRCs.
Purpose of the study: This study aims istoidentify molecular biomarkers that provide early prognostic information.
Methods: in this research, the presence of MSI was determined using Bethesda panel, Multiplex Ligation-dependent Probe Amplification (MLPA) to detect Mismatch-repair (MMR) and BRAF mutation in patients with colorectal cancer, and its importance was acknowledged for patients with suspected hereditary nonpolyposis colorectal cancer (HNPCC) or with suspected Lynch syndrome.
Results: MSI-H was detected in 4 of the 31 cases (12.9%).Genomic rearrangements were not detected (loss or gain of genetic material) in the genes MLH1, MSH2, MSH6, PMS2 and in the 3' region of EPCAM through MLPA in patients presenting MSI-H.The BRAF V600E mutation test was positive in all four patients with MSI-H.
Conclusions: In this research, the molecular markers IMS, MSH6, MLH1, MSH2, PMS2 and BRAF allowed us to set the behavior to be followed.
Therefore, we consider that the implementation of molecular tests is important to determine whether we are facing cases of sporadic CRC or Lynch syndrome.

Key Words: colorectal carcinoma, microsatellite instability, mismatch-repair, BRAF

Introduction

In Argentina, colorectal cancer (CRC) has increased its mortality rate by more than 100% in the past few years[1], which is, unfortunately, bringing us closer to the developed countries where this disease is one of the main three causes of cancer-related deaths[2].
A higher incidence and late diagnosis (metastatic stages) can partly explain this higher mortality rate[3,4].
Over the past few years, great progress has been made in the molecular characterization of CRC in order to try to explain its aggressiveness. However, there is little information as to the host's response to the tumor.
From the molecular viewpoint, it has been possible to characterize different development paths of CRC such as microsatellite instability (MSI), methylation (CIMP), and chromosomal instability (CIN)[5,6].

---

[1] Molecular Genetics Laboratory, University Clinic Reina Fabiola (CURF), School of Health Sciences, Catholic University of Córdoba, Argentina
[2] Molecular Biology Laboratory, Maimónides univesity, Buenos Aires, Argentina
Corresponding author
Dr Daniel Lerda
dlerda@coyspu.com.ar
Tel : +543515918595

Introduction

MSI is observed in approximately 15% of the CRC cases, and it is a reflection of the cancer cell's incapability of correcting deletions-insertions in DNA regions that are repetitive (microsatellites), due to the absence of a DNA repair system, which leads to the accumulation of mutations caused by DNA polymerase, like point mutations. Deletions and mutations occur mainly in microsatellites; they are widely spread in our genome and result in a decrease or increase of said repetitive sequences' size in cancer cells.

This change in microsatellites' length is called microsatellite instability (MSI). Thus, MSI is a genetic fingerprint of the DNA repair system dysfunction. For patients with CRC, having MSI-H (High Microsatellite Instability) would relate to better survival in 5 years compared to patients who have microsatellite stable (MSS)[7-9] tumors, and it has a predictive value of resistance to adjuvant therapy based on 5-fluorouracil[10 -11].

Even so, in both cases of sporadic and hereditary cancer the MSI presents the same biology, and therefore, the benefit of knowing the MSI status would help in the treatment of the patient with CRC. MSI has also been associated with favorable prognosis, related to the high lymphocyte infiltration that is observed in these tumors, which indicates a degree of immune response against the tumor[12]. Tumors with MSI frequently give rise to a leukemoid reaction on the part of the host, in the form of intraepithelial lymphocytosis (INTRATUMORAL) orperitumoral lymphoid reaction. Most intraepithelial or peritumoral lymphocytes are of type T. To analyze the MSI, Boland et al.[13] recommended the use of the Bethesda panel, which consists in the analysis of five microsatellite markers: three dinucleotide repeats (D2S123, D5S346 and D17S250) and two mononucleotide repeats (BAT25 y BAT26.)

Recent research has shown that certain checkpoint inhibitors, called PD-1 or PD-L1 inhibitors, can be effective against one type of MSI-H metastatic colorectal cancer.

The FDA has recently approved pembrolizumab to treat MSI-H tumors. There are ongoing clinical trials that seek to combine checkpoint inhibitors with other drugs or treatments against cancer in order to know whether they can be useful against tumors that are not MSI- H[14].

The proto-oncogene B-RAF encodes a protein belonging to the RAF family of serine/threonine protein kinases which acts in the RAS-RAF-MAPK pathway[15]. Different mutations have been described which activate B-RAF constitutively in many cancers such as melanoma, thyroid cancer and CRC.

These mutations allow for the phosphorylation and activation of proteins MEK and ERK, which leads to an increase in the transcription of various genes that promote higher cell proliferation and survival[15]. In 5% to 15% of CRC cases, mutations in the B-RAF genes have been informed, most of them in exon 15, due to a change of glutamic acid for valine in codon 600 of the protein (V600E)[16].

The objective of the present study is to identify molecular biomarkers that provide early prognostic information.

Materials and Methods

Patients 31 patients diagnosed with colorectal cancer who attended CURF's Oncology Service have been studied under clinical criteria of suspicion of genetic predisposition to colorectal cancer (CRC.) between June 2016 and May 2017 and each patient signed an informed consent form which had been approved previously by CURF's Ethics Committee. Peripheral blood samples as normal tissue and paraffin-embedded tumor samples have been used. With the available data from the patient clinical record, a database was developed with the clinical-pathological characteristics (Table 1).

DNA extraction from peripheral blood was carried out using Promega's WizardGenomic DNA Purification Kit, following the manufacturer's protocol. To the paraffin block sample, a cut was made, stained with Hematoxylin-Eosin to confirm via optical microscopy the presence of the tumor

Averroes European Medical Journal
Open Access Publisher
ISSN: 0772-9200

area, and from one to two unstained cuts to extract genomic DNA. Genomic DNA extraction and purification were carried out using a commercial extraction kit (ReliaPrep FFPE g DNAMiniprep System –Promega-), following the manufacturer's protocol. After DNA isolation, concentration was adjusted through a UV spectrophotometryat 100 - 200 ng/µl per sample (10 – 20 ng/reaction.) DNA samples were stored at –20 °C.

Microsatellite Instability Analysis

Molecular detection in tumor tissue and peripheral blood (normal tissue) of status - microsatellite instability (MSI) and/or loss of heterozygosity (LOH) with microsatellite panel of MMR genes - mismatch repair - h MSH2- h MLH1-h PMS1 (non-coding regions of the genome and repetitive sequences) via PCR and sequencing recommended by the American Joint Commission on Cancer – The International Collaborative Group on HNPCC and the HNPCC Cancer Study Group in Germany. MSI analysis in colorectal tumors was carried out using the following markers: BAT 25 4q (c KIT), BAT 26 2p 16. 3 – 21 (h MSH2), D5S346 5q 21-22 (APC), D17S250 17q 11.2-12 (Mfd 15 CA) BRCA 1, D2S123 2p 16 (h MSH2).

Each microsatellite marker was amplified through PCR with specific fluorochrome primers corresponding to the microsatellite panel of the DNA mismatch repair genes (MMR) MSH2 y MLH1. Fragment analysis of the amplified products was done using ABI Prism 310 Genetic Analyzer, comparing peripheral blood (normal tissue) with tumor tissue, verifying each microsatellite's profile.

Tumors presenting two or more unstable microsatellites (varying in the number of alleles and/or with alterations in the migration pattern) are considered MSI-High tumors, tumors presenting one unstable microsatellite are considered MSI-Low tumors, and tumors presenting all microsatellites in a normal state are considered MSI-Stable[13] tumors (i.e. tumors with two or more markers: MSI-H, and tumors with 1 marker: MSI-L, all WT markers are MSS.)

MMR via MLPA Analysis

The following commercial kits have been used: P072-C1 MSH6, P003-A2 MLH1/MSH2 and P008-C1 PMS2 from MRC-Holland. Fragments obtained were analyzed through automatic sequencer. Genomic rearrangements, duplications and deletions for genes MSH6, MLH1, MSH2 and PMS2 have been studied.

The analysis was carried out using the software Coffalyser.

BRAF Analysis

The amplification of genomic DNA of exon 15 of the BRAF gene (NCBI Ref. Seq. NG_007873.1) was carried out through polymerase chain reaction (PCR) using the pair of primers described by M. Sensiet al.[17].

To prove the sample's integrity, simultaneous detection of the constitutive gene K-RAS was carried out. PCR amplification was done in a final volume of 25 µl, using 2,5 µl of DNA, 200 µM of each dNTPs, 0,5 U of GoTaq DNA Polymerase (Promega), 5 µl bufferGoTaq 5x containing 1,5 mM of MgCl2 (Promega), 0,5 µl of MgCl2 and 0,16 µM of the forward and reverse primers. Amplification conditions were the following: an initial denaturation step at 94 °C for 5 minutes, followed by 35 denaturation steps at 94 °C for 45 seconds, annealing at 58 °C for 45 seconds and a 45- second extension at 72 °C with a final extension of 5 minutes at 72 °C.

The expected PCR product was verified through agarose gel electrophoresis at 1.8% and visualization in a UV transilluminator, isolating the band of interest of 224 bp.

For the DNA fragment purification, the Extraction Kit Wizard Sv Geland PCR Clean Up System (Promega) was used. These quencing reaction of the elution was done using Big Dye Terminator v1.1 (Applied Biosystems), in both directions separately and with the same primers employed in PCR. The product was purified on Sephadex columns (CentriSep Spin Columns – PrincentonSeparations-), and the sequencing was carried out in an ABI Prism 310 Genetic Analyser (Applied Biosystems).

Averroes European Medical Journal
Open Access Publisher
ISSN: 0772-9200

March 2020 • Volume 2 • Number 2

Results

A total of 31 patients, 19 men, with a median age of 62 years and 12 women with a median age of 58 years were analyzed. In men 8/31 (42.1%) had the tumor located on the right side and 11/31 (57.9%) on the left side. Women 7/31 (58.3%) had it on the right side and 5/31 (41.7%) on the left side. (Table 1).

MSI-H was detected in 4 of the 31 cases (12.9%), one was younger than 50 and three were older than 50. D17S250 were unstable in 11/31 (35,5%), D5S346 were unstable in 3/31 (9,7%), D2S123 were unstable in 2/31 (6,4%), BAT 25 and BAT 26 presented instability in 1/31 (3.2%) (Table 2).

Genomic rearrangements were not detected (loss or gain of genetic material) in the genes MLH1, MSH2, MSH6, PMS2 and in the 3' region of EPCAM through MLPA in patients presenting MSI-H(Table 3).

The BRAF V600E mutation test was positive in all four patients with MSI-H (Table 4).

Discussion

In this research, we have detected four patients with MSI-H and V600E mutation of the BRAF gene. When MLPA was carried out to the MSH6, MLH1, MSH2 and PMS2 genes in these patients, to mark the difference between CRC and Lynch syndrome, results were negative and, therefore, these patients were diagnosed with sporadic CRC.

The IMS-high in sporadic CCR tumors, in this work, showed a high response of intratumoral infiltrate, and this should be due to the presence of CD8 + 18- cytotoxic T lymphocytes[18-21].

In addition, the mutation in the BRAF oncogene (V600E) in these patients causes constitutive activation of the EGFR pathway and consequently an inefficient response to treatment with cetuximab or panitumumab.

It should be noted that these patients have a intermediate prognosis[22] and a very poor survival rate after relapse[19]. On the other hand, one of our job limitations was the short follow-up period and the size of our study group.

Conclusions

In this research, the molecular markers IMS, MSH6, MLH1, MSH2, PMS2 and BRAF allowed us to set the behavior to be followed. Thus, we consider that the implementation of molecular tests they provide us with early prognostic information.

Conflict of interests

The authors declare that they have no competing interests.

Acknowledgements

To Lic in Genetic Celeste Goy for critical reading of the work

Averroes European Medical Journal
Open Access Publisher
ISSN: 0772-9200

Table 1. Clinical-pathological characteristics of patients with CRC analyzed

n (%)		Man	Women	Total (n)
		19 (61,3 %)	**12 (38,7 %)**	**31 (100 %)**
Age(medium-min.max)		62 (34-71)	57,8 (26-77)	60,4 (26-77)
Location	**Right**	8(42,1)	7(58,3)	15(48,4)
	Left	11(57,9)	5(41,7)	16(51,6)
Vascular invasion	**Positive**	9(47,4)	5(41,7)	14(45,2)
	Negative	10(52,6)	7(58,3)	17(54,8)
Lymphatic invasion	**Positive**	7(36,8)	4(33,3)	11(35,5)
	Negative	11(57,9)	8(66,7)	19(61,3)
	NI	1(5,3)	0	1(3,2)
Wall invasion	**PT 1-T2**	4(21,05)	3(25)	7(22,6)
	PT3-T4	15(78,9)	9(75)	24(77,4)
Stadium	**I-II**	9(47,4)	5(41,7)	14(45,2)
	III-IV	10(52,6)	7(58,3)	17(54,8)
Differentiation	**moderate**	13(68,4)	9(75)	22(71)
	poor	6(31,6)	3(25)	9(29)
Mucin	**Positive**	5(26,3)	2(16,7)	7(22,6)
	Negative	14(73,7)	10(83,3)	24(77,4)

Min.Max: mínimum – maximum; **NI:** uninformed

Averroes European Medical Journal
Open Access Publisher
ISSN: 0772-9200

Table 2. Microsatellite Instability

Bethesda panel microsatellite markers

Patients	Age/Sex	BAT 25	BAT 26	D5S346	D17S250	D2S123	Result
1	33 - F	S	S	S	S	S	MSS
2	66 - M	S	S	S	I	S	MSI-L
3	62 - M	S	S	S	S	S	MSS
4	56 - M	S	S	S	S	S	MSS
5	26 - F	S	S	S	LOH	LOH	CIN
6	68 - M	S	S	S	I	S	MSI-L
7	67 - M	S	S	S	I	S	MSI-L
8	61 - F	S	S	S	S	S	MSS
9	61 - M	S	S	S	S	S	MSS
10	48 - M	S	S	S	I	S	MSI-L
11	71- M	S	S	S	I	S	MSI-L
12	55 - F	S	S	I	S	I	MSI-H
13	70 - F	S	S	S	S	S	MSS
14	66 - F	S	S	S	S	S	MSS
15	64 -M	S	S	S	S	S	MSS
16	71 - M	S	S	S	I	S	MSI-L
17	57 -M	S	S	I	S	I	MSI-H

18	58 -F	S	S	S	S	S	MSS
19	68 -F	S	S	S	S	S	MSS
20	34 -M	I	I	S	S	S	MSI-H
21	77 -F	S	S	S	S	S	MSS
22	58 -F	S	S	S	S	S	MSS
23	62 -M	S	S	I	I	S	MSI-H
24	65 -M	S	S	S	S	S	MSS
25	56 -M	S	S	S	S	S	MSS
26	70 - M	S	S	S	S	S	MSS
27	59 - M	S	S	S	I	S	MSI-L
28	71 - M	S	S	S	S	S	MSS
29	70 -M	S	S	S	I	S	MSI-L
30	54 -F	S	S	S	I	S	MSI-L
31	68 -F	S	S	S	I	S	MSI-L

CIN: Chromosomal Instability, LOH: Loss of Heterozygosity, S: Stable, I: Unstable, MSS: Microsatellite Stable, MSI-L: low, MSI-H: high.

Table 3. Genomic Rearrangements through MLPA

Patients	Age/Sex	MLH1	MSH2	MSH6	PMS2	3' EPCAM
12	55 -F	Negative	Negative	Negative	Negative	Negative
17	57 -M	Negative	Negative	Negative	Negative	Negative
20	34 -M	Negative	Negative	Negative	Negative	Negative
23	62 -M	Negative	Negative	Negative	Negative	Negative

Table 4. BRAF V600E Mutation

Patients	Age/Sex	V600E B-RAF
12	55-F	Mutated
17	57 -M	Mutated
20	34 -M	Mutated
23	62 -M	Mutated

References

1. National Institute of Cancer, Argentina. Cáncercolorrectal, diagnóstico de situación de la Argentina. 2018.
2. Siegel RL, Miller KD,Fedewa SA, Ahnen DJ, Meester RGS, Barzi A, Jemal A. Colorectal cáncer statistics 2017. CA Cancer J Clin.2017; 67(3)177:193.
3. López F, Rahmer A, Pimentel F, Guzmán S, Llanos O, Ibáñez L.Colorectal cancer. Study of survival and surgical results.RevMed Chile 1993; 121:1142-8.
4. Bannura G, Cumsille MA, Barrera A, Contreras J, Melo C, Soto D. Resultados del tratamientoquirúrgico del cáncer de colon. Análisis de 439 pacientes.Rev Chil Cir. 2010; 62:491-6.
5. Kim JH, Kang GH. Molecular and prognostic heterogeneity of microsatellite-unstable colorectal cancer. World J Gastroenterol. 2014; 20:4230-43.
6. Coppedè F, Lopomo A, Spisni R, Migliore L. Genetic and epigenetic biomarkers for diagnosis, prognosis and treatment of colorectal cancer. World J Gastroenterol. 2014; 20:943-56.
7. Boland CR, Goel A. Microsatellite instability in colorectal cancer.Gastroenterology 2010; 138:73-87.
8. Benatti P, Gafà R, Barana D, Marino M, Scarselli A, PedroniM. Microsatellite instability and colorectal cancer prognosis. Clin Cancer Res. 2005; 11:8332-40.
9. Ward R, Meagher A, Tomlinson I, O'Connor T, Norrie M, Wu R. Microsatellite instability and the clinicopathologicalfeatures of sporadic colorectal cancer. Gut 2001; 48:821-9.
10. Saeterdal I, Bjørheim J, Lislerud K, Gjertsen MK, Bukholm IK, Olsen OC. Frameshift-mutation-derived peptides as tumor-specificantigens in inherited and spontaneous colorectal cancer. Proc Natl AcadSci U S A. 2001; 98(23):13255-60.
11. Ribic CM, Sargent DJ, Moore MJ, Thibodeau SN, French AJ, Goldberg RM. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. N Engl J Med. 2003; 349(3):247-57.
12.Kloor M, Staffa L, Ahadova A, von KnebelDoeberitz M. Clinical significance of microsatellite instability in colorectal cancer. Langenbecks Arch Surg. 2014; 399(1):23-31.
13. Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW.
A National Center Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res. 1998; 58:5248-57.
14. Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, Lu S, Kemberling H, Wilt C, Luber BS, Wong F, Azad NS, Rucki AA, Laheru D, Donehower R, Zaheer A, Fisher GA, Crocenzi TS, Lee JJ, Greten TF, Duffy AG, Ciombor KK, Eyring AD, Lam BH, Joe A, Kang SP, Holdhoff M, Danilova L, Cope L, Meyer C, Zhou S, Goldberg RM, Armstrong DK, Bever KM, Facer AN, Taube J, Housseau F, Spetzler D, Xiao N, Pardoll DM, Papadopoulos N, Kinzler KW, Eshleman JR, Vogelstein B, Andres RA, Diaz La Jr. Mismatch-repair deficiency predicts response of solid tumors to PD-1 blockade. Science. 2017 10.1126/science.aan6733.
15. Sharma SG, Gulley ML. B-RAF mutation testing in colorectal cancer. Arch Pathol Lab Med. 2010 Aug; 134 (8):1225-8.
16. Rozek LS, Herron CM, Greenson JK, Moreno V, Capella G, Rennert G. Smoking, gender, and ethnicity predict somatic B-RAF mutations in colorectal cancer. Cancer Epidemiol Biomarkers Prev. 2010 Mar;19(3):838-43.

References

17. M Sensi , G Nicolini , C Petti , I Bersani , F Lozupone , A Molla , C Vegetti , D Nonaka , R Mortarini , G Parmiani , S Fais and A Anichini. Mutually exclusive NRASQ61R and BRAFV600E mutations at the single-cell level in the same human melanoma. Oncogene 2006 25, 3357–3364
18. Zárate AJ, Álvarez K, Villarroel C, Wielandt AM, Kronberg U, Cavada G, et al. Caracterización del infiltradolinfocitario (cd3, cd4, cd8, cd45ro y foxp3) e inestabilidad microsatelitalenpacientes con cáncercolorrectal. Rev Chil Cir 2015; 67 (1): 43-50. 38.
19. Dunne PD, O'Reilly PG, Coleman HG, Gray RT, Longley DB, Johnston PG, et al. Stratified analysis reveals chemokine-like factor (CKLF) as a potential prognostic marker in the MSI-immune consensus molecular subtype CMS1 of colorectal cancer. Oncotarget 2016; 7 (24): 36632-44.
20. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency N Engl J Med 2015; 372: 2509-20.
21. Becht E, de Reyniès A, Giraldo NA, Pilati C, Buttard B, Lacroix L, et al. Immune and Stromal Classification of Colorectal Cancer Is Associated with Molecular Subtypes and Relevant for Precision Immunotherapy. Clin Cancer Res 2016; 22 (16): 4057-66.
22. Punt C, Koopman M, Vermeulen L. From tumour heterogeneity to advances in precision treatment of colorectal cancer. Nat Rev ClinOncol 2017;14 (4): 235-46

Averroes European Medical Journal
Open Access Publisher
ISSN: 0772-9200
ISSN (print): 2593-1431

March 2020 • Volume 2 • Number 2

Microsatellite instability (MSI) and braf mutation in colorectal carcinomas

Daniel Lerda[1], Edith Illescas[2], Jorge Labrador[2]

ABSTRACT: Background: approximately 15% of colorectal carcinomas (CRC) display high microsatellite instability (MSI-H).
These originate when the DNA mismatchrepair system, known as the MMR system, is dysfunctional, which results in the accumulation of mutations caused by DNA polymerase, like point mutations, deletions and insertions.
Although they are heterogeneous, MSI-H colorectal carcinomas display different biological characteristics when compared to stable or low MSI CRCs.
Purpose of the study: This study aims istoidentify molecular biomarkers that provide early prognostic information.
Methods: in this research, the presence of MSI was determined using Bethesda panel, Multiplex Ligation-dependent Probe Amplification (MLPA) to detect Mismatch-repair (MMR) and BRAF mutation in patients with colorectal cancer, and its importance was acknowledged for patients with suspected hereditary nonpolyposis colorectal cancer (HNPCC) or with suspected Lynch syndrome.
Results: MSI-H was detected in 4 of the 31 cases (12.9%).Genomic rearrangements were not detected (loss or gain of genetic material) in the genes MLH1, MSH2, MSH6, PMS2 and in the 3' region of EPCAM through MLPA in patients presenting MSI-H.The BRAF V600E mutation test was positive in all four patients with MSI-H.
Conclusions: In this research, the molecular markers IMS, MSH6, MLH1, MSH2, PMS2 and BRAF allowed us to set the behavior to be followed.
Therefore, we consider that the implementation of molecular tests is important to determine whether we are facing cases of sporadic CRC or Lynch syndrome.

Key Words: colorectal carcinoma, microsatellite instability, mismatch-repair, BRAF

Introduction

In Argentina, colorectal cancer (CRC) has increased its mortality rate by more than 100% in the past few years[1], which is, unfortunately, bringing us closer to the developed countries where this disease is one of the main three causes of cancer-related deaths[2].
A higher incidence and late diagnosis (metastatic stages) can partly explain this higher mortality rate[3,4].
Over the past few years, great progress has been made in the molecular characterization of CRC in order to try to explain its aggressiveness. However, there is little information as to the host's response to the tumor.
From the molecular viewpoint, it has been possible to characterize different development paths of CRC such as microsatellite instability (MSI), methylation (CIMP), and chromosomal instability (CIN)[5,6].

---

[1] Molecular Genetics Laboratory, University Clinic Reina Fabiola (CURF), School of Health Sciences, Catholic University of Córdoba, Argentina
[2] Molecular Biology Laboratory, Maimónides univesity, Buenos Aires, Argentina
Corresponding author
Dr Daniel Lerda
dlerda@coyspu.com.ar
Tel : +543515918595

Introduction

MSI is observed in approximately 15% of the CRC cases, and it is a reflection of the cancer cell's incapability of correcting deletions-insertions in DNA regions that are repetitive (microsatellites), due to the absence of a DNA repair system, which leads to the accumulation of mutations caused by DNA polymerase, like point mutations. Deletions and mutations occur mainly in microsatellites; they are widely spread in our genome and result in a decrease or increase of said repetitive sequences' size in cancer cells.

This change in microsatellites' length is called microsatellite instability (MSI). Thus, MSI is a genetic fingerprint of the DNA repair system dysfunction. For patients with CRC, having MSI-H (High Microsatellite Instability) would relate to better survival in 5 years compared to patients who have microsatellite stable (MSS)[7-9] tumors, and it has a predictive value of resistance to adjuvant therapy based on 5-fluorouracil[10 -11].

Even so, in both cases of sporadic and hereditary cancer the MSI presents the same biology, and therefore, the benefit of knowing the MSI status would help in the treatment of the patient with CRC. MSI has also been associated with favorable prognosis, related to the high lymphocyte infiltration that is observed in these tumors, which indicates a degree of immune response against the tumor[12]. Tumors with MSI frequently give rise to a leukemoid reaction on the part of the host, in the form of intraepithelial lymphocytosis (INTRATUMORAL) orperitumoral lymphoid reaction. Most intraepithelial or peritumoral lymphocytes are of type T. To analyze the MSI, Boland et al.[13] recommended the use of the Bethesda panel, which consists in the analysis of five microsatellite markers: three dinucleotide repeats (D2S123, D5S346 and D17S250) and two mononucleotide repeats (BAT25 y BAT26.)

Recent research has shown that certain checkpoint inhibitors, called PD-1 or PD-L1 inhibitors, can be effective against one type of MSI-H metastatic colorectal cancer.

The FDA has recently approved pembrolizumab to treat MSI-H tumors. There are ongoing clinical trials that seek to combine checkpoint inhibitors with other drugs or treatments against cancer in order to know whether they can be useful against tumors that are not MSI- H[14].

The proto-oncogene B-RAF encodes a protein belonging to the RAF family of serine/threonine protein kinases which acts in the RAS-RAF-MAPK pathway[15]. Different mutations have been described which activate B-RAF constitutively in many cancers such as melanoma, thyroid cancer and CRC.

These mutations allow for the phosphorylation and activation of proteins MEK and ERK, which leads to an increase in the transcription of various genes that promote higher cell proliferation and survival[15]. In 5% to 15% of CRC cases, mutations in the B-RAF genes have been informed, most of them in exon 15, due to a change of glutamic acid for valine in codon 600 of the protein (V600E)[16].

The objective of the present study is to identify molecular biomarkers that provide early prognostic information.

Materials and Methods

Patients 31 patients diagnosed with colorectal cancer who attended CURF's Oncology Service have been studied under clinical criteria of suspicion of genetic predisposition to colorectal cancer (CRC.) between June 2016 and May 2017 and each patient signed an informed consent form which had been approved previously by CURF's Ethics Committee. Peripheral blood samples as normal tissue and paraffin-embedded tumor samples have been used. With the available data from the patient clinical record, a database was developed with the clinical-pathological characteristics (Table 1).

DNA extraction from peripheral blood was carried out using Promega's WizardGenomic DNA Purification Kit, following the manufacturer's protocol. To the paraffin block sample, a cut was made, stained with Hematoxylin-Eosin to confirm via optical microscopy the presence of the tumor

Averroes European Medical Journal
Open Access Publisher
ISSN: 0772-9200

area, and from one to two unstained cuts to extract genomic DNA. Genomic DNA extraction and purification were carried out using a commercial extraction kit (ReliaPrep FFPE g DNAMiniprep System –Promega-), following the manufacturer's protocol. After DNA isolation, concentration was adjusted through a UV spectrophotometryat 100 - 200 ng/µl per sample (10 – 20 ng/reaction.) DNA samples were stored at –20 °C.

Microsatellite Instability Analysis

Molecular detection in tumor tissue and peripheral blood (normal tissue) of status - microsatellite instability (MSI) and/or loss of heterozygosity (LOH) with microsatellite panel of MMR genes - mismatch repair - h MSH2- h MLH1-h PMS1 (non-coding regions of the genome and repetitive sequences) via PCR and sequencing recommended by the American Joint Commission on Cancer – The International Collaborative Group on HNPCC and the HNPCC Cancer Study Group in Germany. MSI analysis in colorectal tumors was carried out using the following markers: BAT 25 4q (c KIT), BAT 26 2p 16. 3 – 21 (h MSH2), D5S346 5q 21-22 (APC), D17S250 17q 11.2-12 (Mfd 15 CA) BRCA 1, D2S123 2p 16 (h MSH2).

Each microsatellite marker was amplified through PCR with specific fluorochrome primers corresponding to the microsatellite panel of the DNA mismatch repair genes (MMR) MSH2 y MLH1. Fragment analysis of the amplified products was done using ABI Prism 310 Genetic Analyzer, comparing peripheral blood (normal tissue) with tumor tissue, verifying each microsatellite's profile.

Tumors presenting two or more unstable microsatellites (varying in the number of alleles and/or with alterations in the migration pattern) are considered MSI-High tumors, tumors presenting one unstable microsatellite are considered MSI-Low tumors, and tumors presenting all microsatellites in a normal state are considered MSI-Stable[13] tumors (i.e. tumors with two or more markers: MSI-H, and tumors with 1 marker: MSI-L, all WT markers are MSS.)

MMR via MLPA Analysis

The following commercial kits have been used: P072-C1 MSH6, P003-A2 MLH1/MSH2 and P008-C1 PMS2 from MRC-Holland. Fragments obtained were analyzed through automatic sequencer. Genomic rearrangements, duplications and deletions for genes MSH6, MLH1, MSH2 and PMS2 have been studied.

The analysis was carried out using the software Coffalyser.

BRAF Analysis

The amplification of genomic DNA of exon 15 of the BRAF gene (NCBI Ref. Seq. NG_007873.1) was carried out through polymerase chain reaction (PCR) using the pair of primers described by M. Sensiet al.[17].

To prove the sample's integrity, simultaneous detection of the constitutive gene K-RAS was carried out. PCR amplification was done in a final volume of 25 µl, using 2,5 µl of DNA, 200 µM of each dNTPs, 0,5 U of GoTaq DNA Polymerase (Promega), 5 µl bufferGoTaq 5x containing 1,5 mM of MgCl2 (Promega), 0,5 µl of MgCl2 and 0,16 µM of the forward and reverse primers. Amplification conditions were the following: an initial denaturation step at 94 °C for 5 minutes, followed by 35 denaturation steps at 94 °C for 45 seconds, annealing at 58 °C for 45 seconds and a 45- second extension at 72 °C with a final extension of 5 minutes at 72 °C.

The expected PCR product was verified through agarose gel electrophoresis at 1.8% and visualization in a UV transilluminator, isolating the band of interest of 224 bp.

For the DNA fragment purification, the Extraction Kit Wizard Sv Geland PCR Clean Up System (Promega) was used. These quencing reaction of the elution was done using Big Dye Terminator v1.1 (Applied Biosystems), in both directions separately and with the same primers employed in PCR. The product was purified on Sephadex columns (CentriSep Spin Columns – PrincentonSeparations-), and the sequencing was carried out in an ABI Prism 310 Genetic Analyser (Applied Biosystems).

Averroes European Medical Journal
Open Access Publisher
ISSN: 0772-9200

March 2020 • Volume 2 • Number 2

Results

A total of 31 patients, 19 men, with a median age of 62 years and 12 women with a median age of 58 years were analyzed. In men 8/31 (42.1%) had the tumor located on the right side and 11/31 (57.9%) on the left side. Women 7/31 (58.3%) had it on the right side and 5/31 (41.7%) on the left side. (Table 1).

MSI-H was detected in 4 of the 31 cases (12.9%), one was younger than 50 and three were older than 50. D17S250 were unstable in 11/31 (35,5%), D5S346 were unstable in 3/31 (9,7%), D2S123 were unstable in 2/31 (6,4%), BAT 25 and BAT 26 presented instability in 1/31 (3.2%) (Table 2).

Genomic rearrangements were not detected (loss or gain of genetic material) in the genes MLH1, MSH2, MSH6, PMS2 and in the 3' region of EPCAM through MLPA in patients presenting MSI-H(Table 3).

The BRAF V600E mutation test was positive in all four patients with MSI-H (Table 4).

Discussion

In this research, we have detected four patients with MSI-H and V600E mutation of the BRAF gene. When MLPA was carried out to the MSH6, MLH1, MSH2 and PMS2 genes in these patients, to mark the difference between CRC and Lynch syndrome, results were negative and, therefore, these patients were diagnosed with sporadic CRC.

The IMS-high in sporadic CCR tumors, in this work, showed a high response of intratumoral infiltrate, and this should be due to the presence of CD8 + 18- cytotoxic T lymphocytes[18-21].

In addition, the mutation in the BRAF oncogene (V600E) in these patients causes constitutive activation of the EGFR pathway and consequently an inefficient response to treatment with cetuximab or panitumumab.

It should be noted that these patients have a intermediate prognosis[22] and a very poor survival rate after relapse[19]. On the other hand, one of our job limitations was the short follow-up period and the size of our study group.

Conclusions

In this research, the molecular markers IMS, MSH6, MLH1, MSH2, PMS2 and BRAF allowed us to set the behavior to be followed. Thus, we consider that the implementation of molecular tests they provide us with early prognostic information.

Conflict of interests

The authors declare that they have no competing interests.

Acknowledgements

To Lic in Genetic Celeste Goy for critical reading of the work

Averroes European Medical Journal
Open Access Publisher
ISSN: 0772-9200

Table 1. Clinical-pathological characteristics of patients with CRC analyzed

n (%)		Man	Women	Total (n)
		19 (61,3 %)	**12 (38,7 %)**	**31 (100 %)**
Age(medium-min.max)		62 (34-71)	57,8 (26-77)	60,4 (26-77)
Location	**Right**	8(42,1)	7(58,3)	15(48,4)
	Left	11(57,9)	5(41,7)	16(51,6)
Vascular invasion	**Positive**	9(47,4)	5(41,7)	14(45,2)
	Negative	10(52,6)	7(58,3)	17(54,8)
Lymphatic invasion	**Positive**	7(36,8)	4(33,3)	11(35,5)
	Negative	11(57,9)	8(66,7)	19(61,3)
	NI	1(5,3)	0	1(3,2)
Wall invasion	**PT 1-T2**	4(21,05)	3(25)	7(22,6)
	PT3-T4	15(78,9)	9(75)	24(77,4)
Stadium	**I-II**	9(47,4)	5(41,7)	14(45,2)
	III-IV	10(52,6)	7(58,3)	17(54,8)
Differentiation	**moderate**	13(68,4)	9(75)	22(71)
	poor	6(31,6)	3(25)	9(29)
Mucin	**Positive**	5(26,3)	2(16,7)	7(22,6)
	Negative	14(73,7)	10(83,3)	24(77,4)

Min.Max: mínimum – maximum; **NI:** uninformed

Averroes European Medical Journal
Open Access Publisher
ISSN: 0772-9200

Table 2. Microsatellite Instability

Bethesda panel microsatellite markers

Patients	Age/Sex	BAT 25	BAT 26	D5S346	D17S250	D2S123	Result
1	33 - F	S	S	S	S	S	MSS
2	66 - M	S	S	S	I	S	MSI-L
3	62 - M	S	S	S	S	S	MSS
4	56 - M	S	S	S	S	S	MSS
5	26 - F	S	S	S	LOH	LOH	CIN
6	68 - M	S	S	S	I	S	MSI-L
7	67 - M	S	S	S	I	S	MSI-L
8	61 - F	S	S	S	S	S	MSS
9	61 - M	S	S	S	S	S	MSS
10	48 - M	S	S	S	I	S	MSI-L
11	71- M	S	S	S	I	S	MSI-L
12	55 - F	S	S	I	S	I	MSI-H
13	70 - F	S	S	S	S	S	MSS
14	66 - F	S	S	S	S	S	MSS
15	64 -M	S	S	S	S	S	MSS
16	71 - M	S	S	S	I	S	MSI-L
17	57 -M	S	S	I	S	I	MSI-H

18	58 -F	S	S	S	S	S	MSS
19	68 -F	S	S	S	S	S	MSS
20	34 -M	I	I	S	S	S	MSI-H
21	77 -F	S	S	S	S	S	MSS
22	58 -F	S	S	S	S	S	MSS
23	62 -M	S	S	I	I	S	MSI-H
24	65 -M	S	S	S	S	S	MSS
25	56 -M	S	S	S	S	S	MSS
26	70 - M	S	S	S	S	S	MSS
27	59 - M	S	S	S	I	S	MSI-L
28	71 - M	S	S	S	S	S	MSS
29	70 -M	S	S	S	I	S	MSI-L
30	54 -F	S	S	S	I	S	MSI-L
31	68 -F	S	S	S	I	S	MSI-L

CIN: Chromosomal Instability, LOH: Loss of Heterozygosity, S: Stable, I: Unstable, MSS: Microsatellite Stable, MSI-L: low, MSI-H: high.

Table 3. Genomic Rearrangements through MLPA

Patients	Age/Sex	MLH1	MSH2	MSH6	PMS2	3' EPCAM
12	55 -F	Negative	Negative	Negative	Negative	Negative
17	57 -M	Negative	Negative	Negative	Negative	Negative
20	34 -M	Negative	Negative	Negative	Negative	Negative
23	62 -M	Negative	Negative	Negative	Negative	Negative

Table 4. BRAF V600E Mutation

Patients	Age/Sex	V600E B-RAF
12	55-F	Mutated
17	57 -M	Mutated
20	34 -M	Mutated
23	62 -M	Mutated

References

1. National Institute of Cancer, Argentina. Cáncercolorrectal, diagnóstico de situación de la Argentina. 2018.
2. Siegel RL, Miller KD,Fedewa SA, Ahnen DJ, Meester RGS, Barzi A, Jemal A. Colorectal cáncer statistics 2017. CA Cancer J Clin.2017; 67(3)177:193.
3. López F, Rahmer A, Pimentel F, Guzmán S, Llanos O, Ibáñez L.Colorectal cancer. Study of survival and surgical results.RevMed Chile 1993; 121:1142-8.
4. Bannura G, Cumsille MA, Barrera A, Contreras J, Melo C, Soto D. Resultados del tratamientoquirúrgico del cáncer de colon. Análisis de 439 pacientes.Rev Chil Cir. 2010; 62:491-6.
5. Kim JH, Kang GH. Molecular and prognostic heterogeneity of microsatellite-unstable colorectal cancer. World J Gastroenterol. 2014; 20:4230-43.
6. Coppedè F, Lopomo A, Spisni R, Migliore L. Genetic and epigenetic biomarkers for diagnosis, prognosis and treatment of colorectal cancer. World J Gastroenterol. 2014; 20:943-56.
7. Boland CR, Goel A. Microsatellite instability in colorectal cancer.Gastroenterology 2010; 138:73-87.
8. Benatti P, Gafà R, Barana D, Marino M, Scarselli A, PedroniM. Microsatellite instability and colorectal cancer prognosis. Clin Cancer Res. 2005; 11:8332-40.
9. Ward R, Meagher A, Tomlinson I, O'Connor T, Norrie M, Wu R. Microsatellite instability and the clinicopathologicalfeatures of sporadic colorectal cancer. Gut 2001; 48:821-9.
10. Saeterdal I, Bjørheim J, Lislerud K, Gjertsen MK, Bukholm IK, Olsen OC. Frameshift-mutation-derived peptides as tumor-specificantigens in inherited and spontaneous colorectal cancer. Proc Natl AcadSci U S A. 2001; 98(23):13255-60.
11. Ribic CM, Sargent DJ, Moore MJ, Thibodeau SN, French AJ, Goldberg RM. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. N Engl J Med. 2003; 349(3):247-57.
12.Kloor M, Staffa L, Ahadova A, von KnebelDoeberitz M. Clinical significance of microsatellite instability in colorectal cancer. Langenbecks Arch Surg. 2014; 399(1):23-31.
13. Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW.
A National Center Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res. 1998; 58:5248-57.
14. Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, Lu S, Kemberling H, Wilt C, Luber BS, Wong F, Azad NS, Rucki AA, Laheru D, Donehower R, Zaheer A, Fisher GA, Crocenzi TS, Lee JJ, Greten TF, Duffy AG, Ciombor KK, Eyring AD, Lam BH, Joe A, Kang SP, Holdhoff M, Danilova L, Cope L, Meyer C, Zhou S, Goldberg RM, Armstrong DK, Bever KM, Facer AN, Taube J, Housseau F, Spetzler D, Xiao N, Pardoll DM, Papadopoulos N, Kinzler KW, Eshleman JR, Vogelstein B, Andres RA, Diaz La Jr. Mismatch-repair deficiency predicts response of solid tumors to PD-1 blockade. Science. 2017 10.1126/science.aan6733.
15. Sharma SG, Gulley ML. B-RAF mutation testing in colorectal cancer. Arch Pathol Lab Med. 2010 Aug; 134 (8):1225-8.
16. Rozek LS, Herron CM, Greenson JK, Moreno V, Capella G, Rennert G. Smoking, gender, and ethnicity predict somatic B-RAF mutations in colorectal cancer. Cancer Epidemiol Biomarkers Prev. 2010 Mar;19(3):838-43.

References

17. M Sensi , G Nicolini , C Petti , I Bersani , F Lozupone , A Molla , C Vegetti , D Nonaka , R Mortarini , G Parmiani , S Fais and A Anichini. Mutually exclusive NRASQ61R and BRAFV600E mutations at the single-cell level in the same human melanoma. Oncogene 2006 25, 3357–3364
18. Zárate AJ, Álvarez K, Villarroel C, Wielandt AM, Kronberg U, Cavada G, et al. Caracterización del infiltradolinfocitario (cd3, cd4, cd8, cd45ro y foxp3) e inestabilidad microsatelitalenpacientes con cáncercolorrectal. Rev Chil Cir 2015; 67 (1): 43-50. 38.
19. Dunne PD, O'Reilly PG, Coleman HG, Gray RT, Longley DB, Johnston PG, et al. Stratified analysis reveals chemokine-like factor (CKLF) as a potential prognostic marker in the MSI-immune consensus molecular subtype CMS1 of colorectal cancer. Oncotarget 2016; 7 (24): 36632-44.
20. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency N Engl J Med 2015; 372: 2509-20.
21. Becht E, de Reyniès A, Giraldo NA, Pilati C, Buttard B, Lacroix L, et al. Immune and Stromal Classification of Colorectal Cancer Is Associated with Molecular Subtypes and Relevant for Precision Immunotherapy. Clin Cancer Res 2016; 22 (16): 4057-66.
22. Punt C, Koopman M, Vermeulen L. From tumour heterogeneity to advances in precision treatment of colorectal cancer. Nat Rev ClinOncol 2017;14 (4): 235-46