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Mismatch DNA Repair *hMSH2, hMLH1, hMSH6 and hPMS2* mRNA Expression Profiles in Colorectal Carcinomas

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Abstract

Background: Mismatch repair (MMR) deficiency has been related with HNPCCs. So far, there is limited information on MMR mRNA profiles in sporadic colorectal carcinomas (CRCs). We previously showed that distinct MMR mRNA phenotypes were related to tumor stage and survival of patients with lung cancer or urinary bladder carcinomas.

Aim: The aim of this study was to quantify *hMSH2, hMLH1, hMSH6* and *hPMS2* mRNA levels, in CRCs and their adjacent normal tissues (ANTs), using accurate methodology, and to correlate MMR mRNA profiles with patient or tumor characteristics.

Materials and methods: We analyzed 31 fresh frozen tissue specimens of paired CRCs with their ANTs. We evaluated MMR mRNA profiles by a Q-real-time PCR, using *hPBGD* gene as reference control and creating a standard curve. The MMR mRNA levels were assigned as ratios MMR/*hPBGD* mRNAs. Relative expression of each MMR gene was given as ratios of CRCs/ANTs mRNA levels.

Results: All CRCs and their ANTs expressed low *hPMS2* mRNA levels while a significant proportion of CRCs (73%) and their ANTs (82%) presented low *hMSH2* mRNA levels. Analysis of relative expression patterns showed that *hMSH6* and *hMLH1* exhibited the highest percentages of reduction (53% and 45.5%, respectively). We found a correlation of transcriptional levels between *hMSH2* and *hMLH1*, the crucial components of MMR mechanism and between their counterparts, *hMSH6* and *hPMS2*, in CRCs of early stages, related to gender. On the contrary, CRCs of late stages revealed a correlation between reduced levels of *hMSH2* and *hMSH6*, MutSa components, unrelated to gender but related to lymph node metastasis. Also, reduced *hMSH2, hMSH6* and *hMLH1* mRNA phenotypes correlated with advanced stage, and rectal localization.

Conclusion: In this study we demonstrated that MMR mRNA deficiency is a common event in sporadic CRCs. Specific profiles of MMR deficiency maybe related to tumor progression, especially in male patients.

Keywords: Colorectal carcinomas; *hMSH2; hMLH1; hMSH6; hPMS2*; mRNA; Real time PCR; MMR phenotypes

Introduction

Mismatch DNA repair (MMR) mechanism protect cells from replication errors and is important to genome stability [1-3]. Hereditary cancers, like Lynch syndrome, are linked with a deficiency of MMR mechanism [4-13]. Sporadic cancers have also been related with defective MMR mechanisms [14-24]. We have recently demonstrated that distinct MMR mRNA profiles are related to tumor staging and survival of patients with non-small cell lung carcinomas (NSCLCs) [16,17]. We have also showed dependence of *hMSH2* and *hMSH6* mRNA expression in urinary bladder carcinomas (UCCs) and revealed a correlation of *hMSH6* reduction in UCCs (18). So far, there is a limited information on transcriptional levels of MMR genes in sporadic colorectal carcinomas (CRCs) [25,26].

In this study we quantified, with a precise Q-real time PCR method, the transcriptional levels of *hMSH2, hMLH1, hMSH6* and *hPMS2*, MMR genes in CRCs and their paired adjacent normal tissues (ANTs) and we checked for correlations of MMR mRNA CRCs profiles with tumor or patients' characteristics.

Materials and Methods

Tissue collection

Fresh frozen colorectal tissue specimens consisting of paired tumor and their adjacent normal tissues (ANTs), were collected from 31 unselected patients, 17 male and 14 female with an age range 52-92 years (median 74 years), who underwent surgery at the University Hospital of Larissa, Thessaly, Greece. The specimens were immersed immediately after surgery in RNA stabilizer solution (RNAlater®, Life Technologies) and they were preserved in -80°C, deep freezer, till RNA isolation. The localization of the tumor was rectum (11), right colon (10 cases), sigmoid (6) and left colon (1) while in three cases the exact localization was not available (Table 1). The histological review showed that all colorectal tumors of our collection were adenocarcinomas consisting of 1 well differentiated (WD), 24 moderately-differentiated (MD), 3 moderately to poorly differentiated (MD-PD) and 3 poorly differentiated (PD) tumors. Lymph node metastasis, nerve and vascular invasion was observed in 11/31 (35.5%), 18/31 (58%) and 13/31 (42%) out of total 31 of CRC tumors, respectively (Table 1).

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Case no./Age (years)/ sex	Histopathologic characteristics of tumors						*hMSH2*/ control mRNA[a]		*hMLH1*/ control mRNA[a]		*hMSH6*/ control mRNA[a]		*hPMS2*/ control mRNA[a]		Tumor/ANT MMR mRNA expression[b]			
	Grade	Stage	LN	NF	VF	TL	Tumor	ANT	Tumor	ANT	Tumor	ANT	Tumor	ANT	*hMSH2*	*hMLH1*	*hMSH6*	*hPMS2*
1/F/66	WD	I	0/16	no	no	right	1.7	1.73	3.52	4.55	4.81	1.88	0.0478	0.00934	0.982	0.773	2.56	5.12
2/M/80	MD	I	0/1	no	no	rectum	0.0000127	0.0235	0.000139	0.001596	2.27	2.59	0.00339	0.004	0.000539	0.0871	0.878	0.847
3/M/85	MD	I	0/5	Y	no	rectum	3.51	0.911	7.7	1.9	95.1	319	0.722	0.042	3.85	4.05	0.298	17.2
4/F/72	MD	I	0/15	no	no	sigmoid	n.i.	0.00033	4.44	0.0255	2.31	3.7	0.0487	0.0718	0.000	174	0.626	0.678
5/M/64	MD	I	0/23	no	no	rectum	0.0276	n.a.	0.97	36.6	2.67	7.23	0.0015	0.743	na	0.0265	0.369	0.00202
6/M/60	MD	IIA	0/13	Y	no	rectum	2.06	0.828	572	177	0.145	1.54	0.0237	0.0263	2.49	3.22	0.0941	0.901
7/M/79	MD	IIA	0/1	Y	no	rectum	0.00345	0.00146	0.000013	0.0000302	1.08	854	0.00232	0.0000459	2.37	0.43	12.7	50.5
8/M/75	MD	IIA	0/16	no	no	sigmoid	0.0787	n.a.	1.42	1.9	0.198	0.785	0.000518	0.000138	n.i.	0.747	0.252	3.75
9/M/75	MD	IIA	0/22	no	no	right	5.8	0.0000721	33.5	3.6	7.19	1.87	0.043	$0.169*10^{-8}$	80500	9.31	3.83	$254*10^{5}$
10/M/66	MD	IIA	0/21	no	no	right	2.15	1.66	4.12	3.93	184	80.1	0.199	0.116	1.29	1.05	2.3	1.72
11/M/78	MD	IIA	0/0	no	no	sigmoid	44.9	n.a.	762	0.00297	0.0316	n.a.	0.0136	0.000	n.i.	256000	n.i.	n.i.
12/M/74	MD	IIA	0/17	no	no	n.a.	0.091	0.0325	0.000053	0.0000133	0.796	1.91	0.000639	0.000788	2.8	3.98	0.417	0.811
13/F/73	MD	IIA	0/19	no	Y	right	0.0901	0.00286	9.58	2.48	1.01	0.616	0.00428	0.000284	315	3.86	1.64	15.1
14/F/64	MD	IIA	0/4	Y	no	rectum	0.373	0.131	0.00018	0.000111	1.55	1.24	0.00126	0.00138	2.86	1.62	1.25	0.909
15/F/74	MD	IIA	0/31	Y	Y	right	0.0294	0.0000112	7.61	4.71	1.05	0.201	0.00186	0.000171	2620	1.62	5.2	10.9
16/F/75	MD	IIA	0/26	Y	Y	right	0.00843	0.0113	15.3	17	2.89	1.31	0.000334	0.00091	0.748	0.9	2.21	0.367
17/F/83	MD	IIA	0/8	Y	no	rectum	0.939	1.28	1.91	4.31	29	3.37	0.0696	0.0155	0.733	0.442	8.62	4.49
18/M/77	M/PD	IIA	0/4	Y	Y	rectum	$0.155*10^{-7}$	0.0000196	0.0000908	0.000859	0.646	2.76	0.000325	0.0457	0.000788	0.106	0.234	0.00711
19/M/82	PD	IIA	0/38	Y	Y	left	0.0715	0.035	2.52	0.0000194	2.54	13	0.000126	0.000103	2.04	130000	0.195	1.22
20/F/67	MD	IIB	0/1	no	no	rectum	6	0.146	63.7	2.52	0.0367	0.000308	0.00711	0.0000504	41.1	25.3	119	141
21/M/73	MD	IIIB	2/14	Y	Y	right	0.00000788	0.0000187	0.0000242	0.0014	0.284	22	0.000355	0.0408	0.422	0.0173	130	0.0087
22/M/82	MD	IIIB	2/32	Y	Y	right	0.0906	0.216	1.61	2.64	1.96	39.2	0.0482	0.00000169	0.419	0.61	0.0501	286000
23/F/73	MD	IIIB	3/22	Y	Y	right	0.882	0.0577	0.0000293	0.00000928	0.49	1.37	0.000868	0.00172	15.3	3.16	0.357	0.505
24/F/55	MD	IIIB	2/9	Y	no	sigmoid	0.000000285	0.00561	0.16	2.84	0.246	1.06	0.00112	0.00151	0.0000507	0.0563	0.231	0.741
25/F/76	MD	IIIB	3/13	y	y	sigmoid	0.0543	0.00616	0.952	3.04	0.000114	0.00256	0.000121	0.0000114	8.83	0.313	0.044	0.0000114
26/M/67	PD	IIIB	1/14	no	no	sigmoid	2.24	1.95	5.17	3.63	200	31.2	0.0118	0.0943	1.15	1.42	6.41	0.126
27/F/52	MD	IIIC	27/45	y	y	rectum	0.00510	0.00824	0.000000988	0.00000273	1.05	1.84	0.000259	0.000448	0.619	0.362	0.573	0.578
28/F/63	MD	IIIC	10/22	no	no	right	0.834	1.96	879	1560	19.4	49.8	0.0882	0.0649	0.424	0.564	0.39	1.36
29/M/92	M/PD	IIIC	7/13	y	y	rectum	0.446	0.504	0.937	3.45	0.0762	1.27	0.0301	0.0195	0.885	1.41	0.0598	1.54
30/M/67	PD	IIIC	7/8	y	y	n.a.	0.393	0.563	0.963	1.33	5.84	55.1	0.0252	0.0888	0.697	0.725	0.106	0.284
31/F/80	M/PD	IIIC	1/38	y	y	n.a.	0.00028	0.000711	4.05	2.42	3.29	0.487	0.000341	0.000301	0.393	1.67	6.74	1.13

ANT, adjacent normal tissue; WD, well differentiated tumors; MD, moderately differentiated tumors; M/PD, moderately-poorly differentiated tumors; PD, poorly differentiated tumors; LN, lymph node metastasis; NF, nerve infiltration; VF, vessel infiltration; TL, tumor localization; n.a. not availiable; [a]Ratios of mRNA expression; [b]Ratios of tumor to ANT mRNA expression.

Table 1: Quantitative mRNA expression of MMR DNA repair genes in colorectal adenocaricnomas and their adjacent normal tissues.

Quantitative analysis of MMR mRNA expression

We used RNAeasy kit (Qiagen®, USA) for total RNA isolation and Super Script First-Strand Synthesis System (Invitrogen®, Life Technologies, UK) for cDNA synthesis, according the manufacturer's instructions. The qPCR analysis of *hMSH2, hMSH6, hPMS2* and *hPBGD*-control mRNA was performed using specific primers previously published (16,18) and probes for *hMSH2*: 5'-6FAM-CATATAAGGCTTCTCCTGGC-BHQ1-3', for *hMSH6:* 5'-6FAM-CAGGAGCTTTTATCAATGGCTA-BHQ1-3', for *hPMS2:* 5'-6FAM-ACTGCTCTTAACACAAGCGAGATGAAGAA-BHQ1-3' and for *hPBGD*: 5'-6FAM-CCTCGTGCGGTTCCCTCTGCCTGA-BHQ1-3', designed for Rotor Gene 6.1 instrument (CORBETT Research, Australia) using Platinum® Quantitative PCR Super Mix-UDQ (Invitrogen®, Life Technologies, UK) and annealing temperatures at 58°C for *hMSH2* and *hPBGD*, at 54°C for *hMSH6* and *hPMS2*. The qPCR analysis of *hMLH1* was performed using QuantiFast Probe Assay SP kit (Qiagen®, USA) by applying in Rotor Gene 6.1 instrument, according the instructions. The quantitation of mRNAs was achieved creating a standard curve of serial dilutions of *hPBGD* copies, as previously described [16,27]. The mRNA expression of each MMR gene was expressed as a ratio of MMR mRNA to control *hPBGD* mRNAs (MMR/control mRNAs) and defined two major phenotypic groups, the reduced (*r* or *p*) for mRNA ratios <1 and the normal or elevated (*R* or *P*) for ratios ≥1, as previously described [16,18]. Additionally, the MMR gene expression of tumor samples was compared with that of the corresponding ANT samples. This value is indicated as relative mRNA expression of MMR genes between CRC tumors and ANTs (CRCs/ANT) of each patient (Table 1).

Statistical analysis

We used the paired *Student's t-test* to compare ratios of *hMSH2, hMLH1, hMSH6* and *hPMS2* alterations between tumor and matched ANTs for different patient characteristics, including age, gender and clinical or histopathological parameters such as tumor location, grade, stage and lymph node metastasis, vascular invasion or perineurial invasion. The correlation between the mRNA expression ratios of *hMSH2, hMLH1, hMSH6* and *hPMS2* in CRCs and their ANTs for different patient and tumor characteristics was examined by *Pearson test*. The χ^2 *test* was also used to examine the distribution of MMR mRNA phenotypes in tumor and ANT specimens at different tumor histological grades or stages or lymph node or nerve or vessel filtration or tumor location (Tables 2-4).

Results

hMSH2 & hMLH1 & hMSH6 & hPMS2 mRNA quantification in CRCs and their ANTs

We evaluated *hMSH2*, *hMLH1*, *hMSH6* and *hPMS2* mRNA levels in primary CRCs and their corresponding ANTs relative to the reference *hPBGD* control gene by Q-RT-PCR. These data are summarized in Table 1 along with clinical and histopathological data.

Characteristics		Relative copies of *hMSH2* mRNA		Relative copies of *hMLH1* mRNA		Relative copies of *hMSH6* mRNA		Relative copies of *hPMS2* mRNA		Tumor/ANT MMR mRNA gene expression			
	n	Tumor	ANT	Tumor	ANT	Tumor	ANT	Tumor	ANT	*hMSH2*	*hMLH1*	*hMSH6*	*hPMS2*
All patients	31	0.0908[a,f,ib]	0.03375[m]	1.91	0.0908	1.55	1.895	0.00339	0.0908	1.15[u]	1.05	0.5995	0.5995
Gender													
Male	17	0.091[b,g]	0.1255	1.42	1.9	1.96	17.5	0.0118	0.03015	0.791[v]	1.05	0.2145	0.5475
Female	14	0.0722[h]	0.00977	3.785	2.68	1.3[p]	1.275	0.00156	0.001145	1.921[w]	1.26	1.25[ia]	1.0195
Tumor Stage													
I	5 (1WD+4MD)	0.0276[c,]	0.0276	3.52	1.9	2.67[q]	3.7	0.0478	0.042	0.9739	0.773	0.626	0.847
II	15 (13MD+1M/ PD+1PD)	0.232[d,i]	0.0325	4.12	2.48	1.05[r]	1.31	0.00232	0.000788	2.8[x]	1.62	1.64	1.22
III	11(7MD+2M/ PD+2PD)	0.091[j]	0.0577	0.952[n]	2.64	1.05[s]	1.84	0.00112	0.00172	0.619	0.61	0.357	0.578
Lymph node metastasis													
No	19	0.091[e,k,ic]	0.03375	4.12[o]	2.48	2.27[t]	1.275	0.00428	0.000536	2.83[y]	1.62	1.925	2.855
Yes	12	0.07245[l]	0.03297	0.9445	2.53	0.848	2.3	0.000994	0.01061	0.5215[z]	0.587	0.2955	0.5415

Mean age of the patients was 74years; ANT, Adjacent normal tissue; MMR, mismatch repair; WD, well differentiated tumors; MD, moderately differentiated tumors; M/PD, moderately-poorly differentiated tumors; PD, poorly differentiated tumors. [a]*r*=0.574765736, [b]*r*=0.796820522, [c]*r*=0.99204665, [d]*r*=0.794450379, [e]*r*=0.794045972, by *Pearson test*; correlation between *hMSH2/hPBGD* & *hMLH1/hPBGD* mRNA ratios; [f]*r*=-0.008816335, [g]*r*= -0.054661116, [h]*r*=-0.008369458, [i]*r*=-0.066294139, [j]*r*=0.893917164, [k]*r*=-0.054872336, [l]*r*=0.892161159, by *Pearson test*; correlation between *hMSH2/hPBGD* & *hMSH6/hPBGD* mRNA ratios; [m]*r*=0.618906296, by *Pearson test*; correlation between *hMSH2/hPBGD* & *hPMS2/hPBGD* mRNA ratios; [n]*r*=0.819228481, [o]*r*= 0.81809858, *Pearson test*; correlation between *hMLH1/hPBGD* & *hPMS2/hPBGD* mRNA ratios; [p]*r*=0.82653886 [q]*r*=0.99792412, [r]*r*=0.96520875, [s]*r*= -0.0030656, [t]*r*= 0.63185688 by *Pearson test*; correlation between *hMSH6/hPBGD* & *hPMS2/hPBGD* mRNA ratios; [u]*r*=0.999389, [v]*r*=0.999937, [w]*r*=-0.00904, [x]*r*= 0.999477, [y]*r*= 0.999475, *by Pearson test*; correlation between *hMSH2* & *hPMS2* mRNA ratios *of Tumor/ANT;* [z]*r*=0.644378, by *Pearson test*; correlation between *hMSH2* & *hMSH6* mRNA ratios *of Tumor/ANT;* [ia]*r*= 0.992066865, by *Pearson test*; correlation between *hMSH6* & *hPMS2* mRNA ratios *of Tumor/ANT;* [ib]*P*= 0.037994, [ic]*P*=0.03214, by *Student's test*, correlation between *hMSH2/hPBGD* ratios of Tumor/ANT

Table 2: Alterations in *hMSH2, hMLH1, hMSH6* and *hPMS2* mRNA levels between paired colorectal adenocarcinomas tumor and adjacent normal tissue samples relative to their clinical and histopathological parameters.

MMR mRNA phenotype	CRCs (n) / observed phenotypic frequencies	Gender		Tumor Stage (n)			Invasion in						Tumor site					ANT (n) / observed phenotypic frequencies
							lymph node		nerve		vessel							
		M	F	I	II	III	N	Y	N	Y	N	Y	rt	rc	si	lf	n.a…	
hMSH2																		
r_2	23 / 0.7667	12	11	2	11	10	12	11	7	16	10	13	8	8	3	1	3	22 / 0.7857
R_2	7 / 0.2333	5	2	2	4	1	6	1	5	2	7	0	2	3	2	0	0	6 / 0.2143
hMLH1																		
r_1	12 / 0.3871	7	5	1	4	7	4	8	2	10	5	7	2	6	2	0	2	11 / 0.3548
R_1	19 / 0.6129	10	9	4	11	4	15	4	11	8	13	6	8	5	4	1	1	20 / 0.6452
hMSH6																		
r_6	12 / 0.3871	7	5	0	9	6	5	7	4	8	6	6	3	4	3	0	2	6 / 0.2000
R_6	19 / 0.6129	10	9	5	6	5	14	5	9	10	12	7	7	7	3	1	1	24 / 0.8000
hPMS2																		
p_2	31 / 1.0000	16	14	5	15	11	19	12	13	18	18	13	10	11	6	1	3	30 / 1.0000
P_2	0 / 0.0000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0 / 0.0000

n, number of cases, r_2, r_1, r_6, p_2, MMR/control mRNA ratios <1; R_2, R_1, R_6, P_2, MMR mRNA ratios ≥1; M, male; F, female; N, no; Y, yes;, rt, right; rc, rectum; si, sigma; lf, left; n.a., not available.

Table 3: Distribution of individual *hMSH2*, *hMLH1, hMSH6* & *hPMS2* mRNA phenotypes in CRCs and their adjacent normal tissue (ANT).

All CRCs and their ANTs (100%) revealed low mRNA levels (MMR/control mRNAs<1) of *hPMS2* while a significant proportion of CRCs (73.3%) and their ANTs (82%) presented low mRNA levels of *hMSH2*. Also, a smaller proportion of CRCs (42% and 35.5%, respectively) and their ANTs (36% and 20%, respectively) exhibited low mRNA levels of *hMLH1* and *hMSH6*.

Statistical analysis by *Pearson test,* showed a correlation between mRNA expression ratios of *hMSH2* and *hMLH1,* in CRCs (*r*=0.574765736) and ANTs (*r*=0.618906296), that was more intense in males (*r*=0.796820522), in stage I (*r*=0.99204665) or in stage II (*r*=0.794450379) tumors as well as in tumors without lymph node metastasis (*r*=0.794045972) (Table 2). Also, a significant correlation was observed between mRNA levels of *hMSH6* and *hPMS2* in tumors of female patients (*r*=0.82653886), in stage I (*r*=0.99792412) or stage II (*r*=0.96520875) tumors and in tumors without lymph node metastasis (*r*=0.63185688), (Table 2). Additionally, a correlation was observed between mRNA ratios of *hMLH1 and hPMS2* and between *hMSH2* and *hMSH6*, in stage III tumors (*r*=0.819228481 and *r*=0.893917164, respectively) and in tumors metastatic to lymph nodes (*r*=0.81809858 and *r*=0.892161159, respectively).

hMSH2 & hMLH1 & hMSH6 & hPMS2 mRNA relative expression

Calculation of CRCs/ANTs MMR mRNA ratios from quantification

	r_2r_1	r_2R_1	R_2r_1	R_2R_1	r_2r_6	r_2R_6	R_2r_6	R_2R_6	r_6r_1	r_6R_1	R_6r_1	R_6R_1
CRCs	12	11	7	0	10	13	2	5	7	5	5	14
male	5	6	0	2	4	7	1	1	3	2	2	6
female	7	4	0	5	6	4	1	4	4	3	3	6
Stage (type)												
I	1	1	0	2	0	2	0	2	0	0	1	4
II	4	7	0	4	4	9	1	2	4	9	1	2
III	7	3	0	1	5	3	0	1	5	1	2	3
Lymph node metastasis												
Y	8	3	0	1	6	3	0	1	6	1	2	2
N	4	8	0	5	3	9	2	3	1	4	3	11
Vessel infiltration												
Y	10	6	0	2	6	9	1	1	6	2	4	6
N	2	5	0	5	3	4	1	4	1	3	0	8
Nerve infiltration												
Y	7	6	0	0	6	7	0	0	5	1	2	5
N	5	5	0	7	4	6	2	5	2	4	3	9
Tumor Localization												
Rectum	6	2	0	1	2	6	2	1	2	2	4	3
Sigmoid	2	1	0	2	3	0	0	2	2	1	0	3
Right colon	2	6	0	2	3	5	0	2	2	1	0	6
ANTs	10	12	6	0	5	17	0	6	0	6	10	14

*The MMR phenotypes were defined by combination of reduced (r_2, r_1, r_6 <1) or regular (R_2, R_1, R_6 ≥1) *hMSH2*, , *hMLH1* and *hMSH6* mRNA levels relative to reference *hPBGD* mRNA control gene

Table 4: Distribution of combined MMR mRNA phenotypes* in CRCs and their adjacent normal tissues (ANTs).

data (Table 1) revealed reduced mRNA expression ratios (≤0.8) of *hMSH2, hMLH1, hMSH6* and *hPMS2*, with 41% (12/29), 45% (14/31), 53% (16/30) and 36.7% (11/30), respectively. As well as we observed overexpression (CRCs/ANTs ratios ≥1.8) of *hMSH2, hMLH1, hMSH6 and/or hPMS2* in 41% (12/29), 32% (10/30), 36.7% (11/30) and 32% (10/30) of cases, respectively (Table 1).

There was a statistically significant correlation between relative expression levels (T/ANT) of *hMSH2* and *hPMS2* (r=0.999389), notable in male (r=0.999937), in stage II tumors (r=0.999477) and in tumors without lymph node metastasis (r=0.999475). However, a statistically significant correlation was observed between mRNA relative expression levels of *hMSH2* and *hMSH6* in tumors metastatic to lymph nodes (r=0.644378). Also, a correlation was observed between *hMSH6* and *hPMS2* relative expression in female (r= 0.992066865).

Notably, a statistically significant difference was observed between *hMSH2* mRNA levels in CRCs tumors relative to their ANTs (P<0.04, by *Student's test*) specifically in cases without lymph node metastasis (P<0.04, by *Student's test*) (Table 2).

MMR phenotype sorting

We used the ratio of MMR mRNA expression relative to reference mRNA control (MMR/control gene mRNA levels), to adopt a functional unified assessment for our findings, as previously referred [17,19]. We classified our specimens in two major phenotypic groups, one with reduced (r) and the other with regular or elevated (R) ratios of expression (*Materials & methods*) and we subdivided our study group in eight phenotypic entities, r_2 and R_2 for *hMSH2*, r_1 and R_1 for *hMLH1*, r_6 and R_6 for *hMSH6* and p_2 and P_2 for *hPMS2*, DNA repair system components or their combined phenotypes by descending MMR system activity (Table 3 and 4).

All CRCs and their ANTs showed p_2 phenotype. The r_2 was the most commonly founded in CRCs and their ANTs relative to r_6 that was more often in CRCs relative to their ANTs (Table 2).

Specifically, r_2 presented more often in stage II or stage III tumors relative to stage I (p=0.000) and it was more frequently observed in CRCs with lymph node metastasis, perineural or vascular invasion (p<0.009, p=0.000 and p=0.000, respectively) relative to cases without tumor invasion. Also, r_2 was more frequent in CRCs located on the right colon or rectum compared to sigmoid colon tumors (p=0.000) (Figure 1 and 4).

Moreover, r_6 was more common in CRCs compared to their ANTs (p<0.002, χ^2-*test)*, mainly in males (p=0.000). A significant difference was found between male and female patients (p<0.02; χ^2-*test*). Also, r_6 was more often in CRCs of stage II and III relative to I (p=0.000 and p<0.002, respectively) and CRCs of stage III relative to II (p<0.02). Additionally, the r_6 was more common in CRCs with lymph node (p<0.004) or nerve (p<0.002) or vessel infiltration (p<0.0034) compared to their ANTs, with statistically significant difference between the number of cases showed nerve or vessel infiltration and those did not (p<0.0045 and p<0.01, respectively). In addition, r_6 was more frequent in CRCs of rectum (p<0.000) relative to their ANTs with a statistically difference relative to sigmoid or right colon (p<0.03) (Table 3, Figure 2 and 4).

Additionally, r_1 was observed commonly in ANTs of female patients relative to their CRCs (p=0.000), with a significant difference between female and male (p<0.009). However, r_1 found commonly in stage III CRCs (p<0.0068) relative to their ANTs. A significant difference of r_1 phenotype was found between stage III and stage II (p<0.0042) or I (p=0.000) as well as between stage II and I (p=0.000) cases. So, r_1 was more frequently observed in CRCs with lymph node metastasis (p<0.002) relative to their ANTs, with a significant difference between number of cases found with lymph node or nerve or vessel infiltration (p=0.000, p=0000 and p=0.042, respectively) and those without tumor infiltration. In addition, r_1 was more frequent in CRC tumors located to rectum relative to right colon (p=0.000) or to sigmoid (p=0.000) (Table 3, Figure 3 and 4).

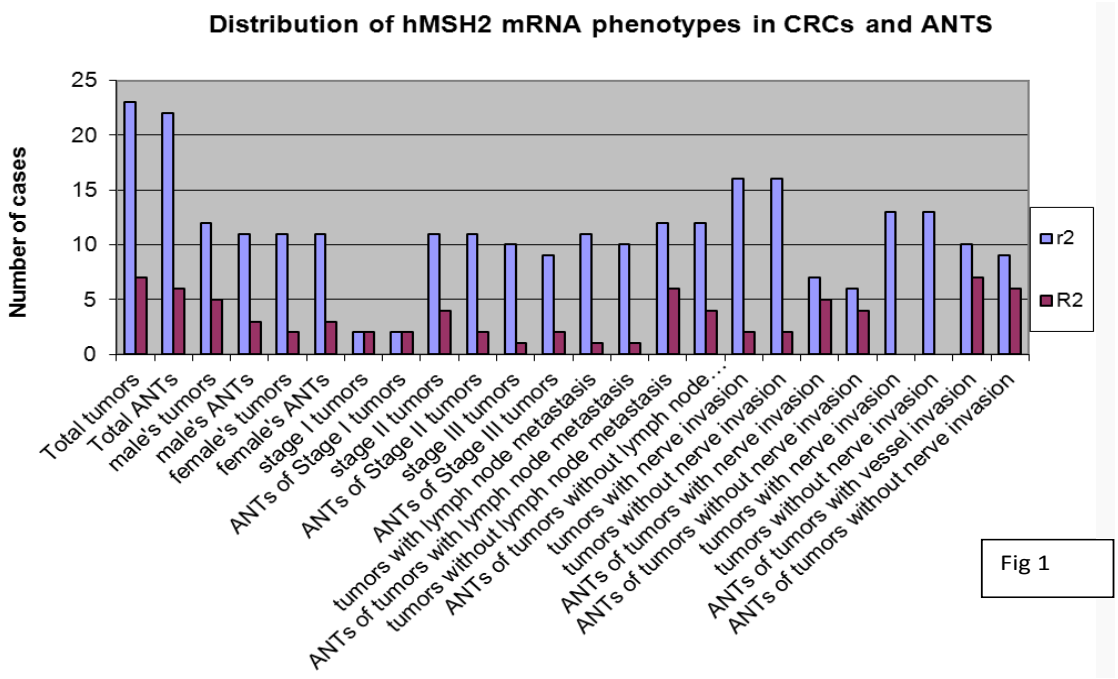

Figure 1: Distribution of hMSH2 mRNA phenotypes in CRCs and their ANTs.

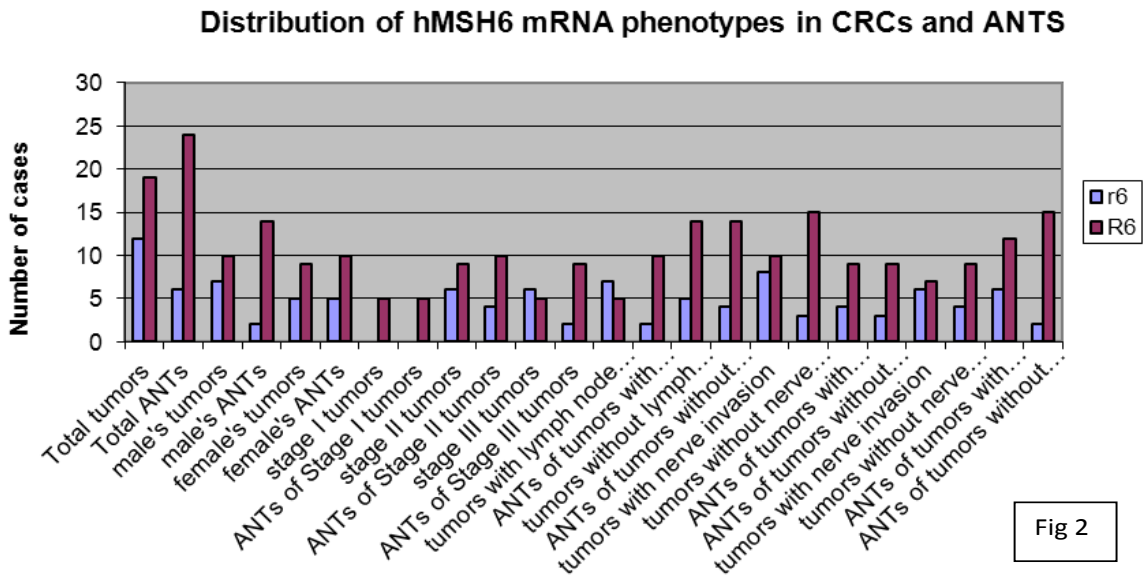

Figure 2: Distribution of hMSH6 mRNA phenotypes in CRCs and their ANTs.

Distribution of combined MMR mRNA phenotypes in CRCs and their ANTs

Table 4 presents the distribution of combined MMR mRNA in CRCs relative to patients and tumors' characteristics. We found a statistically different distribution of r_2r_1, r_2R_1 and R_2R_1 phenotypes i) between CRCs and ANTs in male ($p<0.03$), ii) between male and female patients ($p<0.03$), iii) between CRCs and ANTs in stage III cases ($p<0.026$), iv) between stage III and stage II cases ($p<0.023$), v) between CRCs and their ANTs in cases with lymph node metastasis ($p<0.02$), vi) between cases with lymph node and without tumor metastasis ($p<0.0014$), vii) between CRCs and their ANTs of cases with perineurial invasion ($p<0.01$), viii) between cases with nerve and without nerve invasion ($p=0.000$) and ix) between cases presented vessel invasion relative to cases without vessel invasion ($p=0.000$). Specifically there was a significant different distribution of r_2r_1 and r_2R_1 phenotypes between CRCs and ANTs in cases located on rectum or sigmoid (r_2r_1) relative cases on right colon (r_2R_1) ($p=0.000$ and $p=0.01$, respectively).

The distribution of combined MMR mRNA phenotypes r_2r_6, r_2R_6 and R_2R_6 was statistically significant different i) between CRCs and ANTs in male ($p=0.000$), with a difference between male and female

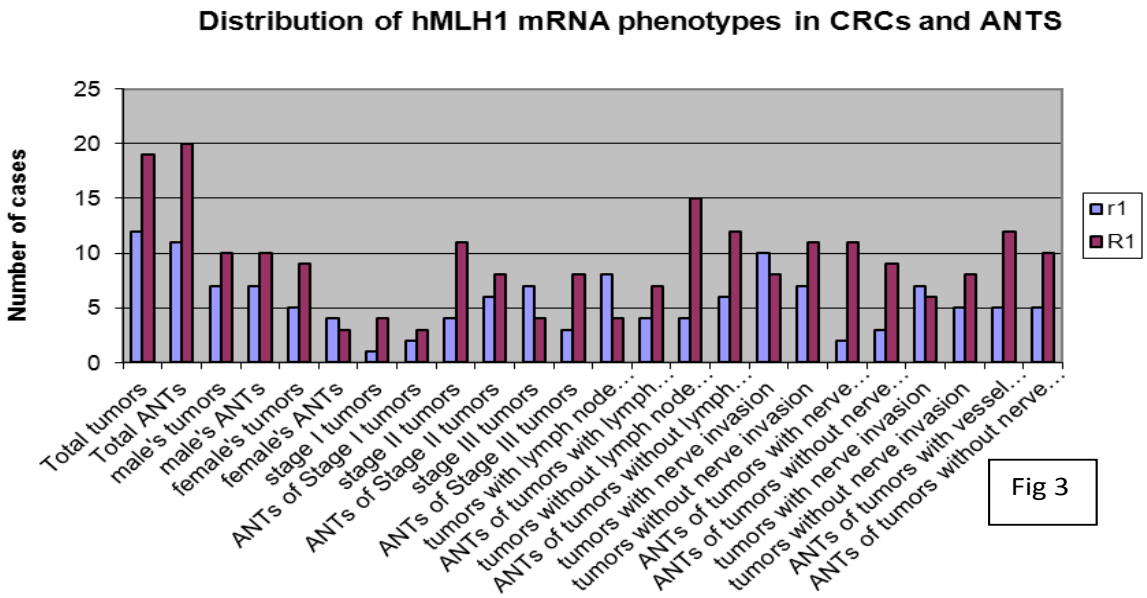

Figure 3: Distribution of hMLH1 mRNA phenotypes in CRCs and their ANTs.

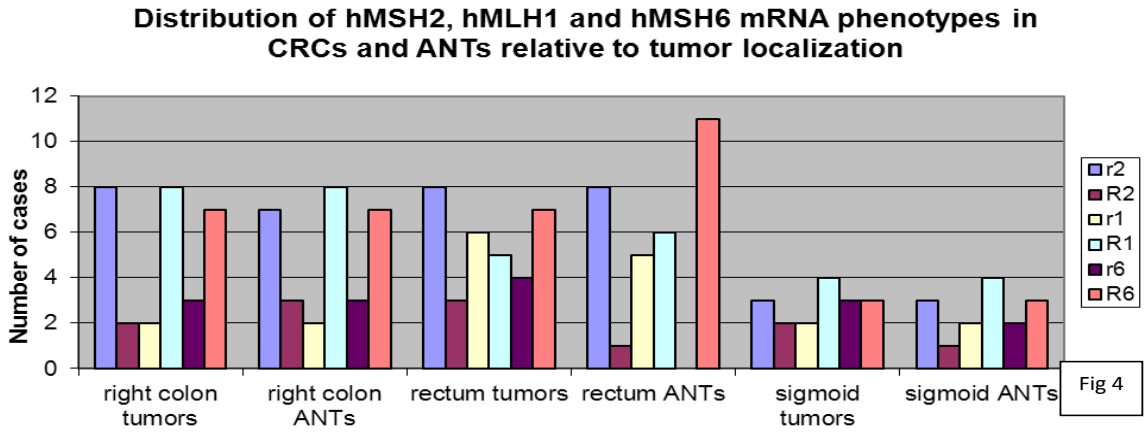

Figure 4: Distribution of hMSH2, hMLH1 and hMSH6 mRNA phenotypes in CRCs and their ANTs relative to tumor localization.

(p=0.000), ii) between CRCs and ANTs in stage III CRCs (p<0.027), with a difference between stage III and stage II (p=0.05) or between stage III and stage I (p=0.000) cases iii) between cases with node metastasis and cases without node infiltration (p<0.002), iv) between CRCs and their ANTs in cases with perineular invasion (p<0.04), v) between cases with perineural invasion compared to cases without perineural invasion (p=0.000) and v) between CRCs and ANTs in vessel filtrated cases (p=0.000), with a difference between vessel filtrated cases and non-vessel filtrated (p<0.005) (Table 4).

The distribution of r_6r_1, r_6R_1 and R_6R_1 was significant different between CRCs and ANTs in stage III cases (p<0.02), with a difference between Stage III and Stage II (p<0.002), and between node (p<0.0024), nerve (p<0.001), vessel (p=0.000) filtrated cases and cases without filtration (Table 4).

Discussion

The aim of this study was to quantify the mRNA levels of h*MSH2, hMLH1, hMSH6* and *hPMS2*, MMR genes in sporadic colorectal carcinomas and their ANTs, by Q-real time PCR and to correlate with clinical and histopathological data. So far, there is limited information on the transcriptional levels of the four major MMR DNA repair mechanism components, in non-hereditary CRCs and their ANTs [25,26]. Despite of the small number of patients [31] included in this study our results could be considered interesting and could give rise in a more extended investigation. We showed that the crucial components of MMR mechanism *hMSH2* and *hMLH1*, and their counterparts *hMSH6* and *hPMS2* exhibited low mRNA expression profiles in a significant proportion of CRCs and their ANTs (58-100%). We also showed that mRNA expression correlated with tumor progression and tumor localization in the colon. Additionally, the transcription of the studied MMR genes was reduced in a significant proportion of CRCs (37-53%) relative to their paired ANTs indicating a possible mechanism of progressive genetic instability [1-3]. We have evaluated various MMR mRNA expression profiles and their relationship to tumor or patients' characteristics.

We observed that CRCs of early histopathological stages (I-II), without lymph node metastasis, exhibited a correlation between the expression status of crucial MMR components, *hMSH2* & *hMLH1*, and between their counterparts *hMSH6* & *hPMS2*, maintained both in CRCs and in their ANTs [27]. Notably, this correlation was reversed between male and female. Surprisingly, cases with lymph node metastasis, revealed a significant positive correlation of mRNA relative expression between *hMSH2* and its counterpart hMSH6. Similarly, late stage (III) CRCs exhibited correlation between mRNA expression levels of *hMLH1* and its counterpart *hPMS2*. Our results show a balanced transcriptional activation between the crucial MMR components *hMSH2* & *hMLH1* and between their counterpart's *hMSH6* and *hPMS2*, in CRCs of early stages related to gender. Surprisingly, later, an upcoming "unbalance" occurs reducing similarly the mRNA expression levels of MutLa or MutSa components in CRCs relative to their ANTs, that seem to related with tumor progression. Our observations could be significant, indicating a mechanism resulting to downregulation of *hMSH2* and *hMSH6* expression during tumor progression. The reduction of mRNA expression of MMR genes has been considered to be caused by gene deletions in hereditary cancers, like Lynch syndrome, or epigenetic modification of genes like methylation of *hMLH1* in sporadic cancers or as recently it has been shown by specific miRNA regulation [28-34]. Cell biological studies have been shown the importance of retainance of MutLa components balance on cell cycle progression or apoptosis procedure, showing that the MutLa protein levels are essential to initiate apotosis and consequentely low expression levels lead to chemo-resistance [35,36].

Phenotypic sorting of our data revealed that the reduced r_2 phenotype of crucial *hMSH2*, MMR mechanism component, was very common both in CRCs and their ANTs indicating a deficiency of MMR mechanism in epithelium of CRCs patients. Significantly, the reduced r_6 phenotype of *hMSH6*, the counterpart of *hMSH2*, was more frequently observed in CRCs relative to their ANTS, supporting an affected MMR mechanism in CRC patients. Moreover, reduced (r_2, r_6 and r_1) mRNA MMR phenotypes are related with tumor invasion, indicating their use as a tumor progressing index. We first observed that the reduced p_2 phenotype of *hPMS2* is a common finding in colorectal epithelium of patients with CRC. It is worthy to mention that *hPMS2* low expression levels have been previously related with hereditary cancers with a late tumor onset [37]. Here, we suggest that tumorigenesis in colon could be probably related with a molecular mechanism including decreased transcriptional activity of *hPMS2*.

Observing the combined phenotypic sorting of our data, we can summarize that reduced r_2r_1, r_2r_6 and r_6r_1 combined MMR phenotypes were related to advanced tumors (stage III) and gender. Specifically, r_2r_1 commonly shows a strong correlation with tumors presented with lymph or nerve or vessel invasion as well as r_2r_6 with nerve or vessel infiltration. In our previous study in lung cancer we suggested that r_2r_1 could be considered as a tumor progression index, while it has been correlated with worst prognosis in squamous cell lung carcinomas. Our data suggests that it could be an indicator of tumor progression in CRC and is in agreement with previous findings [16]. Also, low MMR phenotypic profiles are correlated with male gender.

In conclusion, we presented for the first time a precise quantification of MMR mRNA levels, of *hMSH2, hMLH1, hMSH6* and *hPMS2*, in small number of sporadic CRCs and their ANTs, correlated with clinical and histopathological data. Our findings indicate that tumoral epithelium of CRC patients of our group acquires MMR deficiency, during tumor progression. Distinct MMR mRNA profiles as low *hMSH2, hMLH1* or *hMSH6* mRNA levels (r_2r_1, r_2r_6 or r_6r_1) could be characterized as important indicators of lymph node metastasis and of perineural or vascular invasion. A different expression pattern was found in males relative to females with males showing MMR mRNA profiles related with tumor progressing. All CRCs and their ANTs of our cohort revealed low *hPMS2* mRNA levels that were previously correlated with late tumor onset on hereditary colon cancers [37]. Rectal localization was related with dysregulated MMR mRNA mechanism.

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