Title: Mismatch Repair system protein deficiency as a resistance factor for locally advanced rectal adenocarcinoma patients receiving neoadjuvant chemo-radiotherapy

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Abstract

Background: Available data on Mismatch Repair system deficit and microsatellite instability are conflicting and are generally derived from a small number of patients due to the rarity of this condition in rectal cancer. Our study aimed to evaluate the frequency and therapeutic implications of Mismatch Repair proteins (MMR) status in patients with locally advanced rectal cancer (LARC).

Methods: We retrospectively collected data from 318 patients affected by LARC adenocarcinoma (cT3-4 +/- N1-2) treated at the Medical Oncology Unit of the University Hospital of Cagliari, Italy, the Medical Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy and at the Medical Oncology Unit, AOU Ospedali Riuniti, Ancona. Italy. All patients included in the study underwent neoadjuvant concurrent capecitabine and long-course radiotherapy (RT) (total dose of Gy 50.4), afterwards a total mesorectal excision (TME) was performed. MMR expression was evaluated through immunohistochemistry. The primary objective was major TRG (0-1 Ryan's score) while secondary objectives were pathological complete response, disease-free survival (DFS) and overall survival (OS).

Results: 160 patients (148 pMMR and 12 dMMR) were included in the exploratory cohort and 158 (146 pMMR and 12 dMMR) were included in the validation cohort. A major TRG has been shown in 64/148 (42,6%) and 63/146 (43,1%) patients with pMMR in exploratory cohort and validation cohort, respectively; while no major TRG have been shown in dMMR patients in exploratory cohort nor in validation cohort. Both exploratory and validation cohorts showed a statistically significant

higher median DFS in pMMR patients compared to dMMR ones: NR vs 14 months in exploratory cohort (*p = 0,003*) and NR vs 17 months in validation cohort (*p = 0,02*)

Conclusion: Our retrospective study indicated an association between dMMR and poor or no response to preoperative chemoradiotherapy. These results are consistent with the role of MMR as a predictor of poor-response to chemoradiotherapy and represent a hypothesis-generating study for the selection of these patients for immunotherapy in the neoadjuvant setting, in which the available data are promising.

Keywords: Rectal cancer; Mismatch Repair Deficiency; Microsatellite Instability; Immunotherapy biomarker; Neoadjuvant chemo-radiotherapy; Tumor regression grade; Resistance.

1 INTRODUCTION

Colorectal cancer (CRC) is the third leading cancer and the second cause of cancer-related deaths worldwide (1). A better prognosis in rectal cancer patients than in those with colon cancer was observed (1). Multimodal treatment, consisting of neoadjuvant chemoradiotherapy (CRT) followed by surgical total mesorectal excision (TME) followed by adjuvant chemotherapy, has become the standard of care in patients with locally advanced rectal cancer (LARC) (2). However, the response to neoadjuvant CRT in LARC is variable (3). An alternative strategy, the total neoadjuvant therapy (TNT), was taking root in recent years. TNT consists in administering CRT plus neoadjuvant chemotherapy before surgery to provide uninterrupted systemic therapy to eradicate micrometastasis (4-8).

Furthermore, identifying biomarkers to predict response is desirable to guide treatment decisions and improve CRT outcomes. Recently, several studies have investigated the molecular cancer profiles in different CRC settings to better define prognosis and to find a valid guide in the therapeutic choice, to improve responses. (9-16).

In this context, growing interest in the role played by microsatellite instability (MSI) as a predictor of response to CRT is emerged. MSI, the hallmark of deficient mismatch repair proteins (dMMR) tumors, may be caused by a germline mutation in one of the mismatch repair protein (MMR) genes (MLH1, MSH2, MSH6, PMS2, and EPCAM deletion), consistent with Lynch syndrome, or by epigenetic silencing of the MMR genes promoter region (17). There are two distinct MSI phenotypes: high-MSI (MSI-H) and low-MSI (MSI-L), distinguished based on the type and number of microsatellites analyzed. However, there is no evidence that MSI-L CRC differs in their

clinicopathological or molecular features from stable microsatellite tumors (MSS) (18). Conversely, it is well known that dMMR/MSI-H CRC differs from proficient MMR (pMMRs)/MSS tumors in several aspects, including prognosis, response to treatment, and metastatic spread pattern (19). dMMR occurs in 15-20% of cases, and studies of resected early-stage CRC described a better prognosis and no improvement from adjuvant 5-Fluoruracil therapy in MSI-H patients (20-22). The prognostic impact of dMMR appears to decrease as the stage of the disease progresses. It is considerably less common in the metastatic setting and occurs in 3% -5% of cases. Although data are currently lacking and inconsistent, dMMR tumors appear less responsive to fluoropyrimidines and oxaliplatin chemotherapy, in metastatic CRC (mCRC) (23-24). Furthermore, in this setting MSI/dMMR mCRC achieves long-lasting responses with immune checkpoint inhibitors (25-28). It has been hypothesized that the beneficial effect of immunotherapy in these patients depends on the increased somatic mutational load, the abundant infiltration of immune cells into the tumor microenvironment (TME) and increasing tumor neoantigens (29-31). For this reason, screening for dMMR expression is now recommended for all CRC patients (32).

Compared to colon cancer, the prevalence of dMMR in rectal cancer is less frequent, around 10%. Considering that colon cancer differs from rectal cancer, there are many questions about the role of MMR in LARC, particularly its prognostic and predictive role in response to fluoropyrimidine-based CRT. Currently, we have only a few discordant data in the literature. De Rosa et al. showed that dMMR rectal cancer had an excellent prognosis and pathologic response with fluoropyrimidine-based CRT (33). Conversely, Cercek A et al. demonstrated that (total neoadjuvant) TNT regimen included mFOLFOX or fluoropyrimidine-based CRT is far less efficacious in dMMR than in pMMR rectal cancer (34).

In recent years, there has also been a growing interest in the role of MSI in the neoadjuvant/adjuvant setting in other cancers. For example, some studies suggest a potential lack of benefit of perioperative or adjuvant chemotherapy for patients with MSI gastric cancer undergoing surgery. (35-37).

Based on these considerations, we have conducted a retrospective analysis to evaluate the frequency and therapeutic implications of dMMR status in patients with LARC treated in our Centre.

2 METHODS

2.1 Patients and Methods

We retrospectively collected data from 318 patients affected by LARC adenocarcinoma (cT3-4 +/-N1-2) treated at the Medical Oncology Unit of the University Hospital of Cagliari, Italy, the

Medical Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy and at the Medical Oncology Unit, AOU Ospedali Riuniti, Ancona. Italy.

All patients included in the study underwent neoadjuvant concurrent capecitabine and long-course radiotherapy (RT) (total dose of Gy 50.4). CT and MRI were performed at baseline and before surgery to verify the radiological response, according to RECIST v1.1 criteria. Subsequently, all patients underwent total mesorectal excision (TME) at the local Colo-rectal Surgery Unit.

MMR expression was evaluated through immunohistochemistry. Immuno-histochemical investigations were performed on the surgical samples to evaluate Mismatch Repair Proteins expression (MLH1, PMS2, MSH6, MSH2, and EPCAM).

The primary objective was major TRG (0-1 Ryan's score) while secondary objectives were pathological complete response, disease-free survival (DFS) and overall survival (OS). TRG evaluation was made on the surgical sample according to Ryan's score (38-40) and Dworak's score (41) to describe the tissue response to chemo-radiotherapy. Secondary objectives were disease-free survival (DFS) and overall survival (OS).

2.2 Statistical Analysis

Statistical analysis was performed with the MedCalc Statistical Software Version 20.2016 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2022). The association between categorical variables was estimated by the Fisher exact test for categorical binomial variables or by the chi-square test in all other instances. Survival probability over time was estimated by the Kaplan–Meier method. Significant diferences in the probability of survival between the strata were evaluated by the log-rank test. The independent role of variables that were statistically significant at a univariate analysis was assessed with a logistic regression analysis.

Major Tumor Regression Grade rate was defined as the percentage of patients who achieved a complete (TRG-0) or near complete response (TRG-1) on tissue samples, according to Ryan's score.

Disease free survival was defined as the time from treatment start until the first cancer-related event, second cancer, or death from any cause. Overall survival was defined as the time interval between the date of the treatment start to death or the last follow-up visit for patients who were lost at follow-up. Overall response rate (ORR) is defined as the proportion of patients who have a partial or complete response to therapy. Disease control rate was defined as the percentage of patients with stable disease or partial/complete response to treatment.

Based on the results from the 160 patients of the exploratory cohort, we tried to validate the findings in a validation cohort. Then, we identified the validation group sample size according to

major TRG rate and survival analysis, from the exploratory cohort. To validate the difference in terms of major TRG in pMMR patients (around 40 %) and dMMR patients (around 5%), assuming a probability alpha of 0.05 and a beta of 0.20), with a two group ratio of 12, the required sample size would have been 155 patients (143 + 12), using a "comparison of proportion test".

3 RESULTS

3.1 Patients Characteristics

Globally, 318 patients affected by LARC adenocarcinoma (cT3-4 +/- N1-2) were included in the study, 160 patients (148 pMMR and 12 dMMR) were included in exploratory cohort and 158 (146 pMMR and 12 dMMR) were included in validation cohort. Median age was 68 ± 11 both in exploratory and validation cohort. Stage III patient rate was 64% and 63% in exploratory and validation cohort, respectively. Mismatch Repair system deficiency rate was 7,5% and 7,6% in exploratory and validation cohort, respectively. Patient baseline characteristics are detailed in Tables 1 and 2.

Table n.1 Exploratory cohort: patients baseline characteristics

	pMMR	dMMR	Tot.
N.	148	12	160
M/F	92/56	10/2	102/58
ECOG-PS 0	105	10	115
ECOG-PS 1	41	1	42
ECOG-PS 2	2	1	3
Stage II	53	4	57
Stage III	95	8	103
Upper rectum	15	1	16
Medium rectum	85	7	92
Lower rectum	48	4	52

Table n.2 Validation cohort: patients baseline characteristics

	pMMR	dMMR	Tot.
N.	146	12	158
M/F	95/51	7/5	102/56
ECOG-PS 0	105	11	116
ECOG-PS 1	38	1	39
ECOG-PS 2	5	-	5
Stage II	54	3	47
Stage III	92	9	111
Upper rectum	16	1	17
Medium rectum	81	10	91
Lower rectum	49	1	50

3.2 Tumor Regression Grade and Clinical Outcomes

In the exploratory cohort a major TRG has been shown in 64/148 (42,6%) pMMR patients: 14,2% achieved a TRG-0 and 28,4% achieved a TRG-1; while no major TRG have been shown in dMMR (0%): 2/12 patients achieved a TRG-2 and 10/12 patients achieved a TRG-3. Afterwards, we evaluated the differences in median disease free survival between pMMR and dMMR patients. pMMR showed a statistically significant higher median DFS: NR versus 14 months (p = 0,003).

Results were confirmed in the validation cohort where a major TRG has been shown in 63/146 (43,1%) pMMR patients: 13% achieved a TRG-0 and 30,1% achieved a TRG-1; while no major TRG have been shown in dMMR (0%): 3/12 patients achieved a TRG-2 and 9/12 patients achieved a TRG-3. Then, evaluating the differences in median DFS, pMMR showed a statistically significant higher median DFS: NR versus 17 months (p = 0,02).

At present, the data on median overall survival in both exploratory and validation cohorts are still immature to be able to obtain conclusive results.

**Table n.3 Tumor regression grade according to Ryan's scores.**

		Ryan's score[1]				Tot (n.)
		TRG-0	TRG-1	TRG-2	TRG-3	
Exploratory cohort	pMMR	21	43	75	9	148
	dMMR	-	-	2	10	12
Validation cohort	pMMR	19	44	72	11	146
	dMMR	-	-	3	9	12

*1 Ryan tumor regression (TRG) score: TRG-0 no visible cancer cells (complete response); TRG-1 single cells or small group of cancer cells (near-complete response); TRG-2 residual cancer with evident tumor regression, but more than single cells or rare small group of cancer cells (partial response); TRG-3 extensive residual cancer with no evident tumor regression (poor or no response).

Figure n.1 Exploratory cohort Disease free survival
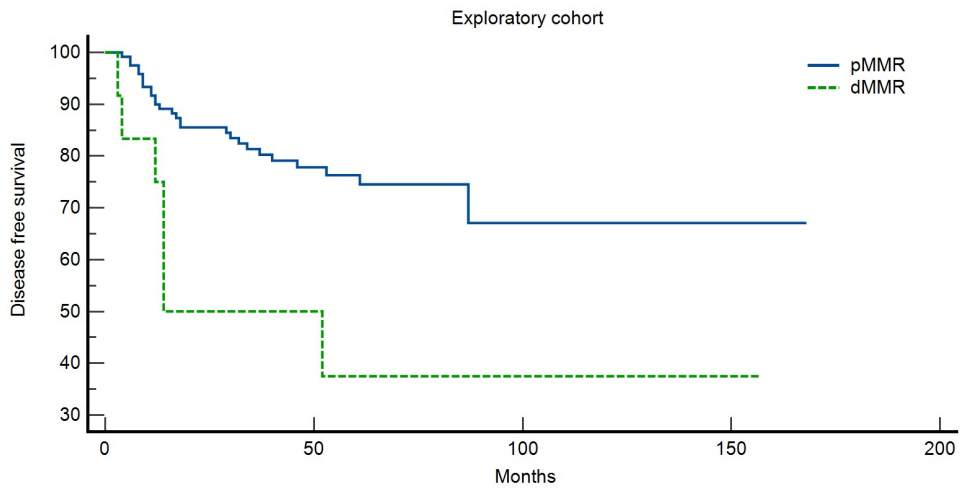
Figure n.2 Validation cohort Disease free survival
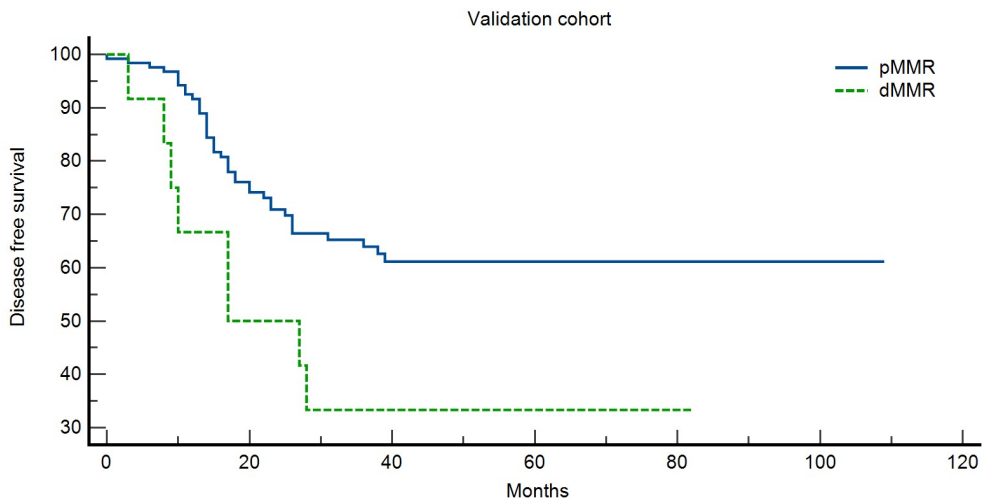
4 DISCUSSION

Our findings show biological resistance to capecitabine-based chemoradiotherapy in patients with LARC adenocarcinoma and dMMR in a real life population. To the best of our knowledge in the literature only few retrospective studies evaluated small groups of dMMR patients, with conflicting results in terms of pCR. De Rosa et al. described the response to multimodality treatment (chemoradiotherapy plus TME) in 62 dMMR rectal adenocarcinomas patients who achieved a high pCR rate (27.6%), however these results could be due to patients selection (stage I patients were also included in this study) (33). On the other hand, Cercek et al. evaluated the outcome of 50 dMMR patients after chemoradiotherapy, compared with a corresponding group of pMMR patients, showing a poor treatment response in the dMMR group (34). These observations laid the basis for the initiation of a prospective phase 2 study in which single-agent dostarlimab, an anti-PD-1 monoclonal antibody was administered every 3 weeks for 6 months in patients with dMMR stage II or III rectal adenocarcinoma. Notably, among the 12 patients who completed treatment with dostarlimab, the authors reported 100% of clinical complete response (42).

A possible biological explanation for these results lies in the MMR protein's biological function. Ten proteins have a role in this process, and all of these combine to obtain two types of functional heterodimer: MutS and MutL (43). MutL proteins are ATPases of the GHKL family, which have ATPase in the N-terminal domain and the dimerization domain at the C-terminal (44-48). Human cells express 4 MutL homologs: MLH1, MLH3, PMS1 and PMS2, which combined into three different heterodimer subtypes: MutLα (MLH1/PMS2), MutLβ (MLH1/PMS1) and MutLγ (MLH1/MLH3). MutLα plays the most important role in MMR deficient cells that exhibit MSI phenotypes, as in the MSH2 mutated cells (49-53).

Several preclinical studies investigated the association between MMR alterations and drug and chemical activity, showing resistance to chemotherapy (fluoropyrimidines and/or oxaliplatin) in dMMR cells (54). Cancer cells deficient in MMR are significantly more resistant to treatment with methylating agents than cancer cells with proficient MMR. The cytotoxic damage induced by these drugs begins with the methylation of specific nucleotide residues that may lead to cell cycle arrest, if intercepted correctly. Instead, in cells with deficient proteins in the repair system, these alterations persist, and the cell survives with a large load of mutations (55-56). These data are consistent with the responses described in our study, where pMMR tumors showed a lower tumor

regression grade (evaluated according to both Ryan's and Dworak's scores). However, few patients with pMMR showed a poor response, whereas few patients with dMMR showed a response to treatment. The reason for these conflicting results could be related to IHC technique and/or tumor biology. IHC detection for MMR proteins has a similar performance to PCR-based analysis for MSI, with a concordance ranging from 90.4% to 99.6%, depending on the case series (57-58). However, other studies evaluating the sensitivity and specificity of detection of MLH1 and MSH2 mutations suggested that these can reach high values (74 and 91% sensitivity and 81 and 90% specificity, respectively) (59). Therefore, despite the excellent performance of IHC, in some cases, the results may not reflect the truthful state of MMR. For example, missense mutations of MMR genes can produce non-functioning proteins that can bind antibodies. In these cases, a percentage of MSI-H ranging from 5% to 11% could still be positive for IHC stains (60). Therefore, despite showing positive staining for MMR proteins, patients may have MSI-H status and may not respond adequately to neoadjuvant treatment. In addition, many other factors can influence the response to treatment, including the extracellular matrix, the immune cell infiltrates, and inter-individual variability.

The study also showed a high frequency of MSH2/MSH6 and MLH1/PMS2 deficiencies, which is consistent with the data available in the literature, which predicts a higher frequency of these mutations in rectal adenocarcinoma rather than colon adenocarcinoma (33-34). Survival data showed a better median DFS in pMMR patients, while OS data are immature for an evaluation.

Our study has some limitations, mainly for its retrospective nature that by definition is hypothesis generating and could represent the first step towards a future validation of these findings in a prospective study. Moreover, MMR proteins were only evaluated in the resected specimen and a comparison with diagnostic biopsy tissue was not performed, implicating a potential bias for a full interpretation of the results. Although exploratory we believe that our findings, along with those deriving from ongoing trials, could represent an important step forward in the definition of the optimal treatment strategy for dMMR/MSI-H locally advanced rectal cancer patients.

5 CONCLUSIONS

Mismatch repair deficient/High Microsatellite Instability locally advanced rectal cancers showed a lower response to standard chemoradiotherapy. Moreover, we now know that they could have an excellent response to treatment with immune checkpoint inhibitors. The emerging picture is showing dMMR/MSI-H rectal cancers as a disease completely different from its pMMR/MSS

counterpart, and furthermore, they should start a completely different diagnostic and therapeutic strategy.

Figure n.3: Graphical abstract (first option)
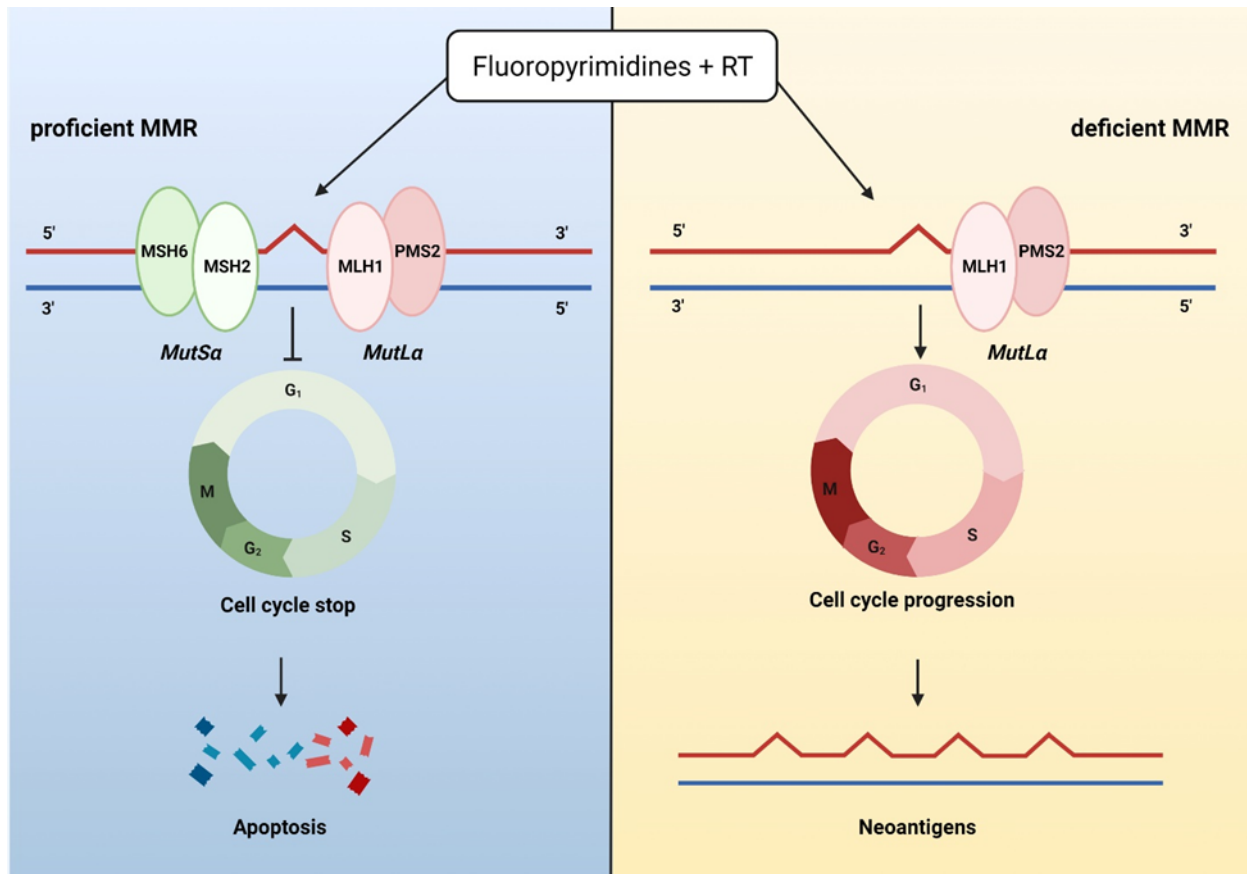
ACKNOWLEDGEMENTS

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Footnotes