Mismatch repair deficiency may affect clinical outcome through immune response activation in metastatic gastric cancer patients receiving first-line chemotherapy

ORIGINAL ARTICLE

Title: Mis-Match Repair (MMR) deficiency may affect clinical outcome through immune response activation in metastatic gastric cancer patients receiving first-line chemotherapy.

Running Head: D-MMR, immune response and gastric cancer survival

By: Riccardo Giampieri[1], Elena Maccaroni[1], Alessandra Mandolesi[2], Michela Del Prete[1], Kalliopi Andrikou[1], Luca Faloppi[1], Alessandro Bittoni[1], Maristella Bianconi[1], Marina Scarpelli[2], Raffaella Bracci[1], Mario Scartozzi[3], Stefano Cascinu[1].

From the Departments of:

1) Medical Oncology, Università Politecnica delle Marche, Ancona, ITALY;

2) Pathology, Università Politecnica delle Marche, Ancona, ITALY;

3) Medical Oncology, Università di Cagliari, Cagliari, ITALY

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Correspondence to:

Elena Maccaroni, MD

Clinica di Oncologia Medica

AO Ospedali Riuniti-Università Politecnica delle Marche, Ancona, ITALY

Tel: +390715963834, Fax: 0715964192, e-mail: elena.maccaroni@live.it

ABSTRACT

Introduction

The microsatellite-instable gastric cancer subtype, due to its supposed high antigenic potential, represents a promising candidate for immunotherapy. We analysed if the presence of defective mismatch repair system (D-MMR) is associated with other markers of immune response and their relationship with outcome in advanced gastric cancer patients.

Methods

We analysed the relationship between clinical outcome and MMR status, tumour infiltrating lymphocytes (TIL), lymphocitosis and neutrophil/lymphocyte ratio (NLR) in metastatic gastric cancer patients treated with a chemotherapy doublet in 1st line setting. Other stratification factors were sex, age, ECOG, adjuvant/neo-adjuvant chemotherapy, metastatic sites, histology.

Results

103 patients were eligible for analysis. D-MMR was found in 15/103 (14%) patients, TILs+ in 18/103 (17%), lymphocitosis in 24/103 (23%) and high NLR in 75/103 (72%). Significant correlations were found between D-MMR and TIL+ (p=0.0004), between D-MMR and lymphocytosis (p=0.0062), between D-MMR and low NLR (p=0.000069), and between TILs+ and lymphocytosis (p=0.000147). All factors had a statistically significant impact on overall survival, although at multivariate analysis only D-MMR (p=0.0001) and TILs+ (p=0.0192) maintained their independent prognostic role. Similar results were observed for progression free survival, with D-MMR (p=0.0001) and TILs+ (p=0.0195) maintaining their prognostic role at multivariate analysis.

Conclusions

Our analysis confirms the favourable prognosis of D-MMR status in metastatic gastric cancer patients and suggests that TILs expression might also be linked to better outcome. Due to the

correlation between D-MMR status and measures of immune system activity, this group of patients would be the best candidate to submit to novel immunotherapy-based therapies.

CONFLICT OF INTEREST STATEMENT/DISCLOSURES:

Authors have no conflict of interest, relevant to the present work, to disclose.

KEYWORDS:

Gastric cancer, mismatch repair, microsatellite instability, chemotherapy, prognosis

INTRODUCTION

In western Countries around a half of gastric tumours are diagnosed at an advanced stage, when a curative surgical resection is no longer possible. As a consequence in most of these patients palliative chemotherapy represents the only treatment option able to improve clinical outcome. Therapeutic approaches have continuously evolved in this setting, particularly with the introduction of targeted agents such as the anti HER-2 monoclonal antibody trastuzumab [1] and anti VEGFR-2 monoclonal antibody ramucirumab [2-3]. Apart from HER-2 characterization, the issue of treatment selection based on clinical and biological factors is still pending, and we are in essentially unable to select the optimal option for each patient.

Recently, researchers from the Cancer Genome Atlas [4] project proposed a categorization of gastric cancer into 4 major subtypes: EBV-related, microsatellite-instable (MSI), genomically stable (GS), and chromosomally instable (CIS). The MSI category, presenting a typical lack of function of the MisMatch Repair (MMR) genes (mainly hMLH1 and hMSH2) is of clinical interest because of the favourable prognostic profile of these tumours than their MMR proficient counterpart.

Several studies have demonstrated that gastric cancer patients presenting lack of MMR genes activity had a better outcome than patients showing a normal function of the MMR machinery.

Similar data have been already found in other tumour types such as colorectal [5-8] and ovarian cancers, thus supporting the observation that MMR deficient tumours have distinctive biological and clinical characteristics. Intriguingly, recent findings also hypothesised that this favourable clinical behaviour might be related to immune response induced by tumor cells in presence of MMR deficiency. In colorectal cancer cells, pre-clinical data suggested that the antigenic load able to activate immune response was significantly higher in high MSI tumours (which is an indirect

indication of dysfunctional MMR). Accordingly only high MSI colorectal tumours seem to derive a relevant benefit from immunotherapy.

A possible role for immunotherapy has been shown also in the treatment of gastric cancer patients, not assessed according to a specific clinical or molecular profile [9]

On the other hand the influence of the MMR genes status on treatment outcome during chemotherapy [10-13] and the potential connection with indirect sign of immune reaction are still lacking in the published literature.

Aim of our study was the assessment of the correlation between the loss of MMR activity, signs of immune response activation (tumour infiltrating lymphocytes, TIL, systemic lymphocytosis and neutrophils to lymphocytes ratio) and clinical outcome in metastatic gastric cancer patients receiving first line chemotherapy.

Secondary objective was to delineate a clinical and molecular profile of gastric tumours in which an underlying mechanism of immune-mediated activity may make these patients optimal candidates for immune therapy.

PATIENTS AND METHODS

Patients' selection

Histologically proven, metastatic gastric cancer patients receiving first-line platinum-based (either cisplatin or oxaliplatin combined with 5FU) chemotherapy at our Institution between January 2007 and December 2013 and whose tumour sample was available for biological analysis, were eligible for our study.

For patients who received adjuvant/neoadjuvant treatment after radical surgical resection, an interval of at least 12 months from the end of treatment was considered necessary for inclusion in the present analysis. Follow-up during chemotherapy was performed according to our Institutional guidelines and consisted of physical examination, full blood count, renal and liver function tests before each cycle. Tumour response was evaluated with CT scan of the chest and abdomen every 8 weeks by clinicians' assessment and according to the RECIST criteria (version 1.1). The site and date of progression and the date of death were recorded. For all patients, we also collected neutrophils, lymphocytes and platelets count within one month before the start of treatment. All blood exams were determined according to IFCC (International Federation of Clinical Chemistry and Laboratory Medicine) method. The assay has been conducted in Institution Laboratories certified for Quality control according to the present rules in Europe.

This analysis was approved by our local Ethical Committee.

Immunohistochemical analysis of hMLH1 and hMSH2

Immunohistochemical investigation of hMLH1 and hMSH2 protein expression was performed on paraffin-embedded tissue sections. Five micron-thick sections containing tumor tissue and normal gastric mucosa (internal control) were removed from the paraffin and rehydrated with xylene and alcohol. Endogenous peroxidase was blocked by dipping the sections in 3% aqueous H2O2 for 10

minutes, and antigen retrieval was performed with a 10-minute microwave treatment in 10 mmol/L citrate buffer, pH 6.00. After antigen retrieval, sections were incubated overnight at 4°C with a mouse monoclonal antibody to the hMLH1 protein (clone G168-728, 1:50 dilution; PharMingen, San Diego, CA) or to the hMSH2 protein (clone FE11, 1:100 dilution; Oncogene Research Products, Cambridge, MA) and lightly counterstained with hematoxylin. Immunostaining was performed by the avidin-biotin peroxidase complex technique; diaminobenzidine was used as a chromogen. The normal staining pattern for both hMLH1 and hMSH2 was assessed, and tumors were considered negative for hMSH2 or hMLH1 expression when there was a complete absence of nuclear staining of tumor cells in the presence of an internal positive control represented by normal epithelial cells, stromal cells, or lymphocytes

Tumours with lack of either hMLH1 or hMSH2 expression were considered MMR-defective (D-MMR) whereas tumours that maintained expression of hHMLH1 and hMSH2 were considered MMR-proficient (P-MMR).

Tumour infiltrating lymphocytes (TILs)

All tumour samples were routinely evaluated for tumour infiltrating lymphocytes by our pathologists and were classified as TIL positive or negative. Tumours were considered positive for TIL if there was documented evidence of lymphocytic response, assessed by presence of CD3+ cells in the histologic specimen. In particular, evaluation and scoring of TILs expression was assessed by the method previously described by Denkert et al. [14]. Presence of more than 50-60% stromal surface area covered by presence of TILs was considered as positive. No evaluation of immune cell type was performed.

Statistical analysis

Statistical analysis was performed with the MedCalc Statistical Software version 14.10.2 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014).

The association between categorical variables was estimated by Fisher exact test for binomial categorical variables and by Chi-square test for all remaining instances. Survival distribution was estimated by the Kaplan-Meier method (Kaplan et al, 1958). For statistical analysis overall survival (OS) and progression free survival (PFS) were defined respectively as the interval between the start of first-line chemotherapy to death or last follow-up visit and as the interval between the start of first-line chemotherapy to clinical progression or death or last follow up visit if not progressed. Significant differences in probability of relapsing between the strata were evaluated by log-rank test. Hazard ratios (HRs) for median PFS and OS between groups were estimated from Cox Regression models.

Cut-off values for lymphocytosis and neutrophil/lymphocyte ratio (NLR) were determined by receiver operating characteristics (ROC) curve analysis in the patients enrolled in the analysis.

The multivariate analysis also included adjustments for other variables such as sex (male vs. female), age (<65 yrs vs. ≥ 65 yrs), ECOG performance status (0-1 vs. ≥ 2), previous adjuvant neo-adjuvant chemotherapy (yes vs. not), metastatic sites (1 vs. ≥ 2). A significant level of 0.05 was chosen to assess the statistical significance. The Holm-Sidak correction was used to adjust multiple comparisons values.

RESULTS

One hundred and three patients were eligible for our analysis. For the entire cohort, median overall survival (OS) was 8.8 months (95%CI: 7.9-9.0 months, range 2.9-18.6 months), whereas median progression free survival (PFS) was 5.8 months (95%CI:4.9-6.0 months, range 0.3-15.6 months). Twenty-seven patients (26%) achieved a partial remission (PR), 58 patients (56%) achieved a disease stabilisation whereas the remaining 18 patients (17%) progressed under treatment.

46/103 (45%) patients had a synchronous metastatic disease at the start of first-line treatment whereas 57/103 (55%) patients had already performed surgery for a primary gastric cancer and started first-line treatment after disease relapse.

Main patients' characteristics have been also summarised in table 1.

Out of 103 patients, analysis of MMR expression and TIL expression was performed on histologic tissue of previous gastric cancer biopsies in 30/103 (29%) patients, whereas in the remaining 73/103 (71%), the analysis was performed on histologic tissue of a previous surgical resection of the primary tumour.

Fifteen patients (14%) showed loss of MMR expression (lack of expression of hMLH1/hMSH2 in the tumour). In this group of patients a significantly improved survival was seen compared with patients who MMR proficient tumours (median OS respectively 14.2 vs. 8.0 months, HR for death: 0.24, 95%CI: 0.16-0.35, p<0.0001) [Fig.1]. An improved progression free survival was also observed (median PFS respectively 11.2 vs. 5.0 months, HR for progression: 0.25, 95% CI: 0.17-0.33, p<0.0001) [Fig.1a, not shown]. Response rate was also better in D-MMR patients, with 10 (66%) patients achieving a partial response compared with 17 (19%) patients with P-MMR tumours (p=0.0004).

Eighteen patients (17%) showed TILs on pathology examination (examples of expression can be seen in Fig.1 supplementary materials). Median OS was significantly better in TILs positive patients vs. TILs negative patients (median OS respectively 12.5 vs. 7.9, HR for death: 0.39, 95%CI: 0.26-0.58, p<0.0001) [Fig.2]. An improved PFS was also evident (median PFS respectively 8.0 vs. 4.9 months, HR for progression: 0.40, 95%CI: 0.26-0.60, p=0.0001) [Fig.2a, not shown]. Fifteen patients (83%) exhibiting pathological TILs achieved a PR whereas only 12 patients (14%) negative for TILs achieved a PR (p<0.0001).

When we compared the results for TILs expression in tumour biopsies or resected gastric cancer specimens, we observed some differences in terms of impact on overall survival: in particular, in resected gastric cancer samples we observed TIL+ status in 14/73 (19%) patients whereas in

biopsies we observed TIL+ status in 4/30 (13%) patients. Overall survival for patients in the resected gastric cancer group was respectively 12.5 vs 7.9 months for TIL+ vs TIL- status (HR:0.37, 95%CI:0.23-0.59, p=0.0002) (Fig.2 supplementary materials). Overall survival for patients in the biopsy group was respectively 9.9 vs 7.9 months for TIL+ vs TIL- status (HR:0.50, 95%CI:0.21-1.17, p=0.1662) (Fig.3 supplementary materials).

Based on ROC analysis results the best cut-off value for lymphocytosis was set at 1.8 the lower-limit-normal. Twenty-four patients (23%) showed lymphocytosis. Median OS was improved in patients with lymphocytosis compared with the remaining patients (median OS respectively 10.8 vs. 7.9 months, HR for death: 0.38, 95%CI: 0.26-0.57, p<0.0001) [Fig.3]. Median PFS resulted improved as well in patients with lymphocytosis (median PFS respectively 7.8 vs. 4.9 months, HR for progression: 0.38, 95%CI: 0.26-0.57, p<0.0001) [Fig.3a, not shown]. Nineteen patients (79%) with lymphocytosis achieved a PR, whereas only 8 patients (10%) without lymphocytosis showed a response to treatment (p<0.0001).

Based on ROC analysis results the best cut-off value for neutrophil/lymphocyte ratio (NLR) was set at 0.4.

A NLR higher than 0.4 was found in 75 patients (72%). Median OS was significantly worse in this group of patients (median OS respectively 7.8 vs. 10.8 months, HR for death: 2.61, 95%CI: 1.77-3.84, p<0.0001) [Fig.4]. Median PFS was also significantly worse in the high NLR group vs. the other (4.8 vs. 7.6 months, HR for progression: 2.51, 95%CI:1.71-3.70, p<0.0001) [Fig.4a, not shown]. Nineteen patients (67%) in the low NLR achieved a partial response compared with 8 patients (11%) in the high NLR (p<0.0001).

A significant relationship was found between MMR status and TILs expression. Ten patients (66%) with MMR defective tumours showed concomitant TILs, compared with 8 patients (9%)

with MMR proficient tumours (p=0.0004). A significant relationship was also found between MMR status and systemic lymphocytosis. Eight patients (53%) with D-MMR also had systemic lymphocytosis, compared with 16 patients (18%) who ha MMR proficient tumours (p=0.0062). In patients showing TILs expression systemic lymphocytosis was more frequently associated. Eleven patients (61%) with TILs showed in fact systemic lymphocytosis, compared with 13 patients (15%) without TILs (p=0.000147). It was also demonstrated an association between MMR status and neutrophil/lymphocyte ratio: in particular, only 4 (27%) patients in the D-MMR group had high neutrophil/lymphocyte ratio compared with 71 (80%) patients in the P-MMR group (p=0.000069).

At multivariate analysis MMR status and TILs expression maintained an independent role for overall survival and progression free survival, whereas the other factors (lymphocytosis, NLR, previous adjuvant, ECOG PS, number of metastatic sites, histotype) were excluded by the model. In particular, D-MMR positive status maintained its prognostic role and was shown to be the strongest predictor of better outcome (Exp(B)=0.41, 95%CI Exp(B):0.2528-0.6772, p=0.0005). TILs+ status also maintained an independent role as predictor of better outcome (Exp(B)=0.49, 95%CI Exp(B):0.2863-0.8688, p=0.0145).

On this basis we identified 3 groups of patients: patients with D-MMR tumours, patients with P-MMR tumours and evidence of TILs and patients with P-MMR tumours without evidence of TILs. Median PFS for the 3 subgroups was respectively 11 vs. 6.9 vs. 4.9 months, p<0.0001 [Fig. 5a, not shown]. Median overall survival analysis also showed significant differences according to the 3 subgroups: 14.2 vs. 9.9 vs. 7.7 months, p<0.0001 [Fig. 5].

DISCUSSION

Although treatment opportunities for metastatic gastric cancer have improved during the years survival outcome is still disappointing. Apart from the need of more effective therapies, a

potential explanation for these unsatisfactory results lies in the substantial lack of reliable predictive and prognostic factors, thus limiting the possibility to select the appropriate treatment for the appropriate patients. A growing body of evidence is now suggesting that gastric cancer is a highly heterogeneous disease with different biological and clinical features [5]. This ultimately leads to a possibly distinctive prognostic stratification and sensitivity/resistance to therapeutic options: specifically, patients in the EBV-related subgroup would appear as the best candidates to receive treatment options based on immunotherapy and inhibition of PI3K-Akt-mTOR pathway due to their relatively high frequence of mutations in PIK3CA gene. On the other hand, while appearing quite homogeneous in their lack of selective mutations (apart from ARID1A), the genomically stable subgroup (GS) would seem as potentially the worst candidate to be the subject of a trial based on a targeted agent.

Our analysis confirmed that MMR defective tumours have an improved clinical outcome compared with their MMR proficient counterparts.

Indeed, we defined MMR-defective tumours on the basis of the lack of expression of MLH1 or MSH2 status in the tumour, thus without information regarding the expression (or lack thereoft) of MSH6 or PMS2. Although it is a well-known fact that MSH6 and PMS2 expression is another crucial factor in the definition of MMR activity in other tumour types (i.e. colon cancer), in gastric cancer it seems that the role of these other 2 genes is less relevant, being MLH1 defective status the most common alteration found in MMR-defective tumours.

Similarly, Shigeyasu [15] et al suggested that metastatic gastric cancer patients showing hMLH1 inactivation via promoter methylation had a significantly improved survival. Unsurprisingly hMLH1 promoter methylation was also associated with the MSI phenotype as commonly found in all tumour types exhibiting a malfunction of the MMR machinery.

A further analysis [16] showed that MSI status and promoter methylation might be associated with improved survival in resected gastric cancer patients, even if the prognostic impact of MMR status was lost at multivariate analysis favouring tumour stage, which is likely to be more prognostically relevant than MSI.

Our analysis confirmed the prognostic role of MMR status and also indicated a potential role of MMR in predicting response to first-line platinum-based chemotherapy as suggested by the advantage in RR and PFS in MMR-defective tumours.

These findings are partly in contrast to previous observations in radically resected patients receiving adjuvant fluoropyrimidines [17]. However both the different setting and type of chemotherapy might explain conflicting results.

We also showed that the presence of TILs is an important, independent prognostic factor. This was particularly evident in MMR-deficient tumours, in which the presence of TILs was linked to MMR status and identified a sub-group of patients with a distinctive favourable outcome in terms of progression-free survival and overall survival.

The clinical implication of tumour infiltrating lymphocytes in gastric tumours has been long debated since early observations in pathology specimens. Although the marked heterogeneity of the published literature analysing TILs in various tumour types [18,19], there is now a general consensus in indicating TILs as a favourable feature particularly in locally advanced, radically resected tumours. Nonetheless data in metastatic patients were lacking as they were lacking the data correlating TILs with response and PFS in this setting. Intriguingly in our experience TILs revealed to be a mixed factor with both a prognostic and a predictive role.

TILs positive status was more strongly associated with an improved prognosis when the evaluation of TIL scoring was performed on resected gastric cancer samples rather than on tumour biopsies:

the results of this difference might be related due to the fact that, in tumour biopsies, the deeper portion of the tumour, mainly comprised of stromal cells (and where we should evaluate TIL expression) could be lacking, thus lessening the reliability of the assessment on simple tumour biopsies. Indeed, that might explain why some conflicting results, in terms of the prognostic role of TIL expression, could be seen by comparison of analyses conducted in patients who underwent surgical resection and who are in adjuvant treatment vs those who have metastatic spread since disease onset. This latter group of patients might be "underevaluated" for TIL expression due to lacking proper tumour tissue, having usually not performed any kind of resection of their primary tumour.

The option to further classify the cellular components of the TILs using immunohistochemical markers such as FoxP3, CD8, CD4 to name few, was not possible in our study. However we believe that our findings of a correlation between MMR status and TILs are already in keeping with our knowledge of the molecular basis underlying MMR defective tumours and their putative antigenic potential. In fact we demonstrated that MMR defective tumours are more frequently associated with TILs and that these peculiar features have a significant impact not only on prognosis but also on RR and PFS. On this basis we can speculate that MMR defective gastric tumours with TILs might be an interesting area of investigation for the immunotherapy options that are emerging in this setting. Although rather limited by the small number of patients, it is interesting to notice how, in our analysis, all patients who had D-MMR gastric cancer and TILs expression had also low neutrophil/lymphocyte ratio (10/10), thus hypothetically suggesting a easily obtainable surrogate for a histological biomarker.

One potential limitation of our work is the relatively small sample size, coupled with the fact that the group of patients who have been enrolled into the analysis is comprised of a heterogeneous population with different types of metastatic gastric cancer (diffuse, intestinal, mixed subtypes)

and who may have already received or not curative surgery before the onset of metastatic disease. This may be at least in part due to the fact of this being a monocentric analysis: because of this, some potential bias regarding different treatment regimens used in the metastatic phase, different expertise of the primary treating surgeon and an overall difference in terms of "behaviour" from the oncologist who is primary responsible for patients' care, and who might be seen in multicentric analyses, are also lessened.

Our findings confirmed that MMR defective gastric tumours are a distinctive biological and clinical category and that TILs might be a relevant pathological feature also in the metastatic setting. Interestingly these 2 factors appeared associated, but still independently able to influence clinical outcome. On the other hand the evident correlation between MMR status and TILs led us to hypothesise that MMR defective gastric tumours might have an intrinsic antigenic activity potentially relevant for immunotherapy.

In our opinion MMR status should then represent a stratification factor for future clinical trials investigating treatment options in metastatic gastric cancer patients. This would let clinicians to individuate specific therapeutic opportunities in specific patients subset, hopefully improving global results. In particular the unexplored area of immunotherapy in MMR defective, TILs positive gastric tumours might allow a better investigation of this novel, although promising, strategy.

Human rights statement and informed consent
All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Informed consent or substitute for it was obtained from all patients for being included in the study.

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Figure Legend:

Figure.1: Overall survival for patients stratified accordingly to MMR status

Figure.2: Overall survival for patients stratified accordingly to TILs expression

Figure.3: Overall survival for patients stratified accordingly to lymphocytosis

Figure.4: Overall survival for patients stratified accordingly to neutrophil-to-lymphocyte ratio (NLR)

Figure.5: Overall survival analysis among the three subgroups identified by multivariate analysis