TITLE:

Correlation between mismatch repair deficiency, microsatellite instability and survival in the MAGIC trial

RUNNING TITLE:

Mismatch repair, microsatellite instability and survival in MAGIC

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PRESENTATIONS

The results relating to microsatellite instability in this manuscript have previously been presented at the ASCO Gastrointestinal Cancers Symposium 2015 (Smyth et al, Abstract 62).

The results relating to mismatch repair deficiency in the manuscript were presented at the ASCO General Meeting 2016 (Smyth et al, abstract 4064).

ABSTRACT

Importance: Mismatch repair deficiency (MMRd) and microsatellite instability (MSI) are prognostic for survival in many cancers and predict resistance to fluoropyrimidines in early colon cancer. However, the effect of MMRd/MSI in curatively resected gastric cancer treated with perioperative chemotherapy is unknown.

Objective: We examined the relationship between MMRd, MSI and survival in patients with resectable gastroesophageal cancer randomised to surgery alone or peri-operative epirubicin, cisplatin and 5-fluorouracil chemotherapy in the MRC MAGIC trial.

Design: Tumour sections were assessed for expression of MMR proteins MLH1, MSH2, MSH6 and PSM2. MSI status was determined using the Promega MSI Analysis System (NR-21, BAT-26, BAT-25, NR-24, MONO-27). The relationship between MSI, MMRd, and survival was assessed.

Setting: MAGIC trial participants who were treated with either surgery alone or peri-operative chemotherapy plus surgery for operable gastroesophageal cancer.

Main Outcome Measure: Interaction between MMRd/MSI status and overall survival

Results: Two hundred and fifty four patients (of 504) had MSI and MMR results available. The concordance rate for MSI-H/MMRd was 97.9%. Patients treated with surgery alone who were either MSI-H/MMRd had a median overall survival (OS) which was not reached (95% CI 11.5 – not reached months), compared to those who were neither MSI-H/ MMRd of 20.5 months (95% CI 16.7 – 27.8 months), HR 0.42 (95% CI 0.15 – 1.15), p= 0.092. In contrast, patients treated with chemotherapy plus surgery who were either MSI-H/MMRd had a median OS of 9.6 months (95% CI 0.1 - 22.5 months), compared to those who were neither MSI-H/ MMRd of 19.5 months (95% CI 15.4 – 35.2 months), HR 2.18 (95% 1.08 – 4.42), p= 0.03. The interaction of both MSI and MMRd with treatment arm was significant.

Conclusions and Relevance: In MAGIC, MMRd and MSI-H status were associated with a positive prognostic effect in patients treated with surgery alone and a differentially negative prognostic effect in patients treated with chemotherapy. If independently validated, MSI or MMRd status on pre-operative biopsies could be used to select patients for peri-operative chemotherapy.

INTRODUCTION

Gastric cancer is the fifth most common cancer and the third most common cause of cancer death globally.[1] In Western countries, patients with operable gastric or gastroesophageal adenocarcinoma frequently undergo neoadjuvant or peri-operative chemotherapy prior to surgical resection.[2,3] This adjunctive chemotherapy is associated with a modest benefit in terms of overall survival (OS) compared to surgery alone but also with toxicities including neutropenia and thromboembolic disease. Unfortunately, following optimal multimodality therapy approximately half of resected patients will relapse and die of their cancer. There are no validated prognostic or predictive biomarkers for gastroesophageal cancer patients who receive neoadjuvant treatment and current patient selection is based purely on pre-operative radiological staging.

Microsatellite instability (MSI) and mismatch repair deficiency are positively prognostic for survival in patients with Stage II colon cancer and negatively predictive for the efficacy of fluropyrimidine adjuvant chemotherapy in the same patient group.[4,5] As a consequence, mismatch repair protein status assessment is recommended by National Comprehensive Cancer Network (NCCN) and European Society for Medical Oncology (ESMO) guidelines for patients with resected stage II colorectal cancer prior to adjuvant chemotherapy.[6,7] For patients with gastric cancer the prognostic and predictive effect of microsatellite instability has been suggested in several studies.[8-11] However, these studies are all retrospective and each lacked a control group.

The UK MRC MAGIC trial was an open label, multicentre, phase III randomised trial comparing the effect of six cycles of peri-operative ECF (epirubicin, cisplatin and infused 5-fluorouracil) chemotherapy (3 cycles pre- and 3 cycles post- resection) chemotherapy plus surgery to surgery alone in patients with resectable gastroesophageal cancer.[2] Patients treated with peri-operative chemotherapy demonstrated improved OS compared to patients treated with surgery alone [5 year OS 36% vs 23%, HR 0.75, (95% CI 0.60-0.93) p=0.009]. As a result, peri-operative ECF chemotherapy became one standard treatment regimen for patients with resectable gastroesophageal adenocarcinoma. The objective of this work was to establish the proportion of patients with MSI-H or MMRd cancer in the MAGIC cohort and to evaluate whether the presence or absence of these biomarkers had a prognostic effect on survival in patients treated with either surgery alone or chemotherapy plus surgery in the MAGIC trial.

METHODS

Microsatellite instability assessment

Genomic DNA was extracted from macrodissected cancer and non-cancer tissue using the QIAamp DNA FFPE Tissue Kit (Qiagen, Germany). MSI status was determined using the Promega MSI Analysis System (NR-21, BAT-26, BAT-25, NR-24, MONO-27) (Promega, WI, USA). A detailed description of microsatellite instability assessment methodology is available in the supplementary methods section.

Tumours were classified as microsatellite stable (MSS) when all markers were stable, as MSI-L when only one marker was unstable and as MSI-H with minimum of instability in two markers[12]. The term "instability" in this context refers to the presence of an increased number of nucleotide repeats in tumour than in the non-tumour control DNA for each sample. MSI-L and MSS tumours were combined for analysis as per previous analyses in gastric cancer.[10,13]

Mismatch repair protein assessment

For mismatch repair protein immunohistochemistry 3- 4µm sections were prepared from the tissue microarray blocks and stained for MLH1, MSH2, MSH6 and PMS2. Please see supplemental methods section for a detailed description of immunohistochemistry methodology.

Loss of MMR protein expression (MMRd) was designated when none of the neoplastic epithelial cells showed nuclear staining while positive internal control nuclei (lymphocytes and stromal cells) were present in the immediate vicinity of the tumour infiltrate. Normal expression was defined as the presence of nuclear staining of tumour cells, irrespective of the proportion or intensity.

Tumour regression grading assessment

Two pathologists, who were blinded to the treatment arm, reviewed the slides from all cases and graded the pathological response using the Mandard tumour regression grading (TRG) system.[14] Differences in opinion were resolved by discussion.

Statistical methods

OS was calculated from surgery to death from any cause or last date of follow up.[2] Progression-free survival (PFS) was calculated from surgery to the first event (i.e., local recurrence or progression, distant recurrence, or death from any cause). Date of surgery

was selected as the baseline for biomarker analysis to reduce potential bias as only patients with a surgical specimen were available for inclusion. Analyses were mainly performed within treatment arms due to the differences in timing of surgery, to reduce potential bias in the estimates of effects. Interactions between treatment arm and biomarker status were used to highlight potential differences in prognostic effect, and were assessed using a Cox model. Date of surgery could not be confirmed for nine patients in the chemotherapy plus surgery arm and these patients were excluded from the survival analyses. Differences in OS by MSI and MMR protein status were assessed using the Kaplan Meier method and compared using Cox regression. The Cox model was univariate for MSI/MMRd status. All MMR proteins were assessed individually and as a group to include "any" MMR protein absent. A p-value of <0.05 was considered significant. All analyses were conducted using Stata version 14.

RESULTS

MSI prevalence and clinical characteristics

MSI results were available for 303 patients (of 456 resected patients). As the data are obtained from resection specimens, and analyses examine survival from the date of surgery, only patients who had surgery (456 of 503 enrolled in the MAGIC trial) are potentially included (Supplementary Figure 1).

There was no significant difference in survival between patients who had tissue available for MSI analysis and those who did not (p=0.48). Twenty (6.6%) and 2 (0.7%) patients were MSI-H and MSI-L respectively. The rate of D2 resection in MSI-H patients was 55% (vs 41% in the entire MAGIC trial population), proportions of D2 resections for MSI-H patients were similar in both arms. Resections were considered by the surgeon to be curative in comparable numbers of MSI-H patients treated with surgery and surgery plus chemotherapy.

All MSI-H tumours were located in the stomach vs. GEJ/oesophagus (p=0.043); 8.5% (20/234) of stomach cancers were MSI-H (Table1); site of tumour was not prognostic for survival. Patients with MSI-H tumours compared to MSS/MSI-L tumours were more frequently female and had an older median age. MSI-H tumours were more frequently Laurens intestinal histological subtype and less commonly had metastatic lymph nodes in the resection specimen. None of these differences were statistically significant. Forty-four percent of MSI-H patients were treated with post-operative chemotherapy, consistent with the proportion of patients in the total trial population.

MSI and pathological response to chemotherapy

No patient with an MSI-H cancer treated with chemotherapy had a significant pathological response as measured by Mandard TRG of 1 or 2 (vs. 3-5) in the resection specimen. In patients with MSS/MSI-L tumours treated with chemotherapy the proportion of patients with a TRG 1 or 2 response was 20/123 (16%), p=0.215 for comparison MSI-H vs MSS/MSI-L patients. The kappa between the two pathologists for TRG assessment was 0.64, which increased to 0.70 when TRG was grouped as TRG 1 and 2 (responders) versus TRG 3 to 5 (nonresponders).

MMRd prevalence and clinical characteristics

Mismatch repair protein assessment was performed in 288 cases for MLH1, 282 cases for MSH2, 281 cases for MSH6 and 273 cases for PSM2. The different numbers of cases assessable for each protein reflect exhaustion of tumour material in selected TMAs and resection blocks. All four mismatch repair proteins were assessable in 268 cases. MLH1 was absent in 15/288 cases (5.2%), PSM2 was absent in 17/273 (6.2%) cases, MSH2 was absent in 3/282 (1.1%) cases, MSH6 was absent in two cases (0.6%).

Association with of MMRd with clinicopathological characteristics was similar to that for MSI (Table 2).

MMRd and pathological response to chemotherapy

No patient with a mismatch repair protein deficient cancer treated with chemotherapy had a good pathological response to chemotherapy (defined as TRG 1 or TRG 2), compared to 14/100 (14%) of patients MMR proficient tumours (p=0.205 for comparison MMR proficient vs. deficient patients).

Correlation of MMR deficiency with MSI status

254 patients had both MSI and MMR results available. Of these, 15/17 MSI-H tumours had MMRd detected. Thirteen of fifteen MLH1 negative tumours (87%) with available MSI results had MSI-H compared to 4/239 (1.7%) of MLH1 positive tumours. This results in a sensitivity of MLH1 deficiency testing for prediction of MSI of 76.5% (50.1% to 93.2%) and a specificity of 99.2 % (97.0% to 99.9%). All patients with absent MSH2 and MSH6 were MSI high. Twelve of 16 (75%) patients with absent PSM2 and MSI results were MSI-H, compared to 4/236 (1.7%) of PSM2 positive tumours. Overall concordance between MSI-H and MMRd status was 97.6% (Supplementary Table 1).

Survival analysis

Microsatellite instability and survival

For patients treated with surgery alone OS for was better for MSI-H patients than MSS/MSI-L patients as median OS was not reached for MSI-H patients (95% CI 4.4 months – not reached); whereas median OS for MSS/MSI-L patients was 20.3 months (95% CI 16.7 - 27.7 months), HR 0.35 (95% CI 0.11 – 1.11), p = 0.076 (Figure 1).

For patients treated with chemotherapy plus surgery, overall survival was better for MSS/MSI-L patients [median OS 22.5 months (95% CI 16.1 – 42.1 months)]; whereas median OS for MSI-H patients was 9.6 months (95% CI 0.1- 21.9 months), HR 2.22 (1.02 – 4.85) p= 0.045 (Figure 1).

The p value for interaction between MSI and treatment for overall survival is 0.007.

Mismatch repair protein deficiency and survival

Patients treated with surgery alone who were deficient in any MMR protein had a median overall survival which was not reached (95% CI 4.4 months – not reached), for patients who were MMR proficient this was 20.7 months (95% CI 17.5 – 28.6 months), HR 0.40 (95% CI 0.13 – 1.26), p=0.118).(Figure 2) Patients treated with chemotherapy plus surgery who were deficient in any MMR protein had a median overall survival of 9.7 months (95% CI 0.2 – 42.4 months); for MMR proficient patients treated with chemotherapy median overall survival was 20.1 months (95% CI 15.5 – 35.7 months) (HR 1.62 (95% CI 0.81 – 3.26), p=0.176.

The p value for interaction between MMR protein status and survival was 0.038.

MSI and/or MMRd and survival

Patients treated with surgery alone who were either MSI-H or MMRd had better OS than patients who were neither MSI-H nor MMRd; median survival was not reached (95% CI 11.5 – not reached months) for MSI-H or MMRd group, compared to those who were MSS/MSI-L who had a median OS of 20.5 months (95% CI 16.7 – 27.8 months), HR 0.42 (95% CI 0.15 – 1.15), p= 0.092

Following treatment with chemotherapy plus surgery patients who were either MSI-H or MMRd had a median survival of 9.6 months (95% CI 0.1-22.5 months), compared to those who were neither MSI-H nor MMRd of 19.5 months (95% CI 15.4 – 35.2 months), HR 2.18 (95% 1.08 – 4.42), p= 0.03

Discussion

Our study is the first to report the differentially prognostic effects of MSI and MMR protein expression on survival in a randomised trial with a non-chemotherapy control arm for peri-operatively treated gastroesophageal cancer. We demonstrate that patients with either MSI-H or MMRd have superior survival to microsatellite stable or MMR proficient patients when treated with surgery alone, and conversely have inferior survival to microsatellite stable or MMR proficient patients when treated with peri-operative chemotherapy plus surgery. These findings are significant because if validated they suggest that patients who are MSI-H or MMRd may not benefit (or have a detriment) from peri-operative chemotherapy and may be better served by a surgery alone approach. As MSI or MMRd tumours comprise up to 10-20% of stomach cancers in some series this has the potential to impact on large numbers of patients.[15]

Our results are consistent with the results of similar previous Asian and Western retrospective studies demonstrating a significant positive prognostic effect of MSI-H status for patients with resected gastric cancer.[8,10,11,13] In our study, MSI and MMRd tumours were only detected in gastric cancer patients; this is commensurate with previous studies which demonstrate a low prevalence of MSI and MMRd in gastroesophageal junction and oesophageal tumours.[15,16] The consistent effect of MSI-H on prognosis is supported by a pooled analysis of 17 studies which demonstrates a HR for OS of 0.76 (95%CI: 0.65–0.88, P =.0003) and limited heterogeneity.[17] In contrast, there are much less data on the interaction between MSI status and chemotherapy. In this our results are comparable to the two largest retrospective Asian studies in which patients with resected gastric cancer were treated with post-operative fluoropyrimidine chemotherapy.[9,13] In these retrospective series, Stage II and III MSS patients derived a benefit from adjuvant 5FU based chemotherapy, whereas MSI-H patients did not. Although our analysis is post-hoc, our study is the first randomised trial with a control group to validate these findings

In colorectal cancer the predictive effect of mismatch repair protein status on benefit from adjuvant chemotherapy is limited to patients with stage II disease.[5] This is hypothesised to be due to the relatively small benefit attributable to adjuvant fluoropyrimidine therapy in Stage II colorectal cancer patients and to the postulated effects of mismatch repair deficiency on the DNA damage response to fluoropyrimidines.[18] Firstly, as the relative benefit of peri-operative chemotherapy for gastroesophageal cancer is greater than the benefit of adjuvant chemotherapy in stage II colorectal cancer and secondly, as cisplatin and epirubicin were used in the MAGIC trial in addition to 5FU, our results are possibly unexpected (although as data on complete nodal staging was absent in a substantial

percentage of patients we cannot definitively stage all patients) One potential explanation for this phenomenon is that the effect of MMRd on the DNA damage response to platinum compounds is differential based on the platinum analogue used.[19] MLH1 deficient cell line models have been reported to be relatively resistant to cisplatin but not oxaliplatin which in turn reflects the differences in platinum compounds used in the MAGIC trial and colorectal cancer. This circumvention of the DNA damage repair mechanism by oxaliplatin may have important clinical implications; since the MAGIC trial was presented, oxaliplatin has been determined to be clinically equivalent to cisplatin and has replaced it in many gastric cancer chemotherapy regimens.[20] A second hypothesis sidesteps the requirement for chemoresistance – MSI-H tumours are associated with a vigorous immune infiltrate which may be responsible for suppression of residual micrometastases following surgery.[21,22] It is possible that chemotherapy may have a negative effect on this immunosurveillance thus reducing the innate benefit of the hypermutated phenotype.

A potential limitation of our analysis is that the entire MAGIC cohort was not analysed, as we did not receive tissue from all patients; this impacts on the numbers analysed in our study, furthermore the low prevalence of MSI/MMRd and number of events limits the statistical reliability of these data, which as a post-hoc analysis should be considered exploratory. However, as survival was not significantly different in those who did not have tissue available for analysis we do not believe there is a significant bias. One potential confounder of our results is that MSI/MMRd tumors were more likely to be of Lauren's intestinal subtype, which may be associated with improved survival outcomes compared to the diffuse subtype.[23,24] However, in multivariate analysis of the MAGIC trial, histological subtype was not an independent predictor of overall survival.[25] As we analysed only resection specimens which were post-treatment in the chemotherapy arm of the study, in order to truly determine the predictive value of MMRd evaluation of biopsy specimens is required. However, there is no evidence that MMRd status changes following chemotherapy: the equivalent proportion of MMRd patients in both arms of the trial support this. There is an imperfect correlation between MMRd and MSI assessment in our study. This may be a result of interobserver variability in immunohistochemistry assessment, heterogeneity of biomarker expression in gastric cancer, the presence of normally translated but non-functional mismatch repair proteins in the setting of a missense MLH1 mutation, or other rare genomic defects resulting in MSI-H status with intact MMRd function such as POLE mutation.[2627,28] Although our overall concordance is high, other studies in gastric cancer have demonstrated lower sensitivities of MMR protein immunohistochemistry for detection of MSI-H mismatch repair deficiency.[29,30] For these reasons, a genomic rather than an immunohistochemistry approach may be preferred for gastric cancer patients. Finally, an alternative hypothesis is

that that MSI-H status might be associated with other molecular changes which predispose to chemotherapy resistance. In pre-clinical gastric cancer models, epigenetic changes such as methylation of *BMP4* are associated with platinum resistance.[31] Clinical data demonstrate that in neoadjuvantly treated gastric cancer patients those with lower levels of promoter gene methylation have improved survival compared to those with more frequent methylation.[32] Promoter methylation of *MHL1* has also been associated with inferior survival of patients with resected gastric cancer treated with oxaliplatin based adjuvant chemotherapy.[33] However, as MSI status is not reported in either of these series the independent contribution of epigenetic changes remains unclear.

In conclusion, we demonstrate for the first time in a randomised trial of operable gastroesophageal cancer patients treated with chemotherapy with a surgery only control group that the presence of MMRd is associated with a positive prognostic effect in patients treated with surgery alone, and a differentially negative prognostic effect in patients treated with chemotherapy plus surgery. If validated, this finding has the potential to improve patient selection for peri-operative chemotherapy, and spare a significant proportion of gastric cancer patients the toxicity of unnecessary treatment. We do not believe that these data justify a change in clinical practice, however we recommend prospective trial validation in order to ascertain the optimal perioperative treatment for MSI-H gastric cancer patients. In the light of the remarkable success of anti-PD1 therapies in MMRd colorectal cancer, alternative treatment strategies could be reasonably be investigated for these patients.[24]

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Table 1. Clinicopathological characteristics MSS/MSI-L vs MSI-H patients				
		MSS + MSI-L N= 283	**MSI-H N= 20**	**p-value**
Age	**Median**	62	66	0.179
	IQR	54 - 69	60 – 69	
	Range	23 - 79	36 - 76	
Sex	**Male**	219 (77%)	14 (70%)	0.449
	Female	64 (23%)	6 (30%)	
Site of tumour	**Stomach**	214 (76%)	20 (100%)	0.043
	Oesophagus	37 (13%)	0 (0%)	
	OG Junction	32 (11%)	0 (0%)	
Histology	**Diffuse**	75 (27%)	2(10%)	0.214*
	Intestinal	163 (58%)	15 (75%)	
	MI + diff/other	35 (12%)	2 (10%)	
	Missing	10 (4%)	1 (5%)	
T-stage	**T1**	12 (4%)	0	0.122*
	T2	88 (31%)	11 (55%)	
	T3	169 (60%)	8 (40%)	
	T4	5 (2%)	0	
	Missing	8 (3%)	1 (5%)	
N-stage	**N negative**	54 (19%)	6 (30%)	0.161*
	N positive	156 (55%)	8 (40%)	
	Missing	73 (26%)	6 (30%	

*excluding those with missing data

Table 2. Clinicopathological characteristics MMRd vs MMR proficient patients				
		MMR proficient N=246	**Any MMRd N=22**	**p-value**
Age	**Median**	61	66	0.194
	IQR	54 - 69	61 - 68	
	Range	23 - 79	36 - 76	
Sex	**Male**	190 (77%)	18 (81%)	0.621
	Female	56 (23%)	4 (18%)	
Site of tumour	**Stomach**	183 (74)	22 (100)	0.025
	Oesophagus	34 (14%)	0 (0)	
	OG Junction	29 (12%)	0 (0%)	
Histology	**Diffuse**	67 (27%)	2 (9%)	0.063*
	Intestinal	138 (56%)	17 (77%)	
	MI + diff/other	32 (13%)	1 (5%)	
	Missing	9 (4%)	2 (9%)	
T-stage	**T1**	10 (4%)	0	0.123*
	T2	72 (29%)	11 (50%)	
	T3	151 (61%)	9 (41%)	
	T4	5 (2%)	0	
N-stage	**N negative**	51 (21%)	3 (14%)	0.855*
	N positive	135 (55%)	9 (41%)	
	Missing	60 (24%)	10 (45%)	

*excluding those with missing data

FIGURE LEGENDS

Figure 1. Overall survival by microsatellite instability (MSI) status and treatment arm in patients treated in the MAGIC Trial. Patients were dichotomized in two groups: MSI-H and MSS/MSI-L which are analysed separately in each treatment arm. Differences in overall survival were assessed using the Kaplan Meier method and compared using the log rank test. A p- value of <0.05 was considered significant.

Figure 2. Overall survival by mismatch repair (MMR) protein status in patients treated in the MAGIC Trial. Patients were dichotomized in two groups: MMR deficient (MMRd) and MMR proficient which are analysed separately in each treatment arm. Differences in overall survival were assessed using the Kaplan Meier method and compared using the log rank test. A p- value of <0.05 was considered significant.

Supplementary Figure 1: CONSORT diagram summarizing the analysis of microsatellite instability testing in the Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) Trial.

Supplementary Figure 2: Overall survival by MLH1 protein status in patients treated in the MAGIC Trial. Patients were dichotomized in two groups: MLH1 absent and MLH1 present which are analysed separately in each treatment arm. Differences in overall survival were assessed using the Kaplan Meier method and compared using the log rank test. A p- value of <0.05 was considered significant.

Supplementary Methods

A. Detailed PCR methodology

The PCR was performed on a 2720 Thermal Cycler (Applied Biosystems, CA, USA) using the following PCR cycling conditions; initial denaturation (11 min at 95°C, 2 min at 96°C), followed by 10 cycles denaturation (1 min at 94°C), annealing (1 min at 58°C) and extension (90 sec at 70°C), followed by 20 cycles denaturation (1 min at 90°C), annealing (1 min at 58°C) and extension (90 sec at 70°C). There was a final step of 30 min at 60°C then hold at 4°C. The samples were run on an Applied Biosystems® 3500 Genetic Analyzer and POP-7™ polymer and analysed using GeneMapper™ v5.0 software (Applied Biosystems, CA, USA).

B. Detailed tissue microarray construction methodology

Tissue microarrays (TMAs) were constructed from representative blocks from the trial specimens, and were composed of replicate 1-mm cores from each case and controls (i.e. samples of non-neoplastic kidney, liver, placenta, small bowel or normal stomach) as previously described.[14] In the case of TMA exhaustion further slides were cut from the resection block if possible.

C. Detailed slide preparation methodology

Sections were deparaffinised using EZ Prep solution (Ventana Corp.). CC1 standard (pH 8.4 buffer containing Tris/borate/ EDTA) was used for antigen retrieval and blocked with inhibitor D (3% H2O2) for 4 min at 37°C. Slides were incubated with primary antibody for 40 min at 37°C followed by a universal secondary antibody for 20 min at 37°C. Slides were incubated with streptavidin-horseradish peroxidase (SA-HRP) D for 16 min at 37°C followed by addition of substrate, 3, 3´-diaminobenzidine tetrahydrochloride (DAB) H2O2 for 8 min. The sections were counterstained by hematoxylin and bluing at 37°C

D. Detailed immunohistochemistry methodology

Immunohistochemical staining was performed using a VentanaXT automated platform (Ventana Corp., Tucson, AZ, USA) with antibodies to hMLH1 (ready to-use, cat number: 790-4535), hMSH2 (ready to-use, cat number: 790-4265), hMSH6 (ready to-use, cat number: 790-4455) and hPMS (ready to-use, cat number: 760-4531).

For cores that showed equivocal staining or suboptimal staining or where the tumour was insufficiently represented the tissue block from which the TMA cores had been derived were

retrieved. Whole sections from these blocks were tested using the following antibodies: mLH1 Leica clone ES05, used at a dilution of 1/200 epitope retrieval: HIER with ER2 solution for 40 min (high pH); mSH2 Leica clone 25D12, used at a dilution of 1/100 epitope retrieval: HIER with ER2 solution for 40 min (high pH); mSH6: Dako clone EP49, used at a dilution of 1/50 epitope retrieval: HIER with ER2 solution for 30 min (high pH) and PMS2 BD Pharmingen clone A16-4, used at a dilution of 1/300 epitope retrieval: HIER with ER2 solution for 40 min (high pH).

Supplementary results

A. Progression free survival analysis in MSI-H vs. MSS/MSI-L patients

Patients treated with surgery alone who were MSI-H had a median progression free survival which was not reached (95% CI 4.4 months – not reached), whereas patients who were MSS/MSI-L who were treated with surgery had a median PFS of 16.7 months (95% CI 13.7 – 20.5 months), HR 0.45 (95% CI 0.17 – 1.22), p= 0.116). In chemotherapy treated patients, progression free survival was 9.6 months (95% CI 0.07 – not reached) for patients who were MSI-H versus 20.5 months for those were MSS/MSI-L, HR 2.07, 95% CI 0.9 – 4.79, p=0.088. See supplementary figure 1.

B. Progression free survival analysis in MMRd vs MMR proficient patients

For patients who were mismatch repair deficient in any protein and treated with surgery alone median progression free survival was not reached (95% CI 4.4 months – not reached), whereas this was 17.4 months (95% CI 14.1 months – 23.7 months for patients who were intact for all mismatch repair proteins, HR 0.51 (95% CI 0.19 – 1.38, p= 0.186).

For chemotherapy treated patients who were mismatch repair deficient median progression free survival was 15 months (95% CI 0.2 – 42.4 months) compared to for mismatch repair proficient patients treated with chemotherapy who had a median progression free survival of 19.6 months (95% CI 15.5-35.2 months), HR 1.47 (95% CI 0.71 – 3.07), p=0.301.

C. Overall survival analysis according to specific MMRd protein

For patients treated with surgery alone, those with MLH1 deficiency had better OS than MLH1 proficient patients as median OS was not reached for MLH1 deficient patients (95% CI 4.4 - not reached months); whereas this was 20.3 (95% CI 16.7 – 27.8 months) months for patients who were MLH1 proficient (HR 0.18 (95% CI 0.02 – 1.27), p=0.085) (supplementary figure 2). Patients treated with surgery alone who were PSM2 deficient had a median OS which was not reached (95% CI 4.4 months – not reached), those who were PSM2 proficient had a median OS of 20.5 months (95% HR 16.7 – 28.6), HR 0.40 (95% CI 0.10-1.63), p=0.203. Median OS for patients with MSH2 and MSH6 deficiency was not calculable due to small numbers of patients in these groups (two and one patients respectively and no deaths).

For patients treated with chemotherapy plus surgery, those with MLH1 deficiency had a median survival of 7.2 months from surgery (95% CI 0.1 – not reached), compared to those with intact MLH1 who had a median OS of 22.3 months (95% CI 16.1-35.7 months) HR 2.73 (95% CI 1.25 – 5.96), p=0.012 (Supplementary Figure 2). Patients treated with

chemotherapy plus surgery who demonstrated PSM2 deficiency had a median OS of 9.6 months (95% CI 0.1 – not reached) compared to 20.1 months (95% CI 15.4 – 35.7 months) for patients who were PSM2 proficient (HR 1.90 (95% CI 0.91-3.97) p= 0.087 . Median OS for patients with MSH2 and MSH6 deficiency was not calculable due to small numbers of patients in these groups (each had one patient and one death).

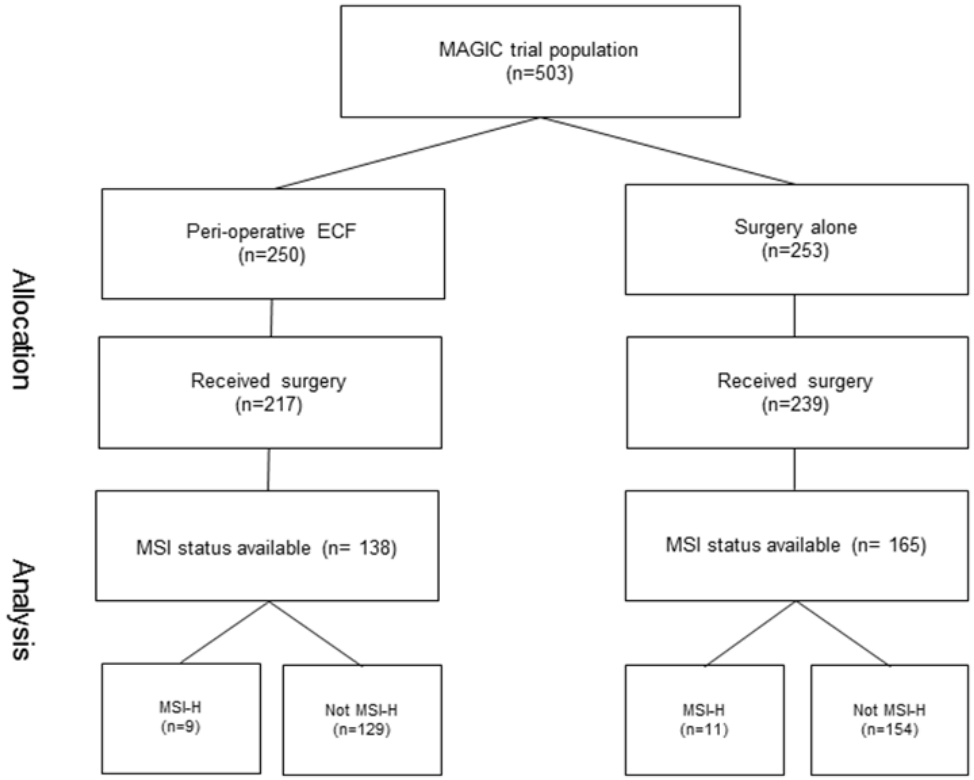
The interaction test between MLH1 and treatment arm was 0.01

D. Supplementary Table 1

Supplementary Table 1: Association between MSI-H and MMRd results								
	MLH1		**MSH2**		**MSH6**		**PMS2**	
	Absent	Present	Absent	Present	Absent	Present	Absent	Present
MSI-H	13	4	2	15	1	16	12	4
MSS/MSI-L	2	235	0	237	0	237	4	232

* different numbers for each assay due to exhaustion of tissue blocks

E. Supplementary Figure 1

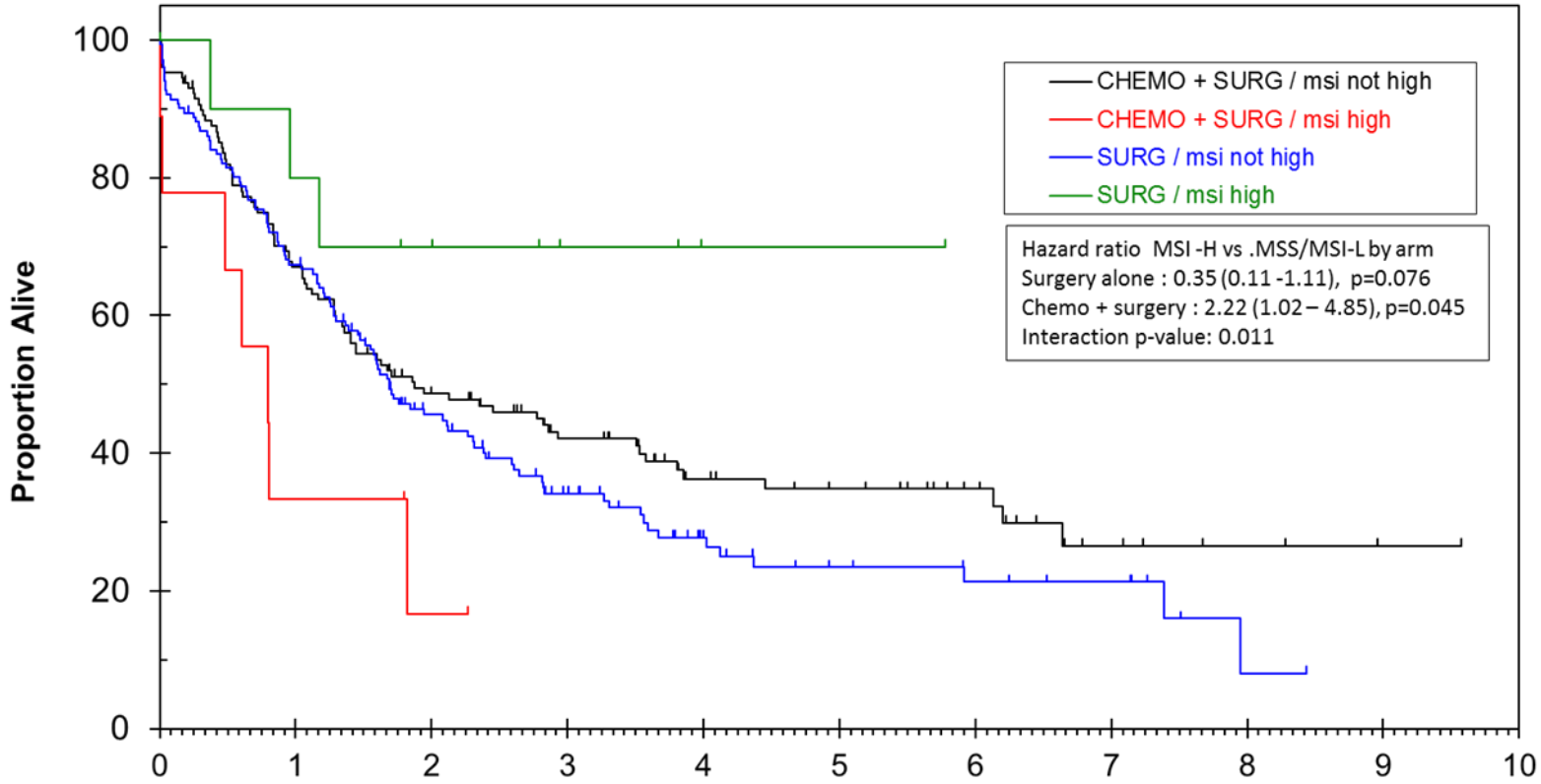


Abbreviations: ECF, epirubicin, cisplatin and capecitabine; MSI-H, microsatellite instability high

F. Supplementary Figure

OS MLH1.eps

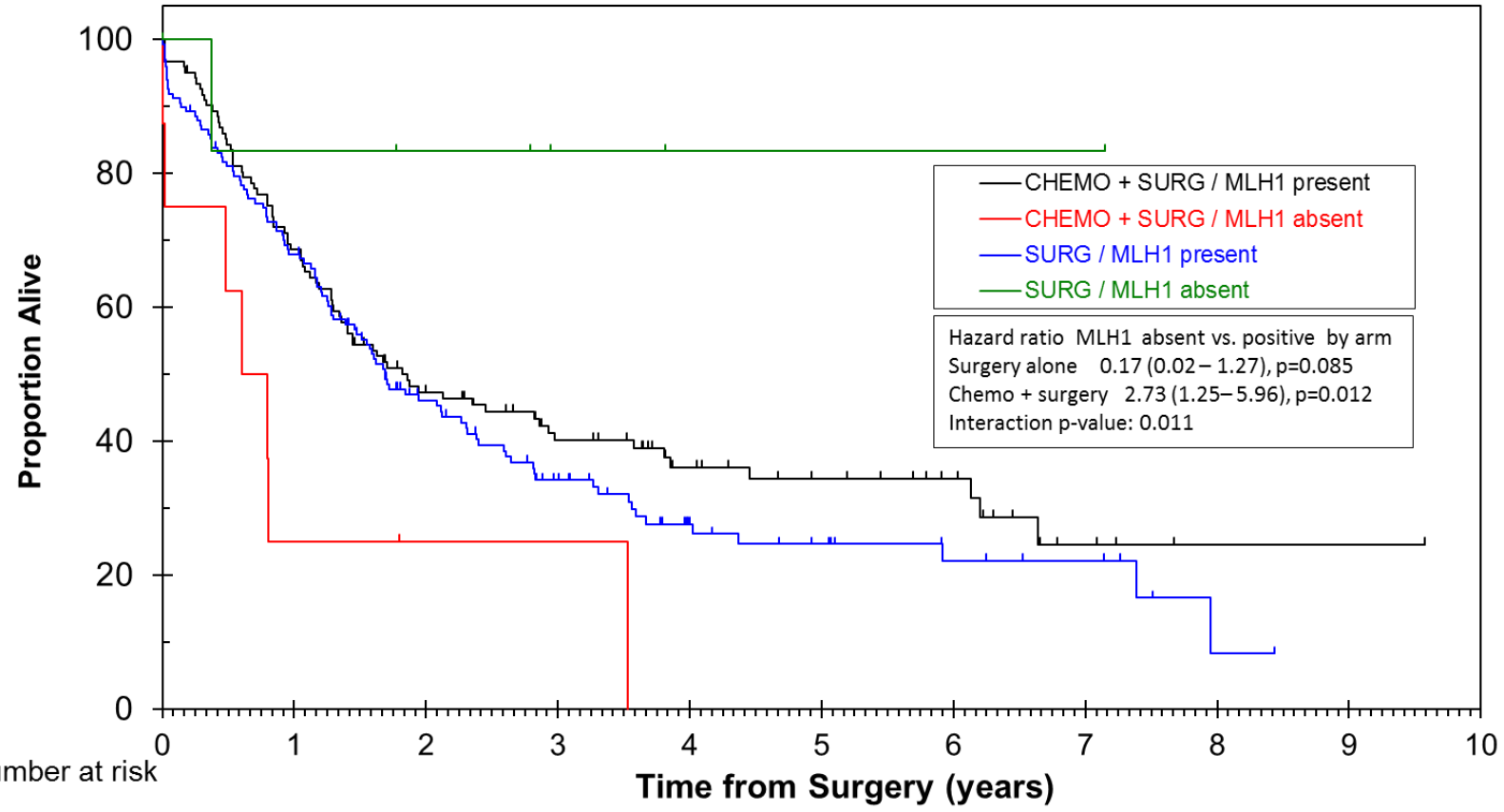
Figure 1: Overall survival by treatment arm & MSI status



Number at risk

CHEMO MSI -	129	85	58	42	27	22	15	6	3	1
CHEMO MSI +	9	3	1							
SURG MSI -	154	100	58	37	21	13	9	7	1	
SURG MSI +	11	8	6	3	1	1				

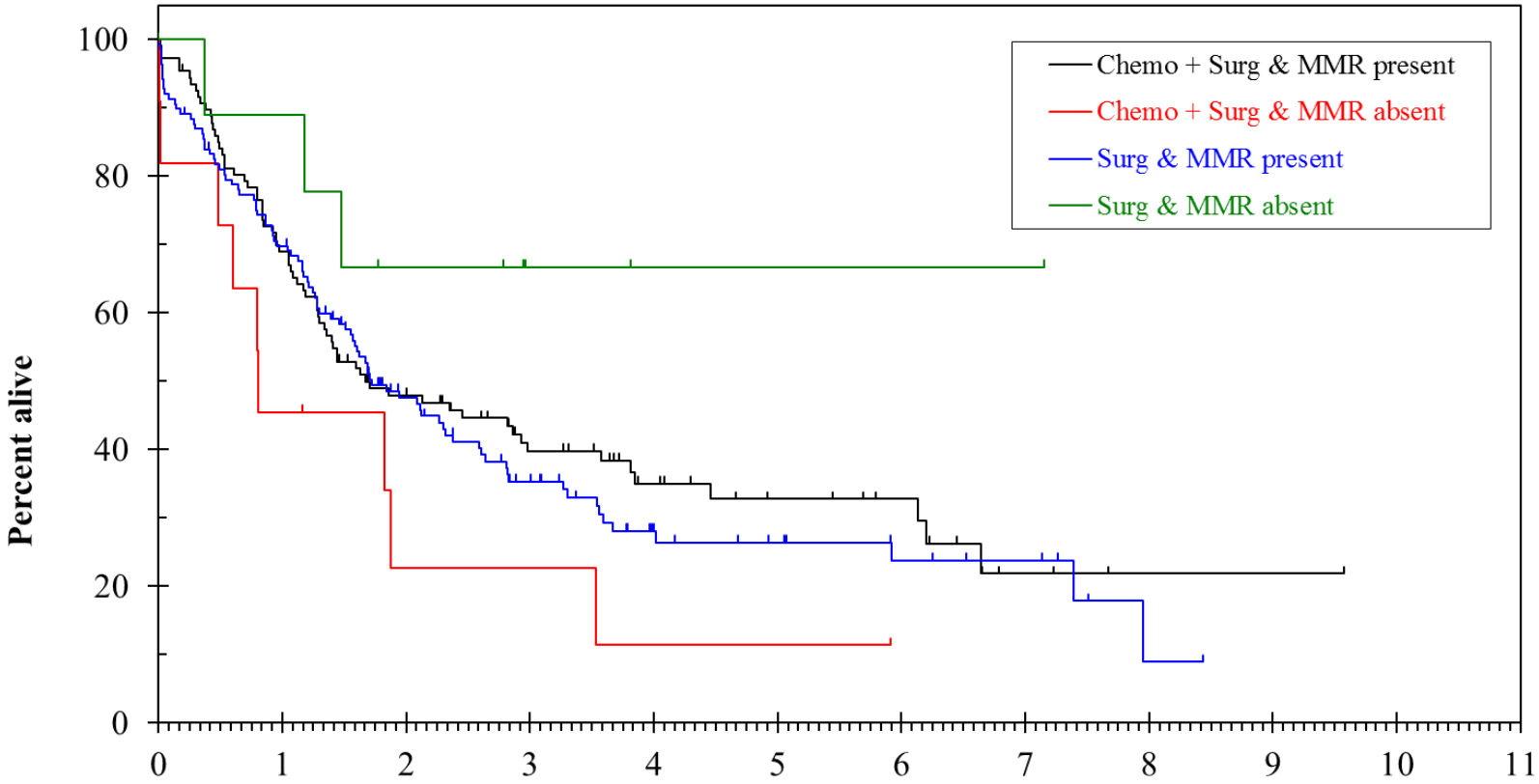
Figure 2:Overall survival by treatment arm & MLH1 status



Number at risk

CHEMO MLH1+	122	83	52	37	24	18	13	4	1	1
CHEMO MLH -	8	2	1	1						
SURG MLH1 +	151	98	56	36	20	14	8	6	1	
SURG MLH1 -	7	5	4	2	1	1	1	1		

Figure X: Overall survival by MMR status and treatment arm



Number at risk										
C +S & MMRp	107	73	47	32	19	13	10	3	1	1
C + S & MMRd	11	5	2	2	1	1				
Surg & MMRp	140	93	52	34	18	13	8	6	1	
Surg & MMRd	10	8	5	2	1	1	1	1		