Clinical effects of Adjuvant Active Specific Immunotherapy differ between Patients with Microsatellite Stable and Microsatellite Instable Colon Cancer

Vincent A. de Weger*1 MD, Annelies W. Turksma*1 MSc, Quirinus JM Voorham*1 MSc, Zelda Euler*1 MSc, Herman Bril2 MD PhD, Alfons J van den Eertwegh3 MD PhD, Elisabeth Bloemena1 MD PhD, Herbert M. Pinedo1 MD PhD, Jan B Vermorken4 MD PhD, Harm van Tinteren5 PhD, Gerrit A. Meijer1 MD PhD, Erik Hooijberg1 PhD.

1 Department of Pathology, VU University Medical Center, de Boelelaan 1117, NL-1081 HV Amsterdam, the Netherlands.
2 Department of Pathology, Kennemer Gasthuis, Boerhaavelaan 22, NL-2035 RC Haarlem, the Netherlands.
3 Department of Medical Oncology, VU University Medical Center, de Boelelaan 1117, NL-1081 HV Amsterdam, the Netherlands.
4 Department of Medical Oncology, Antwerp University Hospital, Wilrijkstraat 10, BE-2650, Edegem, Belgium.
5 The Netherlands Cancer Institute, Plesmanlaan 121, NL-1066 CX Amsterdam, the Netherlands.

* These four authors contributed equally to this work.
Corresponding author:
Erik Hooijberg PhD, De Boelelaan 1117, NL-1081 HV Amsterdam, the Netherlands. PO-box 7057, NL-1007 MB Amsterdam, The Netherlands. Phone number 0031 20 4444041 Email: erik.hooijberg@vumc.nl

Acknowledgements

The authors acknowledge the support of the following pathologist from participating hospitals in retrieving the FFPE material: dr M. Flens, Zaans Medisch Centrum, Zaandam; dr M. Brinkhuis, Stichting Laboratorium Pathologie Oost, Enschede; dr H. Doornewaard, Gelre Ziekenhuis, Apeldoorn; dr A. Uyterlinde, Medisch Centrum Alkmaar, Alkmaar; dr E. Barbé, St Lucas, Amsterdam. The authors would also like to thank the reviewers for their remarks and useful suggestions.

This study was financially supported by a grant from the Dutch Cancer Society (KWF/NKB grant number VU2007-3814), Amsterdam, to A.W. Turksma and a grant from the “Honours Program” of the VU University Medical Center, Amsterdam, to V.A. de Weger.

Previous Presentations and Publications.

Oral presentation at the Dutch Tumor Immunology Meeting June 2010, Breukelen, the Netherlands, no abstract book.
Oral presentation at the European Workshop on Cytogenetics and Molecular Genetics of Solid Tumors, June 2010, Nijmegen, the Netherlands, abstract published in Cancer Genetics and Cytogenetics November 2010, 203(1); 52.

Poster presentation at the 2010 AACR meeting, April 2010, Washington DC, USA, abstract in meeting abstract book.


List of abbreviations: active specific immunotherapy (ASI), microsatellite (MS), microsatellite instable (MSI), microsatellite stable (MSS), miss match repair (MMR), colorectal cancer (CRC), formalin-fixed and paraffin-embedded (FFPE).
Abstract

**Purpose:** Active Specific Immunotherapy (ASI) consisting of an autologous tumor cell vaccine given as adjuvant treatment has been shown to improve recurrence free survival of patients with colon cancer (Vermorken et al, Lancet 1999). The aim of the current retrospective study was to investigate whether the beneficial effects of ASI given as adjuvant treatment correlated with microsatellite instability (MSI) which is considered an important biological determinant of colon cancer.

**Experimental design:** Microsatellite status was assessed on archival tumor material from stage II/III colon cancer patients. Microsatellite status was next associated with clinical outcome in control and ASI treatment groups using Kaplan-Meier analysis.

**Results:** We identified 162 (83%) microsatellite stable tumors (MSS) and 34 (17%) microsatellite unstable tumors. Patients with MSI tumors performed well in RFI as well as disease specific survival (DSS) irrespective of treatment arm and tumor stage. Patients with MSI tumors had significantly less recurrences and prolonged DSS than those with MSS tumors. Patients with MSS Dukes B tumors who received ASI treatment showed a significantly improved recurrence free survival compared to controls. ASI treatment did not improve RFI or DSS for patients with MSS Dukes C tumors.

**Conclusion:** This retrospective study indicated that patients with MSI tumors performed well, irrespective of treatment arm and tumor stage. The data also indicate that the clinical benefit, measured as recurrence free survival, from
adjuvant ASI treatment of patients with colon cancer was restricted to patients with MSS Dukes B tumors.

**Translational relevance**

The majority of colorectal cancers are microsatellite stable (MSS). Whereas a smaller portion (about 15%) of CRC tumors show defects or complete failure of DNA mismatch repair systems and are called microsatellite instable (MSI). The clinical behavior of MSS colon cancer appears to be different from that of MSI cancers. Information about response to (adjuvant) immunotherapy of MSS versus MSI colon cancer was however lacking. Here, we investigated this retrospectively, making use of FFPE material derived from patients who previously participated in an adjuvant Active Specific Immunotherapy trial. From our analyses we concluded that patients with MSI tumors performed well in RFI and DSS, irrespective of tumor stage and treatment arm. Furthermore adjuvant ASI was of benefit for patients with stage II MSS tumors but not stage III. These data provide support for a differentiated approach for future development and clinical testing of adjuvant treatment of colon cancer patients.
Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide, accounting for more than 1 million cases and 600,000 deaths every year. Genomic instability is a key feature of cancer and two main types occur in CRC. About 80-85% of CRCs show chromosomal instability but have functioning DNA mismatch repair systems. These tumors are called microsatellite stable (MSS). Whereas 15-20% of CRC tumors show defects or complete failure of DNA mismatch repair systems and are called microsatellite instable (MSI) (1).

MSS and MSI CRCs differ in many of their biological and clinical features. MSS tumors show aneuploidy, allelic losses, amplifications, translocations and chromosomal gains (2-4). This genetic instability may influence the expression of genes important in the carcinogenesis of CRC, like DCC, SMAD4 (5). Genetic instability may also lead to an increased mutation rate in protein coding sequences potentially giving rise to immunogenic peptides or epitopes (6). Nonetheless MSS tumors are in general not heavily infiltrated by tumor specific T lymphocytes (7;8).

MSI CRCs, on the other hand, are the result of defects or complete failure of the DNA mismatch repair system. Most MSI CRCs are sporadic due to promoter hypermethylation of MLH1. A small sub-set occurs due to germ line mutations in MLH1, MSH2, and/or MSH6, giving rise to the hereditary Lynch syndrome/HNPCC, of which CRC is the most prominent phenotype (9).
the DNA miss-match repair system can lead to frame shift mutations in protein coding sequences, which in turn can lead to the formation of neo-antigens. The exact location of frame shift mutations has been documented for a number of genes e.g. TGF-β receptor II and AIM2. Frame shift mutations have been detected in 60-70% of MSI tumors (10;11). Resulting frame shift peptides have been shown to trigger the immune system in vitro and tumor infiltrating lymphocytes appear to be activated and cytotoxic (8;10-12). Based on histology, MSI CRC show heavy lymphocytic infiltrates at the periphery of the tumor (referred to as Crohn’s like infiltrate) and/or tumor infiltrating lymphocytes. In general, the level of lymphocytic infiltrate has been recognized as a predictor of clinical outcome in CRC (13).

The primary and so far only curative form of therapy in CRC is surgery. Patients at increased risk of disease recurrence, e.g. presence of lymph node metastasis, receive adjuvant chemotherapy. The drug 5-FU has been standard adjuvant treatment for decades, nowadays most often combined with oxaliplatin (14;15). Several studies have indicated that MSI CRC would not benefit from 5-FU based adjuvant chemotherapy, an issue still under debate (16;17).

In addition to adjuvant chemotherapy, immunotherapy has been explored in patients with colon cancer. The study by Vermorken and coworkers, was a multi-center clinical trial on Active Specific Immunotherapy (ASI) for patients with stage II/III (Dukes B/C) colon cancer (18). We will refer to that study throughout the rest
of the text as the original study. The vaccine consisted of irradiated autologous tumor cells admixed with the adjuvant Bacillus Calmette-Guérin bacteria. Aim of the original study was to test whether adjuvant ASI therapy was beneficial in patients with stage II and III colon cancer after surgical resection, in comparison to surgical resection alone. ASI therapy resulted in a significant extension of recurrence free survival for stage II (Dukes B) colon cancer patients at the standard 5 year evaluation point. No statistical significance, however, was reached in stage III (Dukes C) colon cancer patients (18).

At the time the ASI trial was conducted, awareness on the biological heterogeneity of colon cancer and its possible clinical implications was still limited. Since histology clearly shows differences in immune response between MSI and MSS colon tumors, microsatellite status could well affect the outcome of therapies that actually strive to modulate the immune response. Aim of the present study was therefore to retrospectively investigate the association between response to ASI treatment and microsatellite status in colon cancer.
Patients, Materials and methods

The patient population, inclusion criteria and vaccination protocol have been described in detail previously (18). In summary, eligible patients with stage II or stage III resectable adenocarcinoma of the colon and a good performance status were randomly assigned postoperative ASI or no adjuvant treatment.

For the current study, tumor samples for DNA isolation and subsequent microsatellite (MS) analysis were available as frozen single cell suspensions or formalin-fixed and paraffin-embedded material (FFPE). Residual single cell suspensions were not always available from all patients, in particular of the patients who received multiple vaccinations and booster injections no single cell suspensions remained. In those cases we had to make use of FFPE material. In order to validate the procedure of determining micro satellite (in)stability we performed a number of experiments where single cell material and FFPE material from one and the same patient was used. There was complete concordance between the findings with single cell suspensions and FFPE material in the five cases tested. Hence we were confident that we could make use of FFPE material for the remainder of the study.

Samples from single cell suspensions were washed with phosphate buffered saline, cleansing it from dimethylsulfoxide. The cells were then re-suspended in phosphate buffered saline and cellular DNA was isolated with the high pure PCR template Preparation Kit (Roche, Mannheim, Germany) prior to MS analysis. FFPE material was collected from the different hospitals that previously
participated in the clinical trial, and DNA was isolated as described before (4;19).
In summary one section of 4-µm thick was taken and hematoxylin/eosin stained.
In this section the area containing the highest amount of tumor cells was marked
by a pathologist. This section was then used as reference slide to guide macro-
dissection on a series of 5 hematoxylin stained sections of 10 µm. The material
was incubated overnight in 1M NaSCN. After washing, an overnight incubation
with lysis buffer (ATL buffer, QIAmp, DNA micro-kit, Qiagen, Venlo, The
Netherlands) and proteinase K (10 µl of 20 ng/µl) was performed. After the
proteinase K incubation, DNA was isolated using a column based method
(QIAmp, DNA micro-kit, Qiagen, Venlo, The Netherlands). Concentrations and
purities of DNA were measured on a Nanodrop ND-1000 spectrophotometer
(Isogen, IJsselstein, The Netherlands).

For DNA micro satellite analyses of the single cell suspension and the FFPE
material the Promega MSI Analysis System (Version 1.1 and 1.2 Promega,
Madison, United States) was used according to the manufacturer’s instructions.
The protocol makes use of 5 quasi-monomorphic mononucleotide markers (Bat-
26, Bat-25, NR-21, NR-24 and MONO-27). PCR products were separated by
capillary electrophoresis using an ABI 3130 DNA sequencer (Applied Biosystems,
Foster City, CA, USA), and analyzed using GeneScan 3100 (Applied Biosystems,
Foster City, CA, USA), which gives 100% sensitivity for the MS status, obviating
the need to include normal tissue. Tumors showing instability in two or more
markers were designated MSI and tumors with none or one instable marker were designated MSS (20-22).

**Data Analysis**

Data management and initial statistical analyses were performed by an independent monitoring agency (IKA, Comprehensive Cancer Center, Amsterdam). First, analyses were performed to verify that the patient group in the current study was a fair representation of the patients in the original study. Association of MS-status and tumor characteristics were explored by means of chi-square tests or trend tests (Cochran-Armitage Trend Test) in case of ordered categories. Recurrence free survival, defined as time from randomization to recurrence or death, was investigated by means of the Kaplan-Meier analyses and groups were compared by log-rank tests. Survival curves were considered significantly different if the p value is lower than 0.05. Cox-proportional Hazards analyses were performed to test for interaction between several parameters including treatment and MSI-status. Statistical analyses were performed using Microsoft Excel 2003 or Graphpad Prism V5.
Results

Comparison of the current patient sub-set and the original study population

Tumor samples, either single cell suspensions or FFPE material, could be retrieved and analyzed from 196 patients (77%) of the 254 patients included in the original study. Of the remaining 58 cases (23%), 37 samples were not traced back, 4 samples did not contain sufficient tumor tissue anymore, and 17 samples yielded DNA of insufficient quality (Figure 1). Analysis by tumor location, differentiation grade, stage and study arm did not reveal significant differences between the current and original study population (data shown in Supplementary Figures 1, 2, 3 and 4).

Event free survival in the ASI treatment group and the control group was first analyzed at the standard five year evaluation point. Event free survival was comparable for the current subset of 196 patients and the original study population of 254 patients ((current study ASI versus Control at 5 year: HR=0.52 (95% CI: 0.31-0.86), log rank p=0.012) versus (Original study ASI versus Control at 5 year: HR=0.54 (95% CI: 0.34-0.85), log rank p=0.008), respectively).

Next we extended the analysis to 15 year follow up. The event free survival data are presented as Kaplan-Meier plots in Figure 2 for the current MSI/MSS study (subset of 196 patients) and in Figure 3 for the original study, (all 254 patients). Event free survival was comparable for the current subset of 196 patients and the original study population of 254 patients (Current study ASI versus Control at 15 year: HR=0.57 (95% CI: 0.34-0.94), p=0.027) versus ((Original study ASI versus Control at 15 year: HR=0.62 (95% CI: 0.40-0.96), p=0.033), respectively)). Based
on these results we were confident that a selection bias was not introduced for the subset of 196 patients available for the MSI/MSS analysis.

**Micro Satellite status and Recurrence Free Interval over an extended 15 year follow up period**

Of the 196 tumors in the current study, 34 (17%) were MSI and 162 (83%) were MSS (shown in Figure 1). The ASI and control groups of patients with MSS tumors were large and comparable in size, whereas these were smaller and of different size in the patient group with MSI tumors. In the statistical analysis this sometimes resulted in lack of power, especially where a multitude of parameters were included in the comparisons (e.g. MSS, MSI, ASI, Control, and tumor stage II or III).

The RFI data presented in Figure 4A show a clear difference between patients with MSI tumors versus patients with MSS tumors irrespective of treatment group and tumor stage (Dukes A, B, C, or D). Patients with MSI tumors (n=34) showed a significantly better overall recurrence free survival than those with MSS tumors (n=162) at the standard five year evaluation point (MSI versus MSS at 5 years: HR=0.47 (95% CI: 0.24 – 0.91) log rank p-value 0.03) as well as at the extended 15 year follow up (MSI versus MSS at 15 years: HR=0.45 (95% CI: 0.24 – 0.86) log rank p-value 0.016). The RFI data for stage II/III (Dukes B/C) patients with MSI (n=34) versus MSS (n=154) tumors are shown in Figure 4B. The patient group with (stage II/III) MSI tumors clearly performed better than the patient group with (stage II/III) MSS tumors irrespective of treatment arm.
Micro Satellite status and response to ASI treatment in Dukes B and Dukes C.

The original Vermorken study showed significant differences in response to ASI treatment between patients with tumor stage II (Dukes B) and stage III (Dukes C). Careful re-review of the available RFI data in the current study revealed interesting information, although the analysis was somewhat hampered by the low numbers of MSI patients in the current study. For reasons detailed in the discussion section, these numbers can not be raised.

Analysis on RFI for the combined stage II/III (Dukes B/C) subgroups showed a significant difference between MSI and MSS in favor of MSI (as shown in Figure 4B). After separating tumor stages in the analysis, significance was lost in stage II (Dukes B) only (MSI stage II versus MSS stage II: HR=0.43, 95% CI 0.18-1.0, p=0.067) and also in stage III (Dukes C) only (MSI stage III versus MSS stage III: HR=0.60, 95% CI 0.20-1.8, p=0.35) due to the low numbers in the MSI subgroups (Kaplan-Meier data not shown).

Ignoring tumor stage in comparing RFI for MSI ASI (n=14) versus MSI Control (n=20), the HR=0.72, 95% CI 0.01 – 5.26, and log rank p= 0.75 values clearly indicated a lack of significant difference between MSI ASI versus MSI Control (Kaplan-Meier data not shown). Ignoring treatment in comparing RFI for MSI Dukes A/B (n=27) versus MSI Dukes C (n=7), the HR=0.17, 95% CI 0.01 – 1.8, and log rank p= 0.15 values also indicated a lack of significant difference between MSI Dukes A/B versus MSI Dukes C (Kaplan-Meier data not shown).
Based on these analyses we concluded that the data points from the four different MSI groups could be taken together for further comparison with the four different MSS subgroups.

In Figure 5 and Supplementary Table 1, the data on RFI are shown for the combined (stage II/III, control/ASI) MSI group and for the four different MSS groups; MSS Dukes B control, MSS Dukes B ASI, MSS Dukes C control and MSS Dukes C ASI (see the legend to the figure for the numbers of censored subjects and the numbers of events). From these data it is clear that the MSI group and the MSS Dukes B ASI group performed best. No significant difference was found between these two groups. On the other hand significant differences were found between these two best performing groups and the other three MSS subgroups (MSS Dukes B control, MSS Dukes C control and MSS Dukes C ASI). No significant differences were found between the latter three groups.

In Figure 6 Kaplan-Meier plots are shown for Disease Specific Survival (DSS) after review of the data over an extended period of follow up of 15 years. Again the combined MSI group (MSI stage II/III, control/ASI) performed best together with the MSS Dukes B ASI group. Surprisingly the group of patients with MSS Dukes B in the control arm gained in DSS, where it lagged behind in the RFI. The two groups of patients with MSS Dukes C performed relatively poorly again irrespective of adjuvant ASI treatment. Supplementary Table 2 shows all the HR, 95% CI and p values for the DSS data presented in Figure 6.
Discussion

Active Specific Immunotherapy has been explored in patients with colon cancer by Vermorken and co-workers (18). Available tumor samples, in the form of single cell suspension or FFPE material, from a large proportion (77%) of the original patient group has been used in the current study. We evaluated the selection based on availability of tumor material derived from the 196 patients in the current study. The data presented in Figures 2 and 3 and the supplemental Figures 1, 2, 3, and 4 clearly indicated a lack of significant difference between the patient population in the original study and the current study. Hence we were confident a selection bias was not introduced. We next identified patients with either microsatellite instable or microsatellite stable tumors. The percentages of MSI tumors (17%) versus MSS tumors (83%) we found was in the same range as one would expect on the basis of data published by others (17;23;24).

The current retrospective study was limited by the number of patients that could be included, especially for the MSI ASI (n=14) and MSI control (n=20) groups. As indicated in Figure 1, FFPE material from 23% of the original participants was not available (n=37) or of insufficient quality (n=21). Given the current loss rate of 10% due to insufficient quality and the percentage of MSI tumors in our patient population of 17%, no more than 5-6 MSI tumor samples could hypothetically have been added. Since these MSI samples would probably be divided equally
between treatment groups and tumor stages it is not expected that this would give significant differences in the outcome of the current study.

The aim of the current retrospective study was to investigate whether the beneficial effects of ASI given as adjuvant treatment correlated with microsatellite instability (MSI). From our analysis it became clear that patients who received adjuvant ASI treatment had an improved recurrence free survival, when compared to patients receiving surgery alone, irrespective of MS status and tumor stage (Figure 2; n=196, ASI versus control at 15 years of follow up; HR 0.57, 95% CI 0.34 – 0.94, log rank p=0.027). This was equal to the results obtained for the patients in the original study (Figure 3; n=254, ASI versus control at 15 years of follow up; HR 0.62, 95% CI 0.4 – 0.96, log rank p=0.033). Furthermore it became clear that irrespective of adjuvant treatment and tumor stage the patient group with MSI tumors had a better recurrence free survival compared to the group with MSS tumors (Figure 4A; MSI versus MSS for all tumor stages (Dukes A, B, C, D) taken together, and Figure 4B; MSI versus MSS for tumor stages Dukes B and Dukes C taken together).

In the past we have documented delayed type hypersensitivity reactions to tumor-associated antigens in ASI treated colon carcinoma patients (25). We obtained MS status data from six out of ten patients in the DTH study. Five of these patients had an MSS tumor and one had an MSI tumor. All of these patients showed comparable DTH responses to the autologous tumor cells,
which is indicative for systemic anti-tumor reactivity (data not shown). Microscopic analyses by others have shown that MSI tumors are infiltrated by immune cells to a larger extent than MSS tumors (7;26). On the other hand tumor infiltrating CD3/CD8 positive lymphocytes have been suggested to play a role in antitumor immunity in colorectal cancer, irrespective of MSI-status (8). It has been documented that MSI tumors often contain frame shift mutations with immunogenic potential (11;27;28). Unfortunately limited availability of FFPE material in the current study precluded analysis of the extent of T cell infiltration in the tumor epithelium and stroma of patients with MSI or MSS tumors.

Given the low numbers of patients per MSI subgroup and the overall low number of events (4 events on a total of 34 patients) it was not surprising to see that significant differences in RFI were not found within the MSI group as detailed in the results section. The MSI group consisted of the following numbers of censored subjects and events per subgroup; MSI-Dukes A (Control; n=3, events 0; ASI n=0), MSI-Dukes B (Control n=12 events 2; ASI n=10, events 0), MSI-Dukes C (Control n=3, events 0; ASI n=2, events 2), MSI-Dukes D (Control and ASI n=0, no events). Although we do not have supporting data, one could imagine that immune surveillance is already quite active in patients with MSI tumors, thereby masking immune boosting effects of adjuvant vaccination. Raising the numbers of patients substantially would be the only way to proof or disproof the assumption that adjuvant ASI treatment would impact the clinical
outcome for patients with MSI tumors. Since the current study is retrospective in nature we will not be able to further substantiate this.

As we have shown and discussed, the group of patients with MSI tumors did well both in RFI and DSS, irrespective of treatment and tumor stage. Re-review and re-analysis of the available data on patients with MSS tumors clearly showed an effect on RFI of adjuvant ASI treatment for the MSS Dukes B patient group, but not for the MSS Dukes C group. DSS was also high in the MSS Dukes B ASI group of patients and indistinguishable from the MSI patients. Although we do not have direct proof, the immune system of patients with MSS Dukes B tumors has apparently been activated by adjuvant ASI, resulting in an extension of the recurrence free interval. The nature of antigens recognized by pre-existing and induced tumor specific T cells is unknown in these patients, but would probably consist of the normal range of over-expressed tumor antigens in addition to neo-antigens resulting from chromosomal translocations and point mutations in protein coding sequences. Oddly enough the gap seen in RFI between MSS Dukes B ASI versus MSS Dukes B control has disappeared in the disease specific survival. From the data it is suggested that a number of patients (ten in total) in the MSS Dukes B control group experienced a recurrence but did to die from that recurrence. However a closer look at the data revealed that three of these ten patients were lost in follow up relatively soon, three died from disease unrelated courses, leaving four unexplained cases that might have undergone secondary surgery. Secondary surgery of liver metastasis was shown to be
beneficial for patients previously diagnosed with colon cancer (29). It is unknown whether these patients in the original study have indeed undergone secondary surgery in either of the many participating hospitals, since these data were not included in the original database.

From the data presented here we concluded the following; 1) The group of patients with MSI tumors performed well; 2) A potential therapeutic benefit of ASI in patients with MSI tumors remained undetected; 3) Patients with MSS Dukes B ASI gained quality of life since there was an extension in recurrence free interval compared to the control group, despite the notion there was no statistically significant impact on DSS; and 4) The group of patients with MSS Dukes C did not benefit from adjuvant ASI treatment at all.

We did not detect an impact of the autologous tumor vaccine in patients with MSI. It could well be that the immune system has already focused a therapeutic response against the MSI tumor so the addition of a therapeutic vaccine may not give the same effect size as in patients with MSS. At this point it is clear that our original hypothesis that the beneficial effects of ASI given as adjuvant treatment would correlate with microsatellite instability (MSI) of the tumor would have to be rejected. Based on the current data available for the patient group with MSI tumors one could argue that these patients could do without further adjuvant immunotherapy after surgical removal of the primary tumor. Recently this has also been suggested by others (30).
Contradicting results have been published with respect to the predictive value of MS status and the effects of chemotherapy and radiotherapy on colon cancer (17;30-33). It is equally unknown what the predictive value of MS status is on the efficacy of immunotherapy in metastatic colon cancer. In any case we suggest taking MS status of colon cancer into account in new randomized clinical trials as one of the effect modifying factors in the final analyses for the study. Separate approaches might be developed for patients with either MSI or MSS tumors.
Reference List


**Revised CCR-11-1716 Legends to the Figures 1, 2, 3, 4A, 4B, 5 and 6**

**Figure 1) Patient numbers and microsatellite status.**

Diagram showing the number of patients in the original Vermorken study and the number of patients in the current study. Patient derived tumor material was obtained from the archives of participating hospitals and analyzed for Micro Satellite status. The total numbers in each group are indicated in **bold** and the numbers for each subgroup are indicated in *italic*.

**Figure 2) Recurrence Free Interval current MSI/MSS study population.**

Survival time in years on the X-axis and the percentage Recurrence Free Interval on the Y-axis. Kaplan-Meier curves, comparing ASI with the control group in the current study population (n=196), show a significant better prognosis for patients who received adjuvant ASI therapy. (ASI versus Control at 15 year follow up; HR=0.57 (95% CI: 0.34-0.94) log rank p-value=0.027)

**Figure 3) Recurrence Free Interval original study population.**

Survival time in years on the X-axis and the percentage Recurrence Free Interval on the Y-axis. Kaplan-Meier curves, comparing ASI with the control group in the original study population (n=254), show a significant better prognosis for patients who received adjuvant ASI therapy. (ASI versus Control at 15 year follow up; HR=0.62 (95% CI: 0.34-0.96) log rank p-value 0.033)
Figure 4A) Recurrence Free Interval for MSI and MSS patient groups including all tumor stages (Dukes A, B, C and D), independent of ASI therapy.

Survival time in years on the X-axis and the percentage Recurrence Free Interval on the Y-axis. All patients in the current study population were included, irrespective of tumor stage (Dukes A, B, C, and D). Kaplan-Meier curves, comparing the Recurrence Free Interval of the MSI patient group with the MSS patient group, show a significantly better survival for the MSI patient group in comparison to the MSS patient group. (MSI versus MSS; HR=0.45 (95% CI: 0.24-0.86) log rank p-value 0.016). The MSI group (n=34) contained 30 censored subjects and 4 deaths/events, and the MSS group (n= 162) contained 105 censored subjects and 57 deaths/events.

Figure 4B) Recurrence Free Interval for MSI and MSS patient groups with tumor stage Dukes B and Dukes C only, independent of ASI therapy.

Survival time in years on the X-axis and the percentage Recurrence Free Interval on the Y-axis. Only those patients in the current study population with tumor stage Dukes B and C were included in the analysis. Kaplan-Meier curves, comparing the Recurrence Free Interval of the MSI patient group with the MSS patient group, show a significantly better survival for the MSI patient group in comparison to the MSS patient group. (MSI versus MSS; HR=0.47 (95% CI: 0.24-0.92) log rank p-value 0.027). The MSI group (n=31) contained 27 censored
subjects and 4 deaths/events, and the MSS group (n=154) contained 99 censored subjects and 55 deaths/events.

**Figure 5) Recurrence Free Interval for MSI and MSS patient groups with tumor stage Dukes B and Dukes C only, including treatment arm.**

Survival time in years on the X-axis and the percentage Recurrence Free Interval on the Y-axis. Patients in the current study population with either MSI or MSS tumors, initial tumor stage Dukes B or C, control arm or adjuvant ASI treatment were included in the analysis with an extended follow up period of 15 years. For statistical reasons, detailed in the text, the MSI group contained a combination of all Duke B/C patients, irrespective of treatment arm. The numbers of censored subjects and the numbers of deaths/events per subgroup have been indicated below the figure. Hazard ratios, confidence intervals and log rank p values have been mentioned in the text and/or were given in Supplemental Table 1.

**Figure 6) Disease Specific Survival for MSI and MSS patient groups with tumor stage Dukes B and Dukes C only, including treatment arm.**

Survival time in years on the X-axis and the percentage Disease Specific Survival on the Y-axis. Patients in the current study population with either MSI or MSS tumors, initial tumor stage Dukes B or C, control arm or adjuvant ASI treatment were included in the analysis with an extended follow up period of 15 years. For statistical reasons, detailed in the text, the MSI group contained a combination of all Duke B/C patients, irrespective of treatment arm. The numbers
of censored subjects and the numbers of deaths/events per subgroup have been indicated below the figure. Hazard ratios, confidence intervals and log rank p values have been mentioned in the text and/or were given in Supplemental Table 2.
**Figure 1** Patient numbers and microsatellite status.

Original study
Vermorken et al
N = 254

(Control, n=126)
(ASI, n=128)

Analyzed for Micro Satellite status
N = 196
(Control, n=100)
(ASI, n=96)

Not analyzed for Micro Satellite status
N = 58
(Not traced back, n=37)
(Poor quality, n=21)

Micro Satellite Instable
N = 34
(MSI-Control, n=20)
(MSI-ASI, n=14)

Micro Satellite Stable
N = 162
(MSS-Control, n=80)
(MSS-ASI, n=82)
Revised CCR-11-1716 Figure 2
Revised CCR-11-1716 Figure 3
Revised CCR-11-1716 Figure 4A
Revised CCR-11-1716 Figure 4B
Revised CCR-11-1716 Figure 5

<table>
<thead>
<tr>
<th></th>
<th>MSI all</th>
<th>MSS Dukes B Contr</th>
<th>MSS Dukes B ASI</th>
<th>MSS Dukes C Contr</th>
<th>MSS Dukes C ASI</th>
</tr>
</thead>
<tbody>
<tr>
<td># censored subjects</td>
<td>27</td>
<td>29</td>
<td>40</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td># deaths/events</td>
<td>4</td>
<td>19</td>
<td>9</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td># total</td>
<td>31</td>
<td>48</td>
<td>49</td>
<td>28</td>
<td>29</td>
</tr>
</tbody>
</table>
Revised CCR-11-1716 Figure 6

![Graph showing survival rates over time for different groups.](image)

<table>
<thead>
<tr>
<th></th>
<th>MSI all</th>
<th>MSS Dukes B Contr</th>
<th>MSS Dukes B ASI</th>
<th>MSS Dukes C Contr</th>
<th>MSS Dukes C ASI</th>
</tr>
</thead>
<tbody>
<tr>
<td># censored subjects</td>
<td>27</td>
<td>39</td>
<td>42</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td># deaths/events</td>
<td>4</td>
<td>9</td>
<td>7</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td># total</td>
<td>31</td>
<td>48</td>
<td>49</td>
<td>28</td>
<td>29</td>
</tr>
</tbody>
</table>
Clinical Cancer Research

Clinical effects of Adjuvant Active Specific Immunotherapy differ between Patients with Microsatellite Stable and Microsatellite Instable Colon Cancer

Vincent A. de Weger, Annelies W. Turksma, Quirinus JM Voorham, et al.

Clin Cancer Res  Published OnlineFirst December 12, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-1716

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2011/12/12/1078-0432.CCR-11-1716.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.