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Clinical Effects of Adjuvant Active Specific Immunotherapy Differ between Patients with Microsatellite-Stable and Microsatellite-Instable Colon Cancer

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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. 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 biologic determinant of colon cancer.

Experimental Design: Microsatellite status was assessed on archival tumor material from patients with stage II and III colon cancer. 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%) MSI tumors. Patients with MSI tumors did well in recurrence-free interval (RFI) as well as disease-specific survival (DSS) irrespective of treatment arm and tumor stage. Patients with MSI tumors had significantly fewer 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 with controls. ASI treatment did not improve recurrence-free interval or DSS for patients with MSS Dukes C tumors.

Conclusion: This retrospective study indicated that patients with MSI tumors did 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.

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 2 main types occur in CRC. About 80% to 85% of CRCs show chromosomal instability but have functioning DNA mismatch repair systems. These tumors are called microsatellite stable (MSS). Whereas 15% to 20% of CRC tumors show defects or complete failure of DNA mismatch repair systems and are called microsatellite instable (MSI; ref. 1).

MSS and MSI CRCs differ in many of their biologic 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 and 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 because of promoter hypermethylation of mutL homolog 1 (MLH1). A small subset occurs because of germ line mutations in MLH1, mutS homolog 2 (MSH2), and/or MSH6, giving rise to the hereditary Lynch syndrome/hereditary nonpolyposis colorectal cancer (HNPCC), of which CRC is the most prominent phenotype (9). Failure of the DNA mismatch repair system can lead to frameshift mutations in protein-coding sequences, which in turn can lead to the formation of neoantigens. The exact location of frameshift mutations has been documented for a number of genes, for example, TGF-β receptor II and AIM2. Frameshift mutations have been detected in 60% to 70% of MSI tumors (10, 11). Resulting frameshift peptides have been shown to trigger the immune system *in vitro* and tumor-infiltrating lymphocytes appear to be activated and cytotoxic (8, 10–12). On the basis of 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, for example, the 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 colleagues was a multicenter clinical trial on active specific immunotherapy (ASI) for patients with stage II and 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. The 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 with surgical resection alone. ASI therapy resulted in a

significant extension of recurrence-free survival for patients with stage II (Dukes B) colon cancer at the standard 5-year evaluation point. No statistical significance, however, was reached in patients with stage III (Dukes C) colon cancer (18).

At the time the ASI trial was conducted, awareness on the biologic heterogeneity of colon cancer and its possible clinical implications was still limited. Because 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. The aim of the present study was therefore to retrospectively investigate the association between response to ASI treatment and microsatellite status in colon cancer.

Translational Relevance

The majority of colorectal cancers are microsatellite stable (MSS), whereas a smaller portion (about 15%) of colorectal cancer 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 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 did well in recurrence-free interval and disease-specific survival (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 patients with 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 stages II or III resectable adenocarcinoma of the colon and a good performance status were randomly assigned post-operative ASI or no adjuvant treatment.

For the current study, tumor samples for DNA isolation and subsequent microsatellite analysis were available as frozen single-cell suspensions or formalin-fixed and paraffin-embedded (FFPE) material. 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. To validate the procedure of determining microsatellite (in)stability, we carried out 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 5 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 PBS, cleansing it from dimethyl sulfoxide. The cells were then resuspended in PBS and cellular DNA was isolated with the High Pure PCR Template Preparation Kit (Roche) prior to microsatellite 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 mm thickness was taken and hematoxylin and 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 macrodissection on a series of 5 hematoxylin-stained sections of 10 mm. The material was incubated overnight in 1 mol/L NaSCN. After washing, an overnight incubation with lysis buffer (ATL buffer, QIAamp, DNA Micro Kit, Qiagen) and proteinase K (10 µL of 20 ng/µL) was carried out. After the proteinase K incubation, DNA was isolated using a column-based method (QIAamp, DNA Micro Kit, Qiagen). Concentrations and purities of DNA were measured on a NanoDrop ND-1000 spectrophotometer (Isogen).

For DNA MS analyses of the single-cell suspension and the FFPE material, the Promega MSI Analysis System (Version 1.1 and 1.2 Promega) 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), and analyzed using GeneScan 3100 (Applied Biosystems), which gives 100% sensitivity for the microsatellite status, obviating the need to include normal tissue. Tumors showing instability in 2 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 carried out by an independent monitoring agency (IKA, Comprehensive Cancer Center, Amsterdam, the Netherlands). First, analyses were carried out to verify that the patient group in the current study was a fair representation of the patients in the original study. Association of microsatellite status and tumor characteristics was explored by means of χ^2 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 is lower than 0.05. Cox proportional Hazards analyses were carried out to test for interaction between several parameters including treatment and MSI status. Statistical analyses were carried out using Microsoft Excel 2003 or GraphPad Prism v5.

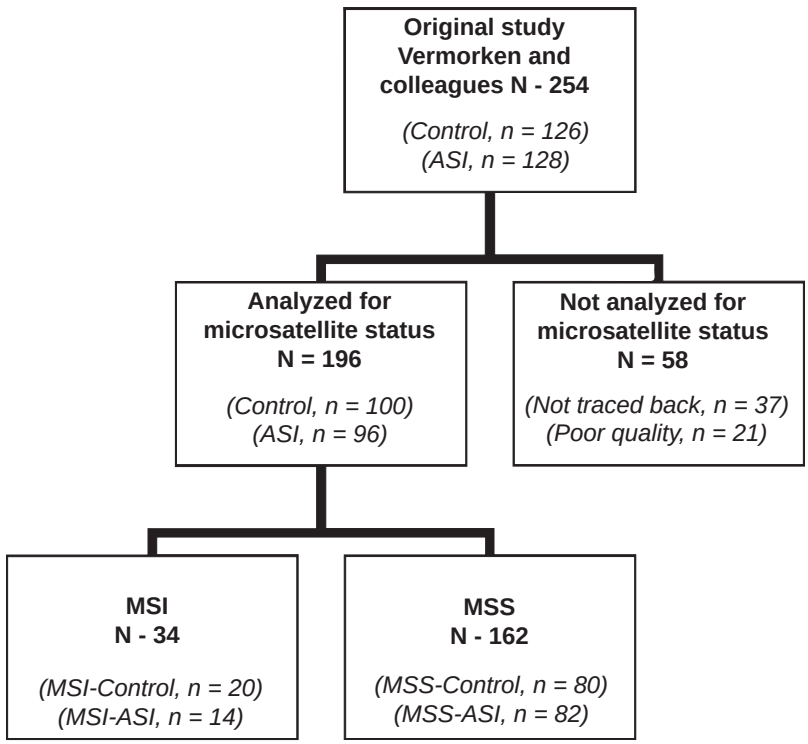


Figure 1. Patient numbers and microsatellite status.
Diagram showing the number of patients in the original Vermorken study and the number of patients in the current study. Patient-derived tumor material was obtained from the archives of participating hospitals and analyzed for microsatellite status. The total numbers in each group are indicated in bold and the numbers for each subgroup are indicated in italic.

Results

Comparison of the current patient subset 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 (Fig. 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 Figs. S1–S4).

Event-free survival in the ASI treatment group and the control group was first analyzed at the standard 5-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 vs. control at 5 years: HR = 0.52 (95% confidence interval, CI, 0.31– 0.86), log-rank P ¼ 0.012] versus [original study ASI vs. control at 5 years: HR = 0.54 (95% CI, 0.34–0.85), log-rank P = 0.008.]

Next, we extended the analysis to 15-year follow-up period. The event-free survival data are presented as Kaplan–Meier plots in Fig. 2 for the current MSI and MSS study (subset of 196 patients) and in Fig. 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 vs. control at 15 years: HR = 0.57 (95% CI, 0.34–0.94), P = 0.027] versus [original study ASI vs. control at 15 years: HR = 0.62 (95% CI, 0.40–0.96), P = 0.033, respectively].On the basis of these results, we were confident that a selection bias was not introduced for the subset of 196 patients available for the MSI/MSS analysis.

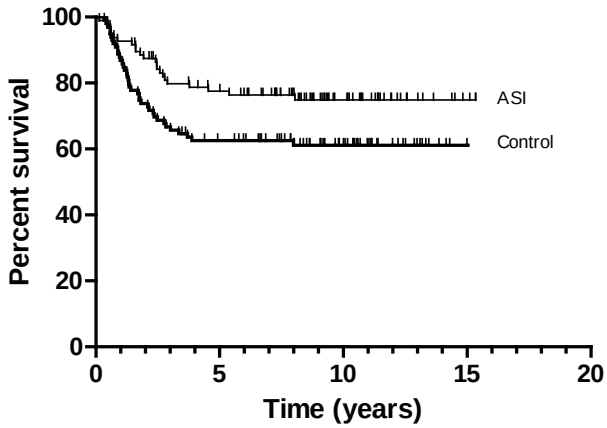


Figure 2. RFI in current MSI/MSS study population.
Survival time in years is on the x-axis and the percentage RFI (survival) is on the y-axis. Kaplan–Meier curves, comparing ASI with the control group in the current study population (n = 196), show a significantly better prognosis for patients who received adjuvant ASI therapy. [ASI vs. control at 15-year follow-up period; HR = 0.57 (95% CI, 0.34–0.94) log-rank P = 0.027.]

Microsatellite 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 Fig. 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 recurrence-free interval (RFI) data presented in Fig. 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 5-year evaluation point [MSI vs. MSS at 5 years: HR = 0.47 (95% CI, 0.24– 0.91) log-rank P = 0.03] as well as at the extended 15-year follow-up period [MSI vs. MSS at 15 years: HR = 0.45 (95% CI, 0.24–0.86) log-rank P = 0.016]. The RFI data for stage II and III (Dukes B/C) patients with MSI (n = 34) versus MSS (n = 154) tumors are shown in Fig. 4B. The patient group with (stage II/III) MSI tumors clearly did better than the patient group with (stage II/III) MSS tumors irrespective of treatment arm.

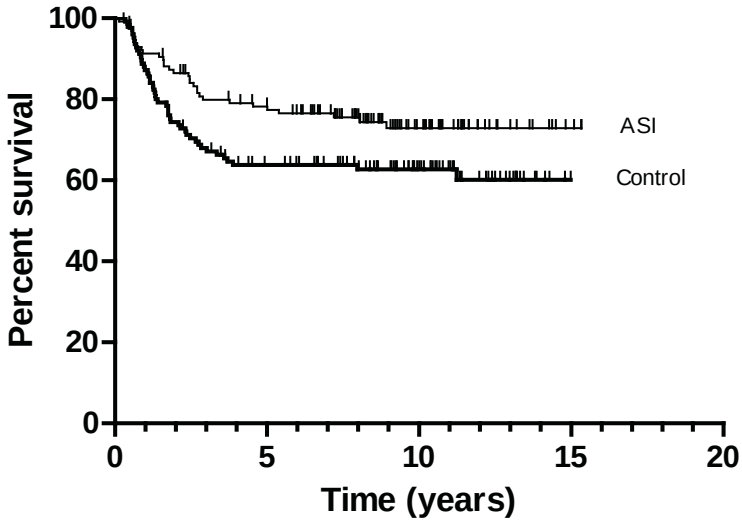


Figure 3. RFI in original study population.
Survival time in years is on the x-axis and the percentage RFI (survival) is on the y-axis. Kaplan–Meier curves, comparing ASI with the control group in the original study population (n ¼ 254), show a significantly better prognosis for patients who received adjuvant ASI therapy. [ASI vs. control at 15-year follow-up period; HR = 0.62 (95% CI, 0.34–0.96) log-rank P = 0.033.]

Microsatellite 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 some- what hampered by the low numbers of patients with MSI 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 and III (Dukes B/C) subgroups showed a significant difference between MSI and MSS in favor of MSI (as shown in Fig. 4B). After separating tumor stages in the analysis, significance was lost in stage II (Dukes B) only (MSI stage II vs. 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 vs. MSS stage III: HR = 0.60; 95% CI, 0.20–1.8, P =

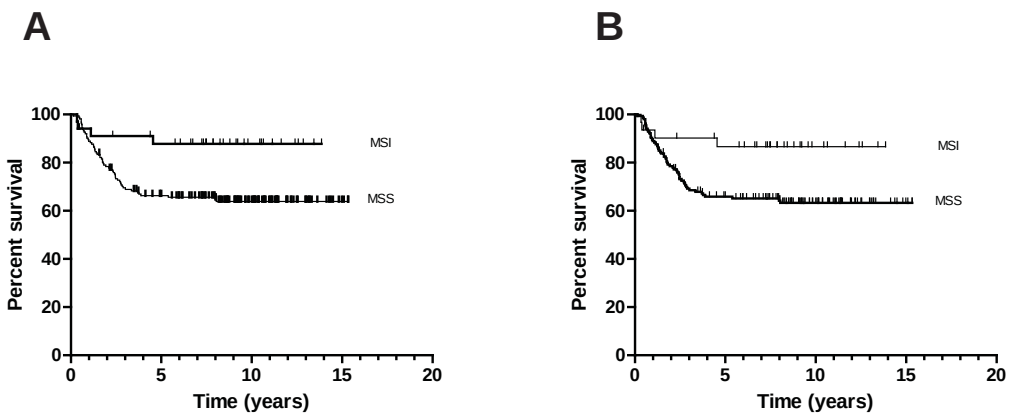
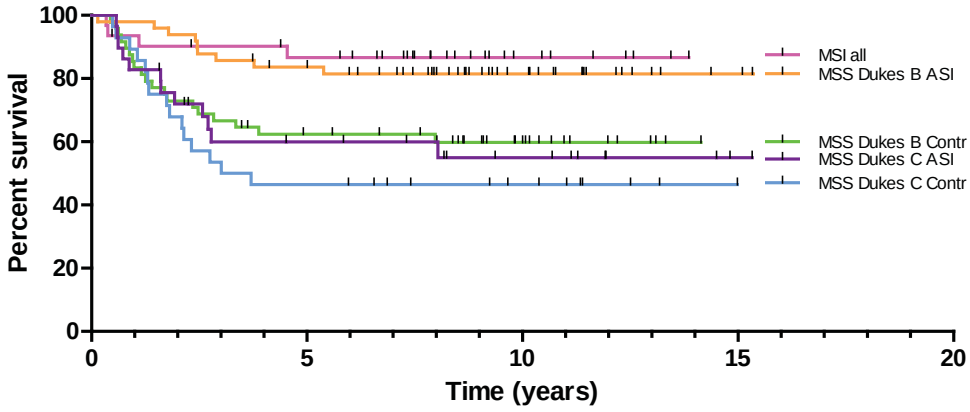


Figure 4. A, RFI for patients with Dukes A, B, C or D tumors, including MS status, but irrespective of treatment arm.
Survival time in years is on the x-axis and the percentage RFI (survival) is on the y-axis. All patients in the current study population were included, irrespective of tumor stage (Dukes A, B, C, and D). Kaplan–Meier curves, comparing the RFI of the patient group with MSI with the patient group with MSS, show significantly better survival for the patient group with MSI than the patient group with MSS [MSI vs. MSS; HR = 0.45 (95% CI, 0.24–0.86) log-rank P = 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.
B, RFI for patients with Dukes B or Dukes C tumors, including MS status, but irrespective of treatment arm. *Survival time in years is on the x-axis and the percentage RFI (survival) is on the y-axis. In the current study population, only those patients with tumor stage Dukes B and C were included in the analysis. Kaplan–Meier curves, comparing the RFI of the patient group with MSI with the patient group with MSS, show a significantly better survival for the patient group with MSI than the patient group with MSS [MSI vs. MSS; HR = 0.47 (95% CI, 0.24–0.92) log-rank P = 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.*

0.35) because of 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 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 and 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 also indicated a lack of significant difference between MSI Dukes A and B versus MSI Dukes C (Kaplan–Meier data not shown). On the basis of these analyses, we concluded that the data points from the 4 different MSI groups could be taken together for further comparison with the 4 different MSS subgroups.

In Fig. 5 and Supplementary Table S1, the data on RFI are shown for the combined (stage II/III, control/ASI) MSI group and for the 4 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 did best. No significant difference was found between these 2 groups. On the other hand significant differences were found between these 2 best performing groups and the other 3 MSS subgroups (MSS Dukes B control, MSS Dukes C control, and MSS Dukes C ASI). No significant differ-

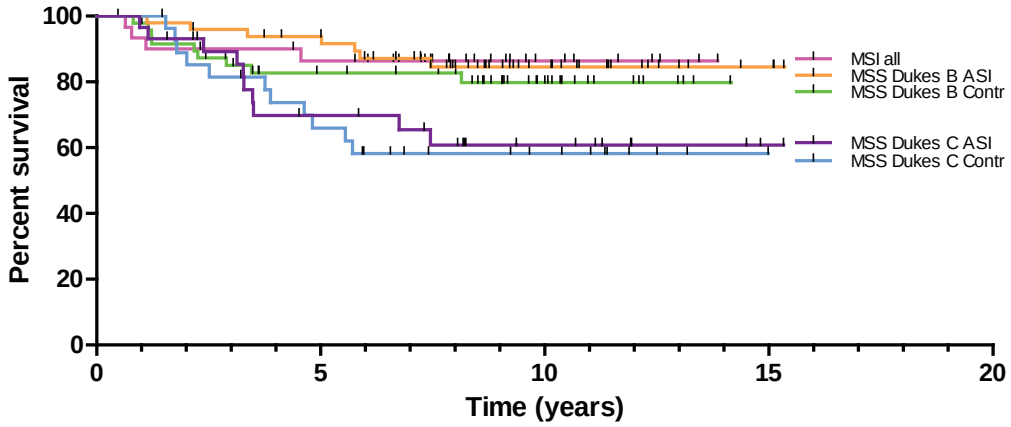


	MSI all	MSS Dukes B Contr	MSS Dukes B ASI	MSS Dukes C Contr	MSS Dukes C ASI
# cencored subjects	27	29	40	13	17
# deaths/events	4	19	9	15	12
# total	31	48	49	28	29

Figure 5. RFI for patients with Dukes B or Dukes C tumors, including MS status and treatment arm. *Survival time in years is on the x-axis and the percentage RFI (survival) is 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 patients with Duke B/C, irrespective of treatment arm. The numbers of censored subjects and the numbers of deaths/events per subgroup have been indicated below the figure. HRs, confidence intervals, and log-rank P values have been mentioned in the text and/or are given in Supplementary Table S1.*

ences were found between the latter 3 groups.

In Fig. 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) did 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 2 groups of patients with MSS Dukes C did relatively poorly again irrespective of adjuvant ASI treatment. Supplementary Table S2 shows all the HR, 95% CI, and P values for the DSS data presented in Fig. 6.



	MSI all	MSS Dukes B Contr	MSS Dukes B ASI	MSS Dukes C Contr	MSS Dukes C ASI
# cencored subjects	27	39	42	17	19
# deaths/events	4	9	7	11	10
# total	31	48	49	28	29

Figure 6. DSS for patients with Dukes B or Dukes C tumors, including MS status and treatment arm. *Survival time in years is on the x-axis and the percentage DSS is 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 patients with Duke B/C, irrespective of treatment arm. The numbers of censored subjects and the numbers of deaths/events per subgroup have been indicated below the figure. HRs, confidence intervals, and log-rank P values have been mentioned in the text and/or are given in Supplementary Table S2.*

Discussion

ASI has been explored in patients with colon cancer by Vermorken and colleagues (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 on the basis of availability of tumor material derived from the 196 patients in the current study. The data presented in Figs. 2 and 3 and the Supplementary Figs. S1–S4 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 MSI or MSS. 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 Fig. 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% because of 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. Because 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 MSI. From our analysis, it became clear that patients who received adjuvant ASI treatment had an improved recurrence-free survival, when compared with patients receiving surgery alone, irrespective of microsatellite status and tumor stage (Fig. 2; n = 196, ASI vs. 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 (Fig. 3; n = 254, ASI vs. 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 than the group with MSS tumors [Fig. 4A; MSI vs. MSS for all tumor stages (Dukes A, B, C, D) taken together, and Fig. 4B; MSI vs. MSS for tumor stages Dukes B and Dukes C taken together].

In the past we have documented delayed-type hyper- sensitivity reactions to tumor-associated antigens in patients with ASI-treated colon carcinoma (25). We obtained microsatellite status data from 6 of 10 patients in the delayed-type hypersensitivity study. Five of these patients had an MSS tumor and one had an MSI tumor. All of these patients showed comparable delayed-type hypersensitivity responses to the autologous tumor cells, which is indicative for systemic antitumor 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 CRC, irrespective of MSI status (8). It has been documented that MSI tumors often contain frameshift 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. Because 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 reanalysis of the available data on patients with MSS tumors clearly showed an effect on RFI of adjuvant ASI treatment for the patient group with MSS Dukes B, but not for the MSS Dukes C group. DSS was also high in the group of patients with MSS Dukes B ASI and indistinguishable from the patients with MSI. 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 RFI. The nature of antigens recognized by preexisting and induced tumor- specific T cells is unknown in these patients, but would probably consist of the normal range of overexpressed tumor antigens in addition to neoantigens 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 DSS. From the data, it is suggested that a number of patients (10 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 3 of these 10 patients were lost in follow-up relatively soon, 3 died from disease unrelated courses, leaving 4 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 under- gone secondary surgery in either of the many participating hospitals, as these data were not included in the original database.

From the data presented here we concluded the following: (i) the group of patients with MSI tumors did well; (ii) a potential therapeutic benefit of ASI in patients with MSI tumors remained undetected; (iii) patients with MSS Dukes B ASI gained quality of life as there was an extension in RFI compared with the control group, despite the notion there was no statistically significant impact on DSS; and (iv) 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 MSI of the tumor would have to be rejected. On the basis of 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 microsatellite status and the effects of chemotherapy and radiotherapy on colon cancer (17, 30– 33). It is equally unknown what the predictive value of microsatellite status is on the efficacy of immunotherapy in metastatic colon cancer. In any case, we suggest taking microsatellite status of colon cancer in to 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.

Note

This work was previously presented as an oral presentation at the Dutch Tumor Immunology Meeting, June 2010, Breukelen, the Netherlands; an oral presentation at the European Workshop on Cytogenetics and Molecular Genetics of Solid Tumors, June 2010, Nijmegen, the Netherlands; a poster presentation at the 2010 AACR Annual Meeting, April 2010, Washington, DC; and a poster presentation at the 2010 Keystone Symposia Conference on Molecular and Cellular Biology of Immune Escape.

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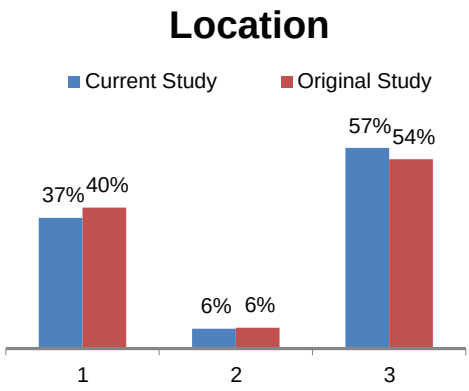
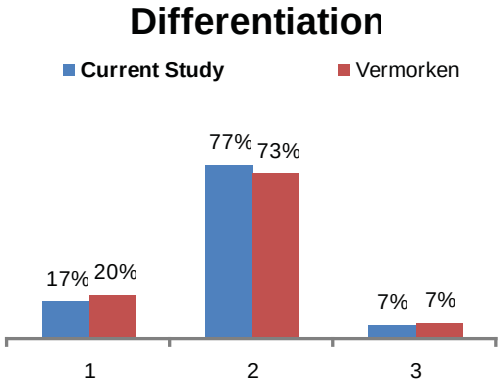
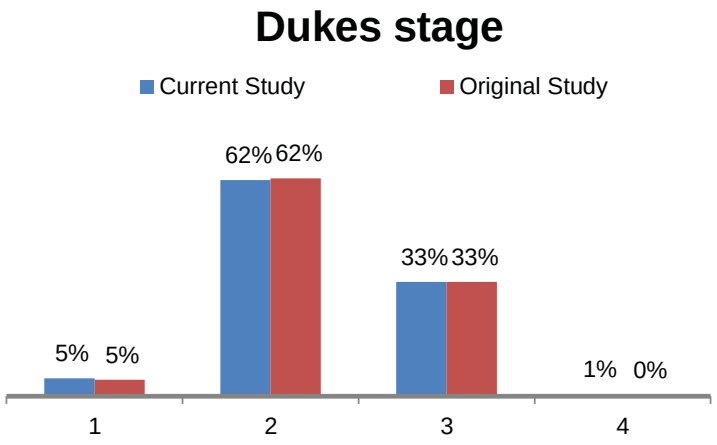
Supplementary figures



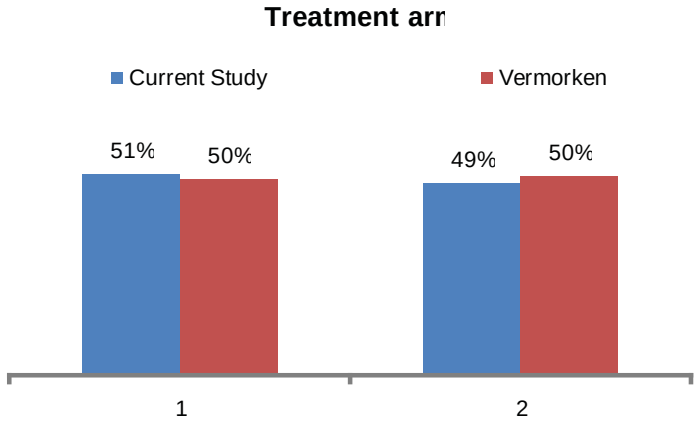
Supplemental Figure 1) Chi-square test for Tumor Location.
The Table indicates the numbers and the percentages for both patient groups included in the Current study and in the Vermorken study. For comparison Chi-square tests were performed for tumor location



Supplemental Figure 2) Chi-square test for Differentiation.
The Table indicates the numbers and the percentages for both patient groups included in the Current study and in the Vermorken study. For comparison Chi-square tests were performed for tumor differentiation.



Supplemental Figure 3) Chi-square test for Tumor Location.
The Table indicates the numbers and the percentages for both patient groups included in the Current study and in the Vermorken study. For comparison Chi-square tests were performed for Stage.



Supplemental Figure 4) Chi-square test for Treatment arm.
The Table indicates the numbers and the percentages for both patient groups included in the Current study and in the Vermorken study. For comparison Chi-square tests were performed for treatment arm.