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The prognostic value of microsatellite instability, *KRAS, BRAF* and *PIK3CA* mutations in stage II colon cancer patients

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ABSTRACT

**Introduction** In the era of personalized cancer medicine, identifying mutations within patient tumors plays an important role in defining high-risk stage II colon cancer patients. The prognostic role of *BRAF* V600E mutation, microsatellite instability (MSI) status, *KRAS* mutation and *PIK3CA* mutation in stage II colon cancer patients is not settled.

**Methods** We retrospectively analyzed 186 patients with stage II colon cancer who underwent an oncological resection but were not treated with adjuvant chemotherapy. *KRAS* mutations, *PIK3CA* mutation, V600E *BRAF* mutation and MSI status were determined. Survival analyses were performed.

**Results** Mutations were found in the patients with each mutation with each mutation in the following percentages: 23% (MSI), 35% (*KRAS*), 19% (*BRAF*) and 11% (*PIK3CA*). A trend toward worse overall survival (OS) was seen in patients with a MSI (5-year OS 74% versus 82%, adjusted hazard ratio (HR) 1.8, 95% CI 0.6-4.9) and a *KRAS*-mutated tumor (5-year OS 77% vs. 82%, adjusted HR 1.7, 95% CI 0.8-3.5). MSI and *BRAF* mutated tumors tended to correlated with poorer disease-free survival (DFS) (5-year DFS 60% vs. 78%, adjusted HR 1.6, 95% CI 0.5-2.1 and 5-year DFS 57% versus 77%, adjusted HR 1.1, 95% CI 0.4-2.6 respectively).

**Conclusions** In stage II colon cancer patients not treated with adjuvant chemotherapy, *BRAF* mutation and MSI status both tended to have a negative prognostic effect on disease-free survival. *KRAS* and MSI status also tended to be correlated with worse overall survival.
INTRODUCTION

Despite advances in diagnosis and treatment, a significant proportion of colon cancer patients who undergo resection with curative intent develop disease recurrence. About 15-30% of the patients with stage II (Dukes B) disease develop recurrent locoregional disease or distant metastases within 5 years and their overall 5-year survival is around 70-80%.

In the era of personalized cancer medicine, identifying mutations within patient tumors might play an important role in defining high risk colon cancer patients. (2;3) The role of the BRAF mutation in colon cancer is one of recent interest. BRAF is a downstream effector molecule of KRAS. One particular missense mutation in BRAF, BRAF V600, accounts for up to 90% of all mutations in human cancers. (4;5) Several studies investigated and confirmed the potential adverse prognostic impact of BRAF mutations but patient categories included in these studies were very heterogeneous. (6-9) As a predictive marker, the BRAF status has been studied in metastatic colorectal cancer, where the presence of the BRAF mutation was correlated with a lower response rate to cetuximab plus chemotherapy. (10;11)

A probably more well known biomarker is microsatellite instability (MSI), which appears in tumors with deficient mismatch repair (MMR). It is the hallmark of Lynch Syndrome, although it is not solely restricted to hereditary colorectal cancer. Although studies have been equivocal concerning proposed survival benefit, some found that MSI is associated with better prognosis. (12) Recent data support a prognostic role for combined MSI/BRAF testing in colorectal cancer. (13;14)

Another potentially promising biomarker is PIK3CA. Mutations in this gene have been identified in colorectal cancer (CRC), with most mutations localized in exon 9 and 20. (15)

Among patients who undergo a curative resection for stage I-III colon cancer, PIK3CA mutation is associated with shorter cancer-specific survival, but the adverse effect of PIK3CA mutation may be potentially limited to patients with KRAS wild-type tumors. (16) KRAS mutations at the codons 12 and 13 are the most frequent alterations in colon cancer, representing more than 90% of all mutations. (17)

To evaluate the prognostic role of above-mentioned mutations in stage II colon cancer, we aimed to determine the status of the BRAF V600E mutation, MSI status, KRAS mutation and PIK3CA mutation in a well-defined group of stage II colon cancer patients who underwent resection but were not treated with adjuvant chemotherapy. The association of the mutations with disease-free survival and overall survival was assessed.
Chapter 6

METHODS

Patients
Patients with stage II (T3N0 or T4N0) colon cancer, diagnosed and surgically treated in one hospital in the southern part of The Netherlands between 2002 and 2008, were included in this study. Patients who received adjuvant chemotherapy were not included.

A tumor was considered right sided when it was located between the cecum and the splenic flexure (C18.0-18.5). The remaining tumors were considered left sided (C18.6-18.9). Demographic and clinical data on the patients were obtained from the medical records of the patients and combined with data from the Netherlands Cancer Registry (NCR) that collects data on all newly diagnosed cancer patients in the Netherlands. Comorbidities are registered according to a slightly modified version of the Charlson Comorbidity index. (18;19)

Patients with insufficient or missing tumor tissue were excluded from analyses. From all patients with sufficient available formalin-fixated paraffin-embedded tumor tissues, DNA was isolated. For this purpose, a tumor area with at least 30% tumor cells from glass slide according to hematoxylin- and eosin (H&E)–stained sections was selected by an experienced pathologist. Subsequently, the selected areas were macrodissected from archival paraffin-embedded tissue after deparaffinization. Subsequently, after proteinase K digestion (1,0 mg/ml TE; overnight 56° C) DNA was extracted using Nucli-SENS® easyMAG® (Biomerieux, Zaltbommel) following manufacturer’s instructions. DNA concentration was measured (BioSpec-nano (Shimadzu, ’s-Hertogenbosch)) and diluted to 10 ng/µl for subsequent analyses.

KRAS analysis
Mutations in exon 2 (codons 12 and 13) and exon 3 (codon 61) of the KRAS gene were detected by PCR high resolution melting (HRM) followed by direct sequencing. Briefly, HRM was performed as previously described (20) using LightScanner Mastermix (Bioke, Leiden, The Netherlands) and LightCycler480 (LC480) Thermal cycler (Roche Diagnostics, Almere, The Netherlands). Positive and equivocal samples in HRM were subjected to Sanger sequencing of the PCR products. Briefly, after the PCR-clean up reaction (Exo-SAP-IT) and purification of the PCR product (MinElute PCR Purification Kit (Qiagen, Venlo, The Netherlands)), the sequence reaction was performed using the same primers independently and the Big Dye reagents (Applied Biosystems, Bleiswijk, the Netherlands). Products were separated on the ABI3100 (Applied Biosystems, Bleiswijk, the Netherlands). The sequences were evaluated with the Sequencing Analysis 5.3.1 software.
**BRAF analysis**

The V600 mutation on the *BRAF* gene was detected by means of a newly developed real-time PCR modified from our previously described V600E assay (4) using the following primers and probes, forward 5’ CTA CTG TTT TCC TTT ACT TAC TAC ACC TCA GA 3’ and reverse 5’ ATC CAG ACA ACT GTT CAA ACT GAT G 3’, wild-type probe VIC-5’ CTA GCT ACA GTG AAA TC 3’ and mutant V600E probe FAM-5’ TAG CTA CAG AGA AAT C 3’. In addition, VIC labeled MGB probes to detect V600R (VIC-5’ TAG CTA CAA GGA AAT C 3’) and V600K (VIC-5’ TAG CTA CAA AGA AAT C 3’) were included, which also detects the V600D mutation. Furthermore, a BRAF-wildtype (wt) locked nucleic acid (LNA) oligonucleotide was used, which supposedly blocks amplification of the wildtype allele during PCR so that mutant DNA can be efficiently amplified. A PCR product of 136 bp was obtained. The assay showed to have a detection limit of at least 1-5% tumor cells in a given specimen. All PCRs were carried out in 10 ul volume using an ABI7500 Fast realtime cycler (Applied Biosystems, Life Technologies, Bleiswijk, the Netherlands).

**PIK3CA analysis**

*PIK3CA* mutations were determined by PCR followed by single nucleotide primer extension assay, as previously described (24) for the hotspots in exon 9, c.1624G>A (p.(E542K)), c.1634A>G (p.(E545G)) and c.1633G>A (p.(E545K)) and in exon 20 the c.3140A>G (p.(H1047R)). Briefly, both exons were amplified by multiplex PCR. After enzymatic purification of the PCR products with EXO SAP IT, the extension reaction was performed using primers published elsewhere (24) and the SNaPshot ready multiplex kit (Applied Biosystems, Life Technologies, Bleiswijk, the Netherlands). Finally, these products were purified and separated by capillary electrophoresis using an ABI 3100 (Applied Biosystems, Bleiswijk, the Netherlands).

**MSI analysis**

Microsatellite instability was detected using only one marker of the Bethesda panel, i.e. the mononucleotide repeat BAT26, as also previously described (4). This marker was chosen because in the Caucasian race, it detects 99% of the MSI high patients and normal DNA is not necessary. (21;22) Briefly, PCR was performed using the following primers, forward VIC-5’ TGA CTA CTT TTG ACT TCA GCC 3’ and reverse 5’ ACC CAT TCA ACA TTT TTA ACC C 3’. Subsequently, PCR products were diluted depending on their intensity and denatured using formamide and incubated at 95°C for 3 minutes. Products size were analyzed using the ABI3100 (Applied Biosystems, Bleiswijk, the Netherlands) and GeneMapper 4.0 software package.
Statistics

Differences in demographic and clinical characteristics between patients with various mutations were analysed using Chi-square tests or Fisher’s Exact tests where appropriate. Crude 5-year overall and disease-free survival were visualised using Kaplan-Meier curves and tested with the Log-Rank test. Overall survival time was defined as the time from primary colon cancer surgery to death or last follow-up date for patients who were still alive. Disease-free survival time was defined as the time from primary colon cancer surgery to recurrence or death or last follow-up date for patients without recurrence or death. Multivariable Cox regression analyses were used to discriminate independent risk factors for death or recurrence and death for the total study population. Besides microsatellite status, *KRAS*, *BRAF* and *PIK3CA*, models were also adjusted for the variables gender, age, comorbidity, surgery, subsite of the tumor, differentiation grade, number of lymph nodes evaluated, tumor obstruction, tumor perforation and lymphangioinvasion. All variables were included in the models at once.

In the period from January to May 2014, data on diagnosis of recurrences were retrospectively collected from the medical records. Date of death is, in addition to passive follow-up via the hospitals, retrieved through linkage with the Municipal Personal Records Database (GBA). This database contains all death or emigrated persons in the Netherlands since October 1994. Date of death was completed until 31 December 2013. 

P values below 0.05 were considered statistically significant. SPSS for Windows (version 16.0, SPSS INC, Chicago, Ill) and SAS/STAT® statistical software (SAS system 9.3, SAS Institute, Cary, NC) were used for all analyses.

RESULTS

The total study population consisted of 211 patients. Twenty-five patients (12%) were excluded owing to insufficient tumor tissue (n=7) and missing tumor tissue in our archive (n=18). Patient and clinicopathological characteristics of the study population are shown in table 1. Of these 211 patients, 43 (23%) had an MSI tumor. *KRAS*, *BRAF* and *PIK3CA* mutations were found in 35%, 19% and 11% of the patients, respectively.

The relationship between various demographic and clinicopathological features and mutational status can be found in table 2. MSI tumors were significantly associated with female sex (p=0.04), right sided location of the tumor (p<0.0001) and poorly differentiated or undifferentiated tumors (p<0.0001). Furthermore, patients with a MSI tumor less often had a *KRAS* mutation (p=0.001), but more often a *BRAF* mutation (p<0.0001). *KRAS* and *BRAF* mutations were mutually exclusive.
Table 1. Demographic and clinicopathological characteristics of stage II colon cancer patients who underwent resection (n=186)

| Characteristic                  | N (%)      |
|--------------------------------|------------|
| **Gender**                     |            |
| Male                           | 99 (53)    |
| Female                         | 87 (47)    |
| **Age**                        |            |
| ≤65                            | 52 (28)    |
| >66-75                         | 62 (33)    |
| ≥76                            | 72 (39)    |
| **Comorbidity**                |            |
| 0                              | 57 (31)    |
| 1                              | 50 (27)    |
| ≥2                             | 67 (36)    |
| Unknown                        | 12 (6)     |
| **Surgery**                    |            |
| Elective                       | 166 (89)   |
| Acute                          | 20 (11)    |
| **Subsite**                    |            |
| Left-sided colon               | 85 (46)    |
| Right-sided colon              | 101 (54)   |
| **Pathological T stage**       |            |
| 3                              | 185 (99)   |
| 4                              | 1 (1)      |
| **Differentiation grade**      |            |
| Well/moderate                  | 114 (61)   |
| Poor/undifferentiated          | 39 (21)    |
| Unknown                        | 33 (18)    |
| **Lymph nodes evaluated**      |            |
| <10                            | 130 (70)   |
| ≥10                            | 56 (30)    |
| **Tumor obstruction**          |            |
| No                             | 164 (88)   |
| Yes                            | 22 (12)    |
| **Tumor perforation**          |            |
| No                             | 179 (96)   |
| Yes                            | 7 (4)      |
| **Lymphangioinvasion**         |            |
| No                             | 180 (97)   |
| Yes                            | 6 (3)      |
| **Microsatellite status**      |            |
| MSS                            | 143 (77)   |
| MSI                            | 43 (23)    |
| **KRAS**                       |            |
| Wild type                      | 121 (65)   |
| Mutant                         | 65 (35)    |
| **BRAF**                       |            |
| Wild type                      | 151 (81)   |
| Mutant                         | 35 (19)    |
| **PIK3CA**                     |            |
| Wild type                      | 165 (89)   |
| Mutant                         | 21 (11)    |
Table 2. Relationship between various demographic and clinicopathological characteristics and mutational status (n=186)

|                        | MSS (n=143) | MSI (n=43) |
|------------------------|-------------|------------|
|                         | n (%)       | n (%)      |
| **Gender**             |             |            |
| Male                   | 82 (57.3)   | 17 (39.5)* |
| Female                 | 61 (42.7)   | 26 (60.5)  |
| **Age**                |             |            |
| ≤65                    | 46 (32.2)   | 6 (14.0)   |
| >66-75                 | 46 (32.2)   | 16 (37.2)  |
| ≥76                    | 51 (35.6)   | 21 (48.8)  |
| **Comorbidity**        |             |            |
| 0                      | 46 (32.2)   | 11 (25.6)  |
| 1                      | 39 (27.3)   | 11 (25.6)  |
| ≥2                     | 48 (33.5)   | 19 (44.2)  |
| Unknown                | 10 (7.0)    | 2 (4.6)    |
| **Surgery**            |             |            |
| Elective               | 126 (88.1)  | 40 (93.0)  |
| Acute                  | 17 (11.9)   | 3 (7.0)    |
| **Subsite**            |             |            |
| Left-sided colon       | 81 (56.6)   | 4 (9.3)**  |
| Right-sided colon      | 62 (43.4)   | 39 (90.7)  |
| **Pathological T stage**|         |            |
| 3                      | 142 (99.3)  | 43 (100.0) |
| 4                      | 1 (0.7)     | 0 (0.0)    |
| **Differentiation grade**|         |            |
| Well/moderate          | 98 (68.5)   | 16 (37.2)**|
| Poor/undifferentiated  | 20 (14.0)   | 19 (44.2)  |
| Unknown                | 25 (17.5)   | 8 (18.6)   |
| **Lymph nodes evaluated**|         |            |
| <10                    | 41 (28.7)   | 15 (34.9)  |
| ≥10                    | 102 (71.3)  | 28 (65.1)  |
| **Tumor obstruction**  |             |            |
| No                     | 125 (87.4)  | 39 (90.7)  |
| Yes                    | 18 (12.6)   | 4 (9.3)    |
| **Tumor perforation**  |             |            |
| No                     | 139 (97.2)  | 40 (93.0)  |
| Yes                    | 4 (2.8)     | 3 (7.0)    |
| **Lymphangioinvasion** |             |            |
| No                     | 138 (96.5)  | 42 (97.7)  |
| Yes                    | 5 (3.5)     | 1 (2.3)    |
| **Microsatellite status**|         |            |
| MSS                    | N/A***      | N/A        |
| MSI                    | 37 (30.6)   | 6 (9.2)    |
| **KRAS**               |             |            |
| Wild type              | 84 (58.7)   | 37 (86.0)* |
| Mutant                 | 59 (41.3)   | 6 (14.0)   |
| **BRAF**               |             |            |
| Wild type              | 134 (93.7)  | 17 (39.5)**|
| Mutant                 | 9 (6.3)     | 26 (60.5)  |
| **PIK3CA**             |             |            |
| Wild type              | 129 (90.2)  | 36 (83.7)  |
| Mutant                 | 14 (9.8)    | 7 (16.3)   |

*p<0.05
**p<0.0001
***N/A = not applicable
BRAF mutations were associated with female sex (p=0.03), comorbidity (p=0.036), right-sided location of the tumor (p<0.0001) and poorly differentiated or undifferentiated tumors (p=0.002).

PIK3CA mutation was associated with female sex (p=0.016).

Survival

For the total study population, 5-year overall survival was 80% and 5-year disease-free survival 74%. In both univariable and multivariable analysis, higher age, more comorbidities, poorly differentiated or undifferentiated tumors and lymphangioinvasion were significantly associated with poorer overall and disease-free survival (tables 3,4). Although not significant, a trend towards worse overall survival was seen in patients with a MSI tumor (5-year overall survival rate of 74% compared with 82% for patients with a MSS tumor, figure 1A), a BRAF-mutated tumor (5-year overall survival rate of 76% compared with 81% for patients with a BRAF wild type tumor, figure 1E) and a KRAS mutated tumor (5-year overall survival rate of 77% versus 82% for KRAS wild type tumors, figure 1C). As 60% of all patients with a MSI tumor was alive and without recurrence at five years versus 78% of patients with a MSS tumor (figure 1B), MSI correlated with poorer disease-free survival. BRAF mutated tumors also correlated with poorer disease-free survival with a 5-years disease-free survival of 57% versus 77% for BRAF wildtype (figure 1F). However, the associations between MSI and disease-free survival and between BRAF and disease-free survival were no longer significant in multivariable analysis (tables 3,4). Not enough patients with PIK3CA mutations were left at the end of follow-up to assess survival for this mutation.
|                                  | Crude 5-year survival (%) | Hazard ratio (95% CI) |
|----------------------------------|---------------------------|----------------------|
| **Gender**                      |                           |                      |
| Male                             | 77                        | 1.0 (reference)      |
| Female                           | 84                        | 2.5 (1.2-5.2)        |
| **Age**                          |                           |                      |
| ≤65                              | 98                        | 1.1 (1.1-1.2)        |
| >66-75                           | 87                        |                      |
| ≥76                              | 61                        |                      |
| **Comorbidity**                  |                           |                      |
| 0                                | 96**                      | 1.0 (reference)      |
| 1                                | 74                        | 3.4 (1.0-10.9)       |
| ≥2                               | 69                        | 4.9 (1.6-15.6)       |
| **Surgery**                      |                           |                      |
| Elective                         | 82                        | 1.0 (reference)      |
| Acute                            | ^                         | 1.5 (0.2-14.4)       |
| **Subsite**                      |                           |                      |
| Left-sided colon                 | 78                        | 1.0 (0.5-2.2)        |
| Right-sided colon                | 82                        | 1.0 (reference)      |
| **Differentiation grade**        |                           |                      |
| Well/moderate                    | 86*                       | 1.0 (reference)      |
| Poor/undifferentiated            | 71                        | 3.9 (1.6-9.3)        |
| **Lymph nodes evaluated**        |                           |                      |
| <10                              | 80                        | 1.4 (0.6-3.0)        |
| ≥10                              | 81                        | 1.0 (reference)      |
| **Tumor obstruction**            |                           |                      |
| No                               | 82                        | 1.0 (reference)      |
| Yes                              | ^                         | 2.1 (0.2-18.4)       |
| **Tumor perforation**            |                           |                      |
| No                               | 82                        | 1.0 (reference)      |
| Yes                              | ^                         | 3.4 (0.8-14.4)       |
| **Lymphangioinvasion**           |                           |                      |
| No                               | 81*                       | 1.0 (reference)      |
| Yes                              | ^                         | 4.8 (1.2-18.7)       |
| **Microsatellite status**        |                           |                      |
| MSS                              | 82                        | 1.0 (reference)      |
| MSI                              | 74                        | 1.8 (0.6-4.9)        |
| **KRAS**                         |                           |                      |
| Wild type                        | 82                        | 1.0 (reference)      |
| Mutant                           | 77                        | 1.7 (0.8-3.5)        |
| **BRAF**                         |                           |                      |
| Wild type                        | 81                        | 1.0 (reference)      |
| Mutant                           | 76                        | 0.7 (0.2-2.0)        |
| **PIK3CA**                       |                           |                      |
| Wild type                        | 80                        | 1.0 (reference)      |
| Mutant                           | ^                         | 0.5 (0.1-1.8)        |

* Adjusted for all variables listed. Included in the analysis but results not shown for comorbidity unknown and differentiation grade unknown.
* *p<0.05
** **p<0.0001
^ Number of patients left <10
### Table 4. Crude 5-year disease-free survival and hazard ratios\(^a\) for recurrence or death for the total study population (n=186)

|                     | Crude 5-year disease-free survival (%) | Hazard ratio for recurrence/death (95% CI) |
|---------------------|---------------------------------------|------------------------------------------|
| **Gender**          |                                       |                                          |
| Male                | 70                                    | 1.0 (reference)                          |
| Female              | 78                                    | 2.1 (1.1-4.0)                            |
| **Age**             |                                       |                                          |
| ≤65                 | 90**                                  | 1.1 (1.0-1.1)                            |
| >66-75              | 75                                    |                                          |
| ≥76                 | 60                                    |                                          |
| **Comorbidity**     |                                       |                                          |
| 0                   | 89*                                   | 1.0 (reference)                          |
| 1                   | 64                                    | 3.3 (1.3-8.1)                            |
| ≥2                  | 64                                    | 3.3 (1.4-7.9)                            |
| **Surgery**         |                                       |                                          |
| Elective            | 75                                    | 1.0 (reference)                          |
| Acute               | ^                                     | 1.7 (0.2-14.5)                           |
| **Subsite**         |                                       |                                          |
| Left-sided colon    | 70                                    | 1.2 (0.6-2.3)                            |
| Right-sided colon   | 76                                    | 1.0 (reference)                          |
| **Differentiation grade** |                                   |                                          |
| Well/moderate       | 82*                                   | 1.0 (reference)                          |
| Poor/undifferentiated | 52                               | 3.7 (1.8-7.4)                            |
| **Lymph nodes evaluated** |                                   |                                          |
| <10                 | 72                                    | 1.1 (0.6-2.2)                            |
| ≥10                 | 76                                    | 1.0 (reference)                          |
| **Tumor obstruction** |                                     |                                          |
| No                  | 75                                    | 1.0 (reference)                          |
| Yes                 | 62                                    | 1.5 (0.2-12.6)                           |
| **Tumor perforation** |                                     |                                          |
| No                  | 75                                    | 1.0 (reference)                          |
| Yes                 | ^                                     | 1.8 (0.5-6.6)                            |
| **Lymphangioinvasion** |                                     |                                          |
| No                  | 75*                                   | 1.0 (reference)                          |
| Yes                 | ^                                     | 6.8 (2.1-21.8)                           |
| **Microsatellite status** |                                   |                                          |
| MSS                 | 78*                                   | 1.0 (reference)                          |
| MSI                 | 60                                    | 1.6 (0.7-3.9)                            |
| **KRAS**            |                                       |                                          |
| Wild type           | 73                                    | 1.0 (reference)                          |
| Mutant              | 75                                    | 1.1 (0.5-2.1)                            |
| **BRAF**            |                                       |                                          |
| Wild type           | 77*                                   | 1.0 (reference)                          |
| Mutant              | 57                                    | 1.1 (0.4-2.6)                            |
| **PIK3CA**          |                                       |                                          |
| Wild type           | 72                                    | 1.0 (reference)                          |
| Mutant              | ^                                     | 0.5 (0.1-1.7)                            |

\(^a\) Adjusted for all variables listed. Included in the analysis but results not shown for comorbidity unknown and differentiation grade unknown.

\(^*p<0.05\)

\(^{**}p≤0.0001\)

\(^\wedge\) Number of patients left <10
In our study we assessed the prognostic value of \textit{BRAF} mutation, \textit{KRAS} mutation, \textit{PIK3CA} mutation and the MSI status with regard to overall and disease-free survival in a well-defined stage II colon cancer cohort of patients who underwent resection but were not treated with adjuvant chemotherapy. \textit{BRAF} mutation and MSI status both tended to have a negative prognostic effect on disease-free survival. \textit{KRAS}, \textit{BRAF} and MSI status also tended to be correlated with worse overall survival.
MSI
MSI positivity was found in 23% of the patients, which is comparable with other subgroup analyses of recent studies reporting 15-25% MSI. (6;8;23;24). Consistent with prior studies (25), MSI status was inversely correlated with the presence of the KRAS mutation. The most remarkable finding in our study is the trend towards a negative prognostic effect of an MSI mutation on disease-free survival and overall survival. Although not all studies have verified the association of MSI mutation and improved overall survival, MSI mutation is generally associated with improved overall and disease-free survival. (26;27) On the other hand, like in our study, MSI is associated with poorly differentiated histology which is a known adverse prognostic factor. (27) This gives rise to a paradoxical situation.

Current treatment protocols recommend adjuvant treatment only to stage II patients with high-risk pathological features (for example, T4 stage, bowel perforation or clinical bowel obstruction, inadequate lymph node sampling, (lymph)angioinvasion and poorly differentiated histology). Exceptions are made for MSI positive colon cancer; the most recent Dutch guideline does not recommend adjuvant chemotherapy in high-risk stage II patients with a MSI tumor. Since we only included stage II patients that did not receive chemotherapy, probably a more favourable group of MSS-tumor patients is analyzed and compared with MSI in our study. Therefore, MSI status might have contributed to a relatively poorer survival in our study population. For the MSI determination, we choose the mononucleotide repeat BAT 26 because it discriminates 99% of MSI in the Caucasian population without the requirement of amplified normal DNA, as previously described. (21) The use of only one marker could have diminished the sensitivity of our analysis but not the specificity. (21;22)

BRAF
The presence of the BRAF mutation varies more widely between recent studies (6-21%), and was 19% in our cohort. (6;8;23;24) A recent meta-analysis found that the risk of mortality in colorectal cancer patients harbouring the BRAF-V600E mutation is more than two times higher than those with wild-type BRAF. (28) Although less strongly, our results show a trend towards the BRAF mutation having a negative prognostic effect on disease-free and overall survival compared with BRAF wild-type tumors.

KRAS
KRAS mutations (codons 12, 13 and 61) were found in 35% of the patients in our study, consistent with other reports. (6;8;24). We found a trend towards worse overall survival for KRAS mutated tumors as compared to KRAS wild-type tumors. In the prognostic setting, there are conflicts about the role of the KRAS mutational status. (6;8;29) A recent large study of more than 1,000 colorectal cancers (stage I through IV) has shown that
**KRAS**

Codon 12 mutation is associated with worse prognosis in *BRAF* wildtype colorectal cancers. However, the study is limited by the lack of information on cancer treatment. (30)

**PIK3CA**

The frequency of *PIK3CA* mutation seems to be dependent on the technique used to evaluate the mutation. (31) We found a *PIK3CA* mutation in 11% of our patients, which is comparable with the literature (10-20%) (31). *PIK3CA*-mutated colorectal tumors have been associated with more proximal location and with a *KRAS* mutation. (31;32) In our study more than 60% of the *PIK3CA* mutated tumors were located in the proximal colon. We found no correlation with *KRAS* mutation. In line with the literature, we did not find a correlation with MSI and *BRAF*. (31;32) *PIK3CA* mutations are more commonly found in exon 9 compared to exon 20. (31) Indeed, 13 of 21 mutations were found in exon 9 in our study. In an earlier report, only a mutation in exon 20 was suggested to be responsible for a worse chance of survival. (33) Because of the small numbers, survival analysis of *PIK3CA* subgroups in our study was not feasible.

A recent prospective study showed that the total number of lymph nodes harvested is highest for colon cancers with MSI. (34) In this study the nodal harvest is associated with MSI influenced by *BRAF* and *KRAS* genotypes. However, we did not find an association with the number of lymph nodes and the mutational status.

As described above, the relationship between the mutational status and various demographic and clinicopathological variables is comparable with the literature. However, our study population is not completely comparable with those from other studies. Most other reports about the prognostic value of molecular markers in colorectal cancer included more heterogeneous groups of colorectal cancer patients, with patients in different stages. (6;8;23;24) Different studies also evaluated the prognostic value of MSI status, *KRAS* and *BRAF* mutational status in stage II colon cancer patients, but in most of them chemotherapy was given to (a partial cohort) of the patients or information regarding adjuvant therapy was lacking. (6;8;23;24;30)

A new way of sub-staging within stage II colon cancer was suggested by a recent report that defined molecular subtypes by genomic instability. For stage II patients, the numerical difference in chromosomal aberrations between recurrence and no recurrence was statistically significant. Further studies with larger patient samples have to confirm these results. (35)

Cancer care is becoming increasingly dependent on tumor markers to diagnose, anticipate prognosis and select optimal therapy for patients. Although biomarker discovery is thriving, incorporation of biomarkers in clinical practice lags behind. It is imperative that the field of oncology works with a common language and clear standards of evidence.
so that the merits of established and emerging biomarkers can be communicated in a clear and unambiguous manner, thereby ensuring that clinicians take full advantage of the current genomic era. (36) Another future direction in (colorectal) cancer research is the host immune response against an invasive tumor process. The recently described ‘Immunoscore’ classification, demonstrating the prevalence of immune infiltrates, was shown to have a superior prognostic significance in colorectal cancer compared to the classical TNM classification. (37)

**Strengths and limitations**

To the best of our knowledge we reported the largest study that analyzed MSI, KRAS, BRAF and PIK3CA mutational status in stage II colon cancer patients who underwent resection but did not receive chemotherapy. Representing 30-40% of all resected colorectal cancers, stage II patients are a very interesting subgroup because clinicians still do not know exactly which of these patients are at high risk of recurrence and therefore may benefit from adjuvant chemotherapy. Furthermore, the percentage of low-stage colon cancers is going to increase because of the screening programmes. (23)

Unfortunately, due to a relative small number of patients, we were not be able to perform adequate subgroup analyses within the different mutations and assess survival for PIK3CA.

**CONCLUSIONS**

The histopathological approach is paramount in colon cancer classification, however, for most patients with stage II disease who are classified as standard risk, there are no additional markers to refine risk assessment. The use of molecular biomarkers in addition to pathological classification will be particularly important in stage II colon cancer, in order to offer the most adequate therapy to each individual patient and avoid unnecessary chemotherapeutic treatment. Our study shows that in stage II patients who have not been treated with chemotherapy, BRAF mutation tended to have a negative prognostic effect on survival and also, in contrast to most other reports, MSI tended to be a poor prognosticator. Further studies are needed to verify and further clarify these results.
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