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The *BRAF* V600E mutation is an independent prognostic factor for survival in stage II and stage III colon cancer patients

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Abstract

Molecular markers in colon cancer are needed for a more accurate classification and personalized treatment. We determined the effects on clinical outcome of the *BRAF* mutation, microsatellite instability (MSI) and *KRAS* mutations in stage II and III colon carcinoma.

Stage II colon carcinoma patients (n=106) treated with surgery only and 258 stage III patients all adjuvantly treated with 5-FU chemotherapy, were included. *KRAS* mutations in codons 12 and 13, V600E *BRAF* mutation and MSI status were determined.

Older patients ($p<0.001$), right sided ($p=0.018$), better differentiated ($p=0.003$) and MSI tumors ($p<0.001$) were significantly more frequent in stage II than stage III.

In both groups, there was a positive association between mutated *BRAF* and MSI ($p=0.001$) and *BRAF* mutation and right sided tumors ($p=0.001$). Mutations in *BRAF* and *KRAS* were mutually exclusive.

In a multivariate survival analysis with pooled stage II and III data *BRAF* mutation was an independent prognostic factor for overall survival and cancer specific survival (HR=0.45 95%CI 0.25 – 0.8 for OS and HR=0.47 95%CI 0.22 – 0.99). *KRAS* mutation conferred a poorer DFS (HR=0.6 95%CI 0.38 – 0.97).

The V600E *BRAF* mutation confers a worse prognosis to stage II and III colon cancer patients independently of disease stage and therapy.

Introduction

Colon carcinoma is classified according to clinical and histopathological criteria. Prognosis and therapy relate to this classification. According to the Dutch treatment guidelines previous to 2006, stage II patients were solely treated with surgery. Stage III patients would receive adjuvant chemotherapy after surgery. Around 20% of stage II patients will develop a relapse in the first five years after surgery. Probably, this group of patients would benefit from adjuvant chemotherapy. On the other hand, 60% of stage III patients are cured after surgery and do not benefit from the adjuvant treatment [1,2]. Hence, other criteria for adjuvant therapy are needed. Molecular markers might prove to be better than clinical and histopathological criteria for therapy selection.

Microsatellite instability (MSI) and *KRAS* mutations have been widely studied in colorectal cancer. Around 20% of the sporadic colon cancers show MSI due to defects in the mismatch repair system (MMR). MSI is associated with a better prognosis[3-6]. Approximately 35% of colon cancers carry a mutation in codons 12 or 13 of the *KRAS* gene leading to the constitutive activation of its downstream pathway and to uncontrolled cell division [7-9]. *BRAF* is recently being studied in relation to prognosis[10-13]. *BRAF* is a downstream effector molecule of *KRAS*. 90% of the *BRAF* mutations consist in a valine to glutamate transition at position 600 of the protein, the so called V600E mutation, which causes the constitutive activation of the protein. This mutation is found in approximately 20% of the colonic tumors.

Mutations in *BRAF* and in *KRAS* are mutually exclusive. Tumors harboring the V600E *BRAF* mutation have other clinical and histopathological features than *KRAS* mutated tumors [14].

The value of *KRAS* mutations in stage II and III is unknown. *BRAF* has been studied only in heterogeneous colon carcinoma patients cohorts including all disease stages [10-12] and recently in a group of stage IV colorectal cancer [13]. To date, it remains unknown what the effect of the *BRAF* mutation is on clinical outcome of patients with either stage II or III disease.

In this study we aimed to determine the status of the V600E *BRAF* mutation and other molecular markers, like MSI status and *KRAS* mutations in two well defined groups of stage II and III colon carcinoma patients who were treated according to the Dutch guidelines previous to 2006 and to assess their effect on patient outcome.

Patients and Methods

Patient population

Three hundred sixty four patients diagnosed at the PAMM Laboratory for Pathology in Eindhoven, the Netherlands and treated in four different regional hospitals in the south of the Netherlands, between 1996 and 2004, were included in this study. We included 106 patients diagnosed with stage II colon carcinoma and treated with surgery only and 258 stage III disease patients treated with surgery followed by adjuvant 5-FU in combination with leucovorin chemotherapy like established by the Dutch guidelines for the treatment of colon cancer previous to 2006. A tumor was considered right sided when it was located between the coecum and the splenic flexure. The remaining tumors were considered left sided. Rectal tumors were not included. Demographic and clinical data on the patients were facilitated by the Cancer Registry of the Comprehensive Cancer Centre South (IKZ, Eindhoven, the Netherlands). In over 93% of the patients data was complete. Follow-up was obtained from the available medical records of the patients.

The use of clinical material for this retrospective study was approved by the institutional review board according to the guidelines of the Dutch Federation of Research Associations.

From all patients with sufficient available material, tumor DNA was isolated. For this purpose, a tumor area with at least 30% tumor cells from glass slide according to HE stained sections was selected by an experienced pathologist. Subsequently, the selected areas were macrodissected from archival paraffin embedded tissue. DNA was purified after proteinase K digestion with the HPPTP kit (Roche, Almere, the Netherlands) following manufacturer's instructions.

From 76 patients data were missing due to different reasons, firstly some tissue blocks were not present in our archive (47.4%), secondly some samples did not reach 30% tumor cells (43.4%) and additionally not all DNA samples could be amplified by PCR (9.2%).

Molecular characterization

BRAF mutation analysis

The V600E mutation on the *BRAF* gene was detected by means of real time PCR 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 probe FAM-5'TAG CTA CAG AGA AAT C 3' like described elsewhere[15]. A PCR product of 136 bp was obtained. The assay showed to have a detection limit of at least 10% tumor cells in a given specimen. All PCR reactions were performed on the Light Cycler v2.0 (Roche, Almere, the Netherlands) using Roche chemistry in a total volume of 20 microliters.

Microsatellite instability

Microsatellite instability was detected using only one marker of the Bethesda panel, i.e. the mononucleotide repeat BAT26. This marker was chosen because in the Caucasian race, it detects 99% of the MSI high patients and normal DNA is not necessary [16,17]. 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´. The expected product length is 116 bp. 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 ABI3130 (Applied Biosystems, Nieuwerkerk aan de Ijssel, the Netherlands) and GeneMapper 4.0 software package.

KRAS mutation analysis

Mutations in codons 12 and 13 of the *KRAS* gene were detected by DNA sequencing. Briefly, PCR amplification of the cited codons was performed using the following primers; forward 5´AGG CCT GCT GAA AAT GAC TG 3´and reverse 5´TCA AAG AAT GGT CCT GCA CC 3´ as previously described by van Zandwijk et al [18]. The expected product length was 172 bp. After purification of the PCR product, the sequence reaction was performed using the same primers independently and the Big Dye reagents (Applied Biosystems, Nieuwerkerk aan de Ijssel, the Netherlands). Products were separated on the ABI3130 (Applied Biosystems, Nieuwerkerk aan de Ijssel, the Netherlands). The sequences were evaluated with the Sequencing Analysis 5.3.1 software.

Statistical Analysis

SPSSv.16 software for Windows (Chicago, IL) was used. X2, Fischer exact tests and Student's t-test were used to analyze the relationship between variables.

Stage II and stage III groups were first analyzed separately and pooled during survival

analysis to increase the sensitivity of the tests. Univariate survival analysis was performed with Kaplan Meier analysis and survival curves were compared by Log-Rank tests. Multivariate analysis was performed with Cox Proportional Hazards regression analysis. T and N stage, but also age, sex, tumour location, differentiation grade, *BRAF*, *KRAS*, and MSI status were included in the model. In case of statistical significant interaction between these variables in the model, we would stratify the analyses accordingly. We considered a minimum of 10 to 15 events per predictor necessary to proceed with multivariate survival analyses [19]. In order to avoid overfitting, all variables were entered and maintained in the model, e.g. not using automated stepwise regression. For the same reason, those variables which did not exhibit a statistically significant relation with survival in the univariate analysis were also entered into the model. Besides, variables in isolation may behave quite differently with respect to the response variable when they are considered simultaneously with 1 or more other variables [20]. Overall survival (OS) was defined as the time between diagnosis and either death of disease or death of other cause, whenever this was specified in the patients' medical record. Disease free survival (DFS) was defined as the time between diagnosis and disease recurrence or development of distant metastasis. Finally, cancer specific survival (CSS) was defined as the period of time between diagnosis and death due to the disease.

Results

Patients' demographic and clinicopathological characteristics

Patients' characteristics according to stage are shown in table 1.

By definition none of the patients diagnosed with stage II disease had tumor positive lymph nodes whereas all of the stage III patients had positive lymph nodes. In both groups a similar number of lymph nodes were examined for diagnosis, median number of 7 in stage II and of 8 in stage III.

In the stage II group median age was 73 years (range 30-94) whereas in the stage III group it was 64 years (range 30-84). This difference was statistically significant ($p<0.001$).

The tumor location was also significantly different between groups, 68% right sided tumors in stage II vs. 54% in stage III ($p=0.018$). Well or moderately differentiated tumors were more frequent in stage II patients than in stage III (87% in stage II vs. 72% in stage III, $p=0.005$).

The cause of death was significantly different between groups. In the stage II group 30% of the patients had died because of reasons other than cancer (as specified in their medical records) and 10% due to cancer related reasons. In the stage III group only 7% had died of non-cancer related causes and 32% died due to cancer related causes ($p<0.001$).

Median follow up of the stage II group was 55 months (0-109) and 46 months (2-133) for the stage III group.

KRAS, *BRAF* and MSI status

Table 2 a&b shows the frequencies of the different mutations in the patient population and the significant associations between variables for the two patients' populations. The percentages of the mutations in *KRAS* and *BRAF* did not differ between the two populations. *KRAS* mutations were found in 33% of stage II patients vs. 35% of stage III. *BRAF* was mutated in 22% of stage II and in 19% of stage III patients. However, the proportion MSI tumors was significantly higher in the stage II group than in stage III (25% vs. 14%, respectively, $p=0.024$).

KRAS and *BRAF* mutations were mutually exclusive ($p<0.001$) in both populations. There was no significant association between *KRAS* mutations and the development of a distant metastasis or local relapse in stage II patients ($p=0.08$). Moreover, it did reach

statistical significance in stage III patients (p=0.014). *KRAS* mutations were associated to better differentiated tumors (p=0.013 stage II and p=0.06 stage III).
The carriage of the V600E *BRAF* mutation was significantly associated with MSI (p<0.001), right side location (p<0.001) in both populations.
In both groups MSI tumors were right sided (p=0.003 stage II and p<0.001 stage III) and poorly differentiated (p=0.024 stage II and p=0.022 stage III).

Survival analysis

In a univariate analysis, in both groups separately the *BRAF* V600E mutation was significantly associated with a shorter CSS in stage II disease (p=0.022) but not in stage III disease (Figure 1). In both groups there was a trend towards a longer OS for the carriers of wild type *BRAF* (p=0.194 stage II and 0.069 stage III) (Figure 2). DFS was not significantly different between *BRAF* mutants and wild type tumors.
When stratifying for MSI status, *BRAF* mutation resulted in shorter survival in MSS patients in both stage II and stage III disease (p=0.011 stage II CSS and p=0.016 stage III OS), but not in the MSI group.
In the stage III group, *KRAS* mutations seemed to confer a significantly worse DFS than *KRAS* wild type (p=0.03) (Figure 3). This effect was not present in the stage II group.

Multivariate analysis

Since results did not significantly differ between both populations, data of both groups were pooled in order to increase sensitivity of the multivariate analysis. A Cox Proportional Hazards model including differentiation grade, age as a continuous variable, sex, tumor location, T-stage, N-stage, *KRAS* status, *BRAF* status and MSI status was used. The results of this model are shown in table 3. Therapy was not included in the model because it covariates linearly with N-stage.
BRAF mutation was as an independent factor for a shorter OS (HR=0.45 95%CI 0.25-0.8), DFS (HR=0.43 95%CI 0.22-0.82) and CSS (HR=0.47 95%CI 0.22-0.99). *KRAS* mutation was an independent prognostic factor for a shorter DFS (HR=0.6 95%CI 0.4-0.97). T-stage was a prognostic factor for DFS, OS and CSS. N-stage, as positive or negative lymphnodes, was prognostic for DFS and CSS. Finally, male gender was a significant variable for a shorter OS (HR=1.84 95%CI 1.19-2.85).

Table 1: Clinicopathological characteristics in stage II and III patients.

Characteristics	Stage II N(%)	Stage III N(%)	p-value
Sex			
Male	54 (51)	144 (56)	0.42
Female	52 (49)	114 (44)	
Location			
Right	69 (68)	137 (54)	**0.018**
Left	33 (32)	117 (46)	
Age			
Mean	71.5	62.5	**<0.001**
Median	73	64	
T-stage			
T1	0	2 (0.8)	0.06
T2	3 (3)	22 (8.5)	
T3	85 (82.5)	186 (72)	
T4	15 (14.5)	48 (18.7)	
Differentiation grade			
Well/moderate	85 (87)	177 (72.5)	**0.005**
Poor/Undifferentiated	13 (13)	67 (27.5)	
Follow up status			
No evidence of disease	52 (50.5)	124 (48.6)	**<0.001**
Alive with disease	10 (9.7)	31 (12.2)	
Death of disease	10 (9.7)	83 (32.5)	
Death of other cause	30 (29.1)	17 (6.7)	

Table 2 a: Patient's characteristics according to disease stage. (wt=wild type mut=mutated).

a) stage II

Stage II		*BRAF*			*KRAS*			MSI		
	N (%)	wt	mut	p	wt	mut	p	MSI	MSS	p
Sex										
Male	54 (51)	38 (81)	9 (19)	0.6	33 (70)	14 (30)	0.5	11 (23)	37 (77)	0.8
Female	52 (49)	35 (76)	11 (24)		28 (62)	17 (38)		12 (26)	34 (74)	
Location										
Right	69 (68)	46 (72)	18 (28)	**0.01**	42 (67)	21 (33)	1.0	21 (32)	44 (68)	**0.003**
Left	33 (32)	25 (96)	1 (4)		18 (69)	8 (31)		1 (4)	25 (96)	
Age										
0-59	12 (12)	11 (100)	0 (0)	0.17	8 (73)	3 (27)	0.4	0 (0)	11 (100)	0.25
60-66	17 (16.5)	12 (80)	3 (20)		9 (60)	6 (40)			11 (73)	
67-72	19 (18.5)	11 (65)	6 (35)		14 (83)	3 (18)		4 (27)	12 (71)	
≥73	55 (53)	39 (78)	11 (22)		30 (61)	19 (39)		5 (29)	37 (72)	
								14 (28)		
Median age	73									
T-status							0.4			
T1	0 (0)				0	0				
T2	3 (3)	2 (67)	1 (33)	0.36	3 (100)	0 (0)		2 (67)	1 (33)	0.24
T3	85 (82.5)	62 (80.5)	15 (19.5)		49 (64.5)	27 (35.5)		18 (23)	59 (77)	
T4	15 (14.5)	9 (64)	5 (36)		10 (71)	4 (29)		4 (27)	11 (73)	
N-status										
N-	106 (100)	73 (78)	21 (22)		62 (67)	31 (33)		24 (25)	71 (75)	
N+	0				0	0		0	0	
Differentiation							**0.013**			0.024
Well/Moderate	25 (25)	63 (81)	15 (19)	0.21	46 (60.5)	30 (39.5)		16 (20)	62 (80)	
Poor/Undiff.	13 (13)	6 (60)	4 (40)		10 (100)	0 (0)		6 (55)	5 (45)	
BRAF							**<0.001**			**<0.001**
wt	73 (78)				41 (57)	31 (43)		8 (11)	65 (89)	
mut	21 (22)				21 (100)	0 (0)		15 (71)	6 (29)	
KRAS				**<0.001**						**0.001**
wt	62 (67)	41 (66)	21 (34)					22 (26)	40 (64)	
mut	31 (33)	31 (100)	0 (0)					1 (3)	30 (97)	
MSI status				**<0.001**			**0.001**			
MSI	24 (25)	8 (35)	15 (65)		22 (96)	1 (4)				
MSS	71 (75)	65 (91.5)	6 (8.5)		40 (57)	30 (43)				

Table 2 b: Patient's characteristics according to disease stage. (wt=wild type mut=mutated).

b) stage III

Stage III		*BRAF*			*KRAS*			MSI		
	N (%)	wt	mut	p	wt	mut	p	MSI	MSS	p
Sex										
Male	144 (56)	95 (83)	20 (17)	0.6	75 (67)	37 (33)	0.65	16 (13)	103 (87)	0.7
Female	114 (44)	70 (79.5)	18 (20.5)		55 (63)	32 (37)		14 (16)	76 (84)	
Location										
Right	137 (54)	76 (70)	32 (30)	**<0.001**	67 (63)	39 (37)	0.55	27 (24)	84 (76)	**<0.001**
Left	117 (46)	89 (95)	5 (5)		62 (67)	30 (33)		2 (2)	94 (98)	
Age										
0-59	83 (32)	51 (82)	11 (18)	0.9	45 (74)	16 (26)	0.3	12 (18)	54 (82)	0.7
60-66	82 (32)	59 (83)	12 (17)		44 (65)	24 (35)		8 (11)	63 (89)	
67-72	61 (24)	36 (78)	10 (22)		28 (61)	18 (39)		7 (15)	40 (85)	
≥73	32 (12)	19 (79)	5 (21)		13 (54)	11 (46)		3 (12)	22 (88)	
Median age	64									
T-status										
T1	2 (0.8)	1 (100)	0 (0)	0.9	0 (0)	1 (100)	0.105	0 (0)	1 (100)	0.6
T2	22 (8.5)	14 (82)	3 (18)		14 (82)	3 (18)		1 (6)	17 (94)	
T3	186 (72)	120 (82)	27 (18)		88 (61.5)	55 (38.5)		22 (14)	131 (86)	
T4	48 (18.7)	30 (79)	8 (21)		28 (74)	10 (26)		7 (19)	30 (81)	
N-status										
N-	0	0	0		0	0		0	0	
N+	255	163 (81)	38 (19)		127 (65)	69 (35)		30 (14)	178 (86)	
Differentiation										
Well/Moderate	29 (12)	121 (87)	18 (13)	**<0.0001**	85 (62.5)	51 (37.5)	**0.036**	16(20.5)	62 (79.5)	**0.022**
Poor/Undiff.	66 (27)	35 (64)	20 (36)		41 (77)	12 (23)		15(10)	129 (90)	
BRAF										
wt	165 (81)				94 (58)	69 (42)	**<0.001**	14 (9)	149 (91)	**<0.001**
mut	38 (19)				36 (100)	0 (0)		14 (37)	24 (63)	
KRAS										
wt	130 (65)	94 (72)	36 (28)	**<0.001**				25 (19)	103 (81)	**0.001**
mut	69 (35)	69 (100)	0 (0)					2 (3)	67 (97)	
MSI status										
MSI	30 (14)	14 (50)	14 (50)	**<0.001**	25 (93)	2 (7)	**0.001**			
MSS	179 (86)	149 (86)	24 (14)		103 (61)	67 (39)				

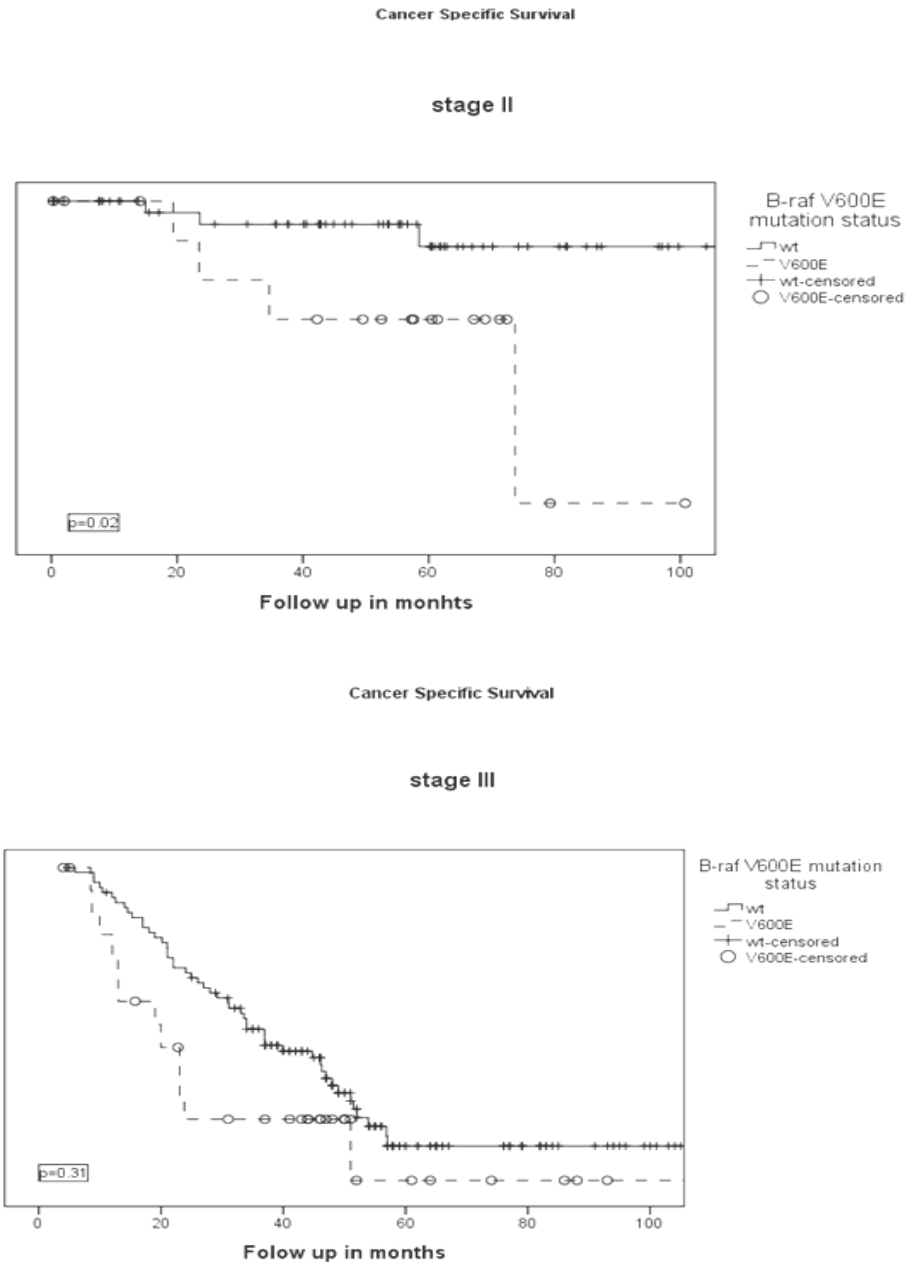

Figure 1: Kaplan Meier plots for CSS in stage II and in stage III patients according to *BRAF* V600E mutational status.

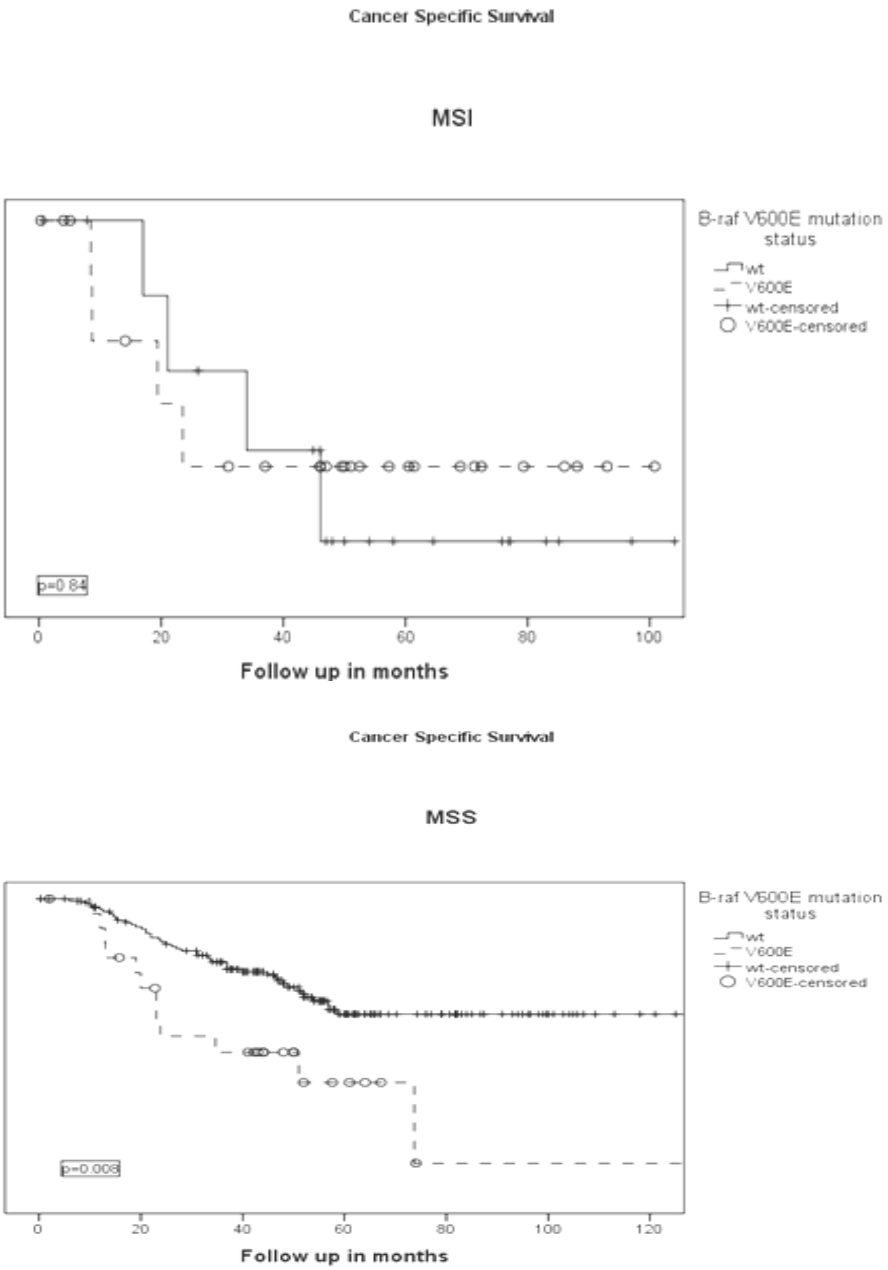

Figure 2: Kaplan Meier plots for CSS according to *BRAF* V600E mutational status in the whole group stratified according to MSI status of the tumor.

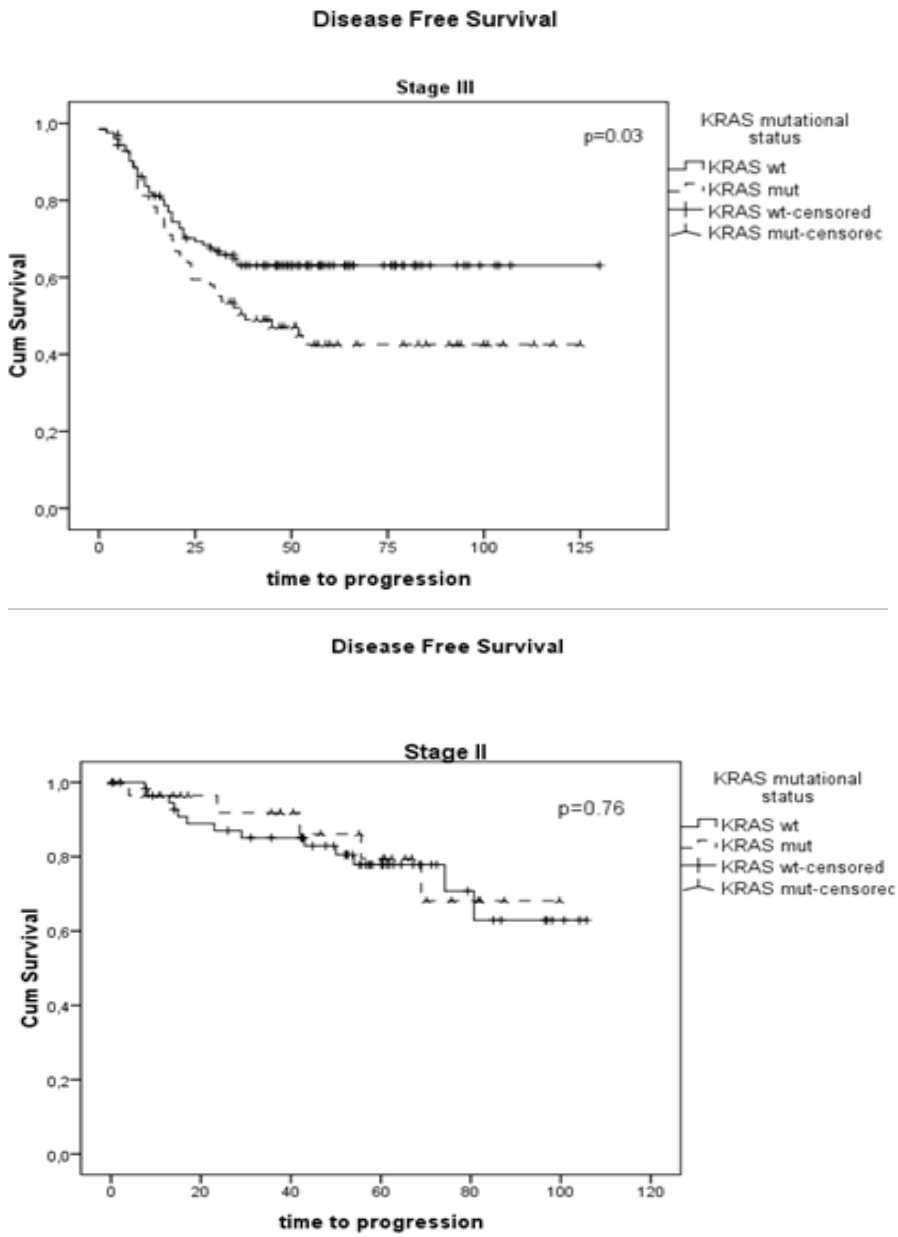

Figure 3: Kaplan Meier plots for DFS according to *KRAS* mutational status in stage II and III independently.

Table 3: Cox proportional hazards model for overall survival, disease free survival and cancer specific survival.

	Overall survival		Disease Free Survival		Cancer Specific Survival	
	HR	(95% CI)	HR	(95% CI)	HR	(95% CI)
Age	1.009	(0.98 – 1.03)	0.99	(0.97 – 1.02)	0.99	(0.97 – 1.02)
Sex	1.84	**(1.19 – 2.85)***	1.1	(0.72 – 1.7)	1.25	(0.73 – 2.15)
Location	0.71	(0.45 – 1.13)	0.7	(0.44 – 1.09)	0.74	(0.42 – 1.31)
***KRAS* status**						
wt	0.83	(0.51 – 1.35)	0.6	(0.38 – 0.97)	0.72	(0.39 – 1.3)
mut	1	(referent)	1	(referent)	1	(referent)
***BRAF* status**						
wt	0.45	**(0.25 – 0.8)***	0.43	(0.22 – 0.82)	0.47	**(0.22 – 0.99)***
mut	1	(referent)	1	(referent)	1	(referent)
MSI status	0.8	(0.42 – 1.5)	0.49	(0.23 – 1.05)	0.6	(0.22 – 1.4)
T stage						
T2	0.2	**(0.04 – 0.8)***	0.07	**(0.009 – 0.51)***	0.001	(0 - >1000)
T3	0.5	**(0.30 – 0.8)***	0.49	**(0.3 – 0.8)***	0.42	**(0.24 – 0.75)***
T4	1	(referent)	1	(referent)	1	(referent)
N-stage						
N-	0.93	(0.56 – 1.52)	0.5	**(0.3 – 0.9)***	0.3	**(0.12 – 0.72)***
N+	1	(referent)	1	(referent)	1	referent
Differentiation grade						
Well/Moderate	0.85	(0.51 – 1.40)	0.99	(0.59 – 1.6)	0.67	(0.37 – 1.22)
Poor/Undifferentiated	1	(referent)	1	(referent)	1	(referent)

* $p<0.05$

OS N=261, CSS & DFS N=252

Discussion

The molecular signature of a tumor will most likely influence patient survival. In stage II and III colon cancer the use of molecular markers might be particularly important in order to offer the most adequate therapy to each patient and avoid unnecessary chemotherapeutic treatment. In this study, we assessed the effect of the V600E *BRAF* mutation, *KRAS* mutations and MSI on patient outcome, in two well defined colon cancer populations of stage II and III patients.

In our population, the V600E *BRAF* mutation is an independent prognostic factor. The carriage of the mutation accounts for a significantly higher risk of dying of cancer related causes, independently of other factors like age, sex, location of the tumor, MSI status, *KRAS* mutational status, differentiation grade, T-stage and N-stage.

Our results agree with recent published studies from Ogino *et al.* and Tol *et al*. However, Ogino *et al.* found a relationship between *BRAF* mutation and CSS in an heterogeneous group of colon cancer patients including all disease stages [11], whereas, our study focus solely on a well described homogeneous stage II and III group. On the other hand, Tol *et al.* demonstrated a positive correlation between the V600E *BRAF* mutation and a shorter survival in a group of metastatic colorectal patients independently of the treatment arm (capecitabine, oxaliplatin, bevacizumab with or without cetuximab) [13]. However, the patients included in that study did all receive palliative chemotherapy and therefore no conclusion could be drawn about either the prognostic or predictive value of the *BRAF* mutation. From our data, we can conclude that the *BRAF* mutation is an independent prognostic factor in all patients with stage II and III colon carcinoma. It could be argued that our selection of patients based on the therapy according to the guidelines could bias the results. However, identical results were obtained in a larger group including stage III patients who did not receive adjuvant chemotherapy (data not shown).

Moreover, concordant with the literature [10,12], the V600E *BRAF* mutation identifies a small group of patients with microsatellite stable tumors who had a poor survival. However, the interaction between MSI, *BRAF* and disease outcome remains subject of study since in the multivariate analysis, MSI seemed to play a marginal role depending on therapy in patients' survival.

The presence of a *KRAS* mutation did not have any effect on patient overall survival in stage II and III disease. However, there was significant difference in DFS between

KRAS mutated and wild type tumors. The prognostic value of *KRAS* mutations in stage II and III colon carcinoma remains controversial. Many studies have reported a prognostic role for *KRAS* and many others failed to report this effect, as reviewed by Castagnola[21]. Based on our results we can conclude that *KRAS* seems to play a role in disease progression, mainly in stage III colon cancer patients, this effect is absent in stage II patients.

In our study, a group of stage II patients, who did not receive adjuvant therapy after surgery and a group of stage III patients who did receive 5-FU based adjuvant chemotherapy according to the Dutch guidelines previous to 2006 were selected. This treatment selection is the major reason for the differences in age and follow up status between patients in the two groups. It is known that only younger patients with a good general condition and little co-morbidity are offered adjuvant chemotherapy. Since all stage III patients in our group received chemotherapy, they were younger and had less comorbidity and thus less non-cancer related deaths than stage II patients, who frequently died of non cancer related deaths like heart failure.

Other significant differences between the two groups were the frequency of MSI and of right sided tumors in the stage II group. 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, like previously described[17]. The use of only one marker could have diminished the sensitivity of our analysis but not the specificity [16,17]. The higher frequency of MSI tumors in stage II is probably due to the significant association of MSI and right sided tumors and the higher proportion of these tumors among stage II patients which in turn can be explained by the shift in tumor location that occurs as patient age increases [22].

Due to the retrospective character of this study, we were not able to test patients who were treated according to the recently published Dutch guidelines where a difference in treatment is made between stage II and high risk stage II. Since 2006, high risk stage II patients receive adjuvant chemotherapy after surgery. High risk stage II patients are defined as having pT4 lesions, lymphovascular invasion, tumor perforation or obstruction, poorly differentiated histology, or less than 10 lymph nodes removed. Eighty four percent of our stage II patients would be nowadays considered as high risk patients. The majority due to the insufficient number of lymph nodes examined. Therefore, we can conclude that the negative effects of the V600E *BRAF* mutation on survival are applicable to this group of patients and that this mutation can be

considered as a prognostic marker.

In conclusion, *BRAF* is an independent prognostic factor in stage II and III colon cancer. These results are promising for the treatment of colon cancer patients since determination of the V600E *BRAF* mutation can discriminate between patients who have a shorter OS, DFS and CSS. The exact effect of MSI and of *KRAS* on survival should be further elucidated. In contrast, this *BRAF* mutation might become an important molecular marker in the future for drug development and in the decision making for patient tailored adjuvant therapy.

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