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Combination of microsatellite instability and *BRAF* mutation status for subtyping colorectal cancer

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Background: The objective of the study was to examine the role of microsatellite instability (MSI) and $BRAF^{V600E}$mutation in colorectal cancer (CRC) by categorising patients into more detailed subtypes based on tumour characteristics.

Methods: Tumour samples from 762 population-based patients with sporadic CRC were analysed for MSI and $BRAF^{V600E}$ by immunohistochemistry. Patient survival was followed-up for a median of 5.2 years.

Results: Compared with microsatellite stable (MSS) CRC, MSI was prognostic for better disease-free survival (DFS; 5 years: 85.8% *vs* 75.3%, 10 years: 85.8% *vs* 72.9%, $P=0.027$; HR 0.49, CI 0.30–0.80, $P=0.005$) and disease-specific survival (DSS; 5 years: 83.2% *vs* 70.5%; 10 years: 83.2 *vs* 65.0%, $P=0.004$). Compared with *BRAF* wild type, $BRAF^{V600E}$ was a risk for poor survival (overall survival; 5 years: 62.3% *vs* 51.6%, $P=0.014$; HR 1.43, CI 1.07–1.90, $P=0.009$), especially in rectal cancer (for DSS, HR: 10.60, CI: 3.04–36.92, $P<0.001$). The MSS/$BRAF^{V600E}$ subtype was a risk for poor DSS (HR: 1.88, CI: 1.06–3.31, $P=0.030$), but MSI/$BRAF^{V600E}$ was a prognostic factor for DFS (HR: 0.42, CI: 0.18–0.96, $P=0.039$). Among stage I–II patients, the MSS/$BRAF^{V600E}$ subtype was independently associated with poor DSS (HR: 5.32, CI: 1.74–16.31, $P=0.003$).

Conclusions: Microsatellite instable tumours were associated with better prognosis compared with MSS. $BRAF^{V600E}$ was associated with poor prognosis unless it occurred together with MSI. The MSI/$BRAF^{V600E}$ subtype was a favourable prognostic factor compared with the MSS/*BRAF* wild-type subtype. $BRAF^{V600E}$ rectal tumours showed particularly poor prognosis. The MSS/$BRAF^{V600E}$ subtype was associated with increased disease-specific mortality even in stage I–II CRC.

Growing evidence suggests that colorectal cancer (CRC) should be subdivided into different prognostic groups defined by molecular biomarker combinations that purportedly reflect the CRC development pathways (Samadder *et al*, 2013; Phipps *et al*, 2015). One such group would be defined by microsatellite instability (MSI) that occurs in ~15% of sporadic CRCs and leads to significant clinical heterogeneity in both phenotype and survival (Ionov *et al*, 1993).

The most common cause of MSI is sporadic hypermethylation of the promoter area of both *MLH1* alleles, resulting in deficient mismatch repair (MMR; Samowitz *et al*, 2005). Microsatellite instability can also occur due to inherited MMR deficiency, such as Lynch syndrome (LS), which involves autosomal-dominant inheritance of a germline mutation in a major MMR gene (*MLH1*, *MSH2*, *MSH6*, or *PMS2*) followed by a second mutation later in life.

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Numerous clinical and histological features have been proposed to differentiate MSI tumours from microsatellite stable (MSS) tumours. However, it remains uncertain whether these features are clinically useful to the extent required for universal MSI screening, and whether such clinical division into subgroups would be beneficial.

In cases of MSI, the presence of the $BRAF^{V600E}$ hotspot mutation practically excludes the possibility of LS, and the clinical utility of the combination of these two markers is well established (Funkhouser *et al*, 2012). $BRAF^{V600E}$ shows an independent negative prognostic association with survival in MSS CRC (Samowitz *et al*, 2005; Ogino *et al*, 2012; Phipps *et al*, 2012), but associations with the combination of MSI and *BRAF* have not been thoroughly investigated. Recent findings indicate that the prognostic potential of MSI overrides the negative prognostic potential of $BRAF^{V600E}$, thus eliminating the deleterious role of $BRAF^{V600E}$ within the MSI subgroup (Hamilton, 2013; Lochhead *et al*, 2013). International guidelines suggest using both MSI and *BRAF* immunohistochemical (IHC) staining for LS screening algorithms; therefore, these markers are increasingly available for clinical use (Palomaki *et al*, 2009; Weissman *et al*, 2012; Vasen *et al*, 2013).

The present study aimed to elucidate the role of MSI and $BRAF^{V600E}$ in a population-based setting using patient material treated according to 21st century guidelines. The findings provide a basis for the routine clinical use of MMR and *BRAF* status.

MATERIALS AND METHODS

Patients. The Central Hospital of Central Finland exclusively serves a defined catchment area of ~274 000 people around Jyväskylä, Finland. The present study included all consecutive patients ($n = 1088$) who underwent major bowel resection for CRC between 2000 and 2010. Tissue microarray (TMA) of a representative tumour sample was available for analysis from 799 patients. Good-quality IHC for MSI were available for 762 patients. Compared with the included subjects, those who dropped out did not substantially differ in age, sex, tumour location, or Union for International Cancer Control (UICC) stage.

Ethical aspects. The study was approved by the Central Finland Central Hospital's ethical committee. Authorisation for use of the patient registry was obtained from the National Supervisory Authority for Welfare and Health (Valvira).

Clinical evaluation. Tumours were classified based on their exact location. Tumours situated from the caecum through the transverse colon were deemed proximal colon tumours. Tumours resected from the descending and sigmoid colon from the splenic flexure down to 15 cm proximal from the anal verge were considered distal colon tumours, and those more distally located were classified as rectal tumours. The specimens were macroscopically examined and histologically studied by an experienced histopathologist following UICC guidelines (sixth edition). The histopathologist also performed pTNM staging regarding tumour size and nodal status. Staging was completed (M) by the treating surgeon based on data from imaging and physical examination (usually a body CT scan). Those patients with inadequate specimen for complete pTNM classification (e.g., transanal extirpation of the tumour, $n = 10$) were excluded from analyses that required stage. For 574 patients, the operation was elective with a radical result (R0).

Follow-up. For study purposes, a surgeon reviewed all stage assignments along with the pathology and radiology statements, and surgery report. Liver and lung metastases found within 6 months of operation were considered synchronous when determining the final pTNM UICC stage.

Medical records were carefully reviewed. We retrieved information regarding type of surgery (laparoscopic or conventional), parameters reflecting surgical quality, surgical result (i.e., radical/palliative), possible special circumstances (e.g. emergency surgery), and surgical complications. The exact date and location of CRC local or distal recurrence, occurrence of metastases or metachronous CRC, and the possible interventions were also recorded. The dates and official causes of death were retrieved from death certificates, with permission from the Finnish Cause of Death Registry (collected and updated by Statistics Finland, a government authority). In cases of postoperative death (within 30 days), the cause of death and the role of cancer in the event were assessed individually. All medical data regarding preoperative diagnosis, surgery, recurrence, clinical staging, adjuvant treatment, clinical follow-up, and cause of death were re-assessed and recorded to our database by a surgery specialist. The current vital status of each patient was reviewed by confirming deaths from the hospital's patient registry or, if uncertain, from the service of the Population Register Centre on the 1 November 2014. Median follow-up was 5.2 years (interquartile range 6.4 years).

Tumour sampling. The resected specimens were delivered to the pathology department as fresh tissue samples. After formalin fixation and macroscopic evaluation, the tissue samples were embedded in paraffin, and TMA blocks were prepared from the formalin-fixed paraffin-embedded tissue (FFPE) samples. From each FFPE sample, 0.6-mm-diameter tissue cylinders were punched out from the previously marked representative tumour areas and set into a recipient paraffin block using the Manual Tissue Microarrayer MTA-1 (Beecher Instruments Inc., Sun Prairie, WI, USA). From these TMA punches, 2-μm-thick sections were cut. Tissue microarray blocks included one punch from normal mucosa for each patient. One to four representative punches were included from tumour tissue. Four punches were taken from majority of the cases. If the tissue sample was unrepresentative, the data were not included to the statistical analyses.

Immunohistochemistry. For all included tumour samples, we performed a universal screening for loss of MMR protein expression. To determine MMR status, IHC analysis was performed for expressions of MLH1, PMS2, MSH2, and MSH6. Following standard procedures, IHC stainings were applied to the 2-μm FFPE TMA sections using the LabVision Autostainer 480 (Thermo Fisher Scientific, Fremont, CA, USA) and BrightVision + polymer detection kit (ImmunoLogic BV, Duiven, The Netherlands). For MLH1, MSH2, MSH6, and PMS2, antigen retrieval was performed by incubation with 1 mM EDTA/10 mM Tris/HCl buffer (pH 9) at 99 °C for 15 min. The utilised antibody dilutions were 1 : 100 for *MLH1* (Novocastra, Leica Biosystems, Nussloch, Germany; NCL-L-MLH1), 1 : 150 for MSH2 (Oncogene Research Products, Cambridge, MA, USA; NA27), 1 : 50 for MSH6 (Cell Marque, Rocklin, CA, USA; 287M-16), and 1 : 400 for *PMS2* (BD Pharmingen, Franklin Lakes, NJ, USA; 556415). A 60-min incubation time was used for all antibodies. Normal MLH1, PMS2, MSH2, and MSH6 expressions in tumour samples were detectable as undisputed nuclear staining in neoplastic epithelial cells. Loss of expression was indicated by a lack of expression in tumour cells combined with the staining of internal positive controls (stromal cells or blood vessels). Tissue samples that exhibited positive staining for all four markers were considered MSS. Cases that were undisputedly negative for at least one of the four markers were classified as MSI (Shia, 2008).

To determine the *BRAF* status, samples were stained for the $BRAF^{V600E}$ hotspot mutation using a mutation-specific antibody (clone VE1, Spring Bioscience, Pleasonton, CA, US). Immunohistochemical staining was performed as described above. Antigen retrieval was performed by incubation with 1 mM EDTA/10 mM Tris/HCl buffer (pH 8) at 99 °C for 25 min. The antibody was

diluted 1 : 100, and a 60-min incubation was used. Positive staining indicated $BRAF^{V600E}$ while the lack of staining indicated wild-type *BRAF* (Thiel *et al*, 2013). $BRAF^{V600E}$ mutation-specific antibody is highly sensitive and specific in comparison with PCR-based methods or sequencing (Thiel and Ristimäki, 2013).

The IHC stains for all protein expressions were evaluated by an experienced histopathologist (JPB). In cases with uncertain staining results, a second opinion was obtained from another histopathologist (THIK). Immunohistochemical stainings were always assessed without awareness of the clinical data.

Statistical analysis. For analysis, the patients were stratified according to the biological subtypes MSI/MSS and $BRAF^{V600E}$/*BRAF* wild type, both separately and in combinations.

For categorical variables, χ^2-tests or contingency tables were used to investigate the differences between MSI, MSS, $BRAF^{V600E}$, and *BRAF* wild-type groups, and the total population. Multiple-group differences were tested using one-way analysis of variances with Bonferroni *post hoc* correction. A *P*-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS Statistics for Mac (release 19.0.0; SPSS Inc., Chicago, IL, USA).

Kaplan–Meier analysis was used to create survival curves, and a log-rank test was used to test the between-subgroup differences in survival curves. Cox regression with proportional hazard analysis was used to evaluate the survival hazard ratio (HR) between groups. Regression models included adjustments for age, sex, tumour location, American Society of Anaesthesiologists (ASA) class, and type of surgery. Separate analyses were conducted to evaluate overall survival (OS), disease-specific survival (DSS), and disease-free survival (DFS; relapse-free survival). Only variables with $P \leq 0.20$ in univariate analysis were entered in the multivariate analysis. For analysis of CRC-specific survival, patients who died from causes other than CRC were censored to the date of death. For analysis of DFS, the date of CRC recurrence after radical surgery was used as an end point. Subjects who were operated with a known non-radical result or who died within 30 days postoperatively for causes other than CRC were excluded from analyses of DSS or DFS.

RESULTS

The final analysis included a total of 762 patients with IHC results for four tumour MSI markers. Of these patients, 111 (14.6%) showed a loss of MMR protein expression that was considered the result of MSI, and 94 (12.3%) had tumours with $BRAF^{V600E}$. $BRAF^{V600E}$ was found in 60 cases with MSI (54%) and 34 cases of MSS (5.2%). Only six out of 191 rectum cancer patients had an MSI tumour, of which three also included $BRAF^{V600E}$.

Age and sex. Tables 1A and 1B present the demographical information of the study population and the subgroups, together with tumour location, staging, molecular markers, lymph node harvest, and follow-up data. Microsatellite instability prevalence increased with age in the study population. Microsatellite instability was strongly associated with female gender, proximal tumour location, and poorly differentiated histology. Compared with men, women showed higher incidences of both MSI and $BRAF^{V600E}$. Microsatellite instability incidence increased with age among women but not in men (Table 2). We identified no sex-related differences in the distribution of tumour stages or differentiation (grade). Patients with tumours with $BRAF^{V600E}$ were older than those with *BRAF* wild type. Accordingly, patients of the MSI $BRAF^{V600E}$ subtype were significantly older than other groups.

Tumour invasion and metastases. In general, MSI tumours were less likely to have metastasised to local lymph nodes (LN) compared with MSS tumours (30.5 *vs* 42.2%). The LN harvest was 15 LN/specimen in MSI tumours, 11 LN/specimen in MSS tumours, 15 LN/specimen in $BRAF^{V600E}$ tumours, and 11 LN/specimen in *BRAF* wild-type tumours. Among MSS/$BRAF^{V600E}$ tumours, 64.7% were nodal positive (N+; Table 1A).

Compared with MSI tumours, MSS tumours were more than three times more likely to have sent distant metastases (M+). M+ cases comprised 47.1% of the MSS/$BRAF^{V600E}$ subtype and only 3.3% of the MSI/$BRAF^{V600E}$ subtype. Accordingly, stage IV was most common in the MSS/$BRAF^{V600E}$ group and least common among MSI cases irrespective of *BRAF* status (Table 1A). Right-sided tumours were associated with MSI and $BRAF^{V600E}$, being most common in the MSI/ $BRAF^{V600E}$ subgroup (Table 1B).

Impact of MSI and *BRAF* status to prognosis. In the total study population, including the patients who underwent operations for emergency conditions or palliation, the overall 5-year survival was 60.8% and DSS was 72.3% (Table 3). Among all electively and radically operated patients (R0; $n=574$) the 5-year DSS was 85.0% and DFS was 78.6%. Compared with the other subtype groups, $BRAF^{V600E}$ MSS tumours were more likely to develop recurrence during follow-up after elective operation with radical outcome, but the subgroups were too small to reliably compare locoregional and distal recurrences (Table 1B).

Compared with MSS cancer, MSI was a prognostic factor for DFS and DSS (5-year DFS: 75.3% *vs* 85.8%, 10-year DFS: 72.9% *vs* 85.8%, $P=0.027$; 5-year DSS: 70.5% *vs* 83.2%, 10-year DSS: 65.0% *vs* 83.2%, $P=0.004$; Figure 1A), but not for OS in univariate analysis. $BRAF^{V600E}$ alone was an adverse prognostic factor for OS (5-year OS: 52.2% *vs* 62.3%, $P=0.014$) but not for CRC-specific survival. For DSS, univariate analysis showed that the HR for MSI *vs* MSS was 0.49 (CI: 0.30–0.80, $P=0.005$). Univariate analysis of $BRAF^{V600E}$ *vs* *BRAF* wild type revealed HR values of 1.14 (CI: 0.98–1.33, $P=0.088$) for DSS and 1.43 (CI: 1.07–1.90, $P=0.015$) for OS.

Prognostic factors in multivariate analysis. UICC stage, operation type, and combinations of MSI/MSS and $BRAF^{V600E}$/*BRAF* wild type were significant in univariate analysis, and therefore entered in all multivariate analyses. In addition, age, sex, and ASA class were included in the model of OS. Both MSI and $BRAF^{V600E}$ alone lost their independent prognostic significance when UICC stage was added to the model. $BRAF^{V600E}$ seemed to show a negative effect on DSS in rectal cancer in a multivariate model, but the numbers were small (for DSS, HR: 10.60, CI: 3.04–36.92, $P<0.001$; for OS, HR: 4.51, CI: 1.92–10.60, $P<0.001$; $n=8$; Figure 1B).

Table 4 presents multivariate analyses for combinations of MSI/MSS and $BRAF^{V600E}$/*BRAF* wild type. The MSS/$BRAF^{V600E}$ subtype was an independent factor associated with poor DSS and OS (for DSS, HR: 1.88, CI: 1.06–3.31, $P=0.030$; Figure 1C; for OS, HR: 1.87, CI: 1.17–3.00, $P=0.009$). Univariate analysis showed a protective effect of the MSI/$BRAF^{V600E}$ subtype (for DSS, HR: 0.47, CI: 0.23–0.96, $P=0.039$) compared with MSS/*BRAF* wild type, but the difference did not reach significance in the multivariate model. However, the MSI/$BRAF^{V600E}$ subtype was an independent prognostic factor for better DFS in the multivariate model (HR: 0.42, CI: 0.18–0.96, $P=0.039$). Among patients with stage I–II tumours, MSS/$BRAF^{V600E}$ subtype was an independent factor for poor DSS compared with MSS/*BRAF* wild type (HR: 5.32, CI: 1.74–16.31, $P=0.003$; Figure 1D).

DISCUSSION

MSI CRCs have a favourable prognosis despite their characteristically poor histological differentiation (Boland *et al*, 1998; Benatti, 2005; Samowitz *et al*, 2005). They also have a reduced

Table 1A. Demographical information stratified by molecular genetic subtypes

Factor	All	%	MSI	%	MSS	%	P	$BRAF^{V600E}$	%	*BRAF* wild type	%	P	MSI/ $BRAF^{V600E}$	%	MSI/*BRAF* wild type	%	MSS/ $BRAF^{V600E}$	%	MSS/*BRAF wild type*	%	P
N	762	100	111	14.6	651	85.4		94	12.3	644	87.7		60	7.9	44	5.8	34	4.5	600	81.3	
Age (years)																					
Mean (s.d.)	70.3	(11.5)	72.1	(12.8)	69.9	(11.2)	0.064	74.1	(10.8)	69.7	(11.5)	<0.001	76.1	(9.0)	66.2	(15.5)	70.4	(12.6)	70	(11.1)	<0.001
<50	37	4.9	9	8.1	28	4.3	0.007	1	1.1	33	5.1	0.117	0	0	9	20.5	1	2.9	24	4.0	0.001
50–60	103	13.5	7	6.3	96	14.7		11	11.7	90	14.0		3	5.0	4	9.1	8	23.5	86	14.3	
60–70	186	24.4	23	20.7	163	25.0		16	17.0	164	25.5		11	18.3	9	20.5	5	14.7	155	25.8	
70–80	274	36.0	38	34.2	236	36.3		35	37.2	230	35.7		25	41.7	11	25.0	10	29.4	219	36.5	
>80	162	21.3	34	30.6	128	19.7		31	33.0	127	19.7		21	35.0	11	25.0	10	29.4	116	19.3	
Sex																					
Male	370	48.6	35	31.5	335	51.5	<0.001	32	34.0	330	51.2	0.005	16	26.7	16	36.4	16	47.1	314	47.7	<0.001
Female	392	51.4	76	68.5	316	48.5		62	66.0	314	48.8		44	73.3	28	63.6	18	52.9	286	52.3	
T																					
1	56	7.3	5	4.5	51	7.8	0.103	2	2.1	51	7.9	0.008	2	3.3	1	2.3	0	0	50	8.3	0.002
2	130	17.1	13	11.7	117	18.0		7	7.4	121	18.8		6	10.0	7	15.9	1	2.9	114	19.0	
3	453	59.4	69	62.2	384	59.0		67	71.3	373	57.9		44	73.3	23	52.3	23	67.6	350	58.3	
4	123	16.1	24	21.6	99	15.2		18	19.1	99	15.4		8	13.3	13	29.5	10	29.4	86	14.3	
N																					
0	447	59.7	76	68.5	371	58.1	0.096	50	53.2	382	60.3	0.106	38	63.3	33	75.0	12	35.3	349	59.2	0.019
1	197	26.2	21	18.9	176	27.5		25	26.6	166	26.2		13	21.7	8	18.2	12	35.3	158	26.8	
2	108	14.4	14	12.6	94	14.7		19	20.2	86	13.6		9	15.0	3	6.8	10	29.4	83	13.8	
+	305	40.6	35	30.5	270	42.2	0.023	44	46.8	252	39.8	0.032	22	36.7	11	25.0	22	34.7	242	40.6	0.005
LN yield																					
Mean (s.d.)	11.6	(12.9)	15.5	(27.2)	11	(8.2)	<0.001	15.1	(28.8)	11.2	(8.4)	0.006	18.2	(35.6)	11.5	(9.5)	9.7	(5.9)	10.4	(11.2)	<0.001
M																					
0	652	86.7	107	96.4	545	85.0	<0.001	76	80.9	554	87.4	0.257	58	96.7	42	95.5	18	52.9	512	86.8	<0.001
1	100	13.3	4	3.6	96	15.0		18	19.1	80	12.6		2	3.3	2	4.5	16	47.1	78	13.2	
UICC stage																					
I	147	19.5	17	15.3	130	20.3	<0.001	8	8.5	137	21.6	0.011	7	11.7	8	18.2	1	2.9	129	21.9	<0.001
II	272	36.2	56	50.5	216	33.7		36	38.3	225	35.5		28	46.7	25	56.8	8	23.5	200	33.9	
III	233	31	34	30.6	199	31.0		32	34.0	192	30.3		23	38.3	9	20.2	9	26.5	183	31.0	
IV	100	13.3	4	3.6	96	15.0		18	19.1	80	12.6		2	3.3	2	4.5	16	47.1	78	13.2	
Tumour location																					
Proximal colon	323	42.4	86	77.5	237	36.5	<0.001	75	79.8	237	36.8	<0.001	53	88.3	26	59.1	22	64.7	211	35.2	<0.001
Distal colon	241	31.7	19	17.1	222	34.2		11	11.7	222	34.5		4	6.7	15	34.1	7	20.6	207	34.5	
Rectum	197	25.9	6	5.4	191	29.4		8	8.5	184	28.6		3	5.0	3	6.8	5	14.7	181	30.2	

Abbreviations: LN = lymph node; MSI = microsatellite instability; MSS = microsatellite stable; UICC = Union for International Cancer Control. BRAF status was missing for 24 subjects. UICC stage, *N* and *M* missing for 10 subjects. Tumour recurrence applicable for those with elective surgery and radical outcome (R0) ($n = 574$). Statistical difference for locoregional/distal recurrence not applicable due to small numbers for MSI/BRAF subtypes. For MSS/BRAFV600e *vs* MSI/BRAFV600E, $P = 0.028$, log-rank test (Mantel–Cox). Exact location was missing for one subject (colon).

Table 1B. Demographical information and follow-up data stratified by molecular genetic subtypes

Factor	All	%	MSI	%	MSS	%	*P*	$BRAF^{V600E}$	%	*BRAF wild type*	%	*P*	MSI/ $BRAF^{V600E}$	%	MSI/*BRAF wild type*	%	MSS/ $BRAF^{V600E}$	%	MSS/*BRAF wild type*	%	*P*
N	762	100	111	14.6	651	85.4		94	12.3	644	87.7		60	7.9	44	5.8	34	4.5	600	81.3	
Tumour location																					
Proximal colon	323	42.4	86	77.5	237	36.5	<0.001	75	79.8	237	36.8	< 0.001	53	88.3	26	59.1	22	64.7	211	35.2	<0.001
Distal colon	241	31.7	19	17.1	222	34.2		11	11.7	222	34.5		4	6.7	15	34.1	7	20.6	207	34.5	
Rectum	197	25.9	6	5.4	191	29.4		8	8.5	184	28.6		3	5.0	3	6.8	5	14.7	181	30.2	
MSI status																					
MSI	111	14.6	0	0	111	100		60	63.8	44	6.8	< 0.001	60	100	44	100	0	0	0	0	<0.001
MSS	651	85.4	651	100	0	0		34	36.2	600	93.2		0	0	0	0	34	100	600	100	
***BRAF* status**																					
$BRAF^{V600E}$	94	12.3	60	54.1	34	5.2	<0.001	94	100	0	0		60	100	0	0	34	100	0	0	
BRAF-wt	644	84.5	44	39.6	600	92.2		0	0	644	100		0	0	44	100	0	0	600	100	
Missing	24	3.1	7	6.3	17	2.6															
Adjuvant chemo																					
Chemo	272	64.3	37	33.3	235	36.1	0.327	36	38.3	225	34.9	0.388	21	35.0	14	31.8	15	44.1	211	35.2	0.707
No chemo	490	35.7	74	66.7	416	63.9		58	61.7	419	65.1		39	65.0	30	68.2	19	55.9	389	64.8	
Cancer recurrence[a]																					
−	449	78.2	80	87.8	369	76.2	0.035	52	82.5	386	78.1	0.452	43	89.6	31	86.1	9	60.0	355	77.5	0.005
+	125	21.8	10	11.4	115	23.8		11	17.5	108	21.9		5	10.4	5	13.9	6	40.0	103	22.5	
Locoregional	36	28.8	5	50.0	31	27.0	NA	2	18.2	32	29.2	0.079	1	20.0	4	80.0	1	16.7	28	27.2	NA
Distant	74	59.2	1	10.0	73	63.5		5	45.5	66	61.1		0	0	1	20.0	5	83.3	65	63.1	
Locoregional and distant	15	12.0	4	40.0	11	9.6		4	36.4	10	9.4		4	80.0	0	0	0	0	10	9.7	
5-Year survival, %																					
OS	60.8		67.1		59.7		0.293	51.6		62.3		0.009	64.1		70.4		29.4		61.8		<0.001
DSS	72.3		83.2		70.5		0.004	69.1		73.3		0.305	84.6		80.7		40.5		72.8		<0.001
DFS	76.9		85.8		75.3		0.027	81.9		76.8		0.334	88.2		82.8		63.1		76.9		0.139[b]

Abbreviations: Chemo = chemotherapy; DFS = disease-free survival; DSS = disease-specific survival; MSI = microsatellite instability; MSS = microsatellite stable; NA = not applicable; OS = overall survival; UICC = Union for International Cancer Control. BRAF status was missing for 24 subjects. UICC stage, *N* and *M* missing for 10 subjects. Exact location was missing for one subject (colon).

[a]Tumour recurrence applicable for those with elective surgery and radical outcome (R0) ($n = 574$). Statistical difference for locoregional/distal recurrence is not applicable due to small numbers for MSI/BRAF subtypes.

[b]For MSS/BRAFV600e *vs* MSI/BRAFV600E $P = 0.028$, log-rank test (Mantel-Cox).

likelihood of both local and distal metastasis, which is suggested by the lower incidence of stages III–IV (Malesci *et al*, 2007; Hutchins *et al*, 2011). Microsatellite instability contributes to improved survival by predicting a lower pathologic stage at diagnosis, and predicts more favourable outcome even within the same stage (Gryfe *et al*, 2000; Popat, 2004; Benatti, 2005).

This study presents the results of analysing tumour samples from a population-based cohort of CRC patients who were residing within a defined catchment area and treated by modern guidelines in the 2000s. Our findings demonstrate the prognostic relevance of MSI/$BRAF^{V600E}$ subtyping in a real-life clinical setting. Our data confirmed some previous inconsistent findings, including the favourable CRC-specific prognosis of MSI/$BRAF^{V600E}$ tumours compared with MSS/*BRAF* wild-type tumours, which was suspected by Lochhead *et al* (Lochhead *et al*, 2013; Toon *et al*, 2013). We also confirmed the previously described negative prognostic effect of $BRAF^{V600E}$ among MSS tumours (Samowitz *et al*, 2005; Ogino *et al*, 2009; Roth *et al*, 2010; Phipps *et al*, 2012; Toon *et al*, 2013; Phipps *et al*, 2015), but we did not find any prognostic significance of $BRAF^{V600E}$ within the MSI group.

Strikingly, among patients with stage I–II CRC, we found a patient subgroup with a significantly worse survival than others, which may impact current adjuvant treatment guidelines. The main unanswered clinical question related to MSI and *BRAF* status relates to the need for and usefulness of postoperative adjuvant therapy in stage I–III CRC. Our present findings indicate that patients with the MSS/$BRAF^{V600E}$ subtype are in danger of increased CRC mortality even in stage I–II and require a more aggressive adjuvant treatment approach (Figure 1D). Combinations with fluorouracil-based (5FU) chemotherapy reportedly do not improve DFS of MSI patients, but improve the course of MSS CRC (Sargent *et al*, 2010). Also, patients with MSI tumours generally do well without chemotherapy (Hutchins *et al*, 2011). In the present study, only stage III and IV patients were referred to an oncologist for adjuvant chemotherapy—primarily FOLFOX (folic acid + 5FU + oxaliplatin) or FOLFIRI (folic acid + 5FU + irinotecan). Among the stage III MSI CRC patients, 74% received adjuvant chemotherapy, without showing any survival benefit compared with those who did not (data not shown). It might be beneficial to further target adjuvant chemotherapy to the stage III MSI CRC patients with the worst prognosis. The rationale behind also administering adjuvant chemotherapy to the stage II MSS CRC patients with the worst prognosis should be studied further.

In our study, the eight subjects with rectal cancer and a $BRAF^{V600E}$ showed extremely poor survival, with an HR of 10.6 for CRC death (Figure 1C). This differs from the findings in a series of 11 patients presented by Phipps *et al* (2012) and from the results presented by Samowitz *et al* (2005), in which the rectum cancer was not reported to stand out with substantially worse prognosis. Most studies include only small numbers of rectal cancer cases, if any, compared with colon cancer cases. Furthermore, $BRAF^{V600E}$ seems to be rare in rectal cancer. The present finding of a potentially worse prognosis in MSS/$BRAF^{V600E}$ rectal cancer compared with MSS/$BRAF^{V600E}$ colon cancer has not been previously reported, and must be verified in a larger setting.

Many of our patients were followed up to over 10 years. No disease-specific CRC deaths occurred in the MSI group after 2.7 years, but events continued to occur even up to 10 years in the MSS group (Figure 1A). In addition, only one recurrence was noted in the MSI group after 2 years of DFS (at 4.8 years), whereas several recurrences were observed in the MSS group even after 5 years. Most previous studies have only reported the 5-year DSS and DFS. These observations may suggest that different follow-up schedules should apply according to MSI status.

We found a significant difference in the LN yield related to MSI, which is a relatively new finding. Earlier reports have described the possible macroscopic growth of LNs (Sloothaak *et al*, 2014) and consequent greater LN harvest (Belt *et al*, 2012; Berg and Guriby, 2013). Despite the higher number of LNs found, there were significantly fewer LN metastases in MSI CRCs (Table 1A). As patients with MSI tumours have better outcome than patients with MSS tumours (Gryfe *et al*, 2000; Popat, 2004; Aparicio *et al*, 2012; Merok *et al*, 2013), the peritumoural immune response may play an imperative role in the defence (Galon *et al*, 2014). The improved survival may be owing to a high number of cytotoxic T lymphocytes and other favourable differences in the tumour microenvironment (Guidoboni *et al*, 2001; Boissière-Michot *et al*, 2014; Richards *et al*, 2014). Our hypothesis is that the more prominent immune response in MSI leads to LN enlargement to a more easily detectable and macroscopically visible size. However, there may be other explanations. The greater LN harvest could also have resulted from the balance between right/left and colon/rectum in our material, as there was a considerable shift towards right-sided colon tumours in MSI. In addition, rectal tumours tend to have fewer investigated LNs.

Table 2. MSI prevalence and case demographical information by gender

	Male Total *N*	MSI *N* (%)	*P*	Female Total *N*	MSI *N* (%)	*P*
Total	370	35 (9.5)		392	79 (19.8)	
Age (years)						
<50	13	3 (23.1)	0.544	24	6 (25.0)	0.011
50–60	49	4 (8.2)		54	3 (5.6)	
60–70	93	9 (9.7)		93	14 (15.0)	
70–80	153	13 (8.5)		121	25 (20.7)	
>80	62	6 (9.7)		100	28 (28.0)	
Tumour location						
Proximal colon	138	26 (18.8)	<0.001	185	60 (32.4)	<0.001
Distal colon	113	6 (5.3)		128	13 (10.2)	
Rectum	118	3 (2.5)		79	3 (3.8)	
Stage						
I	78	6 (7.7)	0.222	69	11 (15.9)	0.008
II	124	16 (12.9)		148	40 (27.0)	
III	108	11 (10.2)		125	23 (18.4)	
IV	57	2 (3.5)		43	2 (4.7)	
Grade						
G1	114	8 (7.0)	0.006	133	20 (15.2)	<0.001
G2	209	16 (7.7)		196	27 (13.8)	
G3 (+mucinous)	47	11 (23.4)		61	28 (45.9)	
G4	0	0		2	1 (50.0)	

Abbreviations: MSI = microsatellite instability; UICC = Union for International Cancer Control. *P*-value for χ^2-tests, MSI subgroup *vs* total within gender. UICC stage missing for 10 subjects (3 males and 7 females). Exact location missing for one subject (male).

Table 3. Colorectal cancer disease-specific 5-year survival in MSI and MSS (%)

	All	MSI	MSS	Colon	Rectum	MSI colon	MSS colon
N	752	111	651	561	191	105	456
Stage I	94.6	100.0	94.0	96.1	92.3	100.0	95.4
Stage II	86.0	92.0	84.4	86.8	82.6	91.7	85.2
Stage III	67.9	71.6	67.4	68.3	66.7	72.8	67.5
Stage IV	14.0	25.0	14.6	12.3	18.2	0.0	13.1
All patients	72.3	83.2	70.5	72.4	72.1	83.3	69.9

Abbreviations: MSI = microsatellite instability; MSS = microsatellite stable. Table includes all elective, palliative and emergency surgery. Results are from Kaplan–Meier analysis. For 10 patients, the TNM stage was incomplete due to transanal or endoscopic removal of the tumor.

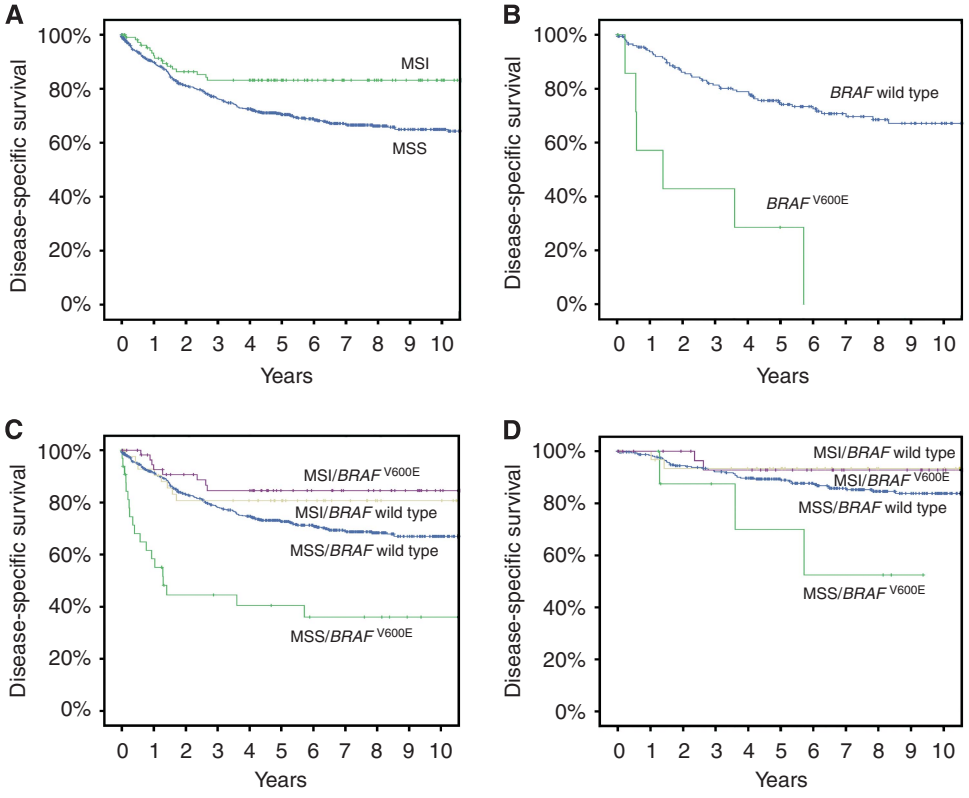

Figure 1. (**A**) Colorectal cancer (CRC) disease-specific survival (DSS) of patients with microsatellite instable (MSI; $n=111$, green line) and with microsatellite stable (MSS) tumours ($n=651$, blue line). Five-year survival: MSI, 83.2%, MSS, 70.5%. Ten-year survival: MSI, 83.2%; MSS, 65.0%. $P=0.004$ for log-rank test (Mantel–Cox). (**B**) Rectal cancer DSS of patients with $BRAF^{V600E}$ ($n=8$, green line) and wild-type *BRAF* tumours (*BRAF* wild type, $n=185$, blue line). Five-year survival: *BRAF* wild type, 73.3%; $BRAF^{V600E}$, 28.6%. Ten-year survival: *BRAF* wild type, 46.3%; $BRAF^{V600E}$, 0%. $P<0.001$ for log-rank test (Mantel–Cox). (**C**) Colorectal cancer DSS of subtypes according to the following combinations of MSI and *BRAF* status: MSI/*BRAF* wild type ($n=44$, yellow line), MSI/$BRAF^{V600E}$ ($n=60$, purple line), MSS/*BRAF* wild type ($n=600$, blue line), and MSS/$BRAF^{V600E}$ ($n=34$, green line). Five-year survival: MSI/*BRAF* wild type, 80.7%; MSI/ $BRAF^{V600E}$, 84.6%; MSS/*BRAF* wild type, 72.8%; MSS/$BRAF^{V600E}$, 40.5%. $P<0.001$ for log-rank test (Mantel–Cox). (**D**) Colorectal cancer DSS of patients with stage I–II disease according to the following combinations of MSI and *BRAF* status: MSI/*BRAF* wild type ($n=33$, yellow line), MSI/$BRAF^{V600E}$ ($n=35$, purple line), MSS/*BRAF* wild type ($n=329$, blue line), and MSS/$BRAF^{V600E}$ ($n=9$, green line). Five-year survival: MSI/*BRAF* wild type 93.4%; MSI/$BRAF^{V600E}$ 92.9%; MSS/*BRAF* wild type 89.0%; MSS/$BRAF^{V600E}$ 70.0%. $P=0.031$ for log-rank test (Mantel–Cox); MSS/$BRAF^{V600E}$ *vs* MSS/*BRAF* wild type.

Table 4. Overall, colorectal cancer disease-specific and disease-free survival by tumor subtype, HRs followed by CIs for Cox regression

Tumour subtype	*N*	Overall survival HR (95 % CI)	*P*	Disease-specific survival HR (95 % CI)	*P*	Disease-free survival HR (95 % CI)	*P*
MSS/*BRAF*-wild-type	600	1		1		1	
MSI/$BRAF^{V600E}$	60	0.83 (0.54–1.27)	0.386	0.58 (0.28–1.18)	0.131	0.42 (0.18–0.96)	0.039
MSI/*BRAF*-wild-type	44	1.16 (0.68–1.97)	0.579	1.04 (0.48–2.25)	0.93	0.86 (0.40–1.85)	0.694
MSS/$BRAF^{V600E}$	34	1.87 (1.17–3.00)	0.009	1.88 (1.06–3.31)	0.03	1.36 (0.59–3.15)	0.468

Abbreviations: ASA = American Society of Anaesthesiologists; CI = confidence interval; HR = hazard ratio; MSI = microsatellite instability; MSS = microsatellite stable; UICC = Union for International Cancer Control. Models were adjusted for age, sex, UICC stage, ASA class and operation type. MSS/BRAF wild type is reference category.

We observed a large overlap between MSI and $BRAF^{V600E}$ tumours, with 60 of the 94 $BRAF^{V600E}$ tumours (64%) also classified as MSI. Interestingly, the poor survival effect caused by $BRAF^{V600E}$ was practically overpowered by the favourable effect of MSI in the MSI/$BRAF^{V600E}$ subgroup. Hence, MSI status should always be included in studies that address *BRAF* mutation status (Hamilton, 2013).

Some clinical features of MSI have been well described over the last two decades. The nearly 15% prevalence of MSI in our study is comparable to previously reported values (Ionov *et al*, 1993) and to a similar series in northern Europe (Merok *et al*, 2013), but not to the prevalence in EPICOLON (7.4%; Moreira *et al*, 2012). Microsatellite instability tumours are more commonly situated in the proximal colon, whereas MSS tumours tend to be equally distributed between proximal and distal sites (Kim *et al*, 1994; Gryfe *et al*, 2000; Yang *et al*, 2010; Corso *et al*, 2013). As found in our present material, histology substantially differs between MSI tumours and those with proficient MMR. Microsatellite instability tumours often have a mucinous phenotype, and show intraepithelial and peritumoural lymphocytic infiltration and prominent inflammatory reaction compared with chromosomally instable tumours (Lothe *et al*, 1993; Thibodeau *et al*, 1993; Gryfe *et al*, 2000; Popat, 2004).

Our findings confirmed that female gender was associated with increasing MSI prevalence with age, while MSI frequency remained

stable in men despite age. Most recent studies have also described an association of female gender with MSI (Malkhosyan *et al*, 2000; Malesci *et al*, 2007; Phipps *et al*, 2015). The increase of incidence has been proposed to be based on the effects of oestrogen withdrawal with increasing age, as oestrogen protects against tumour instability by decreasing the promoter methylation (Miyakura *et al*, 2001; Laghi *et al*, 2003). The female gender predominance may also be linked to a serrated pathway. Serrated pathway proximal adenocarcinomas are concentrated in females (Samadder *et al*, 2013), even though serrated adenomas are more common in males (Tuppurainen *et al*, 2005). In our present study material, female patients had more proximal colon tumours than men, and MSI was significantly more common in proximal tumours. It remains unknown why women have a higher rate of conversing serrated adenomas into adenocarcinomas, but sporadic MSI carcinomas are more frequent in women, occur at an older age, and often have a serrated morphology (Young *et al*, 2001). Patients who exhibit sporadic colon cancer within 5 years after colonoscopy are more likely to have an MSI tumour than those who are diagnosed >5 years after their last colonoscopy (Nishihara *et al*, 2013). They are also more likely to be female and to have tumours at a proximal location in the colon (Erichsen *et al*, 2013). About 18% of proximal adenocarcinomas in women are serrated and they are linked to MSI predominance, which may provide a clinically relevant reason for close follow-up of serrated right adenomas in females (Snover *et al*, 2005; Mäkinen, 2007).

The current study has several limitations. All molecular analyses were conducted blinded to the clinical data, but the patient history and follow-up were obtained from the medical records and are thus retrospective by nature. The subgroup analyses are statistically solid, but the case numbers are small—especially regarding rectal cancer—and should be interpreted with caution. In addition, the clinical relevance of MSI as a prognostic factor has been criticised because most previous population-based studies have analysed samples from the 1980s and 1990s, before many modern developments in tumour staging and adjuvant chemotherapy (Malesci *et al*, 2007). Furthermore, one large well-designed study did not show that MSI influences prognosis (Barnetson *et al*, 2006). It is problematic to analyse the OS advantage of patients with MSI tumours, as many patients develop sporadic MSI CRC at an old age and other causes of death complicate the analysis (Malesci *et al*, 2007). Strengths of our study are that our material was from a defined catchment area and was collected entirely within the era during which staging was based on current guidelines; therefore, our data can be interpreted to corroborate the favourable effect of MSI. We also provided DFS and DSS in addition to OS, as well as an adequate follow-up length compared with studies with OS only (Toon *et al*, 2013). Our cohort of patients is well characterised and the follow-up time is long, but it is evident that further studies using other independent cohorts would be valuable to validate our results and conclusions. Therefore, we are seeking for collaboration with other groups of investigators with large and representative patient materials.

Overall, our data support the easy and inexpensive universal IHC screening of all CRC tumours for MSI and *BRAF* status. Tumour MSI can predict a lower risk of cancer-related death–regardless of standard prognostic factors, including tumour local invasion, but not independently of TNM classification. TNM classification only accounts for the current disease stage without predicting how aggressively the tumour may behave or how effective the immune response will be in defence. Some subgroups of patients may perform worse despite a less advanced TNM stage, thus warranting more specific subtyping of CRC. Microsatellite instability and *BRAF* are likely to complement the TNM classification and provide additional value to clinical prognostic evaluation.

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