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The prognostic role of *KRAS*, *BRAF*, *PIK3CA* and PTEN in colorectal cancer

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Background Mutations in *KRAS*, *BRAF*, *PIK3CA* and PTEN expression have been in focus to predict the effect of epidermal growth factor receptor-blocking therapy in colorectal cancer (CRC). Here, information on these four aberrations was collected and combined to a Quadruple index and used to evaluate the prognostic role of these factors in CRC.

Patients We analysed the mutation status in *KRAS*, *BRAF* and *PIK3CA* and PTEN expression in two separate CRC cohorts, Northern Sweden Health Disease Study (NSHDS; $n=197$) and Colorectal Cancer in Umeå Study (CRUMS; $n=414$). A Quadruple index was created, where Quadruple index positivity specifies cases with any aberration in *KRAS*, *BRAF*, *PIK3CA* or PTEN expression.

Results Quadruple index positive tumours had a worse prognosis, significant in the NSHDS but not in the CRUMS cohort (NSHDS; $P=0.003$ and CRUMS; $P=0.230$) in univariate analyses but significance was lost in multivariate analyses. When analysing each gene separately, only *BRAF* was of prognostic significance in the NSHDS cohort (multivariate HR 2.00, 95% CI: 1.16–3.43) and *KRAS* was of prognostic significance in the CRUMS cohort (multivariate HR 1.48, 95% CI: 1.02–2.16). Aberrations in *PIK3CA* and PTEN did not add significant prognostic information.

Conclusions Our results suggest that establishment of molecular subgroups based on *KRAS* and *BRAF* mutation status is important and should be considered in future prognostic studies in CRC.

Colorectal cancer (CRC) is one of the most common causes of cancer-related deaths in the western world (Jemal *et al*, 2008). Distant metastases represent the greatest threat to patient survival and about 40% of the patients will die from a metastatic disease. Surgical resection is today the basis for curative therapy, but a detailed understanding of the biological processes that regulate the establishment and progression of a malignant tumour may lead to improvements in non-surgical antitumour therapy. Two developmental pathways of sporadic CRC have been identified: chromosomal instability (or microsatellite stable, MSS) and microsatellite instability (MSI). Microsatellite stable tumours are considered to arise by copy number gains of oncogenes and loss of tumour suppressors, due to numerous chromosomal translocations (Grady, 2004). In contrast, MSI tumours show loss of expression of mismatch repair genes. They are less often associated with lymph node metastasis and distant spread, and MSI patients have a better prognosis than stage-matched MSS patients (Gryfe *et al*, 2000; Kohonen-Corish *et al*, 2005; Popat *et al*, 2005; Wright *et al*, 2005). Additionally, MSI tumours have been associated with CpG island methylator phenotype (CIMP) (Ahuja *et al*, 1997), where the groups CIMP-high, CIMP-low or CIMP-negative are based on promoter methylation frequency. We and others have reported a poorer prognosis for CRC patients with CIMP-high or CIMP-low tumours, compared with CIMP-negative tumours, especially in combination with MSS (Van Rijnsoever *et al*, 2003; Ward *et al*, 2003; Samowitz *et al*, 2005; Ogino *et al*, 2007; Shen *et al*, 2007; Barault *et al*, 2008; Dahlin *et al*, 2010).

Signalling through receptor tyrosine kinases in response to cytokines, growth factors and hormones is important for

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maintaining the metabolism, proliferation, survival and motility of a cell (Haglund *et al*, 2007). Many of these signals involve the oncogenic proteins KRAS, BRAF, PIK3CA and the tumour suppressor PTEN which are all downstream effectors of the epidermal growth factor receptor (EGFR) (Siena *et al*, 2009). Treatment targeting EGFR has been found to be efficient only if no mutations are found in *KRAS* or *BRAF* (Lievre *et al*, 2006). Still all patients with wild-type *KRAS* and *BRAF* do not respond to treatment (Amado *et al*, 2008; Bardelli and Siena, 2010; Tol *et al*, 2010). *PIK3CA* and PTEN have been suggested to harbour aberrations in 30–40% of all sporadic CRC cases (Samuels and Ericson, 2006; Frattini *et al*, 2007), which might explain part of this resistance. A recent study suggested that mutations in *PI3K* catalytic subunit (*PIK3CA*) may carry prognostic information in tumour stage I–III (Ogino *et al*, 2009), and that *PIK3CA*/PTEN deregulation, in addition to *KRAS* and *BRAF* mutations, may be a biomarker of resistance (Perrone *et al*, 2009; Sartore-Bianchi *et al*, 2009). Consequently, Sartore-Bianchi *et al* (2009) introduced the Quadruple index as a factor taking aberrations in these four factors into simultaneous consideration. Even though many studies are focusing on the molecules downstream EGFR to estimate benefit from EGFR blocking therapy, it is still not known how the mutations affect patient prognosis and tumour aggressiveness *per se*.

Therefore, we have in the present study analysed the mutational status of *KRAS*, *BRAF*, *PIK3CA* and PTEN expression separately, and combined as Quadruple index, and correlated the results to patient survival. Additionally, we related mutation status to established molecular tumour characteristics such as MSI screening status and CIMP status.

MATERIAL AND METHODS

Patient selection. Colorectal cancer cases from two separate patient groups were included in the present study. Archival paraffin-embedded CRC tissue samples from a total 414 patients were included from the Colorectal Cancer in Umeå Study (CRUMS), all collected during primary tumour surgery over the period 1995–2003 at Umeå University Hospital, Sweden. All routinely stained sections were reviewed by one observer, who performed all histopathological classifications including stage and tumour type (mucinous or non-mucinous). Tissue blocks from the primary tumour were chosen for DNA extraction. When necessary the proportion of tumour cells was maximised by macrodissection and necrotic areas were avoided. Clinical data were obtained by reviewing the patient records and survival data were collected from the Swedish population registry during autumn 2012 with a median follow-up time of 113 months for patients still alive at the end of follow-up.

From the Northern Sweden Health Disease Study (NSHDS), archival paraffin-embedded CRC tissue from a total of 197 patients was included. The NSHDS cohort consists of three separate cohorts: the Västerbotten Intervention Project (VIP), the Northern Sweden WHO Monitoring of Trends and Cardiovascular Disease Study (MONICA) and the local Mammography Screening Project (MSP) (Hallmans *et al*, 2003). The CRC cases in the NSHDS cohort, protocols and selection principles used in the present study have previously been described in detail (Van Guelpen *et al*, 2006). Brief summary of subjects included in the NSHDS cohort: consists of both men and women in the age of 40, 50 and 60 years in VIP; both men and women ages 25–74 years in MONICA; and only women ages ~50–70 years in MSP. Within these cohorts, a total of 226 CRC cases were identified and selected for a previous nested case-referent study (Van Guelpen *et al*, 2006). After exclusion of insufficient or unavailable tumour tissue samples, 197 patients were available for mutation analysis in the NSHDS cohort.

NSHDS patients were followed up until January 2008 with a median follow-up time of 102 months for patients still alive at the end of follow-up. Cancer-specific survival was collected from the Swedish population registry and patient records. Patients originally included in both cohorts were excluded from the CRUMS cohort and only reported once.

The handling of tissue samples and patient data in this study has been approved by the local ethics committee of Umeå University, Umeå, Sweden.

Mutational analysis of KRAS and PIK3CA exon 20. PCR conditions for *KRAS*: 50 ng DNA, 0.5 μg primer, 10 mM dNTP, 1 mM $MgCl_2$ and 0.4U JumpStart Taq (Sigma, Stockholm, Sweden) in a total volume of 20 μl. PCR were run at 95 °C 10 min, 95 °C 15 s, 65–55 °C (− 1 °C/cycle) 72 °C 30 s (touchdown for 10 cycles); 95 °C 15 s, 55 °C 15 s, 72 °C 30 s for 35 cycles and 72 °C 10 min. Primers used:

forward: 5′-tgtaaaacgacggccagtgagtttgtattaaaaggtactgg-3′.

reverse: 5′-caggaaacagctatgacctctgtatcaaagaatggtcct-3′.

PCR conditions for *PIK3CA* exon 20: 50 ng DNA, 0.5 μg primer, 10 mM dNTP, 3 mM $MgCl_2$ and 0.4U JumpStart Taq (Sigma, Stockholm, Sweden) in a total volume of 20 μl. PCR were run at 95 °C 10 min, 95 °C 21 s, 59 °C 21 s, 72 °C 30 s for 40 cycles and 72 °C 10 min. Primers used:

forward: 5′-tgtaaaacgacggccagtctcaatgatgcttggctctg-3′.

reverse: 5′-caggaaacagctatgaccatgctgttcatggattgtgc-3′.

All primers were M13-tagged (forward: 5′-tgtaaaacgacggccagt-3′; reverse: 5′-caggaaacagctatg-3′) to receive a more specific PCR product during the sequencing reaction. Sequencing was performed using Big Dye v. 3.1 according to the manufacture protocol, analysed in a 3730 xl DNA Analyser (Applied Biosystems, Stockholm, Sweden). The results were evaluated in SeqScape v2 1.1 (Applied Biosystem).

BRAF V600E mutational analysis. Detection of *BRAF* V600E mutation was done with the Taqman allelic discrimination assay (reagents from Applied Biosystems), which has been described in detail elsewhere (Benlloch *et al*, 2006).

Immunohistochemical analysis of PTEN expression. Specimens were fixed in 4% formaldehyde and embedded in paraffin, according to routine procedures at the Department of Clinical Pathology, Umeå University Hospital, Sweden. Four micrometre sections were deparaffinized and rehydrated. Antigen retrieval treatment was executed using Borg solution (Biocare Medical, Concord, CA, USA) in a pressure cooker (2100 retriever, Biocare Medical). Primary monoclonal mouse PTEN antibody (Dako, Stockholm, Sweden, clone 6H 2.1, diluted 1 : 50) was used in a semiautomatic staining machine (intelliPATH FLX, Biocare Medical).

The samples were evaluated for cytoplasmic staining, and were graded 0as no staining, 1as weak staining, and 2as moderate-strong staining. Loss of PTEN expression (graded as 0) was considered as abnormal while grade 1 and 2 was considered normal. Nerve tissue and blood vessels were used as positive internal controls in each sample. Cases without internal positive control staining were considered uninformative.

A Quadruple index was created according to Sartore-Bianchi *et al* (2009), where negative specify cases where all selected genes (*KRAS*, *BRAF* and *PIK3CA*) were wild-type and normal expression of PTEN was seen. Quadruple index positivity indicates cases where at least one of the *KRAS*, *BRAF* or *PIK3CA* genes was mutated and/or loss of PTEN expression was found.

Microsatellite instability screening status and CIMP status. Immunohistochemical analyses of mismatch repair proteins were performed as previously described (Dahlin *et al*, 2010). Briefly, expression of four mismatch repair proteins, MLH1, MSH2, MSH6

Table 1a. Clinical characteristics of colorectal cancer cases in the NSHDS cohort

		Quadruple Index			*KRAS*			*BRAF*			*PIK3CA* Exon20			PTEN		
	Total	Negative	Positive	*P-value*	Wt	Mutant	*P-value*	Wt	Mutant	*P-value*	Wt	Mutant	*P-value*	Normal	Loss	*P-value*
Frequency (%)	197	89 (51.7)	83 (48.3)		147 (82.1)	32 (17.9)		161 (82.1)	35 (17.9)		182 (97.8)	4 (2.2)		161 (87.5)	23 (12.5)	
Age, *n* (%)				0.524			0.141			0.451			0.853			0.072
<59	57 (28.9)	24 (27.0)	24 (28.9)		36 (24.5)	13 (40.6)		49 (30.4)	8 (22.9)		53 (29.1)	1 (25.0)		50 (31.1)	4 (17.4)	
60–69	111 (56.3)	50 (56.2)	50 (60.2)		90 (61.2)	14 (43.8)		87 (54.0)	23 (65.7)		102 (56.0)	2 (50.0)		86 (53.4)	18 (78.3)	
70–79	29 (14.7)	15 (16.9)	9 (10.8)		21 (14.3)	5 (15.6)		25 (15.5)	4 (11.4)		27 (14.8)	1 (25.0)		25 (15.5)	1 (4.3)	
>80	—	—	—		—	—		—	—		—	—		—	—	
Sex, *n* (%)				0.319			0.276			0.258			0.191			0.339
Men	85 (43.1)	41 (46.1)	32 (38.6)		66 (44.9)	11 (34.4)		72 (44.7)	12 (34.3)		77 (42.3)	3 (75.0)		67 (41.6)	12 (52.2)	
Women	112 (56.9)	48 (53.9)	51 (61.4)		81 (55.1)	21 (65.6)		89 (55.3)	23 (65.7)		105 (57.7)	1 (25.0)		94 (58.4)	11 (47.8)	
Tumour site, *n* (%)				<0.001			0.033			<0.001			0.894			0.726
Right-sided colon	62 (31.5)	16 (18.0)	41 (49.4)		43 (29.3)	14 (43.8)		37 (23.0)	25 (71.4)		59 (32.4)	1 (25.0)		50 (31.1)	8 (34.8)	
Left-sided colon	57 (28.9)	25 (28.1)	24 (28.9)		40 (27.2)	12 (37.5)		49 (30.4)	8 (22.9)		53 (29.1)	1 (25.0)		48 (29.8)	5 (21.7)	
Rectum	78 (39.6)	48 (53.9)	18 (21.7)		64 (43.5)	6 (18.8)		75 (46.6)	2 (5.7)		70 (38.5)	2 (50.0)		63 (39.1)	10 (43.5)	
Stage, *n* (%)				0.004			0.799			0.001			0.965			0.047
I	36 (18.4)	19 (21.3)	10 (12.0)		28 (19.0)	5 (15.6)		34 (21.3)	2 (5.7)		33 (18.1)	1 (25.0)		29 (18.1)	2 (8.7)	
II	69 (35.2)	36 (40.4)	23 (27.7)		54 (36.7)	10 (31.3)		57 (35.6)	12 (34.3)		67 (36.8)	1 (25.0)		60 (37.5)	4 (17.4)	
III	46 (23.5)	22 (24.7)	20 (24.1)		34 (23.1)	8 (25.0)		41 (25.6)	5 (14.3)		42 (23.1)	1 (25.0)		34 (21.3)	10 (43.5)	
IV	45 (23.0)	12 (13.5)	30 (36.1)		31 (21.1)	9 (28.1)		28 (17.5)	16 (45.7)		40 (22.0)	1 (25.0)		37 (23.1)	7 (30.4)	
Histology type, *n* (%)				0.567			0.526			0.134			0.329			0.846
Non-mucinous	158 (80.6)	71 (80.7)	64 (77.1)		116 (79.5)	27 (84.4)		132 (82.5)	25 (71.4)		146 (80.7)	4 (100.0)		128 (80.0)	18 (78.3)	
Mucinous	38 (19.4)	17 (19.3)	19 (22.9)		30 (20.5)	5 (15.6)		28 (17.5)	10 (28.6)		35 (19.3)	0 (0.0)		32 (20.0)	5 (21.7)	

Abbreviations: NSHDS = Northern Sweden Health Disease Study; Wt = wild-type. Following numbers of missing cases were present in NSHDS: Quadruple Index, 25; *KRAS* mutation status, 18; *BRAF* mutation status, 1; *PIK3CA* mutation status, 11; PTEN mutation status, 13; Stage, 1; Histology type, 1; Adjuvant chemotherapy, 11; Preoperative, 2. Kruskal–Wallis test was used for continuous variables, χ^2-test or Fisher's exact test used for categorical variables.

Table 1b. Clinical characteristics of colorectal cancer cases in the CRUMS cohort

		Quadruple index			*KRAS*			*BRAF*			*PIK3CA*Exon20			PTEN		
	Total	**Negative**	**Positive**	***P-value***	**Wt**	**Mutant**	***P-value***	**Wt**	**Mutant**	***P-value***	**Wt**	**Mutant**	***P-value***	**Normal**	**Loss**	***P-value***
Frequency (%)	414	227 (56.0)	178 (44.0)		331 (80.5)	80 (19.5)		356 (86.8)	54 (13.2)		396 (97.8)	9 (2.2)		352 (85.9)	58 (14.1)	
Age, *n* (%)				0.572			0.287			0.017			0.226			0.807
<59	68 (16.4)	41 (18.1)	23 (12.9)		55 (16.6)	13 (16.3)		64 (18.0)	3 (5.6)		66 (16.7)	0 (0.0)		59 (16.8)	7 (12.1)	
60–69	82 (19.8)	44 (19.4)	36 (20.2)		65 (19.6)	15 (18.8)		70 (19.7)	9 (16.7)		77 (19.4)	4 (44.4)		70 (19.9)	11 (19.0)	
70–79	162 (39.1)	88 (38.8)	73 (41.0)		136 (41.1)	26 (32.5)		131 (36.8)	31 (57.4)		155 (39.1)	3 (33.3)		137 (38.9)	24 (41.4)	
>80	102 (24.6)	54 (23.8)	46 (25.8)		75 (22.7)	26 (32.5)		91 (25.6)	11 (20.4)		98 (24.7)	2 (22.2)		86 (24.4)	16 (27.6)	
Sex, *n* (%)				0.179			0.622			0.313			0.209			0.313
Men	233 (56.3)	135(59.5)	94 (52.8)		188 (56.8)	43 (53.8)		204 (57.3)	27 (50.0)		225 (56.8)	7 (77.8)		201 (57.1)	29 (50.0)	
Women	181 (43.7)	92 (40.5)	84 (47.2)		143 (43.2)	37 (46.3)		152 (42.7)	27 (50.0)		171 (43.2)	2 (22.2)		151 (42.9)	29 (50.0)	
Tumour site, *n* (%)				<0.001			0.100			<0.001			0.700			0.682
Right-sided colon	132 (32.2)	46 (20.5)	83 (46.9)		98 (30.0)	34 (42.5)		88 (25.0)	43 (79.6)		124 (31.6)	4 (44.4)		133 (32.4)	17 (29.8)	
Left-sided colon	126 (30.7)	82 (36.6)	42 (23.7)		104 (31.8)	21 (26.3)		118 (33.5)	6 (11.1)		122 (31.1)	2 (22.2)		110 (31.5)	16 (28.1)	
Rectum	152 (37.1)	96 (42.9)	52 (29.4)		125 (38.2)	25 (31.3)		146 (41.5)	5 (9.3)		146 (37.2)	3 (33.3)		126 (36.1)	24 (42.1)	
Stage, *n* (%)				0.162			0.030			0.744			0.293			0.800
I	63 (15.5)	41 (18.6)	19 (10.8)		57 (17.6)	4 (5.1)		55 (15.8)	7 (13.0)		60 (15.4)	2 (25.0)		53 (15.4)	10 (17.5)	
II	164 (40.4)	88 (39.8)	71 (40.3)		131 (40.4)	32 (40.5)		137 (39.4)	24 (44.4)		152 (39.1)	5 (62.5)		143 (41.4)	20 (35.1)	
III	87 (21.4)	44 (19.9)	43 (24.4)		67 (20.7)	20 (25.3)		74 (21.3)	13 (24.1)		87 (22.4)	0 (0.0)		71 (20.6)	14 (24.6)	
IV	92 (22.7)	48 (21.7)	43 (24.4)		69 (21.3)	23 (29.1)		82 (23.6)	10 (18.5)		90 (23.1)	1 (12.5)		78 (22.6)	13 (22.8)	
Histology type, *n* (%)				0.023			0.515			<0.001			0.239			0.852
Non-mucinous	348 (85.3)	198 (88.8)	142 (80.7)		275 (84.6)	70 (87.5)		310 (88.6)	35 (64.8)		333 (85.2)	8 (100.0)		295 (85.0)	49 (86.0)	
Mucinous	60 (14.7)	25 (11.2)	34 (19.3)		50 (15.4)	10 (12.5)		40 (11.4)	19 (32.2)		58 (14.8)	0 (0.0)		52 (15.0)	8 (14.0)	

Abbreviations: CRUMS = Colorectal Cancer in Umeå Study; Wt = wild-type. Following numbers of missing cases were present in CRUMS: Quadruple Index, 9; *KRAS* mutation status, 3; *BRAF* mutation status, 4; *PIK3CA* mutation status, 9; PTEN mutation status, 4; Tumour site, 4; Stage, 8; Histology type, 6; Adjuvant chemotherapy, 6; Preoperative, 3. Kruskall–Wallis test was used for continuous variables, χ^2-test or Fisher's exact test used for categorical variables.

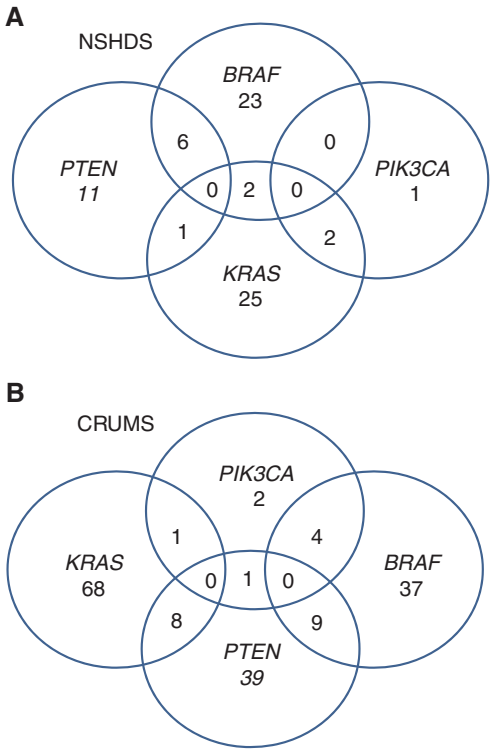

Figure 1. The interrelationship between cases with mutations in *KRAS*, *BRAF*, *PIK3CA* and loss of PTEN expression in the NSHDS and the CRUMS cohorts. Total number of aberrations in NSHDS (**A**); KRAS ($N=30$), BRAF ($N=31$), PIK3CA ($N=3$), PTEN ($N=18$); CRUMS (**B**); KRAS ($N=77$), BRAF ($N=50$), PIK3CA ($N=8$), PTEN ($N=57$). Patients with missing value in any of the marker were excluded from the Figure.

and PMS2 were analysed in formalin-fixed and paraffin-embedded human CRC tissue. Tissue samples lacking nuclear staining in tumour cells for at least one of these proteins were considered to have a positive MSI screening status, referred to as MSI. Negative MSI screening status based on immunohistochemical staining is referred to as MSS.

Methylation analysis to determine tumour CIMP status was performed by the MethyLight method, with primer and probe sequences as previously described (Weisenberger *et al*, 2006; Dahlin *et al*, 2010). The per cent of methylated refence (PMR) value was caluculated for the eight genes included in the CIMP panel (*CDKN2A*, *MLH1*, *CACNA1G*, *NEUROG1*, *RUNX3*, *SOCS1*, *IGF2* and *CRABP1*) (Dahlin *et al*, 2010), and a gene was considered positive for methylation when the PMR >10 (Weisenberger *et al*, 2006).

Tumours with no promoter hypermethylation were classified as CIMP-negative, 1–5 genes methylated as CIMP-low, and 6–8 genes as CIMP-high (Dahlin *et al*, 2010).

Statistical analysis. Clinico-pathological characteristics were compared using Kruskal–Wallis tests for continuous variables and χ^2-tests, or Fisher's exact tests when observed or expected frequencies were less than five for categorical variables. For cancer-specific survival analyses, Kaplan–Meier plots were used, and differences between groups were tested by log-rank tests. Cancer-specific events were defined as death with known disseminated or recurrent disease, and cases were censored at the end of follow-up or at time of death by other causes.

Patients in CRUMS who were deceased with postoperative complications within 1 month after surgery ($n=16$) were excluded from the survival analyses. Deaths due to postoperative complications were not recorded in NSHDS, but only four patients died within 1 month of surgery. To take into consideration other clinico-pathological factors, multivariate Cox proportional hazard models were used. For multivariate analyses, we analysed Quadruple index, *KRAS* and *BRAF* and not *PIK3CA* and PTEN, as the latter two were not significantly associated with prognosis in univariate analyses. The adjusting variables were selected if they affected the risk estimates for *KRAS* and *BRAF* $>10\%$ in bivariate analyses. The final multivariate model included sex, age at diagnosis, stage and tumour site. Other factors tested, but not meeting the criteria for inclusion in the multivariate analyses were aberrant p53 protein expression, mucinous histologic tumour type, preoperative radiotherapy and adjuvant chemotherapy. Microsatellite instability screening status and CIMP status were also tested but excluded due to small subgroups and thereby loss of statistical power. All statistical tests were conducted using PASW Statistics 18 (SPSS Inc., Chicago, IL, USA).

RESULTS

Quadruple index in relation to clinico-pathological variables. We analysed each mutation (*KRAS*, *BRAF* and *PIK3CA*) and PTEN expression as well as the Quadruple Index, in tumours from 197 patients in the NSHDS and 414 patients in the CRUMS cohort with respect to different clinico-pathological characteristics (Tables 1A and 1B). Seven different activating mutations in codon 12 and 13 were analysed in *KRAS*, and the mutation frequency was 17.9% in the NSHDS and 19.5% in the CRUMS cohort. *BRAF* was observed in 17.9 and 13.2% in each study population respectively (Tables 1A and 1B). When combining results from the four studied factors, only two patients had both *BRAF* and *KRAS* mutated in the NSHDS cohort (Figure 1A), while *BRAF* and *KRAS* mutations were mutually exclusive (Figure 1B) in the CRUMS cohort. Four different mutations were analysed in *PIK3CA*, exon 20, where the mutation frequency was 2.2% in both cohorts. Loss of PTEN expression was found in 12.5% in the NSHDS and 14.1% in the CRUMS cohort (Tables 1A and 1B). In the NSHDS cohort mutated *KRAS* and *BRAF* tumours were associated with right colon location, most distinct for *BRAF (NSHDS;* $P<0.001$). In the CRUMS cohort, *BRAF* mutant tumours were significantly correlated to older age (CRUMS; $P=0.017$) and right colon location (CRUMS; $P<0.001$), while *KRAS* mutations were significantly associated with higher tumour stage (CRUMS; $P=0.030$). *BRAF* mutations were most prevalent in mucinous tumours (Tables 1A and 1B).

The frequencies of Quadruple index positivity were 48.3% in the NSHDS and 44.0% in the CRUMS cohort. Quadruple index positivity was correlated significantly to right colon location in both patient groups (NSHDS and CRUMS; both $P<0.001$). Quadruple index positivity, *BRAF* mutations and loss of PTEN expression were significantly associated with higher tumour stage in the NSHDS, but not in the CRUMS cohort (Tables 1A and 1B).

Quadruple index in relation to MSI screening status and CIMP status. Tables 2A and 2B shows Quadruple index and each mutation (*KRAS*, *BRAF* and *PIK3CA*) and PTEN expression in relation to both MSI screening status and CIMP status in the NSHDS and the CRUMS cohort. Quadruple index positivity correlated significantly to CIMP-high status (NSHDS; $P=0.002$ and CRUMS; $P<0.001$) in both the NSHDS and the CRUMS cohort, and to MSI (CRUMS; $P<0.001$) in the CRUMS cohort. *KRAS* mutations were more often seen in patients with MSS (NSHDS; $P=0.031$ and CRUMS; $P=0.002$) and CIMP-low tumours (NSHDS; $P=0.046$ and CRUMS; $P=0.001$). *BRAF* mutations were significantly associated with MSI (NSHDS; $P<0.001$ and CRUMS; $P<0.001$) and CIMP-high (NSHDS;

Table 2a. Molecular characteristics of colorectal cancer cases in the NSHDS cohort

	N	**MSI**	**MSS**	***P-value***	**CIMP-negative**	**CIMP-low**	**CIMP-high**	***P-value***
Frequency (%)	197	24 (12.2)	173 (87.8)		97 (50.0)	70 (36.1)	27 (13.9)	
Quadruple Index				0.384				0.002
Negative	89 (51.7)	9 (42.9)	80 (53.0)		52 (61.9)	31 (50.0)	6 (23.1)	
Positive	83 (48.3)	12 (57.1)	71 (47.0)		32 (38.1)	31 (50.0)	20 (76.9)	
KRAS				0.031				0.046
Wt	147 (82.1)	19 (100.0)	128 (80.0)		68 (79.1)	52 (78.8)	24 (100.0)	
Mutant	32 (17.9)	0 (0.0)	32 (20.0)		18 (20.9)	14 (21.2)	0 (0.0)	
BRAF				<0.0001				<0.0001
Wt	161 (82.1)	13 (54.2)	148 (86.0)		93 (96.9)	57 (81.4)	8 (29.6)	
Mutant	35 (17.9)	11 (45.8)	24 (14.0)		3 (3.1)	13 (18.6)	19 (70.4)	
PIK3CA Exon20				0.448				0.670
Wt	182 (97.8)	23 (100.0)	159 (97.5)		91 (97.8)	63 (96.9)	25 (100.0)	
Mutant	4 (2.2)	0 (0.0)	4 (2.5)		2 (2.2)	2 (3.1)	0 (0.0)	
PTEN				1.000				0.641
Normal	161 (87.5)	21 (87.5)	140 (87.5)		80 (86.0)	58 (90.6)	23 (85.2)	
Loss	23 (12.5)	3 (12.5)	20 (12.5)		13 (14.0)	6 (9.4)	4 (14.8)	

Abbreviations: CIMP = CpG island methylator phenotype; MSS = microsatellite stable; NSHDS = Northern Sweden Health Disease Study; MSI = microsatellite instability; Wt = wild-type. The following numbers of missing cases were present in NSHDS: CIMP status, 3; Quadruple Index, 25; *KRAS* mutation status, 18; *BRAF* mutation status, 1; *PIK3CA* mutation status, 11; PTEN mutation status, 13. Cases lacking nuclear staining of tumour cells for at least one of MLH1, MSH2, MSH6 or PMS2 were considered to have a positive MSI screening status (MSI). CIMP according to an eight-gene panel including *CDKN2A, hMLH1, CACNA1G, NEUROG1, RUNX3, SOCS1, IGF2* and *CRABP1*; CIMP-negative, 0 genes hypermethylated; CIMP-low, 1–5 genes hypermethylated; CIMP-high, 6–8 genes hypermethylated. Kruskall–Wallis test was used for continuous variables, χ^2-test or Fisher's exact test used for categorical variables.

Table 2b. Molecular characteristics of colorectal cancer cases in the CRUMS cohort

	N	**MSI**	**MSS**	***P-value***	**CIMP-negative**	**CIMP-low**	**CIMP-high**	***P-value***
Frequency (%)	414	62 (15.5)	338 (84.5)		209 (50.6)	155 (37.5)	49 (11.9)	
Quadruple Index				<0.0001				<0.0001
Negative	227 (56.0)	19 (31.7)	201 (60.5)		142 (69.3)	82 (54.3)	3 (6.3)	
Positive	178 (44.0)	41 (68.3)	131 (39.5)		63 (30.7)	69 (45.7)	45 (93.8)	
KRAS				0.002				0.001
Wt	331 (80.5)	59 (95.2)	263 (78.3)		174 (83.7)	111 (72.1)	46 (93.9)	
Mutant	80 (19.5)	3 (4.8)	73 (21.7)		34 (16.3)	43 (27.9)	3 (6.1)	
BRAF				<0.0001				<0.0001
Wt	356 (86.8)	27 (44.3)	317 (94.6)		206 (99.0)	143 (92.9)	7 (14.6)	
Mutant	54 (13.2)	34 (55.7)	18 (5.4)		2 (1.0)	11 (7.1)	41 (85.4)	
PIK3CA Exon20				0.013				0.006
Wt	396 (97.8)	55 (93.2)	328 (98.5)		204 (99.0)	150 (98.0)	42 (91.3)	
Mutant	9 (2.2)	4 (6.8)	5 (1.5)		2 (1.0)	3 (2.0)	4 (8.7)	
PTEN				0.719				0.729
Normal	352 (85.9)	52 (83.9)	286 (85.6)		178 (85.6)	134 (87.6)	40 (83.3)	
Loss	58 (14.1)	10 (16.1)	48 (14.4)		30 (14.4)	19 (12.4)	8 (16.7)	

Abbreviations: CIMP = CpG island methylator phenotype; CRUMS = Colorectal Cancer in Umeå Study; MSI = microsatellite instability; MSS = microsatellite stable; Wt = wild-type. The following numbers of missing cases were present in CRUMS: CIMP status,1; Quadruple Index, 9; *KRAS* mutation status, 3; *BRAF* mutation status, 4; *PIK3CA* mutation status, 9; PTEN mutation status, 4. Cases lacking nuclear staining of tumor cells for at least one of MLH1, MSH2, MSH6 or PMS2 were considered to have a positive MSI screening status (MSI). CIMP according to an eight-gene panel including *CDKN2A, hMLH1, CACNA1G, NEUROG1, RUNX3, SOCS1, IGF2* and *CRABP1*; CIMP-negative, 0 genes hypermethylated; CIMP-low, 1–5 genes hypermethylated; CIMP-high, 6–8 genes hypermethylated. Kruskall–Wallis test was used for continuous variables, χ^2-test or Fisher's exact test used for categorical variables.

$P<0.001$ and CRUMS; $P<0.001$). Mutations in the *PIK3CA* gene significantly correlated to MSI (CRUMS; $P=0.013$) and CIMP-high (CRUMS; $P=0.006$) in the CRUMS cohort, but showed no statistical significance in the NSHDS cohort. Loss of PTEN expression did not show significant correlation to MSI screening status or CIMP status in any of the cohorts.

Survival analysis. Cancer-specific survival analyses revealed that Quadruple index positive cases had a significantly worse prognosis compared with negative cases in the NSHDS cohort (Figure 2A; univariate HR 1.98, 95% CI: 1.25–3.13). However, the Quadruple index positive cases had only a slightly poorer, but not statistically significant, prognosis in the CRUMS cohort (Figure 2B; univariate HR 1.22, 95% CI: 0.88–1.69).

When analysing each gene separately only *BRAF* mutations turned out to be of prognostic value in the NSHDS cohort (Figure 2E), a result that retained statistical significant also in a multivariate Cox proportional hazard model (Table 3A).

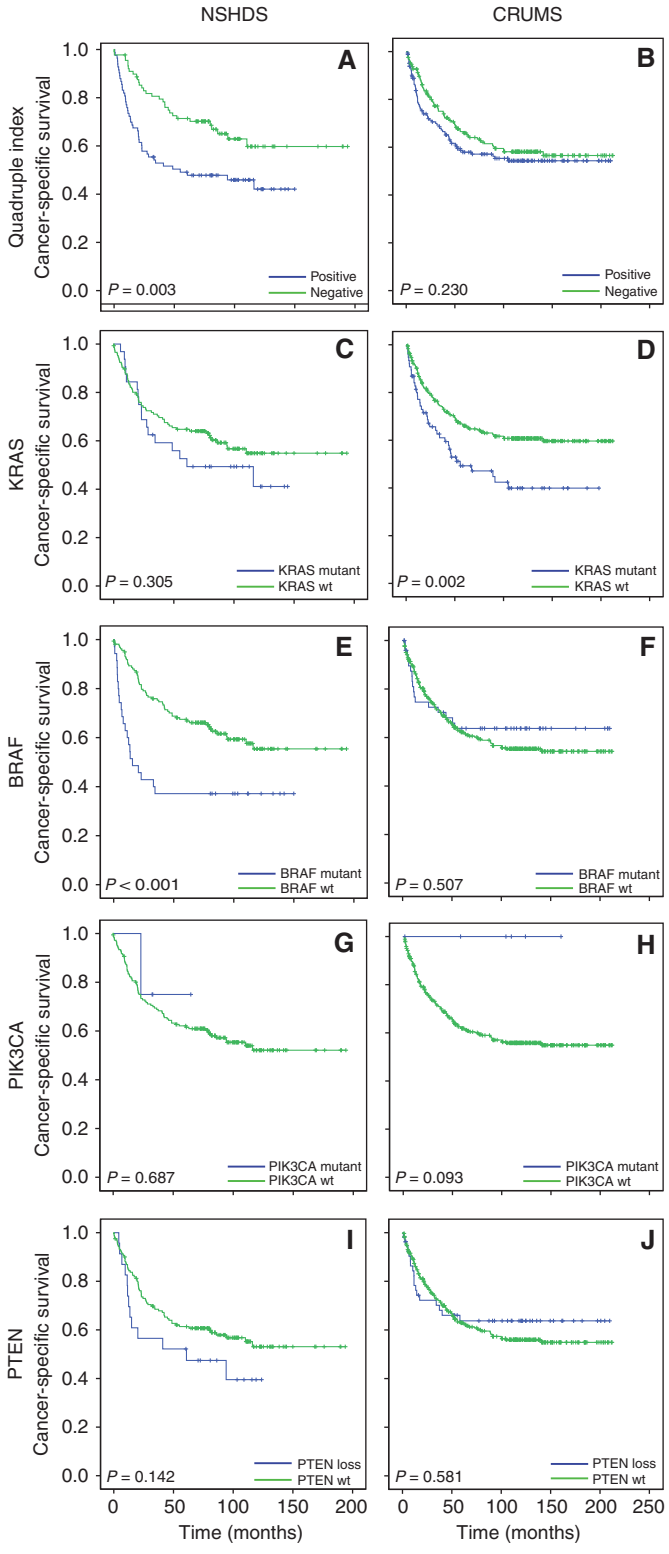

Figure 2. Cancer-specific survival analyses with respect to the Quadruple index and the *KRAS*, *BRAF*, *PIK3CA* and loss of PTEN expression separately.

In the CRUMS cohort, on the other hand, only *KRAS* mutations were of prognostic value (Figure 2D), and this was seen also in multivariate analyses (Table 3B). Neither *PIK3CA* mutations, nor loss of PTEN expression were of prognostic significance in any of the two cohorts when analysed separately (Figure 2G–J).

Table 3a. Cox regression of colorectal cancer cases in the NSHDS cohort

N	Univariate HR (CI 95%)	Multivariate HR (CI 95%)
Quadruple Index		
172	1.978 (1.251–3.128)	1.308 (0.787–2.174)
KRAS		
179	1.325 (0.773–2.271)	0.798 (0.443–1.438)
BRAF		
196	2.428 (1.490–3.956)	1.998 (1.165–3.426)
***PIK3CA* Exon20**		
186	0.657 (0.091–4.739)	0.285 (0.038–2.141)
PTEN		
184	1.555 (0.859–2.816)	1.289 (0.699–2.376)

Abbreviations: CI = confidence interval; HR = hazard ratio, NSHDS = Northern Sweden Health Disease Study. HR determined by Cox proportional hazard models, adjusted for sex, age, tumour site and tumour stage.

Table 3b. Cox regression of colorectal cancer cases in the CRUMS cohort

N	Univariate HR (CI 95%)	Multivariate HR (CI 95%)
Quadruple Index		
372	1.220 (0.881–1.689)	1.157 (0.827–1.619)
KRAS		
378	1.761 (1.220–2.542)	1.485 (1.023–2.155)
BRAF		
377	0.843 (0.508–1.397)	0.914 (0.529–1.576)
***PIK3CA* Exon20**		
372	0.000 (0.000–1.408 E + 122)	0.000 (0.000–1.088E169)
PTEN		
377	0.870 (0.531–1.426)	0.862 (0.519-1.431)

Abbreviations: CI = confidence interval; CRUMS = Colorectal Cancer in Umeå Study; HR = hazard ratio HR determined by Cox proportional hazard models, adjusted for sex, age, tumour site and tumour stage.

Survival analyses stratified for MSI screening status and CIMP status. Patients with Quadruple index positive tumours with MSS (NSHDS; $P = 0.002$), or CIMP-low (NSHDS; $P = 0.022$) or CIMP-high tumours (CRUMS; $P = 0.042$) had a worse prognosis than Quadruple index negative cases. Cancer-specific survival analyses stratified for *KRAS* and *BRAF* is shown in Figure 3. Patients with tumours harbouring *BRAF* mutations together with MSS (NSHDS; $P = < 0.001$) (Figure 3G) or CIMP-low (NSHDS; $P < 0.001$) (Figure 3O) showed an impaired survival in the NSHDS cohort. In the CRUMS cohort, tumours with *KRAS* mutations accompanied with MSS (Figure 3F) (CRUMS; $P = 0.042$) or CIMP-negative (CRUMS; $P = 0.010$) or *BRAF* mutations in CIMP-high tumours (CRUMS; $P = 0.001$) (Figure 3T) showed a poorer patient prognosis. Owing to the loss of statistical power in these small subgroups, a multivariate model was not performed.

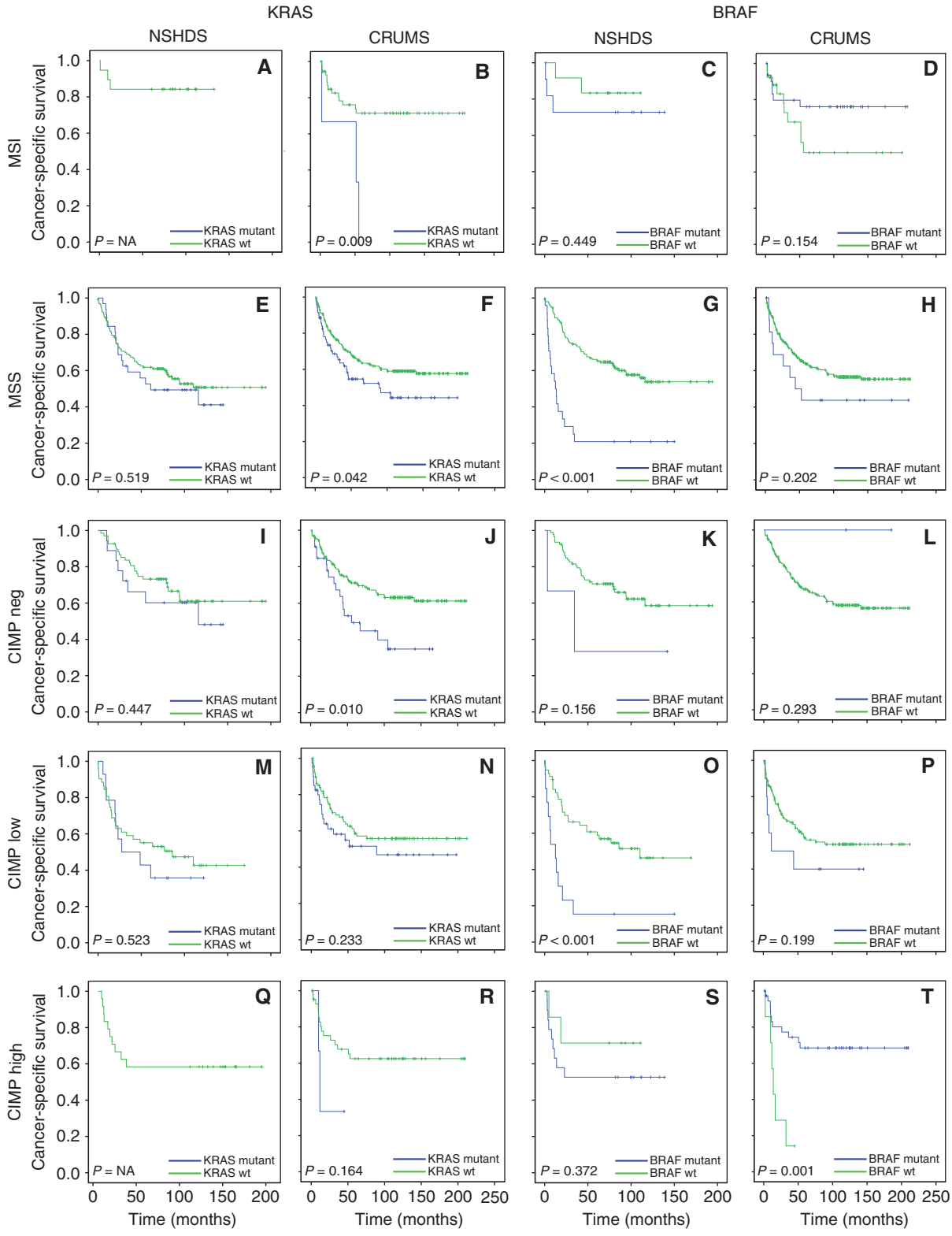

Figure 3. Cancer-specific survival analyses in the NSHDS and the CRUMS, stratified for *KRAS* or *BRAF* mutations, in relation to MSI screening status and CIMP status.

DISCUSSION

In this study archival CRC tissue from two different cohorts from Northern Sweden, NSHDS and CRUMS, were analysed regarding mutations in the genes *KRAS*, *BRAF*, *PIK3CA* and loss of PTEN expression. All four aberrations investigated in this study are part of the same signalling pathway, downstream the EGFR, and to get an increased understanding for how these factors are interconnected in CRC, a Quadruple index as suggested by Sartore-Bianchi

et al (2009) was created, where Quadruple index positive tumours had at least one mutation in any of the genes *KRAS*, *BRAF*, *PIK3CA* and/or loss of PTEN protein expression.

We found a shorter cancer-specific survival in patients with Quadruple index positive tumours in the NSHDS cohort, but the Quadruple index was not statistically significant in the CRUMS cohort. Analysing each gene separately revealed that only mutations in the *BRAF* gene had a significant prognostic value in the NSHDS cohort, especially in combination with MSS or CIMP-low. Only *KRAS* mutations, on the other hand, indicated a significantly poorer patient prognosis in the CRUMS cohort, especially together with MSS or CIMP-negative tumours. Aberrations in *PIK3CA* and PTEN did not add significant prognostic information. Therefore, our results do not support the use of the full Quadruple index but instead emphasise the prognostic information in *KRAS* and *BRAF* mutation status.

Taken together, these results indicate that the establishment of molecular subgroups of CRC based on *KRAS* and *BRAF* mutation status can supply important information, not only in prediction of the EGFR-treatment response but also in prediction of patient prognosis. Importantly, *KRAS* and *BRAF* mutations are nearly mutually exclusive in CRC (Jakubauskas and Griskevicius, 2010; Li *et al*, 2011; Krol *et al*, 2012).

The finding of contrary significances for *KRAS* and *BRAF* mutations in the two cohorts is not easily explained. However, it should be noted that the composition and the underlying design of the two cohorts differs significantly. For example, NSHDS consists of more women than men as a direct result of including the Mammary Screening Project as one of the three subcohorts, and *BRAF* mutations have more often been reported in women (Ogino *et al*, 2012). Furthermore, the age distribution also differs between the two cohorts and might have impact on the results. Not only the *KRAS* and *BRAF* mutations, but also molecular characteristics such as MSI screening status and CIMP status, are well known to correlate with the age and sex distribution (Nosho *et al*, 2009; Kalady *et al*, 2012). The contradictory results, however, emphasise a need for further larger studies on this topic.

One of the main strengths of this study was the two large, non-overlapping, patient groups, which were both from the same northern Swedish population but had different recruitment protocols, age range and sex distributions. The patients in the present study were generally diagnosed previous to the broad introduction of many novel therapies, including successful resection of liver metastases, into clinical practice. Treatment was thus fairly homogeneous within each tumour site and stage. Residual confounding effect due to differences in treatment is therefore unlikely. It is not possible, however, to analyse the predictive value of mutations with respect to EGFR-blocking therapy in our patient cohorts due to the lack of such treatment during the cohort recruitment. Instead, the two cohorts include all tumour stages and are suitable for studies on tumour aggressiveness and prognosis.

The present study is, to the best of our knowledge, the largest study today on this subject. Despite the use of two patient cohorts, a limitation is, however, still the relatively low number of patients, especially when analysing somewhat rare subgroups (e.g., *PIK3CA* mutations, MSI cases or CIMP-high cases). The fact that we could not detect any correlation between loss of PTEN expression or *PIK3CA* mutations and patient prognosis makes us speculate that the need for analysing all four genes, as in the Quadruple index, might be unnecessary when prognosticating cancer-specific survival. There are, however, contradictory reports indicating that both *PIK3CA* mutations and loss of PTEN protein expression do affect patient prognosis (Sawai *et al*, 2008; Li *et al*, 2009; Jang *et al*, 2010; Liao *et al*, 2012).

The mutation frequencies of each analysed gene found in this study were in general similar to previous reports (Rako *et al*, 2012; Soeda *et al*, 2012), except for the *KRAS* gene. We report a frequency of about 20%, while several other reports have reported frequencies of 30–40% (Kim *et al*, 2012). The low mutation frequency of *KRAS* in our studied populations can have several explanations. Our patient cohorts have a rather high proportion of rectal cancers, and rectal cancers have a lower *KRAS* mutation frequency than colon cancers. Technical differences between studies are another likely explanation, and here we have not analysed *KRAS* mutations in exon 61. Furthermore, most studies reporting the frequency of *KRAS* mutations have studied only metastatic CRCs, and *KRAS*-mutated CRC might be more aggressive than their wild-type counterparts.

Previous reports on *PIK3CA* mutation frequencies in CRC have varied considerably. In this study we report a frequency of about 2%. However, we have only analysed mutations in exon 20 in *PIK3CA*, not exon 9, based on recently published data showing that only mutations in exon 20 have a prognostic value (De Roock *et al*, 2010; Farina Sarasqueta *et al*, 2011), probably as this exon translates the kinase domain of *PIK3CA*. Additionally Muller *et al* (2007), recently found a *PIK3CA* pseudogene spanning exons 9–13 located on chromosome 22, which might be the reason for such a high reported frequency of *PIK3CA* exon 9 mutations.

In conclusion, by the use of two patient cohorts we show that mutations in the *KRAS* and *BRAF* genes are of prognostic importance in colorectal cancer. However, adding information on mutation status of *PIK3CA* and loss of PTEN does not add significant prognostic information. These results suggest that establishment of molecular subgroups based on *KRAS* and *BRAF* mutation status is important and should be considered in future prognostic studies in CRC.

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REFERENCES

Ahuja N, Mohan AL, Li Q, Stolker JM, Herman JG, Hamilton SR, Baylin SB, Issa JP (1997) Association between CpG island methylation and microsatellite instability in colorectal cancer. *Cancer Res* **57**: 3370–3374.

Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD, Chang DD (2008) Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* **26**: 1626–1634.

Barault L, Charon-Barra C, Jooste V, de la Vega MF, Martin L, Roignot P, Rat P, Bouvier AM, Laurent-Puig P, Faivre J, Chapusot C, Piard F (2008) Hypermethylator phenotype in sporadic colon cancer: study on a population-based series of 582 cases. *Cancer Res* **68**: 8541–8546.

Bardelli A, Siena S (2010) Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer. *J Clin Oncol* **28**: 1254–1261.

Benlloch S, Paya A, Alenda C, Bessa X, Andreu M, Jover R, Castells A, Llor X, Aranda FI, Massuti B (2006) Detection of BRAF V600E mutation in colorectal cancer: comparison of automatic sequencing and real-time chemistry methodology. *J Mol Diagn* **8**: 540–543.

Dahlin AM, Palmqvist R, Henriksson ML, Jacobsson M, Eklof V, Rutegard J, Oberg A, Van Guelpen BR (2010) The role of the CpG island methylator phenotype in colorectal cancer prognosis depends on microsatellite instability screening status. *Clin Cancer Res* **16**: 1845–1855.

De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, Kalogeras KT, Kotoula V, Papamichael D, Laurent-Puig P, Penault-Llorca F, Rougier P, Vincenzi B, Santini D, Tonini G,

Cappuzzo F, Frattini M, Molinari F, Saletti P, De Dosso S, Martini M, Bardelli A, Siena S, Sartore-Bianchi A, Tabernero J, Macarulla T, Di Fiore F, Gangloff AO, Ciardiello F, Pfeiffer P, Qvortrup C, Hansen TP, Van Cutsem E, Piessevaux H, Lambrechts D, Delorenzi M, Tejpar S (2010) Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol* **11**: 753–762.

Farina Sarasqueta A, Zeestraten EC, van Wezel T, van Lijnschoten G, van Eijk R, Dekker JW, Kuppen PJ, Goossens-Beumer IJ, Lemmens VE, van de Velde CJ, Rutten HJ, Morreau H, van den Brule AJ (2011) PIK3CA kinase domain mutation identifies a subgroup of stage III colon cancer patients with poor prognosis. *Cell Oncol* **34**: 523–531.

Frattini M, Saletti P, Romagnani E, Martin V, Molinari F, Ghisletta M, Camponovo A, Etienne LL, Cavalli F, Mazzucchelli L (2007) PTEN loss of expression predicts cetuximab efficacy in metastatic colorectal cancer patients. *Br J Cancer* **97**: 1139–1145.

Grady WM (2004) Genomic instability and colon cancer. *Cancer Metastasis Rev* **23**: 11–27.

Gryfe R, Kim H, Hsieh ET, Aronson MD, Holowaty EJ, Bull SB, Redston M, Gallinger S (2000) Tumour microsatellite instability and clinical outcome in young patients with colorectal cancer. *N Engl J Med* **342**: 69–77.

Haglund K, Rusten TE, Stenmark H (2007) Aberrant receptor signaling and trafficking as mechanisms in oncogenesis. *Crit Rev Oncog* **13**: 39–74.

Hallmans G, Agren A, Johansson G, Stegmayr B, Jansson JH, Lindahl B, Rolandsson O, Södeberg S, Nilsson M, Johansson I, Weinehall L (2003) Cardiovascular disease and diabetes in the Northern Sweden Health and Disease Study Cohort—evaluation of risk factors and their interactions. *Scand J Public Health Suppl* **61**: 18–24.

Jakubauskas A, Griskevicius L (2010) KRas and BRaf mutational status analysis from formalin-fixed, paraffin-embedded tissues using multiplex polymerase chain reaction-based assay. *Arch Pathol Lab Med* **134**: 620–624.

Jang KS, Song YS, Jang SH, Min KW, Na W, Jang SM, Jun YJ, Lee KH, Choi D, Paik SS (2010) Clinicopathological significance of nuclear PTEN expression in colorectal adenocarcinoma. *Histopathology* **56**: 229–239.

Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ (2008) Cancer statistics, 2008. *CA Cancer J Clin* **58**: 71–96.

Kalady MF, Dejulius KL, Sanchez JA, Jarrar A, Liu X, Manilich E, Skacel M, Church JM (2012) BRAF mutations in colorectal cancer are associated with distinct clinical characteristics and worse prognosis. *Dis Colon Rectum* **55**: 128–133.

Kim SY, Shim EK, Yeo HY, Baek JY, Hong YS, Kim DY, Kim TW, Kim JH, Im SA, Jung KH, Chang HJ (2012) KRAS Mutation Status and Clinical Outcome of Preoperative Chemoradiation With Cetuximab in Locally Advanced Rectal Cancer: A Pooled Analysis of 2 Phase II Trials. *Int J Radiat Oncol Biol Phys* **85**: 201–207.

Kohonen-Corish MR, Daniel JJ, Chan C, Lin BP, Kwun SY, Dent OF, Dhillon VS, Trent RJ, Chapuis PH, Bokey EL (2005) Low microsatellite instability is associated with poor prognosis in stage C colon cancer. *J Clin Oncol* **23**: 2318–2324.

Krol LC, t Hart NA, Methorst N, Knol AJ, Prinsen C, Boers JE (2012) Concordance in KRAS and BRAF mutations in endoscopic biopsy samples and resection specimens of colorectal adenocarcinoma. *Eur J Cancer* **48**: 1108–1115.

Li HT, Lu YY, An YX, Wang X, Zhao QC (2011) KRAS, BRAF and PIK3CA mutations in human colorectal cancer: relationship with metastatic colorectal cancer. *Oncol Rep* **25**: 1691–1697.

Li XH, Zheng HC, Takahashi H, Masuda S, Yang XH, Takano Y (2009) PTEN expression and mutation in colorectal carcinomas. *Oncol Rep* **22**: 757–764.

Liao X, Morikawa T, Lochhead P, Imamura Y, Kuchiba A, Yamauchi M, Nosho K, Qian ZR, Nishihara R, Meyerhardt JA, Fuchs CS, Ogino S (2012) Prognostic role of PIK3CA mutation in colorectal cancer: cohort study and literature review. *Clin Cancer Res* **18**: 2257–2268.

Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Cote JF, Tomasic G, Penna C, Ducreux M, Rougier P, Penault-Llorca F, Laurent-Puig P (2006) KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res* **66**: 3992–3995.

Muller CI, Miller CW, Hofmann WK, Gross ME, Walsh CS, Kawamata N, Luong QT, Koeffler HP (2007) Rare mutations of the PIK3CA gene in malignancies of the hematopoietic system as well as endometrium, ovary, prostate and osteosarcomas, and discovery of a PIK3CA pseudogene. *Leuk Res* **31**: 27–32.

Nosho K, Kure S, Irahara N, Shima K, Baba Y, Spiegelman D, Meyerhardt JA, Giovannucci EL, Fuchs CS, Ogino S (2009) A prospective cohort study shows unique epigenetic, genetic, and prognostic features of synchronous colorectal cancers. *Gastroenterology* **137**: 1609–1620e1-3.

Ogino S, Kawasaki T, Kirkner GJ, Kraft P, Loda M, Fuchs CS (2007) Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. *J Mol Diagn* **9**: 305–314.

Ogino S, Nosho K, Kirkner GJ, Shima K, Irahara N, Kure S, Chan AT, Engelman JA, Kraft P, Cantley LC, Giovannucci EL, Fuchs CS (2009) PIK3CA mutation is associated with poor prognosis among patients with curatively resected colon cancer. *J Clin Oncol* **27**: 1477–1484.

Ogino S, Shima K, Meyerhardt JA, McCleary NJ, Ng K, Hollis D, Saltz LB, Mayer RJ, Schaefer P, Whittom R, Hantel A, Benson 3rd AB, Spiegelman D, Goldberg RM, Bertagnolli MM, Fuchs CS (2012) Predictive and prognostic roles of BRAF mutation in stage III colon cancer: results from intergroup trial CALGB 89803. *Clin Cancer Res* **18**: 890–900.

Perrone F, Lampis A, Orsenigo M, Di Bartolomeo M, Gevorgyan A, Losa M, Frattini M, Riva C, Andreola S, Bajetta E, Bertario L, Leo E, Pierotti MA, Pilotti S (2009) PI3KCA/PTEN deregulation contributes to impaired responses to cetuximab in metastatic colorectal cancer patients. *Ann Oncol* **20**: 84–90.

Popat S, Hubner R, Houlston RS (2005) Systematic review of microsatellite instability and colorectal cancer prognosis. *J Clin Oncol* **23**: 609–618.

Rako I, Jakic-Razumovic J, Katalinic D, Sertic J, Plestina S (2012) Mutation pattern of KRAS and BRAF oncogenes in colorectal cancer patients. *Neoplasma* **59**: 376–383.

Samowitz WS, Albertsen H, Herrick J, Levin TR, Sweeney C, Murtaugh MA, Wolff RK, Slattery ML (2005) Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. *Gastroenterology* **129**: 837–845.

Samuels Y, Ericson K (2006) Oncogenic PI3K and its role in cancer. *Curr Opin Oncol* **18**: 77–82.

Sartore-Bianchi A, Di Nicolantonio F, Nichelatti M, Molinari F, De Dosso S, Saletti P, Martini M, Cipani T, Marrapese G, Mazzucchelli L, Lamba S, Veronese S, Frattini M, Bardelli A, Siena S (2009) Multi-determinants analysis of molecular alterations for predicting clinical benefit to EGFR-targeted monoclonal antibodies in colorectal cancer. *PLoS One* **4**: e7287.

Sawai H, Yasuda A, Ochi N, Ma J, Matsuo Y, Wakasugi T, Takahashi H, Funahashi H, Sato M, Takeyama H (2008) Loss of PTEN expression is associated with colorectal cancer liver metastasis and poor patient survival. *BMC Gastroenterol* **8**: 56.

Shen L, Catalano PJ, Benson 3rd AB, O'Dwyer P, Hamilton SR, Issa JP (2007) Association between DNA methylation and shortened survival in patients with advanced colorectal cancer treated with 5-fluorouracil based chemotherapy. *Clin Cancer Res* **13**: 6093–6098.

Siena S, Sartore-Bianchi A, Di Nicolantonio F, Balfour J, Bardelli A (2009) Biomarkers predicting clinical outcome of epidermal growth factor receptor-targeted therapy in metastatic colorectal cancer. *J Natl Cancer Inst* **101**: 1308–1324.

Soeda H, Shimodaira H, Watanabe M, Suzuki T, Gamoh M, Mori T, Komine K, Iwama N, Kato S, Ishioka C (2012) Clinical usefulness of KRAS, BRAF, and PIK3CA mutations as predictive markers of cetuximab efficacy in irinotecan- and oxaliplatin-refractory Japanese patients with metastatic colorectal cancer. *Int J Clin Oncol*; e-pub ahead of print, 26 May 2012.

Tol J, Dijkstra JR, Klomp M, Teerenstra S, Dommerholt M, Vink-Borger ME, van Cleef PH, van Krieken JH, Punt CJ, Nagtegaal ID (2010) Markers for EGFR pathway activation as predictor of outcome in metastatic colorectal cancer patients treated with or without cetuximab. *Eur J Cancer* **46**: 1997–2009.

Van Guelpen B, Hultdin J, Johansson I, Hallmans G, Stenling R, Riboli E, Winkvist A, Palmqvist R (2006) Low folate levels may protect against colorectal cancer. *Gut* **55**: 1461–1466.

Van Rijnsoever M, Elsaleh H, Joseph D, McCaul K, Iacopetta B (2003) CpG island methylator phenotype is an independent predictor of survival benefit from 5-fluorouracil in stage III colorectal cancer. *Clin Cancer Res* **9**: 2898–2903.

Ward RL, Cheong K, Ku SL, Meagher A, O'Connor T, Hawkins NJ (2003) Adverse prognostic effect of methylation in colorectal cancer is reversed by microsatellite instability. *J Clin Oncol* **21**: 3729–3736.

Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW (2006) CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* **38**: 787–793.

Wright CM, Dent OF, Newland RC, Barker M, Chapuis PH, Bokey EL, Young JP, Leggett BA, Jass JR, Macdonald GA (2005) Low level microsatellite instability may be associated with reduced cancer specific survival in sporadic stage C colorectal carcinoma. *Gut* **54**: 103–108.