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.
maintaining the metabolism, proliferation, survival and motility of a cell (Haglund et al, 2007). Many of these signals involve the oncopgenic 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; Bardellini 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 macrodisection 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 MgCl2 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'-tgaatgagcggcaggttgtctatgaaagtgctggcgt-3'
- reverse: 5'-cagggagacgtgtaatcctcaagggctggtt-3'

PCR conditions for PIK3CA exon 20: 50 ng DNA, 0.5 μg primer, 10 mM dNTP, 3 mM MgCl2 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'-ttggagcagcggcagcctcaagtctgctgccggctgc-3'
- reverse: 5'-ccagggagacgtgtaatcctcaagggctggtt-3'

All primers were M13-tagged (forward: 5'-tgatagcggcagct-3'; reverse: 5'-tgatagcggcagct-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 x1 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) in a total volume of 20 μl. Immunohistochemical analysis of PTEN expression. Primers used:

- forward: 5'-cagggagacgtgtaatcctcaagggctggtt-3'
- reverse: 5'-ccagggagacgtgtaatcctcaagggctggtt-3'

The samples were evaluated for cytoplasmic staining, and were graded 0 as no staining, 1 as weak staining, and 2 as 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**       |      |      |               |      |
| Frequency (%)   | 197  | 89 (51.7) | 83 (48.3) | 147 (79.1) | 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) |
| >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) |
| 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) | 23 (30.0) | 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, \( \chi^2 \)-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 | PIK3CAExon20 | 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 (24.4) | 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 (15.9) | 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 (24.4) |
| 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, \( \chi^2 \)-test or Fisher’s exact test used for categorical variables.
Patients with missing value in any of the marker were excluded.

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; P < 0.001).

Statistical analysis. Clinico-pathological characteristics were compared using Kruskal–Wallis tests for continuous variables and χ² 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).
Role of significantly correlated to MSI (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).

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

Abbreviations: 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, 1; BRAF mutation status, 4; PIK3CA mutation status, 1; PTEN mutation status, 4. 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, χ²-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 (%) | 197   | 24 (12.2) | 173 (87.8) | 0.384 | 97 (50.0) | 70 (36.1) | 27 (13.9) | 0.002 |
| Quadruple Index |       |         |         |         |               |          |           |         |
| Negative | 89    | (51.7) | 9 (42.9) | 12 (57.1) | 80 (53.0) | 71 (47.0) | 52 (61.9) | 31 (50.0) | 6 (23.1) | 20 (6.9) |
| Positive | 83    | (48.3) |         |         |               |          |           |         |
| KRAS   |       |         |         |         |               |          |           |         |
| Wt     | 147   | (82.1) | 19 (100.0) | 0 (0.0) | 128 (80.0) | 32 (20.0) | 68 (79.1) | 18 (20.9) | 52 (78.8) | 14 (21.2) | 24 (100.0) | 0 (0.0) |
| Mutant | 32    | (17.9) |         |         |               |          |           |         |
| BRAF   |       |         |         |         |               |          |           |         |
| Wt     | 161   | (82.1) | 13 (54.2) | 11 (45.8) | 148 (86.0) | 24 (14.0) | 93 (96.9) | 3 (3.1) | 57 (81.4) | 13 (18.6) | 8 (29.6) | 19 (70.4) |
| Mutant | 35    | (17.9) |         |         |               |          |           |         |
| PIK3CA Exon20 |       |         |         |         |               |          |           |         |
| Wt     | 182   | (97.8) | 23 (100.0) | 0 (0.0) | 159 (97.5) | 4 (2.5) | 91 (97.8) | 2 (2.2) | 63 (96.9) | 2 (3.1) | 25 (100.0) | 0 (0.0) |
| Mutant | 4     | (2.2)  |         |         |               |          |           |         |
| PTEN   |       |         |         |         |               |          |           |         |
| Normal | 161   | (87.5) | 21 (87.5) | 3 (12.5) | 140 (87.5) | 20 (12.5) | 80 (86.0) | 13 (14.0) | 58 (90.6) | 6 (9.4) | 23 (85.2) | 4 (14.8) |
| Loss   | 23    | (12.5) |         |         |               |          |           |         |

Abbreviations: MSI = microsatellite instability; MSS = microsatellite stable; Wt = wild-type.
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).

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.

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) |

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; 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.
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.

**DISCUSSION**

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.
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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 (Noshoh 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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