Genomic Profiling of Stage II Colorectal Cancer Identifies Candidate Genes Associated with Recurrence-Free Survival, Tumor Location, and Differentiation Grade

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Keywords

Colorectal cancer stage II · Genomic profiling · *ATM* · *BRAF* · *APC* · *KRAS*

Abstract

Background: Identification of high-risk stage II colorectal cancer (CRC) patients, potential candidates for adjuvant chemotherapy, is challenging. Current clinical guidelines rely mainly on histopathological markers with relatively weak prognostic value. This motivates further search for prognostic markers. **Methods:** This explorative study aimed to identify potential candidate gene mutations to facilitate differentiation between subgroups of patients with CRC stage II. Panel-based massive parallel sequencing was used to genetically characterize tumor tissues from 85 patients radically operated for CRC stage II, of which 12 developed recurrent cancer during follow-up. Genetic data was compared between patients with or without cancer recurrence, between tumors located in colon and in rectum, and for association with tumor differentiation grade. **Results:** Genetic variation in *ATM*, *C11ORF65* was associated with recurrence-free survival. Previous reports regarding the association between

BRAF mutation and a higher age at diagnosis, and tumor location in colon were confirmed. *APC*, *BRAF*, or *KRAS* mutation was associated with tumor differentiation grade. Multiple correspondence analyses revealed no obvious clustering of patients with the studied clinical characteristics, indicating that the genetic signatures observed here were unique for each individual. **Conclusions:** Taken together, we have demonstrated the utility of panel-based massive parallel sequencing to explore the pathogenesis of CRC stage II. We have identified promising candidate gene mutations associated with cancer recurrence, tumor location, and differentiation grade in patients with CRC stage II, which merit further investigation.

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Introduction

Colorectal cancer (CRC) is the most common cancer form after lung cancer, female breast cancer, and prostate cancer [1]. Approximately 25% of the CRC patients are diagnosed as stage II. The majority of these patients are cured by surgery alone and prognosis is relatively good.

However, 15–25% of stage II patients develop a more severe phenotype and may benefit from adjuvant chemotherapy [2–5]. Identification of these patients is challenging.

The risk factors used today for identification of high-risk patients and for medical decision-making comprise histopathological low differentiation grade, lymphovascular or perineural invasion, perforation, T4 tumor invasion, and fewer than 12 lymph nodes removed and examined, in combination with microsatellite instability (MSI) status [2–5]. An MSI stable tumor in CRC stage II is generally associated with a poor recurrence-free survival (RFS) rate [6]. However, the prognostic value of these risk factors is relatively weak [5, 7] leading to potential over- or undertreatment of certain patients.

Technological advances in molecular biology have improved our understanding of genomic changes and signal transduction pathways involved in CRC. Biomarkers at different biological levels have been investigated to distinguish between subgroups of patients within CRC stage II [8–14]. The use of mRNA profiling has shown potential as prognostic tool but still requires special handling to preserve sample stability. Biomarkers based on DNA are generally more stable. Although many of these markers show promising results, they are not included in clinical guidelines for medical decision-making.

Adjuvant chemotherapy in CRC stage II could probably be initiated on a more rational basis if objective and standardized molecular biomarkers were available and combined with the traditional risk factors. Recognizing the complexity of the colorectal carcinogenesis with multi-genetic events and pathways which interact with each other [15–17], we used a panel-based approach to explore whether genetic events could differentiate between subgroups of patients with CRC stage II.

Materials and Methods

Study Population

Eighty-five patients (33 female, 52 male) with radical operation for primary CRC stage II were identified from a local biobank of a total 401 CRC patients who underwent surgical resection for colorectal adenocarcinoma at the Department of Surgery, County Hospital Ryhov, Jönköping, south-eastern Sweden, between 1996 and 2013. Tumor location, differentiation grade, postoperative staging, and other histopathological characteristics were noted. Follow-up for date of recurrence and date and cause of death was obtained through the patients files. Follow-up ended on the date of death or on December 18, 2018. Tumors were classified as stage II (T3 or T4, N0, M0) according to The American Joint Committee on Cancer (AJCC) classification system v.7 [18].

Sampling and DNA Extraction

Tumor tissue samples were snap-frozen in liquid nitrogen and stored at -70°C . Extraction of DNA was done with QIAamp DNA Mini kit (Qiagen, Hilden, Germany). The concentration of DNA was determined with the Qubit dsDNA BR Assay kit and a Qubit 2.0 fluorometer (ThermoFisher Scientific, Waltham, MA, USA).

Massive Parallel Sequencing

DNA libraries were prepared with the TruSeq Amplicon Cancer panel, which targets 48 cancer-related genes (212 amplicons), and the TruSeq Custom Amplicon Index kit (Illumina, San Diego, CA, USA). Library preparation was done according to the manufacturers' instructions with 250 ng of DNA as template. The pooled libraries were then sequenced on a MiSeq sequencer (Illumina). Genes included in the panel are listed in online supplementary Table S1 (see www.karger.com/doi/10.1159/000507118 for all online suppl. material).

Variant Calling and Filtering

Variants were called using MiSeq Reporter version 2.4 (Illumina) and the Human Genome Build 19 (hg19, GRCh37) as the reference genome. Further filtering was done with Variant Studio version 3.0 (Illumina), to meet the following criteria:

- amplicon coverage >300
- allele frequency >5%
- keep all but synonymous variants

The Integrative Genomics Viewer (IGV; Broad Institute, Cambridge, MA, USA) was used to evaluate variants when judged necessary.

Statistical Analysis

Basic statistic is presented as median (lower and upper quartile). For group comparisons, the Mann-Whitney U test was used. For categorical variables, Fisher's exact test of independence was used. Odds ratios (OR) were expressed with 95% confidence intervals (CI). RFS and cancer-specific survival rates were visualized by Kaplan-Meier curves and compared using log-rank tests. Hazard ratios were calculated using Cox-regression analysis. For group comparisons, two-sided tests were used and considered statistically significant if $p < 0.05$. p values were not corrected for multiple testing due to the explorative approach of this pilot study, but also as the gene panel used was designed to cover cancer-related genes only. Many of these genes are involved in signaling pathways of the carcinogenesis which are known to interact with each other and are therefore not considered to be completely independent [15–17].

Multiple correspondence analysis (MCA) was used to study gene signatures. The analyses were based on the pattern of gene mutations per patient.

Mutation count was defined as the sum of all genetic variants detected per gene and patient.

Analysis was done on CRC-associated genes as defined by the gene panel: *AKT1*, *APC*, *BRAF*, *CTNNB1*, *EGFR*, *FBXW7*, *KRAS*, *NRAS*, *MET*, *PIK3CA*, *PTEN*, *TP53*, *SRC*, and on all 48 genes combined.

Statistical analyses were done using Statistica version 13.3 (Statsoft, Inc., Tulsa, OK, USA) and Rstudio version 1.0.143 [19], with the package FactoMineR [20]. Linkage disequilibrium (LD) calculation was done in LD-link version 3.2.0 (National Cancer Institute, Bethesda, MD, USA) [21].

Table 1. Patient characteristics stratified according to cancer recurrence at follow-up

	Cancer recurrence (n = 12)	No cancer recurrence (n = 73)	p value
Gender (female/male)	7/5	26/47	0.20
Age at diagnosis (years)	76.5 (66.5–78.5)	72 (62–78)	0.52
Tumor location (colon/rectum)	8/4	43/30	0.76
<12 lymph nodes examined	5 (42%)	32 (44%)	1.00
Poor tumor differentiation	5 (42%)	15 (21%)	0.14
Mucinous tumor	2 (17%)	13 (18%)	1.00
T4 tumor	3 (25%)	4 (5.5%)	0.05
Postoperative adjuvant therapy planned	1 (8.3%)	3 (4.1%)	0.46
Recurrence-free survival (years)	2.07 (0.89–3.64)		
Survival (years)	6.05 (3.18–9.04)	8.74 (5.87–13.28)	0.07

Results

The median age at diagnosis in the studied population was 72 years (interquartile range 62–78 years). Some 12 patients developed recurrent cancer during follow-up, 8 of which died of a cancer-related cause. There was no difference in the distribution of gender between patients with and without cancer recurrence, between tumor locations, or between tumor differentiation grades (Table 1 and data not shown). All gene mutations found were similarly distributed between gender (data not shown).

The risk factors used today for identification of high-risk stage II patients were similarly distributed between patients with and without cancer recurrence at follow-up (Table 1). Overall, 66% of patients carried one or more risk factors (1 risk factor: 42%; 2 risk factors: 20%; 3 risk factors: 4%). None of the risk factors was associated with RFS, except for tumor T4 (Table 2).

BRAF Mutation Was Associated with Tumor Location

Colon cancer was documented in 51 patients and rectal cancer in 34 patients. *BRAF* mutation was more frequent in colon tumors than in rectal tumors (Table 3) (OR=15.08 [95% CI; 1.89–120.21], $p=0.010$). rs113488022 (*BRAF* p.V600E) was detected in all *BRAF*-positive colon tumors but one (rs121913351; *BRAF* p.G466E).

BRAF mutation was more common in women than in men (30% vs. 13%), but the difference did not reach statistical significance ($p = 0.09$). The age at diagnosis was higher in *BRAF* mutation carriers (median 78, range 73–81 years) than in *BRAF* wild-type patients (median 71, range 61–77 years), ($p = 0.025$). Accordingly, the age at diagnosis was higher in patients with colon cancer (median 75, range 67–80 years) compared with rectal cancer (median 71, range 61–74 years), ($p = 0.048$).

Table 2. Recurrence-free survival versus clinical characteristics and versus gene mutation status (univariate analysis)

Clinical characteristics and gene mutations ¹	HR (95% CI)	p value
Age at diagnosis	1.02 (0.97–1.07)	0.45
Location (colon vs. rectum)	1.56 (0.47–5.21)	0.47
<12 lymph nodes examined	0.91 (0.29–2.88)	0.87
Poor tumor differentiation	2.93 (0.93–9.25)	0.07
Mucinous tumor	0.91 (0.20–4.18)	0.91
T4 tumor	4.50 (1.21–16.65)	0.024
<i>APC</i> ²	1.33 (0.42–4.20)	0.62
<i>ATM</i> , <i>C11ORF65</i>	0.19 (0.06–0.61)	0.005
<i>BRAF</i> ²	2.49 (0.75–8.30)	0.14
<i>CTNNB1</i> ^{2, 4}	1.32 (0.17–10.25)	0.79
<i>FBXW7</i> ²	0.49 (0.06–3.84)	0.50
<i>FGFR1</i>	1.95 (0.58–6.48)	0.28
<i>FGFR3</i>	0.49 (0.06–3.84)	0.50
<i>GNA11</i>	0.83 (0.18–3.83)	0.81
<i>GNAQ</i>	0.58 (0.18–1.94)	0.37
<i>HNF1A</i>	1.11 (0.24–5.09)	0.89
<i>HRAS</i> ⁴	3.66 (0.80–16.73)	0.09
<i>KRAS</i> ²	0.78 (0.21–2.89)	0.71
<i>Chr22 rs35893428</i> ³	0.75 (0.24–2.37)	0.63
<i>Chr2 rs1059524</i> ³	1.84 (0.58–5.79)	0.30
<i>PIK3CA</i> ²	0.47 (0.10–2.16)	0.33
<i>PTEN</i> ²	0.40 (0.12–1.32)	0.13
<i>RB1</i>	1.73 (0.52–5.76)	0.37
<i>APC</i> or <i>CTNNB1</i> ²	1.07 (0.34–3.39)	0.90
<i>KRAS</i> , <i>BRAF</i> , or <i>NRAS</i> ²	1.34 (0.43–4.22)	0.62
<i>KRAS</i> , <i>BRAF</i> , <i>NRAS</i> , or <i>APC</i> ²	3.90 (0.50–30.22)	0.19

¹ Genes with gene mutation present in at least 10 patients were included in the analysis, unless otherwise stated. ² Classified as CRC-associated gene according to the gene panel. ³ No gene assigned. ⁴ Five patients with gene mutation, *CTNNB1* included based on its involvement in the Wnt signaling pathway, *HRAS* based on the significant result in relation to cancer-specific survival (online suppl. Table S5), although few patients with gene mutation. HR, hazard ratio; CI, confidence interval.

Table 3. Gene mutations significantly associated with clinical characteristics

Gene mutation	Samples with gene mutation	%	Samples with gene mutation	%	<i>p</i> value
<i>BRAF</i> ¹	Colon (<i>n</i> = 51) 16	31.4	Rectum (<i>n</i> = 34) 1	2.9	0.002
<i>ABL1</i>	Poor differentiation (<i>n</i> = 20) 3	15.0	Moderate/well differentiation (<i>n</i> = 65) 1	1.5	0.039
<i>APC</i> ¹	5	25.0	39	60.0	0.010
<i>BRAF</i> ¹	13	65.0	4	6.2	<0.001
<i>KRAS</i> ¹	2	10.0	23	35.4	0.047
<i>ATM, C11ORF65</i>	Cancer recurrence (<i>n</i> = 12) 5	41.7	No cancer recurrence (<i>n</i> = 73) 59	80.8	0.008

¹ Classified as CRC-associated gene according to the gene panel.

Fig. 1. Kaplan-Meier curve illustrating a better recurrence-free survival rate in *ATM, C11ORF65* mutation carriers (log-rank *p* = 0.003).

None of the genes frequently studied in CRC, such as *KRAS*, *NRAS*, or *APC*, were associated with tumor location (online suppl. Table S2). The distribution of 0–1 versus 2 *APC* mutations did not differ between tumor locations (*p* = 1.00).

BRAF, KRAS, and APC Gene Mutations Were Associated with Tumor Differentiation Grade

The tumor was of poor differentiation grade in 20 patients (24%) and of moderate/well grade in 65 patients (76%). *BRAF* mutation was more common in poorly differentiated tumors compared with moderate/well-differ-

entiated tumors (OR = 28.32 [95% CI; 7.22–111.07], *p* < 0.001) (Table 3).

KRAS mutation was more frequent in moderate/well-differentiated tumors compared with tumors with poor differentiation grade (OR = 4.92 [95% CI; 1.05–23.15], *p* = 0.043) (Table 3). Overall, *KRAS* mutation was identified in 25 patients (29.4%). The majority of mutations detected were in codons 12 and 13 (84%), whereas sporadic mutations were detected in codons 5, 61, and 117.

APC mutation was noticed in 44 patients (51.8%) and was overrepresented in tumors with moderate/well differentiation grade compared with poorly differentiated tumors (OR = 4.50 [95% CI; 1.46–13.89], *p* = 0.009) (Table 3). The frequency of 0–1 versus 2 *APC* mutations was similar over differentiation grades (*p* = 0.44). Frameshift mutations and stop-gained mutations expected to result in a truncated protein dominated mutations found (91%).

ABL1 mutation was also associated with tumor differentiation grade but identified in a few cases only (Table 3). The distribution of all gene mutations explored in the study population, stratified according to tumor differentiation, is described in online supplementary Table S3.

ATM, C11ORF65 Gene Mutation Was Associated with Cancer Recurrence

ATM, C11ORF65 mutation was detected in 64 patients (75%) and was differently distributed between patients with and without cancer recurrence at follow-up (Table 3). At a median follow-up of 8.33 years (5.26–12.74 years), patients with *ATM, C11ORF65* mutated tumors showed a better RFS than patients with *ATM, C11ORF65* wild-type tumors (Fig. 1; Table 2).

Table 4. Analysis of recurrence-free survival associated with gene mutation status of the four genes strongest correlated with dimension one and two in the MCA, including also genes and clinical characteristics which showed statistical significance in other comparisons in this study (multivariate analysis)

	HR (95% CI)	<i>p</i> value
<i>APC</i>	4.56 (0.94–22.12)	0.06
<i>ATM</i> , <i>C11ORF65</i>	0.14 (0.034–0.53)	0.004
<i>BRAF</i>	2.76 (0.53–14.30)	0.23
<i>CTNNB1</i>	0.96 (0.10–9.38)	0.97
<i>KRAS</i>	0.62 (0.13–3.03)	0.56
<i>PTEN</i>	0.24 (0.06–1.00)	0.05
T4 tumor	7.80 (1.42–42.85)	0.02

HR, hazard ratio; CI, confidence interval; MCA, multiple correspondence analysis.

The genetic variants contributing to the *ATM*, *C11ORF65* results were mainly the intronic variants rs227075 and rs664143, detected in 63 patients. In all but one patient, the same genotype was observed for rs227075 and rs664143. The variants were detected by different PCR products. However, it could be confirmed that the G allele of rs664143 and the C allele of rs227075 were in LD ($r^2 = 1.0$, $p < 0.001$) and redundant based on genotype data of a European dataset originating from Phase 3 (Version 5) of the 1000 Genomes Project, available via LD-link.

ABL1, *APC*, *BRAF*, or *KRAS* mutations were not associated with cancer recurrence (Table 2; online suppl. Table S4). The frequency of 0–1 versus 2 *APC* mutations were similar in patients with and without cancer recurrence at follow-up ($p = 0.15$).

Gene Mutations and Cancer-Specific Survival

Neither gene mutation of *ABL1*, *APC*, *BRAF*, *KRAS*, nor of *ATM*, *C11ORF65* was associated with cancer-specific survival (online suppl. Table S5). However, *HRAS* mutation was significantly associated with cancer-specific survival, but was detected in five patients only (online suppl. Table S5).

Combined Analysis of Gene Mutations and Mutation Count in Relation to Clinical Characteristics

Central pathways in the development of CRC are, among others, the Wnt signaling pathway, the RAS-RAF-MEK-ERK mitogen-activated protein kinase (MAPK) signaling pathway, and the PI3K-PTEN-AKT pathway. These pathways interact with each other in different ways

[15–17]. We found no overlap between *KRAS*, *BRAF*, and *NRAS* mutations, involved in the MAPK pathway, or between *APC* and *CTNNB1* (except for one patient), involved in the Wnt pathway. We analyzed whether defects in any of these pathways separately or combined were associated with RFS (Table 2). *KRAS*, *BRAF*, or *NRAS* mutation and *APC* mutation co-occurred in 27% of patients, but this combination was not more frequent than expected by chance ($p = 1.00$).

Due to the complex nature of interacting gene products in CRC, we next investigated the total number of gene mutations and the total mutation count per patient in relation to RFS, tumor location, and tumor differentiation. No significant results were noticed (online suppl. Table S6a–c).

Analysis of Gene Signatures

In MCA, based on the gene mutation status of each CRC-associated gene per patient, the three first MC dimensions explained 16, 13, and 12% of the total variation in data, respectively (online suppl. Fig. S1a). The gene signatures were not associated with cancer recurrence or with tumor location, based on Mann-Whitney U test of sample coordinates. However, the sample coordinates of the first MC dimension were associated with tumor differentiation grade ($p < 0.0001$). *BRAF*, *APC*, and *KRAS* contributed the most to this dimension, confirming their association with tumor differentiation grade in the univariate analyses. Coordinates of the second dimension were associated with gender ($p = 0.014$). The genes contributing the most to dimension two were *PTEN*, *CTNNB1*, and *MET*. No correlation between samples coordinates and age at inclusion was noticed in any dimension (data not shown).

Including only the six genes contributing the most to dimension one and two, based on correlation, increased the percentage of variation in data explained by the three first MC dimensions to 28, 24, and 16%, respectively, but the statistical results remained the same as with all CRC-associated genes included in the analysis (online suppl. Fig. S1b, and data not shown).

The association between RFS and gene mutation status of the four genes strongest correlated with MC dimension one and two in the MCA, including also genes and clinical characteristics which showed statistical significance in other comparisons in this study, was investigated in a multivariate Cox regression analysis (Table 4). Again, *ATM*, *C11ORF65* mutation and T4 tumor, the only conventional risk factor associated with RFS, were associated with RFS when considering the effect of the other variables.

Discussion

Development in molecular diagnostics holds promise for the delivery of precision medicine in the context of cancer management. In this explorative study we determined the genetic profiles of CRC stage II tumors by panel-based massive parallel sequencing, and evaluated identified gene mutations, and the mutation count, in relation to cancer recurrence, tumor location, and tumor differentiation grade.

With a less stringent filtering approach, our main finding was an association between genetic variation in *ATM*, *C11ORF65* and RFS. The main genetic variants contributing to this result were the intronic variants rs227075 T/C and rs664143 A/G. The two variants were in LD and therefore considered to be redundant. Mutation carriers, as defined by the reference genome (G/A+G/G), had a better RFS. The genomic region of the *ATM* gene harboring these variants overlaps with the 3'-terminal noncoding region of *C11ORF65* on the minus strand of DNA. In silico analysis of rs664143 indicates that genetic variation here may affect a protein binding motif of importance in exon 61 splicing of the *ATM* gene [22]. The putative role of rs227075 remains unclear. *C11ORF65* encodes an uncharacterized protein, with high expression mainly in testis and lower expression in other tissues [23].

ATM (ataxia telangiectasia mutated, a serine/threonine kinase) is widely expressed in vivo [23]. The protein is crucial for maintaining genomic integrity and is a key regulator of cell cycle checkpoint, apoptosis, and a main transducer and sensor in DNA double-strand break repair [24–28]. Reduced protein expression of *ATM* has been suggested as a biomarker of poor RFS in CRC stage II/III [29], supporting the potential importance of *ATM*, *C11ORF65* as a prognostic marker noticed here. In the context of metastatic disease reduced *ATM* protein expression, as well as genetic variants of *ATM*, have been related with increased chemosensitivity to oxaliplatin-based therapy and overall survival [30, 31]. However, the concordance between protein loss and presence of genetic variation, when described, was relatively weak [30].

In summary, *ATM*, *C11ORF65* mutation was associated with RFS in early stage CRC, but not with tumor differentiation or location, and merits further investigation.

Mutations in *KRAS*, *BRAF* and *APC* are important genetic events in the aberrant activation of the MAPK and the Wnt signaling pathways, respectively [15, 32]. The frequency of gene mutations of *BRAF* (20%), *KRAS* (29%), and of *APC* (52%) observed here were of similar

magnitude as those published by others [32–38]. These genes have been studied extensively.

Our results confirm the association between *BRAF* mutation and age at diagnosis, with tumor location in colon [37, 39, 40], and with poor tumor differentiation in CRC stage II [36, 41]. The observed association between *KRAS* and a higher degree of tumor differentiation has been described both in CRC stage II and III [35]. The different associations noticed between *BRAF* and *KRAS* and tumor differentiation are difficult to explain, but suggest that the genes may have different roles although present in a common signaling pathway.

As has been reported by others in CRC stage II [36, 42], *BRAF* mutation here was not associated with RFS or with cancer-specific survival. However, *BRAF* is generally considered an independent predictor of a poor prognosis, and growing evidence suggests that this is particularly true for MSI stable tumors [12, 14, 35, 36, 39, 41].

KRAS seems to be of no prognostic value in CRC stage II, but controversy exists [10, 12, 34–36, 38, 40, 42].

APC is involved in many signaling pathways and its role in the neoplastic process and in different stages of CRC is not fully understood [32]. By stimulation of proteasomal degradation of β -catenin (encoded by *CTNNB1*) *APC* is a main negative regulator of the Wnt signaling pathway. Genetic variation in *APC* or *CTNNB1* may therefore lead to the deregulation of β -catenin/T-cell factor-dependent transcription. Studies have shown that the Wnt and the MAPK signaling pathways interact. Defects in one pathway may enhance the activity in the other [15–17]. Central gene mutations (*KRAS*, *NRAS*, *BRAF*, *APC*, and *CTNNB1*) in these pathways are almost mutually exclusive [33, 43]. Separate and combined analysis of these genes has been associated with RFS in MSI stable stage III patients, but not in MSI stable stage II patients [38, 42]. Similar observations were done here in stage II, although MSI status was unknown in our study population.

Over the last decades, analytical technologies have evolved dramatically allowing for the simultaneous analysis of several markers, for the comparison of genetic signatures, gene-expression profiles, or affected pathways between patient categories. Comparing results between studies based on these new technologies is challenging, as results will depend not only on the study design, the technological platform used, or genomic/proteomic content included, but also on the bioinformatic approaches applied on the data. In our analysis based on gene signatures, no obvious clustering of patients with cancer recurrence, tumor location, or differentiation grade was noticed, indicating that gene signatures here were unique

for each patient or that the population included was too small to find such patterns.

Taken together, of the traditional risk factors used for the identification of high-risk CRC stage II patients, only T4 tumor was associated with RFS. This supports the need for additional objective markers to facilitate identification of these patients. Here, we have demonstrated the utility of panel-based massive parallel sequencing to explore the pathogenesis of CRC stage II. Our results indicate that genetic variation in *ATM*, *C11ORF65* may be prognostic in CRC stage II. *HRAS* mutation was associated with cancer-specific survival, but was detected in few patients only. Previous reports regarding the association between *BRAF* mutation and clinical characteristics were confirmed. Gene mutation of *APC*, *BRAF*, or *KRAS* was associated with tumor differentiation grade. These promising results motivate further studies, including larger cohorts and including associated markers at different biological levels, to assess the true prognostic value and usefulness to refine medical decision-making.

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Statement of Ethics

The study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. It was approved by the Regional Ethical Review Board in Linköping, Sweden, Dnr 2013/271-31, and informed consent was obtained from the participants.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Conception and design: S.H., J.D.; analysis of data: S.H.; interpretation of data: S.H., J.D., R.E.A.; clinical data: R.E.A.; writing – original draft: S.H.; writing – revising, editing for intellectual content: S.H., J.D., R.E.A.; final approval of manuscript: S.H., J.D., R.E.A.; funding acquisition: S.H.

Data Availability

Research materials supporting this publication are not publicly available but may be accessed after reasonable motivation by contacting the corresponding author.

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