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KRAS Mutant Status May Be Associated with Distant Recurrence in Early-stage Rectal Cancer

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Abstract. *Background/Aim: Total mesorectal excision combined with neo-adjuvant chemoradiotherary (CRT) and adjuvant chemotherapy, has been the standard treatment of locally advanced rectal cancer (LARC). Although TNM (Tumor, Node, Metastasis) classification for malignant Tumors is still the cornerstone in rectal cancer staging, there has been an effort to identify molecular biomarkers with additional prognostic or predictive value. Materials and Methods: We retrospectively analyzed molecular biomarkers on prospectively collected histological specimens and clinical data from a cohort of 135 consecutive rectal cancer cases who underwent radical excision in a tertiary center between 2011-2014 (males=87, females=48, age range=22-89 years, mean=64,67 years, SD=13.40). Radiological, histopathological, molecular staging, treatment stratification by the multidisciplinary team (MDT), as well as prognostic outcome data were compared with various biomarkers including KRAS, BRAF, p16, b-catenin, MSI, MMR and MGMT. Results: The mean follow-up was 39.21 months (range=5-83 months, SD=21.34). Twenty-eight cases were Stage I (20.9%), n=30 Stage II (22.4%), n=45 Stage III (33.6%) and n=31 Stage IV (23.1%). Forty specimens were KRAS-mutant (mt) (37.4%) while n=67 (62.6%) wild type (wt). KRAS mt status was associated with female sex (n=20, p=0.021) and older age (69.62 vs. 62.27, p=0.005). Stage I Early Cancer Subgroup analysis showed that KRAS mt status is associated with distant recurrence of disease (n=4, p=0.045). Conclusion: KRAS mt status may affect the prognosis of early rectal cancer, as this is linked with distant recurrence.*

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Treatments for rectal cancer have evolved significantly during the last decade (1). Bowel cancer screening is starting to diagnose tumors at an earlier stage (2, 3). This has notably improved survival and made minimally-invasive treatments more feasible. Neo-adjuvant chemo radiotherapy (CRT), followed by total mesorectal excision (TME) and adjuvant chemotherapy, remains the gold standard in the treatment of locally advanced rectal cancer (LARC) (4). LARC generally refers to Stage III rectal cancer, and it represents 70% of the rectal cancers on presentation (5).

Recent developments in the study of oncogenes and biomarkers, have raised hopes of possible assistance in prognosis and treatment choices (6-8). Molecular biomarkers are grossly divided into prognostic and predictive. Prognostic biomarkers refer to molecules, that could potentially hold important information on life expectancy post diagnosis +/– treatment of disease, whereas predictive biomarkers are used to predict response to a treatment plan (8, 9). Although there is progress in the understanding of the significance of those biomarkers, the complexity and diversity of carcinogenesis pathways in colorectal cancer (CRC), makes it challenging to identify their impact on prognosis, and potential implications to treatment choice (10).

KRAS is a proto-oncogene which seems to be a well-recognized predictive biomarker in CRC (8). Most of the studies comment on KRAS expression as a tool to predict response to anti-EGF chemotherapy, though its prognostic value is still ambiguous (11). *BRAF* V600E mutation has also been well studied and seems to be associated with proximal tumors as well as poorer prognosis (12). Microsatellite instability (MSI) is a part of the molecular phenotype of CRC, and hence has been widely studied in both hereditary and sporadic CRC (1). Methylation products seem to play an increasingly significant role as well. Currently, many studies look at hypermethylation of CpG islands which are generally located at the promoters of various genes, the expression of which can affect CRC outcomes (13-16). For instance, p16 and b-catenin protein expression as well as MMR or MGMT preservation are currently tested routinely in our institution, for completion

Table I. *Patient demographics and treatment*.

Age (years)	Mean age=64.67, SD=13.32			
Gender (%)	Males 87(64.4%)		Female 48(35.6%)	
ASA	Grade I 21 (16.3%)	Grade II 72 (55.8%)	Grade III 35 (27.1%)	Grade IV 1(0.8%)
Follow-up	Mean=39.07 [5, 83], SD=21.3 (months)			
	Treatment			
Surgery	Laparoscopic AP Resection 87 (64.9%)	Open AP resection 41 (30.6%)	Conversion to Open 5 (3.7%)	Endoscopic Treatment 1 (0.7%)
Complications	n=78 (57.8%) uncomplicated		n=57 (42.2%) minor complication	
	Received		Received	
Neo-adjuvant treament	Chemotherapy n=12 (8.9%)	Radiotherapy n=10 (7.4%)	None n=71 (52.6%)	ChemoRadio n=38 (29%)
	Received		Received	
Adjuvant treatment	Chemotherapy n=49 (37.4%)	Radiotherapy n=2 (1.5%)	None n=76 (58%)	ChemoRadio n=4 (3.1%)

of the molecular staging of the CRC cases. Our aim in this study was to examine the significance of certain molecular biomarkers including KRAS, BRAF, MMR, MGMT, p16, MSI, b-catenin in the prediction of recurrence and survival.

Materials and Methods

Samples. One hundred and thirty-five rectal cancer cases with prospective collection of clinical and pathological data (as part of the UK National Bowel Cancer Audit) were used in this study. Analysis of biomarkers was performed on stored tumor specimens. Our center offers treatment options to patients, based on the multidisciplinary team (MDT) approach. Intense follow-up on a 6-month basis, according to local guidelines. This protocol includes CT Abdomen, Thorax and Pelvis, colonoscopy, CEA, and occasionally EUS or MRI of the abdomen. Data on demographics, radiological, histopathology, molecular as well as follow up outcomes are prospectively collected as part of the UK National Bowel Cancer Audit.

Molecular analysis. All biomarkers were assessed on formalin fixed, paraffin embedded (FFPE) samples. B-catenin, MMRs, MGMT and p16 assays were performed using immunohistochemical analyses (IHC). Four-μM sections of the tumor were cut on to coated slides and IHC was performed using standardized protocols for each antibody. The final step was visualization of antigen-antibody complexes by the addition of the chromogen, 3-3'-diaminobenzidine (DAB). The slides were then assessed (by SDC and JM) and scored for percentage of positive tumor cells and protein location.

KRAS mutation analysis was performed on the same tumor samples. H&E-stained sections of the tumor were assessed and marked for tumor content and the tumor was then macro-dissected using serial, unstained section from the non-tumor components, allowing for enrichment of tumor cells. DNA was then extracted from these samples using standardized protocols and quantified by Qubit analysis. PCR using primers either side of the regions of interest, codons 12(35G>A, 35G>T, 34G>T) and 13(38G>A) and codon 61 (182A>T), was performed. After immobilization of the resulting amplicons, single stranded DNA was prepared and the sequencing primer for each region was annealed. The samples were then analyzed on Quigen Q24 pyrosequencer© and the resulting sequence was analyzed using the appropriate Qiagen software (Version 2.07). The mutation status of each tumor was reported according to standard protocols (by SDC and JM).

Appropriate positive and negative controls were included for all assays.

Statistical analysis. Statistical analysis of our results was performed using IBM SPSS for Macintosh version 22 (IBM Corp. Armonk, NY, USA). Univariate correlations (Pearson's and Spearman's rho as well as Chi-square crosstabs) were used to identify any potential links between various parameters and KRAS, BRAF, p16, b-catenin or MGMT. Independent *t*-test associations were used to compare means in different groups. Statistical significant level was set at $p=0.05$.

Results

One hundred and thirty-five patients with confirmed rectal cancer were identified in our cohort (mean age at diagnosis was 64.67 years, range=22-89 years, SD=13.32). Eighty-seven (64.4%) patients were males, and 48 (35.6%) were females. The mean follow-up was 39.21 months (range=5-83 months, SD=21.34). 21 (16.3%) were ASA grade I, 72 (55.8%) ASA grade II, 35 (27.1%) ASA grade III and 1 was ASA grade IV (0.8%) (Table I).

Table II. *Preoperative and pathological staging*.

		Pre-operative Stage (I-IV)		
Stage	I 28 (20.9%)	II 30 (22.4%)	III 45 (33.6%)	IV 31 (23.1%)
		Post-operative Stage (I-IV)		
Stage	I 32(23.7%)	II 38 (28.1%)	III 33(24.4%)	31 (23.0%)
Histology		135 (100%) adenocarcinomas		
Differentiation	23 (17.4%) Well	94 (69.6%) moderately	8 (5.9%) poorly	
pT Stage	T1 9 (7.1%)	T2 34 (26.6%)	T3 69 (53.9%)	T4 16 (12.5%)
pN Stage	N1 78 (61.9%)	N2 26 (20.6%)	N2 22 (17.5%)	
pM Stage	M0 95 (77.3%)		M1 28 (22.7%)	
LN	Mean 15.08 [0.0-41], SD=7.13			
LN +ve	Mean 1.83 [0.0-24], SD=3.73			
LN +ve /LN	Mean 0.107, [0-1], SD=0.2020			
CRM	Clear 77 (71.3%)		Inconclusive 31 (28.7%)	
LVI	Positive 26 (32.9%)		negative 53 (67.1%)	
PNI	Positive 12 (15.2%)		Negative 67 (84.8%)	

Table III. *Molecular staging*.

Molecular Staging (I-IV)				
KRAS	Mutant n=40 (37.4%)		Wild type n=67 (62.6%)	
BRAF	Wild type n=65 (100%)			
MSI	MSI-H n=1 (1%)		Stable n=95 (99%)	
P16	Mean 12.63% [0-70] SD=15.33			
MGMT	Non-preserved 4(6.0%)		Preserved 63 (94%)	
MMR	Non-preserved 1(1.1%)		Preserved 94 (98.6%)	
b-catenin	M 27(42.9%)	M + focal N 22(34.9%)	Mixed N+M 10(15.9%)	N 4(6.3%)

M, Membranous; N, nucleic.

Pre-operative staging (MDT Stage). Twenty-eight were Stage I (20.9%), n=30 were Stage II (22.4%), n=45 were Stage III (33.6%) and n=31 were Stage IV (23.1%). Stage was defined during the MDT meeting based on either radiology or pre-op biopsy if available (Table II).

Neoadjuvant treatment. Seventy-one (52.6%) did not receive any neo adjuvant treatment and went straight to surgery. N=10 (7.4%) received neo-adjuvant radiotherapy only prior to surgery, from whom n=3 (30%) were radiological Stage II and n=7 (70%) were radiological Stage III. N=38 (29%) received neo adjuvant chemo radiotherapy, from whom n=20 (52.6%) were radiological Stage IV, n=15 (39.5%) Stage III, n=2 (5.3%) Stage II and n=1 (2.6%) Stage I. N=12 received neo adjuvant chemotherapy, from whom n=9 (75%) were radiological Stage IV, n=2 (16.7%) Stage III and n=1 (8.3%) Stage II. All decisions were based on MDT pre-operative staging (Table I).

Pathological staging (post-operatively). All 135 cases were adenocarcinomas. N=23 (17.4%) specimens were well differentiated, n=94 (69.6%) were moderately differentiated, and n=8 (5.9%) were poorly differentiated. N=10 (7.3%) were non specified (Table II). N=32 (23.7%) were Stage I, n=38 (28.1%) Stage II, n=33 (24.4%) Stage III and n=31 (23%) Stage IV. This was because some of the patients were offered down-staging neo adjuvant therapy. N=9 were pT1 (7.1%), n=34 were pT2 (26.6%), n=69 were pT3 (53.9%), n=16 (12.5%) were pT4. N=78 were pN0 (61.9%), n=26 were pN1 (20.6%), n=22 were pN2 (17.5%). In n=53 (67.1%) there was LVI negative, whereas, in n=26 (32.9%) it was LVI-positive.

In n=12 there was perineural invasion noted (PNI), whereas in n=67 there was negative PNI. In the rest of the cases there was no PNI or LVI status documented on the biopsy report. Finally, in n=77 the margins were clear

Table IV. *Biomarkers vs. outcomes.*

KRAS Status (I-IV)	*Vs.* Feature No recurrence	Local	Distant	Total/*p*-Value
Wild type				
Mutant	47	3	17	67
	32	1	7	40 p=0.526
	No PNI	PNI		
Wild Type	33	8		41
Mutant	20	2		22 p=0.242
	No LVI	LVI		
Wild Type	25	17		42
Mutant	22	5		27 p=0.017
	Male	Female		
Wild Type	48	19		67
Mutant	20	20		40, p=0.021
		+LN/LN		
Wild Type		0.095		
Mutant		0.1141		p=0.658
		Mean Age		
Wild Type		62.26		
Mutant		69.62		p=0.005
B-Catenin Status (I-IV)	*Vs.* feature No recurrence	Local	Distant	Total/*p*-Value
M	20	1	6	27
M + focal N	19	0	3	22
M + N	9	1	0	10
N	4	0	0	4 p=0.092
	No PNI	PNI		
M	16	3		19
M + focal N	6	2		8
M + N	5	0		5
N	1	0		1 p=0.504
	No LVI	LVI		
M	16	6		22
M + focal N	13	3		16
M + N	8	1		9
N	2	2		4 p=0.952
	Male	Female		
M	16	11		27
M + focal N	15	7		22
M + N	6	4		10
N	4	0		4 p=0.339

(71.3%), whereas in n=31 (28.7%) histology was inconclusive. Regarding the rest n=27 of the cases there was no documentation on the biopsy report. The mean number of the lymph nodes (LN) was 15.08 (range=0-41, SD=7.13), and the mean number of positive LN was 1.83 (0-24, SD=3.73). The mean ratio LN+ve/LN total was 0.107 (range=0-1, SD=0.202).

Molecular staging. Molecular analysis was performed in theatre specimens. All were BRAF wild-type (n=65). There was only n=1 MSI-H and n=95 MSS (MSI Stable). The mean p16 expression was 12.63% (min=0, max=70%, SD=15.33). n=63 (94%) were MGMT preserved whereas n=4 (6.0%) were MGMT non-preserved. In n=94 cases MMR was preserved, whereas in n=1 MMR was not preserved. With regards to beta-catenin, in n=27 it was Membranous (M), in n=22 (34.8%) M and focal Nucleus (N), in n=10 (15.9%) mixed M+N and in n=4 (6.3%) N (Table III). Associations between molecular biomarkers and outcomes are shown in Table IV.

Table V. *Outcomes (recurrence, cancer-related deaths).*

Outcomes	Yes	No
Recurrence	33 (24.4%) Local n=6 (4.4%)	102 (75.6%) Distant n=27 (20%)
Mean time recurrence	14.8 months (2-54), SD=13.44	
Cancer related deaths	n=13 (9.6%)	Mean Time 26.39 (0-53), SD=17.6

Adjuvant treatment. Seventy-six (58%) patients did not receive any adjuvant treatment, while n=49 received adjuvant chemotherapy (37.4%) based on pathological staging. From those 49 patients, n=4 were Stage I (8.2%), n=10 (20.4%) Stage II, n=18 (36.7%) were Stage III and n=17 (34.7%) were Stage IV. n=2 received some additional radiotherapy (1.5%) from whom, n=1 was Stage I and n=1 Stage IV, as they both were not fit for further surgery. N=4 (3.1%) had adjuvant chemo radiotherapy, from whom n=1 was Stage I, n=1 was Stage III and n=2 were Stage IV (Table I).

Outcomes. One hundred and two cancer cases (75.6%) did not recur (Table V). In n=6 there was local recurrence (4.4%), whereas n=27 (20%) suffered distant recurrence. With regards to local recurrence n=1 was pT1, n=1 pT2, n=3 pT3 and n=1 pT4. n=3 were pN0, n=1 pN1 and n=2 pN2. n=5 were pM0 and n=1 pM1. Overall n=1 was Stage I, n=1 Stage II, n=3 Stage III and n=1 Stage IV. Concerning distant recurrence, n=6 were Stage I (22.2%), n=5 (18.5%) Stage II, n=4 (14.8%) Stage III and n=12 (44.4%) Stage IV. The mean time to recurrence was 18.66 months (min=17.00, max=22, SD=2.84) for local recurrence and 10.22 months (range=2-32, SD=8.63) for distant recurrence. In n=13 (9.6%) there was Cancer-related death. The mean time of Cancer-related death was 26.39 months (range=0-52, SD=17.60). No other association between KRAS status and histopathological features (pNI, positive LN/LN, pTNM, LVI), follow up parameters (recurrence, adjuvant/neo-adjuvant chemo/radiotherapy) or radiological staging (uTNM), was noted ($p>0.05$) (Table IV).

In the only MSI-H specimen, there was positive PNI ($p=0.020$), and similarly for the n=1 case with non-preserved MMR expression ($p=0.021$). Furthermore, the mean age of cases with non-preserved MGMT was higher, compared to preserved MGMT (83.50 *vs*. 66.53, $p=0.005$). No other significant association for MGMT status was noted ($p>0.05$ for all associations) (Table IV).

Otherwise, recurrence was positively associated with positive LN [1.09 (for no recurrence) *vs*. 1.33 (for local recurrence) *vs*. 4.68 (for distant recurrence), $p<0.001$], as well as the ratio positive LN/LN [0.066 (no recurrence), *vs*. 0.722 (for local recurrence) *vs*. 0.278 (for distant recurrence) $p<0.001$] and pM Stage ($p=0.005$), as well as overall Stage of the disease as defined by the MDT ($p=0.022$). However, positiveve LN/LN ratio, seems not to be associated directly with any of the molecular biomarkers ($p>0.05$ for all associations) (Table IV).

Stage I cases - sub-group analysis results. In the second part of our analysis we examined biomarkers in stage I rectal cancer exclusively. N=32 cases were identified, and n=20 were male (62.5%) whereas n=12 were female (37.5%). The mean age was 65.15 (min=40, max=85, SD=11.97). N=3 (9.4%) had neo-adjuvant chemo radiotherapy prior to surgery, n=1 (3.1%) chemotherapy and n=1 (3.1%) radiotherapy. N=1 received adjuvant radiotherapy (3.1%), n=4 chemotherapy (12.5%) (Table VI). N=8 were pT1 (27.6%), n=21 were pT2 (65.6%). N=7 (21.9%) were well-differentiated adenocarcinomas, n=20 (62.5%) were moderately (70.0%), n=1 (3.6%) poorly and n=4 were unspecified. The mean ratio positive LN/LN was 0.375 (min=0, max=1, SD=0.20). n=17 had no LVI positive (94.4%), whereas n=1 had positive LVI (5.6%). All the specimens were pNI-negative (n=20). N=21 had complete margins (87.5%), whereas n=3 had inconclusive margins (12.5%), and in the rest n=8 there was no documentation (Table VI). The mean follow-up was 35.25 months (min=11, max=72, SD=20.046), and there was no cancer related death. In n=22 of the cases there was no recurrence (78.6%), n=1 was local recurrence (3.1%) and n=6 distant (18.8%). The mean recurrence time was 11.5 months (range=3-25 months, SD=8.57) (Table VII).

With regards to molecular status, n=12 were KRAS-wt (52.2%), and n=11 (47.8%) were KRAS-mt. MSI was stable in all cases (n=20) and MMR expression was preserved as well (n=20). MGMT status was preserved in n=15 cases and non-preserved in n=1 from the specimens that were analyzed. The mean p16 expression was 16.85% (min=1.00, max=50.0, SD=14.40). B-catenin was M in 53.3% of the cases (n=8), focal N and mainly M in 26.7% (n=4) and mixed N + M in 20.0% (n=3) (Table VII).

KRAS-mt status seems to be associated with distant recurrence (n=5, $p=0.045$). No other significant association was noted ($p>0.05$). Membranous b-catenin seems to be associated with distant recurrence (n=3 distant recurrences/ n=8 M expression), though this did not reach statistical significance due to low numbers ($p=0.098$). However, no association was noted between b-catenin status and any other histopathological, radiological or follow outcomes ($p>0.05$ for all associations). No other significant associations with regards to molecular biomarkers were noted (Table VII).

There were no cancer-related deaths, therefore we cannot comment whether there is any link between KRAS and cancer

Table VI. *Stage I-patient demographics and pathology.*

Age (years)	Mean age=65.15 SD=11.97		
Gender	Male 20 (65.2%)		Female 12 (37.5%)
Follow-up	Mean=35.25 [11, 72], SD=20.046 (months)		
	Treatment		
		Received	
Neo-adjuvant Chemotherapy n=1 (3.1%)	Neo-adjuvant n=3 Chemoradiotherapy n=3 (13.8%)	Neo-adjuvant Radiotherapy n=25	None n=3 (9.3%)
		Pathological Stage I	
Differentiation	n=7 Well	20 moderately	n=1 poorly
pT Stage	T1 8 (27.6%)		T2 21 (65.6%)
LN +ve /LN	Mean 0.375, [0-1], SD= 0.20		
CRM	Clear 21 (87.5%)		Inconclusive 3 (12.5%)
LVI	Positive 1 (5.6%)		negative 17 (94.4%)
PNI	Positive 0 (0%)		Negative 20 (100%)

related deaths (Table VII). Distant recurrence of disease positively associated with the number positive LN (p=0.049) as well as increasing ratio positive LN/LN (p=0.05) as expected. There was no association between KRAS and positive LN or the the ratio positive LN/LN overall (p>0.05) (Table VII).

Discussion

The pathway of carcinogenesis in CRC is a complex multifactorial process (10). Sporadic CRC pathway can result from the down regulation of tumor suppressor genes, activation of oncogenes, or from various discrepancies in the mismatch repair genes (MSI), or even from chromosomal losses (CIN) (6, 7, 10), (17).

KRAS is a proto-oncogene, that is involved in the *mitogen-activated protein kinase* (MAPK) and *phosphoinositide-3-kinase/v-akt murine thymoma viral* oncogene pathways (PI3K/AKT). More specifically KRAS, regulates cellular response to extracellular stimuli. Its mutant status can lead to downstream activation of the latter pathways (MAPK, PI3K/AKT) (9, 18). This is the potential mechanism, through which, mt KRAS can result in resistance against anti-EGFR chemotherapy agents (18).

The incidence of *KRAS* mutations comprises around 35-40% of CRC cases, which is confirmed by our study as well (37.4%) (19). Point-mutations in codons 12, 13 and 61 are considered to be the most common, though approximately 85 different mutations have been identified (6). Rosty *et al.* (19) managed to define various distinct clinicopathological features, which are associated with KRAS mutant status *i.e.* mucinous differentiation, proximal location, certain MSI status, MGMT methylation, and presence of contiguous polyp (p<0.05 for all associations). The same study notes that mt KRAS is associated with female sex, which was the case for our study as well (p=0.021). Notably, in our study, there was also a significant association between older age in mt-KRAS compared to wt (69.62 *vs.* 62.26 years, p=0.005), which could be attributed to the fact that carcinogenesis is a lengthy process, accumulating distinct mutations.

Despite the predictive value of KRAS in better response to anti-EGFR chemotherapy (20), its prognostic value remains debatable (6, 8), and there is not enough evidence with regards to KRAS prognostic value in purely rectal cancer. In our cohort of rectal tumors, KRAS wild type is linked with cancer related death for any stage (n=10, p=0.033). Colorectal cancer follows complex and variable pathways, and in this case, it seems that KRAS may not be involved in the carcinogenesis process.

The most important finding of our study is that KRAS-mt status seems to be associated with distant recurrence in Stage I rectal cancer (p=0.045). This seems to support the assumption that early cancer recurrence can represent the reflection of a specific carcinogenesis pathway where KRAS plays an important role, and it is involved at early stages. Similar findings were reported in one of our recently published study (21), where KRAS is linked with local recurrence in Stage I Rectal Cancer following Transanal Endoscopic Microsurgery (TEMS).

On the other hand, if KRAS is found mutant in more advanced stages of rectal cancer, this may be a consequence

Table VII. *Molecular biomarkers versus outcomes for Stage* I *rectal cancer.*

Outcomes for Stage I	Yes		No	
Recurrence	7		22	
	Local n=1		Distant n=6	
Mean Time Recurrence	11.5 months [3-25], SD=8.57			
Cancer Related Deaths	n=0			
Molecular Staging I				
KRAS	Mutant n=11 (47.8%)		Wild type n=12 (52.2%)	
BRAF				
MSI	MSI-H n=0 (0%)		Stable n=20 (100%)	
P16	Mean 16.85% [1-50] SD=14.40			
MGMT	Non-preserved n=1		n=15, Preserved	
MMR	Non-preserved 0 (0%)		Preserved 20 (100%)	
B-catenin	M 8 (53.3%)	M + focal N 4 (26.7%)	Mixed N+M 3 (20.0%)	N 0 (0%)

KRAS Status I	Vs. Feature No recurrence	Local	Dis. Recurrence	Total/*p*-Value
Wild type				
Mutant	11	0	1	12
	6	0	5	11 p=0.045

B-Catenin Status I	Vs. feature No recurrence	Local	Distant	Total/*p*-Value
M	5	0	3	7
M + focal N	4	0	1	4
M + N	3	0	0	3 p=0.098

of a different molecular pathway, where prognosis is defined by other molecular parameters. No other association was found between KRAS status and distinct histopathological features (pTNM, positive LN/LN, PNI, LVI, $p>0.05$ for all associations) or radiological parameters or survival outcomes ($p>0.05$).

In a recent meta-analysis in 2017, Tosi *et al*., included 1,833 patients (22) and concluded that, KRAS mutant status is negatively associated with overall survival (OS) and relapse-free survival (RFS) in patient who underwent liver resection for metastatic CRC. Brudvik *et al*. (23), reported similar findings in their meta-analysis of 1809 cases, published in 2015. Jones *et al*. (24) evaluated 392 cases of advanced and recurrent colorectal cancer from a UK Centre, and, concluded that codon 12 mutations are independently associated with worse overall survival after diagnosis. Similar findings are reported from another US study (25, 26), where KRAS mutations, seem to be independent risk factors for worse OS. Moreover, the same team, reported in another study(27), that KRAS codon 13 mutation, is associated with higher risk for overall extrahepatic or lung specific recurrence.

BRAF V600E mutation seems to be another valid biomarker in CRC. Its presence has been associated with poorer prognosis (28, 29), and is deemed more as feature of right-sided, advanced CRC (12, 30, 31). In our study, BRAF was found wt and this is explained by its association with proximal colorectal cancer. Recently, there have been studies, that associate *BRAF* V600E mutation with resistance to anti-GFR inhibitors, regardless the presence of wt KRAS (32, 33).

Methylation of CpG islands, and especially, O(6)-methylguanine DNA methyltransferase (*MGMT*) gene promoter methylation has been deemed to play a role in the CRC pathway, however, consensus regarding its prognostic value, has yet to be reached (7, 16, 34). MGMT is a DNA repair enzyme, codified at locus 10q26, which removes alkyl groups from the O6 position, and this leads to irreversible inactivation of the enzyme (34, 35). Hence, loss of MGMT expression, *via* methylation of the CpG islands of its promoter, could be reflected, with alteration of normal DNA (14, 36, 37). Non-preserved MGMT expression is found in 30-40% of metastatic colorectal cancer (34).

In our study, non-preserved MGMT expression was associated with higher age (66.53 *vs*. 83.50, p=0.005), which

should, again, raise a question, whether older age would be deemed as an independent factor to worse prognosis, exclusively based on the accumulation of more genetic events, resulting in a distinct molecular phenotype.

B-catenin is considered an essential molecule, that belongs to Wnt signaling pathway. Wnt/b-catenin pathway is primarily involved in the regulation of oncogenic processes, as well as intracellular adhesion (38). There is still little consensus on the role of b-catenin in CRC pathway. A recent study found that KRAS and Wnt pathway may interact in lung cancer (39). In our study, there was a trend towards an association between M expression of b-catenin and distant recurrence (n=6 p=0.092). In Stage I subgroup analysis, there is a similar trend (n=3, p=0.096). However, these were only trends and none of them reach statistical significant values, therefore their interpretation value is limited. There was no association between b-catenin and neo-adjuvant/adjuvant therapy, and any other histopathological features (pTNM, positive LN/LN, LVI, PNI, p>0.05 for all associations).

With regards to p16 expression, there is a recent meta-analysis (40), that associates various clinico-pathological features with the deregulation of its promoter *via* methylation. In our cohort, there is a trend towards higher p16 expression in M b-catenin (21.66 *vs*. 10.25 *vs*. 5.00, p=0.028). Interestingly, there was no association between the status of any of the biomarkers studied in our cohort (KRAS, BRAF, MMR, MGMT, p16, b-catenin) and the response to neo-adjuvant therapy (p>0.05) or other pathological features.

Finally, we recognize the limitations of this study which is predominantly the relatively small sample size, that does not allow to use multivariate statistics. However, our conclusions can raise interesting questions for further research.

Conclusion

Carcinogenesis of CRC is a complex and multifactorial event, in which various molecular events interfere. We found that when KRAS is mutant in early rectal cancer, this fact may be linked with higher chance of distant recurrence. This is an interesting finding that should be further examined with greater amount of research in hope that it may constitute an additional prognostic factor for early rectal cancer.

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