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ORIGINAL ARTICLE

Role of KRAS mutation as predictor of pathologic response after neoadjuvant chemoradiation therapy for rectal cancer

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Abstract Individual patient response to neoadjuvant treatment is variable and reproducible biomarkers of response are needed. The role of the V-Ki-ras2 Kirsten rat sarcoma viral oncogene (KRAS) in rectal cancer remains equivocal. The aim of the current study was to evaluate the effect of KRAS mutation on outcomes following neoadjuvant chemoradiation therapy (CRT) for rectal cancer. A total of 76 stage II–III rectal cancer patients underwent preoperative CRT followed by surgery. In every patient tumor-related features and outcome results were considered for analysis and correlation with KRAS mutations. Forty-four patients (58 %) obtained a downstaging after CRT, and in 7 patients (9 %) a complete pathological response was found. Twenty-six (33 %) mutations of KRAS were found in 26 patients. Nineteen mutations (73 %) were located in codon 12, 6 in codon 13(23 %) and 1 in codon 61. T-level downsizing and tumor downstaging showed no significant association with KRAS mutation status, except for mutation of codon 13(G13D). No correlation between cancer-associated mortality following CRT and surgery and KRAS mutation was observed. No correlation between pelvic recurrence and KRAS mutation was observed. KRAS mutation also failed to correlate with disease-free survival. No patients with a pCR had a local or distant failure. There appears to be no significant difference in pCR, tumor down-staging, T-downsizing or effects on cancer-associated mortality, overall survival and disease-free survival in patients with KRAS mutations except for patients with KRAS codon 13 mutations that seem to be resistant to neoadjuvant CRT and less likely to achieve a pCR.

Keywords Rectal cancer · Preoperative chemoradiotherapy · Neoadjuvant · KRAS · Response

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Introduction

Currently, the standard treatment of locally advanced rectal cancer consist in a multi-modal treatment with neo-adjuvant chemoradiotherapy (CRT) followed by total mesorectal excision (TME). Improved local control and longer survival have been shown with this approach however, individual patient response is variable.

Approximately, 45–60 % of patients respond to neo-adjuvant CRT with tumor level down-staging [1, 2].

Pathological complete response (pCR) is achieved in 10–30 % of patients while others exhibit an incomplete or no response [3].

Identification of biomarkers predictive of poor response to neo-adjuvant CRT could be used to select optimal treatments for rectal cancer patients and spare significant morbidity in patients who will not benefit from neo-adjuvant treatment.

The search for biomarkers of response to CRT has been extensive. Tumor protein 53 (p53), vascular endothelial growth factor (VEGF), cyclo-oxygenase 2 (COX-2), epidermal growth factor receptor (EGFR) and thymidylate synthase among others have been closely examined [4, 5].

The V-Ki-ras2 Kirsten rat sarcoma viral oncogene (KRAS) is one of the most commonly studied mutations in colorectal cancer, yet its role in rectal cancer remains controversial [6].

Approximately, 30–50 % of colorectal tumors are known to have a mutated (abnormal) KRAS, indicating that up to 50 % of patients with CRC might respond to anti-EGFR antibody therapy. However, 40–60 % of patients with wild-type KRAS tumors do not respond to treatment [7].

In these patients, data suggest that mutated BRAF, which is present in 5–10 % of tumors, can affect response to these agents [8].

The KRAS mutations most commonly implicated in colorectal cancer are mutations located at codons 12 and 13 of the KRAS gene on the short arm of chromosome 12 (exon 2).

Even if in many centers it is routinely tested prior to adjuvant treatment for colon cancer, less is known of its use as a biomarker in rectal cancer and its use as a predictor of response to neo-adjuvant CRT. Several studies have investigated KRAS mutation as a molecular predictor of response to CRT in rectal cancer, however, its use has not been validated and there is no consensus on its predictive value.

Aim of the study was to evaluate the correlation between KRAS mutations and rectal cancer response to CRT, exploring some possible correlation with clinical parameter and survival data.

Methods

Patients

The study included in a prospectively recorded database patients with stage II/III rectal cancer treated in the General, Emergency and minimally invasive surgery unit of Careggi University hospital in Florence between 2007 and 2012.

After a preoperative study protocol including pan-colonoscopy with positive biopsy for rectal adenocarcinoma, a thorax and abdomen CT scan for distant metastasis, Endoscopic Ultrasound (EUS) and in some patients Pelvic Magnetic Resonance Imaging (MRI) for local staging, patients with local advanced rectal carcinoma (T3–T4) or positive lymph nodes (N+) were candidate for neoadjuvant chemoradiation. Radiotherapy was done by 1.8–2 Gy daily in 25–28 fractions for at least 5 weeks to reach 45 Gy plus a 9 Gy boost in some patients. Chemotherapy was done with continuous i.v. infusion of 5-fluorouracil (200–225 mg/m2/day) 5 days a week. After at least 6 weeks from the end of the treatment a local restaging with EUS was performed and after at least 8 weeks patients underwent surgery. EUS was performed by a single experienced surgeon (MJ). MRI were performed by a team of two experienced radiologists. EUS was preferred to MRI for restadiation because of the lower costs, the better availability and the better accuracy, as previously reported [9].

The location of the tumor was considered in the low rectum if it started from <5 cm above the pectineal line and medium if it was between 5 and 10–12 cm (peritoneal reflection). Patients with intraperitoneal rectal tumors or tumors that not required a neoadjuvant treatment were excluded from the present study. The distance of the tumor from the anal verge was measured in all cases using a rigid rectoscope. The cancer stage was defined according to the American Joint Commission on Cancer/International Union for Cancer Control (AJCC/UICC) Tumor Node Metastasis (TNM) classification system [10].

Response was defined as T-category downsizing or as AJCC stage downstaging. Both methods compared the pre-therapeutic assessment determined by EUS with the histopathological diagnosis after surgery. Tumors exhibiting a T-level downsizing or a AJCC downstaging of at least one category were considered responsive. A pCR was defined as the absence of viable tumor in the entire resected surgical specimen and in the regional lymph-nodes evaluated by hematoxylin and eosin staining under microscopy.

The follow-up protocol consisted of a medical examination with rectal exploration and proctoscopy and carcinoembryonic antigen (CEA) assessment every 3–4 months for the first 2 years, then every 6 months up to 5 year and annually until the 10 year. Abdominal ultrasound was performed every 12 months (alternating with CT scan every 6 months). Thorax, abdomen, pelvis CT scan every 12 months up to year 5, then every 3 years and colonoscopy in the 1, 3 and 5 year then every 5 years. Disease-free survival was calculated from the date of surgery to the date disease recurrence or death was first observed or the date of the last follow-up visit.

In every patient tumor-related features (distance from the anal verge, stage, lymph nodes, distant metastases), and outcome results (survival, recurrence, metastases) were considered for analysis and correlation with KRAS mutations.

Mutation analysis

Tumor DNA was obtained from each patient from the surgical specimen and from pre-treatment biopsies. For KRAS mutation analysis the exons 2, 3 and 4 were sequenced to detect the mutation hotspots at codons 12 and 13 as well as to screen for rare mutations such as those in

codons 61 and 146 or additional rare ones previously described in the literature (59/117).

DNA was isolated from three 10 μm formalin-fixed paraffin-embedded tissue sections. After dewaxing and over-night proteinase K (200 μg/ml) digestion at 50 °C, the DNA was heated to 96 °C for 15 min to destroy proteinase K activity. The DNA was purified with MasterPure™ DNA Purification (Epicentre Biotechnologies, WI—USA) according to the manufacturer's protocol and the DNA concentration was assessed spectrophotometrically.

PCR reaction for K-ras gene was performed using a forward primer (ACTGAATATAAACTTGTGGTAGTTG GACCT) and a reverse primer (TAATATGTCGACT AAAACAAGATTTACCTC).

The reaction was carried out in a volume of 50 μl, with 2 mM $MgCl_2$, 0.2 mM dNTPs, 25 pmol of each primer and 2 U Taq polymerase (Applied Biosystems, USA) with 200–350 ng of DNA template. The cycle conditions were: 40 s at 96 °C, 40 s at 55 °C and 30 s at 72 °C for 40 cycles. A 7 min final extension was added. The amplification was performed in a 2,720 Thermal Cycler (Applied Biosystems, USA).

The amplification products were purified with MSB® Vario Cleanup Kit (Invitek, Berlin, DE) according to the manufacturer's protocol.

Subsequently, cycle sequencing reaction of purified PCR products was performed, using the K-ras reverse primer and BigDye® Teminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's protocol. The sequencing products were purified using DyeEx® 2.0 Spin Kit (Qiagen, Hilden, DE).

Then 10 μl of purified sequencing reactions were added to 20 μl formamide and was heated to 95 °C for 5 min to allow the DNA denaturation.

The samples were analyzed using the AbiPrism 310 Genetic Analyzer (Applied Biosystems, USA). The sequence results for each sample were analyzed using Seqscape® Software v2.5 (Applied Biosystems, USA) to verify the sequencing results and identify the possible mutations.

Statistical analysis

To determine differences in clinical and pathological features between pCR and non-pCR patients, the Mann–Whitney *U* test was used for comparing means of continuous variables between groups and the two sided Fisher's Exact test and Chi-square test were used for testing the significance of differences in the distributions of categorical variables.

For genes which could be classified as wild-type or mutant, 2 × 2 analysis tables were constructed and genes were tested for association with tumor response using Fisher's Exact test. A p value <0.05 was considered statistically significant.

Correlation of disease-free survival and overall survival and KRAS mutations was done using Kaplan–Meier curves.

Results

During the study period, 76 patients were enrolled in the study (42 males; 55.2 %). Median age was 61 years (range 31–84). At diagnosis, clinical stage defined with EUS was II in 23 patients (30 %) and III in 53 patients (70 %). Patients' characteristics are described in Table 1. In 32 patients (42 %) the tumor was located in the low rectum and in 44 (58 %) in the medium rectum. Forty-four patients (58 %) obtained a downstaging after CRT, and in 7 patients (9 %) a complete pathological response was found. The association between KRAS mutations and tumor response were reported in Table 2.

In total, 26 (33 %) mutations of KRAS were found in 26 patients. No patients exhibited multiple mutations. Nineteen mutations (73 %) were located in codon 12, 6 in codon 13 (23 %) and 1 in codon 61.

Post-neoadjuvant T-category and lymph node status were compared between patients without KRAS mutation and those with a mutation in either codons 12, 13, 61. None

Table 1 Patients data

	Total 76 pts	$KRAS^{wt}$ 50 pts	$KRAS^{mut}$ 26 pts	p value
Age (median)	61	62	58	0.59
Male	42 (55 %)	27 (54 %)	15 (57.5 %)	0.70
Female	34 (45 %)	23 (46 %)	11 (42.5 %)	
T2	11 (14.5 %)	9 (18 %)	2 (7.5 %)	0.24
T3	53 (70 %)	33 (66 %)	20 (77 %)	0.32
T4	12 (15.5 %)	8 (16 %)	4 (15 %)	0.91
Stage II	23 (30 %)	18 (36 %)	5 (19 %)	0.13
Stage III	53 (70 %)	32 (64 %)	21 (81 %)	
Low rectum	32 (42 %)	23 (46 %)	9 (35 %)	0.34
Medium rectum	44 (58 %)	27 (54 %)	17 (65 %)	

Table 2 Association between KRAS mutations and tumor response

	Total 76 pts	$KRAS^{wt}$ 50 pts	$KRAS^{mut}$ 26 pts	*p* value
T downsizing				
Yes	46 (60 %)	31 (62 %)	15 (58 %)	0.71
No	30 (40 %)	19 (38 %)	11 (42 %)	
Downstaging				
Yes	44 (58 %)	31 (62 %)	13 (50 %)	0.33
No	32 (42 %)	19 (38 %)	13 (50 %)	
pCR				
Yes	7 (9 %)	6 (12 %)	1 (4 %)	0.41
No	79 (11 %)	44 (88 %)	26 (96 %)	

of these comparisons showed a significant difference between the groups. Due to small sample sizes, further analyses of response levels were performed excluding the patient that carried mutations in codons 61. Based on the change of amino acids codon 12 mutations were analyzed separately. Considering the main nucleotide substitutions, in twelve patients (63 %) guanine was replaced by adenine (GGT > GAT; G12D) and in 4 patients (21 %) by thymine (GGT > GTT; G12V). The most common mutation in KRAS codon 13 was G13D (all cases). In these analyses, T-level downsizing and tumor downstaging again showed no significant association with KRAS mutation status (Table 3), except for mutation of codon 13 (G13D).

After a median follow-up of 33 months (range 12–72) 2 patients (2 %) died for a cancer-related cause (24 and 57 months after operation) and one patient for a no-cancer-related cause (cardiac). None of these patients have a mutation of KRAS and no correlation between cancer-associated mortality following neoadjuvant CRT and surgery and KRAS mutation was observed. Five patients (6.5 %) experienced pelvic recurrence (2 with KRAS mutation). No correlation between pelvic recurrence and KRAS mutation was observed. KRAS mutation also failed to correlate with disease-free survival (74 % of patients). No patients with a pCR had a local or distant failure.

Discussion

The response to combined neoadjuvant therapy for advanced stage rectal adenocarcinoma is predictive of outcome. In addition to both clinical and pathological features, the expression of a variety of molecules may provide another method of identifying tumor responsiveness to preoperative therapy.

Acquired mutations in KRAS are an early step in carcinogenesis, identified in approximately, 40 % of colorectal cancers. The most common mutations occur at codons 12 and 13 of exon 2 of the KRAS gene. These mutations in the phosphate-binding loop of Ras deactivate its intrinsic GTPase activity and render it resistant to GAP-mediated GTP hydrolysis, thereby locking Ras into the activated state. Constitutively active Ras is no longer dependent on upstream activation of receptor tyrosine kinases and as a corollary, insensitive to anti-EGFR chemotherapy [11]. An abundance of literature confirming that the presence of KRAS mutation predicts lack of response to anti-EGFR therapy has resulted in the increasing use of KRAS mutation analysis in clinical practice. However, its role as a prognostic marker of overall survival independent of anti-EGFR therapy remains less clear and some studies have shown conflicting results on the predictive value of KRAS mutation status on response to neoadjuvant chemoradiation therapy.

From a biologic standpoint, multiple lines of evidence suggest that tumor behavior and response to therapy of KRAS mutant cells are driven by the relative dominance of individual effector pathways downstream of Ras. Fibroblasts transfected with mutant KRAS codon 12 are more resistant to apoptosis, more predisposed to anchorage—independent growth, and grow more readily as spheroids than fibroblasts transfected with mutant KRAS codon 13 or overexpressed wild-type Kras [12].

Moreover, although the influence of individual KRAS mutations on radiation resistance is inadequately studied, it is well-known that Ras activation induces radiation

Table 3 Analysis of KRAS mutations and tumor response

	Codon 12 19 pts	*p* value	G12D 12 pts	*p* value	G12V 4 pts	*p* value	Codon 13 6 pts	*p* value
T downsizing								
Yes	10 (53 %)	0.39	7 (58 %)	0.95	2 (50 %)	0.73	1 (17 %)	**0.02**
No	9 (47 %)		5 (42 %)		2 (50 %)		5 (83 %)	
Downstaging								
Yes	11 (58 %)	0.97	8 (67 %)	0.39	2 (50 %)	0.73	1 (17 %)	**0.02**
No	8 (42 %)		4 (33 %)		2 (50 %)		5 (83 %)	
pCR								
Yes	1 (5 %)	0.53	1 (8 %)	0.27	0 (0 %)	0.66	0 (0 %)	0.58
No	18 (95 %)		11 (92 %)		4 (100 %)		6 (100 %)	

Statistically significant values are in bold ($p < 0.05$)

resistance [13, 14] and different mutant forms of KRAS codon 12 confer varying levels of chemosensitivity [15].

Despite these results, and the suggestion that patients without mutations in commonly mutated cancer genes may be associated with a higher likelihood of having a pCR after preoperative CR [16], the recent review of Clancy et al. [17] suggested that KRAS mutation does not confer radioresistance in rectal cancer. There appears to be no significant difference in pCR, tumor down-staging or effect on cancer-associated mortality in patients with KRAS mutations whether or not anti-EGFR therapies are used in neo-adjuvant CRT regimens.

The results of the present study confirmed these findings. No statistically significant differences were found about pCR, tumor downstaging or T downsizing, overall survival and disease-free survival. As the results of the current study demonstrated no correlation between pCR or downstaging and KRAS mutation, as would be expected no correlation between cancer-associated mortality following neoadjuvant CRT and surgery and KRAS mutation has been observed, as reported by other Authors [18].

Although these results tend to discourage the use of KRAS mutation as a predictor of response and outcome in patients with rectal adenocarcinoma, several studies suggest that the specific location of KRAS mutation within the gene can affect different downstream pathways, which ultimately could affect tumor response to therapy. Guerrero et al. [12] examined differences between KRAS codon 12 and codon 13 mutations on downstream signaling in transfected NIH3T3 fibroblasts and noted similar KRAS activity and upregulation of the MAPK pathway, but differential activation of the AKT, c-Jun N-terminal kinase (JNK), and focal adhesion kinase (FAK) pathways. The functional consequences of differential downstream signaling manifested as altered apoptotic and mitotic rates.

Divergent signal transduction between KRAS codon 12 and 13 mutations was also demonstrated [19]. In patients who have metastatic colorectal cancer with the *KRAS* G13D mutation appear to benefit more from cetuximab than those who have *KRAS* codon 12 mutations and should be treated differently [20].

An analysis of 94 rectal cancer patients treated with preoperative chemoradiation therapy concluded that G12V mutations appeared to be associated with a lower rate of tumor regression than G13D mutations [21]. Similar results were noted in Dukes B and C colon cancer patients where those harboring G12V mutations had shorter disease-free and overall survival [22].

However, Duldulao et al. [23] demonstrated that rectal cancer patients with KRAS codon 13 mutations are resistant to neoadjuvant CRT and do not achieve a pCR.

Despite the small sample size of this study could represent a limitation, conditioning the account of codon 13 mutation available for analysis, our results seem to confirm these finding. In fact, codon 13 mutations (in particular G13D) was the only statistically significant factor affecting the outcome after neoadjuvant treatment, suggesting that codon 13 mutations could confer resistance to preoperative chemoradiation affecting the possibility of a complete pathological response.

An in vitro study by Guerrero et al. [12] demonstrated that tumors with codon 13 mutations tended to exhibit increased apoptosis. Patients with p.G13D point mutations were diagnosed more commonly as non-metastatic ($p = 0.018$) and tumors with p.G13D point mutations appeared to have latent metastasis due to apoptosis [24]. The higher rates of apoptosis, response to anti-EGFR treatment [12, 19], left-colon localization and diagnosis at non-metastatic stage distinguish p.G13D from the remaining mutations investigated.

However, it is possible that specific mutations in KRAS do not occur in isolation and that the global genetic context modulates the impact of these mutations on overall treatment response of tumors. For example, the association between KRAS mutations, p53 mutations, the cyclin D1 (*CCND1*) G870A (AA) polymorphism and/or the methylenetetrahydrofolate reductase (NAD(P)H) (*MTHFR*) C677T (TT) polymorphism in variable combinations were also associated with a high positive predictive value of non-pCR [25]. Moreover, the presence of KRAS codon 13 mutation correlates with TP53 mutation [23]. Exploring the relationships of KRAS and BRAF status with their possible downstream activation target in case of mutation was also suggested that activation of phosphatidylinositol 3-kinase (PI3K)/AKT and extracellular signal-regulated kinase (ERK) may be beneficial for response to radiation therapy in rectal adenocarcinoma and although KRAS mutation was not associated with lesser response to chemoradiotherapy, high p-ERK or p-ARK expression was associated with better overall survival and response [26].

Thus, the variability in the resistance of KRAS mutant tumors to chemoradiation therapy may be due to inherent differences in downstream signaling by these KRAS mutant tumors and/or differences in prevalence of other mutations in these KRAS mutant tumors.

It was suggested that, as reported for KRAS, patients with mutations of NRAS, BRAF, APC, TP53, and PIK3CA were less likely to achieve a pCR compared to patients whose tumors did not have mutations [16].

Approximately, 5–8 % of colorectal cancers are characterized by a specific mutation in the BRAF gene (V600E) [27]. BRAF mutations are at the moment, for all practical purposes, limited to those tumors that do not have KRAS mutations however, though BRAF mutations are considered as activating mutations of the MAPK pathway and

recent findings indicate that response against anti-EGFR therapy requires the presence of the wild-type allele [8], testing for the mutation would have an impact on therapeutic outcome and on planning individualized therapy concepts. On the contrary, it should also be considered that the frequency of BRAF mutations decreases from the right to the left colon [28] and is not so common in the rectal adenocarcinoma, suggesting that BRAF mutations could play a minor role for rectal carcinogenesis compared to colon carcinogenesis.

Another possible limitation of the present study could be the median follow-up of 3 years. Although the interval until recurrence was longer in rectal cancer respect to colon cancer, the mean recurrence time was 26 months (±24.2) [29], suggesting that this follow-up period could be sufficient to draw valid conclusions, that further studies with larger patients samples and longer follow-up will confirm or refute.

Conclusions

Our findings suggest KRAS mutation does not confer radio-resistance in rectal cancer. There appears to be no significant difference in pCR, tumor down-staging, T downsizing or effects on cancer-associated mortality, overall survival and disease-free survival in patients with KRAS mutations. However, despite the small sample, we also noted that rectal cancer patients with KRAS codon 13 mutations may be resistant to neoadjuvant CRT and less likely to achieve a pCR.

Although KRAS mutation status di per se has shown limited role to select patients for neo-adjuvant chemoradiotherapy for rectal cancer, the analysis of single mutations, different mutations association and downstream effectors could play a potential role in predicting the response to therapy and the oncologic outcomes.

Conflict of interest The authors have no potential conflict of interest or financial ties to be disclosed.

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