Author’s response to reviews

Title: Impact of KRAS, BRAF and PI3KCA mutations in rectal carcinomas treated with neoadjuvant radiochemotherapy and surgery

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Author’s response to reviews: see over
Background: Conventional treatment for locally advanced rectal cancer usually combines neoadjuvant radiochemotherapy and surgery. The tumor downstaging induced by radiochemotherapy, is an important goal for clinicians because it has been shown to predict recurrences and survival. But until now, no clinical or biological factor (easily identifiable at diagnosis) has been established as a significant predictive factor for tumor response. KRAS, BRAF and PIK3CA mutations are commonly found in colon cancers. Only the Dutch trial has found that PI3KCA mutations may play a role in predicting local recurrences in rectal cancer treated with TME surgery. In this study, we aimed to determine the mutation frequencies of KRAS, BRAF and PIK3CA and to establish whether such mutations may be used as prognostic and/or predictive factors in rectal cancer patients.

Materials and Methods: We retrospectively reviewed the clinical and biological data of 98 consecutive operated patients between May 2006 and September 2009. We focused in patients operated in our center after radiochemotherapy and in which tumor samples were available. Mutations detection in KRAS, BRAF and PIK3CA genes was performed using a direct sequencing approach. DNA was extracted from FFPE (formalin fixed and paraffin embedded) samples and PCR amplified with specific primers for exons where "hot-spot" mutations are located.

Results: In the 98 patients with a rectal cancer, the median follow-up time was 28.3 months (4- 74). 8/98 patients experienced a local recurrence (8%) and 17/98 developed distant metastasis (17%). KRAS, BRAF and PIK3CA were identified respectively in 23 (23.5%), 2 (2%) and 4 (4%) patients. As described in previous studies, mutations in KRAS and BRAF were mutually exclusive. No patient with local recurrence exhibited KRAS or PI3KCA mutation and one harbored BRAF mutation (12.5%). Of the seventeen patients with distant metastasis (17%), 5 were presenting KRAS mutation (29%), one BRAF (5%) and one PIK3CA mutation (5%). No relationship was seen between PI3KCA, KRAS or BRAF mutation and local or distant recurrences and neither with overall
Conclusion: The frequencies of \textit{KRAS}, \textit{BRAF} and \textit{PIK3CA} mutations in our study were lower than the average frequencies reported in colorectal cancers and no significant correlation was found between local/distant recurrences or survival and \textit{KRAS}, \textit{BRAF} or \textit{PIK3CA} mutations. Other predictive biomarkers should be studied to predict significantly the response to neoadjuvant radiochemotherapy.

Introduction

\textbf{Over the last decade}, the management of colorectal cancer (CRC) has progressed faster than in any other gastrointestinal tumors (Jemal et al.). These advances have been made especially in metastatic disease, with the introduction of targeted therapies in association with chemotherapy and the development of metastasis surgery (Hurwitz et al., 2004; Abdalla et al., 2004; Adam et al., 2009; Van et al., 2011a). Improvements have also been made in the adjuvant setting with the introduction of the oxaliplatin-based chemotherapy regimen in stage III colon cancer (Andre et al., 2009). Less progress has been made in the management of rectal cancer. Radiochemotherapy based on 5FU regimen, followed by total mesorectum excision (TME) represents the optimal combined treatment for locally advanced rectal cancer (defined as T3 and/or N+ disease) (Heald and Ryall, 1986; Kapiteijn et al., 2001). Neoadjuvant radiochemotherapy has been shown to reduce local recurrences and to increase pathological complete response compared with radiotherapy and surgery (Bosset et al., 2006; Bujko et al., 2006; Gerard et al., 2006). This preoperative modality is currently preferred to the postoperative one because of a significantly lower local recurrence rate, improved sphincter preservation and less toxicity (Pahlman and Glimelius, 1990; Sauer et al., 2004a). Attempts to increase the benefit of radiochemotherapy have been tried, especially with the introduction of oxaliplatin in addition to capecitabine but finally, the 5FU based radiochemotherapy has remained the standard treatment
for patients with locally-advanced rectal cancer (Aschele et al., 2011; Gerard et al., 2010; Sauer et al., 2004b; Bosset et al., 2006). The decision to use neoadjuvant radiochemotherapy is based on a pre-treatment tumor staging defining the T and the N stage with pelvic MRI and endorectal ultrasound. The tumor response is evaluated by the pathological examination of the operative specimen. It is well known that downstaging after radiochemotherapy has been shown to predict less recurrences and better prognosis (Gunderson et al., 2004; de Campos-Lobato et al., 2010). However, this decision-making process seems unsatisfying with open questions. First, the tumor response can be evaluated only after the pathological examination. Secondly, despite low local recurrence rates, patients with initially localized rectal cancer continue to have high mortality because of a secondary metastatic spreading (15-35%). On the other hand, some patients may be overtreated with radiochemotherapy. Therefore, many authors have tried to identify predictive factors to anticipate radiochemotherapy response. In 2012, looking for an accurate early assessment of tumor response with MRI and exploring potential predictive molecular tumor abnormalities are the best available current methods to investigate improved outcomes. Indeed, aggressive behavior and/or chemoresistance may be dependent on some molecular patterns that could be important to identify, in order to adapt management and develop new potent treatment strategies.

**KRAS, BRAF and PIK3CA** mutations are commonly found in colorectal cancers. **KRAS** and **BRAF** genes can harbor oncogenic mutations that yield a constitutively active protein and are found in approximately 30–50% and 10–15% of CRC tumors, respectively (Barault et al., 2008; Schubbert et al., 2007). Several studies have indicated that the presence of mutant **KRAS** in CRC tumors correlates with absence of anti-EGFR’s benefit in metastatic setting (Lievre and Laurent-Puig, 2009; Lievre et al., 2006; Van et al., 2011b). Furthermore, **BRAF** mutations have been incriminated as a poor prognosis factor in
metastatic CRC (Bokemeyer et al., 2011). However, the impact of KRAS and BRAF mutations on clinical outcome of patients with locally advanced CRC are unknown. Regarding PIK3CA, a large cohort study has recently shown that PIK3CA mutation was associated with poor prognosis among patients with resectable stage I to III colon cancer (Ogino et al., 2009a). Another large population-based study in colon cancer suggested that the activation of the PI3K/AKT or the RAS-RAF-MAPK pathway by mutation of at least one of the three genes predicted poor patient outcome, but the effect of mutations in PIK3CA alone was not discussed (Barault et al., 2008). Another previous study of a small cohort of colorectal cancer patients reported that PIK3CA mutation is predictive of poor survival (Kato et al., 2007). These mutation studies make no distinction between rectal and colic cancer, additionally, their prevalence and their prognostic value remain unclear in rectal cancer. Recently, He et al. showed that PIK3CA mutations were strongly associated with a high risk of local recurrences in non irradiated stage I to III rectal cancer patients (He et al., 2009). As their population was heterogeneous in tumor stage (I to III) and was not treated with combined modality therapy, we aimed to corroborate the mutation frequencies of KRAS, BRAF and PIK3CA in rectal cancer and to establish whether such mutations may be used as prognostic and/or predictive factors in multimodal treated rectal cancer patients.

Materials and methods

Patients and tumor samples

The clinical records of all consecutive patients with locally advanced rectal carcinoma (clinical T3 or T4 or node-positive) referred to the Centre Leon Berard between May 2006 and September 2009 were reviewed. The study was approved by the ethic committee of Leon Berard Center. Written informed consent was obtained for all patients. The inclusion criteria were a confirmed diagnosis of rectal
adenocarcinoma and available tumor sample. All patients gave their informed consent for this research. Diagnosis was established on the basis of histological features and was confirmed by immunohistochemical staining. Pathology procedures were standardized and quality controlled. All patients were assessed before treatment, with a history and physical examination, performance status evaluation and assessment of complete blood counts (CBCs), liver function, creatinine and serum carcinoembryogenic antigen. All patients underwent before treatment a rigid rectoscopy and a total colonoscopy. Tumor and nodal stage was evaluated with pelvic MRI and/or an endorectal ultrasound. Metastatic extension was eliminated with a chest-abdomen-pelvis computed tomography. Clinical tumor staging was finally defined with the “i” (MRI) or “u” (ultrasound) tumor-node-metastasis (TNM) classification. Clinical examination and CBCs were repeated every week during radiochemotherapy. Four weeks after the end of radiochemotherapy, clinical tumor stage was re-evaluated with pelvic MRI and CT. After surgery, patients were assessed every 3 months during the first two years and every 6 months during years 3 to 5.

Patients were treated with neoadjuvant radiochemotherapy and TME-surgery. Radiotherapy consisted on 45 to 50 Gy in 25 fractions of 1.8 to 2Gy with concurrent intravenous 5FU or capecitabine. Oral capecitabine 800 mg/m2 twice daily was started on the first day of radiotherapy and given 5 days per week during radiotherapy. When used, infusional 5FU was given at a dose of 350 mg/m2/d from Monday to Friday with leucovorin at a dose of 20 mg/m2/d. Surgery was planned 6 weeks after the end of preoperative chemoradiotherapy. Total mesorectal excision was performed according to a standardized technique.

DNA extraction and mutation analysis

PCR amplified with specific primers for exons where "hot-spot" mutations are located. Mutation detection par sequencing. DNA was extracted from FFPE tumor samples using QIAamp DNA FFPE Tissue Kit
Mutation status of K-RAS gene (exon 2 and 3), PIK3CA gene (exon 9 and exon 20), BRAF gene (exon 15), was investigated by PCR amplification followed by direct sequencing using ABI 3730 automated sequencer (Life Technologies). The oligo sequences of primers used for analyses are available upon request.

Statistical analysis.

Recurrences and survival analyses were done using the Kaplan-Meier method with time of surgery as entry date. Log rank testing was used for comparison of groups.

Results

Patient’s characteristics

Ninety-eight consecutive patients treated at the Centre Leon Berard, Lyon, France for an advanced rectal cancer between May 2006 and September 2009 met the inclusion criteria. Locally advanced rectal cancer was defined as T3 and/or N+ disease with pelvic MRI and/or endorectal ultrasound. Median follow-up was 28.3 months (4-74). Patient’s characteristics are listed in Table 1.

Pathologic characteristics

We correlated the KRAS BRAF and PIK3CA genotypes with clinicopathological features of CRC, including primary tumour location, histological findings, and sites of metastases. There was no correlation between mutations and clinicopathologic features, including age, gender, tumor location, type of resection, circumferential margin (CRM), differentiation grade, lymph node and TNM stage. We also investigated whether KRAS, BRAF or PIK3CA mutations may confer radioresistance and reduced response to CRT. There was no difference in residual tumor status or in chemoradiation’s response as assessed by tumor downstaging, based on mutations status.
Mutations analysis

KRAS, BRAF and PIK3CA were identified respectively in 23 (23.5%), 2 (2%) and 4 (4%) patients. The most frequent mutation at KRAS was G13D which accounted for 43% of KRAS mutations (10/23). The codon 12 mutations were the G12D (5/23), G12V (4/23), G12S (2/23), G12R (1/23) and G12C (1/23). BRAF V600E mutation was identified in one patient (50%). Mutations are summarized in Table 1 and the distribution of the mutations is shown in Figure 1. As described in previous studies, mutations in KRAS and BRAF were mutually exclusive.

Survival

With a median follow-up time of 28.3 months (4-74), 8 patients experienced a local recurrence (8%) and 17 developed distant metastasis (17%). No patient with local recurrence exhibited KRAS or PI3KCA mutation and one harbored BRAF mutation (12.5%). Among the seventeen patients (17%) with distant metastasis, 5 were harboring KRAS mutation (29%), one BRAF (5%) and one KRAS mutation (5%). Survival analyses compared results for patients with each mutation, patients with at least one of the 3 mutations versus those who had no mutations (Table 2 and 3).

No relationship was seen between PI3KCA, KRAS or BRAF mutation and local or distant recurrences and no longer with overall survival (all p< 0.01). The overall survival rate of the total patient group was 96% (95% CI [89- 99]).

Discussion

Despite the recent advances in the management of metastatic colorectal cancer (mCRC) over the last few years, this disease remains a major public health problem in the world (Jemal et al., 2011). Its prognosis has been improved, but recurrences remain a clinical challenge. Local recurrences are a critical issue in rectal cancer and
several tumor characteristics are associated with local recurrence, including depth of tumour invasion into and beyond the bowel wall, number of lymph nodes involved by tumor cells, extramural venous invasion, involvement of the circumferential resection margin (CRM) and the tumor location within 10 cm from anal verge. These factors may be used in therapeutic decisions regarding administration of (neo) adjuvant treatment (Dresen et al., 2009). However, patients with similar clinicopathologic characteristics still display a large variation in prognosis, suggesting that the biology of the tumor may be responsible for the difference. The detection of biological predictive markers may improve the selection of patients who will benefit from (neo) adjuvant treatment and spare patients who will only experience side effects. The MAPK pathway plays a major role in cell proliferation and is involved in up to 30% of CRC (Hoshino et al., 1999). Both KRAS and BRAF are the members of this signalling pathway and are known to be activated by oncogenic mutations. KRAS mutations, are reported in 19 to 48% of patients with rectal cancer (Bengala et al., 2010; He et al., 2009), whereas BRAF mutations are found in 0 to 12% of RC patients (Fransen et al., 2004; Gaedcke et al., 2010). The mutation frequency reported in our study (23% for KRAS and 2% for BRAF) are in concordance with those reported in literature. The rarity of V600E BRAF mutations in rectal cancer has been already described especially in the prior reports from Di Nicolantonio et al (Di et al., 2008) who found 1 V600E allele in 43 rectal samples and Fransen et al. (Fransen et al., 2004) who even found 2 mutations in 55 rectal cancers.

Little is known regarding the impact of PIK3CA mutations in rectal cancer. In our study, we identified a 4% incidence for PIK3CA mutations, which seems lower than the 10% to 30% reported in colon cancer. The first study in colorectal cancer reported that PIK3CA mutations were significantly associated with poor survival (Kato et al., 2007). They further identified PIK3CA mutations as the only independent and significant prognostic factor for relapse-free survival in stage II to III
patients. However, there were only 18 PIK3CA-mutated tumors and the number of deaths was not reported. Another study has shown that the presence of at least one mutation in PIK3CA, BRAF, or KRAS genes predicts poor survival in a population-based colon cancer samples, however, the effect of PIK3CA mutations on survival, independently of clinical and other molecular predictors of outcome, was not described (Barault et al., 2008). In a recent study, Ogino et al. examined the prognostic significance of PIK3CA mutations among 450 patients who had undergone a curative resection of colon cancer. They found that PIK3CA mutations were associated with a decreased survival. The “poor prognosis” effect of PIK3CA mutations seemed to be robust and consistent across most strata of clinical and tumoral predictors of patient outcome, but this effect seems to be limited to patients with KRAS wild-type tumors (Ogino et al., 2009b). A recent study determined the prognostic value of PIK3CA mutations in 240 non-irradiated resectable stages I to III rectal cancer patients from the Dutch TME trial. PIK3CA mutations were identified in 19 (7.9%) of the 240 patients and revealed a strong association with an increased local recurrence rate. Moreover, tumors with PIK3CA mutations showed a tendency to develop local recurrences more rapidly after surgery (He et al., 2009). In our study, no relation was found between KRAS, BRAF or PI3KCA mutations and local recurrence. A second analysis by He et al was published in 2010 concerning the results in irradiated patients from the total mesorectal excision (TME) trial. In this population, they investigated whether PIK3CA mutant patients benefit from preoperative radiotherapy. Although the difference was not statistically significant, it suggests that PIK3CA mutant patients seem to benefit from irradiation in preventing LR (He et al., 2010).

Several studies focused on RAS mutation and radioresistance, with controversial conclusions. Michelassi et al found that tumor downstaging was associated with KRAS wild type tumors (Michelassi et al., 1988) as well as Grana et al reported that KRAS mutations
potentially mediate resistance to ionizing radiation (Grana et al., 2002). Other studies reported no change in downstaging by *KRAS* status (Gaedcke et al., 2010; Bengala et al., 2009; Zauber et al., 2009) and even when adjusting the groups according to the codon which carried the mutation, failed to predict response to preoperative CT/RT. In a recent study, Davies showed that *KRAS* mutational status was not associated with radiosensitivity using more modern sequencing technology in a larger number of patients than previously described (Davies et al., 2011). Interestingly, they reported that activation of AKT and ERK is correlated with response to radiation therapy.

In summary, our retrospective study failed to confirm a significant correlation between *KRAS*, *BRAF* *PIK3CA* mutations as predictive factors of local or distant recurrence, for response to preoperative RT/CT or survival. The exact effects of *KRAS*, *BRAF* and *PIK3CA* mutations on survival require further study with analyses of a larger patient population because the number of relapse events was very small and may represent a sample bias. Finally, the follow-up period was probably too short to drawn definitive conclusions.