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ORIGINAL ARTICLE

Phase II trial of temsirolimus alone and in combination with irinotecan for *KRAS* mutant metastatic colorectal cancer: Outcome and results of *KRAS* mutational analysis in plasma

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Abstract

Background. Patients with chemotherapy refractory metastatic colorectal cancer and *KRAS* mutations have no effective treatment option. The present study evaluated the efficacy of temsirolimus in chemotherapy refractory mCRC with *KRAS* mutations. Furthermore, we wanted to investigate if resistance to temsirolimus could be reversed by the addition of irinotecan. Finally, we analyzed pre-treatment blood samples for *KRAS* mutations to investigate the association between quantitative measures of *KRAS* mutated alleles and clinical outcome. **Material and methods.** Patients received weekly temsirolimus 25 mg until progression. Thereafter patients were treated with combination therapy comprising biweekly irinotecan 180 mg/m² and weekly temsirolimus. A polymerase chain reaction method was used to quantify the *KRAS* mutated alleles in plasma (*pKRAS*). **Results.** Sixty-four patients were included. Treatment was well tolerated. Thirty-eight percent achieved stable disease on monotherapy and 63% on combination therapy. Four and eight patients had a minimal response, respectively. Median overall survival was 160 days. Median time to progression was 45 and 84 days, respectively. The concordance between *KRAS* status in tumor and plasma was 82%. All patients with tumor reduction had low levels of *pKRAS*. Patients with high *pKRAS* had a 77% risk of early progression on monotherapy compared to 43% in patients with lower levels. Multivariate survival analysis confirmed that *pKRAS* was a strong prognostic factor. **Conclusion.** Temsirolimus has limited efficacy in chemotherapy resistant *KRAS* mutant disease, but plasma *KRAS* quantification is a strong predictor of outcome.

A significant proportion of patients with chemotherapy resistant metastatic colorectal cancer (mCRC) are in good performance and third-line treatment is therefore relevant. Therapy with monoclonal antibodies (MoAbs) targeting the epidermal growth factor receptor (EGFR) is confined to patients with *KRAS* wild type tumors [1,2]. Consequently, patients with *KRAS* mutant disease who develop resistance to standard cytotoxic agents have no effective treatment options.

Temsirolimus is a novel inhibitor of mammalian target of rapamycin (mTOR), a central intracellular signaling molecule and a member of the phosphoinositide-kinase-related kinase family. It acts as a component of the phosphoinositide 3-kinase (PI3K)/

Akt signaling pathway that mediates eukaryotic cell growth and proliferation [3]. Preclinical studies have demonstrated that temsirolimus inhibits the proliferation of different malignant tumors, including colorectal cancer cell lines [4–6]. Enhanced antitumor activity has been reported when combined with different cytostatic drugs and recent preclinical data have demonstrated that the combination of rapamycin with irinotecan show potent inhibition of colorectal xenografted tumors [7]. Temsirolimus is presently approved for treatment of renal cell carcinomas and has a favorable toxicity profile [8,9]. A low-dose regimen has shown clinical activity in patients with mantle cell lymphoma [10] and a phase II study in heavily pre-treated metastatic breast cancer reported

an objective response rate of 9.2% [11]. mTOR inhibitor everolimus was evaluated in patients with different advanced tumors and clinical benefit was observed in 4/55 patients including one with mCRC [12]. Taken together the current data hold promise of activity alone or in combination with chemotherapy in patients with CRC, including *KRAS* mutant disease justifying clinical trials.

The identification of *KRAS* mutation as a marker for non-responsiveness to anti-EGFR MoAbs has underlined the need for translational research studies being conducted in parallel to clinical trials. We have developed a highly sensitive method for mutational analysis which enables us to quantify the amount of *KRAS* mutation alleles in plasma samples.

The present study aimed to evaluate the efficacy of temsirolimus in chemotherapy refractory mCRC with *KRAS* mutation. Furthermore, we wanted to investigate if resistance to temsirolimus could be reversed by the addition of irinotecan. Finally, we analyzed pre-treatment blood samples for *KRAS* mutations to investigate the association between quantitative measures of *KRAS* mutated alleles and clinical outcome.

Material and methods

Trial design

The study was conducted as two consecutive non-randomized phase II trials (TIRASMUS, ClinicalTrials.gov identifier; NCT00827684). Patients were treated with monotherapy temsirolimus until disease progression or unacceptable toxicity. Thereafter the patients received combination therapy, which continued until progression or unacceptable toxicity.

The primary endpoints were response to monotherapy and combination therapy according to RECIST. Secondary endpoints included PFS, OS, toxicity and evaluation of the predictive and prognostic value *KRAS* mutation alleles in plasma.

Written informed consent was obtained from each patient. The study followed the Declaration of Helsinki and good clinical practice and was approved by ethics committees and the Danish Medicines Agency.

Patients

Patients met the following criteria: age ≥ 18 and ≤ 70 , histologically confirmed metastatic colorectal adenocarcinoma with *KRAS* mutation and measurable disease as per RECIST. *KRAS* status was determined on archival tumor tissue. All patients had progressed on treatment with fluoropyrimidines, oxaliplatin and irinotecan. Adequate renal, hepatic and bone marrow

function was required, in addition to PS ≤ 2 , cholesterol and triglyceride within normal range.

Treatment schedules

Patients initially received weekly doses of temsirolimus 25 mg IV, followed by response evaluation every six weeks. Combination therapy comprised irinotecan 180 mg/m² IV every second week and weekly temsirolimus. In this step the initial dose of temsirolimus was 15 mg, and if tolerated increased to 25 mg. The initially low dose was chosen to avoid any unexpected toxicity from the combination regimen. Dose reduction to less than 15 mg per week was not allowed and further non-tolerance excluded the patients from therapy. A treatment pause for more than three weeks, regardless of reason also excluded the patient. Similarly, combination therapy was given until progression, unacceptable toxicity, treatment pause or patient's withdrawal of consent.

Evaluation criteria

Computed tomography (CT) scans of chest and abdomen were used for evaluation of response according to RECIST (version 1.0) and performed less than four weeks prior to first treatment given and every six weeks during treatment. Follow-up scans were performed every three months in patients who stopped treatment on stable disease and continued until first sign of progression.

Safety assessment

The NCI-CTCAE version 3.0 was used to assess toxicity and recorded at every visit for treatment until resolved. On combination therapy any dose reduction was applied to both drugs regardless of the type of toxicity.

Biomarker collection and analysis

Archived tumor tissue was obtained and blood samples for translational research drawn prior to therapy. *KRAS* tumor status was determined prior to treatment. Plasma samples were analyzed for *KRAS* mutations.

*DNA purification and *KRAS* mutational analysis in plasma*

DNA was purified from 1.0 ml EDTA-plasma samples using a QIA-symphony virus/bacteria midi-kit on a QIA-symphony robot (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions. Primers and probe for the *KRAS* assay were

developed using the OLIGO 7 software (Molecular Biology Insights Inc., Cascade, CO, USA). The assay utilize an Amplification Refractory Mutation System-Quantitative PCR methodology which detects six mutations in *KRAS* codon 12 (Gly12Ala, Gly12Arg, Gly12Asp, Gly12Cys, Gly12Ser, Gly12Val) and one in codon 13 (Gly13Asp).

A wild type blocking oligo was added to increase the specificity. The oligos were modified by including HyNA nucleotides (Pentabase Aps, Soendersoe, Denmark), which increased the melting temperature and blocked extension. An in-house *cyclophilin* (*gCYC*) qPCR was included as positive control and reference. Final primer mixtures are shown in Supplementary Table I (to be found online at <http://www.informahealthcare.com/doi/abs/10.3109/0284186X.2013.776175>). All qPCR reactions were performed in a volume of 25 µl (duplicates) on an ABI Prism7900HT using ABI Universal Mastermix with UNG. A mixture of patient DNA representing all seven *KRAS* mutations was included as positive controls as well as (negative) water controls and wild type donor DNA controls. The qPCR reaction conditions were: 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 60 s at 60°C.

Quantification of *KRAS* in plasma

From standard curves the slopes were calculated for the *KRAS* (3.6) primer sets. The Y-intercept (cycle threshold (Ct) value) corresponding to one DNA copy of the target DNA were set to 43. Quantification of *KRAS* was done by calculating the copy number of mutated *KRAS* alleles as $10^{((Y\text{-intercept}(KRAS) - \text{meanCt}(KRAS))/\text{slope}(KRAS))}$ and normalization to plasma volume.

Statistics

The study was conducted according to Simon's two-stage design. The initial planned sample size for the first stage was 15 patients. Due to the unknown response rate of mCRC patients to mTOR inhibition a stabilization of disease on monotherapy was considered relevant, whereas response was required for acceptance of the combination regimen. A success rate of 10% was deemed unacceptable and considered interesting if $\geq 30\%$. α was set to 0.05 and $\beta = 0.2$. Consequently, if one or more of the first 15 patients achieved stable disease on monotherapy, enrolment was extended by another 25 patients. Similar conditions were applied to combination therapy, but as no objective response was recorded according to RECIST in the first 15 evaluable patients, further enrolment in the second stage was suspended.

Since not all patients were expected to precede to combination therapy an estimated total sample size of 50 patients was set.

The association between marker status and objective response, baseline characteristics and toxicity

Table I. Patients characteristics.

| Parameter | Number (%) |
|--------------------------------------|-------------|
| Age | |
| median (range) | 62 (50–73*) |
| Gender | |
| Male | 36 (56) |
| Female | 28 (44) |
| ECOG PS at inclusion | |
| 0 | 24 (38) |
| 1 | 33 (52) |
| 2 | 5 (8) |
| ND | 2 (3) |
| Locus primary tumor | |
| Rectum | 30 (47) |
| Colon | 32 (50) |
| ND | 2 (3) |
| Stage at diagnosis | |
| II | 9 (14) |
| III | 12 (19) |
| IV | 36 (56) |
| ND | 7 (11) |
| No. of metastatic sites at inclusion | |
| 1 | 15 (23) |
| 2 | 16 (31) |
| 3 | 22 (34) |
| > 3 | 11 (15) |
| Previous surgery | |
| Yes | 49 (76) |
| No | 15 (24) |
| Previous RT | |
| Yes | 24 (38) |
| No | 39 (61) |
| ND | 1 (2) |
| Previous Anti EGFR | |
| Yes | 6 (9) |
| No | 56 (88) |
| ND | 2 (3) |
| Previous Anti angionenesis | |
| Yes | 56 (88) |
| No | 8 (12) |
| KRAS status primary tumor | |
| 12Ala | 5 (8) |
| 12Arg | 2 (3) |
| 12Asp | 17 (27) |
| 12Cys | 6 (10) |
| 12Ser | 4 (6) |
| 12Val | 15 (24) |
| 13Asp | 10 (16) |
| Not specified | 4 (6) |

N=64 patients included in TIRASMUS.

PS, performance status; ND, not determined; RT, radiotherapy; EGFR, epidermal growth factor receptor.

*Three patients over the age of 70 were included after sponsors acceptance. There was no significant difference in pre-treatment characteristics between the groups of patients who continued onto combination therapy and those who did not proceed to the second part of the study.

rates was determined by two-sided t-tests or χ^2 -test. The Kaplan-Meier method was used to estimate time to progression (TTP) and overall survival (OS). A multivariate Cox regression analysis was performed to examine whether the different variables were associated with reduced survival. P-values referred to two-tailed tests and were considered significant when $p \leq 0.05$. Statistics were carried out using the NCSS statistical software 2007 v.07.1.5 (NCSS Statistical Software, Utah 84037, USA, www.ncss.com).

Results

Patient characteristics

Sixty-four patients were included in two Danish oncology centers. All patients had progressed on fluoropyrimidines, oxaliplatin and irinotecan and 86% had received prior anti-angiogenic therapy. Patient characteristics are listed in Table I. Two patients experienced an anaphylactic reaction at the first infusion and one patient died before commencing treatment, leaving 61 patients having at least one cycle of monotherapy, and a total of 35 patients proceeding to the combination regimen. The reasons for discontinuation of treatment were toxicity (two), patient's wish (five), postponed treatment (two), progression (41) and death (three), other (seven, including one myocardial infarct, and one discovered neuro-endocrine component). At time of this report 52 patients were dead and 12 still alive, including three still on treatment. The cause of death was recorded as mCRC in all deceased patients.

Toxicity

The serious adverse events are presented in Table II. There were no grade 4 events, but two patients discontinued treatment after anaphylactic reaction and three had a mild allergic reaction to temsirolimus. The majority of grade 2–3 GI-related toxicity occurred during combination therapy as expected. Grade 2–3 hematological toxicity from monotherapy was primarily reversible thrombocytopenia, and grade 3 leukopenia occurred only during the combination treatment.

Efficacy monotherapy

The median number of cycles was 3 (range 0–17). None of the patients achieved an objective response according to RECIST but 38% (24/64) of patients achieved SD on monotherapy, when analyzing the overall best responses. Of note, the results showed that four patients achieved a minor response (defined more than 5% tumor shrinkage) including two with reduction $\geq 20\%$ as illustrated in Figure 1A.

Efficacy combination therapy

The median number of cycles was three (range 0–19). Sixty-three percent (22/35) of patients who commenced combination therapy obtained SD, including eight with a minor response, (three with reduction $\geq 10\%$ and three $\geq 20\%$). The maximum changes in target lesions are illustrated in Figure 1B. Three of the patients who achieved tumor reduction

Table II. Reported toxicities possibly or definitely related to treatment in TIRASMUS.

| Adverse events | Grade 1 No. (%) | Grade 2 No. (%) | Grade 3 No. (%) | Grade 4 No. (%) |
|-----------------------------|--------------------|--------------------|--------------------|--------------------|
| Skin toxicity | 26 (40) | 3 (5) | 2 (3) | 0 |
| Epistaxis | 25 (39) | 1 (2) | 0 | 0 |
| Stomatitis | 27 (42) | 6 (9) | 1 (2) | 0 |
| Nausea | 19 (30) | 6 (9) | 2 (3) | 0 |
| Anorexia | 34 (53) | 6 (2) | 0 | 0 |
| Asthenia | 35 (55) | 15 (23) | 1 (2) | 0 |
| Fever | 12 (19) | 6 (9) | 5 (8) | 0 |
| Diarrhea | 24 (38) | 5 (8) | 3 (5) | 0 |
| Edema | 13 (20) | 9 (14) | 0 | 0 |
| Conjunctivitis | 9 (14) | 0 | 0 | 0 |
| Infection | 13 (20) | 8 (13) | 7 (11) | 0 |
| Neutropenia | 2 (3) | 4 (6) | 2 (3) | 0 |
| Leukopenia | 2 (3) | 3 (5) | 3 (5) | 0 |
| Trombocytopenia | 16 (25) | 1 (2) | 5 (8) | 0 |
| Hyperlipidemia | 29 (45) | 6 (9) | 4 (6) | 0 |
| Hyperglycemia | 9 (14) | 6 (9) | 5 (8) | 0 |
| Pneumonitis | 7 (11) | 2 (3) | 4 (6) | 0 |
| Wound healing complications | 4 (6) | 1 (2) | 0 | 0 |
| Arthralgia | 1 (13) | 0 | 0 | 0 |

*Two patients had to discontinue treatment after allergic reaction, and additional four patients experienced a mild allergic reaction to temsirolimus.

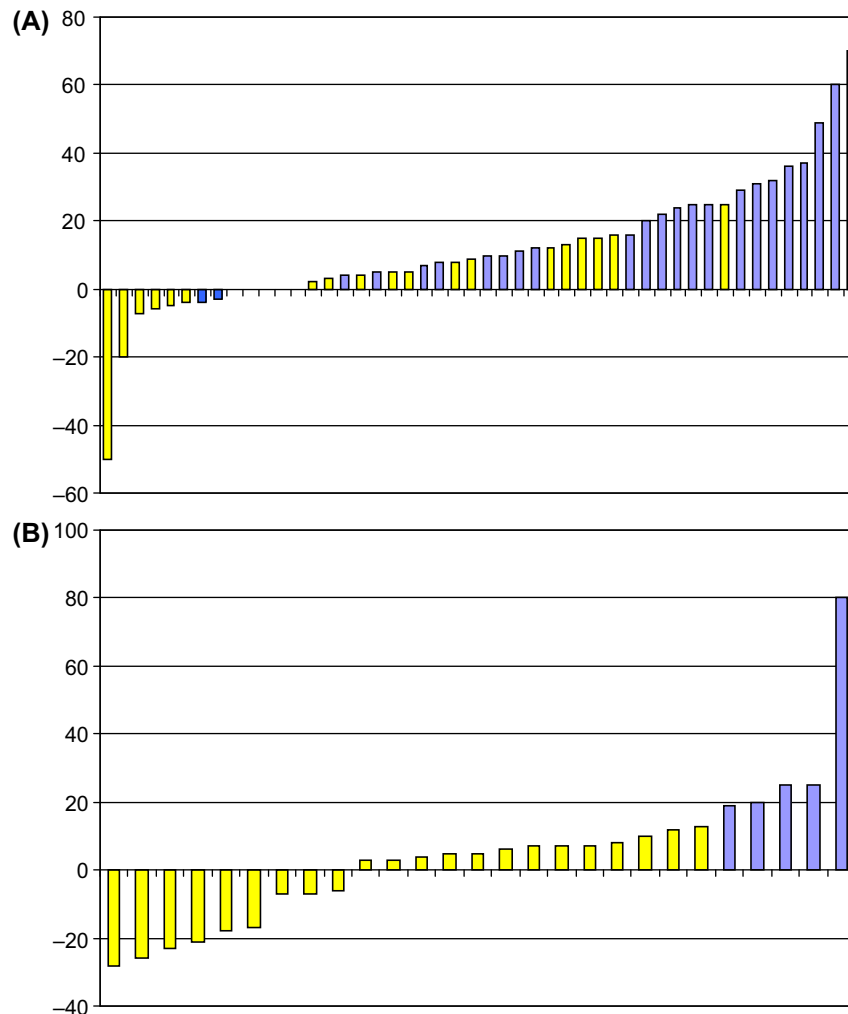


Figure 1. Waterfall plot of changes in target lesions during monotherapy (A) and combination treatment (B). The plot illustrates the best response by changes in target lesions (only) from baseline presented in percentages on the Y-axis. Each patient is represented on the X-axis. Forty-eight patients were eligible for tumor evaluation during monotherapy, whereas 27 patients had evaluation scans performed during combination treatment. The colours illustrate the best response according to RECIST evaluation. Yellow represents the patients with SD during treatment and blue the patients who progressed.

during combination therapy had also shown reduction from monotherapy.

Progression and survival data

The median TTP of monotherapy and combination therapy was 45 days (95% CI 41–67) and 84 days (95% CI 48–120), respectively. The median OS was 160 days in the total cohort (95% CI 103–232).

Biomarker study

Fifty-five patients had available plasma and tumor DNA for comparison. The remaining nine included seven patients who had no baseline sample available, and two which revealed inconclusive results (Supplementary Figure 1, to be found online at <http://informahealthcare.com/doi/abs/10.3109/0284186X.2013.776175>).

The overall concordance between tumor and plasma analysis was 82%.

A high level of pKRAS was associated with early progression (Table III). In patients with a high concentration of baseline pKRAS (above the 75% quartile) only 23% had temporary disease control, and 77% of the patient in this group progressed at time of first tumor evaluation compared to 43% of patients with lower levels ($p = 0.036$). All patients who achieved tumor reduction had low levels of baseline pKRAS.

The median overall survival was significantly longer in patients with pKRAS values below the median (Figure 2). The median survival in patients with levels below the median was 280 days (95% CI 199–406) compared to 99 days (95% CI 71–158) in those with higher levels, HR = 2.3 (95% CI 1.27–4.34, $p = 0.0032$). The overall survival according to the

Table III. Correlation between disease control and plasma *KRAS* levels.

| Best response monotherapy | Quantitative levels of <i>KRAS</i> mutations in plasma | | | | |
|------------------------------|--|---------|----------|----------|----------|
| | Total | < 25% Q | 25–50% Q | 50–70% Q | > 75% Q* |
| DC | 24 | 7 (58%) | 7 (54%) | 7 (58%) | 3 (23%) |
| PD | 26 | 5 (42%) | 6 (46%) | 5 (42%) | 10 (77%) |
| Total | 50 | 12 | 13 | 12 | 13 |

p = 0.22

The sample size was 50 patients (7 patients did not have available plasma samples, 5 were non-evaluable for response, and 2 were excluded, Figure 3), PD progressive disease, DC disease control, Q quartile. Quartiles were used for grouping of the patients and analyzed by χ^2 -test.

*When dichotomizing by the 75% quartile a significantly higher rate of DC was revealed in the patients with level below the 75% ($p = 0.036$) see text. The numbers were too small to do a similar analysis of the combination regime. All patients with tumor reduction had levels below the median.

quartiles of *pKRAS* is illustrated in Figure 2, which shows that patients in the highest quartile had a significantly worse prognosis than those in the lower quartiles.

A Cox regression multivariate survival analysis including PS at inclusion, number of metastatic sites, and *pKRAS* confirmed the independent prognostic value of *pKRAS* in these patients.

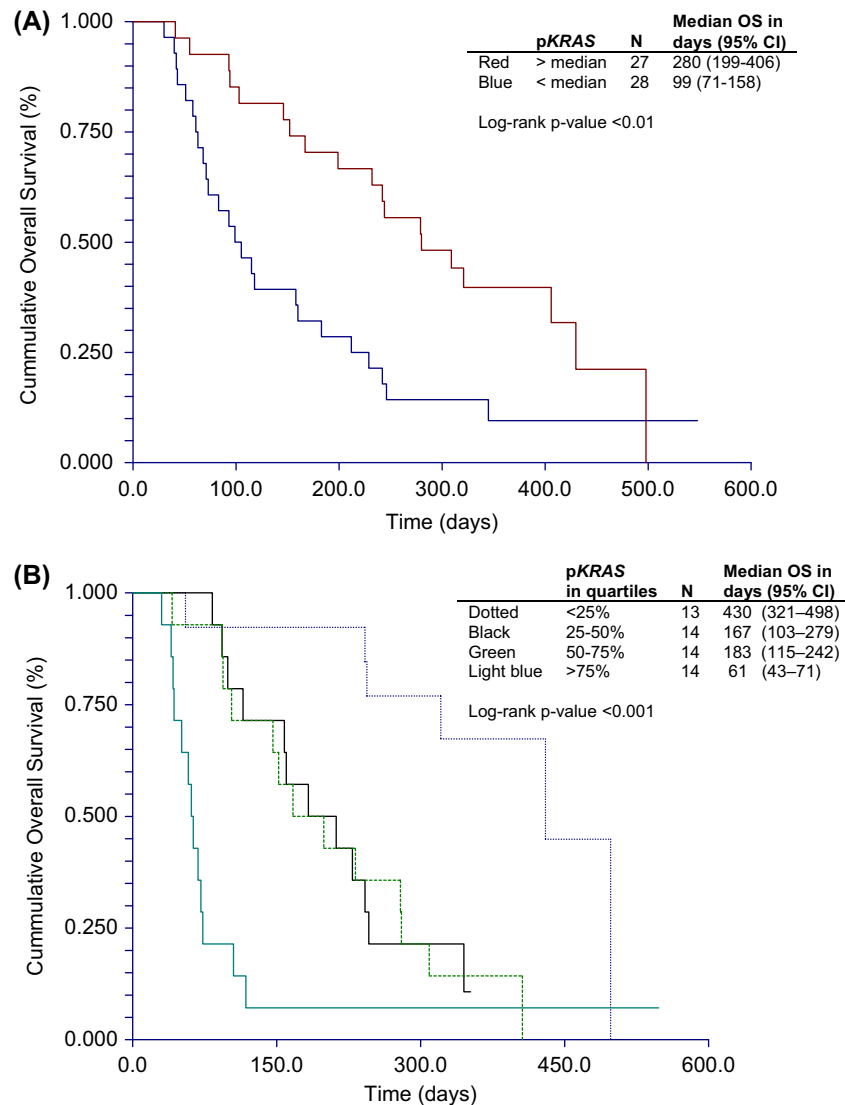


Figure 2. Overall survival and patients divided into groups with *pKRAS* above and below the median level (A) and by *pKRAS* in Quartiles (B).

Discussion

In this phase II study we were unable to demonstrate a clinically meaningful anti-tumor activity of temsirolimus alone. No objective responses according to RECIST were seen, but we did observe stabilization of disease in a proportion of the patients during both treatment regimens (38% and 63%) and tumor reduction in 7% and 23% of patients treated.

It is possible that the minimal responses observed were indicators of potential clinical effect and that therapy was given in insufficient doses. We chose the initial 15 mg and then increased it to 25 mg dose to avoid unexpected severe toxicity during the combination regimen, (which is of high priority in this specific group of incurable patients to whom quality of life is very important). Thus, for safety reasons a low dose approach was chosen and appeared feasible. Data have suggested that, e.g. breast cancer patients seem to benefit from a higher dose [13], and it is also possible that the low-dose regimen used here may be adequate in other tumor types, but not in adenocarcinomas. However, an established dose escalation study was not the aim of this investigation.

The TTP was longer and the proportion of patients with tumor reduction was higher during combination therapy compared to single agent therapy. It is worth noticing that these heavily pre-treated patients after progression on temsirolimus alone achieved a median TTP of nearly three months on the combination regimen. This could indicate a potential effect and it is possible that with proper selection of patients a subgroup of patients would benefit from treatment.

The present study included patients with chemotherapy refractory *KRAS* mutant diseases, which clearly constitutes a major therapeutic challenge. The expected time to progression and clinical deterioration is unfortunately short, and identification of reliable criteria for selection of patients with a good chance of benefit is therefore of major importance.

It is possible that improvement of outcome could be achieved by selection based on mutational status. Nicolantonio et al., demonstrated that cell lines with *PIK3CA* alterations are more sensitive to mTOR inhibition in contrast to *KRAS* mutations, which interestingly seemed to be associated with resistance [14], but another study presented two cases of ovarian cancer with *RAS/RAF* mutations who responded to mTOR inhibition. Further data suggested that mTOR inhibition may reverse colorectal cancer cell lines resistant to EGFR inhibitors [15,16]. Translational research studies of patients treated with mTOR inhibitors are therefore highly relevant.

Many aspects of *KRAS* mutant colorectal cancer need further investigations, including methodological issues, tumor heterogeneity, and different mutational status in primary and metastatic disease [17–19]. Furthermore, results have indicated that clinical outcome could be influenced by the different types of *KRAS* mutations [20]. The present study allowed us to demonstrate that *KRAS* analysis can be performed in the peripheral blood and we confirmed a high concordance between *KRAS* status in the primary tumor and plasma [21,22]. More importantly, we were able to quantify the amount of *KRAS* mutation alleles in plasma and revealed impaired outcome with increasing concentrations. To our best knowledge there is no available literature suitable for comparison, but we have recently reported a similar correlation in patients treated with cetuximab and irinotecan [23]. Patients with a high level of *pKRAS* had a high risk of early progression and poor survival and may therefore not be candidates for third line treatment. We suggest that not the *KRAS* status itself, but rather the quantitative amount of *KRAS* mutational alleles is associated with aggressive disease behavior and could be used for tailoring treatment in mCRC. The obvious limitations of a small sample size and non-randomized study does not allow for a distinction between a potential predictive or prognostic value. Consequently, verification in larger samples sizes and randomized trials are needed to establish the true clinical impact of *pKRAS* as a marker.

In conclusion, temsirolimus alone or in combination with irinotecan showed limited efficacy in patients with *KRAS* mutant chemotherapy resistant mCRC. This study did not, however, investigate the potential efficacy in higher doses or less advanced disease with combination regimens. The measurement of *KRAS* mutations in the plasma is a relevant alternative to tissue analysis, and baseline quantification may potentially help to identify patients with a reasonable chance of benefit from third line treatment.

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Supplementary material available online

Supplementary Table I and Figure 1.