

Aus dem Pathologischem Institut der
Ludwig-Maximilians-Universität
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Häufigkeit und klinische Relevanz von *KRAS* Mutationen im metastasierten Dickdarmkarzinom

Kumulative Dissertation

zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von

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aus

Essen

2012

Mit Genehmigung der Medizinischen Fakultät der Universität München

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Tag der mündlichen Prüfung:

25.10.2012

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1. Einleitung:

1.1. Das kolorektale Karzinom

In den westlichen Industrieländern zählt das kolorektale Karzinom (KRK) immer noch zu den häufigsten zum Tode führenden Tumorerkrankungen. In der Bundesrepublik Deutschland liegt die Inzidenz bei etwa 50000 Erkrankungsfällen pro Jahr. Somit ist das KRK der zweithäufigste maligne Tumor nach dem Mammakarzinom bei der Frau und der dritthäufigste nach dem Prostatakarzinom und dem Bronchialkarzinom beim Mann. (Siegel et al., 2012) Zum Zeitpunkt der Erstdiagnose liegen bei 20-25% der KRK-Patienten bereits Fernmetastasen vor. Weitere 50-60% entwickeln im späteren Verlauf Metastasen, was für die relativ hohe Gesamtmortalität des KRK verantwortlich ist. (La Vecchia et al. 2010)

1.2. Die Adenom-Karzinom-Sequenz

Über 90% aller KRK entwickeln sich auf dem Boden vorbestehender Adenome. Dabei zeigt sich ein kontinuierlicher Übergang einer hyperproliferativen Schleimhaut zum frühen Adenom, intermediären Adenom, späten Adenom bis hin zum Karzinom. Diese sogenannte Adenom-Karzinom-Sequenz konnte in den letzten Jahren auf molekularer Ebene gut charakterisiert werden (Abb. 1). Insbesondere die Arbeiten von Bert Vogelstein und Eric Fearon führten zu dem heute als Konsensusmodell geltenden Mehrstufen Modell der Karzinogenese im Dickdarm (*multistep carcinogenesis model*). (Fearon and Vogelstein, 1990) Dabei kommt es in einem sehr frühen Stadium der Erkrankung zu einer inaktivierenden Mutation im APC-Tumorsuppressorgen (*adenomatous polyposis coli*), was zu einer vermehrten Proliferation des Kolonepithels und zur Ausbildung gutartiger Polypen (frühes Adenom) führt. In einem nächsten Schritt kommt es zu einer aktivierenden Mutation im KRAS-Proto-Onkogen, die eine Störung der Differenzierung nach sich zieht (mittleres Adenom). Noch später folgen Mutationen von Elementen des TGF- β (*transforming growthfactor- β*) Signalwegs, wie SMAD2 oder SMAD 4 (*small mothers against decapentaplegic*) und TP53 (*tumor suppressor protein p53*) (spätes Adenom bzw. Karzinom; Abb. 1).

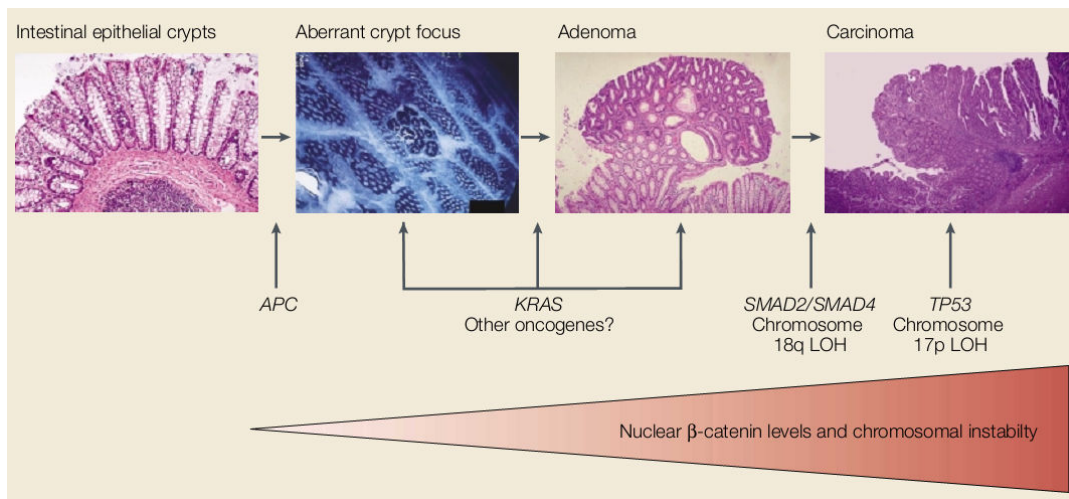


Abb. 1: Schema der Adenom-Karzinom Sequenz kolorektaler Karzinome. Die Karzinogenese kolorektaler Karzinome wird durch die Akkumulation von Mutationen in Tumorsuppressorgenen getrieben (*APC*- adenomatous polyposis coli, *SMAD2/SMAD4* – small mothers against decapentaplegic, *TP53* – tumorsuppressor-protein p53) und Onkogenen (*KRAS* – Kirsten rat sarcoma), die mit distinkten morphologischen Veränderungen einhergeht. Diese führen vom normalen Epithel zu Schleimhauthyperplasien (*aberrant crypt focus*) und weiter über die Entstehung von benignen adenomatösen Polypen mit Dysplasien verschiedener Grade zu nicht invasiven, später invasiven Karzinomen und schließlich zu Metastasen. Dabei ist die Reihenfolge der Mutationen nicht genau festgelegt. (Aus Fodde et al. Nature Reviews Cancer 2001)

1.3. Der EGFR-Signalweg

Der EGF-Rezeptor (EGFR, *epidermal growth factor receptor*) ist ein Transmembranprotein, das zu der Familie der Tyrosinkinase-Wachstumsfaktor-Rezeptoren gehört. Die EGFR-abhängige Signaltransduktion reguliert Prozesse wie Proliferation, Migration, Invasion, Angiogenese und Apoptose. (Ciardiello et al., 2008) Das Ras-Protein spielt in der Signalkaskade des EGFR eine wichtige Rolle (Abb. 2). Durch Mutationen in den Codonen 12 oder 13 des *KRAS*-Proto-Onkogens kommt es zu einer Daueraktivierung des ansonsten inaktiven *KRAS*-Proteins. Bei Patienten mit einem nicht mutierten *KRAS*-Gen (*KRAS*-Wild-Typ) lassen sich oft Mutationen in anderen Genen des EGFR-Signalweges nachweisen. So zeigen bis zu 14% der Patienten mit *KRAS*-Wild-Typ eine Mutation im *BRAF* Gen, einem dem *KRAS* nachgeschaltetes Signalmolekül. (Di Nicolantonio et al., 2008)

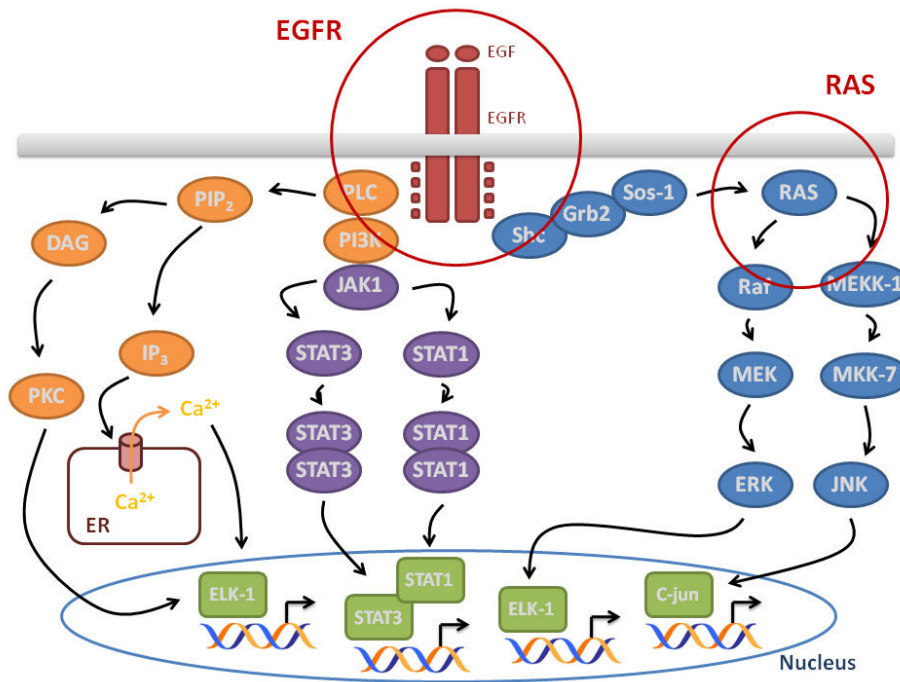


Abb. 2: Der EGF-Rezeptor Signalweg. Durch die extrazelluläre Bindung eines Liganden (*Epidermal Growth Factor*) kommt es zur Dimerisierung und Autophosphorylierung des EGF-Rezeptors. Die Signale werden über intrazelluläre Signalmoleküle in den Zellkern fortgeleitet, wodurch Transkriptionsfaktoren aktiviert werden die das Zellwachstum stimulieren und Apoptose verhindern. Das ubiquitär vorkommende RAS-Protein übernimmt in der Signaltransduktion eine wichtige Rolle.

1.4. Adjuvante therapeutische Konzepte beim kolorektalen Karzinom

In den letzten Jahren konnte das Gesamtüberleben von Patienten mit metastasiertem KKR durch die Verfügbarkeit neuer Substanzen und die Kombination mehrerer Chemotherapeutika (vor allem Fluoropyrimidine in Kombination mit Irinotecan oder Oxaliplatin) in der adjuvanten Therapie deutlich verbessert werden. Zusätzlich haben molekulare zielgerichtete Therapien durch die Entwicklung monoklonaler Antikörper gegen den EGFR oder auch den vaskulären endothelialen Wachstumsfaktor (VEGF) erheblich an Bedeutung gewonnen. (You et al., 2011)

Die beiden gegen den EGFR gerichteten Antikörper Cetuximab (Erbitux[®]) und Panitumumab (Vectibix[®]) haben in zahlreichen Studien ihre klinische Effektivität beim metastasierten KKR bewiesen. (Stintzing et al., 2009; De Roock et al., 2011) Zahlreiche Studien konnten zeigen, dass eine Mutation des *KRAS*-Gens mit einer Resistenz des Tumors gegen EGFR-Inhibitoren assoziiert ist. Dies ist auf die

intrinsische Aktivierung der EGFR-abhängigen Signaltransduktionskaskade infolge einer *KRAS*-Mutation zurückzuführen. Diese Aktivierung ist unabhängig von der EGFR-Expression und kann durch Medikamente, welche am EGFR selbst wirken, nicht gehemmt werden. (Adelstein et al., 2011) Aus diesem Grund wurden die beiden anti-EGFR-Antikörper von der europäischen Zulassungsbehörde (European Medicines Agency, EMA) nur für die Anwendung bei KRK-Patienten mit *KRAS*-Wild-Typ zugelassen. Die von der EMA geforderte Analyse des *KRAS*-Mutationsstatus der Patienten, die für eine anti-EGFR-Therapie in Frage kommen, kann an archiviertem formalinfixiertem Paraffinmaterial erfolgen. Dazu ist Gewebe sowohl vom Primärtumor als auch von Metastasen geeignet. (Dietel et al., 2008)

2. Publikationen:

1.) *Pathology – Research and Practice* 205 (2009):858–862

Frequency and Type of KRAS Mutations in Routine Diagnostic Analysis of Metastasized Colorectal Cancer

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2.) *Anti-Cancer Drugs*, 2011 Oct; 22(9):913-8

Clinical characterization of patients with metastatic colorectal cancer (mCRC) depending on the KRAS-status

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3. Zusammenfassung

3.1. Deutsche Zusammenfassung:

Einleitung:

Seit gezeigt werden konnte, dass bei Patienten mit metastasiertem kolorektalem Karzinom (mKRK) eine Mutation im *KRAS*-Gen mit einem fehlendem Therapieansprechen bei einer Behandlung mit den gegen den Epidermalen Wachstumsfaktor-Rezeptor (EGFR) gerichteten monoklonalen Antikörpern Cetuximab (Erbix®) und Panitumumab (Vectibix®) vorliegt, werden mKRK-Patienten im großen Maßstab auf das Vorliegen einer *KRAS*-Genmutation untersucht. Nichtsdestotrotz fehlen bislang verlässliche Standardwerte für die Häufigkeit und die Art der auftretenden Mutations-Typen in dieser Patientengruppe. Zusätzlich mehren sich in den letzten Jahren Hinweise, dass Patienten mit einer Mutation im Codon 13 des *KRAS*-Gens sowohl klinisch als auch pathologisch eine eigene Gruppe darstellen. Daher war es das Ziel dieser Arbeit ein großes und homogenes Kollektiv von Patienten mit mKRK zu untersuchen und die Ergebnisse der *KRAS*-Mutationsanalyse mit pathologischen und klinischen Parametern zu korrelieren.

Material und Methoden:

Ein populationsbasiertes Patientenkollektiv mit 1018 mKRK-Patienten (davon 879 Primärtumore und 139 Metastasen) wurde unter Anwendung entweder der Didesoxy- oder der Pyro-Sequenzierungs-Technologie hinsichtlich des Vorhandenseins einer *KRAS*-Mutation untersucht. Zusätzlich wurde ein Kollektiv mit 273 mKRK-Patienten, die im Rahmen zweier klinischer Studien (FIRE-3- und AIO KRK-0104-Studie) Cetuximab als Erstlinientherapie erhalten hatten, analysiert.

Ergebnisse:

Im populationsbasierten Kollektiv zeigten 39,3% der mKRK-Patienten eine Mutation im Codon 12 oder 13 des *KRAS*-Gens. Die häufigste Mutation war die Glyzin/Aspartat-Substitution im Codon 12 (p.G12D, 36,0%), die Glyzin/Valin-Substitution im Codon 12 (p.G12V, 21,8%) und die Glyzin/Aspartat-Substitution im

Codon 13 (p.G13D, 18,8%). Diese drei Mutationen machten alleine 76,6% aller Mutationen aus und konnten sowohl in den Primärtumoren als auch in den Metastasen in gleicher Frequenz nachgewiesen werden. Die Korrelation des Mutationsstatus des klinischen Kollektivs zeigte einen signifikanten Unterschied zwischen *KRAS*-Wild-Typ, Codon 12-mutierten und Codon 13-mutierten Tumoren hinsichtlich einer synchronen Lymphknotenmetastasierung ($p=0,018$), Vorhandensein von Organ-Metastasen ($p=0,009$), einer Lebermetastasierung ($p=0,025$), einer Metastasierung in die Lunge ($p=0,041$), singulären Lebermetastasen ($p=0,006$) sowie einer Metastasierung in zwei oder mehr Organsysteme ($p=0,047$). Regressionsberechnungen zeigten einen signifikanten Unterschied zwischen Mutationen im Codon 12 und Codon 13 des *KRAS*-Gens bezüglich einer synchronen Fernmetastasierung ($p=0,01$), einer synchronen Lymphknotenmetastasierung ($p=0,03$) und einen statistischen Trend bezüglich dem Auftreten von Lebermetastasen ($p=0,10$).

Schlussfolgerungen:

Die Frequenz von *KRAS*-Mutationen und die Prädominanz von drei Typen von Mutationen im Codon 12 und 13 in unserem großen und unselektionierten mKRK-Kollektiv bestätigt die bereits publizierten Daten aus kleinen und vorselektionierten Studien in der Literatur. Zusammenfassend kann eine Mutationsfrequenz von 40% und ein Cluster von drei Mutationstypen (p.G12D, p.G12V und p.G13D) als Referenzwert für die *KRAS*-Mutationsanalyse in der Routinediagnostik von Primärtumoren und Metastasen zugrundegelegt werden. Zusätzlich zeigen die Ergebnisse der klinischen Studie, dass mKRK-Patienten in Abhängigkeit vom *KRAS*-Status eine heterogene Gruppe darstellen. Im Vergleich zu *KRAS* Codon 12 Mutationen stellen mKRK mit einer Codon 13 Mutation ein klinisch aggressiveres Krankheitsbild dar, das durch ein vermehrtes Auftreten von loko-regionären Metastasen und Fernmetastasen zum Zeitpunkt der Erstdiagnose charakterisiert ist.

3.2. Englische Zusammenfassung:

Introduction:

Since it could be demonstrated that in metastatic colorectal cancer (mCRC) *KRAS*-mutations are associated with clinical resistance to treatment with monoclonal antibodies targeting the epidermal growth factor receptor (EGFR) such as cetuximab (Erbix[®]) and panitumumab (Vectibix[®]), mCRC patients are widely analyzed for their mutational status of *KRAS*. However, a reliable benchmark for the frequency and types of *KRAS* mutations for the implementation and quality assurance of *KRAS* mutation analysis in routine diagnostics is still missing. Furthermore, within the last years evidence rose that mutations in codon 13 of the *KRAS* gene were associated with different clinical and pathological characteristics. Therefore it was our aim to analyze a large and homogeneous collection of mCRC patients and to correlate the results of the mutational status of *KRAS* with pathological and clinical parameters.

Material and Methods:

A population based collection of mCRC including 1018 cases (879 primary tumors, 139 metastases) were analyzed for *KRAS* mutational status of codon 12 and 13 utilizing either dideoxy- or Pyro-sequencing technology. Furthermore a clinical collection of 273 patients with mCRC receiving Cetuximab as first-line therapy within the FIRE-3 and the AIO KRK-0104 clinical trial were analyzed, respectively.

Results:

In the population based collection of mCRC patients *KRAS* mutations in codon 12 and 13 were present in 39.3%. The most frequent types of mutations were glycine to aspartate on codon 12 (p.G12D, 36.0%), glycine to valine on codon 12 (pG12V, 21.8%) and glycine to aspartate on codon 13 (p.G13D, 18.8%). They account for 76.6% of all mutations, and prevailed in primary tumors as well as distant metastases. The correlation of the mutational status of the clinical collection revealed a significant difference between *KRAS* wild-type, codon 12-mutated and codon 13-mutated tumors with synchronous lymph node metastasis (p=0.018), organ-metastasis (p=0.009), liver-metastasis (p=0.025), lung-metastasis (p=0.041), liver-only-metastasis (p=0.006), and metastatic involvement of two or more organs (p=0.047). Regression models indicate a significant impact of *KRAS* mutation in

codon 12 vs. codon 13 for synchronous organ metastasis ($p=0.01$), synchronous nodal metastasis ($p=0.03$), and a trend for liver metastasis ($p=0.10$).

Conclusions:

The frequency of *KRAS* mutations and the preponderance of three types of mutations in codon 12 and 13 in our large and unselected cohort of mCRC confirm the previous data of small and selected mCRC samples published in the literature. Thus a mutation frequency of 40% and a cluster of three mutation types (p.G12D, p.G12V and p.G13D) in primaries and metastases can be defined as benchmarks for routine *KRAS*-analyses. In addition, the results of the clinical study indicate that mCRC is a heterogenous disease which appears to be defined by *KRAS* mutations of the tumor. Compared to *KRAS* codon 12-mutation, codon 13-mutated mCRC presents as a more aggressive disease frequently associated with local and distant metastasis at first diagnosis.

4. Referenzen:

Adelstein BA, Dobbins TA, Harris CA, Marschner IC, Ward RL. (2011). A systematic review and meta-analysis of KRAS status as the determinant of response to anti-EGFR antibodies and the impact of partner chemotherapy in metastatic colorectal cancer. *Eur J Cancer* 47(9):1343-54.

Ciardello F, Tortora G. EGFR antagonists in cancer treatment. (2008). *N Engl J Med* 358(11):1160-74.

De Roock W, De Vriendt V, Normanno N, Ciardiello F, Tejpar S. (2011). KRAS, BRAF, PIK3CA, and PTEN mutations: implications for targeted therapies in metastatic colorectal cancer. *Lancet Oncol* 12(6):594-603

Dietel M, Tannapfel A, Baretton G, Kreipe H, Kloor M, Gabbert H, Kirchner T. (2009). Molecular pathologic KRAS mutation analysis. A prerequisite of effective antibody treatment for metastasized colorectal cancer. *Chirurg* 79(6):576-9.

Di Nicolantonio, F, Martini, M, Molinari, F, Sartore-Bianchi, A, Arena, S, Saletti, P, De Dosso, S, Mazzucchelli, L, Frattini, M, Siena, S, and Bardelli, A. (2008). Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol* 26, 5705-5712.

Fearon, ER, and Vogelstein, B (1990). A genetic model for colorectal tumorigenesis. *Cell* 61, 759-767.

Fodde R, Smits R, Clevers H. (2001). APC, signal transduction and genetic instability in colorectal cancer. *Nat Rev Cancer* 1(1):55-67.

La Vecchia C, Bosetti C, Lucchini F, Bertuccio P, Negri E, Boyle P, Levi F. (2010). Cancer mortality in Europe, 2000-2004, and an overview of trends since 1975. *Ann Oncol* 21(6):1323-60.

Siegel R, Naishadham D, Jemal A. (2012). Cancer statistics, 2012. *CA Cancer J Clin* 62(1):10-29.

Stintzing S, Heinemann V, Moosmann N, Hiddemann W, Jung A, Kirchner T. (2009). The treatment of colorectal carcinoma with monoclonal antibodies: the importance of KRAS mutation analysis and EGFR status. *Dtsch Arztebl Int* 106(12):202-6.

You B, Chen EX. (2012). Anti-EGFR Monoclonal Antibodies for Treatment of Colorectal Cancers: Development of Cetuximab and Panitumumab. *J Clin Pharmacol* 52 (2):128-155

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ORIGINAL ARTICLE

Frequency and type of *KRAS* mutations in routine diagnostic analysis of metastatic colorectal cancer

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ARTICLE INFO

Article history:

Received 18 June 2009

Accepted 9 July 2009

Keywords:

Colorectal cancer

KRAS

Mutation analysis

Routine diagnostic

Molecular pathology

ABSTRACT

Mutation analysis of the *KRAS* oncogene is now established as a predictive biomarker in colorectal cancer (CRC). Large prospective clinical trials have shown that only CRCs with wild-type *KRAS* respond to anti-epidermal growth factor receptor (EGFR) treatment. Therefore, mutation analysis is mandatory before treatment, and reliable benchmarks for the frequency and types of *KRAS* mutations have to be established for routinely testing large numbers of metastatic CRCs.

A thousand and eighteen cases (879 primary tumors and 139 metastases) of metastatic colorectal cancer were analyzed for the *KRAS* mutational status of codons 12 and 13 of the *KRAS* gene by genomic sequencing in a routine setting. Results were analyzed separately for specimens derived from primary tumors and metastases.

KRAS mutations in codons 12 and 13 were present in 39.3% of all analyzed CRCs. The most frequent types of mutations were glycine to aspartate on codon 12 (p.G12D, 36.0%), glycine to valine on codon 12 (p.G12V, 21.8%), and glycine to aspartate on codon 13 (p.G13D, 18.8%). They account for 76.6% of all mutations and prevail in primary tumors and distant metastases, indicating a robustness of the *KRAS* mutational status during neoplastic dissemination.

The frequency of *KRAS* mutations and the preponderance of three types of mutations in codons 12 and 13 in a large, unselected cohort of metastatic CRC confirm the previous data of small and selected CRC samples. Thus, a mutation frequency of 40% and a cluster of three mutation types (p.G12D, p.G12V, and p.G13D) in primaries and metastases can be defined as benchmarks for routine *KRAS* analyses.

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Introduction

Anti-EGFR-targeted therapies with monoclonal antibodies such as cetuximab (Erbix[®]) or panitumumab (Vectibix[®]) are a successful strategy for the treatment of metastatic CRC in addition to or after failure of conventional chemotherapy. However, the EGFR-targeted therapies accomplish the partial response or stabilization of disease only in a subgroup of patients [1,10,11,13,15,23,25,27]. Prospective clinical trials have clearly shown that only CRCs with wild-type *KRAS* proto-oncogene, a small G-protein downstream in the EGFR signaling cascade, respond to anti-EGFR treatment, whereas no response is observed in CRC with *KRAS* mutation. Consequently, the European Medicines Agency (EMA) approved panitumumab (Vectibix[®]) and cetuximab (Erbix[®]) only for the treatment of metastatic CRC with intratumorous wild-type *KRAS* status.

Activating mutations of the *KRAS* gene, resulting in EGFR-independent activation of the mitogen-activated protein kinase

pathway (MAPK), have been reported in 30–54% of metastatic colorectal tumors. The most frequent alterations are detected in codon 12 (approximately 82% of all reported *KRAS* mutations) and codon 13 (approximately 17%). Mutations in other positions, such as codons 61 and 146, have also been reported. However, these alterations account for a minor proportion (1–4%) of *KRAS* mutations, and their clinical relevance in CRC still remains unclear [2–5,7–9,12,16–20,26,30,31].

Until now, the overall frequency of *KRAS* mutations has been estimated according to the results of clinical studies, drug admission trials, or retrospective multi-center studies with different inclusion criteria and variable ethnical backgrounds, whereas large cohorts of unselected metastatic CRC with a demand for routine EGFR-targeted therapy have not been analyzed. Meanwhile, large numbers of metastatic CRCs (UICC Stage IV) have to be routinely tested for *KRAS* mutations. Therefore, reliable benchmarks for the frequency and types of *KRAS* mutations are needed for the implementation and quality assurance of *KRAS* mutation analysis of metastatic CRC in routine diagnosis. Moreover, primary tumors and metastases have to be compared to evaluate the robustness or variation of the *KRAS* mutation status during neoplastic dissemination.

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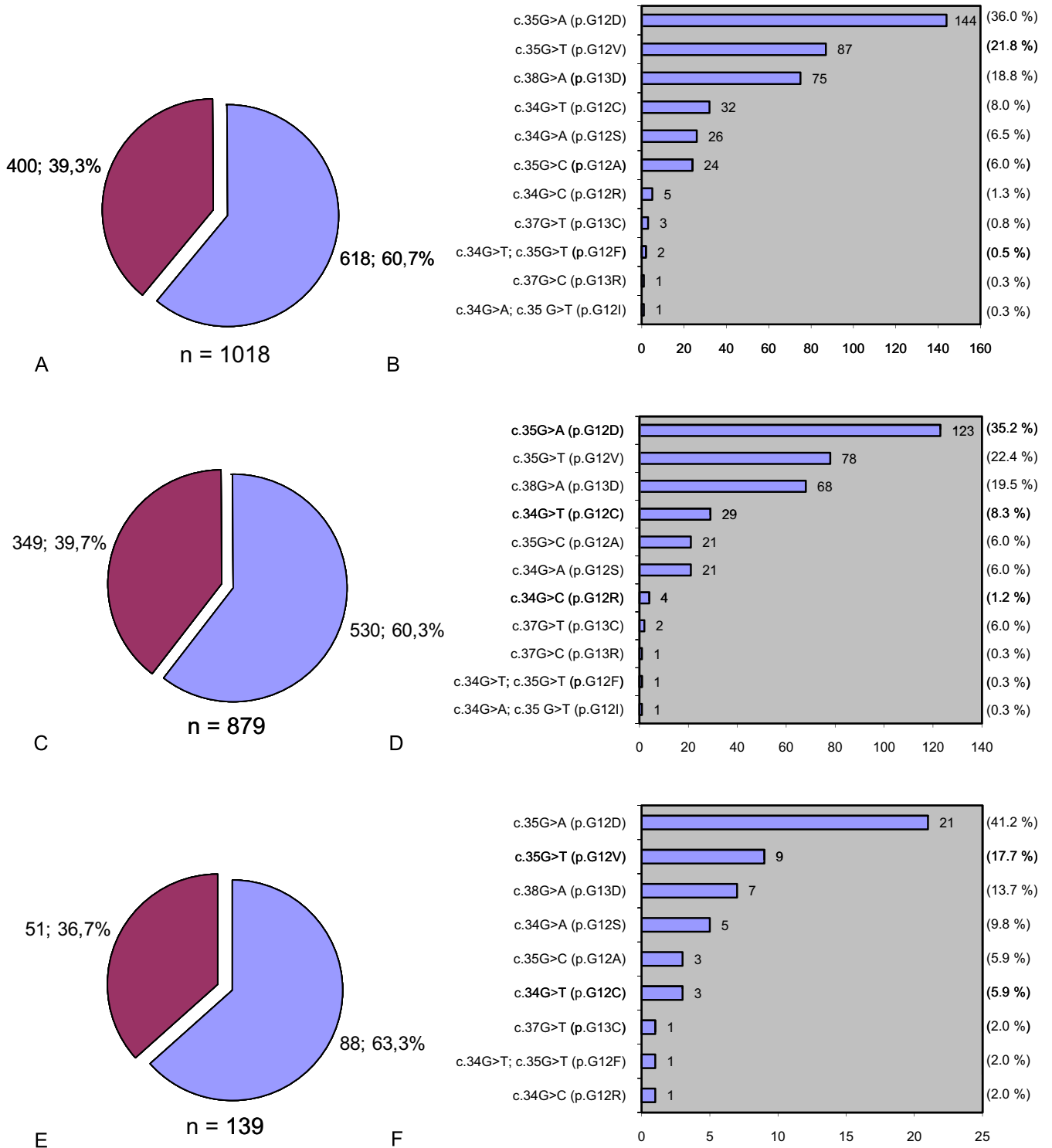


Fig. 1. In Panel A, the overall mutation frequency of *KRAS* oncogene in all analyzed metastatic CRCs is shown ($n = 1018$). In Panel B, the frequency and type of mutations, affected codons, and corresponding altered amino acids in exon 2, codons 12 and 13 of the *KRAS* gene, are shown in descending order. Percentages refer to the group of mutated tumors. Panel C shows the overall mutation frequency of *KRAS* within the group of analyzed primary tumors ($n = 879$). In Panel D, the frequency and type of mutations, affected codons, and corresponding altered amino acids in exon 2, codons 12 and 13 of the *KRAS* gene in primary tumors, are shown in descending order. Percentages refer to the group of mutated tumors. Panel E shows the number of *KRAS* gene mutations analyzed separately for CRC metastases ($n = 139$). In Panel F, the frequency and type of mutations, affected codons, and corresponding altered amino acids in exon 2, codons 12 and 13 of the *KRAS* gene in metastases, are presented in descending order. Percentages refer to the group of mutated tumors.

In conclusion, our study is the first to provide data on the frequency and types of *KRAS* mutations from a large homogeneous Caucasian cohort of unselected metastatic CRC tested in a routine setting.

Methods

Tissue selection

At the Department of Pathology, Ludwig-Maximilians-Universität München, in the period from January to November 2008, we analyzed the *KRAS* mutation status in 1018 unselected cases of metastatic CRC (UICC Stage IV), for which anti-EGFR-therapy was intended. The mean age at the date of receipt of the patients' specimens was 63.8 years (± 10.87). 62.4% of the patients were male, and 37.6% were female. For all analyses, formalin-fixed and paraffin-embedded tissue specimens were used. Four hundred and seventy-five (475) of the specimens were processed and diagnosed primarily at the Department of Pathology, Ludwig-Maximilians-University of Munich. Five hundred and forty-three (543) cases were derived from external histopathological laboratories sent to our laboratory for analysis. Eight hundred and seventy-nine (879) cases were primary tumor specimens (86.4%), 139 cases were metastases of CRC predominantly from liver (56%), lymph node (10%), lung (9%), or other sites of resection (25%) such as omentum majus, peritoneum, or brain. In all cases, paraffin sections and H&E stains were prepared according to standard protocols and re-evaluated by an experienced histopathologist to verify the histological diagnosis, to revise the adequacy of specimen, and to select areas for microdissection.

Mutational analysis

For enrichment of tumor tissue, an H&E-stained histologic serial section was inspected and used as the blue-print for defining areas containing tumor from that DNA was isolated. DNA was purified using DNA-Micro-Amp[®] Kits (Qiagen, Hilden) following the user's handbook. For the subsequent detection of the mutational status of the *KRAS* proto-oncogene, either dideoxy- or Pyro-sequencing was employed. Briefly, for dideoxy-sequencing, a semi-nested procedure was applied using 2 μ l of the DNA as the template [29]. In the first reaction, a fragment of 211 bp was generated, using a primer pair covering exon 2 of the *KRAS* gene (GTAAAACGACGGCCAGTTTATAAGGCTGCTGAAAATGACTG, TCATGAAAATGGTCAGAGAAACC). The annealing temperature was 58 °C. The resulting fragment was used as a template to amplify a 214 bp fragment using the first primer from the first reaction together with TAATACGACTCACTATAGGGCAAA-GAATGGTCTGCACCACTAAT at an annealing temperature of 60 °C.

After checking the success of the PCR using agarose-gel electrophoresis, dideoxy-sequencing was done, employing Big-Dye terminator sequencing kits v.1.0 (Applied Biosystems) together with the universal M13 primer (GTAAAACGACGGCCAGT) or T7-phage promoter primer (TAATACGACTCACTATAGGGU), which were part of the PCR primers (underlined), explaining why the nested PCR product was larger than that from the first PCR. Then, a purification step employing DyeEx v2.0 (Qiagen, Hilden) and, finally, the analysis on a Genetic Analyzer 3130 (Applied Biosystems) together with the Seqscape software were done. All steps were performed according to the user's instructions.

Pyro-sequencing was performed using the DNA together with the Pyro-Gold kit (Qiagen, Hilden) and HotStar Taq-Polymerase

(Qiagen, Hilden). In case of sequencing the counter-strand, we used the sequencing primer PF2 employing a PyroMark Q24 device (Qiagen, Hilden) and, subsequently, the PyroMark[™] Q24 software according to the manufacturer's recommendations [21,24].

Twenty-five cases were analyzed independently with either of the two methods, leading to the same results, suggesting that the methodology was correct.

Statistics

Basic data analysis and statistical calculations were done using Microsoft[®] Excel 2007. Differences in mutation frequencies between the tumor specimens derived from primary tumors and metastases were tested for significance by applying Student's t-test. $p < 0.05$ (two-sided) was considered significant.

Results

Of all cases investigated, 39.3% showed mutations in exon 2 codons 12 and 13 of the *KRAS* proto-oncogene. Conversely, 60.7% showed a wild-type (WT) sequence (Fig. 1). Three patients presented two mutations in codon 12. The most common mutations were glycine to aspartate on codon 12 (p.G12D, 36.0% of mutated tumors; 144 of 1018), glycine to valine on codon 12 (p.G12V, 21.8% of mutated tumors; 87 of 1018), and glycine to aspartate on codon 13 (p.G13D, 18.8% of mutated tumors; 75 of 1018). These three types account for 76.6% of all mutations. Other types were less frequent and were detected only in a few single cases (Table 1). All mutations found had previously been described to be oncogenically active and were found in the COSMIC (catalog of somatic mutations in cancer)-database (Sanger Institute, Cambridge, UK) [14].

When analyzing specimens separately derived from primary tumors, an overall mutation frequency of 39.7% of mutated tumors (349 of 879) was found (Fig. 1). In 35.2% of mutated tumors (123 of 879), glycine to aspartate mutations on codon 12 (p.G12D) were detected. In addition, 22.4% (78 of 879) and 19.5% of mutated tumors (67 of 879) showed glycine to valine on codon 12 (p.G12V) and glycine to aspartate on codon 13 (p.G13D) mutations, respectively. In two primary CRC specimens, a double mutation was found in codon 12.

When investigating metastases separately, an overall mutation frequency of 36.7% (51 of 139) was calculated (Fig. 1). The frequency of glycine to aspartate on codon 12 mutations (p.G12D)

Table 1

Number and type of mutations, affected codons, and corresponding altered amino acids in exon 2, codons 12 and 13 of the *KRAS* gene detected in 1018 metastatic colorectal cancers.

Codon	Type of point mutation	Number of point mutations (% of all tumors)
12	c.35G>A (p.G12D)	144 (14.1%)
	c.35G>T (p.G12V)	87 (8.5%)
	c.34G>T (p.G12C)	32 (3.1%)
	c.34G>A (p.G12S)	26 (2.6%)
	c.35G>C (p.G12A)	24 (2.4%)
	c.34G>C (p.G12R)	5 (0.5%)
	c.34G>T, c.35G>T (p.G12F)	2 (0.2%)
	c.34G>A, c.35G>T (p.G12I)	1 (0.1%)
	13	c.38G>A (p.G13D)
c.37G>T (p.G13C)		3 (0.3%)
c.37G>C (p.G13R)		1 (0.1%)
	Wild type	618 (60.7%)

Table 2

Number and type of mutations, affected codons, and corresponding altered amino acids in exon 2, codons 12 and 13 of the *KRAS* gene analyzed separately for specimens derived from primary tumors and metastases.

Codon	Type of point mutation	Frequency of mutations in primary tumors (<i>n</i> = 879) (% of all tumors)	Frequency of mutations in metastases (<i>n</i> = 139) (% of all tumors)	<i>p</i> -Value
12	c.35G>A (p.G12D)	123 (14.0%)	21 (15.1%)	0.9421
	c.35G>T (p.G12V)	78 (8.9%)	9 (6.5%)	0.8974
	c.34G>T (p.G12C)	29 (3.3%)	3 (2.2%)	0.7227
	c.34G>A (p.G12S)	21 (2.4%)	5 (3.6%)	0.5648
	c.35G>C (p.G12A)	21 (2.4%)	3 (2.2%)	0.7465
	c.34G>C (p.G12R)	4 (0.5%)	1 (0.7%)	0.7218
	c.34G>A, c.35G>T (p.G12I)	1 (0.1%)		
	c.34G>T, c.35G>T (p.G12F)	1 (0.1%)	1 (0.7%)	0.7653
13	c.38G>A (p.G13D)	68 (7.7%)	7 (5.0%)	0.8701
	c.37G>T (p.G13C)	2 (0.2%)	1 (0.7%)	0.3948
	c.37G>C (p.G13R)	1 (0.1%)		
	Wild type	530 (60.3%)	88 (63.3%)	0.3672

When comparing primary tumors with metastases, there was no significant difference between both sets with respect to type or frequency of mutations in the *KRAS* proto-oncogene.

was 41.2% (21 of 139). Furthermore, glycine to valine on codon 12 mutations (p.G12V) was detected in 17.7% (9 of 139) of the metastases analyzed. Glycine to aspartate mutations on codon 13 (p.G13D) were found in only 13.7% (7 of 139) of cases (see also Table 2). Neither a significant difference in mutation frequencies between the group of tumor specimens derived from primary tumor sites or metastases ($p = 0.3672$) nor a clustering of particular mutations in one of these conditions was observed. No correlation of gender, age, and mutation frequency or type of mutation was detected.

To survey the analytical reliability of the *KRAS* Pyro-sequencing method, mutation analysis was performed both by Pyro-sequencing and by Big-Dye terminator sequencing for 25 tumor specimens. No differences between Big-Dye- and Pyro-sequencing results could be detected (data not shown).

Discussion

We present the results of routine *KRAS* mutation analyses in a large cohort of unselected metastatic CRCs (UICC Stage IV) showing a mutation frequency of 39.3%. This confirms the data of previous studies of selected cohorts, in which the frequency ranges from 30% to 54%. Up to now, the estimates of the mutation frequency of *KRAS* for metastatic CRCs have been based on selective clinical studies or drug admission trials with variable inclusion criteria. Most of the studies have evaluated smaller numbers of selected CRC patients and did not specify the type of mutation [3,17,18]. Large population-based studies, like the Netherlands Cohort Study, analyzing 737 CRC specimens, include only a small subgroup (85 cases) of metastatic CRC [8]. Even the 2nd RASCAL collaborative study, collecting 4268 patients from 42 different centers in 21 countries, assembled only 525 cases of metastatic CRC and covered a wide ethnical background, as well as numerous laboratories for *KRAS* mutation analysis without standardization [4].

In accordance with our data, previous studies have usually identified the glycine to aspartate transition on codon 12 (p.G12D) as the most frequent mutation of *KRAS* [1–5,7–9,12,16–20,26,30]. Three types (p.G12D, p.G12V and p.G13D) accounted for 76.6% of all *KRAS* mutations in our study. Thus, the majority of mutations could be detected by simplified methods, e.g. mutation-specific antibodies for immunohistochemistry, concentrating on these types.

The frequency and the predominant types of *KRAS* mutations did not differ between primary tumors and metastases of CRCs in

our study. This indicates the robustness of the *KRAS* mutation status during tumor dissemination. Moreover, a selection of certain types of *KRAS* mutations during the progress of dissemination is unlikely. Based on the design of our study, we were not able to analyze matched pairs of primary tumors and metastases from the same patient. However, the high number of primary tumors (879 cases) and metastases (139 cases), which we analyzed, provides a reasonable basis to exclude a clustering of *KRAS* mutation or certain types of *KRAS* mutations during the metastatic process. Other studies, including the analyses of matched pairs from primary tumors and metastases, also indicate a high concordance of the *KRAS* mutation status [2,6,22,28]. Therefore, either the primary tumor or the metastases seem to be appropriate for the routine analysis of the *KRAS* mutation status of a metastatic CRC before anti-EGFR antibody therapy.

At present, the demand for routine *KRAS* mutation testing in metastatic CRC is rapidly increasing worldwide, since the testing becomes a standard for CRC with UICC Stage IV disease to evaluate the options of anti-EGFR target-orientated treatment. It is estimated that up to 30% of new CRC cases have to be tested per year, which means a test load of about 20,000 cases per year in Germany. The data of our study are the first to provide reliable benchmarks for the implementation and quality assurance for the increasing number of *KRAS* mutation analysis in routine laboratory practice.

Competing interests

Thomas Kirchner and Andreas Jung received financial support from AMGEN GmbH Germany and Merck-Serono KG for the implementation of a quality assurance system in Germany for the routine molecular-pathological analysis of *KRAS* mutations, which is under the auspices of the QuIP (Quality Initiative in Pathology) structure. QuIP is a structure founded and supported by the Deutsche Gesellschaft für Pathologie (German Society for Pathology) and the Bundesverband Deutscher Pathologen (Federation of the German Pathologists).

Acknowledgments

We thank Mrs. Gaby Charell, Jutta Hügel-Tegge, Nicole Perera, Irina Redich, and Karina Windhorst for their excellent technical assistance.

References

- [1] R. Adams, A. Meade, H. Wasan, G. Griffiths, T. Maughan, Cetuximab therapy in first-line metastatic colorectal cancer and intermittent palliative chemotherapy: review of the COIN trial, *Expert Rev. Anticancer Ther.* 8 (2008) 1237–1245.
- [2] F. Al-Mulla, J.J. Goings, E.T. Sowden, A. Winter, I.R. Pickford, G.D. Birnie, Heterogeneity of mutant versus wild-type Ki-ras in primary and metastatic colorectal carcinomas, and association of codon-12 valine with early mortality, *J. Pathol.* 185 (1998) 130–138.
- [3] R.G. Amado, M. Wolf, M. Peeters, E. Van Cutsem, S. Siena, D.J. Freeman, T. Juan, R. Sikorski, S. Suggs, R. Radinsky, et al., Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer, *J. Clin. Oncol.* 26 (2008) 1626–1634.
- [4] H.J. Andreyev, A.R. Norman, D. Cunningham, J. Oates, B.R. Dix, B.J. Iacopetta, J. Young, T. Walsh, R. Ward, N. Hawkins, et al., Kirsten ras mutations in patients with colorectal cancer: the 'RASCAL II' study, *Br. J. Cancer* 85 (2001) 692–696.
- [5] H.J. Andreyev, A.R. Norman, D. Cunningham, J.R. Oates, P.A. Clarke, Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" study, *J. Natl. Cancer Inst.* 90 (1998) 675–684.
- [6] S. Artale, A. Sartore-Bianchi, S.M. Veronese, V. Gambi, C.S. Sarnataro, M. Gambacorta, C. Lauricella, S. Siena, Mutations of KRAS and BRAF in primary and matched metastatic sites of colorectal cancer, *J. Clin. Oncol.* 26 (2008) 4217–4219.
- [7] L. Barault, N. Veyrie, V. Jooste, D. Lecorre, C. Chapusot, J.M. Ferraz, A. Lievre, M. Cortet, A.M. Bouvier, P. Rat, et al., Mutations in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in a population-based series of colon cancers, *Int. J. Cancer* 122 (2008) 2255–2259.
- [8] M. Brink, A.F. de Goeij, M.P. Weijnenberg, G.M. Roemen, M.H. Lentjes, M.M. Pachen, K.M. Smits, A.P. de Bruine, R.A. Goldbohm, P.A. van den Brandt, K-ras oncogene mutations in sporadic colorectal cancer in the Netherlands cohort study, *Carcinogenesis* 24 (2003) 703–710.
- [9] J.P. Cerottini, S. Caplin, E. Saraga, J.C. Givel, J. Benhattar, The type of K-ras mutation determines prognosis in colorectal cancer, *Am. J. Surg.* 175 (1998) 198–202.
- [10] F. Ciardiello, G. Tortora, EGFR antagonists in cancer treatment, *N. Engl. J. Med.* 358 (2008) 1160–1174.
- [11] J. Dancy, E.A. Sausville, Issues and progress with protein kinase inhibitors for cancer treatment, *Nat. Rev. Drug Discovery* 2 (2003) 296–313.
- [12] F. Di Fiore, F. Blanchard, F. Charbonnier, F. Le Pessot, A. Lamy, M.P. Galais, L. Bastit, A. Killian, R. Sesboue, J.J. Tuech, et al., Clinical relevance of KRAS mutation detection in metastatic colorectal cancer treated by Cetuximab plus chemotherapy, *Br. J. Cancer* 96 (2007) 1166–1169.
- [13] N.E. Hynes, H.A. Lane, ERBB receptors and cancer: the complexity of targeted inhibitors, *Nat. Rev. Cancer* 5 (2005) 341–354.
- [14] COSMIC (Catalogue Of Somatic Mutations In Cancer) Database, S. Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, <<http://www.sanger.ac.uk/genetics/CGP/cosmic/>>.
- [15] A.E. Karnoub, R.A. Weinberg, Ras oncogenes: split personalities, *Nat. Rev. Mol. Cell Biol.* 9 (2008) 517–531.
- [16] U. Kressner, J. Bjorheim, S. Westring, S.S. Wahlberg, L. Pahlman, B. Glimelius, G. Lindmark, A. Lindblom, A.L. Borresen-Dale, Ki-ras mutations and prognosis in colorectal cancer, *Eur. J. Cancer* 34 (1998) 518–521.
- [17] A. Lievre, J.B. Bachet, V. Boige, A. Cayre, D. Le Corre, E. Buc, M. Ychou, O. Bouche, B. Landi, C. Louvet, et al., KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab, *J. Clin. Oncol.* 26 (2008) 374–379.
- [18] A. Lievre, J.B. Bachet, D. Le Corre, V. Boige, B. Landi, J.F. Emile, J.F. Cote, G. Tomic, C. Penna, M. Ducreux, et al., KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer, *Cancer Res.* 66 (2006) 3992–3995.
- [19] S.G. Martinez-Garza, A. Nunez-Salazar, A.L. Calderon-Garciduenas, F.J. Bosques-Padilla, A. Niderhauser-Garcia, H.A. Barrera-Saldana, Frequency and clinicopathology associations of K-ras mutations in colorectal cancer in a northeast Mexican population, *Dig. Dis.* 17 (1999) 225–229.
- [20] P. Moerkerk, J.W. Arends, M. van Driel, A. de Bruine, A. de Goeij, J. ten Kate, Type and number of Ki-ras point mutations relate to stage of human colorectal cancer, *Cancer Res.* 54 (1994) 3376–3378.
- [21] S. Ogino, T. Kawasaki, M. Brahmandam, L. Yan, M. Cantor, C. Namgyal, M. Mino-Kenudson, G.Y. Lauwers, M. Loda, C.S. Fuchs, Sensitive sequencing method for KRAS mutation detection by Pyrosequencing, *J. Mol. Diagn.* 7 (2005) 413–421.
- [22] J.J. Oudejans, R.J. Slebos, F.A. Zoetmulder, W.J. Mooi, S. Rodenhuis, Differential activation of ras genes by point mutation in human colon cancer with metastases to either lung or liver, *Int. J. Cancer* 49 (1991) 875–879.
- [23] M. Peeters, J. Balfour, D. Arnold, Review article: panitumumab – a fully human anti-EGFR monoclonal antibody for treatment of metastatic colorectal cancer, *Aliment. Pharmacol. Ther.* 28 (2008) 269–281.
- [24] A. Poehlmann, D. Kuester, F. Meyer, H. Lippert, A. Roessner, R. Schneider-Stock, K-ras mutation detection in colorectal cancer using the Pyrosequencing technique, *Pathol. Res. Pract.* 203 (2007) 489–497.
- [25] M. Raponi, H. Winkler, N.C. Dracopoli, KRAS mutations predict response to EGFR inhibitors, *Curr. Opin. Pharmacol.* 8 (2008) 413–418.
- [26] M. Span, P.T. Moerkerk, A.F. De Goeij, J.W. Arends, A detailed analysis of K-ras point mutations in relation to tumor progression and survival in colorectal cancer patients, *Int. J. Cancer* 69 (1996) 241–245.
- [27] J.P. Spano, G. Milano, S. Vignot, D. Khayat, Potential predictive markers of response to EGFR-targeted therapies in colorectal cancer, *Crit. Rev. Oncol. Hematol.* 66 (2008) 21–30.
- [28] B. Suchy, C. Zietz, H.M. Rabes, K-ras point mutations in human colorectal carcinomas: relation to aneuploidy and metastasis, *Int. J. Cancer* 52 (1992) 30–33.
- [29] M. Tartaglia, C.M. Niemeyer, A. Fragale, X. Song, J. Buechner, A. Jung, K. Hahlen, H. Hasle, J.D. Licht, B.D. Gelb, Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia, *Nat. Genet.* 34 (2003) 148–150.
- [30] N. Urosevic, K. Krtolica, A. Skaro-Milic, S. Knezevic-Usaj, A. Dujic, Prevalence of G-to-T transversions among K-ras oncogene mutations in human colorectal tumors in Yugoslavia, *Int. J. Cancer* 54 (1993) 249–254.
- [31] J.H. van Krieken, A. Jung, T. Kirchner, F. Carneiro, R. Seruca, F.T. Bosman, P. Quirke, J.F. Flejou, T. Plato Hansen, G. de Hertogh, et al., KRAS mutation testing for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for an European quality assurance program, *Virchows Arch.* 453 (2008) 417–431.

Clinical characterization of patients with metastatic colorectal cancer depending on the *KRAS* status

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This retrospective study investigated the clinical characteristics of patients with metastatic colorectal cancer (mCRC) depending on the *KRAS* status, thereby differentiating *KRAS* exon 2 mutations in codon 12 versus codon 13. In total, 273 patients with mCRC receiving first-line therapy were analyzed. One hundred patients were treated within the FIRE-3 trial (FOLFIRI plus cetuximab or bevacizumab), 147 patients within the AIO KKR-0104 trial (cetuximab plus CAPIRI or CAPOX), and further 26 patients received therapy outside the study. Thirty-eight tumors with *KRAS* mutation in codon 13, 140 tumors with mutation in codon 12, and 95 tumors with *KRAS* wild type as a comparison were included in this analysis. Bivariate analyses demonstrated significant differences between *KRAS* wild-type, codon 12-mutated, and codon 13-mutated tumors with regard to synchronous lymph node metastasis ($P=0.018$), organ metastasis (76.8% vs. 65.9% vs. 89.5%, $P=0.009$), liver metastasis (89.5% vs. 78.2% vs. 92.1%, $P=0.025$), lung metastasis (29.5% vs. 42.9% vs. 50%, $P=0.041$), liver-only metastasis (48.4% vs. 28.8% vs. 28.9%, $P=0.006$), and metastases in two or more organs (49.5, 61.4, 71.1, $P=0.047$). Regression models indicated a significant impact of *KRAS* mutations in codon 12 versus

codon 13 for synchronous organ and nodal metastasis ($P=0.01, 0.03$). This pooled analysis indicates that mCRC is a heterogeneous disease, which seems to be defined by *KRAS* mutations of the tumor. Compared with *KRAS* codon 12 mutations, codon 13-mutated mCRC presents as a more aggressive disease frequently associated with local and distant metastases at first diagnosis. *Anti-Cancer Drugs* 22:913–918 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2011, 22:913–918

Keywords: clinical characterization, codon 13, *KRAS* mutation, metastatic colorectal cancer

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Received 14 April 2011 Revised form accepted 25 May 2011

Introduction

KRAS mutation was identified as a negative prognostic factor for disease recurrence more than a decade ago in patients undergoing surgery for primary colorectal cancer (CRC) [1–4]. First indications of the therapeutic impact of this mutation were described during the last years when mutations in the *KRAS* protooncogene were identified as determinants of poor response to anti-EGFR treatment for metastatic colorectal cancer (mCRC). Therefore, patients with *KRAS* mutations were excluded from treatment with cetuximab or panitumumab [5–9].

Although the *KRAS* mutational status has become a widely accepted tool to guide anti-EGFR therapy, it seems that the group of patients with mCRC cannot longer be seen as a uniform collective. Specifically, *KRAS*-mutated tumors obviously represent a heterogeneous group with regard to their clinical outcome.

Evidence for clinically relevant subgroups of *KRAS* mutations was presented in 1998 when *KRAS* mutations

in codon 13 were described as a negative prognostic marker during postoperative follow-up [1]. This observation was later confirmed by an analysis demonstrating that codon 13 mutation of the *KRAS* oncogene was associated with advanced Dukes' stage, lymph node metastasis, a higher recurrence rate, and shorter survival when compared with other *KRAS* mutations or *KRAS* wild type [10]. In advanced disease, the poor outcome of patients with *KRAS* codon 13 mutations was also described by other investigators [4,11], but in one case, it was not confirmed by a multivariate analysis [11]. However, two large studies focusing on all stages of colorectal cancer and only stage II and III, respectively, did not identify the *KRAS* codon 13 mutation to correlate with the clinical outcome [12,13].

In 2010, De Roock *et al.* [14] investigated patients with chemotherapy-refractory mCRC and identified the codon 13 mutation to be correlated with poor survival in patients undergoing the best supportive care. However, a benefit from treatment was noted when codon

13-mutated patients received the anti-EGFR antibody cetuximab. This observation was substantiated by results obtained from in-vitro studies and from mouse models that clearly indicated that codon 13-mutated tumor cells were sensitive to cetuximab [14].

This retrospective study evaluated patients receiving first-line chemotherapy for mCRC; the aim of this investigation was to correlate *KRAS* mutational status (wild type, codon 12 mutations, codon 13 mutations) with specific clinical patient characteristics associated with the presentation or course of the disease. The results obtained are clinically and scientifically relevant, as only limited information is currently available regarding the clinical course of mCRC patients with codon 13-mutated tumors. We tested the hypothesis that codon 13 mutations are correlated with synchronous metastasis in both lymph nodes and distant organs at the time of first diagnosis and with adverse allocation of metastasis in mCRCs when compared with codon 12 mutations or wild-type tumors.

Methods

KRAS mutation detection

KRAS testing was performed centrally in a German reference laboratory for *KRAS* analysis (Institute of Pathology, University of Munich). After dissection of tumor-containing areas, DNA was isolated using Qiagen DNA Micro-Amp kits (Qiagen, Venlo, Netherlands). Detection of mutations in codons 12 and 13 of the *KRAS* protooncogene then was performed by pyrosequencing using Qiagen's PyroMark Gold kits (Qiagen) together with a Q24 pyrosequencer device. This procedure resulted in a specificity of 0.98 and a sensitivity of 0.99 for the detection of mutations in the *KRAS* protooncogene.

Patients

For this study, we analyzed 273 patients suffering from mCRC with a proven *KRAS* status. One hundred patients were treated within the FIRE-3 trial (NCT00433927-comparing FOLFIRI plus cetuximab to FOLFIRI plus bevacizumab) before an amendment determined that only patients with *KRAS* wild type could be included into the study. A further 147 patients received first-line treatment within the AIO KRK-0104 trial (NCT00254137; comparing CAPIRI plus cetuximab to CAPOX plus cetuximab [15]), whereas 26 patients were treated outside clinical trials at the University of Munich. All patients had a histologically confirmed diagnosis and received first-line treatment for metastatic disease. The protocols of the clinical trials were approved by an independent ethics committee and governmental authorities. The trials were conducted in accordance with the Declaration of Helsinki (1996). All patients provided written informed consent to be treated within a clinical trial.

End points

This investigation was performed as an exploratory, retrospective pooled analysis. Initial tumor stage was

documented according to the tumor nodes metastasis classification of malignant tumors developed by the international union against cancer. pT and pN stage were noted if surgery was performed on the primary tumor or cT and cN stages in rare cases if radiologic images gave a clear impression of these parameters. For this investigation, clinical and pathological stages of tumor and lymph nodes were summarized. Allocations of metastasis at the beginning of palliative treatment were detected by spiral computed tomographic scans of the thorax and abdomen. The performance status was determined using the Eastern Cooperative Oncology Group (ECOG) scale. When performance status was indicated as the Karnofsky index, values of 90–100% were as attributed to ECOG 0, 80% to ECOG 1, and 70% to ECOG 2. Patient characteristics were presented as valid percentages based on nonmissing data.

Statistical analysis

Data were summarized by adequate measures of location and spread for continuous variables and by proportions for discrete variables. Adequate tests for continuous data (Mann–Whitney *U*-test) and for discrete data (χ^2 test) were used. For modelling the dichotomous outcomes 'synchronous organ metastasis', 'liver metastasis', 'pulmonary metastasis', 'liver-only metastasis', 'synchronous nodal metastasis', and 'two or more organs with metastasis', we used the logistic regression model where covariates were selected from a set of candidate variables (*KRAS* status, sex, age, ECOG, grading, and 'primary tumor site') relying on a backward elimination algorithm using likelihood ratio tests. For each outcome, we used complete cases and the variable selection level for all variables was set to 0.05. For each final model, the *P* values of the likelihood ratio tests are reported as they especially allow assessing the impact of a covariate that has several categories. In addition, the odds ratio, 95% confidence intervals and *P* values resulting from the Wald tests are reported as they allow a *P* value to be provided for each category (except the chosen reference) of a covariate with several categories. All statistical tests were performed two-sided, and a *P* value of 0.05 or less was considered as statistically significant. All statistical analyses were performed by using *R* (version 2.12.2).

Results

Study population

This pooled analysis included 273 patients with mCRC who received first-line treatment. Among this population, 38 patients showed a mutation in codon 13 (c.38G > A) of exon 2, whereas the other 140 patients presented with mutations in codon 12 (c.34G > A, c.34G > C, c.34G > T, c.35G > A, c.35G > C, c.35G > T). In addition, 95 patients with *KRAS* wild-type mCRC were included for comparison. The mutation frequency of codon 13 mutations within the *KRAS*-mutated subgroup of our study population was 21.3%, whereas a mutation in codon 12 was observed in 78.7% of the tumors. Demographical, pathological, and

Table 1 Patient characteristics

Baseline characteristics	KRAS wild type		KRAS mutation codon 12		KRAS mutation codon 13		P value
	N	%	N	%	N	%	
Number of patients	95		140	78.7	38	21.3	χ^2 test
Age (years)							
Median		62.0		64.0		64.0	NS
Range		33–75		36–76		28–76	
Sex							
Female	30	31.6	42	30.4	14	36.8	NS
Male	65	68.4	96	69.6	24	63.2	
Not reported	0	0	2		0	0	
ECOG							
0	71	76.3	80	58.0	20	52.6	
1	19	20.4	53	38.4	14	36.8	0.009
2	3	3.2	5	3.6	4	10.5	
Not reported	2		2		0		
Primary tumor site							
Colon	66	69.5	82	59.4	27	71.1	
Rectum	29	30.5	55	39.9	11	28.9	NS
Colon and rectum	0	0	1	0.7	0	0	
Not reported	0	0	2		0	0	
Prior therapy							
Adjuvant chemotherapy	14	14.7	36	26.3	4	10.5	0.03
Not reported	0	0	3		0	0	
Prior radiotherapy	7	7.4	27	19.9	1	2.7	0.003
Not reported	0	0	4		1		

Percentages are calculated based on non-missing data.

ECOG, Eastern Cooperative Performance Score; NS, not significant.

clinical data were documented in all patients. Baseline characteristics of the patients analyzed in this study do not show significant differences with regard to age, sex, and location of the primary tumor when compared between patients with wild-type, codon 12%-mutated, or codon 13-mutated tumors. Patients with wild-type tumors seemed to present in a better performance status (ECOG) at treatment initiation for mCRC compared with patients with *KRAS*-mutated tumors ($P = 0.009$) (Table 1).

Impact of *KRAS* mutation status on initial tumor nodes metastasis status and tumor grading

Although the *KRAS* mutation status did not correlate with the status of the primary tumor, patients with a *KRAS* mutation in codon 13 had a statistically significant higher rate of synchronous nodal metastasis (93.6% vs. 71.3%, $P = 0.03$) and organ metastasis (89.5% vs. 65.9%, $P = 0.01$) at the time of first diagnosis of CRC compared with patients with codon 12-mutated tumors (Tables 2 and 3). Correspondingly, the rate of patients previously treated with systemic chemotherapy ($P = 0.03$) or local radiation therapy ($P = 0.003$) was significantly lower for patients with *KRAS* codon 13-mutated tumors (Table 1). No association of *KRAS* status with tumor grading was observed in our patient population (Table 2). A higher rate of synchronous nodal and organ metastasis was detected when *KRAS* wild-type tumors were compared with codon 12-mutated tumors (Table 2).

Impact of the *KRAS* mutation status on distant metastasis

Patients with *KRAS* wild-type or codon 13-mutated tumors had a higher rate of liver metastasis at the

beginning of palliative treatment compared to the subgroup of patients with codon 12-mutated tumors (codon 12 vs. codon 13, $P = 0.1$; codon 12 vs. wild type, $P = 0.02$) (Tables 2 and 3). Moreover, patients with *KRAS*-mutated tumors tended to have a higher rate of at least two organs involved with metastasis when starting palliative treatment ($P = 0.047$; Tables 2 and 3). Correspondingly, the rate of pulmonary metastasis was significantly higher for *KRAS*-mutated patients ($P = 0.041$), showing no difference between codon 12-mutated and codon 13-mutated tumors (Tables 2 and 3). Despite the high frequency of synchronous metastasis, patients with *KRAS* wild-type tumors had a significantly higher probability of having hepatic metastasis only ($P = 0.006$) (Tables 2 and 3).

Discussion

This analysis was stimulated by a recent study indicating that patients with *KRAS* codon 13-mutated mCRC may belong to a specific subgroup of mCRC. Chemotherapy-refractory patients with *KRAS* codon 13 mutations had a very poor survival when treated with best supportive care only [14]. However, a marked benefit was obtained from anti-EGFR-directed treatment with cetuximab, whereas this effect was not observed in *KRAS* codon 12-mutated patients. Accordingly, it may be concluded that different mutations of the *KRAS* oncogene may confer specific patterns of response to treatment. This investigation was set out to define the phenotype of mCRC disease associated with *KRAS* codon 13 mutation.

According to the published literature, codon 13 mutations occur at an overall frequency of 8%, and at a rate of

Table 2 Tumor characteristics

Tumor characteristics	KRAS wild type		KRAS mutation codon 12		KRAS mutation codon 13		P value
	N	%	N	%	N	%	
Number of patients	95		140		38		χ^2 test
Initial T stage							NS
T1	3	3.3	3	2.4	0	0	
T2	2	2.1	13	10.6	4	13.3	
T3	59	65.6	73	57.7	16	53.3	
T4	26	28.9	36	29.3	10	33.3	
Not reported	5		17		8		
Initial N stage							0.018
N0	17	19.3	35	28.7	2	6.5	
N1	23	26.1	42	34.4	11	35.5	
N2	48	54.5	45	36.9	18	58.1	
Not reported	7		18		7		
Initial M stage							0.009
M0	22	23.2	47	34.1	4	10.5	
M1	73	76.8	91	65.9	34	89.5	
Not reported	0	0	0	0	0	0	
Tumor grading							NS
G1	0	0	2	1.7	0	0	
G2	59	67.8	87	71.9	27	81.8	
G3	28	32.2	32	26.4	6	28.2	
Not reported	8		19		5		
Metastatic disease site							
Liver	85	89.5	104	78.2	35	92.1	0.025
Liver only	46	48.4	38	28.8	11	28.9	0.006
Lung	28	29.5	57	42.9	19	50.0	0.041
Peritoneum	15	15.8	14	10.5	5	13.2	NS
Other including lymph nodes	37	38.9	50	37.6	11	28.9	NS
Two or more organs involved	47	49.5	81	61.4	27	71.1	0.047

Percentages are calculated based on non-missing data. NS, not significant.

Table 3 Logistic regressions

Variables	N	Odds ratio (95% CI)	P value (Wald)	P value (LR)
Synchronous organ metastasis (initial M1 status)				
codon 12 vs. wild type	239	1.9 (1.0–3.6)	0.04	0.003
codon 12 vs. codon 13		5.5 (1.6–19.1)	0.01	
Synchronous nodal metastasis (initial N Status)	219			
codon 12 vs. wild type		2.0 (0.98–4.2)	0.06	0.02
codon 12 vs. codon 13		5.3 (1.2–24.3)	0.03	
ECOG 0 vs. 1		2.4 (1.1–5.4)	0.04	0.04
ECOG 0 vs. 2		0.4 (0.1–2.1)	0.3	
Liver metastasis at the beginning of palliative treatment				
codon 12 vs. wild type	234	2.8 (1.2–6.5)	0.02	0.01
codon 12 vs. codon 13		2.9 (0.8–10.2)	0.1	
Pulmonary metastasis at the beginning of palliative treatment				
codon 12 vs. wild type	234	0.48 (0.3–0.9)	0.02	0.02
codon 12 vs. codon 13		1.4 (0.6–3.1)	0.4	
PTS (colon vs. rectum)		2.4 (1.4–4.3)	0.003	0.002
Liver-only metastasis at the beginning of palliative treatment				
codon 12 vs. wild type	233	2.8 (1.5–5.2)	0.001	0.003
codon 12 vs. codon 13		1.3 (0.5–3.0)	0.6	
PTS (colon vs. rectum)		0.5 (0.2–0.8)	0.01	0.01
Two or more organs with metastasis at the beginning of palliative treatment				
codon 12 vs. wild type	233	0.5 (0.3–0.9)	0.02	0.03
codon 12 vs. codon 13		1.3 (0.5–3.0)	0.6	
ECOG 0 vs. 1		1.0 (0.6–1.8)	1.0	0.05
ECOG 0 vs. 2		7.8 (0.95–63.6)	0.06	
PTS (colon vs. rectum)		1.9 (1.0–3.3)	0.04	0.03

Logistic regressions, only significant 'candidate variables' are shown.

Odds ratio >1 means the second parameter is more likely to be coexisting.

Codon 12, patients with a KRAS mutated tumors, mutation located in codon 12; codon 13, patients with a KRAS mutated tumors, mutation located in codon 13; ECOG, Eastern Cooperative Performance Score; LR, likelihood-ratio test; PTS, primary tumor site colon vs. rectum; Wald, Wald-test; wild type, patients with KRAS wild-type tumors.

approximately 20% in KRAS-mutated patients [16]. In this analysis of KRAS-mutated tumors, a frequency of 21.3% was observed. Ninety-five patients with KRAS

wild-type tumors treated inside the AIO KKK 0104 trial were included in this investigation to provide a comparison for the mutant subgroups. Therefore, the rate of

KRAS mutations in our study population appears to be higher compared with a nonpooled analysis. *KRAS* mutation status was neither correlated to sex or age. ECOG performance status, however, was superior in *KRAS* wild-type patients when compared with *KRAS* mutant patients, which is possibly explained by the differing number of organs involved in the respective subgroups. This finding differs from the results reported by Yokota *et al.* [11], who – in a smaller analysis – failed to observe a correlation between *KRAS* mutation status and performance status.

In this study, we observed a higher rate of synchronous nodal and organ metastasis in *KRAS* codon 13-mutated patients compared with patients with codon 12 mutations. An increased rate of synchronous nodal metastasis was also observed in a study by Bazan *et al.* [10], who described an association of lymph node involvement and codon 13 mutations in a collective of patients undergoing surgery for primary CRC. In accordance with Yokota *et al.* [11], we did not find a correlation between *KRAS* mutation status and tumor grading.

In this study, patients with *KRAS* codon 13 mutation showed a trend for a higher rate of liver metastasis than patients with *KRAS* codon 12-mutated tumors (92% vs. 78%, $P = 0.1$). Possibly because of the smaller number of patients investigated, no correlation between the rate of liver involvement and the subtype of *KRAS* mutation was described by Yokota *et al.* [11].

Tie *et al.* [17] reported that in recurrent CRC *KRAS* mutations were specifically associated with lung and brain metastasis. Our study reveals a significantly higher rate of pulmonary metastasis for patients with *KRAS* mutant tumors compared with wild-type tumors. No significant difference between the subtypes of *KRAS* mutation was observed in our study, although the rate of pulmonary metastasis was higher in *KRAS* codon 13-mutated tumors (50% vs. 43%).

Within our study population, the metastatic involvement of at least two organs was significantly associated with a *KRAS* mutation with a higher rate for codon 13 compared with codon 12-mutated patients (71% vs. 61%). Again, this observation points to the high malignant potential derived from a *KRAS* oncogene alteration. A comparable observation was also reported by Yokota *et al.* [11]; they found the highest rate of more than two organs involved in patients with *KRAS* codon 13 mutations.

Although the character of *KRAS* codon 13-mutated mCRC seems aggressive in our analysis and codon 13 mutations were identified as a poor prognostic markers in several investigations [1,4,10,14], conflicting data exist [12,13]. Treatment regimens for codon 13-mutated mCRC may play an important role with regard to the outcome of these patients and may explain the inconsistent data. As De Roock *et al.* [14] suggest, the application of cetuximab changes the outcome of patients

with codon 13 mutant mCRC significantly. The precise predictive and prognostic impact of codon 13 mutations in patients with mCRC has to be investigated by large studies with defined treatment strategies containing anti-EGFR treatment such as the PRIME study or the CRYSTAL study [9,18]. This analysis is limited by the retrospective nature, which only allows an explorative analysis of data. Moreover, this analysis included only patients with disease progression into a metastatic situation. The character of resectable codon 13-mutated tumors might differ from our population. Moreover, the number of codon 13-mutated patients was limited.

In conclusion, this study demonstrated that *KRAS* mutation status may significantly be associated with different clinical characteristics of mCRC. *KRAS* mutations in codon 13 were associated with a significantly higher rate of synchronous nodal and organ metastases compared with codon 12 mutations, and with a higher frequency of multiorgan involvement. Although patients with *KRAS* wild-type tumors also had a high rate of synchronous metastasis, they presented with a lower rate of multiorgan involvement. Baseline characteristics of patients with mCRC showed a trend toward a higher rate of liver metastasis in *KRAS* codon 13-mutated compared with codon 12-mutated mCRC.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

References

- 1 Cerottini JP, Caplin S, Saraga E, Givel JC, Benhattar J. The type of K-ras mutation determines prognosis in colorectal cancer. *Am J Surg* 1998; **175**:198–202.
- 2 Benhattar J, Losi L, Chaubert P, Givel JC, Costa J. Prognostic significance of K-ras mutations in colorectal carcinoma. *Gastroenterology* 1993; **104**:1044–1048.
- 3 Tortola S, Marcuello E, González I, Reyes G, Arribas R, Aiza G, *et al.* P53 and K-ras gene mutations correlate with tumor aggressiveness but are not of routine prognostic value in colorectal cancer. *J Clin Oncol* 1999; **17**:1375–1381.
- 4 Samowitz WS, Curtin K, Schaffer D, Robertson M, Leppert M, Slattery ML. Relationship of Ki-ras mutations in colon cancers to tumor location, stage, and survival: a population-based study. *Cancer Epidemiol Biomarkers Prev* 2000; **9**:1193–1197.
- 5 Allegra CJ, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, Hayes DF, *et al.* American Society of Clinical Oncology provisional clinical opinion: testing for *KRAS* gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. *J Clin Oncol* 2009; **27**:2091–2096.
- 6 Bokemeyer C, Bondarenko I, Makhson A, Hartmann JT, Aparicio J, de Braud F, *et al.* Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. *J Clin Oncol* 2009; **27**:663–671.
- 7 Jonker DJ, O'Callaghan CJ, Karapetis CS, Zalcborg JR, Tu D, Au HJ, *et al.* Cetuximab for the treatment of colorectal cancer. *N Engl J Med* 2007; **357**:2040–2048.
- 8 Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, *et al.* K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 2008; **359**:1757–1765.
- 9 Van Cutsem E, Köhne CH, Hitre E, Zaluski J, Chang Chien CR, Makhson A, *et al.* Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med* 2009; **360**:1408–1417.

- 10 Bazan V, Migliavacca M, Zanna I, Tubiolo C, Grassi N, Latteri MA, *et al.* Specific codon 13 K-ras mutations are predictive of clinical outcome in colorectal cancer patients, whereas codon 12 K-ras mutations are associated with mucinous histotype. *Ann Oncol* 2002; **13**:1438–1446.
- 11 Yokota T, Ura T, Shibata N, Takahari D, Shitara K, Nomura M, *et al.* BRAF mutation is a powerful prognostic factor in advanced and recurrent colorectal cancer. *Br J Cancer* 2011; **104**:856–862.
- 12 Andreyev HJ, Norman AR, Cunningham D, Oates J, Dix BR, Iacopetta BJ, *et al.* Kirsten ras mutations in patients with colorectal cancer: the RASCAL II study. *Br J Cancer* 2001; **85**:692–696.
- 13 Roth AD, Tejpar S, Delorenzi M, Yan P, Fiocca R, Klingbiel D, *et al.* Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: results of the translational study on the PETACC-3, EORTC 40993, SAKK 60-00 trial. *J Clin Oncol* 2010; **28**:466–474.
- 14 De Roock W, Jonker DJ, Di Nicolantonio F, Sartore-Bianchi A, Tu D, Siena S, *et al.* Association of KRAS p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. *JAMA* 2010; **304**:1812–1820.
- 15 Moosmann N, Von Weikersthal LF, Vehling-Kaiser U, Stauch M, Hass HG, Dietzfelbinger H, *et al.* Cetuximab plus capecitabine and irinotecan compared with cetuximab plus capecitabine and oxaliplatin as first-line treatment for patients with metastatic colorectal cancer: AIO KRK-0104—a randomized trial of the German AIO CRC Study Group. *J Clin Oncol* 2011; **29**:1050–1058.
- 16 Neumann J, Zeindl-Eberhart E, Kirchner T, Jung A. Frequency and type of KRAS mutations in routine diagnostic analysis of metastatic colorectal cancer. *Pathol Res Pract* 2009; **205**:858–862.
- 17 Tie J, Lipton L, Desai J, Gibbs P, Jorissen RN, Christie M, *et al.* KRAS mutation is associated with lung metastasis in patients with curatively resected colorectal cancer. *Clin Cancer Res* 2011; **17**: 1122–1130.
- 18 Douillard JY, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, *et al.* Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. *J Clin Oncol* 2010; **28**:4697–4705.