Concordance of KRAS/BRAF Mutation Status in Metastatic Colorectal Cancer before and after Anti-EGFR Therapy

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Anti-EGFR targeted therapy is a potent strategy in the treatment of metastatic colorectal cancer (mCRC) but activating mutations in the KRAS gene are associated with poor response to this treatment. Therefore, KRAS mutation analysis is employed in the selection of patients for EGFR-targeted therapy and various studies have shown a high concordance between the mutation status in primary CRC and corresponding metastases. However, although development of therapy related resistance occurs also in the context of novel drugs such as tyrosine kinase-inhibitors the effect of the anti-EGFR treatment on the KRAS/BRAF mutation status itself in recurrent mCRC has not yet been clarified. Therefore, we analyzed 21 mCRCs before/after anti-EGFR therapy and found a pre-/posttherapeutic concordance of the KRAS/BRAF mutation status in 20 of the 21 cases examined. In the one discordant case, further analyses revealed that a tumor mosaicism or multiple primary tumors were present, indicating that anti-EGFR therapy has no influence on KRAS/BRAF mutation status in mCRC. Moreover, as the preselection of patients with a KRASwt genotype for anti-EGFR therapy has become a standard procedure, sample sets such ours might be the basis for future studies addressing the identification of potential anti-EGFR therapy induced genetic alterations apart from KRAS/BRAF mutations.

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1. Introduction

Colorectal carcinoma (CRC) is one of the most common forms of malignant neoplasia and frequently takes a fatal course following metastasis [1]. CRC is a multipathway disease involving dysregulatory phenomena in a number of signal transduction pathways [2]. The epidermal growth factor receptor (EGFR), a tyrosine kinase receptor belonging to the ErbB family, is overexpressed in 25%–80% of CRCs and has been found to play a major role in the pathogenesis of CRC by inducing downstream signaling pathways such as the phosphatidylinositol-3-kinase/Akt and Ras/Raf/mitogen-activated protein kinase (MAPK) pathways, which are crucial in the regulation of cell growth, proliferation, apoptosis, invasion, migration, and angiogenesis [3]. Consequently, antibodies targeting EGFR, such as cetuximab and panitumumab, have been examined for therapeutic efficacy in CRC patients [4]. Although it was determined that combination therapy of irinotecan and cetuximab is significantly more successful in the treatment of metastatic CRC (mCRC) than irinotecan alone, the overall therapeutic response rate to combined cetuximab therapy is less than 30%, suggesting that there are escape mechanisms present in many cases of CRC [5, 6]. Among others, mutation of the genes encoding the Kirsten rat sarcoma viral oncogene homologue (KRAS) and the V-raf murine sarcoma viral oncogene homolog B1 (BRAF) was established as two of these mechanisms and preselection of CRC patients with a KRASwt genotype have been shown to increase the therapeutic efficacy of anti-EGFR therapy [7, 8]. Therefore, clinical trials involving anti-EGFR therapy are now commonly conducted with patients preselected for KRASwt mutation status...
Table 1: Primer data for sequencing and allele specific PCR for KRAS exon 2 and BRAF exon 15 mutation analysis.

<table>
<thead>
<tr>
<th></th>
<th>Primer data</th>
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<tbody>
<tr>
<td>KRAS d1</td>
<td>5′-GAG TTT GTA TTA AAA GGT ACT</td>
</tr>
<tr>
<td></td>
<td>GG-3′</td>
</tr>
<tr>
<td>d2</td>
<td>5′-TAC TGG TAG AGT ATT TGA TAG</td>
</tr>
<tr>
<td>r1 + r2</td>
<td>5′-CTG TAT CAA AGA ATG GTC CTG-3′</td>
</tr>
<tr>
<td>BRAF</td>
<td>5′-TGC TTG CTC TGA TAG GAA AAT-3′</td>
</tr>
</tbody>
</table>

To ensure that therapy targeting EGFR is effective in primary CRC as well as in corresponding metastases, various studies have examined the concordance or discordance of KRAS and BRAF mutation status in primary CRC and corresponding metastases. Although the results of these studies appear contradictory in part, the majority of authors report high rates of concordance between the mutation status of KRAS in primary tumors and corresponding metastases [4, 9, 11–16]. In a study published recently by our group, KRAS mutation status was monitored in the primary tumors and corresponding metastases of 106 cases of mCRC [17]. Here, we found concordance in the mutation status of KRAS in 105 of 106 cases (Figure 1) and were able to show that the only case of discordance was due to a tumor mosaic or the coexistence of multiple primary tumors (Figure 2), a fact that could help in explaining the partially contradicting results reported in the past [13, 17]. Moreover, in analogy to other types of cancer [18–20], therapy-related resistance based on a treatment-induced shift in KRAS and/or BRAF mutation status could also play a role in explaining the low therapeutic efficacy of anti-EGFR therapy in mCRC by rendering tumor cells initially responsive to anti-EGFR mAbs resistant to this therapeutic regimen [9].

2. Materials and Methods

49 individual specimens of 21 metastatic CRCs and corresponding metastases collected before and after combined therapy with cetuximab were examined using CGH, certified PCR/DNA sequencing protocols (KRAS exon 2, G12/13; BRAF exon 15, V600E) as well as allele-specific PCR [8].

The majority of samples analyzed in this approach derived from the sample pool of 106 mCRC with 270 syn-/metachronic metastases used in our earlier study on the concordance of KRAS mutation status in primary CRC and corresponding metastases [17]. Biopsy sets were collected before and after combined cetuximab therapy, whereby the samples collected before therapy were taken from primary CRCs and/or liver metastases, while those gathered after therapy were from metastases in different locations, predominantly the liver (Table 2). Each CRC studied was clinically documented as a single primary malignant tumor in the colon/rectum and all metastases were identified as such by a characteristic “CRC-like” immunohistochemical profile.
(cytokeratin 20 positive, cytokeratin 7 negative). Following
pathohistological characterization, tumor cells were enriched
to >90% from 4–6 10 μm slices using microdissection. Two
independent samples from each specimen were incubated
overnight in lysis buffer containing proteinase K at 56°C
and DNA was subsequently extracted by column affinity
chromatography (Qiagen DNA Minikit, Cat No 51306).

CGH was performed as previously described [17].
Briefly, DNA was labeled by nick translation with biotin-
16-dUTP (Roche Diagnostics, Mannheim, Germany). After
inactivation of DNase I (Roche Diagnostics, Mannheim,
Germany) equal amounts (1 μg) of tumor and refer-
ence DNA (DIG-labeled DNA from placental tissue of a
healthy newborn) were cohybridized on metaphase slides
(Vysis, Downers Grove, IL). Signals were visualized with
a Zeiss Axioshot fluorescence microscope and analyzed
with the ISIS digital image analysis system (MetaSystems,
Altluessheim, Germany).

K R A S and B R A F mutation analyses were performed on
two independent samples from all primary tumors/
metastases using protocols described previously [8]. Briefly,
DNA was amplified using Taq DNA Polymerase (Invitrogen
Cat. No. 10342-020) and allele specific primers (Eurofins, see
Table 1). Amplificates were visualized in 2% agarose gels and
purified from the gels using column affinity chromatography
(QIAquick Gel Extraction Kit, Qiagen Cat. No. 28704).
Sequencing PCR was performed using PCR primers (see
Table 1) and ABI BigDye Terminator v3.1 Cycle Sequencing
RR-100 (ABI Heidelberg, Germany) as described [17].

3. Results

In the first part of our study we analyzed 106 metastatic CRCs
with at least 2 multifocal and syn-/metachronic metastases
(n = 270) using PCR/DNA sequencing protocols certified
by the German Society for Pathology (exon 2, Glycin12 and
Glycin13) as well as allele-specific PCR approaches. As shown
in Figure 1, we observed concordance of the K R A S mutation
status between primary CRCs and all corresponding metas-
tases in 105 of 106 patients. However in one case (Figure 1
#43) of a K R A S mutation G12V positive moderately dif-
ferentiated and undifferentiated primary CRC (Figure 2(a)),
the mutation was detectable in soft tissue and peritoneal
metastases with infiltrates from the undifferentiated tumour
fraction (Figures 2(b) and 2(c)), but not in moderately
differentiated lymph node and liver metastases (Figures
2(e) and 2(f)). Microdissected subfractions of the primary
heterogeneous CRC showed a corresponding mutational
mosaicism with detection of K R A S mutation G12V only in
the undifferentiated tumour areas (Figures 2(d) and 2(g)).

In the second part of the study we addressed the
question, whether combined cetuximab therapy might influence
the K R A S/B R A F mutation status in mCRC. Therefore
16 patients from the described sample pool (Figure 1) as
well as 5 novel patients (including case #4, see Table 2)
suffering from mCRC were analyzed for the K R A S/B R A F
mutation status using the above mentioned molecular and
immunohistochemical techniques.

Figure 2: Morphological changes and results of K R A S mutation
analyses in case #43 of a heterogeneously differentiated CRC with
mosaicism for K R A S mutation G12D. In Figure 2(a) moderately
(right half) and undifferentiated (left half) tumor areas reveal
positive detection of K R A S mutation G12V (inset). In contrast,
only in soft tissue (b) and peritoneal (c) metastases harbouring
exclusively undifferentiated tumor infiltrates an identical K R A S
mutation was detectable ((b) and (c) insets), whereas in lymph
node (e) and liver (f) metastases with moderately differentiated
tumor infiltrates only, no K R A S mutation was found ((e) and
(f) insets). After microdissection of undifferentiated (d) and
moderately differentiated (g) areas from primary CRC the K R A S
mutation G12V was only detectable in the undifferentiated fraction
((d), inset). Images were produced with a B × 50 microscope
(Olympus, Hamburg, Germany) and a DP50 digital camera with
DP-Soft 5.0 software (Olympus, Hamburg, Germany).
Table 2: Overview of the clinical patient data. 20 of 21 patients show concordance of the KRAS (exon 2, Gly 12/13) and BRAF (exon 15, V600E) mutation status between samples of primary CRCs and/or corresponding metastases before and after combined cetuximab therapy. In one case (#4), the primary CRC had a mutated KRAS gene (Gly12Asp), while the liver metastasis biopsied after combined cetuximab therapy showed a KRAS<sup>wt</sup> genotype. BRAF mutation status in this case was concordant between the samples gathered before and after anti-EGFR therapy.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Sex/Age</th>
<th>Date and localisation of tumor manifestation</th>
<th>KRAS Gly12/13 (exon2)</th>
<th>BRAF V600E (exon15)</th>
<th>Anti-EGFR therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/51 y</td>
<td>09/05 primary CRC</td>
<td>Gly12Val</td>
<td>WT</td>
<td>01/07–04/07 Folfiri/Cetuximab (PD)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11/07 small bowel metastasis</td>
<td>Gly12Val</td>
<td>WT</td>
<td>dead 05/08</td>
</tr>
<tr>
<td>2</td>
<td>M/71 y</td>
<td>11/02 soft tissue</td>
<td>Gly12Cys</td>
<td>WT</td>
<td>09/04–08/05 Folfiri/Cetuximab (PR)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>03/06 mesocolon transversum metastasis</td>
<td>Gly12Cys</td>
<td>WT</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>M/46 y</td>
<td>03/07 primary CRC</td>
<td>WT</td>
<td>WT</td>
<td>08/07–06/08 Folfiri/Cetuximab (PR)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>02/09 peritoneal carcinoma</td>
<td></td>
<td>WT</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M/68 y</td>
<td>11/06 primary CRC</td>
<td>Gly12Asp</td>
<td>WT</td>
<td>04/07–02/08 Fufox/Cetuximab (PR)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>03/08 liver metastasis</td>
<td>WT</td>
<td>WT</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>F/56 y</td>
<td>07/08 primary CRC</td>
<td>Gly12Asp</td>
<td>WT</td>
<td>07/08–09/08 Fufox/Cetuximab (PD)</td>
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<tr>
<td></td>
<td></td>
<td>12/08 peritoneal carcinoma</td>
<td>Gly12Asp</td>
<td>WT</td>
<td>dead 12/08</td>
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<tr>
<td>6</td>
<td>M/71 y</td>
<td>12/05 primary CRC</td>
<td>Gly12Val</td>
<td>WT</td>
<td>01/06–07/06 Folfiri/Cetuximab (PD)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12/06 liver metastasis</td>
<td>Gly12Val</td>
<td>WT</td>
<td>dead 04/08</td>
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<tr>
<td>7</td>
<td>F/58 y</td>
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<td>WT</td>
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<tr>
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<td></td>
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<td>WT</td>
<td>WT</td>
<td>dead 07/08</td>
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<tr>
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<td>WT</td>
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<td></td>
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<td>WT</td>
<td>WT</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>F/66 y</td>
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<td>Gly12Ser</td>
<td>WT</td>
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<tr>
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<td></td>
<td>11/05 + 04/06 liver metastasis</td>
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<td>WT</td>
<td>07/06–09/06 Folfiri/Cetuximab (PD)</td>
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<td></td>
<td></td>
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<td>Gly12Ser</td>
<td>WT</td>
<td>09/06–11/06 Folfiri/Avastin (PD)</td>
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<td>Gly13Asp</td>
<td>WT</td>
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<td></td>
<td></td>
<td>07/06 lymph node</td>
<td>Gly13Asp</td>
<td>WT</td>
<td>10/06–02/07 Folfiri/Avastin (PD) dead 05/07</td>
</tr>
<tr>
<td>11</td>
<td>M/63 y</td>
<td>05/07 primary CRC</td>
<td>WT</td>
<td>WT</td>
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<tr>
<td></td>
<td></td>
<td>01/08 liver metastasis</td>
<td>WT</td>
<td>WT</td>
<td>10/08–05/09 Folfiri/Avastin (PR)</td>
</tr>
<tr>
<td>12</td>
<td>M/67 y</td>
<td>07/07 liver metastasis</td>
<td>WT</td>
<td>WT</td>
<td>08/07–04/08 Folfiri/Cetuximab (PR)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>01/08 liver metastasis</td>
<td>WT</td>
<td>WT</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>M/59 y</td>
<td>09/07 primary CRC</td>
<td>WT</td>
<td>WT</td>
<td>11/07–03/08 Folfiri/Cetuximab (PR)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10/07 liver metastasis</td>
<td>WT</td>
<td>WT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>05/08 liver metastasis</td>
<td>WT</td>
<td>WT</td>
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</tbody>
</table>
We hereby observed a concordance in the \textit{KRAS} and \textit{BRAF} mutation status between samples from primary CRCs and/or corresponding metastases before and after combined cetuximab therapy in 20 of 21 patients (Table 2). The timespan between first and last sampling ranged between 4 and 81 months (average 25.8 months) and the patients involved in the study received 1 to 5 rounds of therapy (average 1.8 rounds) during this period.
Figure 3: Further analysis of biopsies from the patient with a discordant KRAS mutation status before and after anti-EGFR therapy (#4, see Table 2) showing varying morphology between primary CRC with papillary tissue organization (a) and liver metastasis with tubular tissue organization (b). KRAS mutation analysis of corresponding specimens demonstrate detection of KRAS mutation exon 2 Gly12D (c) in primary CRC whereas an unmutated KRAS status in the liver metastasis was detectable (d). In fluorescence scan and data analysis of CGH only in the liver metastasis biopsied after treatment (f) but not in the primary CRC (e) a decrease in fluorescence of chromosomes 4 and 6 and an increase of chromosome 20 (arrows) could be seen.
In the one case of discordance between KRAS mutation status before and after combined cetuximab therapy (case #4), a mutated KRAS gene (Exon 2 G12D) was found in the primary CRC, while no KRAS mutation was observed in a liver metastasis sample obtained after combined cetuximab therapy (Table 2). Further analysis of the biopsy samples in this patient revealed that the primary CRC sampled before therapy and the liver metastasis biopsied after therapy showed differences not only in their KRAS mutation status but also in morphology and overall genetic composition (Figure 3). While the primary tumor was predominantly organized in a papillary fashion (Figure 3(a)), the liver metastasis displayed a distinctly tubular organization (Figure 3(b)). As mentioned above, microdissected samples gathered from each of these specimens showed distinct genetic sequences in exon 2 of the KRAS gene. While a mutated KRAS gene (Exon 2 G12D) was observed in the primary CRC (Figure 3(c)), a KRAS WT genotype was found in the liver metastasis (Figure 3(d)). Moreover, comparative genomic hybridization performed on the samples revealed that each of these displayed different genetic alterations. In comparison to the primary CRC (Figure 3(e)), samples from the liver metastasis showed sequence losses in chromosomes 4 and 6, while gains were observed in chromosome 20 (Figure 3(f)).

4. Discussion

Development of therapy-related resistance is a frequent phenomenon in cancer and also occurs in the context of novel drugs such as monoclonal antibodies and tyrosine kinase inhibitors. In gastrointestinal stromal tumors (GISTs), the most common mesenchymal neoplasm of the gastrointestinal tract, mechanisms of resistance to imatinib mesylate (Gleevec®) include both de novo and, more frequently, acquired resistance, which may occur after several months of drug administration and most often is based upon an acquired second mutation in the c-kit and PDGFRα genes [18, 19]. In B-cell lymphoma, resistance to the chimeric anti-CD20 monoclonal antibody rituximab, the first monoclonal antibody to have been registered for the treatment of B-cell lymphomas, is suggested to be due to reduced expression of CD20, the failure of rituximab to trigger the cells leading to inhibition of antibody-dependent and complement-dependent cell toxicity (ADCC and CDC), as well as hyperactivation of antiapoptotic signaling pathways such as p38 MAP kinase, NF-kappaB, ERK1, and AKT [20].

In the first part of our study we analyzed a well-documented cohort of 106 metastatic CRCs with 270 synchronous/metachronous metastases concerning the KRAS mutation status showing an overall concordance of the KRAS mutation status in primary CRCs and metastases in 99% of the cases examined that stands in good accordance to previous reports [21]. However, apart from potentially coexisting secondary malignancies, the KRAS mutation mosaicism found in one heterogeneous differentiated CRC (#43, Figures 1 and 2) may explain the discordant results concerning this trait in primary CRCs and metastases reported previously [12, 13, 22] and underlines the necessity for diligent clinical and histological characterization of any atypical tumour manifestation in mCRC to prevent misleading results with negative impact on anti-EGFR targeted therapies.

The second and major part of our study was to investigate whether therapy-related resistance due to acquired second KRAS/BRAF mutations also occurs in metastatic colorectal cancer after anti-EGFR therapy. Therefore we analyzed 49 individual specimens from 21 mCRC collected before and after combined cetuximab therapy. Of the 21 patients examined in this study, 20 showed concordance between the KRAS mutation status before and after combined cetuximab therapy, while the rate of concordance for BRAF was 100%. In one patient (case #4, Table 2) that did not derive from the above mentioned sample pool of 106 mCRC, the KRAS mutation status was discordant between the samples collected before and after cetuximab therapy, but due to further analysis of these samples with clearly different morphological and genomic features (Figure 3) it might be suggested that populations of carcinoma cells heterogeneous with respect to wild-type and mutant KRAS were probably present in the primary carcinoma, but the metastatic clone derived from a KRAS negative population, as reported previously [22, 23].

The results of this study provide first evidence that secondary KRAS/BRAF mutations do not play a major role in therapy-related resistance to anti-EGFR antibody treatment in mCRC, although it cannot be excluded that KRAS/BRAF mutations beyond Glycin12/13 KRAS exon2 and V600E BRAF exon15 as well as secondary resistance due to combinational chemotherapies (as in most patients investigated) are responsible for therapy-related resistance in mCRC. Moreover, as the preselection of patients with a KRAS WT genotype for cetuximab therapy has become a standard procedure, sample sets such as the one used in this study will have to be conserved carefully for use in future studies particularly with respect to analyses addressing the identification of anti-EGFR therapy-induced genetic alterations apart from KRAS/BRAF mutations.

Acknowledgments

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