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Original article

Influence of mucinous and necrotic tissue in colorectal cancer samples on *KRAS* mutation analysis

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ABSTRACT

Evaluation of the *RAS* mutation status is necessary for patients with advanced colorectal cancer to predict the response to anti-EGFR therapy. In routine diagnostics, FFPE tissue samples are tested by sequencing (amplicon-based NGS and Sanger) to obtain the *RAS* status of the patient. Samples that are collected after chemotherapy occasionally contain necrotic tissue. Furthermore, colorectal cancer tissue sometimes has mucinous components. This may pose a challenge to molecular analysis because mucinous tumor samples often contain only few tumor cells compared to solid tumor samples. Therefore, the aim of this study was to explore if mucin or necrosis affect mutation analysis and if mucinous tumor samples contain enough tumor cells for reliable mutation detection. To this end, we analyzed *KRAS* status in 10 samples showing mucin production and 10 samples with necrosis. In all 20 samples the tissue areas with the highest amount of mucin or necrosis were used for re-evaluation. These results show no differences with those obtained during routine diagnostics, where analysis of mucinous or necrotic areas was tried to avoid. Our study thus shows that mucin and necrosis have no influence on *KRAS* mutation analysis. Furthermore we were able to demonstrate that mucinous adenocarcinoma contains enough tumor cells for a valid mutation analysis. In addition, we also observed only minor differences in *KRAS* status results when comparing Sanger sequencing with NGS. Both methods detected the *KRAS* mutation in 19 of 20 tested samples, even for mutated samples with low allele-frequencies.

1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies in Europe and the third most common type of cancer in the world. There are more than one million new cases every year [1]. Based on the knowledge of predictive or prognostic gene mutations there are several personalized therapies recommended for CRC patients. The major targeted therapeutic approach is still to block the epidermal growth factor receptor (EGFR) [2]. Cetuximab or panitumumab are examples for EGFR antibodies, which are accepted by the WHO since 2008 as treatment of metastatic CRC without an activating mutation in the *KRAS* gene [3–5]. In CRC, the frequency of *KRAS* mutations in exon 2 is about 40% [4,6,8]. Later studies showed that *KRAS* exons 3, 4 and *NRAS* exons 2, 3 and 4 can also hold an activating mutation in colorectal cancer that affects about 5–10% of CRCs. [8–11]. Thus, approximately 50% of the patients may benefit from an anti-EGFR therapy. Therefore, evaluation of the *RAS* mutation status is necessary to predict the response to anti-EGFR agents.

The gold standard to detect mutations in FFPE material used to be Sanger sequencing. However, Sanger sequencing has already been replaced by Next Generation sequencing (NGS) in many routine laboratories to improve the detection limit and to facilitate the parallel evaluation of multiple genes.

Many studies exist that deal with improving the detection limit of mutation analyses [12–15] and it is already known that many factors have a negative influence on DNA/RNA-based techniques. One of the most important factors is the tissue fixation time in formalin [16], because formalin treatment can cause DNA-fragmentation and DNA-crosslinking [17]. Further factors affecting the quality of the tested tissue with respect to sequencing results, are melanin, collagen or hematin [18,19]. Biological parameters such as sample size, tumor viability, number of tumor cells or technical factors such as decalcification can influence the mutation analysis as well [20].

Furthermore, 10–15% of colorectal cancers are mucinous [21]. Mucinous adenocarcinoma is defined by a mucinous tumor com-

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ponent of at least 50% of the whole tumor. These mucin-rich tumor regions typically contain only very few tumor cells. Another property potentially relevant to sequencing analysis is necrosis. Necrotic tumor tissue often contains cell debris, non-viable tumor cells and fragmented DNA. Therefore, mucinous and necrotic cancer samples often contain only few viable tumor cells compared to solid tumor samples.

Most of the mentioned parameters are well investigated and many recommendations exist on how to remove, for example, potential PCR inhibitors [18,19] or how to avoid fixation artefacts by using novel methods instead of formalin fixation [17]. Nevertheless, only limited data are available about the influence of mucin and cell debris/necrosis on mutational analysis.

The aim of this retrospective study was to explore if mucin and necrosis affect *KRAS* mutation analysis and whether these samples contain enough tumor cells to detect a mutation. To this end, we re-evaluated 10 CRC samples with mucin and 10 CRC samples with necrosis with known mutations of the *KRAS* gene, exon 2, which had been previously detected during routine diagnostics, where the tissue area with as many viable tumor cells as possible were used and mucinous or necrosis areas were tried to avoid.

2. Materials and methods

2.1. Materials

Specimens were selected from FFPE colorectal tissue samples, which had undergone routine analyses for *KRAS* or *RAS* mutations between June 2011 and November 2015 in the laboratory for molecular pathology, Institute of Pathology, Charité, Berlin. During this period, 844 in house resection CRC samples had been analyzed out of which 340 harbored a mutation in the *RAS* gene. Among these 340 mutated CRC samples, 20 representative samples were selected for re-evaluation: 10 mucinous samples and 10 samples with necrosis.

2.2. Sample preparation

According to *RAS* routine diagnostic procedure, a pathologist marked the area with highest density of tumor cells on a haematoxylin & eosin (H&E) stained slide of the FFPE tissue block. The H&E stained slide of each sample was used to estimate the tumor cell content and the amount of tumor cells in the encircled area for routine diagnostic and for re-evaluation. For re-evaluation of the necrotic tumor samples only the vital tumor cell content and the amount of viable tumor cells has been estimated.

For re-evaluation of the *RAS* mutational status of the 20 selected samples, the pathologist identified and encircled the tumor region with the maximum amount of mucin or necrosis present in the tissue sample.

For the 10 selected mucinous carcinoma samples only for samples 1–6 a new area has been marked. Samples 7–10 were pure mucinous carcinoma samples and contained more than 80% mucin within the tumor area. Therefore, the area used for routine diagnostic included already the maximum amount of mucin and only few tumor cells.

For the 10 selected necrotic carcinoma samples in all 10 samples a new area with a high amount of necrosis has been encircled for re-evaluation. In the samples 11–14 and 19–20 the new marked area was already part of the larger area used during routine diagnostic. For the samples 15–18 the new marked areas were completely different from those used for routine diagnostic.

For samples 17–20 the re-evaluation of the small high necrotic area was first analyzed by NGS, followed by Sanger Sequencing, whereas samples 11–16 were re-evaluated first by Sanger sequencing followed by NGS.

2.3. DNA preparation

2.3.1. For sanger sequencing

Depending on the size of the encircled tissue area, three to fifteen 3 μm thick unstained paraffin sections were incubated at 80 °C for 15 min and afterwards twice in xylene for 5 min and twice in ethanol for 5 min to remove the paraffin. The encircled tissue area was transferred into a 1.5 ml tube with 180 μl ATL buffer and the DNA preparation was performed in accordance with tissue protocol QIAamp DNA Mini Kit (Qiagen). Finally, the DNA was eluted in a volume of 50–100 μl elution buffer depending on the size of the microdissected tumor area. The DNA content was determined by Nanodrop spectrophotometry.

2.3.2. For amplicon-based parallel sequencing (Next Generation Sequencing, NGS)

Two to twelve 3 μm thick unstained paraffin sections were incubated at 80 °C for 15 min and afterwards in xylene for 5 min and in ethanol for 5 min to remove the paraffin. The encircled tissue area was transferred into a 1.5 ml tube with 180 μl incubation buffer and the DNA preparation was performed using Maxwell 16 System Instrument together with the Maxwell 16 FFPE Plus LEV DNA purification Kit (Promega) in accordance with manufacturer's instructions. After the elution of the DNA in a volume of 30–50 μl elution buffer, the DNA content was determined by Qubit™ Fluorometer by using Qubit™dsDNA high sensitivity assay according to manufacturer's instruction.

2.4. Sanger sequencing

A 190 bp PCR fragment of the *KRAS* exon 2 gene was generated with the specific primer pair including a T7 universal primer at the forward primer (by Tib Molbiol Berlin, Germany):

KRAS-T7-F: 5′-GCGTAATACGACTCACTATAAGGCCTGCTGAAAATGACT-3′ and

KRAS R: 5′-AGAATGGTCCTGCACCAGTAA-3′

2.5 μg of genomic DNA was used for the PCR under following conditions: initial denaturation for 5 min at 95 °C, annealing at 60 °C for 1 min, elongation at 72 °C for 1 min, denaturation at 94 °C for 1 min, 40 cycles, final elongation for 7 min at 72 °C.

PCR fragments were purified by MSB Spin PCRAPACE (Stratec, Berlin, Germany) or by QIAquick PCR Purification Kit (Qiagen) according to the user manual. To check the quality of the PCR product 10 μl of the PCR product were loaded on a 3.3% agarose gel. Depending on the thickness of the DNA fragment on the agarose gel 0.5 μl–5 μl of the PCR product were used for the following sequencing reaction with Big Dye Terminator cycle sequencing mix v1.1 (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions and by using the following sequencing primers (by Sigma-Aldrich, Taufkirchen, Germany) for *KRAS*-F: 5′-GCGCGTAATACGACTCACTATA-3′ and *KRAS*-R: 5′-AGAACTGGTCCTGCACCAGTAA-3′ for both DNA strands. The PCR was performed under the following conditions: initial denaturation for 1 min at 96 °C, denaturation at 96 °C for 10 s, annealing and elongation at 60 °C for 2 min, 35 cycles. Dye purification was done by alcohol/sodium acetate precipitation.

Sequencing analysis was performed on a 3100 AVANT or ABI 3730XL genetic analyzer.

The raw data of Sanger sequencing were analyzed using the software sequencing analysis v5.1.1 and SeqScape v2.1.1 or v2.6 (both Applied Biosystems).

2.5. Next generation sequencing

For NGS the Ion AmpliSeq™ Colon and Lung Cancer Panel (Life Technologies) was used. This panel includes 22 genes and 90 ampli-