Original Article

# KRAS and aneusomy of chromosomes 4, 10 and 12 in colorectal carcinomas

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ABSTRACT

*Aims:* KRAS mutation status has predictive significance in EGFR-antibody treatment of colorectal adenocarcinoma. The aim of the study was the evaluation of KRAS mutation status in correlation to KRAS copy numbers and ploidy status.
*Methods:* Colorectal adenocarcinomas ($n = 52$) were assembled into a TMA and analyzed by FISH. Probes for centromeres 4 and 10 were applied as surrogate markers for the ploidy status. In addition, a dual color FISH probe set for the centromere of chromosome 12 and the KRAS gene was applied to the TMA to analyze numerical alterations and KRAS gene copy numbers. Further we analyzed DNA sequence profiles of KRAS codons 12 and 13 to assess the allele status of the mutation within the tumor samples.
*Results:* KRAS mutation was confirmed in 24 cases, while 28 cases showed a wild-type KRAS status. The majority of cases showed diploid FISH signals for chromosomes 4 and 10. Near triploid FISH signals were observed in only 2 cases, 12 cases were hypodiploid, and 8 cases were hyperdiploid. In 6 cases, trisomy 12 could be ascertained. In total, aneuploidy could be detected in 28 cases, including cases with trisomy 12 and hyposomy 10. Tumors with aneuploid chromosomal content had a worse prognosis compared to euploid tumors, however, without reaching statistical significance ($p = 0.231$). Hypodiploid carcinomas carried the worst prognosis. Specifically, monosomy 10 was significantly associated with reduced survival ($p = 0.039$). Increased FISH signals of KRAS did not correlate significantly with relapse ($p = 0.916$).
*Conclusions:* FISH analysis can be used as a surrogate marker for the ploidy status. Loss of chromosome 10 may serve as a potential adverse prognostic marker being indicative for tumor progression.

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## 1. Introduction

The development of colorectal carcinoma (CRC) is a multistep process known as adenoma-carcinoma sequence. It is initiated by benign adenoma, leading to malignant invasive and finally metastatic cancer. Cancer progression is characterized by an accumulation of mutations of proto-oncogenes and tumor suppressor genes, as well as the emergence of chromosomal instability [6].

Aneuploidy is commonly understood as an abnormal number of chromosomes in a cell, but the term may also describe structural aberrations of chromosomes that lead to gain or loss of genetic material being detectable by DNA content analysis [20]. Aneuploidy is a synonym for genomic imbalances being distinct from normal or polyploid chromosome numbers. In general, the extent of aneuploidy is associated with a poor prognosis in carcinomas [17]. With regard to colorectal cancer, a meta-analysis suggested that aneuploid tumors are associated with a more advanced pathologic stage and a higher risk of death [3].

KRAS is a small GTPase, playing a major role in the EGFR-MEK-MAP kinase pathway. The KRAS gene belongs to the family of RAS oncogenes and is located on the short arm of chromosome 12 (12p.12.1). Gain of function mutations impair the intrinsic GTPase activity of KRAS and lead to constitutive, receptor-independent activation of downstream signaling.

As a crucial part of the EGFR-signaling pathway, RAS is not only involved in transmitting growth signals but also in the regulation of the cell cycle by controlling cyclin-levels via the MAP kinase pathway [2]. Defects in cell-cycle checkpoint components contribute to genetic instability [26].

KRAS mutations are detectable relatively early in colon carcinogenesis [16] and are found in about 58% of adenomas larger than
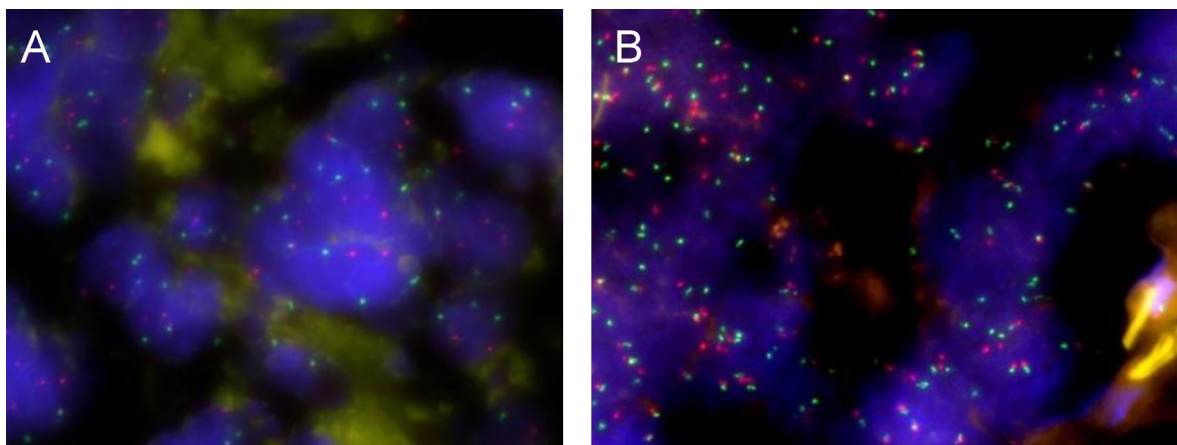
**Fig. 1.** Representative examples of the FISH-analysis. (A) KRAS wild-type triploid colon carcinoma showing three CEN4 (green) and CEN10 (orange) signals; (B) KRAS mutated colon carcinoma with polysomy of chromosome 12 showing increased KRAS signals (green) and CEN12 signals (orange). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

1 cm [22]. In addition, they may be present in lesions that have only minimal potential to develop into a malignant tumor, i.e. hyperplastic polyps or aberrant crypt foci [7]. However, KRAS mutations are also relevant in tumor progression and seem to be associated with distant metastases [14].

A meta-analysis concluded that KRAS mutation status does not correlate with prognosis [16]. Nevertheless, subgroup analyses could show a significant reduction of the overall survival in patients with mutated KRAS codon 12. So far, the role of KRAS mutation status in tumor progression and survival time is inconclusive.

Multiple molecular markers in colorectal adenocarcinomas have been identified, but so far only KRAS mutation status has predictive significance being related to the treatment with EGFR-antibodies [6]. The possibility of this treatment modality seems to have a favorable prognostic impact in patients with left-sided primary tumors [23].

The main objective of our study was to gain a better understanding for the pathology of colorectal cancer regarding the impact of chromosomal changes. We therefore aimed to analyze the correlation between KRAS mutation status, KRAS copy number and ploidy. In order to assess the importance of chromosomal changes in tumor characteristics and progression, we correlated the KRAS status and ploidy with clinical parameters, such as tumor staging, grading and overall survival time.

## 2. Materials and methods

### 2.1. Patient eligibility

Formalin-fixed, paraffin-embedded tumor samples were derived from 52 patients who mostly underwent surgical tumor resection at the clinics for visceral surgery of the Jena University Hospital and the St. Georg Klinikum Eisenach between 2004 and 2010.

The patients were between 40 and 89 years of age and had histologically proven colorectal adenocarcinomas. The mean age was 68. Among them, 38% of the patients were female. The KRAS mutation status was determined by polymerase chain reaction (PCR) following Sanger DNA sequencing. The patient collective was selected to create almost equal sample sizes for mutant and wild-type KRAS status.

### 2.2. Methods

Genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tumor samples by using a QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Before DNA isolation, suitable areas in hematoxylin and eosin (H&E) sections were marked by a pathologist. Manual microdissection was performed to minimize the contamination from normal tissues.

Genomic DNA isolated from the patient samples was subjected to mutation analysis. The sequences containing the hotspot mutations including exon 2 (codons 12 and 13) of KRAS were amplified by PCR using gene-specific primers (sense: 5′-GTGTGACATGTTCT-AATATAG-3′; anti-sense: 5′-TTGTTGGATCATATTCGTCC-3′).

PCR conditions were 95 °C for 15 min, 38 cycles of 94 °C for 1 min, 55 °C for 45 s and 72 °C for 30 s with a final extension at 72 °C for 10 min. The PCR products were purified using a PCR purification kit (ZYMO Research, Germany), and 100 ng of purified PCR products were bidirectionally sequenced based on capillary electrophoresis (LGC Genomics, Germany).

The paraffin embedded tumor samples were then assembled into a tissue microarray (TMA) [5] and analyzed by fluorescence in situ hybridization (FISH). We conducted an enzymatic digestion for six minutes in order to minimize connective tissue-related background artifacts.

A KRAS/Centromere (CEN) 12 dual color FISH probe (ZytoLight SPEC KRAS/CEN 12 dual color probe, ZytoVision, Bremerhaven, Germany) was used to detect numerical alterations of chromosome 12 as well as the copy number status of the KRAS gene. In addition, a CEN4 and CEN10 dual color FISH probe set (ZytoLight CEN 4/10 dual color probe, Zytovision, Bremerhaven, Germany) was applied as a surrogate marker for the ploidy status (Fig. 1). We assumed aneuploidy when the mean number of CEN4 and CEN10 signals per cell fell below 1.8 or exceeded 2.2 (hypodiploid: ≤1.8; diploid: >1.8 and ≤2.2; hyperdiploid: >2.2 and ≤2.5; triploid: >2.5).

We counted 20–30 cell nuclei of every tumor sample to establish mean values of the centromere probes and the KRAS gene.

Further we analyzed DNA sequence profiles of KRAS codons 12 and 13 to assess the allele status of the mutation (heterozygous or homozygous, relative quantitation of both alleles by comparison of the profiles of each nucleotide) within the tumor sample (Fig. 2).

### 2.3. Statistical analysis

The clinical information was derived retrospectively from the patients' medical records. All but three patients were observed for at least 4 years after primary tumor resection or until death.

We used the log-rank test to analyze differences in overall survival. Associations between KRAS mutation status and tumor