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ORIGINAL ARTICLE

Mutant KRAS Status Is Associated with Increased KRAS Copy Number Imbalance: a Potential Mechanism of Molecular Heterogeneity

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Abstract Mutation rates determined by allele-specific PCR can be highly different in KRAS exon 2 mutant colorectal carcinoma (CRC) samples suggesting intratumoural heterogeneity. To address the effect of KRAS gene copy number on the relative mutant allele frequency the KRAS locus was individually quantified following FISH analysis in 36 cases. We observed, that mutant KRAS status was associated with an elevated KRAS locus number (2.36 ± 0.42 vs 2.63 ± 0.75; $p = 0.037$) reflecting an increased aneuploidy status but no true amplification of the locus. In parallel, KRAS locus copy numbers showed significant intercellular variability (1–16 copies/cell nucleus) within individual tumours also indicating to a dynamic intratumoural oscillation of the mutant allele copy number. In conclusion, aneusomy is a common feature of KRAS mutant CRC and KRAS copy number variations may have an impact on the relative mutant allele frequency detected by allele specific PCR/sequencing), potentially leading to subclonal diversity and influencing tumour behaviour.

Keywords Amplification · CEP12 · Colorectal cancer · Heterogeneity · KRAS

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Introduction

RAS oncogenic mutations are supposed to be an early event during carcinogenesis leading to a significantly increased RAS-GTPase activity and ultimately resulting in the constant activation of the RAS/MAPK pathway. Activating exon 2 mutations of the KRAS gene belong to the most frequent oncogenic molecular alterations in many different types of cancer including adenocarcinoma of the colon [1]. The link between *KRAS* gene mutations and decreased response to anti-EGFR agents was repeatedly confirmed [2–4]. Colorectal cancer (CRC) patients with no abundance of RAS mutations on the other hand might benefit from EGFR antibody therapy [4, 5]. KRAS mutation testing is available in many forms and became a standard requirement in the clinical management of CRC. Followed by others, Yu and co-workers described the utility of the BNA-clamped allele specific-PCR method allowing the relative quantification of KRAS mutant alleles in tumour cell populations [6]. However, clamped PCR based mutation quantification demonstrates highly different mutation rates hardly to explain with individual tumour cell content variability present in the samples. Similarly, direct sequencing frequently reflects disproportional mutant/wild type allele composition [7].

In addition to oncogenic mutations, copy number aberrations (CNA) of whole chromosomes or chromosomal regions may occur reflecting chromosomal instability and mitotic failures as important mechanisms in colorectal cancer development [8]. Chromosome instability and missegregation related aneuploidy is frequent in CRC potentially also resulting in disproportional copy number changes of the KRAS gene locus located at the chromosome arm 12p. Ploidy status could therefore also affect the intensity of RAS-GTPase activity through the mutant KRAS gene dosage.

Currently little is known about the correlation between ploidy and the KRAS status in CRC. In our evolving concept

the proportion of the wild type and the mutant KRAS allele within single tumour cell nuclei can be different due to dynamic copy number changes and thus allelic heterogeneity may occur as a consequence of ploidy alterations. Hyperdiploidy is a common feature of CRC which can be demonstrated at single chromosomal level. Fluorescence in situ hybridization (FISH) is a potent tool to quantify the copy number of specific chromosomal loci in individual tumour cells, allowing the detailed characterization of ploidy status and intratumoural heterogeneity.

The aim of our study was to identify the relationship between KRAS copy number alterations individually determined in tumour tissue samples by FISH and evaluate its potential associations with the presence and rate of exon 2 codon 12/13 activating KRAS mutations in mCRC.

Methods

Patients and Samples

36 samples from patients with the diagnosis of metastatic CRC were divided into two study groups according to KRAS exon 2 codon 12–13 mutation status: 23 tumours carrying KRAS exon 2 codon 12/13 mutations and 13 control tumours with KRAS wild-type status in the primary tumour samples were compared. Male:female ratio was 1:1, median age of patients was 58 years. Clinicopathologic data including histologic grade, TNM stage, KRAS mutation status, mutation rate and KRAS genotypes were balanced between study groups

Surgical diagnostic samples were handled according to rules and regulations of the University of Debrecen, Medical Center, with the approval of the local ethics committee (file number: RKEB/IKEB 3856–2013; ETT HRB 48369–2/2013-her). Histological examination and material selection was done by trained pathologists. Tumour samples with the highest quality containing a large tumour proportion were included in the study.

RAS Mutation Analysis and Genotyping

Samples with tumour area ~ 1 cm^2 were selected for RAS mutation analysis. The tumour cell content was determined histologically as percentage of tumour cells within the selected sample.

DNA isolation was carried out from a 1x5μm thick FFPE slides after xylene deparaffination using a commercially available kit (Quick Gene DNA Tissue Kit, KURABO INDUSTRIES LTD., Osaka, Japan) according to manufacturer's instructions (OD260/OD280 = 1.5–2.1).

RAS mutation panel testing was performed by BNA (Bridged Nucleic Acid) clamped real time-PCR assay described by Orum et al. with modifications [9]. BNA/DNA complex can effectively block the formation of a PCR product when targeted against one of the intact PCR primer sites. On the contrary, amplification is enabled when target sequences differ by at least one base pair [9]. For PCR clamping 600 nM BNA (Bio-Synthesis Inc., Lewisville, TX 75057) specifically designed to identify exon 2 codon 12/13 nucleotide sequence of the wild type KRAS was added to the PCR mixture. PCR was performed on a LC480 Real Time PCR System (Roche Diagnostics GmbH, Mannheim, Germany) for 45 cycles followed by a 10 min elongation step at 72 °C. DNA samples were considered mutant when amplification occurred in the BNA clamped reaction. Mutation rate was calculated based on the parallel amplification curves as percentage of clamped (KRAS exon 2 mutant) and total KRAS PCR product. The percentage mutant DNA sequences was calculated as amount of KRAS mutant DNA/amount of total DNA [6].

Sanger sequencing of PCR products was performed in cases with ΔCt value higher than 5.26 (cutoff determined by ROC analysis) on a 310 Genetic Analyzer (Applied Biosystems, Foster City, Ca., USA) using the forward PCR primer and Big Dye Terminator chemistry. To exclude KRAS exon 3 and 4 as well as NRAS exon 2, 3 and 4 mutations, Sanger sequencing was performed in all wild type samples lacking KRAS exon 2 mutations following conventional PCR amplification using appropriate primers in a routine setting.

KRAS/CEP12 Copy Number Analysis by FISH

KRAS/CEP12 dual color FISH analysis was performed in samples processed to tissue microarray (TMA Master 1.0, 3D Histech, Budapest, Hungary). Three cores (Ø 1 mm) from different intratumoural localizations were processed from FFPE donor block obtained from all 34 cases. KRAS/CEP12 FISH analysis was successfully performed using KRAS locus and CEP12 centromere specific oligonucleotide based high-resolution FISH probes (Agilent Technologies Inc., USA) according to the manufacturer's protocol. Deparaffinised 5 μm thick TMA sections (NeoClear xylene substituent, Merck KGaA, Darmstadt, Germany) were pre-treated with MES (2-(N-morpholino)ethanesulphonic acid, DAKO, Glostrup, Denmark) buffer followed by proteolytic digestion using pepsin (DAKO). After washing in 1 × wash buffer (DAKO) slides were dehydrated through a graded series of ethanol and the probe hybridization mixture was applied to the sections, coverslipped and sealed. Denaturation and hybridization were performed in a hybridization chamber (StatSpin ThermoBrite, Abbott Laboratories, Abbott Park, Illinois, USA). Slides were denatured at 90 °C for 5 min. Hybridization was performed overnight at 37 °C. Stringent wash was performed at 65 °C for 10 min (1 × stringent wash buffer, DAKO). Slides were dehydrated and covered with FISH Mounting Buffer (with DAPI, Agilent, Waldbronn, Germany) and coverglass. Signals were counted in 100 tumour cells/tissue core using Zeiss Axio Imager Z2

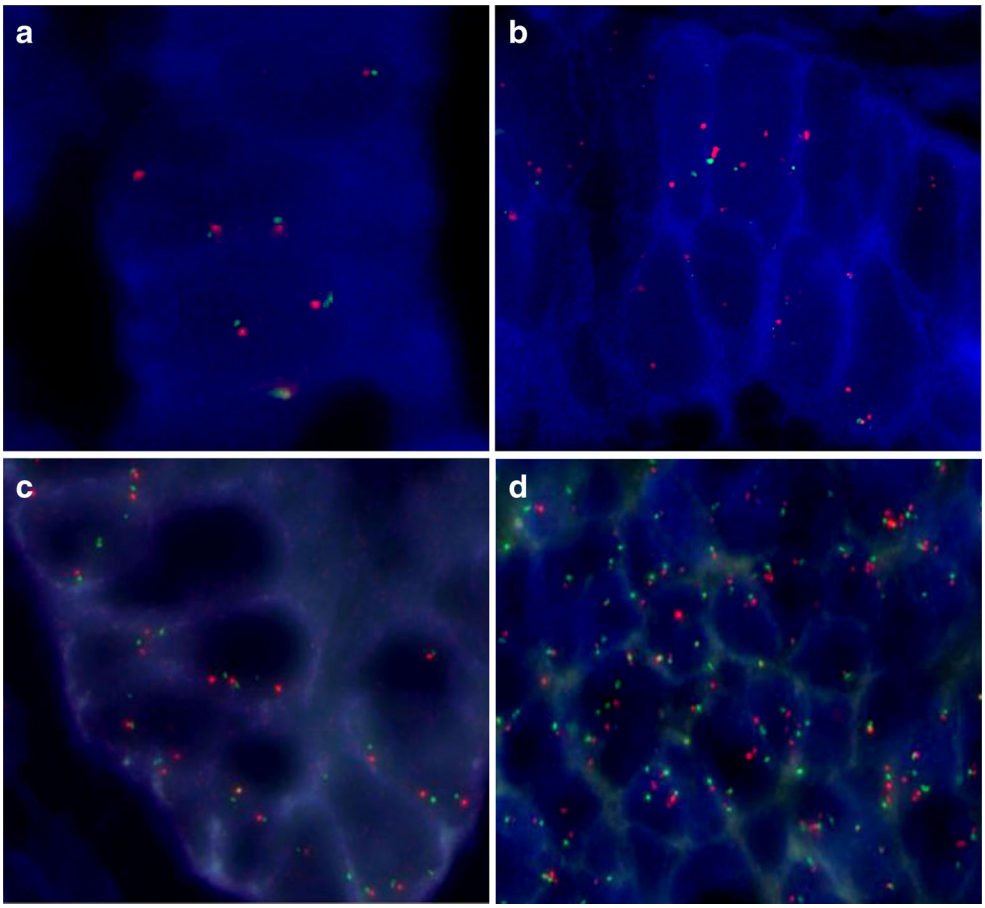

Fig. 1 Colorectal tumour samples analysed by FISH using KRAS (red) and CEP12 probes (green). (**a**) normal KRAS/CEP12 copy number, tumour is negative for KRAS gene amplification; (**b**) elevated KRAS copy number observed within the sample; (**c**) balanced KRAS/CEP12 copy gain; (**d**) imbalanced KRAS locus gain observed in presence of elevated CEP12 number

fluorescence microscope equipped with DAPI, FITC and Texas Red filters in a blind fashion. Fluorescence images were archived with the Isis imaging system (MetaSystems, Altlussheim, Germany).

Ploidy status was defined following the evaluated FISH findings as published earlier [10] (Table 1).

Table 1 Definition of polysomy/aneusomy classes based on the distribution of the FISH findings. Patients were classified into six FISH strata with ascending number of copies of the KRAS gene per cell according to the frequency of tumour cells with specific number of copies of the KRAS gene at 12p and chromosome 12 centromere

Cytogenetic category	Number of copies of the KRAS gene per cell according to the frequency of tumour cells
Disomy	≤2 copies in >90 % of cells
Low trisomy	≤2 copies in ≥40 % of cells, 3 copies in 10 %–40 % of the cells, ≥4 copies in <10 % of cells
High trisomy	≤2 copies in ≥40 % of cells, 3 copies in ≥40 % of cells, ≥4 copies in <10 % of cells
Low polysomy	≥4 copies in 10 %–40 % of cells
High polysomy	≥4 copies in ≥40 % of cells
Gene amplification	KRAS/CEP12 ≥ 2, or ≥ 15 copies per cell in ≥10 % cells

Statistical Analysis

Clinicopathologic data were analysed using median, ±SD, mean, unpaired t-test and compared with literature data. Statistical analysis was carried out by GraphPad Prism 6.04 Trial (GraphPad Software, Inc. La Jolla, CA, USA).

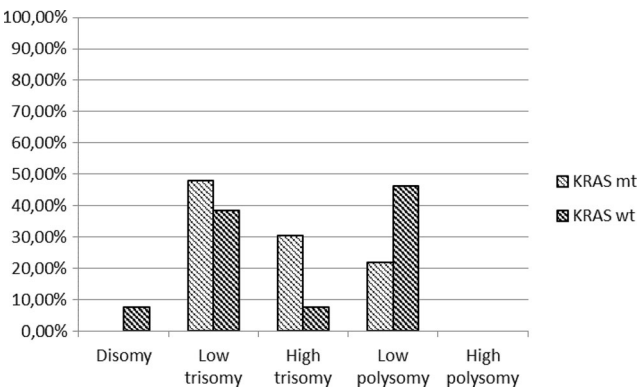

Fig. 2 Distribution of KRAS locus (chromosome 12p) ploidy groups according to the KRAS mutation status in CRC. No gross difference in the proposed copy number classes could be stated in relation with the mutation status. Both disomy and high-level gain/gene amplification of the KRAS locus are rare in mutant cancers

Table 2 Number and percentages of CRC cases within cytogenetic categories according to the chromosome 12 FISH characteristics of the samples. While disomy and real amplification were not detected, KRAS high trisomy and polysomy were more frequent in KRAS mutant samples

n (%)	Disomy	Low trisomy	High trisomy	Low polysomy	High polysomy	Amplification
KRAS mt	0	11 (47.82)	7 (30.43)	5 (21.73)	0	0
KRAS wt	1 (7.69)	5 (38.46)	1 (7.69)	6 (46.15)	0	0
Sum	1 (2.78)	16 (44.44)	8 (22.22)	11 (30.56)	0	0

Results

KRAS Locus Copy Number Is Increased in KRAS Mutant Tumours

FISH analysis of the KRAS locus on chromosome 12p was evaluable in all 36 cases (Fig. 1). Classification of ploidy status was done according to Cappuzzo et al. [10]. Disomy of chromosome 12p was observed in only 1/36 case (2.78 %), while low trisomy in 16/36 (44.44 %) and high trisomy in 7/36 (22.22 %) could be detected. Low polysomy was observed in the remaining 11/36 (30.56 %), while high polysomy was not found. According to its definition, gene amplification was not detected in our samples (Fig. 2). Detailed cytogenetic characteristics according to KRAS mutational status are shown in Table 2.

The KRAS exon 2 codon 12/13 mutation rate defined by BNA-clamped allele specific PCR as the percentage of mutant/total KRAS in tumour samples varied from 6 % to 50 %; however, the tumour cell content of samples was highly different, ranging between 10 and 100 %. The relative mutation frequency (mutation rate/tumour cell content) was 0.54 (range: 0.11–2.45). Gain of function KRAS mutations other than exon 2 and NRAS mutations could not be presented in any of the samples included in this study by sequential direct sequencing (KRAS exon 3 and exon 4, as well as NRAS exon 2, 3 and 4).

As next KRAS FISH data were correlated with the mutational status. The mean KRAS copy/nucleus value was 2.36 ± 0.42 for KRAS wild type and 2.63 ± 0.75 for KRAS mutant (statistically significant, $p = 0.037$) (Fig. 3a*)*. The KRAS/CEP12 ratio was 1.25 ± 0.04 for KRAS wild type and 1.29 ± 0.03 for KRAS mutant samples ($p = 0.422$) (Fig. 3b*)*.

Intercellular Heterogeneity of Mutant KRAS Allele Density

The generally elevated mean values of the KRAS copy and the KRAS/CEP12 ratio in KRAS mutated samples covered a high degree of variability between and within individual tumour samples. This directs to potential mutant allele heterogeneity at the single cell level.

To express tumour cell related allele frequencies more accurately the tumour contents of the individual samples were also considered. Relative mutational rates (RMR) were calculated by dividing the mutation rate as the result of the clamped/total reaction curves following the parallel PCR reactions by the tumour cell counts obtained following histology.

Relative KRAS mutation rates and KRAS copy numbers were individually correlated. RMR higher than 1.0 was observed in 2/23 (8.7 %) samples and further 9 cases (39.1 %) showed RMR between 0.5–1.0 (Fig. 4.). According to this figure altogether 11/23 (47.8 %) of the evaluated CRC cases presented with an elevated RMR referring to allelic imbalance. Median KRAS copy values on the other hand did not directly correlate with the increase of RMR (Fig. 4.). While both the median KRAS copy number increase and the extended spectrum of the copy number changes was obvious in the mutated compared to the wild type tumours, the level of ploidy did not refer to the mutation rate, even when the highest

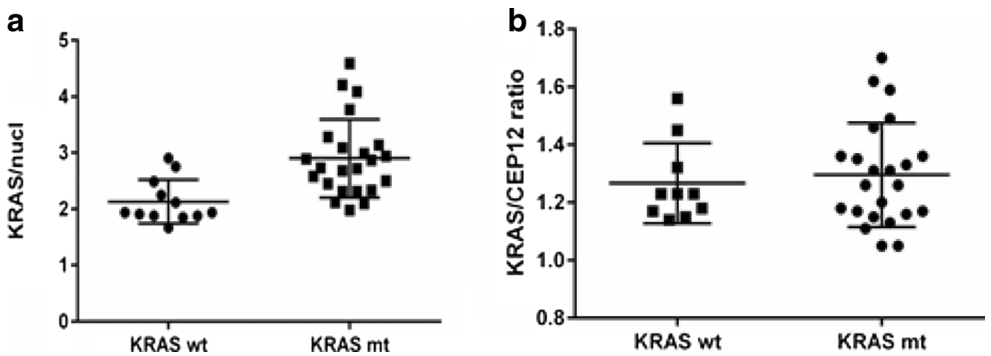

Fig. 3 Mean KRAS (**a**) and KRAS/CEP12 (**b**) values in wild-type and KRAS mutant tumours. A significant increase in KRAS copy number was observed in association with KRAS mutation (2.36 vs 2.63, $p = 0.037$) (A) while no change in the KRAS/chromosome 12 ratio was obvious

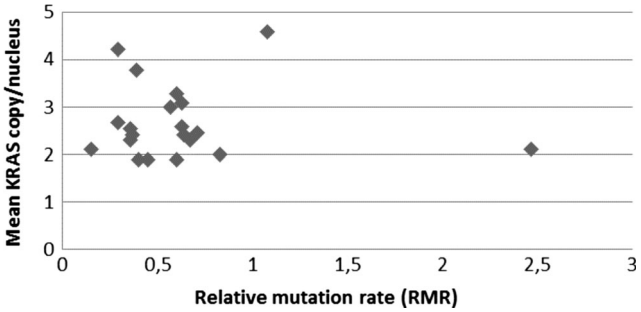

Fig. 4 Tumour content corrected relative mutation rate (RMR) and mean KRAS copy number changes determined in KRAS mutant colorectal carcinomas (n = 23). RMR and copy numbers proved to be highly different in the individual tumours

RMRs were frequently associated with extreme KRAS copy numbers (up to 16 copies/cell nucleus) (Fig. 5.). Table 3.

Table 3 Summary of the chromosome 12p FISH results according to the KRAS mutational status in CRC

	KRAS wt		KRAS mt		
	mean	±SD	mean	±SD	p value
Mutation rate	-		29.28	10.67	
Tumour cell ratio (%)			60.77	20.59	
Mut rate/Tu. cell ratio	-		0.63	0.29	
Mean KRAS/nucl	2.36	0.42	2.63	0.75	p = 0.037
Mean CEP12/nucl	1.98	0.29	2.4	0.48	p = 0.134
Mean KRAS/CEP12	1.25	0.04	1.29	0.03	p = 0.4226

Mean KRAS copy numbers proved to be higher in KRAS mutant samples (p = 0.037) while mean centromeric chromosome 12 signals and the KRAS/CEP12 values did not differ

Discussion

Tumour cells frequently acquire a wide range of chromosomal aberrations, with gains and/or losses of whole or fragment chromosomes leading to complex DNA copy imbalances. Moreover, tumour samples are mixtures of genetically variable cancer cells and normal cells. If the proportion of tumour cells within a sample is low, specific aberrations, including gene mutations may remain undetected. DNA based techniques currently reflect dominant molecular changes and do not allow deeper insight in processes occurring at the individual tumour cell level. The quantity of the mutant allele in KRAS exon 2 (and other) mutated CRC seems to be highly variable and the intratumour heterogeneity associated with aggressive/resistant subclone selection was repeatedly proposed [11–13]. Despite its potential clinical effect, however, the biological causes and consequences of KRAS mutational heterogeneity were considered in a limited fashion.

The BNA-clamped PCR method is able to detect the relative amount of mutant KRAS copies (mutation rate) within a tumour mass. In our CRC samples diagnosed with KRAS exon 2 mutation the mutation rate - calculated as the ratio of mutant (clamped) and total KRAS amplicon - varied from 6 % to 50 %, while the tumour cell content of the same samples was between 10 and 100 %. The mean mutation rate/tumour cell content ratio (relative mutation rate, RMR) was 0.54, however stretched over a wide range (0.11–2.45), reflecting totally different mutation frequencies in CRC tumours declared as KRAS mutant. A potential interference with other known KRAS activating mutations (exon 3 and 4) could be excluded by additional sequencing of the target regions in all exon 2 wild type and mutant cases.

The number of mutant alleles (gene dosage) is potentially dependent on the total gene copy number determined by aberrant cytogenetic mechanisms related to tumour progression.

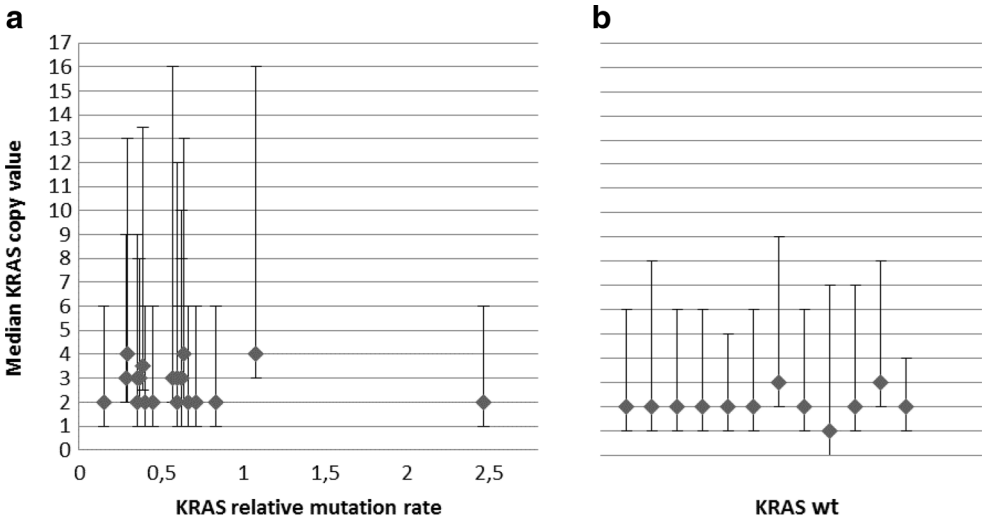

Fig. 5 Median with minimum and maximum values of KRAS copy numbers detected in individual KRAS mutant (**a**) and KRAS wild-type (**b**) tumour cell nuclei. Median KRAS copy number was higher in KRAS mutant samples (2.67 vs 2.08) and intratumour variability of FISH signals proved to be also highly different (1–16 vs. 1–9). KRAS copy number was independent of the RMR in the mutant cases

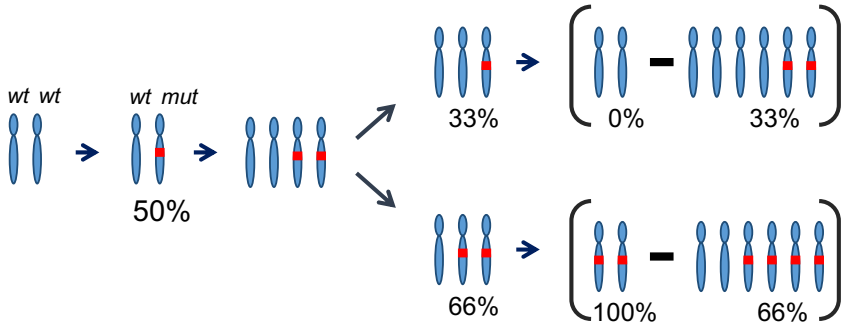

Fig. 6 Schematic presentation of the potential effect of the copy imbalance on the actual mutation status. The distribution of chromosomes carrying KRAS mutant genes is dynamically variable as a consequence of continuous gains and losses due to mitotic failures. Cyclic variation in ploidy is therefore a possible mechanism of gross allelic heterogeneity also reflected by the broad spectrum of the relative mutation rates (0.2–2.5)

The increase in gene dosage may result primarily from polysomy/aneusomy or gene amplification. The role of gene dosage on the behaviour of the tumour cells was intensively studied [2, 14, 15]. Seth and co-workers also concluded that homozygous KRAS mutations and concomitant KRAS/BRAF mutations may be indicative of a gene dosage effect [13].

According to the highly different values of RMR considering both BNA-clamped PCR data and tumour ratio in colorectal carcinoma the aim of our study was to correlate the mutation rate with copy number changes of the KRAS locus at 12p. Cytogenetic characteristics defined by FISH analysis suggest that trisomy/aneusomy of KRAS gene is frequent in mCRC while gene amplification is virtually absent. This is in agreement with literature data, Valtorta et al. reported KRAS amplification only in 7/1039 (0.67 %) of CRC cases [14].

Our results suggest that there is a statistically significant correlation between KRAS exon 2 activating mutations and KRAS copy number increase. KRAS copy gain (KRAS signals >2/cell) was found to be significantly more frequent in KRAS mutant tumours (p = 0.037). However, polysomy/aneusomy related copy number changes presented with marked heterogeneity and reached up to 16 copies/cell nucleus in KRAS mutant tumours. The significant diversity and individual ploidy level of the tumour cells may serve as a good explanation for the missing linear regression in the comparison with the RMR.

As generally accepted, cyclic chromosome missegregation events lead to aneuploidy, which is a dynamic process reorganizing the genetic material in a complex fashion. Polysomy/aneusomy of the chromosome region 12p observed in the majority of the evaluated samples indicate to an imbalanced allele distribution serving with a profound explanation of the variability of the mutation rates obtained by parallel BNA-clamped allele specific PCR. It is technically most challenging to obtain information regarding the true effect of aneusomy on the mutational frequency at the single cell level. In theory, every mitotic cycle associated with pre-existing aneusomy may further influence the distribution of mutant alleles and thus generate new allelic composition. Mitotic failures responsible for numeric chromosomal aberrations are frequently combined with chromosome fragility and further mechanisms of chromatin instability in most malignancies and dominantly in CRC [16]. Regulatory changes of cell cycle or cell division initiation such as Aurora B kinase activation or overexpression are key elements of polyploidization [17]. Our scheme drawn to highlight the full spectrum of the process, proposes the significance of ploidy changes as series of improportional gains or losses that may directly influence the actual density of the mutant allele (Fig. 6.). This model concerns that the mutation rate can be only conceived at the gross tumour cell population level but also considers that ploidy related imbalances potentially enable a complete shift in the mutational status.

Conclusions

In summary, KRAS copy number is significantly elevated in KRAS exon 2 mutant CRC cases. Both copy changes and relative mutation rates were found to be highly heterogeneous and may be interrelated within one tumour sample interfering with the general KRAS mutation status. Aneuploidy raises the scale of intercellular variability and thus has the potential to influence the mutation rate in any direction.

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