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ORIGINAL ARTICLE

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Numerical and structural centrosome aberrations are an early and stable event in the adenoma–carcinoma sequence of colorectal carcinomas

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Abstract *Aims:* Numerical and structural centrosome changes have been described for and linked with genetic instability in solid tumors. Here, we specifically address centrosome aberrations in the adenoma–carcinoma sequence of colorectal cancer by detailed evaluation of γ-tubulin staining patterns. *Methods:* Formalin-fixed and paraffin-embedded specimens (normal colonic epithelium n=21; low-grade intraepithelial neoplasia n=27, high-grade intraepithelial neoplasia n=16 and invasive adenocarcinomas n=33) were stained by an anti-γ-tubulin antibody using standard immunofluorescence. Three-dimensional image stacks of the stainings were recorded (Zeiss LSM510 confocal microscope), followed by numerical and structural data analysis (DIAS software package) and statistical evaluation (NCSS-software). *Results:* The mean centrosome signal per cell differed significantly (P<0.0001) between normal colonic epithelium (0.8775) and each low-grade intraepithelial neoplasia (1.787), high-grade intraepithelial neoplasia (2.259) and invasive carcinomas (2.267). Similarly, both the centrosomes' structural entropy (SE) and minimal spanning tree (MST) differed significantly (P< 0.001) between normal (SE=3.956, MST=38.78) and each low- (SE=6.39, MST=26) and high-grade intraepithelial neoplasia (SE=5.75, MST=26.97) and invasive carcinoma (SE=6.86, MST=28.08). *Conclusion:* Numerical and structural centrosome dysregulation is seen as early as in low-grade dysplastic lesions of the adenoma–carcinoma sequence of colorectal carcinomas and may, as such, play an initial role in the carcinogenic process.

Keywords Centrosomes · Adenoma–carcinoma sequence · Colon cancer · Genetic instability · Syntactic structure analysis

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Introduction

The formation and organization of centrosomes is essential for proper cell division and the generation of two diploid daughter cells [1, 25]. During interphase, centrosomes are involved in the organization of the cytoplasmatic microtubule network and thereby also influence cell movement and cell polarity. Parallel to the cell cycle, centrosomes duplicate, mature and separate by moving to the opposite poles of the cell, ensuring mitotic-spindle formation and attachment. At mitosis, centrosomes are directly involved in proper chromosome segregation. Changes in either the number and/or location of centrosomes may negatively influence chromosomal segregation and cell ploidy and thereby promote chromosomal instability in malignancy [1, 25].

Indeed, centrosome aberrations have been demonstrated in several types of cancer [2, 3, 4, 18, 22], both in cell lines [7, 23, 27, 32] as well as in solid [8, 17, 19, 26] and hematopathological [5, 21] tumors. In fact, both supernumerary and structurally altered centrosomes have been

described for especially aneuploid cell lines [7] and tumors [18, 19]. Moreover, direct manipulation of centrosome regulation in cell culture models was shown to induce aneuploidy and transformation [32], supporting the concept of a potentially causative role of centrosome aberrations in malignant progression. A recent in situ study of precursor lesions implicated centrosome defects in the carcinogenesis of tumors of the uterine cervix, prostate and breast [24].

In colorectal cancer, concomitant phenotypic and genotypic changes characterize the progression of normal epithelium to adenoma to invasive carcinoma [6, 11, 12, 16]. The genetic alterations accompanying the adenoma–carcinoma sequence are generally divided into two pathways, resulting in either chromosomal unstable, frequently aneuploid tumors (CIN) or microsatellite unstable (MIN), mostly diploid tumors. Specific small genetic alterations, such as point mutations, and/or gains and losses of entire genes have been associated with chromosomal and microsatellite instability [9]. However, whether or not centrosome aberrations and the associated chromosome missegregation are primarily involved in or contribute to the carcinogenesis of colorectal tumors, particularly the CIN-type aneuploid carcinomas, is still unclear.

In the present study, we have therefore investigated centrosome alterations in the adenoma–carcinoma sequence of colorectal cancer by examining numerical and structural changes of the centrosome-specific marker γ-tubulin in situ, using formalin-fixed and paraffin-embedded tissue sections of normal epithelial cells, low-grade intraepithelial neoplasia (LGIN), high-grade intraepithelial neoplasia (HGIN) and invasive adenocarcinoma.

Materials and methods

Tissue specimens

Colorectal specimens (n=76; surgically removed polypectomies and small biopsies), all formalin fixed and paraffin embedded, were selected from the archives of the Pathological Institute of Freiburg. Upon reclassification (GK, MW) according to the World Health Organization 2000 [10] guidelines, these specimens included 27 samples of LGIN, 16 samples of HGIN and 33 samples of adenocarcinomas of the colon. Normal epithelial samples (n=21) were analyzed in some of the above specimens from adjacent tissues.

Immunohistochemistry

Tissue sections (8 μm) were deparaffinized, hydrated using standard measures and subjected to antigen retrieval using Pronase E (0.05%). Subsequently, tissue sections were incubated with a primary mouse anti-γ-tubulin antibody (1:100; clone GTU-88, Sigma), followed by incubation with a fluorescein isothiocyanate (FITC)-labeled goat-anti mouse IgG (1:200; Dianova). Finally, nuclei were counterstained with 4′6′-diamidino-2-phenylindole-2-hydrochloride (DAPI).

Data collection and in-situ parameter analysis

For data collection, five microscopic areas were selected (63 objective, zoom 2), and three-dimensional image stacks, consisting of 16 planes with a distance of 0.5 μm in the z-axis, were acquired with a confocal laser-scanning microscope (Zeiss LSM 510Meta, PlanNeofluar 63×/1.2 water, zoom 2). Excitation of fluorescent dye was at 351 nm for DAPI and 488 nm for FITC-labeled γ-tubulin. Image stacks were converted into a projection image of all recorded slices and were analyzed semi-automatically with respect to the number of cells (DAPI) and γ-tubulin signals by programs based on the DIAS software package [13, 14]. Furthermore, structural image analysis was performed by measuring the structural entropy of γ-tubulin signals, which quantifies the degree of signal perturbation within a given environment (i.e. selected tissue area), as well as the minimum spanning tree (MST) of γ-tubulin signals, which quantifies the shortest connection of all γ-tubulin signals as mean distance within the selected tissue area [15, 31]. The total number of analyzed cells was between 250 and 300 cells per case.

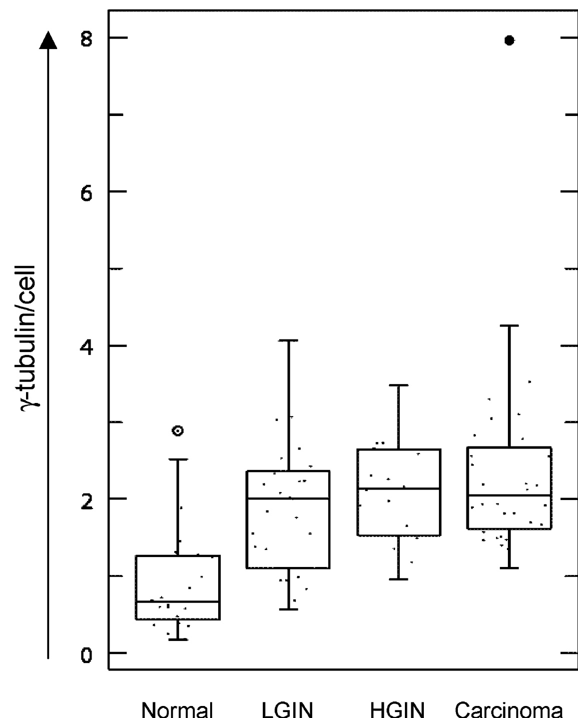

Fig. 1 Summary of the numerical changes of centrosomes along the adenoma–carcinoma sequence of colorectal adenocarcinoma. *Box plots* depict the measured γ-tubulin signals per cell for normal epithelial cells, low- and high-grade intraepithelial neoplasia (LGIN and HGIN) and invasive carcinoma. Each individual sample is indicated by a *dot*, minimum and maximum data points are indicated (*bar*, linked to the box by a line), and one statistical "outsider" (carcinoma) is shown by the *closed circle*

Table 1 Summary of the numerical changes of centrosomes within the adenoma–carcinoma sequence. *DAPI* 4′6′-diamidino-2-phenyl-indole-2-hydrochloride, *LGIN* low-grade intraepithelial neoplasia, *HGIN* high-grade intraepithelial neoplasia

Histology	γ-Tubulin/ DAPI (per cell)	Fold increase (relative to normal)	Significance (*P*) (relative to normal)
Normal	0.878		
LGIN	1.787	2.035	<0.0001
HGIN	2.259	2.573	<0.0001
Carcinoma	2.267	2.582	<0.0001

Statistical analysis

Statistical analysis was performed on the entire data set collected from all tissue samples using a commercially available software package (NCSS 2000, Number Cruncher Statistical Systems, Kaysville, Utah, USA) and included analysis of variance, the Mann-Whitney U test, the Wilcoxon-Rank test and the Kolmogorov-Smirnov test.

Results

Numerical changes of γ-tubulin staining in the adenoma–carcinoma sequence

In normal colonic epithelial cells (*n*=21), γ-tubulin staining showed a mean number of 0.88+/−0.6 signals per cell. In LGIN, an increase of γ-tubulin signals to 1.79+/−0.8 per cell was observed. These changes were even more obvious in HGIN, with 2.26+/−0.6 signals per cell, and invasive carcinomas, with 2.28+/−1.2 signals per cell (Fig. 1).

Statistical analysis (summarized in Table 1) of the numerical changes seen in γ-tubulin immunofluorescence stain revealed a significant alteration ($P<0.0001$) between: (1) normal cells and LGIN, (2) normal cells and HGIN and (3) normal and invasive carcinoma cells. However, the changes of γ-tubulin signals between LGIN and HGIN and invasive carcinoma showed no statistical significance.

Structural changes of γ-tubulin staining in the adenoma–carcinoma sequence

Whilst a regular, apical-orientated distribution of γ-tubulin was seen in normal epithelial cells, a progressively increasing sterical distortion of the γ-tubulin signals was observed in LGIN and HGIN and was most prominent in invasive carcinomas, with a complete loss of orientation (Fig. 2). To quantitatively measure these observations, we performed quantitative and syntactic analysis of: (1) the structural entropy of the γ-tubulin signals and (2) the MST between all γ-tubulin signals within the tissue images.

The structural entropy of the centrosomes within normal epithelium was 3.96+/−1.4 and was raised to 6.39+/−1.6 in LGIN, 5.75+/−1.6 in HGIN and to 6.86+/−2.1 in invasive carcinomas. As seen in the numerical γ-tubulin changes, the structural entropy measurements were also significantly different between normal and neoplastic epithelium (*P*<

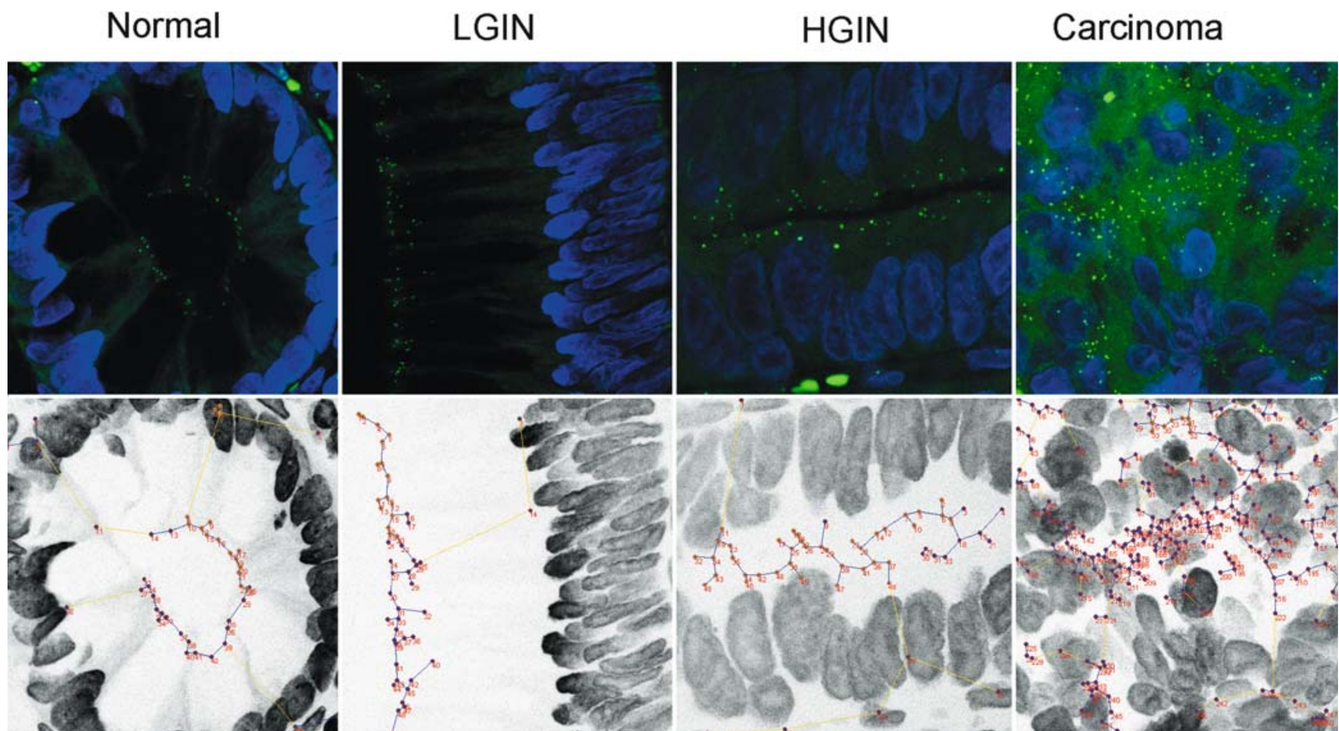

Fig. 2 Changes of centrosomes along the adenoma–carcinoma sequence of colorectal adenocarcinoma. Representative staining of centrosomes by γ-tubulin immunofluorescence (*top row*) and structural analysis of centrosome location by minimal spanning tree (*bottom row*). *Panels from left* normal epithelium, low- and high-grade intra-epithelial neoplasia and far right invasive adenocarcinoma. Magnification: 1260 (63 objective, zoom 2, camera ×10). γ-Tubulin signals are shown in *green*

Table 2 Summary of the structural changes of centrosomes within the adenoma–carcinoma sequence. *MST* minimal spanning tree, *LGIN* low-grade intraepithelial neoplasia, *HGIN* high-grade intraepithelial neoplasia

Histology	Entropy	Significance (relative to normal)	MST	Significance (relative to normal)
Normal	0.0396		38.78	
LGIN	0.0639	<0.001	26.00	<0.001
HGIN	0.0575	<0.001	26.968	<0.001
Carcinoma	0.0686	<0.001	28.08	<0.001

0.001) (Table 2). Together with an elevated number of γ-tubulin signals, this parallel increase of structural entropy may lead to a reduction of the mean distance between γ-tubulin signals, i.e., the MST. Indeed, the measurement of the MST of γ-tubulin signals also revealed significant differences between normal and neoplastic cells ($P<0.001$): the mean MST length in normal mucosa was 38.8+/−13.4 and shortened to 26.0+/−4.0 in LGIN, 27.0+/−3.3 in HGIN and 28.1+/−5.3 in invasive carcinoma.

Discussion

Centrosome dysfunction is closely associated with aneuploidy and chromosomal instability in cell-culture models [32], including colorectal cell lines [7], and has been implicated in a variety of human solid tumors [8, 17, 19, 26] as well as in lymphomas and leukemias [5, 21]. However, the association with and/or involvement of centrosome aberrations at early stages of malignant transformation, such as in dysplastic precursor lesions, have thus far only been addressed in a single study [24].

The present study is, therefore, the first in situ analysis of centrosomes within a large series of normal colonic epithelium, LGIN, HGIN and invasive carcinoma samples of colorectal cancer patients. By visualizing centrosomes with γ-tubulin, a highly conserved component of centrosomes [29, 30], we found not only numerical but also structural aberrations of centrosomes within the adenoma–carcinoma sequence. Moreover, these numerical and structural centrosome changes were already evident at the transition of normal epithelium to LGIN, suggesting that centrosome alterations may represent a very early event in the carcinogenesis of colorectal cancer. In fact, centrosome signals increased twofold from normal to LGIN and threefold from normal to HGIN and invasive carcinoma (Table 1). These observations are in accordance with earlier studies demonstrating that supernumerary centrosomes may occur in preinvasive lesions of the uterine cervix, prostate and female breast [24], as well as in pre-invasive lesions of the cervix in association with human papilloma virus infections [28].

Centrosome dysfunction may occur not only at the level of numerical changes but also in the failure of structural organization [17, 20, 23]. We, therefore, evaluated whether structural and/or sterical alterations might additionally be involved in centrosomal dysfunction in colorectal carcinogenesis. Indeed, semi-automated image analysis of the structural entropy and MST [13, 14, 15, 31] of γ-tubulin-stained tissues revealed statistically significant changes in centrosome orientation and localization. Concomitantly with the numerical changes, these structural alterations were already present in LGIN.

Finally, the numerical and structural centrosome alterations observed from the transition of normal colonic epithelium to LGIN did not progress further along the adenoma–carcinoma sequence, remaining equally elevated in HGIN and invasive carcinomas.

Together, these results suggest early and stable numerical as well as structural alterations of centrosomes in the carcinogenesis of colorectal cancer. Whether these centrosome alterations are simply a consequence of the well-characterized genetic events of the adenoma–carcinoma sequence [6, 11, 12, 27] or whether they are a primary cause for the progression of a normal epithelium into an (aneuploid) invasive carcinoma remains an open question. However, the early occurrence of centrosome alterations, as seen in our samples of LGIN, may point toward a causative role of centrosome dysfunction in malignant transformation.

In fact, we have obtained further data supporting an early role of centrosome alteration in colorectal carcinogenesis by mRNA expression analysis of STK15, a serine-threonine kinase previously shown to be essential for centrosome function [32]. Similar to the pattern of γ-tubulin staining, STK15 mRNA increased significantly already in LGIN without any further increases in HGIN and carcinoma (Gerlach et al., unpublished observations). Moreover, our preliminary data also suggest that this increase of STK15 mRNA occurs preferentially in the carcinogenesis of chromosomal unstable colorectal tumors, and this appears to also be correlated with the number of γ-tubulin signals/cells. However, further studies have to prove the link of STK15 mRNA and γ-tubulin, i.e., centrosome aberrations, particularly in the development of chromosomal unstable colorectal cancers.

In summary, we have shown that centrosome aberrations are an early event in the adenoma–carcinoma sequence of colorectal adenocarcinomas. These changes are characterized by supernumerary centrosomes and an increasing perturbation in the arrangement of centrosomes within the cells. Both of these alterations may be associated with the induction of aneuploidy in colorectal carcinomas, particularly those that are chromosomally unstable.

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