

Aurora A is differentially expressed and regulated in chromosomal and microsatellite instable sporadic colorectal cancers

Silke Lassmann¹, Mihai Danciu^{1,2}, Matthias Müller¹, Roland Weis¹, Frank Makowiec³, Jürgen Schulte-Mönting⁴, Ulrich T Hopt³ and Martin Werner¹

¹Institute of Pathology, University Medical Center, Albert-Ludwigs University, Freiburg, Germany; ²Pathology Department, Faculty of Medicine, University of Medicine and Pharmacy, 'Gr T Popa', Iasi, Romania; ³Department of Surgery, University Medical Center, Albert-Ludwigs University, Freiburg, Germany and ⁴Institute for Medical Biometry and Informatics, University Medical Center, Albert-Ludwigs University, Freiburg, Germany

The centrosome-associated kinase aurora A has been shown to be involved in genetic instability and to be (over)expressed in several human carcinomas. This study investigated *aurora A* gene copy numbers, mRNA and protein expression as well as tumour cell proliferation and aneuploidy in chromosomal and microsatellite instable sporadic colorectal cancers. Case-matched tissues of normal ($n=71$) and dysplastic ($n=49$) colorectal epithelium and invasive carcinomas ($n=71$) were included in this study. PCR-based microsatellite analysis classified 14/71 (20%) of carcinomas as microsatellite instable. A stepwise increase of aurora A mRNA expression ($P<0.0001$; quantitative RT-PCR) and aurora A protein expressing tumour cells ($P=0.0141$; immunohistochemistry) occurred in the adenoma-carcinoma sequence. Within invasive carcinomas, aurora A mRNA levels ($P=0.0259$) and aurora A positive tumour cells ($P<0.0001$) were closely associated with tumour cell proliferation (Ki-67 specific immunohistochemistry). Compared with chromosomal instable carcinomas, microsatellite instable carcinomas had significantly more aurora A positive tumour cells ($P=0.0043$) and a higher tumour cell proliferation ($P=0.0335$). In contrast, only chromosomal instable carcinomas exhibited marked tumour cell aneuploidy ($P=0.0004$, fluorescence *in situ* hybridization) and significantly higher *aurora A* gene copy numbers ($P=0.0206$) as compared with microsatellite instable carcinomas. This study further supports a role of aurora A in the carcinogenesis of sporadic colorectal cancers. Moreover, it demonstrates that in a minority of predominantly microsatellite instable carcinomas the presence of aurora A positive tumour cells is merely reflecting tumour cell proliferation. In contrast, the large majority of chromosomal instable carcinomas shows additional (de)regulation of aurora A by gene amplification and concomitant tumour cell aneuploidy. Thus, sporadic colorectal cancers exhibit different mechanisms of aurora A regulation and this may impact the efficacy of aurora-targeted therapies.

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The development and progression of colorectal cancer is closely linked to increased genetic instability in the malignant cells.^{1–3} In the large majority of sporadic colorectal cancers, genetic

instability manifests itself by multiple numerical chromosomal alterations (gene amplifications, deletions) and is referred to as chromosomal instability.^{4–6} In a smaller group of sporadic colorectal cancers, defects of the DNA mismatch repair system lead to microsatellite instability.^{7,8} Chromosomal, but less frequently microsatellite instable colorectal cancers exhibit frequent tumour cell aneuploidy.^{4,9,10}

Whereas the cause for microsatellite instability has clearly been defined to reside in mutations of

Correspondence: PD Dr S Lassmann, Institute of Pathology, University Medical Center, Albert-Ludwigs University, Breisacherstr. 115a, Freiburg 79106, Germany.
E-mail: silke.lassmann@uniklinik-freiburg.de
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the mismatch repair genes, the underlying mechanism(s) of chromosomal instability still remain to be fully elucidated. Besides chromosome non-disjunction and/or telomere-related alterations,^{11,12} gross defects in chromosome segregation during cell division may contribute to the development of chromosomal instability and aneuploidy.¹³ Indeed, changes in proteins involved in chromosome segregation—so-called chromosomal passenger proteins^{14–18}—and/or proteins involved in mitotic spindle^{19–21} have been associated with the development of aneuploidy in cancer cells. Also, the adenomatous polyposis coli protein, whose alteration is observed in early colorectal carcinogenesis,^{22,23} has recently been implicated in the control of chromosome segregation during mitosis.^{24–27} Deregulation of chromosome segregation, either directly or indirectly linked to expression and/or modification of adenomatous polyposis coli,^{24,25,28} may contribute to the characteristic broad genomic alteration of tumour cell aneuploidy or polyploidy. Moreover, it is by now well established that deregulation of centrosomes may be a key event that contributes to or primarily induces aneuploidy.^{29,30} In particular, supernumerary centrosomes, caused by overexpression of (the numerous) proteins of the centrosome complex, may promote a failure in appropriate chromosome segregation and result in tetra-, poly and/or aneuploid daughter cells.

The protein family of aurora kinases (-A, -B, -C) is involved in the centrosomal complex (aurora A) and in the chromosomal passenger complex (aurora B)^{31–34} and has gained substantial attention in view of its potential therapeutic value in diverse solid tumour entities.^{35–38} In particular, overexpression of aurora A induces supernumerary centrosomes and aneuploidy.^{31–34,39}

In tissue specimens of colorectal cancer, aurora A is amplified and overexpressed as determined by Southern and northern blotting of tissue extracts,⁴⁰ by quantitative PCR⁴¹ as well as immunohistochemistry.^{42–44} We recently showed by quantitative RT-PCR that step-wise aurora A mRNA upregulation occurs during colorectal carcinogenesis, especially in chromosomal unstable carcinomas.⁴⁵ In addition, our array-based comparative genomic hybridisation analysis suggested that chromosomal unstable carcinomas exhibit preferential amplification of the *aurora A* gene locus at 20q13.⁴⁶ Similarly, Nishida *et al*⁴¹ found that microsatellite unstable colorectal cancers rarely have *aurora A* gene amplification, as measured by PCR. By mRNA expression profiling of microdissected invasive cancer cells from formalin-fixed and paraffin-embedded colorectal cancers, we recently also detected aurora A as one prominently expressed gene in chromosomal unstable tumours.⁴⁷ Finally, *in vitro*, ectopic aurora A overexpression in tumour cells induces aneuploidy³⁹ and may convert microsatellite unstable into aneuploid (chromosomal unstable like) colorectal tumour cells.⁴⁸

These data clearly point towards differential regulation and expression of aurora A in subtypes of colorectal cancers. Still, the clinically relevant aspect of aurora A 'over'expression has so far been mostly defined by the presence of an elevated number of aurora A positive tumour cells. As aurora A expression also changes tightly within the cell cycle,^{49,50} the previously observed *aurora A* gene amplification^{40,41} and particularly the aurora A overexpression in colorectal cancers^{40,42–44} may have been influenced by the fraction of proliferating tumour cells.

The aim of this study was therefore to analyse aurora A expression and regulation in the adenoma-carcinoma sequence of sporadic colorectal cancers. Specifically, we tested whether sporadic chromosomal and microsatellite unstable carcinomas display differences in aurora A regulation and expression by gene amplification and/or proliferation and whether this is linked to differences of chromosomal and microsatellite instability associated (aneu-)ploidy.

Materials and methods

Patients

The study included formalin-fixed and paraffin-embedded specimens of 71 patients resected for sporadic colorectal cancer (median age of patients = 72 years; Table 1). Samples of normal colonic mucosa ($n = 71$, obtained from resection margins), low ($n = 36$)- and high ($n = 13$)-grade intraepithelial neoplasia and invasive colorectal carcinomas ($n = 71$) were included.

Normal epithelium, dysplastic epithelium (adenoma) and invasive carcinoma tissue samples were obtained from primary resection specimens of the same cases. Normal epithelium was obtained from resection margins at least 15 cm away from the invasive carcinomas. Adenomas—if present in the resection specimen—were derived from separate locations at distance from the invasive carcinomas, thereby ruling out a bias due to carcinoma-associated dysplastic lesions.

Data of low- and high-grade intraepithelial neoplasia samples were combined as 'dysplastic epithelium' for statistical purposes. The histopathological characteristics of all patients are displayed in Table 1, with specific reference to the subgroups of cases with chromosomal and microsatellite unstable carcinomas. For classification of microsatellite unstable carcinomas, microsatellite analysis was performed by analysis of five microsatellite loci in DNA derived from microdissected tumour cells and normal epithelial cells as before.⁴⁵ Three of 71 cases yielded uninformative data and were excluded from statistical analyses addressing differences between chromosomal and microsatellite instability. Appropriate tissues samples were selected after re-classification^{51,52} of hematoxylin and eosin-stained sections by an experienced pathologist (MD), as

Table 1 Clinicopathological parameters of sporadic colorectal cancer patients investigated

	All cases	Chromosomal instable cases*	Microsatellite instable cases*
Number of cases (N)	71	54	14
Sex			
Male	44	35	6
Female	27	19	8
Age (median, range)	72 (46–90)	72 (46–88)	76 (67–90)
Location			
Colon	63	46	14
Rectum**	8	8	0
Tissue samples (N)			
Normal epithelium	71	54	14
Dysplastic epithelium (<i>LGIN</i> , <i>HGIN</i>)	49 (36, 13)	40 (30, 10)	6 (4, 2)
Invasive carcinoma	71	54	14
Tumour classification			
pT1	3	3	0
pT2	9	7	2
pT3	53	38	12
pT4	6	6	0
pN0	46	34	10
pN1	11	10	1
pN2	11	8	2
pNx	3	2	1
Tumour grading			
G1	5	5	0
G2	48	37	8
G3	18	12	6

The table summarizes the major clinicopathological parameters of the investigated cases and the tissue materials used for all cases as well as for chromosomal and microsatellite instable cases separately.

Note: *For three cases, no MSI-status could be obtained; **no neoadjuvant radio/chemotherapy.

LGIN and *HGIN* = low- and high-grade intraepithelial neoplasia.

described before for 41 of the cases.^{45,46} The study had been approved by the local Ethics Committees (#251/04 Ethik-Kommission, Albert-Ludwigs-Universität, Freiburg, Germany; and the University Medicine and Pharmacy, Department of Pathology, Iasi, Romania).

Analysis of Aurora A mRNA Expression

We had previously determined aurora A mRNA levels in 41 cases⁴⁵ and in this study examined aurora A mRNA levels of the additional 30 new cases. For this, and as before,⁴⁵ RNA extracts of microdissected cell populations (normal or dysplastic epithelial cells, invasive tumour cells) of formalin-fixed and paraffin-embedded tissue speci-

mens were prepared and subjected to quantitative RT-PCR (qRT-PCR) analysis using established protocols^{45,47,53} and the comparative Ct-method⁵⁴ for final calculation of aurora A mRNA expression levels. Of all tissue samples analysed, 183/191 (96%) yielded sufficient RNA of appropriate quality for qRT-PCR analysis (acceptable signals for both reference gene/*TATA box binding protein*, target gene/*aurora A*). Failures to obtain sufficient mRNA and/or appropriate signals in qRT-PCR analysis (reference gene/*TATA box binding protein*, target gene/*aurora A*) were observed in 1/71 (1%) normal epithelium samples, 3/49 (6%) dysplastic samples and 5/71 (7%) invasive carcinoma samples.

Analysis of Aurora A Protein Expression and Tumour Cell Proliferation

For analysis of aurora A protein expression and tumour cell proliferation, tissue microarrays were constructed from representative tissue areas (normal and dysplastic epithelium, invasive carcinoma) by punching 0.2 cm cores per tissue sample. Comparative analyses of entire tissue sections and 0.2 cm cores demonstrated that staining patterns were homogeneous throughout the selected tissue blocks. Serial tissue microarray sections of 4 µm thickness were subjected to immunohistochemistry with aurora A⁵³ and Ki67^{55,56}-specific antibodies. In brief, sections were cut, dried and deparaffinized. Antigen retrieval was done by boiling sections in Tris-EDTA buffer pH9 (aurora A) or citrate buffer pH6 (Ki67), followed by incubation with primary antibodies for aurora A (1:50, aurora kinase 2, clone JLM28, Loxo/Novocastra, Dossenheim, Germany) and Ki67 (clone MIB-1, 1:500; DakoCytomation) for 60 min. Secondary antibodies were incubated for 30 min and detection was with the LSAB-Fast red System (DakoCytomation). Positive controls for aurora A⁴⁴ and Ki67 protein stainings were routinely processed formalin-fixed and paraffin-embedded tonsils and negative controls sections were stained by omission of the primary antibody. All stainings were performed on a DAKO Autostainer (DakoCytomation) and evaluated semi-quantitatively in tumour cells with scores for cytoplasmic and nuclear aurora A staining as before⁵³ (Figure 1) and scores for nuclear Ki67 staining: score 0 = all tumour cells negative, score 1 = <10% tumour cells positive, score 2 = 10–50% tumour cells positive and score 4 = >50% positive.⁵⁶

Analysis of Aurora A Gene Copy Numbers and Aneuploidy by Fluorescence *In Situ* Hybridisation

Aurora A gene copy numbers^{46,53} and tumour cell aneuploidy were assessed as follows: Serial tissue microarray sections of 5 µm thickness were subjected to deparaffination, pre-treatment in pH6 citrate buffer in a microwave oven (180 W) for

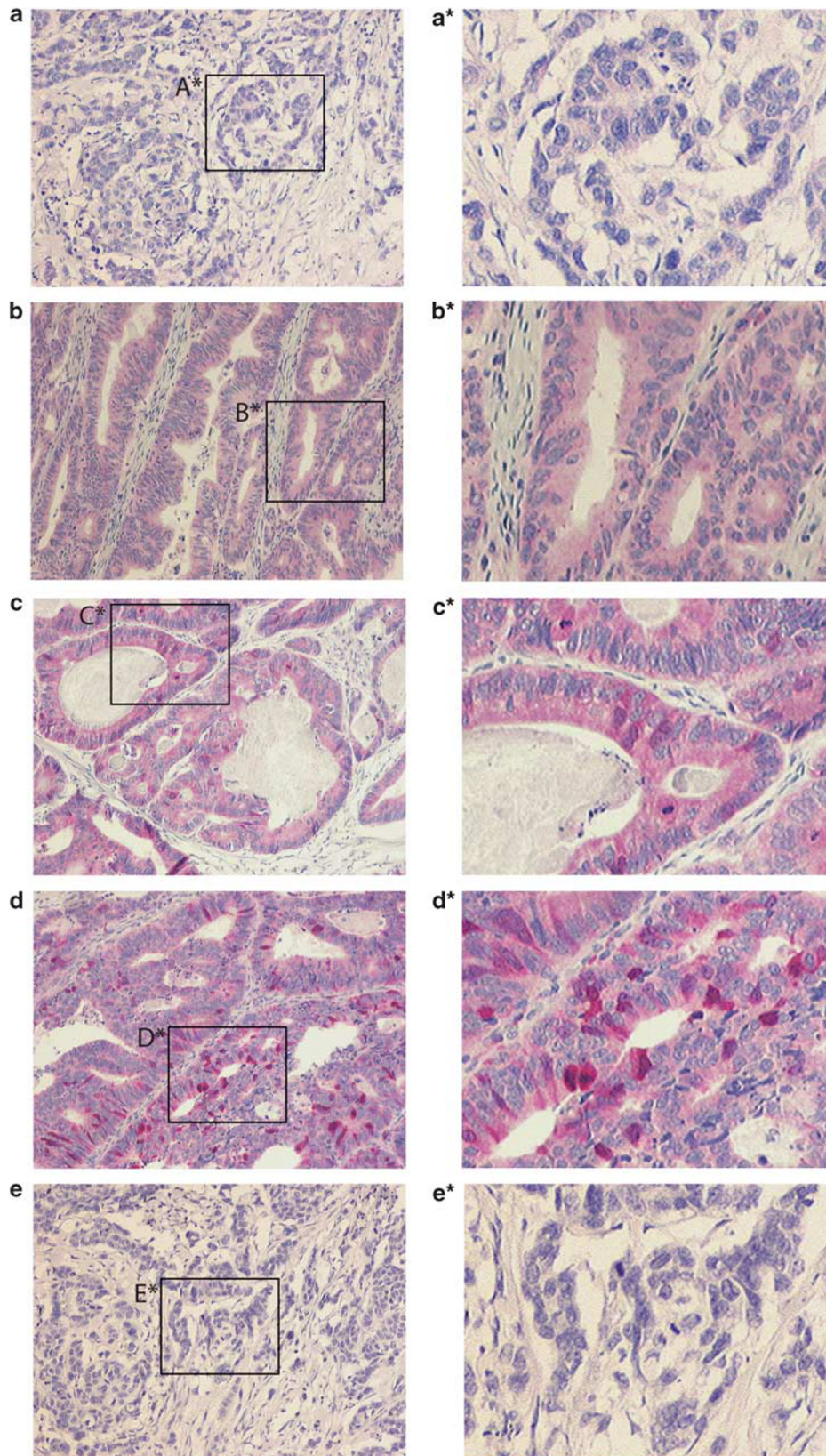


Figure 1 Representative immunohistochemical stainings of aurora A protein expression in sporadic colorectal cancers. The figure shows representative photographs of sections stained with an aurora A specific antibody. (**a–d**) (magnification $\times 100$): Four examples of invasive carcinomas with immunohistochemistry score 0 (**a**; case #23; chromosomal instable), 1 (**b**; case# 13; microsatellite instable), 2 (**c**; case #16; chromosomal instable) and 3 (**d**; case # 5; chromosomal instable). (**e**) Negative control staining (no primary antibody; case# 23). **A*–E*** (magnification $\times 200$): enlarged inserts from **a–e**.

20 min and Pronase E (0.05%) digestion for 3 min at 37°C. Subsequently, sections were denatured in 2 × SSC/50% Formamide (room temperature), and 2 × SSC/70% Formamide (75°C, 15 min), immersed in ice cold ethanols (70, 95 and 100%, 5 min each) and dried at 37°C. In the meantime, the fluorescence *in situ* hybridization (FISH) probes had been denatured (75°C, 5 min) and were hybridised to the dried sections (16 h, 37°C) as follows: Section (i) probe specific for the *aurora A* gene and centromere 20 (chromosome enumeration probe, CEP20; Chrombios, Raubling, Germany)^{46,53} and section (ii) to (v) probes specific for CEP7, CEP8, CEP11 (Abbott Vysis) and CEP13 (QBiogen). After hybridization, sections were washed in 2 × SSC (room temperature) and in 2 × SSC (73°C, for 2 min), cover-glassed with DAPI-containing mounting medium (Vector) and stored at -20°C until analysis. Stained sections were evaluated on a fluorescence microscope (AxioPlan 2 imaging with ApoTome system, Carl Zeiss MicroImaging GmbH, Göttingen, Germany) by taking image stacks at 0.5–1.0 μm intervals for each three representative areas per case (63 × magnification). Image stacks were converted into 3D view by AxioVision software and then assessed for *Aurora A*- (section i) and centromere- (sections i–v) specific signals.

For *aurora A* gene copy numbers, FISH data are presented as the mean number of *aurora A* gene- and CEP-specific signals per tumour cell per case. A mean of 67 ± 21 tumour cells (range, 13–100) were counted per case. The ratio of the mean *aurora A* gene- to CEP20-specific signals per cell was calculated to assess *aurora A* gene amplification for each case.

For determination of tumour cell aneuploidy by FISH,⁵⁷ first 20 samples of normal colonic epithelium were analysed with FISH probes specific for CEP7, CEP8, CEP11, CEP13, CEP20. The mean CEP-specific signals per normal epithelial cell were (number of cells counted in brackets): CEP7: 2.13 ± 0.2 (*n* = 131); CEP8: 2.17 ± 0.12 (*n* = 140); CEP11: 2.23 ± 0.07 (*n* = 140); CEP13: 2.1 ± 0.18 (*n* = 130) and CEP20: 2.01 ± 0.11 (*n* = 98). The mean (2.15) ± 3 standard deviations (3 × 0.15) of all five CEP-signals per normal epithelial cell was 2.6 and this was set as cut-off for classification of aneuploidy in invasive tumour cells. In invasive carcinomas, a mean of 88 ± 25 tumour cells per case (range, 2–105) were evaluated for the five CEP-signals and the corresponding mean calculated.

Statistics

Statistical evaluation included experimental data of *aurora A* mRNA (qRT-PCR: dCT values) and protein (immunohistochemistry: scores 0–3) expression, *aurora A* gene- and CEP20-specific FISH signals, tumour cell proliferation (Ki67-specific immunohistochemistry: scores 0–2), ploidy (diploid or aneuploid)

and microsatellite status as well as clinicopathological parameters of tumour T and N category and tumour grading. Samples of low- and high-grade intraepithelial neoplasia were combined as 'dysplastic epithelium' for these analyses and three cases were excluded from comparisons of chromosomal and microsatellite instable cases, due to lacking microsatellite status. Correlations between parameters were performed using the Spearman correlation coefficient. Wilcoxon signed rank test was applied to compare continuous parameters of paired samples. Frequency tables were tested by Fisher's exact test for comparison of binary parameters. A *P*-value of <0.05 was considered significant.

Results

Aurora A Expression in the Adenoma-Carcinoma Sequence of Sporadic Colorectal Cancer

Aurora A mRNA and protein expression was determined in case-matched normal and dysplastic epithelium and invasive carcinomas of 71 cases with sporadic colorectal cancer.^{45,46}

As shown in Figure 2a and Table 2, significant upregulation of *aurora A* mRNA expression occurred between normal and dysplastic epithelium (*P* = 0.0008) as well as between dysplastic epithelium and invasive carcinoma (*P* = 0.0001). *Aurora A* mRNA expression was significantly higher in invasive carcinomas than normal epithelium (*P* < 0.0001). Similarly, the frequency of *aurora A* protein expressing (hereafter referred to as 'aurora A positive') cells increased significantly from dysplastic epithelium to invasive carcinoma (*P* = 0.0027) and was markedly higher in invasive carcinomas as compared with normal epithelium (*P* = 0.001) (Figures 1 and 2b; Table 2). There were, however, no significant differences in the number of *aurora A* positive cells in normal and dysplastic epithelium (*P* = 1.000).

In invasive carcinomas, *aurora A* mRNA expression and *aurora A* positive tumour cells were significantly correlated between each other (*P* = 0.0151). High numbers of *aurora A* positive tumour cells were preferentially found in node-negative cases (pN0 category; *P* = 0.0419) but were not associated with pT category (*P* = 0.9316) or tumour grading (*P* = 0.5864).

Analysis of *Aurora A* Gene Copy Numbers and Tumour Cell (Aneuploidy)

To examine *aurora A* gene copy numbers and tumour cell ploidy,^{4,9,10,34,35,39,40} serial sections of 39/71 invasive carcinomas in which *aurora A* expression had been measured, were subjected to established FISH-based analyses.^{46,53,57}

Table 2 Summary of aurora A mRNA and protein expression in the carcinogenesis of sporadic colorectal cancers

	Normal epithelium	Dysplastic epithelium		Invasive carcinoma
		(LGIN)	(HGIN)	
<i>Aurora A</i> mRNA				
n/N (%)	70/71 (99%)	34/36 (94%)	12/13 (92%)	66/71 (93%)
Mean \pm s.d.	1	1.59 (\pm 1.1)	7.41 (\pm 15.8)	8.08 (\pm 15.2)
<i>Aurora A</i> protein				
Score 0	6/64 (9%)	6/31 (19.4%)	1/12 (8%)	4/68 (6%)
Score 1	58/64 (91%)	25/31 (80.6%)	9/12 (75%)	29/68 (43%)
Score 2	—	—	2/12 (17%)	23/68 (34%)
Score 3	—	—	—	12/68 (17%)

The table summarizes the findings of qRT-PCR and immunohistochemical analyses for aurora A mRNA and protein expression in normal and dysplastic epithelium and invasive carcinomas. **Aurora A mRNA:** The number of cases (*n/N*, percentage) with acceptable qRT-PCR results and mean relative aurora A mRNA levels (mean \pm s.d.) are given for low- (LGIN) and high (HGIN)-grade intraepithelial neoplasia and invasive carcinomas as compared with normal epithelium (set to '1'). **Aurora A protein** expression is presented as the frequency, given as number/*N* (percentage), of samples for each immunohistochemistry score. Note that there were no marked differences between samples of low- and high-grade intraepithelial neoplasia (mRNA: *P*-value = 0.530; protein: *P*-value = 0.085), which were therefore combined as 'dysplastic epithelium' for further analyses (Figure 2, main text).

passenger complex and/or proteins involved in mitotic spindle function have been associated with the development of aneuploidy in cancer cells.^{13–21} Moreover, proteins of the centrosomal complex have received substantial experimental attention as key regulators of cellular ploidy.^{29,30} Alteration of the centrosomal kinase aurora A^{31–34} by gene amplification and/or overexpression has been associated with induction of supernumerary centrosomes and/or aneuploidy in several gastrointestinal, eg esophageal,⁵⁹ gastric,⁶⁰ as well as gynaecological^{153,61,62} carcinomas.

In particular, aurora A overexpression has also been observed in colorectal cancer cell lines and/or tissues, in which it is linked to aneuploidy.^{39–48} However, a precise investigation of aurora A expression at the RNA and protein level within the adenoma-carcinoma sequence, and/or within the different subtypes of chromosomal and microsatellite instable sporadic colorectal cancers has so far not been performed. Moreover, it is still unclear whether aurora A overexpression in colorectal cancers is merely a reflection of tumour cell proliferation^{49,50} and, in this context, has no effects on chromosomal instability and/or aneuploidy. This central question still needs to be answered in view of the ongoing discussion of therapeutically targeting aurora kinases in solid tumours.^{35–38} It is conceivable that differential aurora A regulation and expression may effect the efficacy of aurora inhibitors: for example, colorectal cancers with high numbers of tumour cells only seemingly 'over'expressing aurora A due to active proliferation, may respond differently to aurora inhibitors as compared with colorectal cancers with few aurora A positive tumour cells, but in which aurora A expression is associated with gene amplification, aberrant centrosome function and/or chromosomal instability. In this study, we therefore addressed these aspects in 71 cases of sporadic

colorectal cancers, focussing on chromosomal and microsatellite instable invasive carcinomas.

Our current data supports previous findings^{39–48} that, in general, aurora A overexpression occurs at the mRNA and protein level in invasive colorectal cancers. In the adenoma-carcinoma sequence, the first increase of aurora A mRNA expression occurs at the transition of normal to dysplastic epithelium, but significant aurora A protein overexpression does only take place at the transition from dysplastic epithelium to invasive carcinoma. Statistical comparison of aurora A mRNA or protein expression between the samples of low- and high-grade intraepithelial neoplasia revealed no significant differences and these tissue samples had therefore been combined as 'dysplastic epithelium'. The observed lack of a close association of aurora A mRNA and proteins levels in dysplastic epithelium may be due to potential post-transcriptional modifications and, in particular, aurora A protein dynamics.^{63,64} Together, these results may in part reflect a general increase of proliferating dysplastic and/or invasive tumour cells. However, as low level of *aurora A* gene amplification (*aurora A* /CEP20 ratio > 1.25) was clearly present in 40% of invasive carcinomas, additional levels of aurora A regulation appear to be involved in sporadic colorectal cancers.

Indeed, comparative analyses between chromosomal and microsatellite instable invasive carcinomas strengthened the hypothesis that (de)regulation of aurora A expression occurs—uncoupled from proliferation—also at the level of *aurora A* gene copy numbers. Our detailed FISH analysis of serial tissue specimens of invasive carcinomas revealed that this occurs exclusively in those with chromosomal instability^{45,46} and correlates with aneuploidy.^{39,41,46,48} Surprisingly, our analyses revealed that, despite aneuploidy and increased *aurora A* gene copy numbers in chromosomal

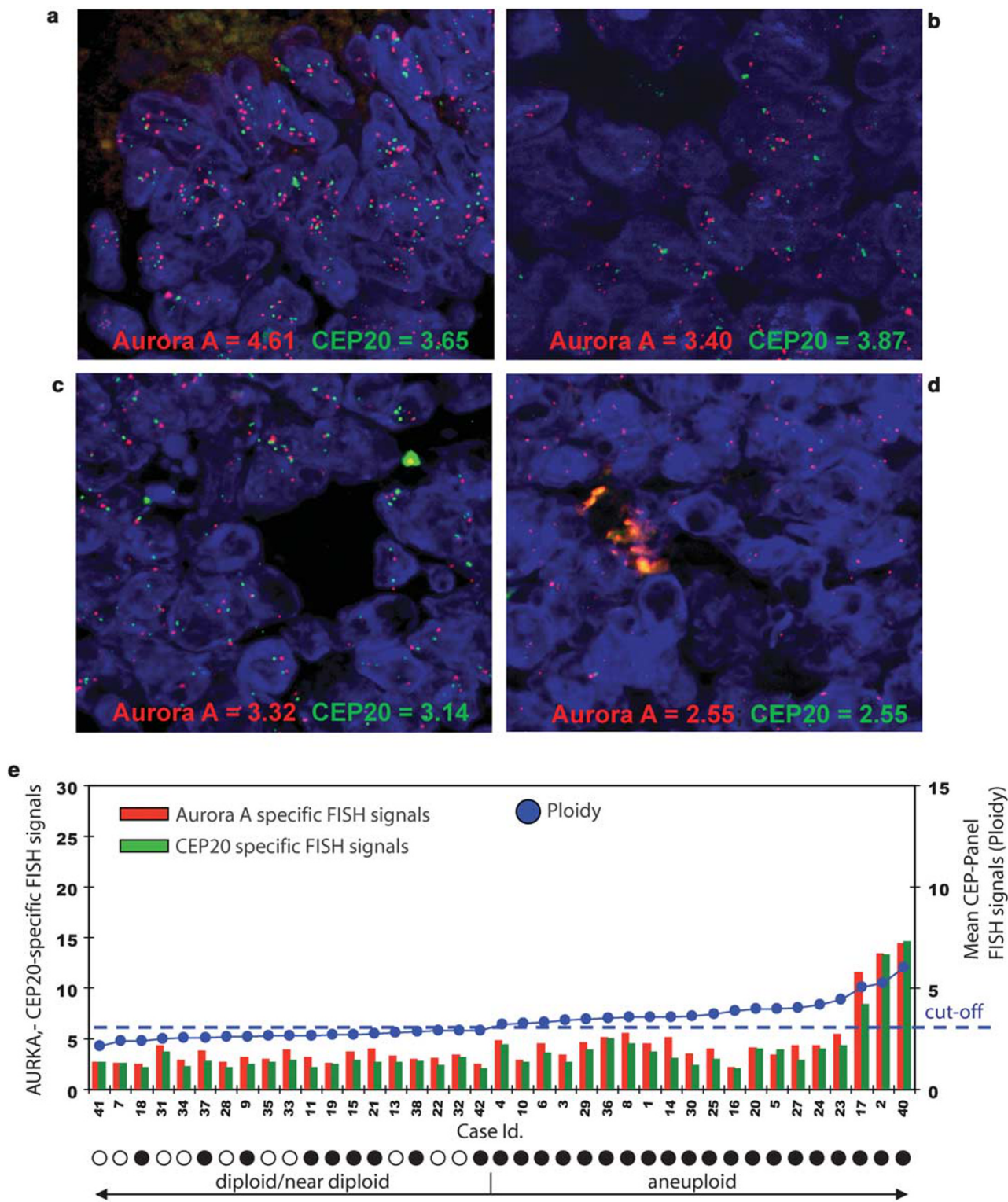


Figure 3 *Aurora A* gene- and centromere 20-specific fluorescence *in situ* hybridization analysis and summary of ploidy in sporadic colorectal cancers. (a–d) Show four representative 3D-image stacks of *aurora A*—(red signals) and CEP20- (green signals) specific fluorescence *in situ* hybridization (FISH) analysis (cell nuclei counterstained with DAPI/blue), with chromosomal instable invasive carcinomas in a (case #29) and b (case #5) and microsatellite instable invasive carcinomas in c (case #32) and d (case #7). *Aurora A* gene- and CEP20-specific FISH signals (mean of 67 ± 21 tumour cells per case) are given below each panel. (e) Summarizes the *aurora A* gene- (red bars) and CEP20- (green bars) specific FISH signals (left y-axis) and the mean count of the multi-centromere FISH panel ('ploidy'; blue closed circles, right y-axis). The cut-off for aneuploidy was 2.6 (dashed blue line, right y-axis). Note that aneuploid invasive carcinomas were exclusively chromosomal instable (black closed circles below x-axis) and that microsatellite instable invasive carcinomas (black open circles below x-axis) were diploid/near diploid with generally lower *aurora A* gene- and CEP20-specific FISH signals as compared with chromosomal instable invasive carcinomas.

Table 3 Case-specific summary of clinicopathological parameters and experimental data of sporadic chromosomal and Microsatellite instable invasive colorectal carcinomas

<i>A: Chromosomal instable</i>					<i>FISH analysis</i>			<i>Q-RT-PCR analysis</i>		<i>IHC analysis</i>	
<i>Case Id.</i>	<i>pT</i>	<i>pN</i>	<i>G</i>	<i>Histology</i>	<i>Ploidy</i>	<i>Aurora A</i>	<i>CEP20</i>	<i>Aurora A:CEP20</i>	<i>Aurora A</i>	<i>Aurora A</i>	<i>Ki67</i>
23	4	2	3	Tub	Aneuploid	5.36	4.27	1.26	3.41	0	3
40	3	0	2	Tub	Aneuploid	14.40	14.62	0.98	9.65	0	0
42	3	0	2	Tub	Diploid	2.50	2.00	1.25	1.00	1	1
19	3	0	2	Tub	Diploid	2.58	2.47	1.04	2.55	1	2
11	2	0	1	Tub	Diploid	3.21	2.11	1.52	7.78	1	0
30	3	2	3	Tub	Aneuploid	3.47	2.38	1.46	1.83	1	1
15	3	2	2	Muc	Diploid	3.71	2.81	1.32	2.60	1	3
37	3	0	2	Muc	Diploid	3.79	2.77	1.37	0.23	1	3
25	2	0	2	Tub	Aneuploid	3.95	2.97	1.33	2.17	1	3
27	1	2	2	Tub	Aneuploid	4.24	2.87	1.48	8.22	1	2
24	3	1	2	Tub	Aneuploid	4.28	4.00	1.07	4.96	1	1
8	3	0	2	Tub	Aneuploid	5.48	4.45	1.23	2.11	1	3
39	3	0	2	Tub	ND	ND	ND	ND	ND	1	0
16	3	0	2	Tub	Aneuploid	2.18	2.00	1.09	0.27	2	0
38	3	1	3	Tub	Diploid	3.01	2.74	1.10	ND	2	2
9	2	0	2	Tub	Diploid	3.16	2.43	1.30	5.82	2	1
21	2	0	2	Tub	Diploid	3.98	2.66	1.50	4.03	2	ND
20	3	0	3	Tub	Aneuploid	4.13	4.00	1.03	7.11	2	2
1	3	0	2	Tub	Aneuploid	4.44	3.71	1.20	9.00	2	2
6	3	0	2	Tub	Aneuploid	4.54	3.55	1.28	0.30	2	ND
29	3	0	2	Tub	Aneuploid	4.61	3.85	1.20	3.51	2	2
4	4	0	2	Tub	Aneuploid	4.84	4.39	1.10	2.43	2	1
14	3	1	2	Tub	Aneuploid	5.07	3.05	1.66	1.96	2	ND
17	3	1	2	Tub	Aneuploid	11.54	8.32	1.39	21.56	2	2
2	2	0	2	Tub	Aneuploid	13.35	13.29	1.00	12.30	2	0
18	3	0	3	Tub	Diploid	2.42	2.19	1.11	7.62	3	3
10	3	0	2	Tub	Aneuploid	2.89	2.62	1.10	9.13	3	2
3	3	0	2	Tub	Aneuploid	3.33	2.69	1.24	2.55	3	3
5	3	0	2	Tub	Aneuploid	3.40	3.87	0.88	23.59	3	4
36	4	0	2	Tub	Aneuploid	5.15	5.02	1.03	3.23	ND	0
26	3	2	2	Tub	ND	ND	ND	ND	19.84	ND	ND
<i>B: Microsatellite instable</i>					<i>FISH analysis</i>			<i>Q-RT-PCR analysis</i>		<i>IHC analysis</i>	
<i>Case Id.</i>	<i>pT</i>	<i>pN</i>	<i>G</i>	<i>Histology</i>	<i>Ploidy</i>	<i>Aurora A</i>	<i>CEP20</i>	<i>Aurora A:CEP20</i>	<i>Aurora A</i>	<i>Aurora A</i>	<i>Ki67</i>
13	3	1	2	Tub	Diploid	3.30	2.67	1.24	3.14	1	ND
28	3	0	2	Muc	Diploid	2.70	2.15	1.26	0.83	2	3
41	3	x	2	Tub	Diploid	2.70	2.62	1.03	10.13	2	3
22	3	0	2	Tub	Diploid	3.06	2.36	1.30	8.00	2	1
32	3	0	3	Tub	Diploid	3.32	3.14	1.06	0.57	2	1
31	3	0	3	Muc	Diploid	4.30	3.66	1.17	1.01	2	1
7	3	0	3	Muc	Diploid	2.55	2.55	1.00	4.03	3	1
34	2	0	2	Muc	Diploid	2.81	2.27	1.24	2.14	3	3
35	2	0	3	Tub	Diploid	2.93	2.70	1.09	3.51	3	3
33	3	0	2	Tub	Diploid	3.89	2.90	1.34	0.38	3	3
12	3	2	3	Tub	ND	ND	ND	ND	1.10	ND	ND

Abbreviations: G = tumour grading; Tub = tubular carcinoma; Muc = mucinous carcinoma; ND = not determined/not detectable.

The table summarises case-specific clinicopathological parameters and experimental data for **A** = chromosomal instable ($n = 31$) and **B** = Microsatellite instable ($n = 11$) invasive carcinomas investigated in detail.

Note: fluorescence *in situ* hybridization (FISH) = mean signals per cell (*Aurora A*, CEP20) and ratio (*Aurora A* signals divided by CEP20 signals); Q-RT-PCR = relative mRNA expression; immunohistochemistry (IHC) = scores for *Aurora A* and Ki67 (see also Figure 1 for examples of IHC and Figure 3 for examples of FISH).

instable invasive carcinomas, the microsatellite instable invasive carcinomas had significantly more aurora A positive tumour cells, whilst there were no significant differences between chromosomal and microsatellite instable invasive carcinomas at the aurora A mRNA level. Aurora A expression and associated regulatory mechanisms of post-transcrip-

tional modifications and/or protein stability may be distinct in chromosomal and microsatellite instable invasive carcinomas, especially in view of *aurora A* gene amplification in chromosomal instable invasive carcinomas. However, more importantly, we asked whether this frequently described aurora A 'over' expression⁴⁰⁻⁴⁴ is regulated by tumour cell

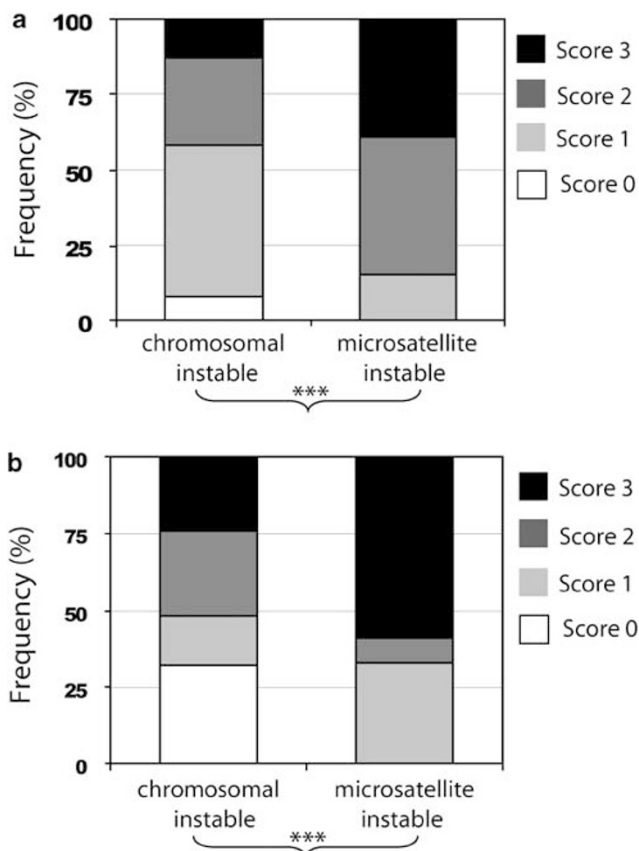


Figure 4 Comparison of aurora A protein expression and tumour cell proliferation in chromosomal and microsatellite instable sporadic colorectal cancers. The figure summarizes results of aurora A- (a) and Ki67- (b) specific immunohistochemistry in chromosomal and microsatellite instable invasive carcinomas. Note that high aurora A- and Ki67-scores were significantly more frequent in microsatellite instable as compared with chromosomal instable invasive carcinomas (marked by brackets and ***; *P*-values are given in the main text).

proliferation, especially since aurora A is an essential part of the cell cycle machinery^{49,50} and since microsatellite instable colorectal cancers show an increased rate of tumour cell proliferation.⁵⁸ This increase in proliferation may be accompanied by increased aurora A protein activity and/or stability explaining why only aurora A protein, but not mRNA levels were different between chromosomal and microsatellite instable invasive carcinomas. Indeed, also in our series of sporadic colorectal cancers microsatellite instable invasive carcinomas exhibited a higher frequency of Ki67 positive tumour cells, respective increased tumour cell proliferation on comparison to chromosomal instable invasive carcinomas. Moreover, we showed that the presence of aurora A positive tumour cells was significantly correlated with tumour cell proliferation. Taken together, we reason that aurora A (over)expression is tightly associated and regulated by tumour cell proliferation in microsatellite instable sporadic colorectal cancers, whereas an additional level of (de)regulation of aurora A (over)-

expression occurs at the DNA level in chromosomal instable sporadic colorectal cancers. In fact, strong aurora A expression can be seen in both microsatellite and chromosomal instable invasive carcinomas—only in a different frequency of tumour cells. This might introduce a measurements bias of aurora A levels obtained from whole populations of colorectal cancer cell lines and/or entire tumour extracts.^{41,48}

Further to the difference of aurora A expression and regulation at the level of specific molecular subtypes of colorectal cancer cells, the heterogeneity of aurora A within individual colorectal cancers requires further attention. In particular, it has been recently shown that aurora A is functionally linked with signalling mechanisms of cell polarity,⁶⁵ suggesting that aurora A may also play a role at the invasive tumour edge. Thus, further studies, for example, clinical phase trials of aurora inhibitors, should also include early/prospective *in situ* analyses of individual tumour cells in tissue specimens.

Clearly, as suggested before,^{44,66,67} strong aurora A expression alone does not suffice to induce chromosomal instability and/or aneuploidy, as observed in this study for diploid microsatellite instable sporadic colorectal cancers. In contrast, the close association of—and rather moderate—aurora A expression with aneuploidy in chromosomal instable sporadic colorectal cancers points to the involvement of e.g. additional defects of checkpoint controls. Thus, within the chromosomal instable invasive carcinomas it might be those with defective p53 signalling^{66,67} or other checkpoint controls,⁴⁴ in which—also marginally—elevated aurora A expression is able to trigger overt aneuploidy. In this case, the effect of aurora (-A) inhibitors may be less efficient than in aurora A expressing tumour cells without additional defective checkpoint controls. In addition, it is reasonable that the activation status of aurora A, controlled by phosphorylation as well as protein stability⁶⁴, may introduce a further functional level affecting successful aurora (-A) inhibition and tumour cell death.⁶⁸ In this context, functional investigations of inactive/active aurora A protein levels as well as other centrosomal and chromosomal passenger complex^{13–21} proteins in chromosomal and microsatellite instable colorectal tumour cell lines with specific reference to their interaction with the p53⁶⁶ and adenomatous polyposis coli^{24,25} pathways, will certainly be a worthwhile effort and are subject of our ongoing *in vitro* investigations.

Irrespective of the potential differential therapeutic response of chromosomal and microsatellite instable colorectal cancers to aurora A inhibitors, this study shows that between 42 and 85% of sporadic colorectal cancer cases may be considered for novel, aurora-targeted therapies.^{35–38} Whereas previous studies have analysed the antitumoural effect of aurora inhibitors, targeting both aurora A and aurora B, in a recent study, Manfredi *et al*⁶⁹ have

evaluated the antitumour activity of an aurora A specific inhibitor, which has also entered phase I clinical trials for solid tumours. Specifically, the authors had included three colorectal cancer cell lines in their study, two of which are microsatellite instable (HCT-116, DLD-1) and one of which is chromosomal instable (SW480). In HCT116 cells, the mechanism of action of the tested compound was a reduction of aurora A activity, but not aurora A degradation or down-regulation. The reduced phosphorylation of aurora A led to cell cycle progression delay and tumour cell growth inhibition *in vitro* as well as to a high efficacy for growth inhibition of *in vivo* HCT116 xenografts. This is in accordance with our data on aurora A expression and deregulation in microsatellite instable sporadic colorectal cancers. A detailed comparison of the effects of this aurora A inhibitor in chromosomal and microsatellite instable colorectal cancer cell lines, potentially also showing differences in mitotic checkpoint controls,^{44,66,67} will be a valuable experimental setup before further clinical testing.

Besides, aurora A expression in colorectal cancers may also be a valuable predictive marker for current therapeutic strategies, as shown for other epithelial tumours^{53,67,70,71} or in the neoadjuvant setting of rectal cancer.⁷² In fact, we found a weak association of high aurora A protein expression with node-negative colorectal cancer cases. One explanation may be that during migration (tumour) cells down-regulate their proliferative activity and hence aurora A expression. Therefore, the high proliferation rate and/or presence of high numbers of aurora A positive cells may reflect a non-migratory phenotype of colorectal cancer, ie the node-negative cases. Clearly, further studies need to address the prognostic and predictive impact of aurora A expression in view of clinical UICC stages.

In summary, this study further supports a role of aurora A in the carcinogenesis of sporadic colorectal cancers. Moreover, it demonstrates that in a minority of predominantly microsatellite instable sporadic colorectal cancers, the presence of aurora A positive tumour cells is merely reflecting tumour cell proliferation. In contrast, the large majority of chromosomal instable sporadic colorectal cancers shows additional (de)regulation of aurora A by gene amplification and concomitant tumour cell aneuploidy. Thus, sporadic colorectal cancers exhibit different mechanism of aurora A regulation and this may impact the efficacy of aurora-targeted therapies and/or may influence the associated therapeutic protocols.

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Disclosure/conflict of interest

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