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ORIGINAL ARTICLE

# Expression and localization of axin 2 in colorectal carcinoma and its clinical implication

**Ute Schaal · Sandra Grenz · Susanne Merkel · Tilman T. Rau · Michel V. Hadjihannas · Elisabeth Kremmer · Priya Chudasama · Roland S. Croner · Jürgen Behrens · Michael Stürzl · Elisabeth Naschberger**

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**Abstract**
*Purpose* Aberrant activation of the Wnt/β-catenin pathway plays a major role in the development of colorectal carcinoma (CRC). Axin 2 is a key protein of this pathway and is upregulated in CRC. Here, we investigated RNA- and protein expression of axin 2 in CRC tissues at the single cell level. Moreover, the association of axin 2 with prognosis and survival was investigated in a large cohort of CRC patients ($n$=280).
*Methods* Localization and expression of axin 2 and β-catenin was investigated using in situ hybridization and immunohistochemical staining. The quantitative expression levels of axin 2 were determined using RT-qPCR. The association of axin 2 expression with prognosis and survival of the patients was determined by statistical analysis (logrank test, Kaplan–Meier).
*Results* Our results confirmed the upregulation of axin 2 in CRC and showed that it is broadly expressed in the cytoplasm of the tumor epithelial cells both, in the tumor center and at the invasion front. Axin 2 was rarely expressed by tumor stromal cells and only weakly by normal colonic epithelial cells. Staining of β-catenin and axin 2 in consecutive CRC tissue sections revealed that nuclear translocation of β-catenin in the tumor front was not associated with changes in the cytoplasmic localization of axin 2. Axin 2 did not show any association with proven prognostic factors or survival of the CRC patients.
*Conclusion* The generally increased expression of axin 2 in all tumor stages as compared to normal tissue suggests an initiating pathogenic function in the development of CRC.

**Keywords** Wnt/β-catenin signaling · Axin 2 · β-Catenin · Colorectal carcinoma

U. Schaal · S. Grenz · P. Chudasama · M. Stürzl · E. Naschberger
Division of Molecular and Experimental Surgery, University Medical Center Erlangen, Erlangen, Germany

S. Merkel · R. S. Croner
Department of Surgery, University Medical Center Erlangen, Erlangen, Germany

T. T. Rau
Institute of Pathology, University Medical Center Erlangen, Erlangen, Germany

M. V. Hadjihannas · J. Behrens
Nikolaus-Fiebiger-Center of Molecular Medicine, University of Erlangen, Erlangen, Germany

E. Kremmer
Institute of Molecular Immunology, Helmholtz Center Munich, Marchioninistr. 25, Munich, Germany

E. Naschberger (✉)
Division of Molecular and Experimental Surgery, Department of Surgery, University Medical Center Erlangen, Schwabachanlage 10, 91054 Erlangen, Germany
e-mail: Elisabeth.Naschberger@uk-erlangen.de

## Introduction

Colorectal carcinoma (CRC) is the second most common cancer worldwide with more than one million new cases per year [1]. The cancer-related mortality is about 529,000 deaths per year worldwide with about 75 % of them being sporadic cases caused by somatic mutations. The cancer-related 5-year survival rate is between 30 % in India and 65 % in North America [1]. The majority of the sporadic cases show mutations in genes involved in Wnt/β-catenin

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signaling, such as the tumor suppressor adenomatous polyposis coli (APC) or β-catenin. Mutations in these genes lead to an aberrant activation of the Wnt/β-catenin pathway [2, 3]. The Wnt/β-catenin pathway plays a key role in several developmental processes in embryogenesis, such as axis formation and segmentation but is also involved in a number of physiological and pathophysiological processes [4, 5]. A key function of Wnt/β-catenin signaling is the maintenance of the stem cell-like character of crypt cells in the colon [6]. These cells differentiate into various cell types that constitute the colonic epithelium [7, 8]. A recent study showed that the Wnt/β-catenin signaling is altered in 93 % of colorectal cancer cases, including mutations in 16 Wnt β-catenin pathway genes. This fact underlines the essential role of Wnt/β-catenin signaling in diseases like cancer [9].

In humans, 19 different Wnt proteins are known and may bind to their respective receptors namely frizzled (Fz) and LDL receptor-related protein 5/6 (Lrp5/6). Subsequently, the transduced signals lead to the stabilization of β-catenin and ultimately to the transcription of β-catenin target genes [10]. The level of cytoplasmic β-catenin is constantly balanced by the so called "destruction complex" in the absence of Wnt proteins. The destruction complex is composed of the scaffold proteins axin 1 or axin 2/conductin, APC, casein kinase 1 (CK1) and glycogen synthase kinase-3beta (GSK-3β). The complex continuously phosphorylates β-catenin inducing its ubiquitination and subsequent proteasomal degradation [11, 12]. In contrast, after Wnt protein binding and activation of the Wnt receptors, axin proteins are recruited to the phosphorylated Lrp5 receptor by the protein dishevelled (Dsh). Thus axin is no longer available as scaffold protein, resulting in inactivation of the destruction complex [13, 14]. Consequently, β-catenin is stabilized and can shuttle into the nucleus, where it interacts with the transcription factors T-cell factor/lymphoid enhancer-binding factor (Tcf/Lef) initiating transcription of the Wnt target genes [15, 16]. Among these target genes are c-myc, cyclin D1, MMP7, and axin 2 [17, 18]. Of note, axin 2 regulates the level of nuclear β-catenin in a negative-feedback loop thereby being a negative regulator and target gene at the same time [18, 19]. There are two axin genes in humans, axin and axin 2 which are considered to be functionally homologous. However, only axin 2 is expressed in a Wnt/β-catenin signaling dependent manner, while axin is constitutively expressed [20]. Mutations in axin 2 are found in familial tooth agenesis and predispose to colorectal cancer [21]. It has previously been shown, that axin 2 is overexpressed in different tumor tissues such as colorectal or liver carcinomas and tumor cell lines (e.g., colon carcinoma, hepatoblastoma, and lung carcinoma cells) due to aberrant activation of the Wnt/β-catenin pathway [19, 22]. However, all of these studies employed small cohorts of patient samples and did not analyze the possible association with clinical parameters [22].

Moreover, an important aspect of Wnt/β-catenin function in CRC tissue is the nuclear distribution of β-catenin. Nuclear β-catenin is an accepted marker for an active Wnt/β-catenin pathway and is correlated with a poor prognosis for CRC patients [8, 23, 24]. However, several studies show that nuclear β-catenin is heterogeneously distributed in CRC tumors, even though the Wnt/β-catenin pathway is constitutively activated [25–27]. This phenomenon is called "β-catenin paradox", and was speculated to be influenced by a yet unknown function of axin 2 [20, 28, 29].

Here, we investigated the expression of axin 2 at the single cell level in CRC tissues in relation to β-catenin localization and in relation to clinical parameters of the patients.

## Material and methods

### Study participants

The study is based on specimen and prospectively collected data from well-documented colorectal carcinomas who underwent surgery at the Department of Surgery (University Medical Center Erlangen). In a pilot experiment, 29 colorectal carcinomas with the following International Union against Cancer (UICC) stages [30] were included: stage I ($n$=8), stage II ($n$=6), stage III ($n$=6), and stage IV ($n$=9).

### Tissue microarray

The tissue microarray (TMA) was described earlier [31]. Patients were included who underwent surgery at the Department of Surgery (University Medical Center Erlangen) in between 1991 to 2001. Median follow-up was 125.10 months (range 0.56–230.49 months). Patients with the following criteria were included: solitary invasive colon carcinoma UICC stage II–IV, no appendix carcinoma, no other previous or synchronous malignant tumors except basalioma of the skin, no familial adenomatous polyposis, ulcerative colitis or Crohn's disease, no neoadjuvant therapy, treatment with colon resection with regional lymph node dissection and no local residual tumor (classification R0). Cases of hereditary nonpolyposis colorectal cancer (HNPCC) were not excluded. Patients who died postoperatively and patients with unknown tumor status (with respect to local and distant recurrence) at the end of the study were excluded. Altogether, 133 of 280 patients died and from these 65 patients died because of the CRC. Histopathological grading was divided in the categories low and high grade.

Three punch biopsies from the tumor center and three punch biopsies from the tumor invasion front of each patient were integrated in 14 paraffin array blocks. In total, punches of 280 patients were available for immunohistochemical

staining. At the end, total punches from 234 patients were valid for evaluation for the tumor center and punches from 222 patients of the invasion front (Table 2).

Scoring was performed as described earlier [31]. Two independent investigators were evaluating the TMA. An average value of the three punches for the tumor center or the invasion front was calculated and resulted in one final score for each patient, with respect to intensity, cell count, tumor center, and invasion front.

### Immunohistochemical staining

Immunohistochemical staining was performed as described earlier [32, 33]. In brief, paraformaldehyde fixed paraffin embedded (FFPE) tissue was cut in 4-μm sections, dewaxed in xylene and rehydrated. Antigen retrieval was performed in a 95 °C water bath using Target Retrieval Solution pH 6 (Dako, Hamburg, Germany). The following primary antibodies were used: polyclonal rabbit anti-human axin 2 (ab32197, Abcam, Cambridge, UK, 1:10,000) and monoclonal mouse anti-human β-catenin (#610154, BD Transduction Laboratories, Heidelberg, Germany, 1:750). The polyclonal rabbit anti-human axin 2 antibody is specific for axin 2 and does not cross-react with axin 1. The ABC Vectastain Elite kit (PK-6101 and PK-7200, Vector Laboratories, Burlingame, CA) and NovaRed substrate (SK-4800, Vector Laboratories) were used for detection according to the manufacturers' instruction. Counterstaining was performed using Gill-III hematoxylin (Merck, Darmstadt, Germany). Microscopic pictures were taken with a Leica DM6000 and Leica Aristoplan (Leica, Mannheim, Germany).

### In situ hybridization with S35-labeled probes

Specimens were processed as previously described [34]. As a template for in vitro transcription a specific sequence (957 bp) of axin 2 cDNA (NM_004655) was cloned in the pcDNA3 expression vector in sense and antisense orientation. Primer forward: 5′-AATGGTACCTCCCCGGACCACCACC-3′; Primer reverse: 5′-AAAGGTACCCTTCAGCATCC-TCCGG-3′. T7 Polymerase (Agilent, Böblingen, Germany) was used for transcription of RNA. After 4 weeks of exposure the sections were counterstained with Gill-III hematoxylin (Merck) and eosin (Sigma-Aldrich, Taufkirchen, Germany). Positive signals appear as black silver grains in the bright field and as white grains in the dark field due to light scattering. Microscopic pictures were taken using a Leica DM6000 (Leica).

### Cell culture and immunocytochemical staining

The CRC cell lines SW480 and DLD-1 were purchased from ATCC (Reference number CCL-228 and CCL-221). SW480 were cultured in DMEM (PAA, Pasching, Austria) supplemented with 10 % (*v*/*v*) fetal bovine serum (FBS, PAA). DLD-1 cells were cultured in RPMI 1640 (PAA) supplemented with 10 % (*v*/*v*) FBS (PAA). For immunocytochemical staining cells were seeded on glass slides and fixated in 4 % PFA. The cells were permeabilized with 0.1 % saponin and then immunocytochemically stained using polyclonal rabbit anti-human axin 2 (ab32197, Abcam, 1:5,000). The anti-rabbit-ABC Vectastain Elite kit and NovaRed substrate (SK-4800) were used for detection according to the manufacturers' instruction. Counterstaining was performed using Gill-III hematoxylin. Microscopic pictures were taken with a Leica DM6000 (Leica).

### Western blot

Western Blot was performed as previously described [31, 35]. Two anti-axin 2 antibodies were used to prove specificity: polyclonal rabbit anti-human axin 2 (ab32197, Abcam, 1:20,000) and monoclonal rat anti-human axin 2 clone Cond4 1D5 (E. Kremmer, hybridoma supernatant, 1:5).

### RNA extraction

RNA was extracted from 80 CRC samples and 20 normal mucosa samples from prospectively collected patients who underwent surgery at the Department of Surgery (University Medical Center Erlangen). Total RNA was isolated from 10 μm whole FFPE tissue sections of CRC samples with a fully automated method based on silica-coated iron oxide beads (Tissue Preparation System with VERSANT Tissue Preparation Reagents, Siemens Healthcare Diagnostics, Tarrytown, NY) [36, 37]. In the last step, an automatic DNase I digestion (Life Technologies, Carlsbad, CA) was also performed on the system.

### Reverse transcription-kinetic PCR (RT-qPCR)

RT-qPCR analysis was performed as described earlier [36, 38, 39]. Briefly, samples were analyzed using the SuperScript III Platinum One-step Quantitative RT-PCR System including ROX (Life Technologies) according to manufacturer's instructions. Each sample was included in triplicates on the Siemens VERSANT ® kPCR Molecular System (Siemens Healthcare Diagnostics). Normalization of the signal was performed using the expression levels of the housekeeping gene ribosomal protein L37A (RPL37A). DNA contamination of isolated RNA was estimated with a DNA specific primer set of progestagen-associated endometrial protein (PAEP). The expression levels between tumor and normal tissue samples were calculated according the ΔΔCt method with ΔCt=average of $Ct_{axin\ 2}$− average of $Ct_{RPL37A}$ [40]. Fold change values indicated the upregulation of expression normalized to normal tissue samples. The sequences of primers and probes were as follows:

Axin 2 forward: 5′-GTCTCTACCTCATTTCCCGAG AAC-3′, reverse: 5′-CGAGATCAGCTCAGCTGC AA-3′, probe: 5′-Fam-ACCCCCGTGGAACCCG CC-Tamra-3′;
RPL37A forward: 5′-TGTGGTTCCTGCAT GAAGACA-3′, reverse: 5′-GTGACAGCGGAA GTGGTATTGTAC-3′, probe: 5′-Fam-TGGCTGGC GGTGCCTGGA-Tamra-3′;
PAEP forward: 5′-CACAGAATGGACGCCATGAC-3′, reverse: 5′-AAACCAGAGAGGCCACCCTAA-3′, probe: 5′-Fam-AAGCCCTCAGCCCTGCTCTCCATC-Tamra-3′.

## Statistical analysis

Statistical analysis for the tissue microarray was performed according to earlier description [31]. The $\chi^2$ analysis for linear trend was used to examine the association between the expression of axin 2 and clinical parameters. The Kaplan–Meier method was used to calculate the survival of patients. Four different kinds of survival defined by different events were calculated. Overall survival includes all events of death whereas cancer-specific survival is related to patients' death due to CRC. Additionally, the survival rates regarding locoregional recurrence or development of distant metastasis were used. The

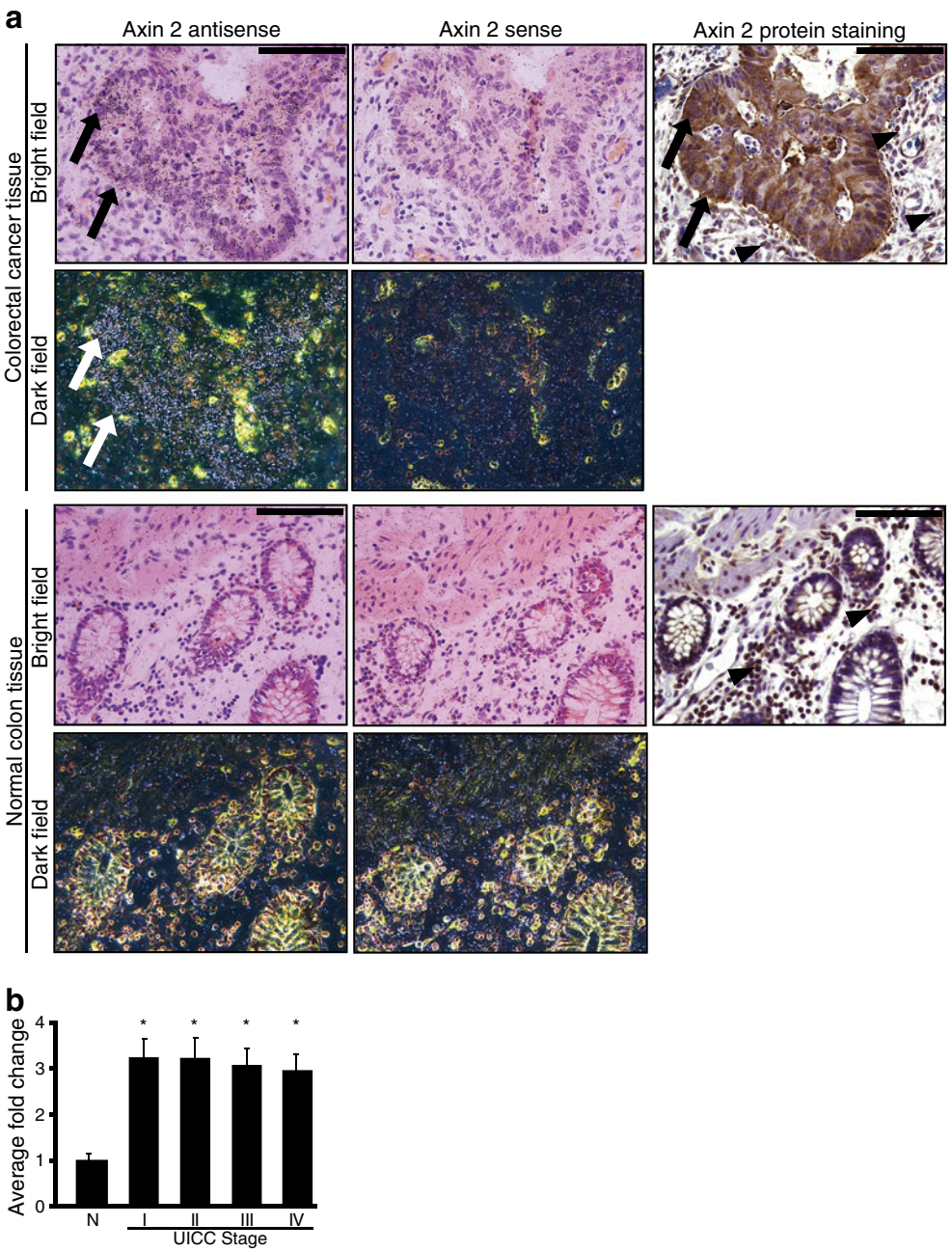


**Fig. 1** Axin 2 is overexpressed by tumor cells in the colorectal carcinoma. In situ hybridization of CRC tissue sections and normal colon tissue with S35-radiolabelled axin 2 antisense (*left panel*) and sense (*middle panel*) RNA strand hybridization probes. Signals were obtained with the antisense hybridization probe (complementary to axin 2 mRNA) in the epithelial cells of CRC tissue (**a**, *arrows*) and normal colon tissue (**a**), both in the bright field (*black grains*) and in the dark field (*white grains*). Control hybridization with the axin 2 sense strand RNA probe did not show specific signals (*middle panel*). Immunohistochemical staining of axin 2 on consecutive sections in CRC and normal colon tissue (**a**, *right panel*). Examples of axin 2 positive tumor cells (*brown*) are indicated by arrows and positive infiltrating immune cells are marked by *arrowheads*. *Scale bars* correspond to 100 μm. Axin 2 expression levels were quantitatively determined with specific probes using RT-qPCR (**b**). The indicated values are average values of the tumor samples normalized to average values of the normal samples. RPL37A was used as endogenous expression control. *Asterisks* indicate significant differences in relation to normal samples ($p<0.001$)

logrank test was used to compare survival rates. The statistical significances were calculated using Student's *t* test for paired samples. *p* values below 0.05 were considered to be statistically significant. All analyses were performed using SPSS software version 18 (IBM, Ehningen, Germany).

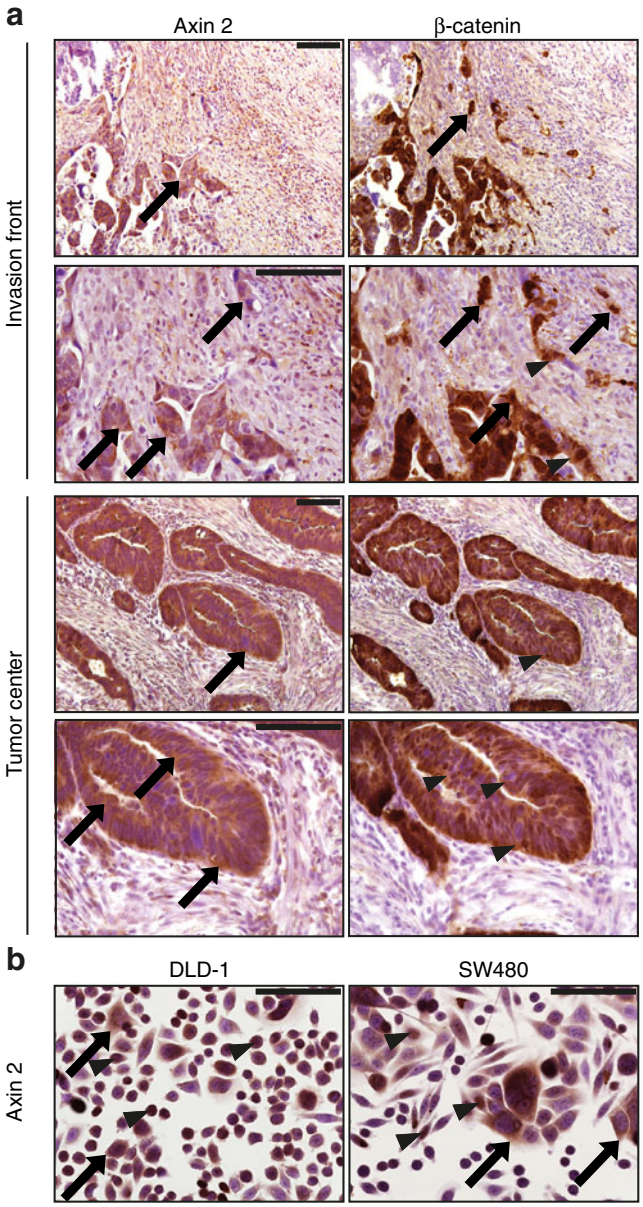


**Fig. 2** Axin 2 expression and subcellular localization is not affected by nuclear β-catenin. Immunohistochemical staining of axin 2 (**a**, *left panel*) and β-catenin (**a**, *right panel*) on consecutive sections at the invasion front and in the tumor center of CRC. Positive (*brown*) staining of axin 2 and nuclear staining of β-catenin at the invasion front are indicated by *arrows*. Cytoplasmic staining of β-catenin is indicated by arrowheads. Immunocytochemical staining of axin 2 in DLD-1 cells (**b**, *left panel*) and SW480 cells (**b**, *right panel*). Positive (*brown*) staining of axin 2 in the cytoplasm is indicated by *arrows*. Positive staining in the nucleus is indicated by *arrowheads*. *Scale bars* correspond to 100 μm

## Results

### Axin 2 is overexpressed by tumor cells in colorectal carcinoma tissue

In order to determine the expression of axin 2 in CRC tissue on RNA- and protein level axin 2 in situ hybridization and immunohistochemical staining on consecutive sections of CRC and normal colon tissue were performed.

**Table 1** Clinical characteristics of the colorectal carcinoma patients included in the tissue microarray analysis ($n$=280)

| | *n* | % |
|---|---|---|
| Sex ratio (male / female) | 171/109=1.57 | |
| Age median / range (years) | 63.54/28–91 | |
| Tumor site | | |
| Cecum | 30 | 10.7 |
| Ascending colon | 44 | 15.7 |
| Hepatic flexure | 19 | 6.8 |
| Transverse colon | 24 | 8.6 |
| Splenic flexure | 14 | 5 |
| Descending colon | 14 | 5 |
| Sigmoid colon | 135 | 48.2 |
| Stage (UICC 2002) | | |
| II | 139 | 49.6 |
| III | 116 | 41.4 |
| IV | 25 | 8.9 |
| Primary tumor | | |
| pT2 | 23 | 8.2 |
| pT3 | 225 | 80.4 |
| pT4 | 32 | 11.4 |
| Regional lymph nodes | | |
| pN0 | 147 | 52.5 |
| pN1 | 86 | 30.7 |
| pN2 | 47 | 16.8 |
| Distant metastasis | | |
| M0 | 255 | 91.1 |
| M1 | 25 | 8.9 |
| Histopathological grading | | |
| Low grade (G1/G2) | 236 | 84.3 |
| High grade (G3/G4) | 44 | 15.7 |
| Venous invasion | | |
| Negative | 232[a] | 82.9 |
| Positive | 47[a] | 16.8 |
| Lymphatic vessel invasion | | |
| Negative | 107 | 38.2 |
| Positive | 173 | 61.8 |

[a] Venous invasion of one patient was unknown

Axin 2 was robustly expressed by epithelial tumor cells as compared to cells of the surrounding stroma (Fig. 1a). The upregulation of axin 2 expression in tumor cells was detected both, at the RNA level (Fig. 1a, left panel, arrows), as well as at the protein level (Fig. 1a, right panel, arrows). In contrast, axin 2 RNA and protein were only very weakly expressed in normal colon tissue (Fig. 1a). Infiltrating immune cells expressed axin 2 on protein level (Fig. 1a, right panel, arrowheads). In these cells, no axin 2 expression was detectable on RNA level (Fig. 1a, left panel). Of note, antibody specificity was proven by western blot in addition (data not shown).

Furthermore, RT-qPCR was used to quantitatively determine axin 2 expression levels in 80 CRC patients in different UICC stages of the disease (20 patients per stage) and 20 normal colon samples. Axin 2 expression was significantly increased in tumors of all stages as compared to normal colon tissue (Fig. 1b). Between the different tumor stages axin 2 expression was constant (Fig. 1b).

## Axin 2 subcellular localization is not affected by nuclear translocation of β-catenin

Recent studies investigated the "paradox" of β-catenin localization in CRC tissue [29, 41]. It has been speculated that the axin 2 localization might influence the nuclear/cytoplasmic distribution of β-catenin. This was addressed by staining of axin 2 and β-catenin in consecutive CRC tissue sections. In agreement with previous reports [25, 26] β-catenin was localized in the nuclei and the cytoplasm of the tumor cells at the invasion front (Fig. 2a, right panel, arrows) whereas it was only present in the cytoplasm of tumor cells in the tumor center (Fig. 2a, right panel, arrowheads). In contrast, axin 2 was consistently located in the cytoplasm both, at the invasion front and in the tumor center (Fig. 2a, left panel, arrows).

Nuclear translocation of axin has been described [42]. To verify that this can be detected with the technology used, an immunocytochemical staining of the colorectal cell lines SW480 and DLD-1 was performed. Axin 2 was detected both in the cytoplasm (Fig. 2b, arrows) and in the nucleus (Fig. 2b,

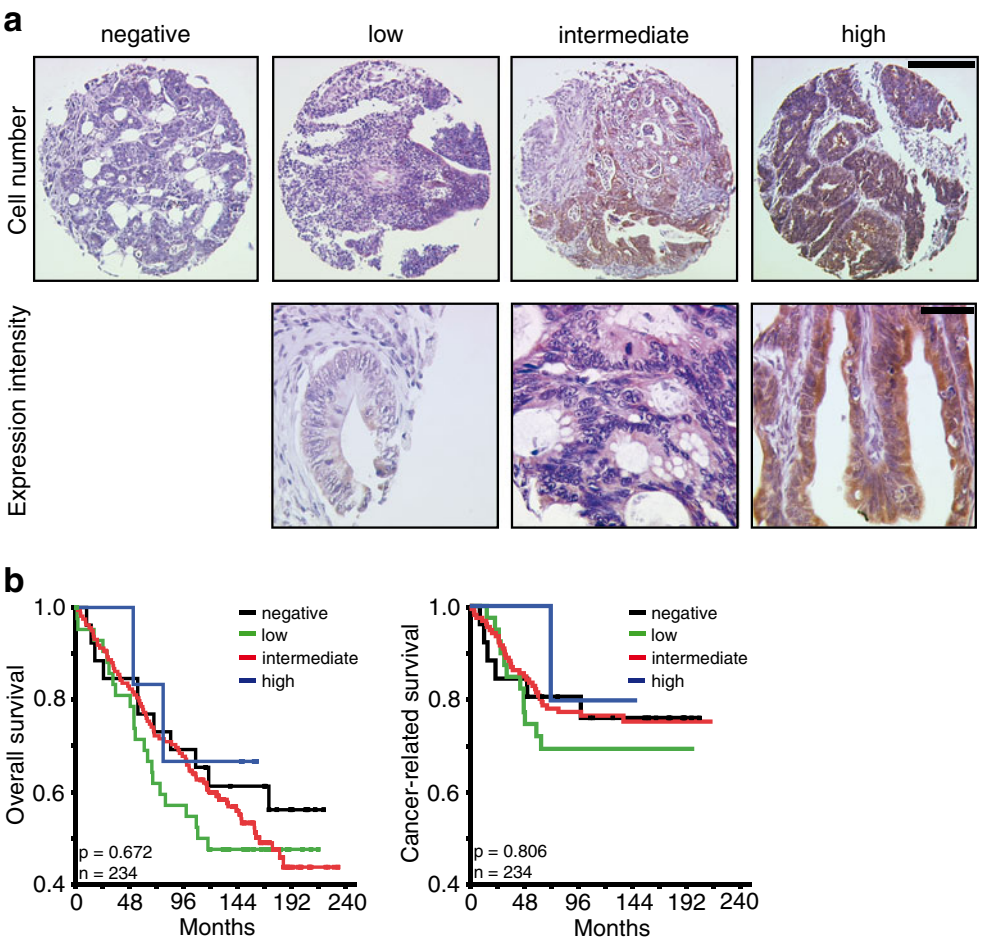


**Fig. 3** Axin 2 expression is not associated with clinical prognosis in CRC. Tissue microarray punches were stained for axin 2 by immunohistochemical staining. The punches were scored for positive cells (**a**, *cell number*) and expression intensity (**a**, *expression intensity*) using different categories (*negative*, *low*, *intermediate*, and *high*). *Scale bars* correspond to 250 μm (*upper panel*) and 50 μm (*lower panel*). Kaplan–Meier analysis was performed and curves for the evaluation of axin 2 staining in the tumor center regarding overall survival (**b**, *left side*) and cancer-related survival (**b**, *right side*) are depicted as examples

arrowheads). This experiment demonstrated that nuclear axin 2 can be detected using the applied methodology and that apparently subcellular translocation of axin 2 and β-catenin are not related in CRC tissues in vivo.

## Axin 2 expression is not associated with clinical prognosis in CRC

The strong overexpression of axin 2 in tumor cells and its key role in the regulation of the Wnt/β-catenin pathway led to the question whether expression of the axin 2 protein may correlate with survival, locoregional recurrence or distant metastasis development in CRC patients.

Therefore, axin 2 was immunohistochemically stained using a tissue microarray of 280 CRC patients with well-documented clinical parameters. Non-metastasizing (UICC Stage II, 49.6 %) and metastasizing CRC (UICC Stage III/IV, 50.3 %) were matched to prevent metastasis-dependent bias (Table 1). Standard characteristics such as sex ratio (1.57) and mean age (63.54 years) were consistent with published CRC statistics (Table 1) [31]. The number of stained cells and their expression intensity was evaluated for each patient (three punches of tumor center and invasion front each) by two independent investigators (Fig. 3a and Table 2). Punches lost during the staining procedure were declared as "missing values" (Table 2).

Axin 2 was predominantly expressed by the epithelial tumor cells both, at the tumor center and at the invasion front in comparison to stromal cells ($\chi^2$ test: $p<0.001$) (Table 2). More precisely, in the tumor center, 88.4 % of the tumor cells but only 35.8 % of the stromal cells were axin 2 positive (Table 2; low, intermediate and high). Similarly, at the invasion front 76.4 % of the tumor cells compared to 35.0 % of the stromal cells were axin 2 positive. The number of cells expressing axin 2 are comparable between the tumor center and the invasion front ($p=0.351$). With respect to expression intensity all of the cells expressed axin 2 either at low or intermediate levels. High expression intensity of axin 2 was less frequently found (tumor center, 18.4 % tumor cells and 2.2 % stromal cells (high vs. low/intermediate: $p=0.002$); invasion front, 6.1 % tumor cells and 0.5 % stromal cells [high vs. low/intermediate: $p<0.001$]) (Table 2). Of note, among the 280 CRC patients only 11 patients (=3.9 %) were completely negative.

Finally, Kaplan–Meier analysis and correlation studies were performed. In the correlation studies T-, N-, M-category, histopathological grading, venous invasion, lymphatic vessel invasion, tumor site and UICC stage was analyzed in correlation to axin 2 expression. None of the analyzed clinical parameters showed a significant association with axin 2 expression levels (data not shown). For Kaplan–Meier analysis, axin 2 staining was classified in different categories. The number of positive cells and their staining intensity was analyzed in the tumor center versus the invasion front. None of these parameters alone or in combination did show a significant correlation between axin

**Table 2** Axin 2 is mainly expressed by tumor cells in the tumor center and at the invasion front with similar expression levels. The distribution of the axin 2 staining is expressed by tumor and stromal cells in the tumor center and in the invasion front is given by cell number and expression intensity using different categories

| | | |
|---|---|---|
| Tumor center | | |
| Tumor cells | Cell number (*n*/%) | Intensity (*n*/%) |
| Negative | 27/11.6 | 27/11.6 |
| Low | 9/3.9 | 65/27.9 |
| Intermediate | 43/18.4 | 98/42.1 |
| High | 154/66.1 | 43/18.4 |
| Missing[a] | 47 | 47 |
| Stromal cells | | |
| Negative | 147/64.2 | 147/64.2 |
| Low | 28/12.2 | 37/16.2 |
| Intermediate | 42/18.3 | 40/17.4 |
| High | 12/5.3 | 5/2.2 |
| Missing[a] | 51 | 51 |
| Invasion front | | |
| Tumor cells | | |
| Negative | 50/23.6 | 50/23.6 |
| Low | 12/5.7 | 82/38.7 |
| Intermediate | 59/27.8 | 67/31.6 |
| High | 91/42.9 | 13/6.1 |
| Missing[a] | 68 | 68 |
| Stromal cells | | |
| Negative | 143/65.0 | 143/65.0 |
| Low | 30/13.6 | 45/20.4 |
| Intermediate | 41/18.7 | 31/14.1 |
| High | 6/2.7 | 1/0.5 |
| Missing[a] | 60 | 60 |

[a] Missing values were not included in the calculation of relative percentages in order to show the relative distribution of axin 2 expression in the stained punch biopsies only

**Table 3** Axin 2 expression is not significantly associated with the survival of CRC patients. *p* values of Kaplan–Meier analysis for the indicated types of survival are given

| | Tumor center | Invasion front | Cell number | Intensity |
|---|---|---|---|---|
| Overall survival | 0.672 | 0.639 | 0.213 | 0.823 |
| Cancer-specific survival | 0.806 | 0.897 | 0.781 | 0.828 |
| Locoregional recurrence | 0.633 | 0.996 | 0.500 | 0.336 |
| Distant metastasis | 0.674 | 0.681 | 0.709 | 0.680 |

2 expression and survival rate (Fig. 3b, Table 3). Of note, survival analysis with patients older than 50 years only ($n$=251, 89.6 %) thereby excluding potential HNPCC cases yielded identical results (data not shown). In conclusion, axin 2 expression was not associated with classical clinical parameters and survival of CRC patients.

## Discussion

The Wnt/β-catenin signaling cascade is an important pathway involved in initiation and maintenance of CRC [43]. Therefore, the understanding of the function and clinical relevance of this pathway is mandatory.

Axin 2 is a key regulator in Wnt/β-catenin signaling and a target gene at the same time. It acts in a negative-feedback loop to regulate the stability of β-catenin [18, 19]. Axin 2 is known to be overexpressed in different tumors and cancer cell lines [19, 22]. In agreement with this, we showed at single cell level that axin 2 is overexpressed in the tumor epithelial cells of CRC in contrast to colonic normal tissue where axin 2 expression is low. Axin 2 expression was not related to tumor stage, indicating that this protein may not be engaged in the regulation of tumor malignancy including metastasis but may regulate the maintenance of the tumorigenic phenotype.

An open issue in understanding Wnt/β-catenin signaling on CRC is the so called "βcatenin paradox". This addresses the fact that nuclear β-catenin, an accepted marker for an active Wnt/β-catenin pathway, is heterogeneously distributed in CRC tumors, whereas the Wnt/β-catenin pathway was found to be constitutively activated [25, 26]. Our study was in accord with these results and a further in vitro study [44], showing that the β-catenin localization was not affected by the expression of axin 2. This indicated that the intracellular localization of β-catenin is not regulated by axin 2. Recently, an influence of MAPK signaling [29] and phosphorylation of β-catenin at tyrosine 654 [41] have been suggested to be involved in this regulation, which warrants further investigation.

Earlier studies investigating the cell biological functions of axin 2 showed that it is localized at the centrosomes and mitotic spindles. Overexpressed axin 2 was shown to induce chromosomal instability (CIN) [45, 46]. CIN is believed to be a driving force during carcinogenesis [47]. In consequence, it was of high interest to analyze the impact of endogenous axin 2 on classical clinical parameters and the survival of patients in a large cohort ($n$=280) of CRC. From a total of 280 patients only 11 patients were negative for axin 2 expression in the tissue microarray. Tumor cells expressed axin 2 significantly higher than stromal cells ($\chi^2$ test: $p$<0.001). This is in agreement with the fact, that axin 2 is a target gene of Wnt/β-catenin signaling [17, 18], which is aberrantly activated in the tumor epithelial cells in CRC. Recently, an in vitro study showed a positive influence of axin 2 on the invasive capacity of CRC cell lines [48]. However, in a clinical retrospective study, we did not detect an association of axin 2 expression with clinical parameters such as UICC stage, histopathological grading or survival of the patients. This indicates that the in vitro effects of axin 2 on invasion may be masked by microenviromental effects in the tumors.

The general upregulation of axin 2 in CRC suggests that this protein may be an early marker for malignant transformation and it may be interesting to investigate axin 2 expression in benign adenomas of the colon. Assuming a general tumorigenicity maintenance function of axin 2 in CRC, it may be speculated that this protein may be an early tumor-specific target for therapy of CRC.

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**Ethical standards** All human studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All patients gave their informed consent prior to their inclusion in the study.

**Conflict of interest** The authors declare that they have no conflict of interest.

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