Pathol. Oncol. Res. (2016) 22:95–102
DOI 10.1007/s12253-015-9977-9

ORIGINAL ARTICLE

Loss of H2Bub1 Expression is Linked to Poor Prognosis in Nodal Negative Colorectal Cancers

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Received: 15 June 2015 / Accepted: 25 August 2015 / Published online: 7 September 2015

Abstract To correlate H2Bub1 expression with outcome in colorectal cancer, H2Bub1 expression was analyzed by immunohistochemistry on a tissue microarray containing 1800 colorectal cancers. Results were compared to clinicopathological parameters.

H2Bub1 IHC was seen in 1256 (79.3 %) of 1584 interpretable CRC and was considered weak in 26.2 % and strong in 53.1 % of cancers. H2Bub1 expression was completely lost in 20.7 % of the cases. Loss of H2Bub1 expression was associated with high tumor grade (p = 0.0211), high tumor stage (p = 0.0003), positive nodal status (p = 0.0139) and histological tumor type (p = 0.0202). No link was found between H2Bub1 expression and tumor localization (p = 0.1262), peritumoral lymphocytic infiltration (p = 0.2523) or vascular invasion (p = 0.5970).

Loss of H2Bub1 expression in CRC was strongly associated with poor patient survival (p = 0.0006). This observation held true also in a subset survival analysis of nodal negative (N0) and nodal positive (N1) cancers (p = 0.0296 and p = 0.0197, respectively). In the subgroup of p53 negative cancers no prognostic impact of H2Bub1 staining was seen (p = 0.1924), whereas in p53 positive CRC H2Bub1 expression loss was associated with poor prognosis (p = 0.0031). Strikingly worsened outcome was found for nodal negative cancers presenting with accumulation of p53 when H2Bub1 expression was lost (p = 0.0006).

Our data demonstrate that a reduced H2Bub1 expression is a strong prognostic biomarker both in nodal negative and nodal positive CRC. H2Bub1 expression measurement might help to select nodal negative CRC patients that may benefit from adjuvant therapy.

Keywords Tissue microarray · Gastrointestinal cancer · Colorectal cancer · Biomarker

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Introduction

Colorectal cancer (CRC) is the fourth most common malignant disease with over one million novel cases and over 500.000 deaths each year worldwide [1]. Although recent advances in the management of the disease have improved outcomes, CRC remains the second leading cause of cancer-related death in Western countries [1]. In advanced metastatic colorectal cancer (mCRC), surgery alone is not curative and therefore adjuvant chemotherapy is needed. Nodal negative status is a generally favorable prognostic sign. However, approximately one third of node-negative CRCs recurs or shows progressive disease, suggesting failure to detect occult disease [2]. Because aberrant genetic changes occur early in tumor progression and are associated with lymphatic metastases, molecular profiling of specific tumor markers in the primary tumor might predict the tumor's metastatic potential. Thus,

patients with a potentially aggressive tumor, although not yet metastasized at time of surgery might also benefit from adjuvant therapy.

Recent studies have uncovered a potential tumor suppressor role for histone H2B monoubiquitination (H2Bub1). Like many other histone modifications, H2Bub1 has diverse functions and plays roles both in transcriptional activation and repression as well as in controlling mRNA processing and directing DNA repair processes [3–12].

Identification of H2Bub1 as a key transcriptional regulator raised the likelihood that it may be altered in cancer development, and the possibility that H2Bub1 itself may have tumor suppressive roles [13]. Recently, H2Bub1 has been shown to be lost in advanced cancers, including breast, parathyroid and lung cancer [14–16].

To further elucidate the clinical relevance of H2Bub1 as either a diagnostic or prognostic tumor marker we performed an immunohistochemical (IHC) study on a large cohort of CRC patients utilizing our pre-existing CRC microarray containing 1800 CRC specimens. The results of this study show that H2Bub1 expression loss is a predictor of poor outcome in CRC.

Materials and Methods

Patients and Tissue Microarray (TMA) Construction

Two different TMAs with a total of 1800 CRC samples were included in this study. The first TMA was manufactured from resection specimens of 1420 CRC patients at the Institute of Pathology of the University Hospital of Basel. Raw survival data were obtained from the responsible physicians for all of the 1420 patients. The median follow up time was 46 months (range 1–152 months).

The second TMA included samples from 380 CRC patients, whose tumor resection specimens were examined at the Institute of Pathology of the University Medical Center, Hamburg-Eppendorf. For this TMA too, raw survival data were available for all of the 380 patients with a median follow up period of 36 months (range 1–179 months). There were no differences between the two patient cohorts concerning epidemiology and cancer related parameters. TMA construction was as described [17]. In brief, hematoxylin and eosin-stained sections were made from each block to define representative tumor regions. One tissue cylinder with a diameter of 0.6 mm was then punched from the tumor on the "donor" tissue block using a home made semi-automated precision instrument and brought into empty recipient paraffin blocks. Four μm sections of the resulting TMA blocks were transferred to an adhesive coated slide system (Instrumedics Inc., Hackensack, New Jersey). Patient information and clinical data such as age, sex, localization and type of the tumor, pTNM-stage and carcinoma grade were retrospectively retrieved from clinical and pathological databases (Table 1). All tumors were re-classified by two pathologists (PS, AM). For statistical analyses, tumor localizations were grouped as follows: right-sided cancer (cecum, ascending colon), cancer of the transverse colon, cancer of the left-sided colon (descending and sigmoid colon), and rectum. For internal controls, each TMA block also contained various control tissues, including 55 specimens of normal colonic mucosa. The molecular database attached to this TMA contained results on p53 expression in 665 cases. Follow-up data were obtained from local cancer register boards or via attending physicians. The use of tissues and clinical data were according to the Hamburger Krankenhaus Gesetz (§ 12) and approved by our local ethical committee.

Immunohistochemistry

Freshly cut TMA sections were analyzed on 1 day and in one experiment. Slides were deparaffinized and exposed to heat-induced antigen retrieval for 5 min in an autoclave at 121 C in pH 7.8 Tris–ethylenediaminetetraacetic acid–citrate buffer. Primary antibody specific for H2Bub1 (monoclonal rabbit, #5546, Cell Signaling Technology, Danvers, Massachusetts, USA; 1/4050 dilution) was applied at 37 °C for 60 min. Bound antibody was then visualized using the EnVision Kit

Table 1 Clinical and pathological features of colorectal cancers

clinical/pathological features		n available
gender	female	898
	male	893
age	mean: 69 yrs. (29–96)	
tumor grade	G1	21
	G2	1184
	G3	168
tumor stage	pT1	62
	pT2	231
	pT3	873
	pT4	212
nodal status	pN0	730
	pN1	347
	pN2/N3[a]	283
tumor type	tubular carcinoma	976
	mucinous carcinoma	75
	others	12
localization	right-sided colon	331
	transverse colon	354
	left-sided colon	276
	rectum	91
total number of patients		1584

[a] only one case with pN3

(Dako, Glostrup, Denmark) according to the manufacturer instructions. H2Bub1 was analyzed by one person (NM) experienced in IHC. Staining was localized in the nucleus of invasive colon cancers. No accompanying cytoplasmic staining was seen. For statistical analyses, the staining results were categorized into three groups: Tumors without any staining were considered H2Bub1 "negative". Tumors with 1 + or 2 + staining in up to 50 % of cells or 3 + staining in up to 20 % of cells were considered "weakly positive". Tumors with 2 + staining in >50 % or 3 + staining in >20 % were considered "strongly positive". This categorization had also successfully been used in an earlier study of our group and has proved to yield data with high inter-observer consistency [18].

Statistics

Statistical calculations were performed with JMP® 10.0.2 software (2012 SAS Institute Inc., NC, USA). Contingency tables and the chi-squared test were performed to search for associations between molecular parameters and tumor phenotype. Survival curves were calculated according to Kaplan–Meier. The Log-Rank test was applied to detect significant survival differences between groups. Cox proportional hazards regression analysis was performed to test the statistical independence and significance between pathological, molecular and clinical variables.

Results

Technical Issues

A total of 1584 (88.0 %) of tumor samples were interpretable in our TMA analysis. Reasons for non-informative cases (216 spots; 12.0 %) included lack of tissue samples or absence of unequivocal cancer tissue in the TMA spot.

H2Bub1 Expression in Normal Colonic Mucosa and Colorectal Cancer

In all 55 specimens of normal colonic mucosa a strong H2Bub1 expression was observed. Positive H2Bub1 staining was seen in 1256 (79.3 %) of 1584 interpretable CRC and was considered weak in 26.2 % and strong in 53.1 % of cancers. Representative images of positive and negative H2Bub1 IHC are given in Fig. 1.

H2Bub1 expression loss was associated with high tumor grade ($p = 0.0211$), high tumor stage ($p = 0.0003$), positive nodal status ($p = 0.0139$) and histological tumor type ($p = 0.0202$; Table 2). No link was found between H2Bub1 expression and tumor localization ($p = 0.1262$), peritumoral lymphocytic infiltration ($p = 0.2523$) or vascular invasion ($p = 0.5970$, Table 2).

Survival Analysis

As expected, high tumor grade and stage as well as advanced nodal status were associated with poor patient survival (Figs. 2a-c; $p < 0.0001$ each), while histological tumor type was unrelated to clinical outcome (Fig. 2d; $p = 0.6240$). Left-sided CRC (distal to the splenic flexure) was associated with a better prognosis (Fig. 2e; $p = 0.0449$). Loss of H2Bub1 expression in CRC was strongly associated to poor patient survival (Fig. 2f; $p = 0.0006$). H2Bub1 expression loss was associated with poor prognosis independent of the nodal stage, with $p = 0.0296$ in node negative (N0, Fig. 2g) and $p = 0.0197$ in node positive (N1, Fig. 2h) disease. In addition, loss of H2Bub1 expression was linked to poor prognosis in the subset of cancers with nuclear p53 accumulation ($p = 0.0031$; Fig. 2k), which is indicative of dominant-negative p53 mutations [19], but not in the subset of immunohistochemically p53 negative cancers ($p = 0.1924$; Fig. 2i). Interestingly, combined subgroup analysis comparing both nodal and p53 status in the tumors revealed that negative staining for H2Bub1 was strongly linked to earlier death in the subgroup of nodal negative disease with accumulation of p53 ($p = 0.0006$; Fig. 2l). This phenomenon was borderline in cancers with nodal metastases and negative p53 staining ($p = 0.0606$; Fig. 2m).

Multivariate Analysis

In a multivariate analysis including pT, pN, tumor grade, tumor localization and H2Bub1 expression, pT and pN showed independent prognostic relevance ($p < 0.0001$ each, Table 3). In contrast, H2Bub1 expression proved not to be an independent prognostic biomarker ($p = 0.1690$).

Discussion

The results of our study demonstrate that loss of H2Bub1 expression is linked to poor prognosis in CRC. In this immunohistochemical study, loss of H2Bub1 expression (weak expression and negative cases) was found in 46.9 % of CRC, while normal colonic mucosa showed constantly high levels of H2Bub1 staining. This finding suggests that a decreased function of H2Bub1 contributes to tumor development. Only one earlier study had analyzed H2Bub1 expression in colorectal cancer. In this study, a decreased H2Bub1 expression level was found in cancer cells as compared to their stromal cells in 31 of 36 cases [16]. Intra-tumoral heterogeneity of H2Bub1 expression has not been reported and therefore sampling issues of our TMA approach are unlikely. Furthermore, considering that a clinical biomarker must be analyzed on biopsy material and before treatment decisions are taken, it is of note, that our approach of analyzing molecular features on 1 min TMA tissue specimen measuring 0.6 mm in diameter closely

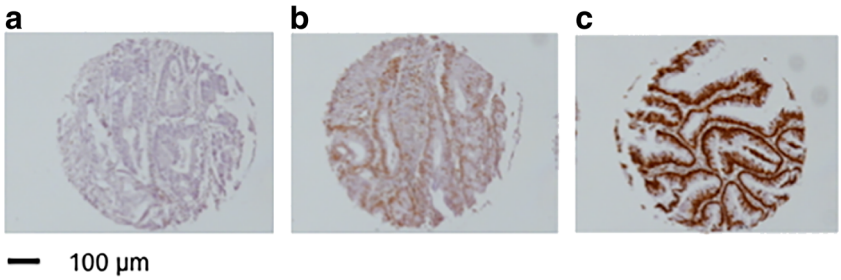

Fig. 1 H2Bub1 immunohistochemistry. Representative pictures of H2Bub1 immunostaining in colorectal cancer with **a** negative, **b** weak and **c** strong staining, magnification: 50 × each

models the molecular analyses of core needle biopsies where comparable amounts of tissues are evaluated. As our TMA samples were not exactly taken from the "worst" area of each tumor but randomly from within a representative cancer area,

Table 2 Associations between H2Bub1 expression, clinicopathological and molecular data in all cancers

	H2Bub1				
Parameter	**n evaluable**[a]	**negative (%)**	**weak (%)**	**strong (%)**	***p* value**
All cancers	1584	20.7	26.2	53.1	
Tumor stage					
pT1	62	17.7	12.9	69.4	0.0003
pT2	231	19.0	23.4	57.6	
pT3	873	19.9	25.5	54.5	
pT4	212	27.4	32.5	40.1	
Lymph node metastasis					0.0139
pN0	730	20.7	25.9	53.4	
pN1	347	17.9	23.9	58.2	
pN2/N3[b]	283	27.0	28.4	44.7	
grading					0.0211
G1	21	4.8	9.5	85.7	
G2	1184	20.7	26.2	53.1	
G3	168	24.4	25.6	50.0	
tumor localization					0.1262
right	331	19.0	23.0	58.0	
transverse	354	22.6	28.5	48.9	
left	276	22.1	29.0	48.9	
rectum	91	27.5	28.6	44.0	
histological type					0.0202
adenocarcinoma	976	21.9	27.7	50.4	
mucinous	75	14.7	20.0	65.3	
others	12	50.0	25.0	25.0	
peritumoral lymphocytes					0.2523
absent	585	19.7	27.4	53.0	
present	467	23.8	27.0	49.3	
vascular invasion					0.5970
no	584	20.5	26.9	52.6	
yes	467	22.7	27.6	49.7	
p53 status					0.3251
negative	381	21.5	26.0	52.5	
positive	284	16.9	27.1	56.0	

[a] Numbers do not always add up to 1584 in the different categories because of cases with missing data

[b] Only one case with pN3

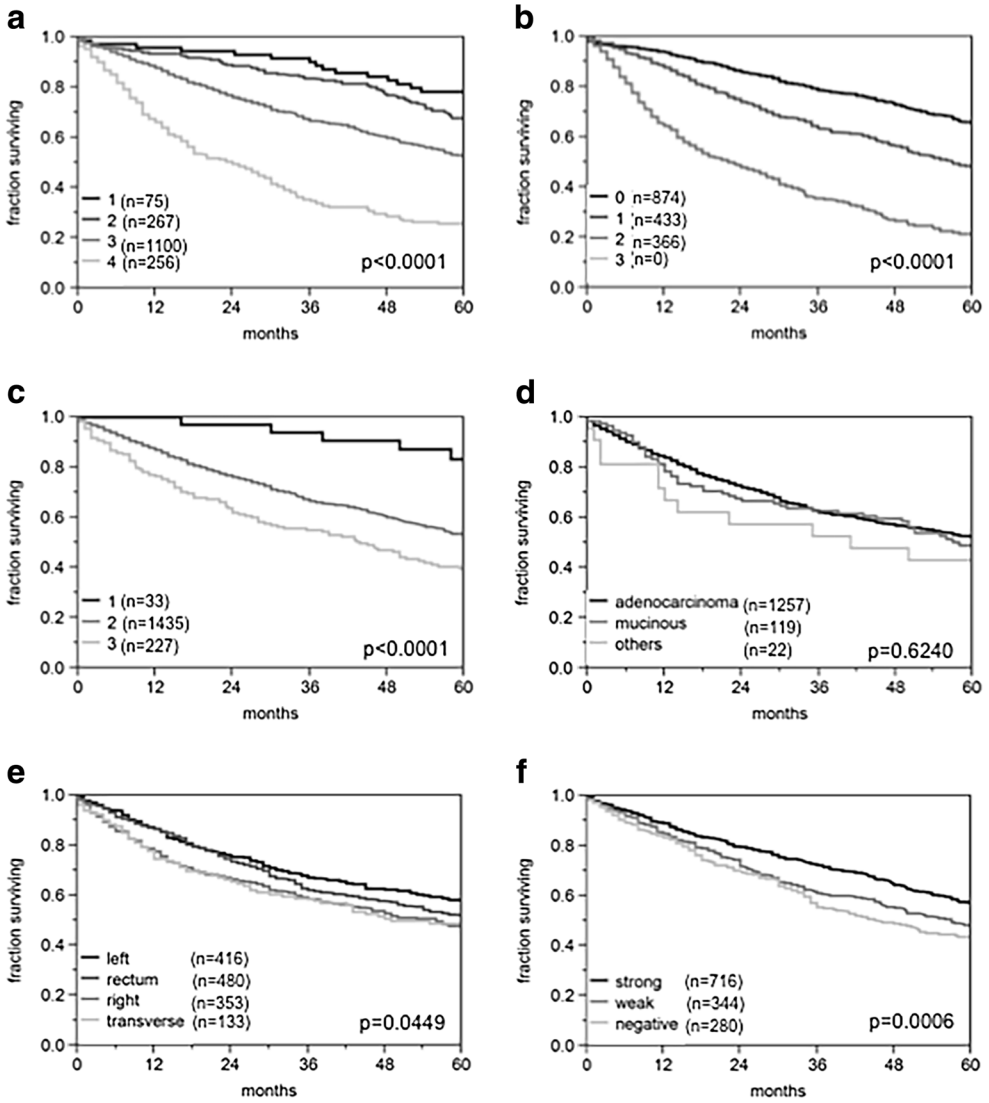

Fig. 2 H2Bub1 and pathological features. Association between H2Bub1 expression and cancers with **a** tumor stage, **b** nodal status, **c** grading, **d** histological type, **e** localization and **f** H2Bub1 expression in colon cancer, **g** negative nodal status, **h** positive nodal status, **i** negative p53, k positive p53, **l** negative nodal status and positive p53, **m** positive nodal status and negative p53

our TMA spot might be as representative as possible of the "worst" area of a clinical cancer identified in a set of cancer biopsies.

Loss of H2Bub1 detected by immunohistochemical staining has been reported for a number of cancers, including breast (reduced expression in 68 %), colorectal (reduced expression in 86 %), lung (reduced expression in 97 %) and parathyroid cancer (reduced expression in 52 %) [14–16], suggesting that loss of H2Bub1 expression is a common feature of many human solid cancers.

Loss of H2Bub1 staining was linked to features of aggressive cancers in our study including advanced stage, high grade, metastastic growth, and poor prognosis. These results argue for a relevant role of impared H2B monoubiquitination for the progression of colorectal cancers. The mechanism of how H2Bub1 expression levels influence tumor aggressiveness is not clear. There is, however a large array of H2Bub1-related mechanisms that normaly function to regulate transcription [3, 20], genomic stability [21], DNA damage repair [9, 22] and cell cycle progression [20], which may also impact tumorigenesis. In this respect, two specific mechanisms may be of particular interest. Urasaki et al. have earlier demonstrated a tight link between H2Bub1 expression and glucose metabolism in cancer cells and hypothesized that glucose is as strong inducer of H2B monoubiquitination [16]. Moreover, the known link of H2B monoubiquitination with p53 activation - the most frequently altered tumor suppressor gene in cancer - could represent an alternative mechanism. It has been shown that both monoubiquitination of H2B and expression of p53 are governed by the RING finger E3 ubiquitin ligase RNF20 [23]. Mutations of RNF20 have been shown to occur in colorectal cancers [24] and may, thus,

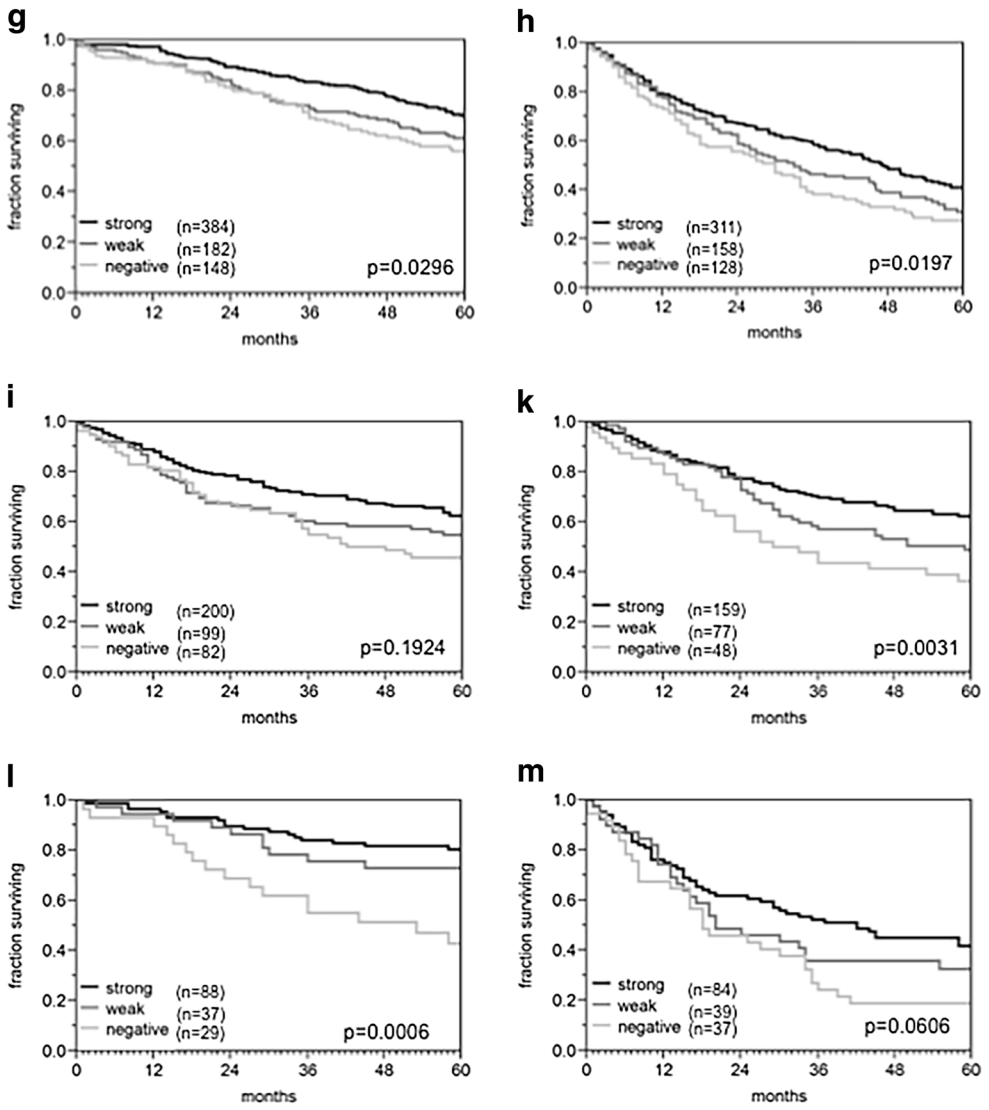

Fig. 2 continued.

account for both reduced p53 activity and loss of H2B monoubiquitination. In addition, deficiencies of both p53 and RNF20 have been linked to defective DNA repair, replication stress, chromosomal instability and cancer progression [8, 9, 20–22, 25, 26].

Moreover, also improper phosporylation of H2B has been associated with disturbed cytokinesis and tetraploidization [27]. These findings suggest an important role of H2Bub1 for maintainance of genome integrity. A clinically important role for a p53-H2Bub1 interaction is also supported by the striking difference in the prognostic impact of H2Bub1 expression between tumors without and with nuclear p53 accumulation in our study (Figs. 2i and k).

Thus, we were able to show that H2Bub1 expression loss is linked to poor survival not only in the whole patient cohort but also in the clinically relevant subgroups of cancers without lymph node metastases and especially without lymph node metastases but altered p53 (Figs. 2g and l). This is rather interesting for stratification of nodal negative patients into groups of high and low risk disease, which might make adjuvant therapy necessary in the high risk group. Limitations of our study are possible inter- and intra-observer issues known for TMA approaches. Furthermore, the relevance of H2Bub1 would surely be even higher if it proved to be independent of established prognostic parameters. However, the strengths are

Table 3 Multivariate analysis of H2Bub1 expression in colorectal cancer

n analyzable	p -value				
	pT	pN	grading	tumour localization	H2Bub1-expression
1000	<0.0001	<0.0001	0.5838	0.2320	0.1690

the large case load and thus power of our TMA, the long-term follow-up, the close modeling of preoperative biopsies using tissue cores and the potential role of H2Bub1 for personalizing therapy.

In summary, our data demonstrate that a reduced H2Bub1 expression is a strong prognostic biomarker both in nodal negative and nodal postive CRC and in clinically relevant subgroups. H2Bub1 expression measurement might help to select nodal negative CRC patients that may benefit from adjuvant therapy.

Conflict of Interest The authors declare no conflict of interest.

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