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ORIGINAL ARTICLE

Prognostic significance of the frequency of primary cilia in cells of small bowel and colorectal adenocarcinoma

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Summary

Purpose: *The primary cilium is a solitary, sensory, immotile microtubule-based structure that arises from the centrosome and is projected from the surface of most human cell types. It has been hypothesized that primary cilia could serve as a tumor suppressor organelle. The objective of this pilot study was to investigate the presence and frequency of primary cilia in cells of small bowel and colorectal adenocarcinoma and to evaluate the prognostic significance of their frequency.*

Methods: *The presence of primary cilia in cells in samples of small bowel (8 patients) and colorectal adenocarcinoma (32 patiens) was evaluated. The primary cilia of cells were immunofluorescently labeled using primary monoclonal anti-acetylated a-tubulin antibody and cell nuclei were labeled using DAPI.*

Results*: Primary cilia were identified in all examined specimens. The median frequency of primary cilia was 0.49% in cells of small bowel cancer and 0.22% in cells in colorectal cancer. Overall survival according to frequency of primary cilia in all intestinal adenocarcinomas was significantly longer in patients with higher frequency (≥ 0.187) than in patients with lower frequency of primary cilia (< 0.187) in univariate analysis (p=0.007) and also in the Cox proportional hazard model (p=0.032). Overall survival according to frequency of primary cilia in colorectal adenocarcinoma was significantly longer in patients with higher frequency (≥ 0.187) than in patients with lower frequency of primary cilia (< 0.187) (p=0.028).*

Conclusions: *The present pilot study provides the first evidence of the prognostic significance of the frequency of primary cilia in small bowel and colorectal adenocarcinoma. Because of significantly higher median frequency of primary cilia in the rare small bowel adenocarcinoma than in the frequent colorectal adenocarcinoma (p<0.001), the results of this study support a potential role for primary cilia as a biomarker in these types of cancer.*

Key words*: colorectal adenocarcinoma, primary cilia, prognosis, small bowel adenocarcinoma*

Introduction

The primary cilium is an immotile solitary sensory microtubule-based organelle protruding from the surface of the majority of human cells [1]. As post-mitotic cellular structures primary cilia are present during G_0/G_1 and the outset of S phase. Primary cilia disassemble at late S phase

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or start of G_2 when centrioles, including the centriole functioning as a basal body, are required to organize the mitotic spindle [2,3], and resorption of primary cilium is essential for cell cycle re-entry [4].

Primary cilia respond to a variety of mechanical and molecular cues in the extracellular environment and transmit signals to the cell [5]. Primary cilia play an important role in epidermal growth factor (EGF), Hedgehog, Wnt and mammalian target of rapamycin (mTOR) signaling pathways, and also cause alterations of cytosolic calcium fluxes [2,6]. Although the numbers and distribution depend on tissue type, primary cilia are detected in most tissues [7]. Defects in the structure and/or function of primary cilia have been shown to be associated with the development of a broad range of human diseases [8,9].

The tumor microenvironment plays a major role in cancer progression and metastasis [10,11]. Although the molecular biology of small bowel [12] and colorectal adenocarcinoma [13] has been studied in depth, to the best of our knowledge, there are no data in the literature about the presence and frequency of primary cilia of cells of these types of cancer in different regions of human small intestine, colon and rectum. Information on the presence of cilia in the healthy small intestine and colon is limited to the reported observation of numerous ciliated cells in the intestinal mesenchyme, but not in the mucosa, of rodent small intestine and colon [7]. Primary cilia are present in cancer stromal cells such as cancer-associated fibroblasts but not in small bowel and colorectal epithelial adenocarcinoma cells. It was hypothesized that primary cilia could serve as a tumor suppressor organelle [14,15].

The objective of this pilot study was to investigate the presence and frequency of primary cilia in cells of the small bowel and colorectal adenocarcinoma originating from all anatomical parts of the small intestine, colon and rectum and to evaluate the prognostic significance of their frequency.

Methods

Patients

The presence of primary cilia in cells was retrospectively evaluated in tumor tissue blocks of 8 patients with small bowel adenocarcinoma, 7 males and 1 female, with a median age of 67 years (range 50-79) and 32 patients with colorectal adenocarcinoma, 15 males and 17 females, with a median age of 69 years (range 48-88) (Table 1). The anatomical location of tumors included duodenum in 3 patients, jejunum in 4 patients, ileum in 1 patient, cecum in 4 patients, ascending colon in 4 patients, right colic flexure in 4 patients, transverse colon in 4 patients, left colic flexure in 3 patients, descending colon in 4 patients, sigmoid colon in 6 patients and rectum in 3 patients. Each patient had a histologically verified adenocarcinoma. All patients with small bowel adenocarcinoma and 26 patients with colorectal adenocarcinoma had grade 2 and 6 patients with colorectal adenocarcinoma had grade 3 tumors. All patients were treated at Charles University Medical School Teaching Hospital's Department of Oncology and Radiotherapy in Hradec Kralove, Czech Republic.

Immunofluorescence

To detect primary cilia in formalin-fixed, paraffin-embedded tissues using immunofluorescence, histological sections were deparaffinized in xylene followed by rehydration and antigen retrieval for 40 min at 96°C in 0.1M citrate buffer (pH adjusted to 6) [16]. Following light washing, sections were treated with 0.05% Tween 20 for 5 min, followed by incubation in blocking solution (1% goat serum, 0.1% Triton X-100 in PBS) for 30 min. The next step was to incubate the sections in primary antibody (monoclonal mouse anti-acetylated α-tubulin antibody produced, dilution 1:4000, clone 6-11B-1, Sigma-Aldrich) for 60 min at room temperature. After washing in PBS, sections were incubated in secondary antibody (Alexa fluor 594 donkey anti-mouse IgG (H+L) conjugate, Invitrogen) at room temperature for 1 hr. The samples were again washed gently in PBS, before slices were mounted using a mounting medium containing 4´,6-diamidino-2-phenylindole (VECTASHIELD Mounting Medium with DAPI, Vector Laboratories) for nuclei labelling (Figures 1,2).

Assessment

Samples were evaluated by an immunofluorescence microscope Nikon Eclipse 80i (Nikon Corporation, Tokyo, Japan). Fifteen photos per case with at least 60 cells (DAPI positive nuclei) were taken with Quad Scan XC-HR300 camera (Sony Corporation, Tokyo, Japan) using immersion lens, magnification 100x. Primary cilia and cell nuclei were counted manually because of the 3-dimensional location of primary cilia and cell nuclei. The percentage of primary cilia on cells was counted as a primary cilia to cell nuclei ratio [16].

Statistics

Standard descriptive statistics were used to study the cohort. Categorical variables were summarized by absolute (relative) frequencies and differences between small intestine and colorectum were tested by Fisher's exact test. Continuous variables were described by median (minimum;maximum) and mean (standard deviation), and differences between small intestine and colorectum were tested by nonparametric Mann-Whit-

Table 1. Patient and disease characteristics

Characteristics	*Total (N=40) N (%)*	*Small intestine (N=8) N (%)*	*Colorectum (N=32) N (%)*	*p value*
Sex				0.054
Female	18 (45.0)	1 (12.5)	17 (53.1)	
Male	22 (55.0)	7 (87.5)	15 (46.9)	
Age, years, median (range)	68.0 (48.0-88.0)	67.0 (50.0-79.0)	69.0 (48.0-88.0)	0.542
Mean (±SD)	68.6 (9.5)	66.0 (8.1)	69.3 (9.8)	
PT				0.700
1	2 (5.0)	1 (12.5)	1 (3.1)	
2	7 (17.5)	1 (12.5)	6 (18.8)	
3	23 (57.5)	5 (62.5)	18 (56.3)	
4	8 (20.0)	1 (12.5)	7 (21.9)	
pN*				0.274
0	21 (53.8)	6 (85.7)	15 (46.9)	
1	11 (28.2)	1 (14.3)	10 (31.3)	
2	7 (17.9)	0 (0.0)	7 (21.9)	
Stage				0.131
I	9 (22.5)	2 (25.0)	7 (21.9)	
II	12 (30.0)	5 (62.5)	7 (21.9)	
III	13 (32.5)	1 (12.5)	12 (37.5)	
IV	6 (15.0)	0 (0.0)	6 (18.8)	
Frequency of cilia				
Median (range)	0.25 (0.06-0.71)	0.49 (0.29-0.71)	0.22 (0.06-0.60)	< 0.001
Mean (±SD)	0.29 (0.17)	0.50 (0.14)	0.24 (0.13)	

* No information about pN in 1 patient with small intestine disease

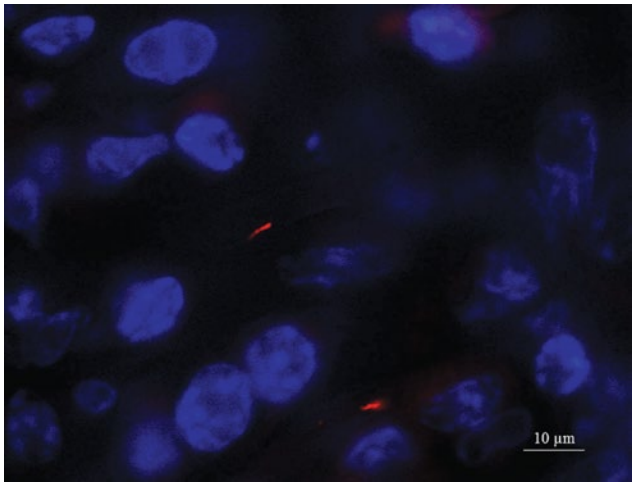
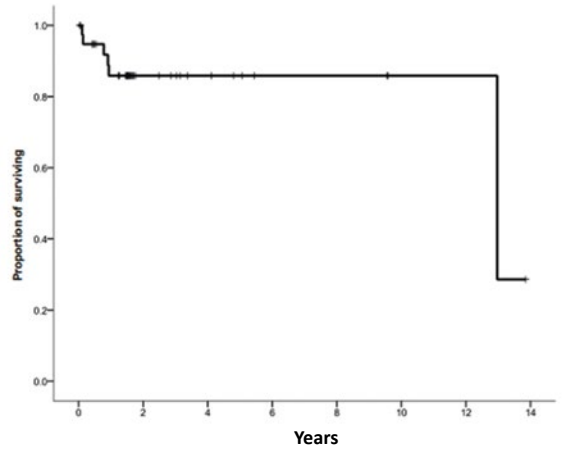


Figure 1. Primary cilia of cells of adenocarcinoma of duodenum immunofluorescently labeled using anti-acetylated tubulin-alpha antibody and cell nuclei labeled using DAPI, magnification 100x. Scale bar 10 µm.



	1 year	2 years
Probability of survival (95&CI)	0.859 (0.743; 0.974)	0.859 (0.743; 0.974)

Figure 2. Overall survival of all patients with intestinal adenocarcinoma (N = 40)

ney U test. The frequency of primary cilia was estimated with corresponding 95% confidence intervals (95% CI). Differences in categorical variables were assessed with Fisher's exact test and the differences between continuous variables were assessed with the Mann-Whitney U test or with the Kruskal-Wallis test. The survival outcomes were estimated using the Kaplan-Meier method. Log-rank test and Cox proportional hazard regression models were used for univariate and multivariate analyses of time-to-event data, respectively. Resulting estimates of hazard ratio (HR) were supplied with corresponding 95% CI.

A p value <0.05 was considered statistically significant.

Table 2. Association of patient characteristics on with frequency of cilia

Characteristics		*Frequency of cilia*			*p value*
		Valid N	*Median (range)*	*Mean (SD)*	
Sex	Female	18	0.21 (0.06-0.48)	0.22 (0.12)	0.030[1]
	Male	22	0.27 (0.09-0.71)	0.34 (0.18)	
Age, years	< 60	4	0.27 (0.09-0.64)	0.32 (0.24)	0.588[2]
	60 – 69	21	0.26 (0.06-0.71)	0.32 (0.18)	
	70 – 79	8	0.21 (0.14-0.49)	0.27 (0.13)	
	≥ 80	7	0.25 (0.07-0.32)	0.21 (0.09)	
Diagnosis	Small intestine	8	0.49 (0.29-0.71)	0.50 (0.14)	< 0.001[1]
	Colorectum	32	0.22 (0.06-0.60)	0.24 (0.13)	
Stage – small intestine	I	2	0.45 (0.43-0.48)	0.45 (0.04)	0.184[2]
	II	5	0.59 (0.36-0.71)	0.56 (0.14)	
	III	1	0.29 (0.29-0.29)	0.29 (-)	
	IV	0	-	-	
Stage – colorectum	I	7	0.39 (0.06-0.60)	0.33 (0.21)	0.380[2]
	II	7	0.21 (0.12-0.36)	0.24 (0.09)	
	III	12	0.19 (0.07-0.32)	0.19 (0.08)	
	IV	6	0.23 (0.17-0.35)	0.24 (0.06)	

[1] Differences tested by Mann-Whitney U test
[2] Differences tested by Kruskal-Wallis test

Table 3. Diagnostic tests – prediction of mortality by frequency of cilia

	Valid N	*Cut-off**	*AUC*	*p value*	*Sensitivity*	*Specificity*	*PPV*	*NPV*	*Accuracy*
Total	40	0.187	0.619	0.328	0.571	0.788	0.364	0.897	0.750
Small intestine	8	0.614	0.583	0.739	1.000	0.333	0.333	1.000	0.500
Colorectum	32	0.187	0.741	0.092	0.800	0.741	0.364	0.952	0.750

*Values smaller than cut-off correspond to events (deaths).
AUC : Area under the curve, PPV : Positive predictive value, NPV : Negative predictive value

Results

Primary cilia were observed in all examined specimens. The median frequency of primary cilia of cells of the entire cohort (irrespective of cancer origin) was 0.25% (range 0.06-0.71) (Table 2). The probability of survival (95% CI) of all intestinal adenocarcinomas was 85.9% (range 74.3-97.4) at one year and 85.9% (range 74.3-97.4) at two years (Figure 2).

The presence of primary cilia was assessed in a total of 10,131 cells of small bowel adenocarcinomas (Table 1). In 10,131 cell nuclei a total of 50 primary cilia were observed. Thus, the median frequency of primary cilia cells in small bowel adenocarcinoma was 0.49% (range 0.29-0.71), indicating the presence of approximately one primary cilium per 203 cells of small bowel adenocarcinoma.

The presence of primary cilia was evaluated in a total of 53,555 colorectal carcinoma cells (Table 1). In 53,555 cell nuclei a total of 126 primary cilia were detected. Therefore, the median frequency of primary cilia in cells of colorectal adenocarcinoma was 0.22% (range 0.06-0.60), i.e. approximately one primary cilium per 425 cells of colorectal adenocarcinoma.

The median frequency of primary cilia was significantly higher in small bowel adenocarcinoma than in colorectal adenocarcinoma (p<0.001). The median frequency of primary cilia in the entire cohort (N=40) was significantly higher in men than in women (p<0.030) (Table 2). Overall survival according to frequency of primary cilia in all patients with intestinal adenocarcinoma (N=40) was significantly longer in patients with higher frequency (≥ 0.187) than in patients with lower frequency of primary cilia (< 0.187) (log rank test; p=0.007). The difference was significant also in the Cox proportional hazard model (p=0.032) (Tables 3 and 4a-c, Figures 3a and 4a). The cohort of small bowel adenocarcinoma patients was too small (N=8) for prediction of overall survival according to frequency of primary cilia (Figure 3b). Overall

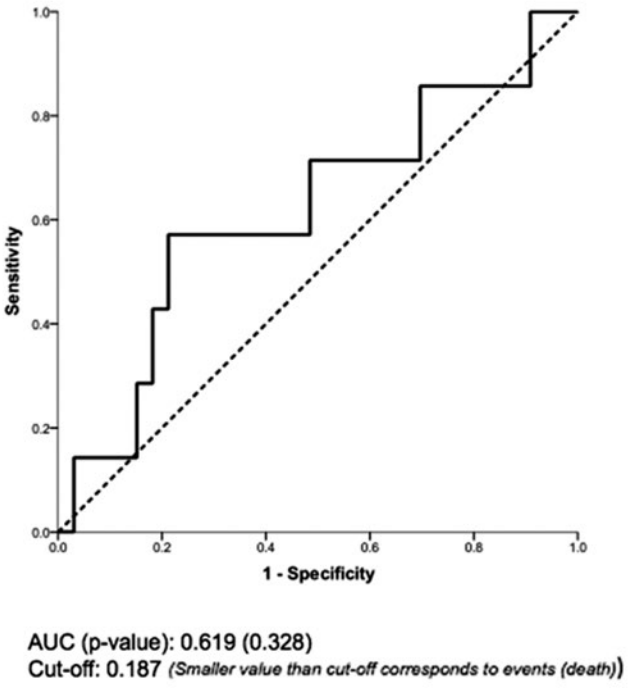


Figure 3a. Receiver operating characteristic (ROC) analysis - prediction of mortality by frequency of cilia of all patients with intestinal adenocarcinoma (N = 40).

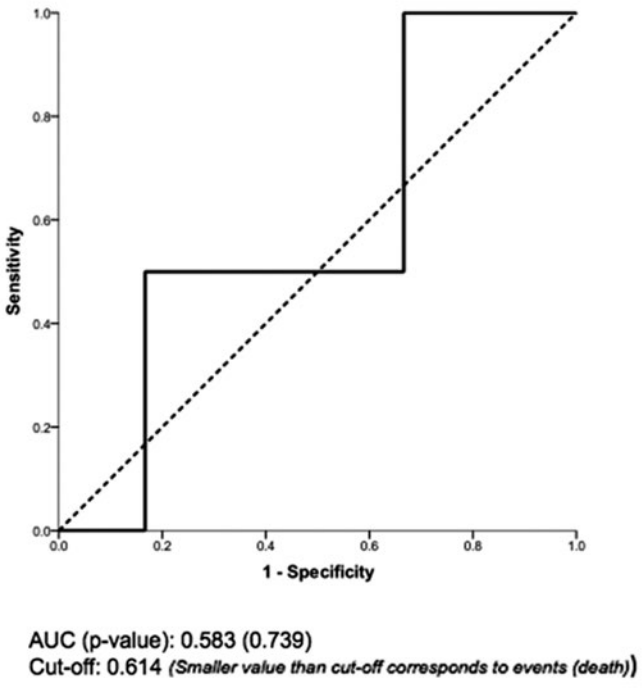


Figure 3b. Receiver operating characteristic (ROC) analysis - prediction of mortality by frequency of cilia of patients with small bowel adenocarcinoma (N = 8).

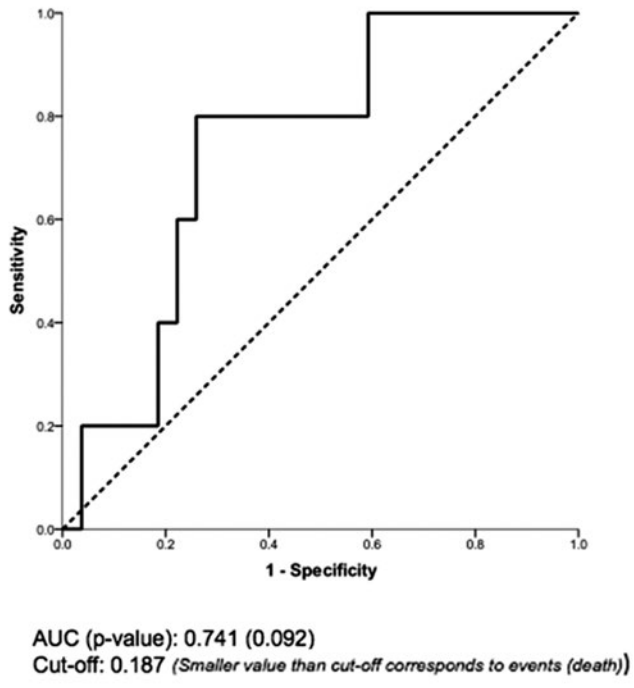


Figure 3c. Receiver operating characteristic (ROC) analysis - prediction of mortality by frequency of cilia of patients with colorectal adenocarcinoma (N = 32).

survival according to frequency of primary cilia in colorectal adenocarcinoma (N=32) was significantly longer in patients with higher frequency (≥ 0.187) than in patients with lower frequency of primary cilia (< 0.187) (p=0.028) (Tables 3 and 4a-c, Figures 3b and 4b).

The median frequency of primary cilia was not significantly higher in left-sided colorectal cancer (0.23%) compared with right-sided disease (0.19%).

Discussion

To the best of our knowledge, the present study provides the first information about the frequency of primary cilia in cells of the small bowel and colorectal adenocarcinoma originating from all anatomical regions of the small intestine and colorectum and also provides the first evidence of the prognostic significance of their higher frequency.

In an era characterized by the advent of targeted agents, medical oncology is increasingly relying on the use of biomarkers [17]. Biomarker determination is crucial for the estimation of patient prognosis that guides the management of individual patient. The present data indicate that primary cilia could represent a biomarker in intestinal carcinomas.

The frequency of primary cilia has been characterized in several other tumor types. For exam-

Table 4a. Prognostic significance of the frequency of cilia: Univariate analysis

	HR (95% CI)	*p value*
Total (N = 40)		
Frequency of cilia (continuous) (range)	0.004 (0.000-6.292)	0.143
Frequency of cilia < 0.187 (range)	11.072 (1.235-99.251)	0.032
Colorectum (N = 32)		
Frequency of cilia (continuous) (range)	0.002 (0.000-35.688)	0.210
Frequency of cilia < 0.187 (range)	7.892 (0.881-70.713)	0.065

HR : hazard ratio, based on Cox proportional hazard model

Table 4b. Prognostic significance of the frequency of cilia: Multivariate analysis*

		HR (95% CI)	*p value*
Frequency of cilia + sex			
Frequency of cilia (range)		0.001 (0.000-3.953)	0.105
Sex: men (range)		2.377 (0.363-15.578)	0.367
Frequency of cilia + age			
Frequency of cilia (range)		0.007 (0.000-17.996)	0.217
Age (continuous) (range)		1.063 (0.964-1.171)	0.220
Frequency of cilia + age (categorical)			
Frequency of cilia (range)		0.009 (0.000-18.300)	0.224
Age (cat) – ref. cat. (< 60) (range)	60 – 69	0.231 (0.017-3.045)	0.265
	70 – 79	0.504 (0.036-7.104)	0.611
	≥ 80	0.921 (0.083-10.223)	0.947

* Not calculated for stage and diagnosis – coefficients do not converge.
HR : hazard ratio, based on Cox proportional hazard model

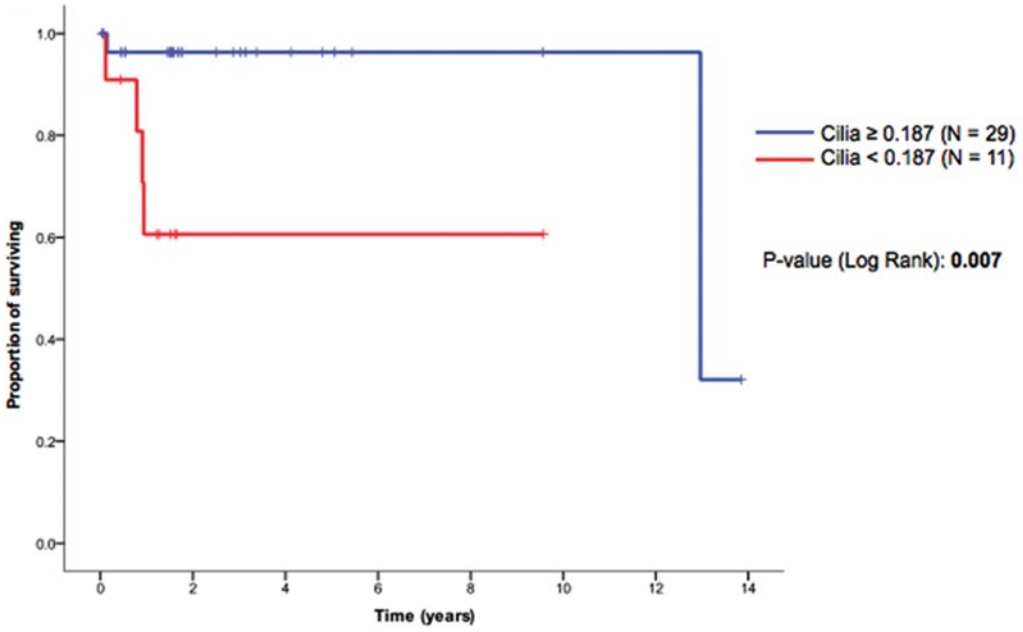
Table 4c. Survival analysis: frequency of cilia (categorical) as risk factor in multivariate analysis*

		HR (95% CI)	*p value*
Frequency of cilia + sex			
Frequency of cilia < 0.187 (range)		12.709 (1.378-117.219)	0.025
Sex: men (range)		2.016 (0.327-12.411)	0.450
Frequency of cilia + age			
Frequency of cilia < 0.187 (range)		9.165 (0.981-85.608)	0.052
Age (continuous) (range)		1.053 (0.960-1.155)	0.272
Frequency of cilia + age (categorical)			
Frequency of cilia < 0.187 (range)		9.195 (0.912-92.681)	0.060
Age (categorical) – ref. categorical (< 60) (range)	60-69	0.401 (0.030-5.417)	0.492
	70-79	1.028 (0.068-15.531)	0.984
	≥ 80	1.064 (0.095-11.893)	0.960

* Not calculated for stage and diagnosis – coefficients do not converge.
HR : hazard ratio, based on Cox proportional hazard model

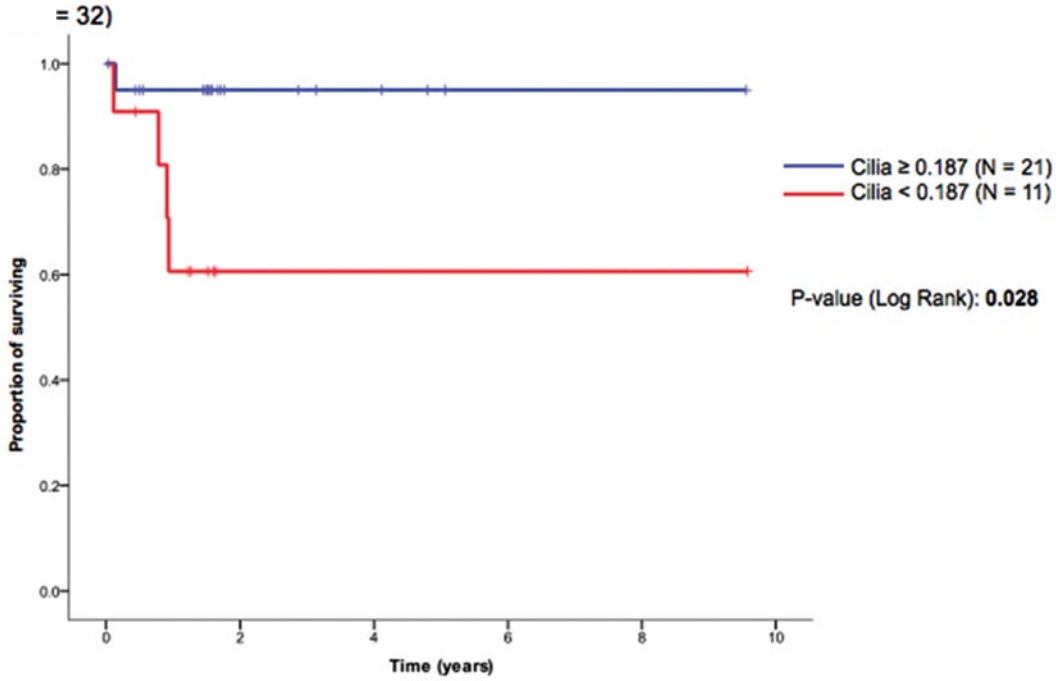
ple, in a study of human prostate cancer the frequency of primary cilia on stromal cells associated with prostatic intraepithelial neoplasia (5.8%), invasive cancer (5.2%) and perineural invasion (5.9%) was not different compared to normal tissue surrounding stromal cells (5.9%) [18]. In a study of gastrointestinal stromal tumors (GIST) the median frequency of primary cilia on GIST cells was 4.26% [16].

In human breast cancer cell lines, primary cilia of breast cancer associated fibroblasts were reported to be more than two orders of magnitude more frequent (73%) than in the present study of cells in small bowel and colorectal adenocarcinoma. The frequency of primary cilia in normal breast associated fibroblasts was 68% [19]. There are at least two possible explanations for these differences. First, there may be a difference be-



	Cilia ≥ 0.187	Cilia < 0.187
Probability of survival – 1 year (95% CI)	0.963 (0.892; 1.000)	0.606 (0.304; 0.908)
Probability of survival – 2 years (95% CI)	0.963 (0.892; 1.000)	0.606 (0.304; 0.908)

Figure 4a. Overall survival according to the frequency of cilia of all patients with intestinal adenocarcinoma (N = 40).



	Cilia ≥ 0.187	Cilia < 0.187
Probability of survival – 1 year (95% CI)	0.950 (0.854; 1.000)	0.606 (0.304; 0.908)
Probability of survival – 2 years (95% CI)	0.950 (0.854; 1.000)	0.606 (0.304; 0.908)

Figure 4b. Overall survival according to the frequency of cilia of patients with colorectal adenocarcinoma (N = 32).

tween the percentage of primary cilia in cultured cells and in tissue blocks. Second, the presence of primary cilia of cells of different cancer types can vary similarly to the presence of primary cilia in different tissue types. Indeed, primary cilia on fibroblasts in tissue blocks of histologically normal mammary gland primary cilia are found in 42% [19] in comparison with the very rare primary cilia on fibroblasts in the healthy mice colon [8]. Similarly, primary cilia on fibroblasts in tissue blocks of breast cancer are relatively frequent [19] compared with the rarity of primary cilia in cells of small bowel and colorectal cancer observed in the current study.

Tumor tissue comprises a heterogeneous cell population. Prior studies have demonstrated that primary cilia are present in stromal cells [7], not neoplastic cells, tumor-infiltrating lymphocytes or endothelia. In the present study, the frequency of primary cilia was calculated as a ratio of the number of primary cilia and the number of cell nuclei counted. The present study identified cilia in the whole tumor tissue and it is possible that the suggested prognostic impact of the presence of cilia reflects different composition of tumor stroma. Future studies should identify which cell types in tumor stroma express primary cilia.

Based on data from proteomics and comparative genomics studies it is estimated that the ciliome is comprised of approximately 1,000 proteins [20,21]. Different receptors, ion channels and signal pathways are present in primary cilia in different tissues. Future studies should address the molecular composition of primary cilia of cells of small bowel and colorectal adenocarcinoma. So far there is no information about receptors, ion channels or signal pathways specific for primary cilia on cells of these types of cancer. Genes and proteins involved in the forming, structure or function of the primary cilia may represent new therapeutic targets [21]. Marked differences were observed in the frequency of primary cilia cells in some patients. Future studies should address the association between the presence of primary cilia and biology of the tumor. Because of the association of primary cilia with several molecular pathways targeted by novel antineoplastic agents, the possible use of primary cilia as a predictive or prognostic biomarker in cancer patients should also be investigated.

Because of significantly higher median frequency of primary cilia in the rare small bowel adenocarcinoma than in the much more common colorectal adenocarcinoma, the results of this study support a potential association of primary cilia with favorable prognosis in these types of cancer. Due to the limited number of patients (40) in this retrospective pilot study, the beneficial prognostic significance of primary cilia of cells of intestinal adenocarcinoma should be investigated prospectively in a large prospective cohort of patients. Primary cilia as a prognostic biomarker should also be compared with other prognostic biomarkers in this patient population.

In conclusion, primary cilia are present in cells of small bowel and colorectal adenocarcinoma with median frequency of 0.49% and 0.22% of cells, respectively. These primary cilia were present in all samples of small bowel and colorectal carcinomas originating from all anatomical regions of small bowel, colon and rectum that were examined. Overall survival according to the frequency of primary cilia in all intestinal adenocarcinomas was significantly longer in patients with higher frequency than in patients in with lower frequency of primary cilia in both univariate and multivariate analyses. Overall survival according to the frequency of primary cilia in colorectal adenocarcinoma was also significantly longer in patients with higher frequency of primary cilia.

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Conflict of interests

The authors declare no confict of interests.

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