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ORIGINAL ARTICLE

# Association of the combined parameters including the frequency of primary cilia, CD8+ tumor infiltrating lymphocytes and PD-1 expression with the outcome in intestinal cancer

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## Summary

***Purpose:*** *Primary cilium (PC) is considered to be a functional homologue of the immune synapse. Microtubule structures, PC of cancer associated fibroblasts and immune synapses between cytotoxic CD8+ tumor infiltrating lymphocytes (TILs) and cancer cells, are regularly found in varying amounts in the microenvironment of solid tumors. The purpose of this study was to find out the potential association and combined prognostic significance of the frequency of PC, PD-1 and CD8+ TILs in patients with intestinal cancer.*

***Methods:*** *The frequency of PC, programmed cell death protein-1 receptor (PD-1) expression and the frequency of stromal and intraepithelial CD8+TILs were evaluated in samples of colorectal adenocarcinoma (32 patiens) and small bowel cancer (8 patients).*

***Results:*** *The median frequency of PC was 0.25%. The expression of PD1 was <5% in 34 patients, 5-25% in 5 patients and 26-50% in 1 patient. The frequency of stromal CD8+ TILs was negative in 3 patients, <25% in 26, 26-50% in 10 and >50% in 1 patient, respectively. Intraepithelial CD8+ TILs were not detectable in 14, <25% in 24 and 26-50% in 2 patients, respectively. Statistically, the frequency of PC and PD-1 positivity were significantly associated (p=0.004). An association between the PC frequency and intraepithelial CD8+ TILs was of borderline statistical significance (p=0.059). An index combining the frequency of PC and stromal CD8+ TILs, but not the combination of frequency of PC and intraepithelial CD8+ TILs, was of borderline prognostic significance (p=0.067).*

***Conclusions:*** *The present study provides the first data on the potential association and combined prognostic significance of frequency of PC, PD-1 and CD8+ TILs in patients with intestinal cancer.*

***Key words:*** *CD8+ tumor infiltrating lymphocytes, colorectal adenocarcinoma, primary cilia, small bowel adenocarcinoma, programmed cell death protein 1*

## Introduction

Primary cilium is considered to represent a functional homologue of the immune synapse due to morphological and functional similarities in architecture. In both microtubule structures (primary cilium and immune synapse), the exocytosis and endocytosis are focused at the point

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of centrosome docking [1]. Ciliary intraflagellar transport and Hedgehog proteins are found in T cells [2], and both structures form important signaling platforms [3,4]. This highlights a potential origin of the immune synapse as a modified primary cilium [5].

The primary cilium is an immotile solitary sensory microtubule-based organelle which protrudes during the quiescent phase of cell cycle from the surface of most human cells with the exception of hematopoetic cells (lymphocytes) [6].

Favorable prognostic and predictive significance of high density of CD8+TILs has been repeatedly demonstrated across a spectrum of different primary tumors [7,8]. Recently we reported positive prognostic significance of primary cilia in tumor microenvironment in intestinal cancer [9]. A hypothesis that primary cilia could serve as a tumor suppressor organelle has been proposed [10,11]. However, so far both parameters have not been evaluated simultaneously within the same group of patients. Both microtubule structures, i.e. primary cilia of cancer-associated fibroblasts and immune synapses between cytotoxic CD8+ TILs and antigen-presenting or cancer cells, are regularly found in varying amounts in the microenvironment of solid tumors. These could, in fact, represent two sides of the same coin. To investigate solely CD8+ TILs frequency may provide only a part of the information. The second part of the information may be the frequency of primary cilia in tumor microenvironment, which is not routinely investigated, though it is not difficult to assess nor expensive.

The final effector mechanism of antitumor immune response relies mainly on cytotoxic T lymphocytes recognizing nonself antigens, ultimately leading to tumor cell killing via immune synapse [12]. The immune synapse is a temporary interface between an antigen-presenting or cancer cell and the effector lymphocyte [13]. One mechanism by which cancer cells limit the formation of immune synapse is via upregulation of programmed death-1 ligand PD-L1 and its ligation to programmed death protein-1 receptor (PD-1) on CD8+ TILs (termed adaptive immune resistance) [14-16]. Immune checkpoint blockade using anti-PD-1 antibodies, such as nivolumab and pembrolizumab, seems to be an approach with the most potential [17].

While primary cilia can be directly displayed and quantified [9,18], it is difficult to do the same with CD8+ TILs at the moment of forming the immune synapse and attacking the cancer cells. Closest approximation is thus the simultaneous immunohistochemical demonstration of CD8+ TILs and PD-1 expression.

The aim of this pilot study was to investigate the relationships and correlated prognostic significance of the frequency of primary cilia, PD-1 and CD8+ intraepithelial and stromal TILs in the same group of patients with intestinal cancer.

## Methods

*Patients*

In a previous study we reported the prognostic significance of primary cilia in 40 patients with intestinal cancer [9]. In the present investigation we used this prior cohort of patients with already determined frequency of primary cilia and added the examination of the PD-1 as well as intraepithelial and stromal CD8+ TILs expression. We retrospectively evaluated tumor tissue blocks of 8 patients, 7 males and 1 female, with small bowel adenocarcinoma, median age of 67 years (range 50-79) and 32 patients, 15 males and 17 females, with colorectal adenocarcinoma, median age of 69 years (range 48-88). All patients were treated at

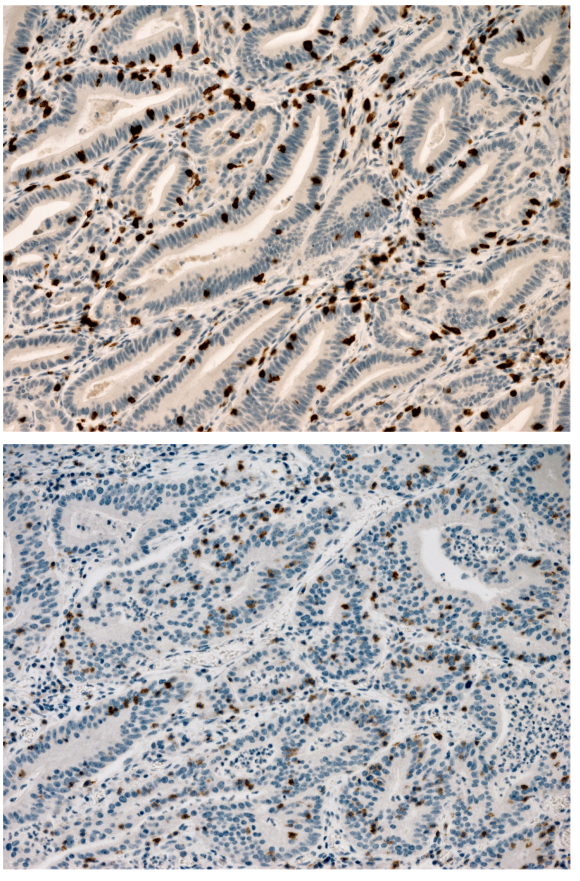


**Figure 1. A:** PD-1 expression using mouse monoclonal primary antibody in colorectal cancer. Indirect immunohistochemistry was used with mouse monoclonal primary antibody against PD-1 (NAT-105, Cell Marque,Darmstadt, Germany). Original magnification 100x. **B:** CD8+ expression of cytotoxic T lymphocytes using human antibody in stromal areas of colorectal cancer. Indirect immunohistochemistry was used with mouse monoclonal primary antibody against CD8 (M7103, DAKO, Glostrup, Denmark). Original magnification 100x.

the Department of Oncology and Radiotherapy, Charles University Medical School Teaching Hospital, Hradec Kralove, Czech Republic. The prognostic significance of PC was previously studied in the same cohort [9].

*Immunofluorescence*

Primary cilia of cells were demonstrated by immunofluorescence using anti-acetylated tubulin-alpha antibody and the nuclei of the cells were visualised using DAPI labeling. The percentage of primary cilia on cells was counted as a primary cilia to cell nuclei ratio as described previously [9,18].

*Immunohistochemistry*

An indirect immunohistochemistry using mouse monoclonal primary antibodies against PD-1 (NAT105, Cell Marque, Darmstadt, Germany; Figure 1A) and CD8 (M7103, Dako, Glostrup, Denmark; Figure 1B) was used. All slides were assessed for PD-1 and for quantity of intraepithelial and stromal CD8+ TILs by an experienced pathologist, ignorant of the treatment results of the patients. The immunohistochemical evaluation of PD-1 expression and CD8+ TILs was scored semi-quantitatively. The extent of PD-1 staining was evaluated as follows: 0 (<5%), 1 (5-25%), 2 (26-50%), 3 (51-75%) and 4 (>75%). Presence of intraepithelial/stromal CD8+TILs compared with the total amount of nucleated cells was quantified as follows: 0 - negative CD8+ TILs, 1 - <25% CD8+ TILs, 2 - 25≤50% CD8+ TILs and 3 - >50% CD8+ TILs.

*Statistics*

Standard descriptive statistics were used to characterized the cohort. Categorical variables were summarized by absolute (relative) frequencies while median (range) and mean (standard deviation) were used to summarize the continuous variables. The relative frequency of primary cilia was estimated with the corresponding 95% confidence intervals (95% CI). Differences in categorical variables were assessed with Fisher 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 calculated with corresponding 95% CI.

## Results

Primary cilia were detected in all adenocarcinoma samples analyzed. As previously reported, the median frequency of the primary cilia was 0.25% (0.07-0.71%) [9]. The expression of PD-1 was (<5%) in 34 patients, 5-25% in 5 patients, and 26-50% in 1 patient. The expression of stromal CD8+ TILs was negative in 3 patients, <25% in 26 patients, 26-50% in 10 patients and >50% in 1 patient, while intraepithelial CD8+ TILs were negative 14 patients, <25% in 24 patients and 26-50% in 2 patients.

Descriptive statistics are summarized in Table 1. Association of the patient characteristics with the frequency of primary cilia is shown in Table 2a, with stromal CD8+ TIL in Table 2b, with intraepithelial CD8+ TIL in Table 2c and with PD-1 positivity in Table 2d, respectively. A statistically significant association was noted between the frequency of primary cilia and PD-1 positivity (p=0.004; Table 2a) and between CD8+ stromal TILs and CD8+ intraepithelial TILs (p=0.005; Table 2b and 2c).

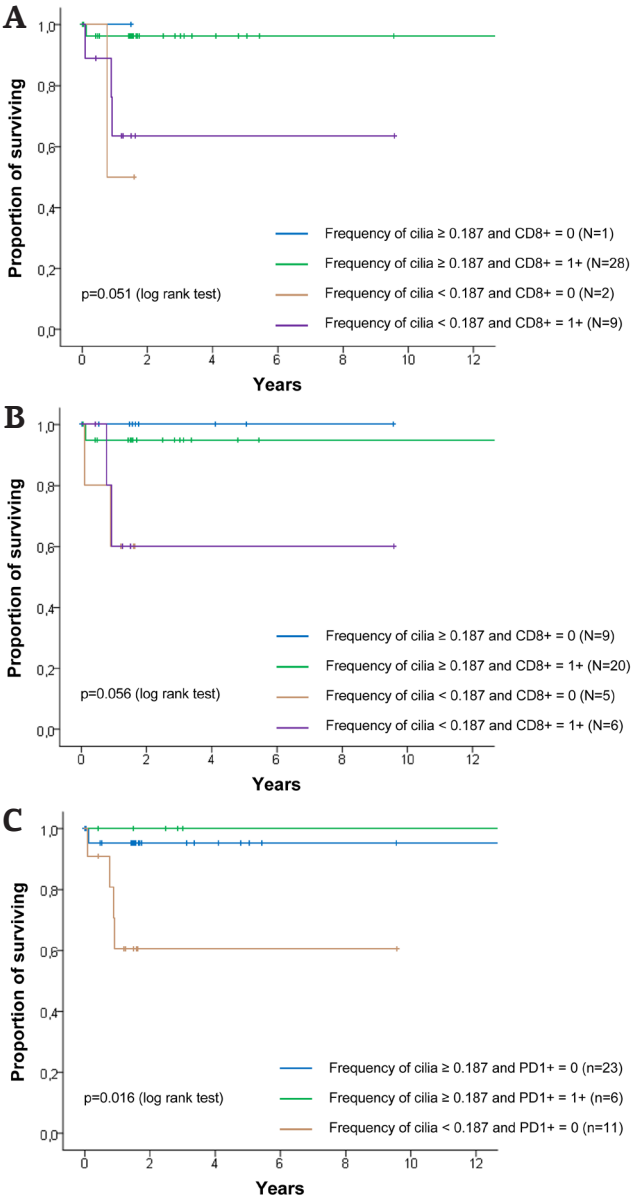


**Figure 2. A:** Survival according to combination of the frequency of primary cilia and stromal CD8+ TILs of all patients with intestinal adenocarcinoma (n= 40). **B:** Survival according to combination of the frequency of primary cilia and intraepithelial CD8+ TILs of all patients with intestinal adenocarcinoma (n=40). **C:** Survival according to combination of the frequency of primary cilia and PD1+ of all patients with intestinal adenocarcinoma (n= 40).

When evaluating all patients with intestinal adenocarcinoma (n=40), overall survival was significantly longer in patients with higher frequency of primary cilia than in patients with lower frequency in the Cox proportional hazard model (p=0.032; Table 1 and 3a). Tables 3b, 3c and 3d show that neither PD-1 nor CD8+ TILs were significantly associated with survival in univariate analysis in this cohort of patients.

Table 4 shows borderline prognostic significance of the combination of frequency of primary cilia and expression of stromal CD8+ TILs (p=0.067), but not of the combination of frequency of primary cilia and expression of intraepithelial CD8+ TILs.

A trend indicating prognostic significance of an index combining the frequency of primary cilia and stromal CD8+ TILs (p=0.051; Figure 2A), frequency of primary cilia and intraepithelial CD8+ TILs (p=0.056; Figure 2B) and frequency of primary cilia and PD-1 (p=0.016; Figure 2C) was observed.

**Table 1.** Characteristics of the cohort

| | *All (n=40) n (%)* | *Small intestine (n=8) n (%)* | *Colorectum (n=32) n (%)* | *p value* |
|---|---|---|---|---|
| Gender | | | | 0.054 |
| Female | 18 (45.0) | 1 (12.5) | 17 (53.1) | |
| Male | 22 (55.0) | 7 (87.5) | 15 (46.9) | |
| Age (years)* | 68.0 (48.0;88.0)<br>68.6 (9.5) | 67.0 (50.0;79.0)<br>66.0 (8.1) | 69.0 (48.0;88.0)<br>69.3 (9.8) | 0.542 |
| 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) | |
| TNM 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* | 0.25 (0.06;0.71)<br>0.29 (0.17) | 0.49 (0.29;0.71)<br>0.50 (0.14) | 0.22 (0.06;0.60)<br>0.24 (0.13) | **< 0.001** |
| Stromal CD8+ TILs | | | | 0.163 |
| 0 | 3 (7.5) | 0 (0.0) | 3 (9.4) | |
| 1 | 26 (65.0) | 4 (50.0) | 22 (68.8) | |
| 2 | 10 (25.0) | 3 (37.5) | 7 (21.9) | |
| 3 | 1 (2.5) | 1 (12.5) | 0 (0.0) | |
| Intraepithelial CD8+ TILs | | | | **0.026** |
| 0 | 14 (35.0) | 0 (0.0) | 14 (43.8) | |
| 1 | 24 (60.0) | 7 (87.5) | 17 (53.1) | |
| 2 | 2 (5.0) | 1 (12.5) | 1 (3.1) | |
| PD1+ expression | | | | 0.082 |
| 0 | 34 (85.0) | 5 (62.5) | 29 (90.6) | |
| 1 | 5 (12.5) | 2 (25.0) | 3 (9.4) | |
| 2 | 1 (2.5) | 1 (12.5) | 0 (0.0) | |

Categorical variables described by absolute (relative) frequencies and differences between small intestine and colorectal primary are tested by Fisher exact test. Continuous variables described by median (minimum; maximum), mean (standard deviation) and differences between small intestine and colorectum are tested by Mann-Whitney test.
*Median (range) and mean (SD); ** No information about pN in 1 patient (with small intestine). Bold numbers denote statistical significance.

**Table 2a.** Association of patient characteristics and the frequency of primary cilia

| | | *Frequency of cilia* | | *p value* |
|---|---|---|---|---|
| | *n* | *Median (range)* | *Mean (SD)* | |
| Gender | | | | **0.030**[1] |
| Female | 18 | 0.21 (0.06;0.48) | 0.22 (0.12) | |
| Male | 22 | 0.27 (0.09;0.71) | 0.34 (0.18) | |
| Age (years) | | | | 0.588[2] |
| <60 | 4 | 0.27 (0.09;0.64) | 0.32 (0.24) | |
| 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) | |
| Primary | | | | **<0.001**[1] |
| Small intestine | 8 | 0.49 (0.29;0.71) | 0.50 (0.14) | |
| Colorectum | 32 | 0.22 (0.06;0.60) | 0.24 (0.13) | |
| Stage - small intestine | | | | 0.184[2] |
| I | 2 | 0.45 (0.43;0.48) | 0.45 (0.04) | |
| 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 | | | | 0.380[2] |
| I | 7 | 0.39 (0.06;0.60) | 0.33 (0.21) | |
| 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) | |
| Stromal CD8+ | | | | 0.239[2] |
| 0 | 3 | 0.19 (0.08;0.36) | 0.21 (0.14) | |
| 1 | 26 | 0.24 (0.06;0.71) | 0.26 (0.14) | |
| 2+ | 11 | 0.43 (0.08;0.64) | 0.37 (0.21) | |
| Intraepithelial CD8+ TILs | | | | 0.059[1] |
| 0 | 14 | 0.20 (0.08;0.49) | 0.22 (0.10) | |
| 1+ | 26 | 0.32 (0.06;0.71) | 0.33 (0.18) | |
| PD1+ expression | | | | **0.004**[1] |
| 0 | 34 | 0.23 (0.06;0.71) | 0.26 (0.15) | |
| 1+ | 6 | 0.45 (0.26;0.64) | 0.46 (0.15) | |

[1]Differences tested by Mann-Whitney test; [2]Differences tested by Kruskal-Wallis test; Bold numbers denote statistical significance.

**Table 2b.** Association of patient characteristics and stromal CD8+ TILs

| | | *CD8+ stromal* | | | *p value* |
|---|---|---|---|---|---|
| | *n* | *0 (n=3) n (%)* | *1 (n=26) n (%)* | *2+ (n=11) n (%)* | |
| Gender | | | | | 1.000[1] |
| Female | 18 | 1 (33.3) | 12 (46.2) | 5 (45.5) | |
| Male | 22 | 2 (66.7) | 14 (53.8) | 6 (54.5) | |
| Age (years) | | | | | |
| <60 | 4 | 1 (33.3) | 1 (3.8) | 2 (18.2) | 0.162[1] |
| 60-69 | 21 | 2 (66.7) | 12 (46.2) | 7 (63.6) | |
| 70-79 | 8 | 0 (0.0) | 6 (23.1) | 2 (18.2) | |
| ≥80 | 7 | 0 (0.0) | 7 (26.9) | 0 (0.0) | |
| Primary | | | | | 0.264[1] |
| Small intestine | 8 | 0 (0.0) | 4 (15.4) | 4 (36.4) | |
| Colorectum | 32 | 3 (100.0) | 22 (84.6) | 7 (63.6) | |
| Stage - small intestine | | | | | 0.429[1] |
| I | 2 | 0 (0.0) | 0 (0.0) | 2 (50.0) | |
| II | 5 | 0 (0.0) | 3 (75.0) | 2 (50.0) | |
| III | 1 | 0 (0.0) | 1 (25.0) | 0 (0.0) | |
| IV | 0 | 0 (0.0) | 0 (0.0) | 0 (0.0) | |
| Stage - colorectum | | | | | 0.279[1] |
| I | 7 | 0 (0.0) | 3 (13.6) | 4 (57.1) | |
| II | 7 | 1 (33.3) | 5 (22.7) | 1 (14.3) | |
| III | 12 | 1 (33.3) | 9 (40.9) | 2 (28.6) | |
| IV | 6 | 1 (33.3) | 5 (22.7) | 0 (0.0) | |
| Frequency of cilia* | 40 | 0.19 (0.08;0.36) | 0.24 (0.06;0.71) | 0.43 (0.08;0.64) | 0.239[2] |
| | | 0.21 (0.14) | 0.26 (0.14) | 0.37 (0.21) | |
| Intraepithelial CD8+ | | | | | **0.005**[1] |
| 0 | 14 | 1 (33.3) | 13 (50.0) | 0 (0.0) | |
| 1+ | 26 | 2 (66.7) | 13 (50.0) | 11 (100.0) | |
| PD1+ expression | | | | | **0.016**[1] |
| 0 | 34 | 2 (66.7) | 25 (96.2) | 7 (63.6) | |
| 1+ | 6 | 1 (33.3) | 1 (3.8) | 4 (36.4) | |

Categorical variables are described by absolute (relative) frequencies.
Continuous variables are described by median (range) and mean (standard deviation).
[1]Differences tested by Fisher exact test; [2]Differences tested by Kruskal-Wallis test; *Median (range) and mean (SD); Bold numbers denote statistical significance.

**Table 2c.** Association of patient characteristics and intraepithelial CD8+ TILs

| | | *CD8+ epithelial* | | *p value* |
|---|---|---|---|---|
| | *Valid n* | *0 (n=14) n(%)* | *1+ (n=26) n(%)* | |
| Gender | | | | 0.510[1] |
| Female | 18 | 5 (35.7) | 13 (50.0) | |
| Male | 22 | 9 (64.3) | 13 (50.0) | |
| Age (years) | | | | 0.200[1] |
| <60 | 4 | 0 (0.0) | 4 (15.4) | |
| 60-69 | 21 | 6 (42.9) | 15 (57.7) | |
| 70-79 | 8 | 4 (28.6) | 4 (15.4) | |
| ≥80 | 7 | 4 (28.6) | 3 (11.5) | |
| Primary | | | | **0.034**[1] |
| Small intestine | 8 | 0 (0.0) | 8 (30.8) | |
| Colorectum | 32 | 14 (100.0) | 18 (69.2) | |
| Stage - small intestine | | | | - |
| I | 2 | 0 (0.0) | 2 (25.0) | |
| II | 5 | 0 (0.0) | 5 (62.5) | |
| III | 1 | 0 (0.0) | 1 (12.5) | |
| IV | 0 | 0 (0.0) | 0 (0.0) | |
| Stage - colorectum | | | | 0.621[1] |
| I | 7 | 2 (14.3) | 5 (27.8) | |
| II | 7 | 3 (21.4) | 4 (22.2) | |
| III | 12 | 7 (50.0) | 5 (27.8) | |
| IV | 6 | 2 (14.3) | 4 (22.2) | |
| Frequency of cilia | 40 | 0.20 (0.08;0.49) | 0.32 (0.06;0.71) | 0.059[2] |
| | | 0.22 (0.10) | 0.33 (0.18) | |
| Stromal CD8+ TILs | | | | **0.005**[1] |
| 0 | 3 | 1 (7.1) | 2 (7.7) | |
| 1 | 26 | 13 (92.9) | 13 (50.0) | |
| 2+ | 11 | 0 (0.0) | 11 (42.3) | |
| PD1+ expression | | | | 0.074[1] |
| 0 | 34 | 14 (100.0) | 20 (76.9) | |
| 1+ | 6 | 0 (0.0) | 6 (23.1) | |

Categorical variables are described by absolute (relative) frequencies.
Continuous variables are described by median (range) and mean (standard deviation).
[1]Differences tested by Fisher exact test; [2]Differences tested by Mann-Whitney test; Bold numbers denote statistical significance.

**Table 2d.** Association of patient characteristics and PD1+ expression

| | | *PD1+* | | *p value* |
|---|---|---|---|---|
| | *Valid n* | *0 (n=34) n(%)* | *1+ (n=6) n(%)* | |
| Gender | | | | 0.673[1] |
| Female | 18 | 16 (47.1) | 2 (33.3) | |
| Male | 22 | 18 (52.9) | 4 (66.7) | |
| Age (years) | | | | 0.913[1] |
| <60 | 4 | 3 (8.8) | 1 (16.7) | |
| 60-69 | 21 | 18 (52.9) | 3 (50.0) | |
| 70-79 | 8 | 7 (20.6) | 1 (16.7) | |
| ≥80 | 7 | 6 (17.6) | 1 (16.7) | |
| Primary | | | | 0.082[1] |
| Small intestine | 8 | 5 (14.7) | 3 (50.0) | |
| Colorectum | 32 | 29 (85.3) | 3 (50.0) | |
| Stage - small intestine | | | | 0.107[1] |
| I | 2 | 0 (0.0) | 2 (66.7) | |
| II | 5 | 4 (80.0) | 1 (33.3) | |
| III | 1 | 1 (20.0) | 0 (0.0) | |
| IV | 0 | 0 (0.0) | 0 (0.0) | |
| Stage - colorectum | | | | 0.171[1] |
| I | 7 | 6 (20.7) | 1 (33.3) | |
| II | 7 | 5 (17.2) | 2 (66.7) | |
| III | 12 | 12 (41.4) | 0 (0.0) | |
| IV | 6 | 6 (20.7) | 0 (0.0) | |
| Frequency of cilia* | 40 | 0.23 (0.06; 0.71) | 0.45 (0.26; 0.64) | **0.004**[2] |
| | | 0.26 (0.15) | 0.46 (0.15) | |
| Stromal CD8+ TILs | | | | **0.016**[1] |
| 0 | 3 | 2 (5.9) | 1 (16.7) | |
| 1 | 26 | 25 (73.5) | 1 (16.7) | |
| 2+ | 11 | 7 (20.6) | 4 (66.7) | |
| Intraepithelial CD8+ TILs | | | | 0.074[1] |
| 0 | 14 | 14 (41.2) | 0 (0.0) | |
| 1+ | 26 | 20 (58.8) | 6 (100.0) | |

Categorical variables are described by absolute (relative) frequencies.
Continuous variables are described by median (range) and mean (standard deviation).
[1]Differences tested by Fisher exact test; [2]Differences tested by Mann-Whitney test; *median (range) and mean (SD); Bold numbers denote statistical significance.

**Table 3a.** Frequency of primary cilia as risk factor for overall survival*

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

* Not calculated for small intestine because of small patient number. HR: hazard ratio, based on Cox proportional hazard model. Bold number denotes statistical significance.

**Table 3b.** Stromal CD8+ TILs as risk factor for overall survival*

| | *HR (95% CI)* | *p value* |
|---|---|---|
| Total (n= 40) | | |
| CD8+ = 1+ | 0.440 (0.049;3.996) | 0.466 |
| CD8+ = 2+ | 0.099 (0.005;1.966) | 0.130 |
| CD8+ = 1+ & 2+ | 0.333 (0.037;2.989) | 0.326 |
| Colorectum (n= 32) | | |
| CD8+ = 1+ | - | 0.879 |
| CD8+ = 2+ | 0.567 (0.063;5.093) | 0.613 |
| CD8+ = 1+ & 2+ | 0.431 (0.048;3.868) | 0.452 |

* Not calculated for small intestine because of small patient number. HR: hazard ratio, based on Cox proportional hazard model.

**Table 3c.** Intraepithelial CD8+ TILs as risk factor for overall survival*

| | *HR (95% CI)* | *p value* |
|---|---|---|
| Total (n= 40) | | |
| Intraepithelial CD8+ = 1+ | 0.771 (0.129;4.619) | 0.776 |
| Colorectum (n=32) | | |
| Intraepithelial CD8+ = 1+ | 1.134 (0.189;6.796) | 0.890 |

* Not calculated for small intestine because of small patient number. HR: hazard ratio, based on Cox proportional hazard model.

**Table 3d.** PD1+ as risk factor for overall survival*

| | *HR (95% CI)* | *p value* |
|---|---|---|
| Total (n= 40) | | |
| PD1+ = 1+ | 0.032 (0.000;86.227) | 0.394 |
| Colorectum (n=32) | | |
| PD1+ = 1+ | 0.043 (0.000;32 174.966) | 0.649 |

* Not calculated for small intestine because of small patient number. HR: hazard ratio, based on Cox proportional hazard model.

**Table 4.** Survival analysis: combined risk index

| | *HR (95% CI)* | *p value* |
|---|---|---|
| Frequency of cilia + stromal CD8+ | | |
| Frequency of cilia < 0.187 and CD8+ = 1+ | 5.343 (0.892;32.012) | 0.067 |
| Frequency of cilia + intraepithelial CD8+ | | |
| Frequency of cilia < 0.187 and CD8+ = 1+ | 3.858 (0.643;23.147) | 0.140 |

HR: hazard ratio, based on Cox proportional hazard model.

## Discussion

To the best of our knowledge, the present study provides the first information about an association and correlated prognostic significance between the frequency of primary cilia, PD-1 expression and stromal or intraepithelial CD8+ TILs in intestinal cancer.

Tumor tissue represents a heterogeneous population of various components. Prior studies have demonstrated that primary cilia are present on stromal cells [19], but not on neoplastic cells, TILs or endothelia. Our previous study identified cilia in the intratumoral compartment and it is possible that the suggested prognostic impact of the presence of cilia reflects differences in the composition of the tumor stroma [9].

Significant association between the presence of CD8+ TILs and PD-1 expression was observed, indicating that an index combining these variables could increase the power to predict prognosis. The frequency of primary cilia correlated significantly only with PD-1, but not with the presence of CD8+ TILs. Therefore, PD-1 in combination with CD8+ TILs could be mutually independent predictors, in particularly stromal CD8+ TILs.

Immune cells are among the important components of the tumor stroma. Intraepithelial TILs are in direct cell contact with cancer cells, while stromal TILs are dispersed in the stroma. Despite the somewhat controversial results in the literature, the latter seems to be superior and more reproducible than intraepithelial TILs when used to quantify TILs in cancer patients [12]. The present study shows that combining the frequency of primary cilia and stromal CD8+ TILs in an index results in a complex biomarker of borderline statistical significance that, however, did not outperform the primary cilia frequency.

Heavy infiltration by CD8+ TILs is quite characteristic in colorectal cancers with microsatellite instability, which represents approximately 15%

of sporadic colorectal cancer cases. Microsatellite instability is associated with 10-50 fold higher mutational load [14,20]. As previously shown, cancers with high gene mutational load have better response to immune checkpoint inhibitor therapy [14,20,21]. However, because of the limited number of patients, microsatellite instability was not investigated in the present study.

Marked differences were observed in the frequency of primary cilia, PD-1 and CD8 TILs in individual patients. Because of an 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 the limited size of the patient cohort in this retrospective pilot study, the potential prognostic significance of primary cilia of cells of intestinal adenocarcinoma should be investigated prospectively in a large and more homogeneous prospective cohort of patients.

Concluding, the present pilot study provides the initial information about a statistically significant association between the frequency of primary cilia and PD-1 positivity and borderline prognostic significance of an index combining the frequency of primary cilia and expression of stromal CD8+ TILs.

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## Conflict of interests

The authors declare no conflict of interests.

## References

1. Griffiths GM, Tsun A, Stinchcombe JC. The immunological synapse: a focal point for endocytosis and exocytosis. J Cell Biol 2010;189:399-406.
2. Finetti F, Paccani SR, Riparbelli MG et al. Intraflagellar transport is required for polarized recycling of the TCR/CD3 complex to the immune synapse. Nat Cell Biol 2009;11:1332-9.
3. Stinchcombe JC, Majorovits E, Bossi G, Fuller S, Griffiths GM. Centrosome polarization delivers secretory granules to the immunological synapse. Nature 2006;443:462-5.
4. Singla V, Reiter JF. The primary cilium as the cell's antenna: signaling at a sensory organelle. Science 2006;313:629-33.
5. de la Roche M, Ritter AT, Angus KL et al. Hedgehog signaling controls T cell killing at the immunological synapse. Science 2013;342:1247-50.
6. Satir P, Pedersen LB, Christensen ST. The primary cilium at a glance. J Cell Sci 2010;123:499-503.
7. Melichar B, Študentová H, Kalábová H et al. Predictive and prognostic significance of tumor-infiltrating lymphocytes in patients with breast cancer treated with neoadjuvant systemic therapy. Anticancer Res 2017;34:1115-26.
8. Galon J, Mlecnik B, Bindea G et al. Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. J Pathol 2014;232:199-209.
9. Dvorak J, Hadzi Nikolov D, Dusek L et al. Prognostic significance of the frequency of primary cilia in cells of small bowel and colorectal adenocarcinoma. JBUON 2016;21:1233-41.
10. Mans DA, Voest EE, Giles RH. All along the watchtower: is the cilium a tumor suppressor organelle? Biochim Biophys Acta 2008;1786:114-25.
11. Gradilone SA, Lorenzo Pisarellob MJ, LaRussob NF. Primary cilia in tumor biology: the primary cilium as a therapeutic target in cholangiocarcinoma. Curr Drug Targets. 2015 Feb 23. [Epub ahead of print].
12. Migali C, Milano M, Trapani D et al. Strategies to modulate the immune system in breast cancer: checkpoint inhibitors and beyond. Ther Adv Med Oncol 2016;8:360-74.
13. Grakoui A, Bromley SK, Sumen C et al. The immunological synapse: a molecular machine controlling T cell activation. Science 1999;285:221-7.
14. Le DT, Uram JN, Wang H et al. PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med 2015;372:2509-20.
15. Richter I, Jirasek T, Dvorak J, Cermakova E, Rehakova P, Bartos J. The prognostic effect of neoadjuvant chemoradiotherapy on the change of PD-L1 expression in patients with locally advanced rectal adenocarcinoma. JBUON. (Accepted for publication 20.1.2017).
16. Tumeh PC, Harview CL, Yearley JH et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature 2014;515:568-71.
17. Yuasa T, Masuda H, Yamamoto S, Numao N, Yonese J. Biomarkers to predict prognosis and response to

checkpoint inhibitors. Int J Clin Oncol. 2017 Apr 5. doi: 10.1007/s10147-017-1122-1. [Epub ahead of print]

18. Dvorak J, Sitorova V, Nikolov DH et al. Primary cilia in gastrointestinal stromal tumors. Neoplasma 2014;61:305-8.
19. Saqui-Salces M, Dowdle WE, Reiter JF, Merchant JL. A high-fat diet regulates gastrin and acid secretion through primary cilia. FASEB J 2012;26:3127-39.
20. Sun X, Suo J, Yan J. Immunotherapy in human colorectal cancer: Challenges and prospective. World J Gastroenterol 2016;22:6362-72.
21. McGranahan N, Furness AJ, Rosenthal R et al. Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. Science 2016;351:1463-9.