

ORIGINAL ARTICLE

The prognostic effect of neoadjuvant chemoradiotherapy on the change of PD-L1 expression in patients with locally advanced rectal adenocarcinoma

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Summary

Purpose: To evaluate the prognostic effect of neoadjuvant chemoradiotherapy on the change of programmed death ligand 1 (PD-L1) expression in patients with locally advanced rectal adenocarcinoma, by comparing PD-L1 expression in pretreatment biopsies and PD-L1 expression in pathological specimens after neoadjuvant chemoradiotherapy.

Methods: A total of 25 patients with rectal adenocarcinoma were evaluated. Patients were treated by neoadjuvant chemoradiotherapy (radiotherapy:44Gy normofractionation; chemotherapy: capecitabine 825 mg/m² in two daily doses). Surgery was performed 6-8 weeks after the chemoradiotherapy completion. PD-L1 expression was determined in endoscopic biopsies and in resected specimens with immunohistochemistry.

Results: All 25 patients received radiotherapy without interruption, while concomitant chemotherapy was discontinued prematurely in one patient because of hematologi-

cal toxicity. In 13 patients sphincter-saving surgery were performed, and 12 patients underwent rectum resection. Downstaging was noticed in 17 patients. Stable disease was found in 5 patients, and progression in 3. The median disease free survival (DFS) was not reached. Three-year DFS was 54.3% (95% CI 34.3-74.2). The median overall survival (OS) was 60 months (95% CI 48-60). Three-year OS was 75% (95% CI 57.7-92.3). No PD-L1 expression was noticed in pretreatment biopsy and in resected tissue after chemoradiotherapy.

Conclusion: No prognostic effect of neoadjuvant chemoradiotherapy on the change of PD-L1 expression was demonstrated in patients with locally advanced rectal adenocarcinoma.

Key words: neoadjuvant chemoradiotherapy, PD-L1, rectal adenocarcinoma

Introduction

Malignant tumors of the colon and rectum are the most commonly occurring cancers in Europe and North America. Rectal adenocarcinoma represents approximately 30% of these tumors. Neoadjuvant chemoradiotherapy followed by total mesorectal excision is the current standard of treatment of locally advanced adenocarcinoma of the rectum. Neoadjuvant chemoradiotherapy has shown a lower incidence of local recurrence and better toxicity profile compared to postoperative

adjuvant therapy [1]. The treatment potentiation with 5-fluorouracil or capecitabine demonstrated a higher number of pathological complete remissions and lower incidence of local recurrences compared to the treatment with radiotherapy alone [1-6].

Strategies to improve the long-term outcomes that should be investigated in future clinical trials include incorporation of more active agents. One of very actual topic in oncology is immuno-

therapy, which is used in various types of tumors such as melanoma, non-small cell lung cancer, renal cell carcinoma and others [7-9]. The immune checkpoint inhibitors are becoming mainstream systemic cancer treatments, but as for now we have less information about the combination of radiotherapy and immune checkpoint inhibitors. Programmed cell death-1 (PD-1) and its ligand (PD-L1) belong to one type of immune-inhibitory checkpoint molecules that suppress T cell-mediated immune response, leading to the development of tumors [10]. PD-1 (CD279) is a cell surface receptor that belongs to the immunoglobulin superfamily and also a member of the extended CD28/CTLA-4 family. PD-1 is mainly expressed on activated T cells [11,12]. PD-L1 (B7-H1, CD274) has been identified as a cell-surface glycoprotein belonging to the B7 family [13]. PD-L1 is mainly expressed on the surface of tumor cells and antigen-presenting cells in various solid malignancies [14-17] and is rarely expressed on normal tissues [18]. PD-1/PD-L1 pathway plays a prominent role in immune regulation by delivering inhibitory signals to maintain the balance in T-cell activation, tolerance and immune-mediated tissue damage. It exerts significant inhibitory activity in persistent antigenic stimulation environment such as exposure to self-antigens, chronic viral infections, and tumors [19,20]. Cancer treatment like radiotherapy and chemotherapy exert very complex immunomodulatory effects [21].

Positive PD-L1 expression has been reported to be associated with better clinical outcome in some types of tumors. Clinical trials demonstrated that monoclonal antibodies that target PD-L1 or its receptor PD-1 prevent the inhibitory effects of PD-1/PD-L1 pathway and enhance T cell functions, leading to impressive outcomes in patients with melanoma, renal cell carcinoma, NSCLC and bladder cancer [22-24]. However, for the moment only scarce information exists over the usage of checkpoint inhibitors and the combination of neoadjuvant chemoradiotherapy in rectal cancer.

The aim of our study was to evaluate the prognostic effect of neoadjuvant chemoradiotherapy on the change of PD-L1 expression in patients with locally advanced rectal adenocarcinoma, by comparing PD-L1 expression in pretreatment biopsies and in pathological specimens after neoadjuvant chemoradiotherapy.

Methods

Patients

A total of 25 patients (18 men, 7 women) with rectal adenocarcinoma were evaluated. Patients were

treated between January 2008 and December 2012 in the Department of Oncology, Regional Hospital Liberec, Czech Republic. The mean patient age was 61.1 years (range 40-74). Tubular adenocarcinoma was identified in all 25 patients in the pretreatment biopsy. The tumor was well-differentiated in 3 patients, moderately differentiated in 19 patients, and poorly differentiated in 3 patients. The anatomical tumor localization was as follows: 7 patients lower rectum (< 5 cm from the anal verge), 17 patients middle rectum (< 5-10 cm) and one patient upper rectum (above 10 cm from the anal verge). Nine patients had clinical stage II and 16 had clinical stage III according to TNM classification. The median pretreatment hemoglobin concentration was 144 g/l (range 108-166), the median pretreatment leukocytes' number was $8.3 \times 10^9/l$ (range 3.5-13.1) and the median pretreatment platelets' number was $260 \times 10^9/l$ (range 152-388). Preoperative CEA was examined in 23 patients and the median concentration was 3.5 $\mu g/l$ (0.5-46.8). Eight patients (34.8 %) had elevated CEA.

Treatment

Patients were treated by neoadjuvant chemoradiotherapy. The source of radiation was a linear accelerator Elekta Precise or Elekta Synergy (Elekta, Sweden). We used ionizing photon radiation with energy 15 MV. Patients were irradiated by 3D conformal radiotherapy technique or intensity modulated radiotherapy (IMRT) using segmented fields. All of the patients received a total dose of 44 Gy (2 Gy per day) in 22 fractions to the tumor area, mesorectum and pelvic lymph nodes. Capecitabine was concomitantly administered at a dose of 825 mg/m² in two daily oral doses for the whole duration of radiotherapy (including the weekends). Surgery was performed 6-8 weeks after the chemoradiotherapy completion.

Immunohistochemical determination of PD-L1 expression

Twenty five primary carcinomas of the rectum were examined in this study. The pathological diagnosis of carcinoma was performed on the tissue obtained during endoscopy and the resected specimen tissue after neoadjuvant radiotherapy, and was available in all patients. Paraffin-embedded tissue samples were obtained from the archive of the Department of Pathology, Regional hospital Liberec. Tissue of human tonsilla palatina and squamous cell carcinoma of head & neck region were used as positive controls. All biopsied specimens were fixed in buffered formalin and embedded in paraffin. Five μm thin sections were stained with haematoxylin and eosin. Histologic grading was assessed from resected specimens only. For immunohistochemical purposes the sections were placed on poly-D-lysine-coated glass slides. Rabbit monoclonal antibody recognizing PD-L1, clone 28-8 (Abcam, Cambridge, UK) was diluted 1:400. Standard immunohistochemical procedure was applied to all specimens using Ventana BenchMark XT autostainer following antibody data-sheet recommended procedure. Pre-treatment with cell conditioning solution (CC1) and OptiView detection system with 3,3-di-

aminobenzidine (all obtained from Roche, Prague, Czech Republic) were used to visualize immunohistochemical reactions. Immunostaining results were evaluated independently by two experienced histopathologists (P.R and T.J) semiquantitatively in the whole tissue sections in both endoscopic biopsy and resection specimens as follows: 0: no positive staining; 1: up to 1% positive cells; 2: 1-10% positive cells; 3: 10-50% positive cells. Consensus was achieved viewing slides with multihead microscope (Olympus, Prague, Czech Republic) in few controversial cases.

Statistics

The statistical evaluation was performed using the Number Cruncher Statistical Systems 9 NCCS (Kaysville, Utah, USA) program. OS (time from diagnosis till death of any cause or the date of the last follow up visit) and DFS (time from surgery to recurrence or the last follow up visit without recurrence) were assessed using Kaplan-Meier analysis. Multivariate analysis was performed using the Cox regression model. All the statistical tests were significant at level $\alpha = 0.05$.

Results

All of the 25 patients received radiotherapy without interruption up to the total planned dose. The neoadjuvant chemoradiotherapy was relatively well tolerated. No patient died during treatment. Concomitant chemotherapy was discontinued prematurely in one patient because of grade III thrombocytopenia. No patient was hospitalized because of acute treatment toxicity and no grade III or IV non-hematological toxicity was encountered. The most common type of non-hematological toxicity was gastrointestinal in 20 patients and genitourinary in 13 patients according to RTOG scale. Grade I-II anaemia was found in 12 patients and grade III in 3 patients. Grade I-II leukopenia was found in 7 cases. Grade I-II thrombocytopenia was seen in 2 patients and grade III in one patient (Table 1). The median of hemoglobin nadir was 112 g/l (range 79-154), for

leukocytes it was $4.8 \times 10^9/l$ (range 2.4-7.1), and for platelets $166 \times 10^9/l$ (range 58-257). Surgery was indicated in all patients following 6-8 weeks after neoadjuvant chemoradiotherapy completion. The median time between chemoradiotherapy completion and surgery was 50 days (7.1 weeks). In 13 patients sphincter-saving surgery were performed while 12 patients underwent rectal resection. No patient was assessed as inoperable by the surgeons. R0 resection was performed in 23 (92%) patients, microscopically positive margin was described in 2 patients. The pathologic TNM stage after chemoradiotherapy was as follows: 9 patients stage I, 9 patients stage II and 5 patients stage III. One patient achieved pathological complete remission. Preoperative metastases were seen in the liver in one patient. Downstaging was achieved in 17 patients, stable disease in 5, and progression in 3 patients.

At the time of assessment (30 September 2016), recurrence occurred in 11 (44%) patients. Local recurrence was found in 7 patients, and generalized disease was reported in 7 patients. The most common sites of metastases were the lungs (3 patients) and liver (2 patients). The median of DFS was not reached. Three-year DFS was 54.3% (95% CI 34.3-74.2). A total of 12 patients died. The median of OS was 60 months (95% CI 48-60). Three-year OS was 75% (95% CI 57.7-92.3).

No PD-L1 expression in pretreatment biopsy and no PD-L1 expression in the resected specimen tissue after chemoradiotherapy was found (Figure 1). In 4 patients expression PD-L1 was <1% of tumor cells in the resected specimen (Figure 2). Therefore, no prognostic effect of neoadjuvant chemoradiotherapy to change of PD-L1 expression in patients with rectal adenocarcinoma was detected.

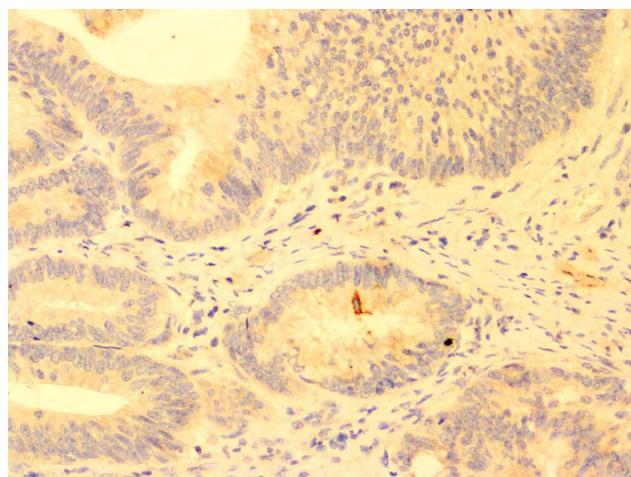


Figure 1. No PD-L1 expression in resected specimen (immunostaining x200).

Table 1. Treatment toxicity according to RTOG scale

Toxicity	Grade I-II n (%)	Grade III – IV n (%)
Anaemia	12 (48)	3 (12)
Leukopenia	7 (28)	0
Thrombocytopenia	2 (8)	1 (4)
Gastrointestinal	20 (80)	0
Genitourinary	13 (52)	0

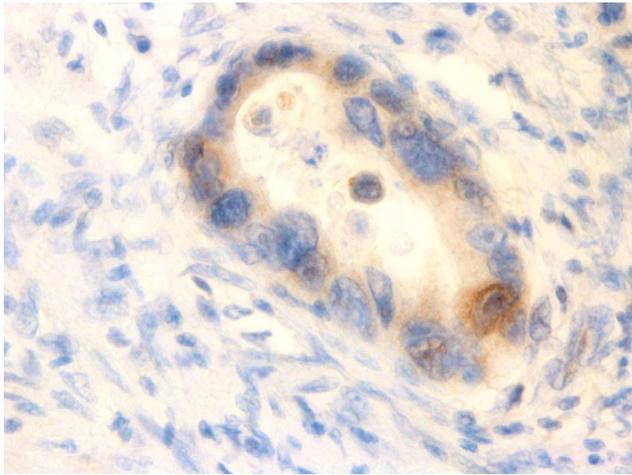


Figure 2. PD-L1 expression in less than 1% of tumor cells (immunostaining x200).

Discussion

No PD-L1 expression was detected in endoscopic biopsies of the resected specimens according to the defined criteria.

In 4 patients we found PD-L1 expression in less than 1% of tumor cells of resected specimens. In the analysis of this group of patients we failed to demonstrate prognostic influence on OS (HR 1.74, 95% CI 0.37–8.11, $p=0.3985$) and DFS (HR 2.16, 95% CI 0.41–11.40, $p=0.2421$) compared with patients lacking any expression (Figures 3 and 4). We chose the evaluation of PD-L1 expression according to the KEYNOTE-012 trial with positivity of at least 1% of tumor cells [25]. Therefore the group of 4 patients with PD-L1 expression less than 1% was characterized as negative.

We discussed the possibility of laboratory error in the determination of PD-L1 expression. Therefore we performed an immunohistochemical assessment of PD-L1 expression in squamous cell carcinoma of the head and neck, where the expression of PD-L1 has been reported to reach approximately 50% of the cases [26] and demonstrated strong (3+) expression of PD-L1 (Figure 5) in these cells.

This type of detection of PD-L1 expression has limitations due to its subjectivity in determining a clear definition of positive tumor PD-L1 staining [27,28]. Immunostaining results in our study were evaluated independently by two experienced histopathologists who were ignorant of the treatment results. The other possible problem with the determination of PD-L1 expression is the usage of different PD-L1 antibodies. Another limitation of diagnostic biopsy is that it offers only focal analysis of the tumor, while the major parts of tumor cells may remain unexamined.

PD-L1 expression has two patterns: focal expression and diffuse expression. Even from the

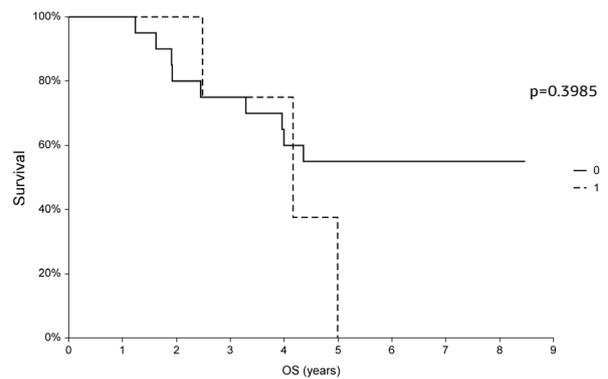


Figure 3. Kaplan-Meier overall survival (solid line: no PD-L1 expression in resected specimens; dashed line: PD-L1 expression in less than 1% of tumor cells in resected specimens).

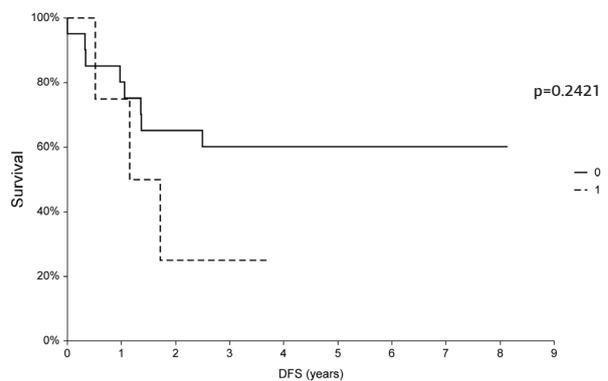


Figure 4. Kaplan-Meier disease free survival (solid line: no PD-L1 expression in resected specimens; dashed line: PD-L1 expression in less than 1% of tumor cells in resected specimens).

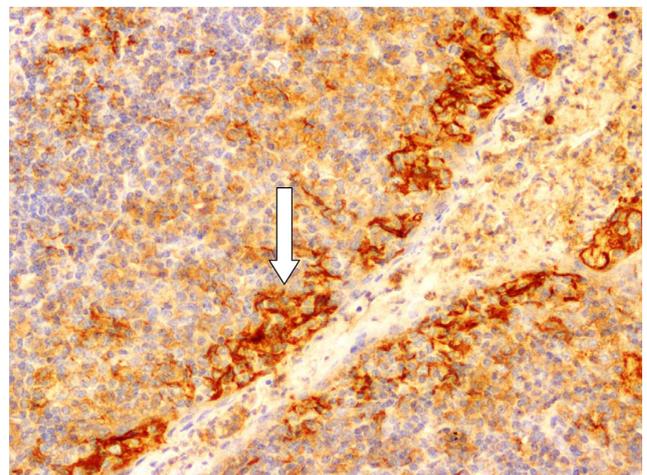


Figure 5. Strong (3+) PD-L1 expression in squamous cell carcinoma of the tonsil marked by arrow (immunostaining x200).

same sample, biopsy may result in a bias due to the focal nature of PD-L1 expression in many tumors [29]. The above factors are possible reasons for different patterns of PD-L1 expression in some studies. We evaluated the membrane positivity of PD-L1 in tumor cells. Several studies have demonstrated that only cell membrane-expressed PD-L1 has biological significance [30].

The PD-L1 expression has been investigated in certain studies. PD-L1 expression was described in a number of gastrointestinal tumors [31-36]. On other hand PD-L1 has only rarely been expressed in colorectal adenocarcinoma cells (12.8% of the cases, with 5-100% of positive cells, median 40%) [37]. Some published studies demonstrated correlation between expression of PD-L1 and prognosis and response to immunotherapy [31,33,38,39], while others demonstrated efficacy in patients deemed as PD-L1 negative [40]. Therefore the relationship between PD-L1 expression and outcome is yet to be clearly established. In patients with colorectal cancer the PD-L1 expression in tumor cells was positively associated -with OS ($p=0.002$) and DFS ($p=0.004$) [41].

Little data concerning the correlation between radiotherapy and PD-L1 expression are available. In our study we hoped to demonstrate an influence of chemoradiotherapy to changes of PD-L1 expression and treatment outcomes in patients with rectal adenocarcinoma. There were two pre-clinical studies published testing radiotherapy in combination with PD-1/PD-L1 inhibition. Deng et al. demonstrated superior tumor control of implanted breast and colorectal carcinomas when anti-PD-L1 antibodies were used in combination with radiotherapy [42]. In mouse glioma models, the combination of radiation with anti-PD-1 therapy significantly increased the median survival, with some mice exhibiting cure and development of antitumor memory responses [43]. More studies would be required to precisely determine this outcome.

In colorectal cancer, the expression of PD-L1 differs in microsatellite instability (MSI) and microsatellite stability (MSS). MSI colorectal tumors show increased PD-L1 expression when compared to MSS tumors (56 vs 21%, $p=0.007$) [44]. According to another study, seventeen percent of colorectal cancer cases showed mismatch

repair deficiency (MMRD) and statistically positive correlation was detected between the MMRD and PD-L1 expression [37].

On the other hand, Droeser et al. analyzed the PD-L1 expression in two subtypes of colorectal cancer, including 1197 mismatch repair proficient (MMRP) and 223 MMRD colorectal carcinoma cells. They detected strong PD-L1 expression in 37% of MMRP and in 29% of MMRD colorectal carcinoma cells. In this study, PD-L1 expression was paradoxically associated with improved survival in patients with MMRP, which might be due to a concomitant increase of CD8+ T cells infiltration ($p=0.0001$) [45]. Only a minority of patients with colorectal cancer demonstrated significant response to PD-1 blockade.

A phase II study looked at 296 patients with multiple tumor types (colorectal cancer, non-small cell lung cancer, castrate-resistant prostate cancer, renal cell carcinoma, melanoma). Response rates for non-small cell lung cancer, melanoma and renal cell carcinoma were 18,28 and 27% respectively, while no objective response was seen in patients with colorectal and prostate cancer [46]. This trial investigated pembrolizumab in patients with MMRD colorectal cancer. Pembrolizumab in doses of 10 mg/kg was administered to patients with MSI and patients with MSS colorectal cancer. The immune-related 20-week PFS was 78% in the MSI group vs 0% in the MSS group.

Correlation analysis suggests the mutational load in the tumors of MSI patients may be the mechanism through which the PD-1 blockade exerts its effect, leading to a response in such cohorts [47]. Another two studies showed that positive PD-L1 expression in tumor is an independent predictor of poor prognosis [48,49].

Conflict of interests

The authors declare no conflict of interests.

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