PD1-positive tumor-infiltrating lymphocytes are associated with poor clinical outcome after pulmonary metastasectomy for colorectal cancer

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ABSTRACT

Pulmonary metastasectomy (PM) is routinely performed in colorectal cancer (CRC) patients with oligometastatic spreading to the lungs. Patients with an aggressive tumor phenotype should be excluded from PM, since its benefit is outweighed by early tumor recurrence and impaired prognosis. Expression of PD-1 and its ligands are prognostic factors in a variety of primary tumors. However, their impact on patients’ outcome in the setting of PM for CRC has not been evaluated before.

53 CRC patients with pulmonary metastases receiving PM with curative intent were included in this study. Tissue samples of resected pulmonary metastases and available corresponding primary tumors were collected and assessed for PD-1, PD-L1 and PD-L2 expression by tumor-infiltrating lymphocytes (TILs) and tumor cells. Expression patterns were correlated with clinical outcome parameters.

PD-1 and PD-L1 expression was commonly found in TILs and tumor cells. Expression levels significantly differed between metastases and primary tumors. High PD-1 expression by TILs was associated with impaired overall survival (low vs high expression (mean, 95% CI): 78 mo (60–96) vs 35 mo (25–44); p = 0.011). Additionally, the subgroup of patients, who experienced an upgrading in their TILs/PD1 status between primary and metastasis had a worse survival outcome compared with patients with the same grade or a downgrading (34 mo (26–42) vs 96 mo (72–120); p = 0.004).

Thus, PD-1 expression by TILs is a strong prognostic marker in CRC patients with pulmonary spreading treated by PM. Moreover, this study provides a rationale for a therapeutic PD-1 pathway blockade in the treatment of CRC lung metastases. Future, large-scale studies are warranted to validate the findings of this single-center, retrospective analysis.

Introduction

Pulmonary metastasectomy (PM) is one of the corner stones in the treatment of oligometastatic colorectal cancer (CRC). By removing all gross tumor spreading to the lungs, 5-y survival rates of up to 50–70% can be achieved. This is in strong contrast to survival rates of patients, who do not qualify for a surgical treatment, with only 1 out of 10 patients being alive at 5 y. One of the main problems in PM is the selection of patients for the procedure. Most thoracic surgeons are reluctant to offer PM to patients with an aggressive tumor phenotype. In these cases, a removal of pulmonary metastases is frequently challenged by an early tumor recurrence, thus exposing patients mainly to the risks of a surgical procedure without a clear oncological benefit. To date there is a lack of clear selection criteria for PM in patients with CRC. Recently, markers that determine tumor behavior and aggressiveness have been in the spotlight of research. Programmed death-1 (PD-1) and its ligands PD-L1 and PD-L2 are important immune checkpoints. PD-1 is an inhibitory co-signal on activated lymphocytes and plays a crucial role in regulating the magnitude and quality of T-cell responses. Immuneoncogenic tumors can escape immune surveillance by an upregulation of PD-1 ligands and thus leading to an inactivation of the endogenous antitumor immune defense. Blocking of the PD-1 pathway by therapeutic antibodies is a novel targeted therapy, which counteracts PD-1 dependent tumor immune escape mechanisms. In clinical trials, PD-1 inhibitors have been proven effective in patients with advanced melanoma, renal cell carcinoma, non-small-cell lung cancer and colorectal carcinoma.
A prognostic impact of PD-1 and PD-L1 expression has been described for several tumor entities, e.g., breast cancer, hepatocellular carcinoma and esophageal cancer.⁷⁻⁹ However, in primary CRC PD-1 and PD-L1 expressions are paradoxically associated with favorable outcome parameters. PD-1 and PD-L1 expressing tumor-infiltrating lymphocytes (TILs) at the primary tumor site lead to prolonged recurrence free and overall survival (OS).¹⁰⁻¹¹ This unique finding in CRC has been controversially discussed in the literature and is explained by the gut specific microenvironment and particular features of the intestinal immune system.

To the best of our knowledge the expression of PD-1 and its ligands has not been described in CRC lung metastases. Furthermore, its prognostic impact on outcome parameters in patients receiving curative PM has not been evaluated before.

**Material and methods**

**Study population**

53 CRC patients with pulmonary metastases receiving PM with curative intent between April 2009 and November 2013 were included in this single-center study. In case a PM had been performed before the inclusion period, the specimen of the first PM was also assessed. For 30 (57%) patients paraffin embedded specimens from the primary tumor were available. Tumor staging before metastasectomy was performed by abdominal and thoracic computed tomography (CT) scans. In case of an inconclusive CT, positron emission tomography (PET) was added to exclude extrathoracic spreading. Patients received surgery through a muscle-sparing anterolateral or posterior incision. Lungs were bimanually palpated for occult lesions and a lymph node sampling was performed. Video-assisted thoracoscopic metastasectomy was applied in patients with a singular subpleural metastasis. Complete resection (R0) was achieved in all patients. After surgery patients were followed-up in 3 mo intervals with chest and abdominal CT scans during the first year and were seen every 6 mo thereafter.

Lung metastasis free survival was defined as the time between the diagnosis of the primary tumor and the diagnosis of metastatic spread to the lungs. Time to recurrence represented the time between PM and the first evidence of metastatic recurrence at any site. OS was defined as the period of time between PM and death of any cause.

This study was approved by the ethics committee of the Medical University of Vienna (EK#: 1097/2014) and was performed according to the Declaration of Helsinki and the Good Scientific Practice guidelines of the Medical University of Vienna.

**Immunohistochemistry**

3–5 μm thick sections were deparaffinized and rehydrated in graded series: X-TRA-Solv 8 (Medite, # 41-5212-00) – 15 min at 68°C; Xylol – 5 min room temperature (RT), 100% EtOH – 5 min RT; 96% EtOH – 5 min RT; 80% EtOH – 5 min RT; distilled water – 2 min RT. Antigen retrieval with target retrieval solution (pH 9) was performed. For this purpose, the slides were heated to 115°C for 10 min in a Dako Cytomation Pascal Pressure Cooker. After cooling to RT and washing with TBS buffer, 3% hydrogen peroxide in distilled water was used to block the endogenous peroxidase activity (10 min, RT). The following antibodies were used for PD stains: mouse anti-human PD1 (R&D systems, # AF 1086, dilution 1:20), PD-L1 (Cell signaling, clone: E1L3N, dilution: 1:25), PD-L2 (Cell signaling, clone: D7U8C, dilution: 1:25). The slides were automatically processed with a Dako Autostainer Plus System. Visualization was implemented with streptavidin conjugated to alkaline phosphatase. Cell nuclei were counterstained by Mayer’s hematoxylin.

For each slide, four different areas of tumor tissue were randomly selected for analysis. Tumor cells and TILs in these areas were independently evaluated by two investigators blinded to the clinical data. If the rating differed, the slide was re-discussed and a consensus was found using a multi-head microscope. Slides were examined at 400× magnification, and the staining rate (percentage of tumor cells and lymphocytes showing positive staining) was determined. PD-1, PD-L1 and PD-L2 expression was categorized into 0: no positive cells, 1+: 5–25% of cells, 2+: 26–50% of cells, 3+: 51–75% of cells and 4+: 76–100% of cells. For some analyses a dichotomization was used. 0–50% positive PD-1 cells were defined as PD-1low, 51–100% positive PD-1 cells as PD-1high.

Immunostaining for CD3 (clone SP7, #RM9107-S1, Thermo Fisher Scientific, Cheshire, UK), CD8+ (clone C8/144B, #M7103, Dako), CD45RO (clone UCHL1, #M074201, Dako) were performed using an autostainer (Benchmark Ultra, Ventana Medical Systems), as described previously.¹² In negative controls the primary antibody was omitted. A mediastinal lymph node served as positive control. As previously published, TILs were classified semi-quantitatively by two independent observers.¹² The following score for TIL was used: scattered (+), intermediate (2+), dense infiltrate (3+), very dense infiltrate (4+). Grades of none (−), sparse (1+), intermediate (2+), high (3+) were used to classify the proportion of CD8+ or CD45RO+ cells in the immune infiltrate.

**Statistical analysis**

All data were evaluated using SPSS 23. Nominal variables were compared using Fisher’s exact test and chi-square test. Correlations were calculated using the Kendall-Tau equation. Survival curves were estimated by Kaplan–Meier plots and the differences between the groups were compared using the log-rank test. All performed tests were two-sided. p-values <0.05 were considered statistically significant. Due to the hypothesis generating approach of the study no correction for multiple testing was applied.¹³

**Results**

**Patients’ characteristics**

A total of 53 (30 male and 23 female) patients were included in the study. The primary tumor site was colon in 57% and rectum for 43% of patients. Most patients were in UICC stage III (n =
24) and IV (n = 9) at the time of diagnosis with a high number of primary lymph node positive tumors (n = 29), 16 patients had a previous liver metastasectomy before they were operated for their pulmonary nodules. The majority of patients (76%) presented with a singular lung metastasis. Uniformly, a high number had received chemotherapeutic treatment(s) before metastasectomy. 40 out of 53 patients were treated with pseudo-adjuvant chemotherapy after resection of pulmonary metastases. None of the patients received a PD-1 blocking agent during the study period. The median follow-up after metastasectomy was 35 mo (range 4–137). Patients’ characteristics are summarized in Table 1.

**Characterization of the immune-infiltrate**

A lymphocytic immune infiltrate was seen in nearly all metastases with a broad distribution ranging from scattered to very dense infiltrates. In most patients, the immune infiltrate consisted of a high proportion of CD8+ and CD45RO+ lymphocytes. A detailed description of TILs is presented in Table 2.

| Table 1. Patients’ characteristics. |
|-----------------------------------|
| **Total study cohort (n = 53)**    |
| Median age at surgery (years, range) | 64 (33–79) |
| Median follow-up after metastasectomy (months, range) | 35 (4–137) |
| **Sex**                           |
| Male                              | 30 | 56.6 |
| Female                            | 23 | 43.4 |
| **Localization of primary tumor** |
| Colon                             | 30 | 56.6 |
| Rectum                            | 23 | 43.4 |
| **T stage**                       |
| −1                                 | 1  | 2.0  |
| −2                                 | 6  | 12.0 |
| −3                                 | 36 | 72.0 |
| −4                                 | 7  | 14.0 |
| Unknown                            | 3  | —    |
| **N stage**                       |
| −0                                 | 21 | 42.0 |
| −1                                 | 14 | 28.0 |
| −2                                 | 15 | 30.0 |
| Unknown                            | 3  | —    |
| **UICC stage of primary tumor**   |
| I                                  | 4  | 8.0  |
| II                                 | 13 | 26.0 |
| III                                | 24 | 48.0 |
| IV                                 | 9  | 18.0 |
| Unknown                            | 3  | —    |
| **Previous liver metastasis**     |
| Yes                                | 16 | 30.2 |
| No                                 | 37 | 69.8 |
| **Lung metastasis free survival** |
| <36 mo                             | 32 | 60.4 |
| 36–60 mo                           | 8  | 15.1 |
| >60 mo                             | 9  | 17.0 |
| **No. of pulmonary metastases**   |
| Singuler                           | 40 | 75.5 |
| Multiple                           | 13 | 24.5 |
| **Chemotherapy before metastasectomy** |
| Yes                                | 42 | 79.2 |
| No                                 | 11 | 20.8 |
| **Chemotherapy after metastasectomy** |
| Yes                                | 40 | 75.5 |
| No                                 | 13 | 24.5 |

**Density and distribution of PD-1, PD-L1 and PD-L2 in pulmonary metastases**

Quality of immunohistochemical stainings was sufficient in 52/53 (98.1%) for PD-1, 51/53 (96.2%) for PD-L1 and 52/53 (98.1%) for PD-L2, respectively. A detailed list of the density of PD-1 and its ligands in TILs and tumor cells is shown in Table 2 and representative images are provided in Fig. 1. A high number of TILs were positive for PD-1. Although staining intensity was generally lower in tumor cells, PD-1 expression was also a commonly found feature on malignant cells. PD-L1 was frequently expressed by TILs as well as tumor cells. However, PD-L2 expressing tumor cells were only found in three patients and TILs were all negative for PD-L2. There was no association of PD-1 and PD-L1 expression on tumor cells and TILs with clinicopathological characteristics of the patients (Table 3).

**Correlation of PD-1 and PD-L1 expression in pulmonary metastases and corresponding primary CRC**

The pattern of PD-1 and PD-L1 expression was distinct between pulmonary metastases and their corresponding primaries (Fig. S1). There was an upgrading in TILs/PD-1
expression in 14 patients (48%), 7 patients (24%) had a similar expression and 8 patients (28%) had a lower PD-1 expression in their metastasis compared with the primary tumor. Contrarily, the expression of PD-L1 in TILs was less variable between primary tumor and metastasis. 15 patients (54%) were classified the same grade, but only five and eight patients were upgraded and downgraded, respectively. Differences between PD-L1 expression of TILs and tumor cells between primary tumors and metastases are summarized in Table S1.

Impact of PD-1 and PD-L1 expression on outcome parameters

Univariate outcome analyses of recurrence-free survival and OS after PM were calculated by log-rank tests. Neither the expression of PD-1 nor its ligands by TILs were prognostic for time to recurrence (Fig. 2A, Fig. 3A). However, PD-1 expression on TILs was associated with impaired OS (low vs high (mean, 95% CI): 78 mo (60–96) vs 35 mo (25–44); \( p = 0.011 \); Fig. 2B, Table 4). Additionally, the subgroup of patients, who experienced an upgrading in their TILs/PD1 status had a worse survival outcome compared with patients with the same grade or a downgrading (34 mo (26–42) vs 96 mo (72–120); \( p = 0.004 \), Fig. 2C). The prognostic impact of PD-L1 expression on TILs was less obvious (Fig. 3B). However, an impaired survival was observed in patients with TILs/PD-L1 upregulation compared with patients with the same or less PD-L1 positive TILs (26 mo (19–34) vs 82 mo (60–103); \( p = 0.045 \), Fig. 3C). Expression of PD-1 and PD-L1 on tumor cells was not associated with outcome parameters (Table 4).

Discussion

The aim of this study was to evaluate the expression of PD-1 and its ligands PD-L1 and PD-L2 in pulmonary metastases from CRC and to correlate the expression pattern with clinical outcome parameters. We found that PD-1 and PD-L1 was highly abundant in TILs in pulmonary nodules as well as in corresponding primary tumors, whereas PD-L2 was only found sporadically. Interestingly, there was a significant heterogeneity of PD-1 and PD-L1 expression between pulmonary metastases and corresponding primaries. High PD-1 expression of TILs in pulmonary metastases was a predictor of impaired survival, with the worst prognosis in patients who had an upgrading in

![Figure 1. Shows representative slides of PD-1 and PD-L1 expressing tumor-infiltrating lymphocytes. Patients were dichotomized into PD1low (0–50% positive cells) PD1high (51–100% positive cells).](image-url)
their TILs/PD-1 status between primary and metastasis. PD-L1 expression did not impact OS; however, patients with an upgrading of their TILs/PD-L1 also had a worse outcome.

To the best of our knowledge this is the first structured evaluation of PD-1 expression and its ligands in pulmonary metastases from CRC. The PD1/PD-L1 axis is considered an essential immune checkpoint. PD-1 is primarily expressed by activated lymphocytes and upon triggering by its ligands (PD-L1 and PD-L2) it can repress Th1 cytotoxic immune responses.14

TILs are an important endogenous defense mechanism against cancer. High levels of TILs are associated with a favorable prognosis in various malignancies including lung, kidney, breast and CRC.15-18 Especially in CRC, CD3+ , CD8+ and CD45RO+ TILs have been extensively studied, recently leading to the foundation of an international consortium to implement a novel staging system including the immune infiltrate (Immunoscore).19 High expression of PD-L1 in the tumor microenvironment can lead to an escape from tumor-specific T-cell immunity. Consequently, therapeutic PD-1 blocking antibodies have been developed to counteract this phenomenon. In clinical trials, PD-1 inhibitors were successfully tested in patients with advanced melanoma, renal cell carcinoma, non-small-cell lung cancer, as well as subsets of colorectal carcinoma.6 This concept of TILs re-sensibilization by PD-1 pathway blockers has recently been challenged by the observation that anti-PD-1 cancer therapies are also effective in low immunogenic tumors. An explanation for this could be the finding that PD-1 is also expressed by cancer cells, although to a lesser extent. Engaging of tumor-harbored PD-1 results in the activation of the mTOR pathway and augments tumor growth.20,21 Based on these findings, we also evaluated PD-1 and PD-L1 on cancer cells. Both proteins were present in a high percentage of CRC tumor cells. Despite this fact, expression levels did not correlate with clinical outcome parameters (Table 4). Nevertheless, the high number of PD-1 and PD-L1 positive tumor cells in CRC lung metastasis provides a further rationale for a future application of therapeutic PD-1 blockage in the setting of pulmonary spreading.

PD-L1 is expressed by a variety of cell types, including lymphocytes, endothelial and epithelial cells. Contrarily, PD-L2 expression is limited to antigen presenting cells and macrophages.22 Most recently, some solid cancers as endometrium cancer and hepatocellular cancer has been shown to express PD-L2.23,24 In our patients, PD-L2 was absent in the lymphocytic infiltrate and only an insignificant expression was found on CRC tumor cells. We, therefore, excluded PD-L2 from further analysis, since its impact on tumor biology and immunosurveillance can be considered minimal.

Linking the number of TILs with their PD-1/PD-L1 expression status is an aspect, which has not been addressed
sufficiently in the previous studies. The impact of PD-1/PD-L1 axis on immunogenic tumors should theoretically correlate with the density of the immune infiltrate. Despite the fact, that our study population was small, patients with a high number of CD8\(^+\) TILs but low expression of PD-1 had the best prognosis with a mean survival of 64 mo (48–79). Patients with only few CD8\(^+\) PD-1\(^{\text{high}}\) cells had the worst prognosis of only 28 mo (22–34). Patients with low CD8\(^+\) numbers but low expression of PD-1 accordingly showed intermediate outcome with a mean survival of 41 mo (24–59).

It is commonly accepted within the thoracic surgical and oncological community that patients, who have a high likelihood of early tumor recurrence, should not undergo PM. Despite this general agreement, there is a lack of knowledge on predictive factors for this group of patients. The indication for PM is still based on clinical features, which have been proposed in the 1970s.\(^4\) The disease-free interval between primary and pulmonary metastasis, the number of pulmonary nodules and available alternative treatment regimens are mostly used selection criteria. Recently, several attempts have been made to link markers of tumor biology with patients’ outcome. We have previously shown that the immune cellular infiltrate as well as markers of inflammation are strongly associated with recurrence free and OS of PM for CRC.\(^12,25\) This study extends these findings by showing that the PD-1 pathway is a valid prognostic factor in those patients. Additionally, it provides a rationale for the therapeutic feasibility of PD-1 blockage in the subset of CRC patients with pulmonary spreading.

The prognostic role of PD-1 and PD-L1 expression in primary CRC is still a matter of discussion.\(^10,26\) Unlike most tumors, in which PD-1 overexpression clearly correlates with impaired survival, PD-1 expressions seems to be paradoxically
associated with improved outcome in primary CRC.\textsuperscript{10,11} This is possibly based on specific features of the intestine immune system, which is constantly exposed to commensal gut flora. Furthermore, expression of PD-L1 in primary rectal cancer has been shown to be rare or even absent.\textsuperscript{27} A similar paradox is the fact that in primary CRC regulatory T-cells are associated with improved survival, whereas they are a poor prognostic factor in most other solid tumors.\textsuperscript{28} The local immune response in the lung seems to be profoundly different from the intestine immune system and PD-1/PD-L1 expression of lung cancer infiltrating TILs is associated with poor clinical outcome.\textsuperscript{29} This spatial effect on PD-L1 expression has also been highlighted in a study on lung cancer patients with brain metastasis. Dong and colleagues found substantially altered PD-L1 levels at different cancer sites and concluded that the PD-1/PD-L1 axis is strongly influenced by the tumor surrounding microenvironment.\textsuperscript{30}

There are several limitations to this study. First, it is a single-center, retrospective analysis with only a limited number of patients, thus deductions have to be interpreted with caution. We currently recruit patients within an international multi-institutional study protocol to confirm the impact of proposed prognostic markers in CRC PM in a larger cohort. Second, a selection bias cannot be excluded. Patients undergoing PM are a highly selected subset, who do not necessarily represent the whole spectrum of CRC patients with lung metastases. One strength of this study is that IHC analysis were performed on the whole sections of slides, rather than using tissue microarrays (TMAs).\textsuperscript{11,19} Although TMAs facilitate the analysis of a large number of samples, tissue analysis is limited to a small

\textbf{Figure 3.} Kaplan–Meier plots of PD-L1/TILs on patients’ outcome. In contrast to PD-1, PD-L1 was only loosely associated with outcome parameters. A non-significant trend toward worse overall survival in the group of PD-L1 high-expressing TILs was observed (B). Additionally, upgrading of PD-L1/TIL resulted in an impaired prognosis (C).
cylinders of less than 1 mm in diameter. In our study, the size of obtained tissue samples was approximately 10–20 mm and four different random spots were used for analysis. This technique minimized the possibility of bias due to tumor heterogeneity.

In conclusion, this study shows that PD-1 and PD-L1 are uniformly expressed in tumor cells and TILs of resected CRC pulmonary metastases. High expression of PD-1 in TILs reflects an aggressive tumor biology with impaired OS. Further studies are warranted to confirm these findings in a larger study cohort.

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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References
1. Fiorentino F, Hunt I, Teoh K, Treasure T, Utley M. Pulmonary metastasectomy in colorectal cancer: a systematic review and quantitative synthesis. J R Soc Med 2010; 103:60-6; PMID:20118336; https://doi.org/10.1258/jrsm.2009.090299
2. Pfannschmidt J, Hoffmann H, Dienemann H. Reported outcome factors for pulmonary resection in metastatic colorectal cancer. J Thorac Oncol 2010; 5:S172-8; PMID:20502257; https://doi.org/10.1097/JTO.0b013e3181dca330
3. Suzuki H, Kiyoshima M, Kitahara M, Asato Y, Amemiya R. Long-term outcomes after surgical resection of pulmonary metastases from colorectal cancer. Ann Thorac Surg 2015; 99:435-40; PMID:25499475; https://doi.org/10.1016/j.athoracsur.2014.09.027
4. Schweiger T, Lang G, Klepetko W, Hoetzenecker K. Prognostic factors in pulmonary metastasectomy: spotlight on molecular and radiological markers. Eur J Cardiothorac Surg 2014; 45:408-16; PMID:23729747; https://doi.org/10.1093/ejcts/ezt288
5. Schweiger T, Hagedus B, Nikolowsky C, Hagedus Z, Szirtes I, Mair R, Birner P, Dome B, Lang G, Klepetko W et al. EGFR, BRAF and KRAS status in patients undergoing pulmonary metastasectomy from primary colorectal carcinoma: a prospective follow-up study. Ann Surg

Table 4. Univariate outcome analysis of recurrence-free survival and overall survival after pulmonary metastasectomy.

|                      | Recurrence-free survival | Overall survival |
|----------------------|--------------------------|-----------------|
|                      | Mean survival (months)   | p-value         | Mean survival (months) | p-value |
| **Sex**              |                          |                 |                          |         |
| Male                 | 17 (9–25)                | 0.081           | 67 (49–84)               | 0.685   |
| Female               | 31 (17–33)               |                 | 66 (44–87)               |         |
| **Age (years)**      |                          |                 |                          |         |
| <64 y                | 22 (13–32)               | 0.857           | 51 (39–62)               | 0.714   |
| ≥ 64 y               | 26 (15–38)               |                 | 69 (49–89)               |         |
| **Location**         |                          |                 |                          |         |
| Colon                | 19 (13–25)               | 0.796           | 68 (48–88)               | 0.743   |
| Rectum               | 28 (16–40)               |                 | 53 (41–65)               |         |
| **UICC stage**       |                          |                 |                          |         |
| I + II               | 23 (12–35)               | 0.721           | 43 (32–55)               | 0.061   |
| III + IV             | 26 (17–36)               |                 | 87 (66–107)              |         |
| **Chemotherapy before metastasectomy** | | | | |
| Yes                  | 24 (16–33)               | 0.683           | 70 (53–87)               | 0.538   |
| No                   | 26 (10–41)               |                 | 45 (30–59)               |         |
| **Chemotherapy after metastasectomy** | | | | |
| Yes                  | 22 (15–29)               | 0.589           | 59 (46–71)               | 0.269   |
| No                   | 29 (10–48)               |                 | 97 (70–125)              |         |
| **Previous liver metastasis** | | | | |
| Yes                  | 14 (7–21)                | 0.055           | 49 (34–63)               | 0.447   |
| No                   | 30 (20–40)               |                 | 70 (52–88)               |         |
| **Lung metastasis free survival** | | | | |
| <36                  | 23 (15–31)               | 0.952           | 69 (50–89)               | 0.931   |
| ≥36                  | 22 (12–32)               |                 | 55 (41–69)               |         |
| **Number of metastasis** | | | | |
| Singular             | 20 (14–25)               | 0.448           | 72 (54–89)               | 0.551   |
| Multiple             | 33 (14–53)               |                 | 56 (28–84)               |         |
| **PD1 – Tumor-infiltrating lymphocytes** | | | | |
| Low                  | 24 (16–33)               | 0.766           | 78 (60–96)               | 0.011   |
| High                 | 24 (12–35)               |                 | 35 (25–44)               |         |
| **PD1 – Tumor cells** | | | | |
| Low                  | 28 (14–41)               | 0.918           | 83 (57–109)              | 0.425   |
| High                 | 21 (15–28)               |                 | 59 (45–74)               |         |
| **PD-L1 – Tumor-infiltrating lymphocytes** | | | | |
| Negative             | 15 (6–23)                | 0.310           | 38 (29–46)               | 0.307   |
| Positive             | 26 (17–35)               |                 | 73 (55–90)               |         |
| **PD-L1 – Tumor cells** | | | | |
| Negative             | 16 (10–23)               | 0.538           | 74 (51–98)               | 0.628   |
| Positive             | 26 (17–35)               |                 | 71 (52–89)               |         |
10. Droeser RA, Hirt C, Viehl CT, Frey DM, Nebiker C, Huber X, Zlobec I, Eppenberger-Castori S, Tzankov A, Rosso R et al. Clinical significance of programmed death-ligand 1 expression in colorectal cancer. Eur J Cancer 2013; 49:2233-42; PMID:23478000; https://doi.org/10.1016/j.ejca.2013.02.015

11. Li Y, Liang L, Dai W, Cai G, Xu Y, Li X, Li Q, Cai S. Prognostic impact of programmed cell death-1 (PD-1)-positive tumor-infiltrating lymphocytes is associated with poor prognosis in human breast cancer. Breast Cancer Res Treat 2013; 139:667-76; PMID:23756627; https://doi.org/10.1007/s10549-013-2581-3

12. Schweiger T, Berghoff A, Winkelmann J, Abbott ME, Hamid O, Carvalho R, et al. Overexpression of PD-L1 significantly associates with tumor aggressiveness and postoperative recurrence in human hepatocellular carcinoma. Clin Cancer Res 2009; 15:971-9; PMID:19188168; https://doi.org/10.1158/1078-0432.CCR-08-1608

13. Veenstra DL, Schubert M, Heidenreich KA, Panetta JC, Chiaretti S, et al. Prognostic and predictive value of tumor-infiltrating lymphocytes and programmed death receptor-ligand expression in muscle-invasive bladder cancer. Nat Clin Pract Urol 2005; 2:570-8; PMID:15950361; https://doi.org/10.1038/ncpurnol0030

14. Chen L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat Rev Immunol 2004; 4:336-47; PMID:15122199; https://doi.org/10.1038/nri1349

15. Donnem T, Hald SM, Paulsen EE, Richardsen E, Al-Saad S, Kilvaer TK, Brustugun OT, Helland A, Lund-Iversen M, Poehl M et al. Stromal CD8+ T-cell density – a promising supplement to TNM staging in non-small cell lung cancer. Clin Cancer Res 2015; 21:2635-43; PMID:25680376; https://doi.org/10.1158/1078-0432.CCR-14-1905

16. Miyashita M, Sano S, Shi Y, Sato H, Saito M, et al. Prognostic significance of tumor-infiltrating CD8+ and FOXP3+ lymphocytes in residual tumors and alterations in these parameters after neoadjuvant chemotherapy in triple-negative breast cancer: a retrospective multicenter study. Breast Cancer Res Treat 2015; 17;124; PMID:26341640; https://doi.org/10.1007/s10549-015-0632-x

17. Giraldo NA, Becht E, Pages F, Skliris G, Verkarre V, Vano Y, Mejean A, Saint-Aubert N, Lacroy L, Natario I et al. Orchestration and prognostic significance of immune checkpoints in the microenvironment of primary and metastatic renal cell cancer. Clin Cancer Res 2015; 21:3031-40; PMID:25688160; https://doi.org/10.1158/1078-0432.CCR-14-1924

18. Meclinik B, Tosolini M, Kirillovskiy A, Berger A, Bindea G, Mechtchi T, Brunnert P, Trajanoski Z, Fridman WH, Pages F et al. Histopathologic-logic prognostic factors of colorectal cancers are associated with the state of the local immune reaction. J Clin Oncol 2011; 29:610-8; PMID:21245428; https://doi.org/10.1200/JCO.2010.30.5425

19. Galon J, Mlecnik B, Bindea G, Angell HK, Berger A, Lagorce C, Lugli A, Zloboe I, Hartmann A, Bifulco C et al. Towards the introduction of the ‘Immunoscore’ in the classification of malignant tumours. J Pathol 2014; 232:199-209; PMID:24212236; https://doi.org/10.1002/path.4287

20. Klefors S, Posch C, Barthel SR, Mueller H, Slabachb C, Guenova E, Eclo CP, Lee N, Juneca VR, Zhan Q et al. Melanoma cell-intrinsic PD-1 receptor functions promote tumor growth. Cell 2015; 162:1242-56; PMID:26359984; https://doi.org/10.1016/j.cell.2015.08.052

21. Clark CA, Gupta HB, Sareddy G, Pandeswara S, Lao S, Yuan B, Drrerp JM, Padron A, Conejo-Garcia J, Murthy K et al. Tumor-Intrinsic PD-L1 signals regulate cell growth, pathogenesis, and auto-agphy in ovarian cancer and melanoma. Cancer Res 2016; 76:6964-74; PMID:27671674; https://doi.org/10.1158/0008-5472.CAN-16-0258

22. Ohaegbulam KC, Assal A, Lazar-Molnar E, Yao Y, Zang X. Human cancer immunotherapy with antibodies to the PD-1 and PD-L1 pathway. Trends Mol Med 2015; 21:24-33; PMID:25440900; https://doi.org/10.1016/j.molmed.2014.10.009

23. Mo Z, Liu J, Zhang Q, Chen Z, Mei J, Liu L, Yang S, Li H, Zhou L, You Z. Expression of PD-1, PD-L1 and PD-L2 is associated with differentiation status and histological type of endometrial cancer. Oncol Lett 2016; 12:944-50; PMID:27446374; https://doi.org/10.3892/ol.2016.4744

24. Jung HI, Jeong D, Ji S, Ahn TS, Bae SH, Chin S, Chung JC, Kim HC, Lee MS, Baek MF. Overexpression of PD-L1 and PD-L2 is associated with poor prognosis in patients with hepatocellular carcinoma. Cancer Res Treat 2017; 49:246-54; PMID:27456947; https://doi.org/10.4143/crt.2016.066

25. Ghanim B, Schweiger T, Jedamzik J, Glocue O, Glogner C, Lang G, Klepetko W, Hoetznecner K. Elevated inflammatory parameters and inflammation scores are associated with poor prognosis in patients undergoing pulmonary metastasectomy for colorectal cancer. Interact Cardiovasc Thorac Surg 2015; 21:616-23; PMID:26242317; https://doi.org/10.1093/ictv/ictv2016.167

26. Saigusa S, Toiyama Y, Tanaka K, Inoue Y, Mori K, Ide S, Imaoka H, Kawamura M, Mohri Y, Kusunoki M. Implication of programmed cell death ligand 1 expression in tumor recurrence and prognosis in rectal cancer with neoadjuvant chemoradiotherapy. Int J Clin Oncol 2016; 21:946-52; PMID:26919982; https://doi.org/10.1016/j.ijrobp.2016.0962-4

27. Jomrich G, Silberhumer GR, Marion B, Beer AM, Mullauer L. Programmed death-ligand 1 expression in rectal cancer. Eur Surg 2016; 48:352-6; PMID:28058043; https://doi.org/10.1007/s11353-016-0447-8

28. Ladoire S, Martin F, Ghiringhelli F. Prognostic role of FOXP3+ regulatory T cells infiltrating human carcinomas: the paradox of colorectal cancer. Cancer Immunol Immunother 2011; 60:909-18; PMID:21644034; https://doi.org/10.1007/s00262-011-1046-y

29. Shimoji M, Shimizu S, Sato K, Suda K, Kobayashi Y, Tomizawa K, Takemoto T, Mitsudomi T. Clinical and pathologic features of lung cancer expressing programmed cell death ligand 1 expression. Ann Oncol 2016; 27:1953-8; PMID:27393509; https://doi.org/10.1016/j.annonc.2016.04.021

30. Mansfield AS, Aubry MC, Moser JC, Harrington SM, Dronca RS, Park SS, Dong H. Temporal and spatial discordance of programmed cell death-ligand 1 expression and lymphocyte tumor infiltration between paired primary lesions and brain metastases in lung cancer. Ann Oncol 2016; 27:1953-8; PMID:27393509; https://doi.org/10.1016/j.annonc.2016.04.021

31. Russo G, Zegar C, Giordano A. Advantages and limitations of microarray technology in human cancer. Oncogene 2003; 22:6497-507; PMID:14528274; https://doi.org/10.1038/sj.onc.1206865