The clinical significance of PD-L1 in advanced gastric cancer is dependent on ARID1A mutations and ATM expression

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ABSTRACT

Whether PD-L1 expression is associated with survival outcomes in gastric cancer (GC) is controversial. The inhibition of the PD-1/PD-L1 pathway is effective against genomically unstable tumors. Hypothesizing that also the clinical significance of PD-L1 might be dependent on the activation of molecular circuits ensuring genomic stability, we evaluated PD-L1 expression in tissue samples from 72 advanced GC patients treated with first-line chemotherapy. Samples were already characterized for DNA damage repair (DDR) component expression (pATM, pChk1, pWee1, γ-H2AX and pRPA2) along with mutations in DDR-linked genes (TP53 and ARID1A). Overall, PD-L1 expression was not associated with progression-free survival (PFS) and overall survival (OS), independently on whether we considered its expression in tumor cells (PD-L1-TCs) or in the immune infiltrate (PD-L1-TILs). In subgroup analysis, positive PD-L1-TC immunostaining was associated with better PFS in patients whose tumors did not carry DDR activation (multivariate Cox; HR 0.34, 95%CI: 0.15–0.76, p = 0.008). This subset (DDRoff) was characterized by negative pATM expression or the presence of ARID1A mutations. Conversely, the relationship between PD-L1-TC expression and PFS was lost in a molecular scenario denoting DDR activation (DDRon), as defined by concomitant pATM expression and ARID1A wild-type form. Surprisingly, while PD-L1-TC expression was associated with better OS in the DDRoff subset (multivariate Cox; HR 0.41, 95% CI: 0.17–0.96, p = 0.039), in the DDRon subgroup we observed an opposite impact on OS (multivariate Cox: HR 2.56, 95%CI: 1.06–6.16, p = 0.036). Thus, PD-L1-TC expression may impact survival outcomes in GC on the basis of the activation/inactivation of genome-safeguarding pathways.

ARTICLE HISTORY

Received 9 February 2018
Revised 15 March 2018
Accepted 19 March 2018

KEYWORDS

Gastric cancer; chemotherapy; PD-L1; DNA damage repair; genomic stability; ARID1A; ATM

INTRODUCTION

Antibodies directed against the programmed cell death protein-1 (PD-1)/programmed cell death ligand-1 (PD-L1) pathway demonstrated significant efficacy in a variety of malignancies. Expression of PD-L1, assessed by immunohistochemical assays, is widely used in clinical trials in the attempt of delineating the patient population with greater likelihood of response to PD-1-blocking approaches. More recently, genetic biomarkers gained popularity, as accumulating evidence conveyed the message that abnormalities leading to log-fold increases in tumor mutational burden predict responsiveness to immune checkpoint blockade. Consistently, pembrolizumab was found to be active across a range of solid tumors harboring mismatch-repair (MMR) deficiency (microsatellite instability, MSI), a condition stemming from epigenetic inactivation or germline mutations in the MMR machinery that gives rise to replication infidelity, a hypermutated phenotype and formation of neoantigens. At the same time, a wave of studies strived to investigate the prognostic significance of PD-L1 expression, independently of the administration of PD-1-targeting agents. In gastric cancer (GC), efforts toward understanding whether PD-L1 affects survival outcomes yielded mixed and in some
instances opposite findings, as emerging when evaluating results from individual studies included in three recent meta-analyses.\textsuperscript{18-20} Moreover, available evidence did not point to the advanced setting.

Taking into account the inconsistencies emerging from studies evaluating the relationship between PD-L1 expression and survival outcomes in GC, and considering that the efficacy of immune checkpoint inhibitors is tied to genomic instability, it is plausible to hypothesize that PD-L1 is not a standalone prognostic marker, but it should rather be framed into a broader molecular context mirroring the activation/inactivation status of genome-protecting pathways.

The transmission of an unaltered genetic code to the progeny is ensured by DNA damage repair (DDR) pathways.\textsuperscript{21} When DNA single- and double-strand breaks (SSBs and DSBs) arise, the DDR network halts the cell cycle and coordinates DNA repair or self-elimination of irreversibly damaged cells.\textsuperscript{21} Central to this process are two crosstalk signaling avenues: i) ATM-Chk2 (Ataxia-Telangiectasia Mutated-Checkpoint Kinase 2), and ii) ATR-Chk1-Wee1 (Ataxia Telangiectasia and Rad3-related Protein-Checkpoint Kinase 1-Weel-like Protein Kinase).\textsuperscript{22-24} This system is often aberrantly activated in cancer cells, being a protective response against a number of extracellular and intracellular cues that threaten the genome.\textsuperscript{25-27} These include cytotoxic therapies, reactive oxygen species, the replicative stress generated by mutations in oncogenes that control cell proliferation, and mutations in genes that control cell-cycle checkpoints.\textsuperscript{25-28}

On this premise, we herein investigated the connection between PD-L1 expression, DDR pathways and survival outcomes exploiting tissue samples from 72 advanced GC patients treated with first-line chemotherapy in prospective phase II trials or in routine clinical practice.\textsuperscript{29-32} The present patient population represents a subset of a wider cohort already characterized for the expression levels of DDR kinases (pATM, pChk1 and pWee1) and DNA damage markers, namely the DNA DSB marker phosphorylated H2A Histone Family Member X (γ-H2AX) and the single-stranded DNA/SSB marker phosphorylated replication protein A2 (pRPA2, best known as pRP32).\textsuperscript{33} Mutational status of TP53 and ARID1A, two recurrently mutated genes in GC that intersect the DDR at the protein level, was also available.\textsuperscript{33} Indeed, TP53 mutations hinder correct function of the G1-S checkpoint, thus rendering cancer cells reliant on intact cell cycle control systems to deal with DNA damage.\textsuperscript{27} The nexus between ARID1A and the DDR machinery was more recently described, in a model envisioning the recruitment of ARID1A to DNA DSBs via ATM/ATR signaling.\textsuperscript{34} Consistently, ARID1A-deficient cells are characterized by impaired initiation of the G2-M checkpoint and reduced non-homologous end joining activity.\textsuperscript{34,35} Overall, the present study was designed with the following goals: i) investigating the connection between PD-L1 expression and survival outcomes in advanced GC patients treated with chemotherapy, with a special focus on progression-free survival (PFS), the most direct indicator of efficacy/inefficacy of anticancer treatments, and ii) investigating whether underlying molecular backgrounds mirroring the activation of genome-protecting molecular circuits impact the clinical significance of PD-L1 expression.

\textbf{Results}

\textbf{Baseline characteristics of the patients and PD-L1 expression pattern}

Baseline characteristics of the 72 patients included in the present analysis are summarized in Table 1. In this subset of the original cohort, 40 (55.6%) patients received three-drug regimens, taxane-containing chemotherapy was administered to 38 (52.8%) patients, and 36 (50%) patients were treated in prospective phase II trials. PD-L1 expression in tumor cells (PD-L1-TCs) was detected in 41 samples (57%), whereas 30 samples (41.5%) displayed PD-L1 expression in the immune infiltrate (TILs, PD-L1-TILs). Associations between PD-L1 and baseline characteristics of the patients are provided in Supplementary Table 1, whereas associations between PD-L1 and DDR biomarkers are detailed in Supplementary Table 2. Immunohistochemical staining of four representative cases is presented in Supplementary Fig. 1.

\textbf{The relationship between PD-L1 expression and progression-free survival is dependent on DDR markers}

In the entire cohort, PD-L1 expression was neither significantly associated with PFS nor with OS (Fig. 1, panel A-B) for PD-L1-TCs and C-D (right) for PD-L1-TILs. Reasoning that the significance of PD-L1 might be dependent on the molecular contexts denoting inactivation/over-activation of genome-protecting mechanisms, we verified whether PD-L1 was associated with PFS in a DDR-dependent way. Thus, univariate Cox regression analyses were carried out by stratifying for DDR marker status (positive

| Table 1. Baseline characteristics of gastric cancer patients included in this study (N = 72). |
|-------------------|---------------|----------|
| Characteristics   | N (%)         |
| Age at diagnosis  | Median (min-max) [IQ range] |
| Gender            | 60.4 (28.79) [51.6-67.5] |
| Male              | 36 (50.0)     |
| Female            | 36 (50.0)     |
| ECOG PS           |               |
| 0                 | 43 (59.7)     |
| 1-2               | 29 (40.3)     |
| Stage             |               |
| Locally advanced  | 34 (47.2)     |
| Metastatic        | 38 (52.8)     |
| Previous surgery  |               |
| No                | 20 (27.8)     |
| Yes               | 52 (72.2)     |
| Neoadjuvant/adjuvant chemotherapy |     |
| No                | 46 (63.9)     |
| Yes               | 26 (36.1)     |
| Lauren classification |            |
| Intestinal        | 29 (40.3)     |
| Diffuse           | 35 (48.6)     |
| Mixed             | 8 (11.1)      |
| Grade             |               |
| G2                | 19 (26.4)     |
| G3                | 51 (70.8)     |
| Unknown           | 2 (2.8)       |
| Localization      |               |
| Esophago-gastric junction (EOJ) | 4 (5.6) |
| Stomach           | 68 (94.4)     |
| Agents (N)        |               |
| 2                 | 32 (44.4)     |
| 3                 | 40 (55.6)     |
| Taxanes           |               |
| No                | 34 (47.2)     |
| Yes               | 38 (52.8)     |
vs negative for protein biomarkers, wild-type vs mutated for ARID1A and TP53). While PD-L1-TILs immunostaining pattern was not associated with PFS even when stratifying for DDR marker status (data available upon request), patients with PD-L1-TC-positive tumors had a decreased risk of disease progression exclusively in the subgroups with DDR-negative biomarkers (Fig. 2). A similar pattern was documented in the ARID1A mutated background, whereas TP53 mutational status seemed to be irrelevant (Fig. 2). Prompted by the observation that the protective significance of PD-L1-TCs emerges in DDR-negative

Figure 1. Kaplan-Meier survival curves of progression-free survival (PFS) and overall survival (OS) comparing PD-L1-TC-positive versus negative cases (panel A and B); and PD-L1-TIL-positive versus negative cases (panel C and D).

Figure 2. Forest plot illustrating the relationship between PD-L1-TCs expression and progression-free survival (univariate Cox regression analyses) by stratifying for DDR markers (positive versus negative for protein biomarkers, wild-type versus mutated for ARID1A and TP53). Statistically significant analyses are indicated with red arrows.
tumors, and considering that both ATM and ARID1A participate in the repair of DNA DSBs, we then focused on a molecular scenario reflecting this process. On this ground, we separately analyzed two distinct molecular subgroups: i) tumors in which ARID1A wild-type co-existed with the expression of pATM (DDRon). This molecular context plausibly reflects conserved ARID1A/ATM function and then an efficient processing of DNA lesions, and ii) their negative counterparts, defined by the presence of ARID1A mutations or negative nuclear pATM expression (DDRoff). As illustrated in Fig. 3, PD-L1-TC expression was

Figure 3. Kaplan-Meier survival curves of progression-free survival (PFS) comparing PD-L1-TC-positive versus their negative counterparts in the DDRoff subgroup (panel A, N = 40) and in the DDRon subset (panel B, N = 32).
Table 2. Uni- and multivariate Cox regression models for progression-free survival (PFS) performed in the DDRon group (left columns) and in the DDRoff group (right columns).

|                  | DDRon                    | DDRoff                   |
|------------------|--------------------------|--------------------------|
|                  | Univariate Cox regression model | Multivariate Cox regression model | Univariate Cox regression model | Multivariate Cox regression model |
|                  | HR (95% CI) p-value       | HR (95% CI) p-value       | HR (95% CI) p-value | HR (95% CI) p-value |
| PD-L1-TCs       |                          |                          |                          |                          |
| Positive vs Negative | 1.21 (0.56–2.63) | 0.624 | — | 0.42 (0.20—0.89) | 0.023 |
| Stage           |                          |                          |                          |                          |
| Metastatic vs locally advanced | 0.60 (0.27–1.34) | 0.214 | — | 1.69 (0.81—3.52) | 0.161 |
| N° metastatic sites | 2—3 vs 1 | 1.08 (0.43–2.73) | 0.865 | — | 1.65 (0.79—3.44) | 0.180 |
| Taxanes         |                          |                          |                          |                          |
| Yes vs No        | 0.97 (0.44—2.13) | 0.937 | — | 0.78 (0.38—1.59) | 0.501 |

*Backward stepwise exclusion.

Table 3. Uni- and multivariate Cox regression models for overall survival (OS) performed in the DDRon group (left columns) and in the DDRoff group (right columns).

|                  | DDRon                    | DDRoff                   |
|------------------|--------------------------|--------------------------|
|                  | Univariate Cox regression model | Multivariate Cox regression model | Univariate Cox regression model | Multivariate Cox regression model |
|                  | HR (95% CI) p-value       | HR (95% CI) p-value       | HR (95% CI) p-value | HR (95% CI) p-value |
| PD-L1-TCs       |                          |                          |                          |                          |
| Positive vs Negative | 1.61 (0.73–3.55) | 0.237 | — | 2.56 (1.06–6.16) | 0.036 |
| Stage           |                          |                          |                          |                          |
| Metastatic vs locally advanced | 1.12 (0.50–2.47) | 0.787 | — | 1.58 (0.78—3.17) | 0.201 |
| N° of metastatic sites | 2—3 vs 1 | 1.98 (0.80–4.95) | 0.141 | — | 1.85 (0.91—3.75) | 0.088 |
| Taxanes         |                          |                          |                          |                          |
| Yes vs No        | 0.62 (0.26—1.46) | 0.274 | — | 0.72 (0.36—1.46) | 0.368 |
| Second-line chemotherapy Yes vs No | 0.32 (0.13—0.78) | 0.012 | — | 0.23 (0.09—0.60) | 0.003 |

*Backward stepwise exclusion.

associated with better PFS exclusively in the DDRoff subgroup (log-rank \( p = 0.020 \)), whereas its protective effect was no longer evident in the subset of patients with DDRon GC (log-rank \( p = 0.622 \)). Results from uni- and multivariate Cox regression models confirmed that PD-L1-TCs expression was an independent predictor of better PFS only in the DDRoff subset (multivariate Cox: HR 0.34, 95%CI: 0.15–0.76, \( p = 0.008 \)) (Table 2). These findings suggest that PD-L1-TC expression is associated with better PFS only in specific molecular contexts that may denote suboptimal processing of DNA DSBs.

**DDR status is an effect modifier of the relationship between PD-L1 expression and overall survival**

We next investigated whether PD-L1-TC expression was associated with overall survival (OS). As expected, the multivariate Cox regression model for OS indicated that PD-L1-TC expression conferred a decreased risk of death in the DDRoff population (HR 0.41, 95%CI: 0.17–0.96, \( p = 0.039 \), Table 3). Surprisingly, the multivariate Cox regression models carried out in the subgroup of patients with DDRon tumors showed that PD-L1-TC expression was associated with an increased risk of death (HR 2.56, 95%CI: 1.06–6.16, \( p = 0.036 \), Table 3), even though PD-L1 expression did not test statistically significant at the univariate assessment. Given that positive PD-L1-TC immunostaining oppositely impacted OS in an ARID1A- and pATM-dependent manner, DDR activation status may represent an effect modifier of the relationship between PD-L1-TC and OS in patients with advanced GC.

**Discussion**

In the present study, we examined the expression pattern of PD-L1 in a relatively large series of advanced GC patients treated with first-line therapy. Collectively, our results suggest that: i) PD-L1 expression in cancer cells is associated with better PFS exclusively in a subset of patients whose tumors did not harbor biomarkers reflecting the activation of genome-protecting mechanisms (DDRoff), ii) while this pattern was retained when evaluating OS, the relationship between PD-L1-TCs and OS was inverted in the DDRon subgroup, configuring a role of effect modifiers for DDR markers, and iii) the assessment of PD-L1 in the immune infiltrate did not yield any additional information. To our knowledge, this is the first report striving to tie PD-L1, pathways that ensure the correction of DNA lesions and survival outcomes in advanced GC patients, and with special emphasis on PFS. We are aware that this study is hypothesis-generating in nature owing to its retrospective design. Another aspect that needs to be mentioned is that both in this study and in our previous report,\(^{33}\) we did not evaluate pATR expression levels. This choice is rooted in the fact that tissue samples were characterized for both protein biomarkers and genomic alterations. Beyond DDR-related markers, in the same cohort we also evaluated another oncogenic cooperation, namely the Hippo-Wnt pathway crosstalk.\(^{36}\) Given this fairly wide characterization, we preferred to evaluate other effectors, such as pChk1 and pWee1, which act downstream of pATR and whose pharmacological inhibitors are at a more advanced stage of clinical development, as highlighted by the recent dissemination of final results from phase II trials.\(^{37,38}\) It is also worth mentioning that the model exploited for testing the significance of PD-L1 expression, namely combined pATM expression and ARID1A mutations, beyond being biology-driven, applies to all clinical endpoints (PFS and OS). Indeed, PD-L1 expression did not achieve full statistical significance for both PFS and OS when stratifying by individual DDR biomarkers (PFS data are reported in Fig. 2, OS data are available...
upon request). Our belief is that this is attributable to the size of analyzed subgroups, that enabled us to exclusively uncover the strongest association. Consistently, in a prospective study with biomarker validation purposes that we have recently initiated and that is mentioned below, we considered an expanded set of DDR- and immune-related markers.

On the other hand, this study has some important strengths, such as that half of the patients were treated in prospective phase II trials. Moreover, we did not record any marked differences in the distribution of PD-L1 expression when comparing DDR marker positive vs negative tumors (supplementary Table 2). This observation, in association with the results from multivariate Cox models, allows us to exclude potential confounding factors.

From a molecular perspective, the characterization of GC carried out by the Cancer Genome Atlas Research (TCGA) Network assists us in framing these results. Indeed, ARID1A mutations were significantly more frequently observed in hypermutated GC as compared with non-hypermutated tumors (44% vs 14%). This suggests that ARID1A mutations may be a genomic trait characterizing hypermutated GC, thus enforcing our hypothesis that PD-L1-TCs is a protective factor exclusively in a molecular scenario characterized by defective activation of the DDR machinery, replication infidelity and accumulation of DNA lesions. More recently, Sato and colleagues described a model envisioning an increase of PD-L1 accumulation of DNA lesions. In particular, we are focusing on alterations involved in deregulated G1-S transition (e.g. TP53 and CCNE1), oncogene-induced replication stress (e.g. MYC and PTEN), and ATM/ATR-initiated DNA repair (e.g. ATM and ARID1A). Results from this study will provide further knowledge on how two intertwined phenomena, namely the anticancer immune response and DNA repair deficiency, impact survival outcomes in advanced GC.

In conclusion, our data point to PD-L1-TC expression as a potential biomarker of better PFS in advanced GC patients treated with chemotherapy in the first-line setting. This link relies on the activation of genome-protecting pathways, being exclusively observed when tumors did not carry biomarkers denoting activation of these mechanisms. Conversely, PD-L1-TC expression apparently influences OS in a more complex, DDR-dependent, way.

Patients and methods

For this analysis, 72 patients with histologically confirmed, inoperable locally advanced or metastatic GC or esophagogastric junction (EOJ) cancer who received first-line chemotherapy (August 2001-August 2014) were included. Median follow-up was 12 months (IQR 5–23 months). Patients were considered eligible on the following basis: i) complete data on clinical features and treatment outcomes, ii) complete data on PD-L1 immunostaining, and iii) complete data on DDR biomarkers, including the expression of DDR kinases/DNA damage markers and mutations in the TP53 and ARID1A genes. Chemotherapy regimens and schedules were already detailed elsewhere. Tumor responses were evaluated by Response Evaluation Criteria in Solid Tumors (RECIST) criteria v.1.1. PFS was calculated as the time between the first cycle of chemotherapy until radiological evidence of disease progression or death due to any cause. OS was computed as the time from the first cycle of chemotherapy to death due to any cause. Written informed consents were obtained from all the participants. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the “Regina Elena” National Cancer Institute of Rome.

Study procedures

The immunohistochemical assessment of PD-L1 was performed in formalin-fixed paraffin-embedded (FFPE) tissues with the monoclonal mouse anti-PD-L1 antibody clone 22C3 (1:20). PD-L1 expression was evaluated both in tumor cells and in the immune infiltrate (identified by morphological assessment), and considered positive when expressed in >1% of the respective cellular compartment. A detailed description of reagents and procedures used for evaluating DDR markers (proteins and genes) was already provided. Shortly, DNA damage markers (γ-H2AX and pRPA32) were classified as positive/negative using the median score of all tumors as the cut-off points, whereas DDR kinases
were considered positive when ≥20% of the neoplastic cells showed a distinct nuclear immunoreactivity of any intensity. Targeted DNA deep sequencing was carried out on a NextSeq 500 (Illumina, Inc., San Diego, CA, USA). TP53 and ARID1A mutations were filtered on the basis of variant allele frequencies (≥10%), and considering the established or predicted oncogenicity of the detected mutations.33

Statistical analysis

Descriptive statistics were computed for all the variables of interest. The relationship between categorical variables was investigated with the Pearson’s Chi-squared test of independence (2-tailed) or the Fisher Exact test, depending upon the size of the groups compared. Survival curves were estimated through backward elimination. The related estimates were tested in univariate Cox proportional hazard models. Variables potentially affecting PFS and OS were considered positive when 20% of the neoplastic cells showed a distinct nuclear immunoreactivity of any intensity. Targeted DNA deep sequencing was carried out on a NextSeq 500 (Illumina, Inc., San Diego, CA, USA).

The list of abbreviations

ATM ataxia-telangiectasia mutated
ATR ataxia telangiectasia and Rad3-related protein
Chk1 checkpoint kinase 1
Chk2 checkpoint kinase 2
DDR DNA damage and repair
DSBs double-strand breaks
GC gastric cancer
MMR mismatch-repair
OS overall survival
γ-H2AX phosphorylated H2A Histone Family Member X
PFS progression-free survival
PD-1 programmed cell death protein-1
PD-L1 programmed cell death ligand-1
PD-L1-TCs PD-L1 expression in tumor cells
PD-L1-TILs PD-L1 expression in tumor-infiltrating lymphocytes
pRPA32 phosphorylated replication protein A2
SSBs single-strand breaks
TILs tumor-infiltrating lymphocytes
Wee1 wee1-like protein kinase.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Tania Merlino for technical assistance.

Funding

This study was supported by an intramural research grant to the "Gastrointestinal Tumors Translational Research Group".

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